Entwicklung eines Screeningsystems zur Identifizierung hochaktiver und selektiver Hemmstoffe der 17β-Hydroxysteroid-Dehydrogenase Typ 1 (17βHSD1)

Dissertation
zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III
-Chemie, Pharmazie, Bio- und Werkstoffwissenschaften-
der Universität des Saarlandes

von
Patricia Kruchten
Saarbrücken
2009

Tag des Kolloquiums:	30.10.09
Dekan:	Prof. Dr.-Ing. Stefan Diebels
Berichterstatter:	Prof. Dr. Rolf W. Hartmann
	Prof. Dr. Alexandra K. Kiemer

Diese Arbeit entstand unter der Anleitung von Prof. Dr. R. W. Hartmann in der Fachrichtung 8.2 Pharmazeutische und Medizinische Chemie der Naturwissenschaftlich-Technischen Fakultät III der Universität des Saarlandes von Januar 2006 bis Juni 2009

Meiner Familie

Danksagung

Herzlich bedanken möchte ich mich bei:

Prof. Dr. Rolf W. Hartmann für die interessante Thematik, das entgegengebrachte Vertrauen, die gute Betreuung und die überlassene Freiheit.

Prof. Dr. Alexandra K. Kiemer für die Übernahme des Koreferats.

Dr. Martin Frotscher für die Übernahme des Beisitzers in der Prüfungskomission sowie für die einzigartige Leitung der 17βHSD1-Projektgruppe.

Dr. Sandrine Oberwinkler-Marchais für interessante Denkansätze und Diskussionen sowie für die Leitung des 17βHSD1-Projekts.

Dr. Ursula Müller-Vieira für eine kompetente Einführung in die Zellkultur, die Betreuung meiner Diplomarbeit, für Rat und Tat und dafür, dass sie mir den Spaß an der Wissenschaft vermittelt hat.

Allen aktuellen und ehemaligen Mitglieder der 17βHSD1-Gruppe für die konstruktive und erfolgreiche Zusammenarbeit.

Ruth Werth, Christina Ries und Claudia Henn für offene Ohren, die fabelhafte Zusammenarbeit im „Biologenteam“ sowie für interessante Diskussionen, bei denen kreative Ideen entstanden. Dr. Emmanuel Bey für den Spaß bei den nächtlichen Gewebepräparationen, seine Unterstützung und die heißen HSD-Diskussionen. Alex Oster für die hervorragende Zusammenarbeit und seine fortwährend gute Laune und Alessandro Spadaro dafür, dass er mir den „italian way of life“ nähergebracht hat. Danke für Eure Freundschaft!

Dr. Anke Steinbach, Ruth Werth, Claudia Henn, Markus Altmeyer und Alex Oster für die gemeinsame, reibungslose Gestaltung und Betreuung des Arzneibuch-Praktikums. Mit Euch hat es Spaß gemacht!

Dr. Stefan Boettcher für die Unterstützung bei HPLC-Problemen und unbürokratische Problemlösungen.

Matthias Negri für die anschaulichen Modelling-Studien und neue Denkansätze, Kerstin Esch für ihre Arbeit in der Entwicklung des 17βHSD1-Tests und eine wunderbare Diplomzeit und Dr. Christiane Scherer für die Entwicklung des CaCo2-Tests.

Beate Geiger für die riesige Unterstützung bei der Routinetestung, für flinke Finger und eine einmalige Organisation und Jannine Ludwig, Anja Palusczak und Martina Jankowski für die Übernahme der Routinetestung.

Dr. Emmanuel Bey, Alex Oster, Claudia Henn, Alessandro Spadaro, Erika Ziegler, Marie Lordon, Yaseen Al-Soud und Kuiying Zhou für die Synthese der zu testenden Verbindugen.

Corina Przybyla und Martina Schwarz für die außerordentlich gute Organisation im Sekretariat.

Lothar Jager, dem Mann für alle Fälle, für technische Problemlösungen bei Hilferufen aller Art und Angelika Tehrani für den Überblick im Lager und Schwerstarbeit während des Praktikums, sowie Gerhard Müller für seinen Einsatz rund um den Computer.

Maria-Christina Scherzberg, meinen Wahlpflichtpraktikanten Kristina Hüsecken, Jenny Gorges, Diana Schäfer, Daniela Hodapp, sowie meiner Schülerpraktikantin Annika Demuth für die fleißige Arbeit und die schönen Ergebnisse.

Allen Insassen der Räume 5/6 und 0.32 für eine angenehme Arbeitsatmosphäre sowie allen ehemaligen und aktuellen Mitgliedern des Arbeitskreises für Spaß, gute Stimmung und Unterstützung bei der Arbeit.

Der Postgraduate School GRK532 für die Gewährung eines Stipendiums, Dr. Markus Ehses und Dr. Thorsten Voigt für die Mühe in der Planung der GRK-Einsätze und Ulli Hille, Hedrik Hähl, Doro Loytved, sowie Daniela Summa für viel Spaß und gute Laune während der Tagungen.

Den Mitarbeitern von Pharmacelsus CRO für die Durchführung von PK-Studien und eine gute Zusammenarbeit.

Meiner Familie für Geduld, Rat und Unterstützung, meinem Vater Hans-Josef Kruchten für die gewissenhafte Durchsicht des Manuskripts. Ohne Euch hätte ich das nie geschafft.

Eingeflossene Publikationen

Die vorgelegte Arbeit gliedert sich in sechs Publikationen, welche durch römische Zahlen gekennzeichnet sind:

I. Development of a biological screening system for the evaluation of highly active and selective 17β-HSD1-inhibitors as potential therapeutic agents

Patricia Kruchten, Ruth Werth, Sandrine Marchais-Oberwinkler, Martin Frotscher, Rolf W. Hartmann
Molecular and Cellular Endocrinology (**2009**) *301*, 154-157.

II. New Insights into the SAR and Binding Modes of Bis(hydroxyphenyl)thiophenes and Benzenes: Influence of Additional Substituents on 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) Inhibitory Activity and Selectivity

Emmanuel Bey, Sandrine Marchais-Oberwinkler, Matthias Negri, Patricia Kruchten, Alexander Oster, Ruth Werth, Martin Frotscher, Barbara Birk, Rolf W. Hartmann
Journal of Medicinal Chemistry (**2008**), Manuskript eingereicht.

III. Substituted 6-phenyl-2-naphthols. Potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation, and pharmacokinetics

Sandrine Marchais-Oberwinkler, Patricia Kruchten, Martin Frotscher, Erika Ziegler, Alexander Neugebauer, Uma Devi Bhoga, Emmanuel Bey, Ursula Müller-Vieira, Josef Messinger, Heinrich Thole, Rolf W. Hartmann
Journal of Medicinal Chemistry (**2008**) *51,* 4685-4698.

IV. Aging reduces the efficacy of estrogen substitution to attenuate cardiac hypertrophy in female spontaneously hypertensive rats.

Virginija Jazbutyte, Kai Hu, Patricia Kruchten, Emmanuel Bey, Sebastian Maier, Karl-Heinrich Fritzemeier, Katja Prelle, Christa Hegele-Hartung, Rolf W. Hartmann, Ludwig Neyses, Georg Ertl, Theo Pelzer
Hypertension (**2006**) *48*, 579-586.

V. Development of biological assays for the identification of selective inhibitors of estradiol formation from estrone in rat liver preparations

Patricia Kruchten, Ruth Werth, Sandrine Marchais-Oberwinkler, Emmanuel Bey, Erika Ziegler, Alexander Oster, Martin Frotscher, Rolf W. Hartmann
Comptes Rendus Chimie (**2009**), Manuskript akzeptiert.

VI. Selective inhibition of 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) reduces estrogen responsive cell growth of T47-D breast cancer cells

Patricia Kruchten, Ruth Werth, Emmanuel Bey, Alexander Oster, Sandrine Marchais-Oberwinkler, Martin Frotscher, Rolf W. Hartmann
Journal of Steroid Biochemistry and Molecular Biology (**2009**) *114*, 200-206.

Stellungnahme über die Beiträge der Autorin

Die Autorin möchte zu ihren Beiträgen zu den Veröffentlichungen I – VI in der Dissertation stellungnehmen.

I. Patricia Kruchten trug wesentlich zum Konzept des Screeningsystems bei und etablierte und validierte fünf der beschriebenen Tests. Sie konzipierte und verfasste das Manuskript.

II. Patricia Kruchten war für die Durchführung der Routinetests und für die Interpretation der erhaltenen Ergebnisse verantwortlich. Die Autorin trug zur Konzeption der Inhibitoren, zur Evaluierung der Verbindungen sowie zur Erstellung des Manuskripts bei.

III. Patricia Kruchten war für die Durchführung der Routinetests und für die Interpretation der erhaltenen Ergebnisse verantwortlich. Die Autorin trug zur Konzeption der Inhibitoren, zur Evaluierung der Verbindungen sowie zur Erstellung des Manuskripts bei.

IV. Patricia Kruchten evaluierte das Ausmaß der altersabhängigen E1-Aktivierung bzw. E2-Inaktivierung in Lebergewebe hypertensiver Ratten. Die Autorin plante und konzipierte die durchgeführten Tests. Sie trug zur Interpretation der Ergebnisse und zur Erstellung des Manuskripts bei.

V. Patricia Kruchten etablierte und validierte die zur Evaluation notwendigen Tests. Die Autorin war zuständig für die Durchführung der Routinetests sowie für die Interpretation der erhaltenen Ergebnisse. Die Autorin konzipierte und verfasste das Manuskript.

VI. Patricia Kruchten etablierte und validierte die zur Evaluation notwendigen Tests. Die Autorin war zuständig für deren Durchführung sowie für die Interpretation der erhaltenen Ergebnisse. Die Autorin konzipierte und verfasste das Manuskript.

INHALTSVERZEICHNIS

1 EINLEITUNG

1.1 Estrogene

1.1.1 Estrogene als Steroidhormone

Estrogene gehören zu den Steroidhormonen. Unter diesem Begriff werden Derivate des Sterans zusammengefasst, welche als Hormone wirken. Bezüglich des Hauptbildungsortes lassen sich die Steroidhormone einteilen in Corticosteroide, welche größtenteils in der Nebenniernenrinde gebildet werden, und Sexualhormone mit den Gonaden als Hauptentstehungsort.
Die Corticosteroide werden weiterhin unterteilt in Mineralocorticoide, welche Salz- und Wasserhaushalt aufrechterhalten, und Glucocorticoide, welche für die Glucosehomöostase benötigt werden. Unter den Sexualhormonen lassen sich drei Steroidhormonklassen zusammenfassen: Gestagene, Androgene und Estrogene. Gestagene werden auch als C21-Steroide bezeichnet und haben im humanen Organismus einen wesentlichen Vertreter, das Progesteron. Bei den Androgenen handelt es sich im Allgemeinen um C19-Steroide. Hauptvertreter der Androgene sind Dihydroepiandrosteron (DHEA), Androstendion (Adion), Androsteron, Testosteron (Testo) und Dihydrotestosteron (DHT). Ein weiteres humanes C19-Steroid stellt das Androstendiol dar, welches jedoch estrogene Wirkung besitzt. Durch Aromatisierung des steroidalen A-Rings entstehen aus den Androgenen Estrogene, deren Hauptvertreter die C18-Steroide Estron (E1), das am stärksten wirksame Estradiol (E2) und Estriol (E3) sind. Wesentlicher Bildungsort der Estrogene sind die Ovarien in der prämenopausalen Frau (Sasano *et al.*, 1989). Es gibt jedoch weitere Gewebe, welche in geringerem Ausmaß Estrogene produzieren. Zu diesen Geweben gehören Leber, Fettgewebe, Gehirn (Bulun *et al.*, 2001), Haut (Nelson und Bulun, 2001), Knochen, Plazenta (Simpson, 2003) und die Zona reticularis der Nebennieren (Moreau *et al.*, 2008; Wasada *et al.*, 1978).

1.1.2 Biosynthese und Regulation der Estrogene

Wie auch Androgene und Gestagene leiten sich Estrogene als Derivate des Sterans vom Cholesterol ab, dessen Seitenkette zunächst in einer katalytischen Reaktion durch das mitochondriale CYP11A1 (side chain cleaving enzyme) abgespalten wird (Abbildung 1).

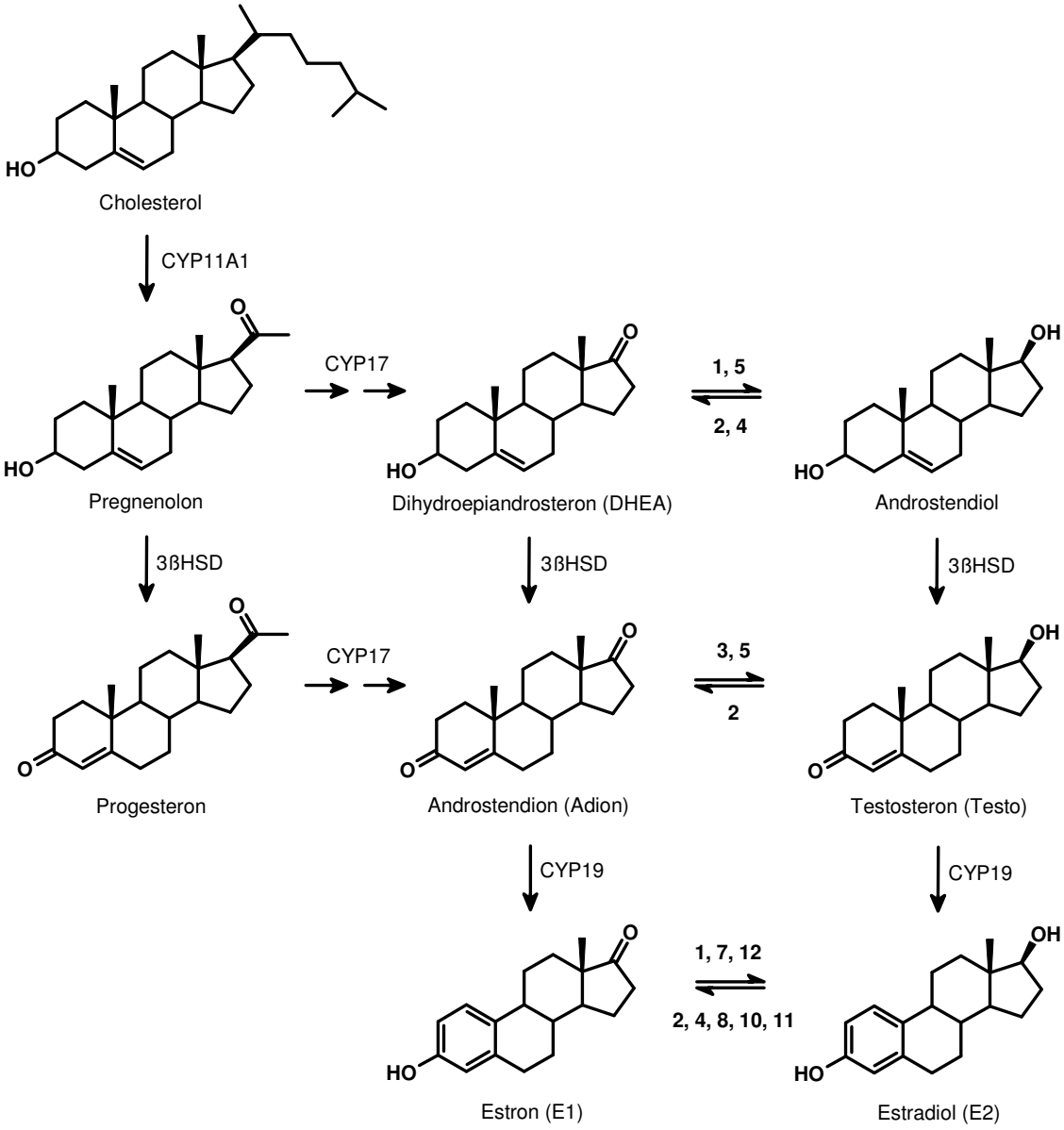


Abbildung 1: Estrogenbiosynthese ausgehend vom Cholesterol.

Das aus dieser Reaktion entstehende Pregnenolon kann unter Einfluss der 3β-Hydroxysteroid-Dehydrogenase (3βHSD), welche im endoplasmatischen Retikulum lokalisiert ist, durch Oxidation in Position 3 und Verschieben der Doppelbindung in Progesteron umgewandelt werden. Beide Steroide, Pregnenolon und Progesteron, können in Position 17α hydroxyliert werden. Vermittelt wird diese Reaktion durch das bifunktionale Enzym CYP17 (17α-Hydroxylase-C17/20-Lyase), welches zunächst in der Hydroxylasereaktion die Bildung von 17-Hydroxy-Pregnenolon und 17-Hydroxyprogesteron katalysiert, aus welchen dann in der nachfolgenden Lyasereaktion DHEA und Adion dargestellt werden (Hall, 1991). Prinzipiell kann E2 nun auf zwei Wegen gebildet werden. Die erste Möglichkeit ist die reversible Reduktion der Androgene DHEA und/oder Adion durch 17βHSDs in Position 17 mit nachfolgender, irreversibler Aromatisierung des steroidalen A-Rings durch das microsomale CYP19 (Aromatase). Alternativ kann auch die Reihenfolge von Aromatisierungsschritt und

Reduktion in Position 17 vertauscht sein. Die Konversion der 17-Ketosteroide zu den 17-Hydroxysteroiden kann grundsätzlich durch unterschiedliche 17β-Hydroxysteroid-Dehydrogenasen (17βHSD) katalysiert werden (Luu-The, 2001).

Die einzelnen Subtypen haben jedoch unterschiedliche Expressionsmuster und Substratpräferenzen (Kap. 1.2.2). Der bevorzugte Weg der E2-Synthese verläuft über Adion (Brueggemeier, 2001; Bulun *et al.*, 2000), welches aus DHEA unter Einfluss der 3βHSD gebildet werden kann und durch CYP19 zum E1 aromatisiert wird (Thompson und Siiteri, 1974). Im letzten Schritt wird das entstandene E1 in einer katalytischen Reaktion durch die 17βHSD1 in Position zum E2 reduziert (Gangloff *et al.*, 2001) (Abbildung 1).

Die Estrogenproduktion unterliegt der endokrinen Regulation des hypothalamisch-hypophysären Systems (Robker und Richards, 1998) (Abbildung 2). In der hypophysiotropen Zone des Hypothalamus (kleinzellige Kerne) wird Gonadotropin Releasing Hormon (GnRH) gebildet und pulsatil in den hypophysären Pfortaderkreislauf sezerniert. Dadurch wird in der Adenohypophyse (Hypophysenvorderlappen) die Freisetzung von Follikelstimulierendem Hormon (FSH) und Luteinisierendem Hormon (LH) stimuliert. FSH bewirkt in den Ovarien die Follikelreifung (Richards *et al.*, 1998). LH löst die Ovulation aus und leitet die Bildung des Corpus luteum (Gelbkörper) ein (Rao *et al.*, 1978), welcher Progesteron produziert. Außerdem stimuliert LH in den Thekazellen des Follikels die Bildung von Androgenen, welche in den Granulosazellen unter Einfluss von FSH zu Estrogenen konvertiert werden (Richards, 1980).

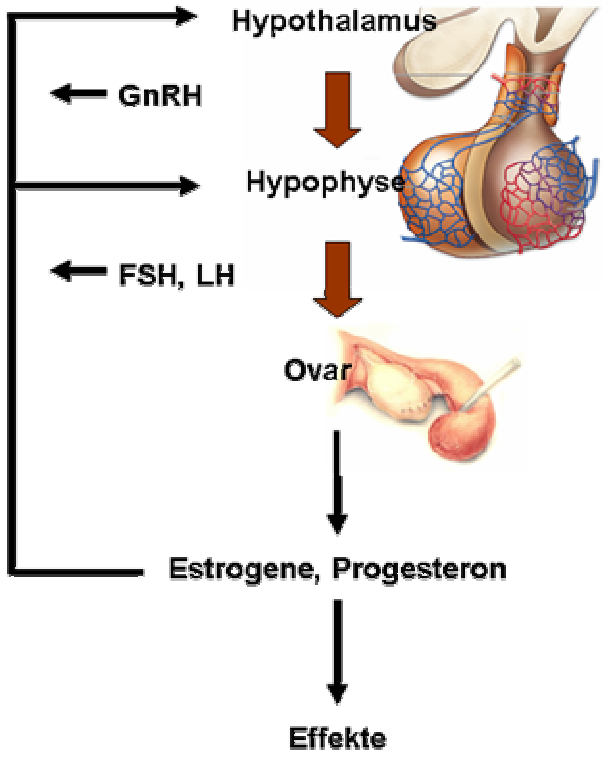


Abbildung 2: Zentrale Regulierung der Ausschüttung von Estrogenen und Progesteron. GnRH, Gonadotropin Releasing Hormon; FSH, Follitropin, LH: Lutropin.

Darüberhinaus wird auch in den Nebennieren die Bildung von Androgenen gesteigert, welche nachfolgend aromatisiert werden. Der Hormontransport vom Bildungsort ins Zielgewebe erfolgt teilweise gebunden an Transportproteine und teilweise in der freien oder sulfatierten Form im Blut (Suzuki *et al.*, 2005). Durch Feedback-Mechanismen wird die Versorgung der Gewebe mit Estrogenen kontrolliert.

Prämenopausal unterliegt die Estrogenkonzentration im Blut also starken Schwankungen. In der ersten Zyklushälfte steigt die E2-Plasmaspiegel an bis sie kurz vor der Ovulation ihren Maximalwert erreicht. Nach dem Eisprung sinkt der Plasma-E2-Spiegel zunächst ab, steigt aber schnell wieder auf ein hohes Niveau an, da der Gelbkörper neben Progesteron auch E2 bildet. Am Zyklusende werden wieder basale Werte erreicht. Da postmenopausal die ovarielle Estrogenproduktion eingestellt wird, unterliegt die Blutkonzentration, die nun hauptsächlich durch die Nebennieren aufrechterhalten wird, wesentlich geringeren Schwankungen. Außerdem sinkt die Estrogenkonzentration im Blut deutlich ab. So kann postmenopausal nur ca. ein Zehntel der prämenopausal beobachteten Werte detektiert werden (Miller und O'Neill, 1987; van Landeghem *et al.*, 1985).

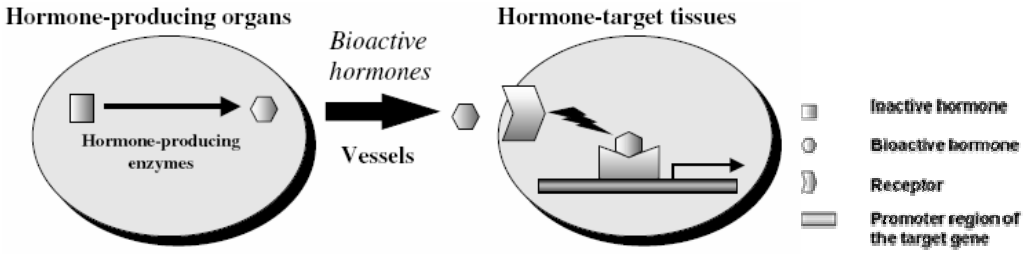


Abbildung 3: Endokrine Hormonsekretion (Suzuki et al., 2005).

Diesem endokrinen Mechanismus, bei dem die aktiven Steroidhormone an einem Ort produziert und danach zum Zielgewebe transportiert werden (Abbildung 3), steht nun die intrakrine Versorgung des Zielgewebes gegenüber (Abbildung 4). Dieser Begriff wurde 1991 von F. Labrie geprägt (Labrie, 1991) und besagt, dass lokal produzierte Androgene und/ oder Estrogene ihre Wirkung in derselben Zelle entfalten, wo ihre Synthese stattgefunden hat, ohne vorher in den extrazellulären Raum abgegeben worden zu sein (Labrie *et al.*, 2000).

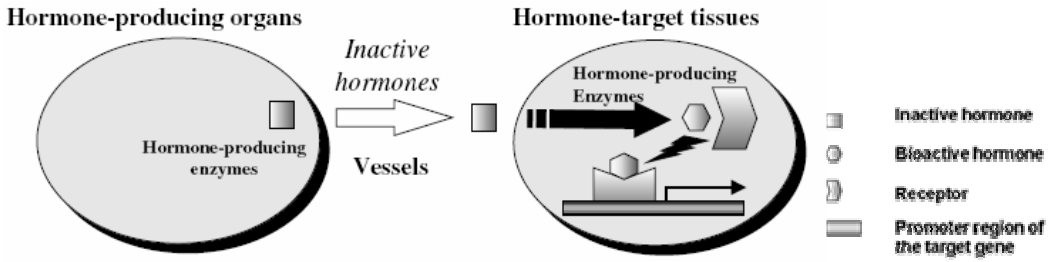


Abbildung 4: Intrakrine Hormonsekretion (Suzuki et al., 2005).

Unterstützt wird die Theorie der E2-Aktivierung im erkrankten Gewebe auch dadurch, dass in postmenopausalen Frauen ähnlich hohe E2-Konzentrationen in Brusttumorgewebe gefunden wurden wie bei prämenopausalen, obwohl nach der Menopause die Blut-E2-Konzentration stark

vermindert ist (Gunnarsson *et al.*, 2005; Miyoshi *et al.*, 2001; van Landeghem *et al.*, 1985). Außerdem wurde in Brusttumoren eine zehnfach höhere E2-Konzentration festgestellt als im Blut (Suzuki *et al.*, 2000).

Zieht man nun in Betracht, dass im Blut wesentlich mehr E1 vorliegt als E2, kommt der 17βHSD1 unter dem Aspekt der Intrakrinologie besondere Bedeutung zu. Sie ist nämlich in der Lage, das aus der Transportform freigesetzte E1 direkt im Zielgewebe zu E2 zu aktivieren (Lukacik *et al.*, 2006; Luu-The *et al.*, 1995).

1.1.3 Physiologische Wirkungen und Wirkmechanismus der Estrogene

Estrogene greifen in unterschiedlichen Zielgeweben an. Dabei zeigen sie hauptsächlich genitale Wirkung. Das heißt, sie wirken auf die Sexualorgane wachstumsfördernd und sind für die Prägung der weiblichen sekundären Geschlechtsmerkmale zuständig. Ebenso finden auch der Aufbau der Uterusschleimhaut (Groothuis *et al.*, 2007), die Bildung der Endometriumdrüsen, sowie die Viskositätserniedrigung des Zervikalschleims unter Einfluss der Estrogene statt. Durch E2 wird die Uterusschleimhaut für den Einfluss von Progesteron sensitiviert und damit für die Implantation vorbereitet (Navot und Bergh, 1991; Riesewijk *et al.*, 2003). Auch für die Aufrechterhaltung der Schwangerschaft und die fetale Entwicklung ist E2 essentiell (Albrecht *et al.*, 2000; Pepe und Albrecht, 1995).

Daneben entfalten Estrogene auch extragenitale Effekte. Im Knochen bewirken sie durch Schluss der Epiphysenfuge die Beendigung des Längenwachstums und steigern die Resorption von Kalzium sowie dessen Einlagerung in den Knochen (Turner *et al.*, 1994). Neben der Retention von NaCl und Wasser (Mendelsohn und Karas, 1999) sorgen sie für die Vergrößerung der subkutanen Fettdepots und zeigen schwache anabole Effekte.

Estrogene entfalten ihre Wirkung im Wesentlichen durch Aktivierung der Estrogenrezeptoren (ER). Derzeit sind zwei Subtypen, ERα (Walter *et al.*, 1985) und ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996), im humanen Organismus bekannt. Prinzipiell zeigen sie denselben Aufbau (Kumar *et al.*, 1987) aber eine unterschiedliche Gewebeverteilung (Nilsson und Gustafsson, 2000). So ist der ERα vorwiegend in Uterus, Leber, Niere und Herz exprimiert, während der ERβ hauptsächlich in Ovarien, Prostata, Lunge, Gastrointestinaltrakt, Blase und im zentralen Nervensystem überwiegt. In zahlreichen Geweben sind beide Rezeptortypen coexprimiert, beispielsweise in Brustdrüse, Schilddrüse, Nebennieren und Knochen (Kuiper *et al.*, 1997). Auch in malignen Tumoren der Brust, Endometrium und endometriotischem Gewebe wurden beide Subtypen detektiert (Cotterchio *et al.*, 2003; Deroo und Korach, 2006; Dotzlaw *et al.*, 1997; Jensen *et al.*, 2001; Maaroufi *et al.*, 2000; Palmieri *et al.*, 2002).

Beide Rezeptorsubtypen gliedern sich in sechs funktionelle Domänen A bis F (Abbildung 5). Die A/B-Domäne am aminoterminalen Ende enthält die ligandenunabhängige

Transaktivierungsfunktion 1 (Kumar *et al.*, 1987). Im mittleren Bereich der C-Region befindet sich die DNA-Bindungssequenz, welche den Rezeptor nach Dimerisierung in Wechselwirkung treten lässt. Hier sind beide Rezeptorsubtypen hochhomolog. Domäne D, eine flexible Region, enthält das Kernlokalisationssignal und außerdem für die Dimerisierung wichtige Aminosäuren (Picard und Yamamoto, 1987). In Domäne E mit einer Homologie von lediglich 59 % findet die Ligandenbindung statt. C-terminal gelegen ist die ligandenabhängige Aktivierungsfunktion lokalisiert (Pratt *et al.*, 1988; Webster *et al.*, 1988), welche in die variable F-Domäne übergeht (Vegeto *et al.*, 1992).

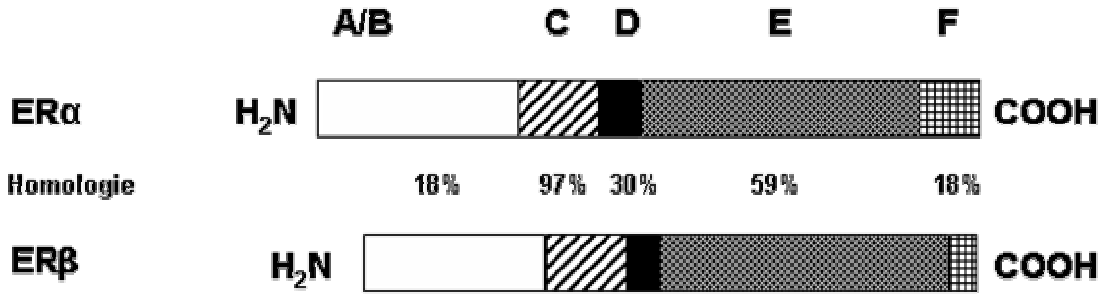


Abbildung 5: Vergleich der humanen ER.

Ebenso wie im Expressionsmuster unterscheiden sich die Rezeptortypen auch in der Art der vermittelten Effekte (Gustafsson, 1999; Ham und Parker, 1989; Katzenellenbogen, 1996; Katzenellenbogen *et al.*, 1993; Montano *et al.*, 1996). So ist der bereits länger bekannte ERα für die Auslösung der klassischen und proliferationsfördernden estrogenen Effekte verantwortlich, während dem ERβ ein antiproliferativer, sogar antagonistischer Effekt zum ERα zugeschrieben wird (Hall und McDonnell, 1999; Ström *et al.*, 2004; Weihua *et al.*, 2000). Diese antagonistische Wirkung soll durch Heterodimerisierung zustande kommen (Lindberg *et al.*, 2003; Matthews und Gustafsson, 2003). Außerdem scheint der ERβ wichtig zu sein für die Funktion der Ovarien und damit für die Fertilität.

Estrogene Effekte können molekular auf unterschiedlichen Wegen ausgelöst werden (Abbildung 6). Man kann vier verschiedene Wege der ER-Aktivierung unterscheiden: die ligandenabhängige, direkte Aktivierung, die ligandenabhängige, indirekte Aktivierung, die ligandenunabhängige Aktivierung (Murdoch und Gorski, 1991), welche die Aktivierung von Genen auslösen, und nichtgenomische Effekte (Morani *et al.*, 2008; Nilsson und Gustafsson, 2000).

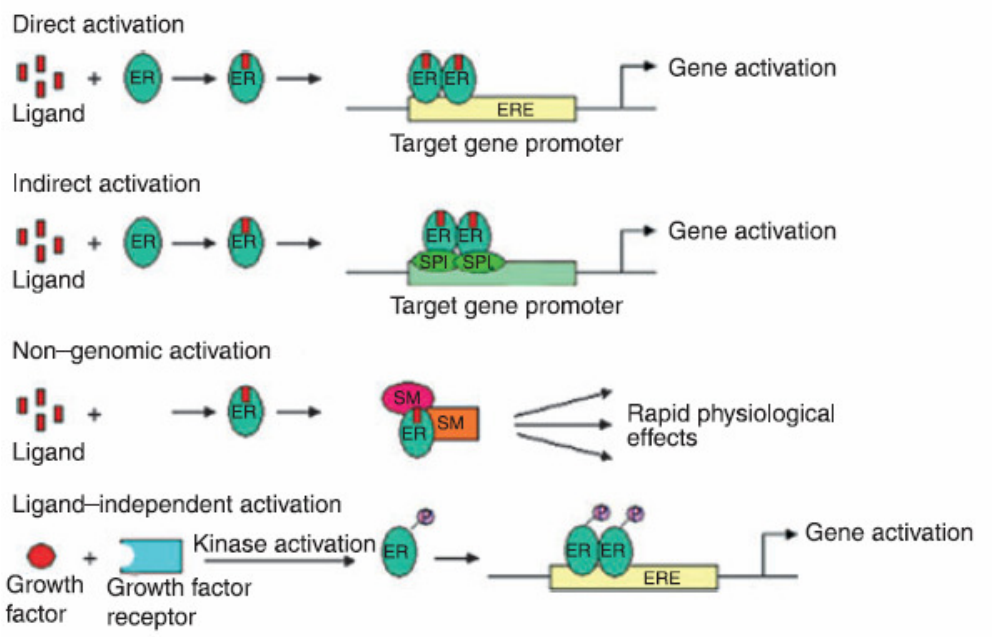


Abbildung 6: Molekulare Wege der Effektauslösung durch den ER.

Bei der ligandenabhängigen, direkten Rezeptoraktivierung laufen bis zur Transkription mehrere Prozesse ab. Nach Ligandenbindung erfolgt zunächst eine Rezeptordimerisierung. Dabei können Homo- und Heterodimere gebildet werden. Daraufhin transloziert das Dimer in den Nucleus und bindet an das estrogen-responsive element (ERE) in der Promotorregion des Targetgens (Nilsson und Gustafsson, 2000). Eine weitere Regulationsinstanz stellen die Coaktivatoren und -repressoren dar, welche einen Komplex mit Dimer und DNA eingehen und dadurch die mRNA-Produktion anregen oder unterdrücken können (Heldring *et al.*, 2007; Ozers *et al.*, 2005; Smith *et al.*, 1997a).

Die ligandenabhängige, indirekte Rezeptoraktivierung läuft ähnlich wie die direkte Aktivierung ab. Der Unterschied besteht darin, dass hier non-ERE-Reportergene durch Aktivierung von Transkriptionsfakoren wie SP1 abgelesen werden (Saville *et al.*, 2000).

Im Falle des ligandenunabhängigen Weges sind Wachstumsfaktoren Auslöser der Rezeptoraktivierung. Nach Bindung der Wachstumsfaktoren an ihren Rezeptor kommt es zur Aktivierung von Kinasen, welche den ER phosphorylieren. Das phosphorylierte Protein ist nun ebenfalls in der Lage zur Dimerisierung und nachfolgender ERE-Bindung. Dieser Mechanismus scheint am Wachstum hormonunabhängiger Tumoren beteiligt zu sein (Coutts und Murphy, 1998; Shim *et al.*, 2000).

Der Mechanismus der nichtgenomischen Effekte unterscheidet sich von den übrigen Aktivierungswegen, da die Wirkung hier nicht über die Ablesung von Targetgenen vermittelt wird. Auf diesem Wege können schnelle Effekte der Estrogene ausgelöst werden, da hier nicht die DNA, sondern second messenger und Strukturproteine zur Verfügung stehen. Die Auslösung nichtgenomischer Effekte wurde in neueren Arbeiten auch als „membrane-initiated steroid signalling“ (MISS) bezeichnet, da offenbar eine Assoziation von Rezeptor und

Membran bedeutend ist (Daufeldt *et al.*, 2003; Song, 2007). Im Gegensatz dazu wurde vorgeschlagen, die genomischen Effekte unter dem Begriff NISS für „nucleus-initiated steroid signalling“ zusammenzufassen (Nemere *et al.*, 2003). Letztlich wird vermutet, dass die MAP-Kinase (mitogen-activated protein kinase) und/ oder Wachstumsfaktoren wie EGF (epidermal growth factor) oder IGF-1 (insulin-like growth factor 1) aktiviert werden (Heldring *et al.*, 2007; Márquez und Pietras, 2001). Darüberhinaus sind Estrogene in der Lage, durch Interaktion mit Ionenkanälen sehr schnelle Effekt auszulösen. Außerdem kann offenbar auch über die Bildung von NO eine Vasodilatation hervorgerufen werden (Chen *et al.*, 1999).

1.2 17βHSD1

1.2.1 17βHSD1 als Hydroxysteroid Dehydrogenase

Die Hydroxysteroid Dehydrogenasen sind Enzyme, welche stereospezifische Reaktionen an unterschiedlichen Positionen des Steroidgrundgerüstes katalysieren (Penning, 1996). Sie können zwei unterschiedlichen Enzymfamilien zugeordnet werden (Penning, 1997). Zu den Aldo-Keto Reduktasen (AKR) gehören die 17βHSD5, 3αHSDs und 20αHSDs (Jez *et al.*, 1997). Die Enzyme dieser Familie liegen als Monomere vor und zeigen circa 67 % Sequenzidentität der Aminosäuren. Für die AKR wird der Hydridtransfer nach dem 4-pro-*R*-Mechanismus postuliert (Schlegel *et al.*, 1998).

Die zweite Enzymfamilie, welche Hydroxysteroid Dehydrogenasen umfasst, sind die Short-Chain Dehydrogenase/ Reduktasen (SDR) (Jornvall *et al.*, 1995). Zu dieser Familie gehören die 3βHSDs, die 11βHSDs und die 17βHSDs (Peltoketo *et al.*, 1999a; Wu *et al.*, 2007) mit Ausnahme der 17βHSD5 (Dufort *et al.*, 1999). Die Enzyme liegen als Mono- Di-, oder Multimere vor. Charakteristisch für die SDR ist der so genannte Rossman fold mit der konservierten Struktur aus alternierenden β-Faltblättern und α-Helices (β-α-β-α-β), einem Proteinabschnitt, in dem die Cosubstratbindung erfolgt. Außerdem ist ein Tyr-X-X-X-Lys-Motiv konserviert, dessen Tyrosin an der katalytischen Reaktion beteiligt ist (Puranen *et al.*, 1994). Die Sequenzidentität der Aminosäuren liegt bei circa 25 % innerhalb der Familie. Im Falle der SDR wird die Hydridübertragung nach dem 4-pro-*S*-Mechanismus vorgeschlagen (Penning, 2003).

Die Funktion der Dehydrogenasen ist es, hochaktive Hydroxy- und weniger aktive Ketosteroide ineinander zu überführen (Penning, 2003). Innerhalb der SDR lassen sich weiterhin die echten Dehydrogenasen, welche bevorzugt die Substratoxidation katalysieren, von den Reduktasen unterscheiden, welche die Substratreduktion präferieren (Kap. 1.2.2).

1.2.2 allgemeine Aspekte der 17βHSDs

Die Familie der 17βHSDs umfasst Enzyme, welche in der Lage sind, stereospezifisch oxidoreduktive Reaktionen in Position 17 des Steroidgrundgerüstes zu katalysieren. Sie zeigen jedoch eine relativ geringe Sequenzidentität von 25 bis 30 % innerhalb der Familie (Lukacik *et al.*, 2006). Zur Zeit sind fünfzehn Subtypen bekannt (Luu-The *et al.*, 2008). Die Nummerierung erfolgt chronologisch nach dem Zeitpunkt der Erstbeschreibung. Zwölf der fünfzehn Subtypen kommen im humanen Organismus vor (Tabelle 1 und Tabelle2). 17βHSD6, 9 und 13 wurden in Nagetieren gefunden (Biswas und Russell, 1997; Möller und Adamski, 2006; Su *et al.*, 2007). Die humanen nächsten Homologe zu 17βHSD6 und 9 sind – anders als in den Nagetieren – wahrscheinlich in den Retinoidstoffwechsel involviert.

Obwohl alle Vertreter der 17βHSDs prinzipiell zur Katalyse beider Reaktionsrichtungen in der Lage sind, können die Enzyme dieser Familie in oxidativ und reduktiv wirkend unterteilt werden, da sie intrazellulär eine Präferenz der Katalyserichtung zeigen (Miettinen *et al.*, 1996). Dies ist durch zwei Faktoren erklärbar (Abbildung 7, Abbildung 8).

Zum einen ist intrazellulär NADPH die Hauptelektronenquelle für reduktive Reaktionen. Die Konzentration innerhalb der Zelle liegt im millimolaren Bereich, da die kontinuierliche Regeneration aus $NADP^+$ über den Pentosephosphatweg gewährleistet ist. Dagegen sind intrazellulär lediglich micromolare Konzentrationen an NADH und $NADP^+$ vorhanden. Als Elektronenakzeptor für oxidative Reaktionen stehen dagegen millimolare Mengen an NADH zur Verfügung, welches ständig über die Zellatmung erneuert wird (Williamson *et al.*, 1967). Zusammenfassend wird also intrazellulär ein Ungleichgewicht der Cosubstrate aufrechterhalten, in dem ca. 500mal mehr NADPH als $NADP^+$ für reduktive Prozesse vorhanden ist und ca. 700mal mehr NAD^+ als NADH vorliegt, um oxidative Reaktionen zu gewährleisten (Agarwal und Auchus, 2005; Sherbet *et al.*, 2007) (Abbildung 7).

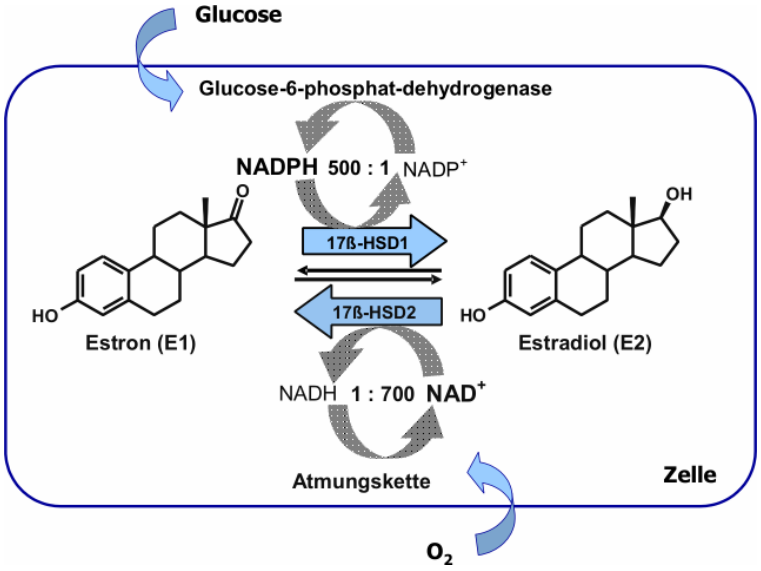


Abbildung 7: Intrazelluläre Determinierung der Präferenz der Katalyserichtung von 17βHSD1 und 17βHSD2.

Der zweite Faktor, welcher zur Determinierung der Katalyserichtung beiträgt, ist eine Cofaktor-Präferenz der einzelnen 17βHSD-Subtypen (Vihko *et al.*, 2001). In kinetischen Studien wurde beobachtet, dass die K_m-Werte für NADP(H) und NAD(H) sich subtypabhängig stark unterscheiden. Beispielsweise zeigt die 17βHSD1 eine Präferenz für NADPH (K_m = 0,07 µM) im Vergleich zu NADH (Km = 0,42 µM) (Gangloff *et al.*, 2001; Jin und Lin, 1999; Mazza *et al.*, 1998). Analysen der Röntgenkristallstrukturen zeigten, dass in der N-terminalen Region des Rossman fold reduktiver 17βHSDs ein Arginin-Rest vorhanden ist, dessen positiv geladene Guanidinium-Fuktion eine Salzbrücke mit dem 2´-Phosphatrest des NADPH ausbilden kann (Duax *et al.*, 2003; Pletnev *et al.*, 2004; Sawicki *et al.*, 1999). Dadurch wird die Affinität zum phosphorylierten Cosubstrat stark erhöht. Im Gegensatz dazu enthalten oxidative 17βHSDs eine negativ geladene Aminosäure anstelle des oben erwähnten Arginins, welche die Ladung des 2´-Phosphatrestes der phosphorylierten Cofaktor-Form abstoßen würde. Andererseits ist sie geeignet, um Wasserstoffbrücken mit der 2´-OH-Funktion des NAD^+ auszubilden und damit die Affinität zur nicht phosphorylierten Form erhöht (Chen *et al.*, 1991; Huang *et al.*, 2001).

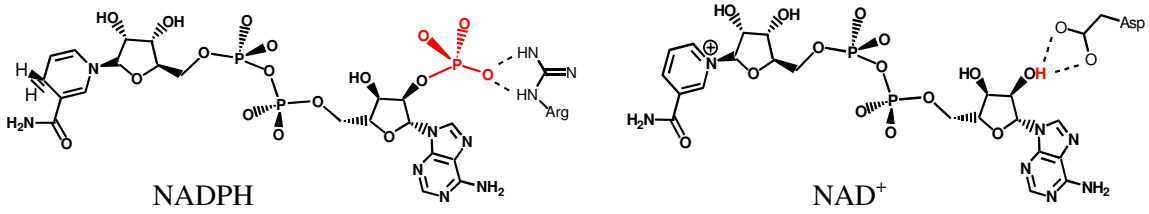


Abbildung 8: Cosubstratpräferenz reduzierender bzw. oxidierender HSD.

Die bevorzugte Katalyserichtung beinhaltet, dass die reduktiven Subtypen eine aktivierende Funktion innehaben, da sie die weniger wirksamen 17-Ketosteroide in die hochaktiven 17-Hydroxysteroide umwandeln (Gangloff *et al.*, 2001). Dagegen wirken die oxidativen 17βHSDs, welche die Bildung der 17-Ketosteroide katalysieren, inaktivierend und damit möglicherweise protektiv (Miettinen *et al.*, 1996).

Die 17βHSDs unterscheiden sich nicht nur bezüglich der Präferenz von Cosubstrat und Katalyserichtung, sondern auch bezüglich der Hauptexpressionsorte, der subzellulären Lokalisation und der Substratpräferenz (Puranen *et al.*, 1997). Dadurch kommt jedem einzelnen Enzym eine bestimmte physiologische Rolle zu.

Tabelle 1 fasst die wichtigsten Charakteristika der reduktiven humanen 17βHSDs zusammen (Möller und Adamski, 2006; Peltoketo *et al.*, 1999a; Prehn *et al.*, 2008). Sie zeigt auf, dass die Expression sowohl in hormonabhängigen als auch in hormonunabhängigen Geweben erfolgt. Darüberhinaus wird deutlich, dass die Subtypen 1, 7 und 12 prinzipiell zur Aktivierung von Estrogenen in der Lage sind (Krazeisen *et al.*, 1999; Luu-The *et al.*, 2006; Peltoketo *et al.*, 1999b). Die tatsächliche physiologische Rolle der Typen 7 und 12 wird jedoch in Cholesterol- und Lipidbiosynthese gesehen (Blanchard und Luu-The, 2007; Day *et al.*, 2008; Marijanovic *et al.*, 2003; Moon und Horton, 2003; Ohnesorg und Adamski, 2006).

Tabelle 1: Charakteristika der humanen, reduktiven 17βHSDs.

Enzym	**Vorkommen**	**Substrate**	**(Patho)physiologische Rolle**	*Literatur*
17βHSD1	Brust, Endometrium, Ovarien, Plazenta (Cytosol)	Estrogene, Androgene	Bereitstellung hoher Konzentrationen an aktiven Steroiden (E2, Testo) im Zielgewebe	(Langer und Engel, 1958; Peltoketo *et al.*, 1988; Poutanen *et al.*, 1993)
17βHSD3	Testes (Microsomen)	Androgene	Bildung von Testo; Defizienz: Pseudo-hermaphroditismus	(Geissler *et al.*, 1994)
17βHSD5	Leber, Prostata (Cytosol)	Androgene	Aktivierung von Androgenen, steigende Androgenspiegel in der Pubertät bei Pseudo-hermaphroditismus	(Dufort *et al.*, 1999; Khanna *et al.*, 1995; Penning *et al.*, 2000)
17βHSD7	Brust, Leber, Plazenta (Membran)	Estrogene, Zymosteron	Cholesterolbiosynthese, Estrogenaktivierung, Defizienz: CHILD-Syndrom	(Krazeisen *et al.*, 1999; Marijanovic *et al.*, 2003; Törn *et al.*, 2003)
17βHSD12	Brust, Niere, Leber, Plazenta (Microsomen)	Estrogene, langkettige Fettsäuren	Regulation der Lipidbiosynthese	(Blanchard und Luu-The, 2007; Luu-The *et al.*, 2006; Moon und Horton, 2003)
17βHSD15	Prostata	Androgene	Pathogenese des Prostatakarzinoms	(Luu-The *et al.*, 2008)

Tabelle 2 gibt einen Überblick über die oxidativen humanen 17βHSDs (Möller und Adamski, 2006; Peltoketo *et al.*, 1999a; Prehn *et al.*, 2008). Hier wird klar, dass vor allem Typ 2 und 4 für die Inaktivierung von Estrogenen von Bedeutung sein können (Leenders *et al.*, 1994). Typ 4 wird jedoch in Verbindung mit der β-Oxidation und dem Gallensäurenmetabolismus gebracht (Adamski *et al.*, 1995; Leenders *et al.*, 1996; Normand *et al.*, 1995), während der 17βHSD2 eine protektive Rolle bei estrogenabhängigen Erkrankungen zugeschrieben wird.

Zusammenfassend kann man aus Tabellen 1 und 2 entnehmen, dass 17βHSDs im Körper in vielen Geweben exprimiert werden. Es gibt jedoch subtypabhängige Expressionsmuster. Darüberhinaus sind die 17βHSDs in der Lage, verschiedene Substrate zu akzeptieren, wobei die einzelnen Enzyme bestimmte Präferenzen aufweisen und unter anderem deshalb mit bestimmten Erkrankungen assoziiert werden können.

Tabelle 2: Charakteristika der humanen, oxidativen 17βHSDs.

Enzym	Vorkommen	Substrate	(Patho)physiologische Rolle	*Literatur*
17βHSD2	Brust, Niere, Lunge, Plazenta, Prostata (Microsomen)	Estrogene, Androgene, Progesteron	Gewebeprotektion vor zu hohen Spiegeln an aktivem Steroid	(Blomquist *et al.*, 1985; Moghrabi, 1998; Puranen *et al.*, 1999; Wu *et al.*, 1993)
17βHSD4	Brust, Leber, Lunge, Plazenta (Peroxisomen)	Gallensäuren, Estrogene	Inaktivierung von Steroidhormonen, β-Oxidation, Gallensäuren-metabolismus; Defizienz: Zellweger Syndrom	(Adamski *et al.*, 1992; Adamski *et al.*, 1995; van Veldhoven *et al.*, 1996)
17βHSD8	Leber, Ovarien (Microsomes)	Androgene, Estrogene	Inaktivierung von Steroidhormonen; möglicherweise bedeutend für Fettstoffwechsel	(Fomitcheva *et al.*, 1998; Ohno *et al.*, 2008; Pelletier *et al.*, 2005)
17βHSD10	Zentrales Nervensystem (Mitochondrien)	Androgene, Estrogene, Gallensäuren	Pathogenese von Morbus Alzheimer	(He *et al.*, 1999; He *et al.*, 2001; He *et al.*, 1998; Shafqat *et al.*, 2003)
17βHSD11	Niere, Leber, Lunge (Microsomen)	Lipide, Estrogene, Gallensäuren	Regulation der Lipidbiosynthese, Inaktivierung von Steroidhormonen	(Chai *et al.*, 2003; Keller *et al.*, 2006)
17βHSD14	Prostata	Androgene	Pathogenese des Prostatakarzinoms	(Jansson *et al.*, 2006; Lukacik *et al.*, 2006)

1.2.3 Strukturelle und biologische Merkmale der 17βHSD1

Die 17βHSD1 wurde erstmals von Langer und Engel 1958 als „plazentare Estradiol-17β-dehydrogenase 1“ beschrieben (Langer und Engel, 1958) und nachfolgend durch unterschiedliche Gruppen charakterisiert (Luu-The *et al.*, 1995; Peltoketo *et al.*, 1988; Tseng und Mazella, 1981). Nach der Enzymnomenklatur der International Union of Biochemistry and Molecular Biology wird sie unter der Abkürzung EC1.1.1.62 eingeordnet.

Das entsprechende Gen ist auf Chromosom 17q12.1 lokalisiert und 3,2 kbp groß. Es besteht aus sechs Exons und fünf Introns (Luu-The *et al.*, 1990; Luu The *et al.*, 1989; Peltoketo *et al.*, 1999b). Die 17βHSD1 wird in einer Reihe von Geweben exprimiert. Die stärkste Expression findet sich jedoch in Brustgewebe, Endometrium, den Ovarien und in der Plazenta (Martel *et al.*, 1992) (Tabelle 1).

Die erste Röntgenkristallstruktur der 17βHSD1 wurde 1995 publiziert (Ghosh *et al.*, 1995). In diesem Fall handelte es sich um das native Enzym. Seit dem wurden jedoch auch mehrere

Kristallstrukturen mit verschiedenen Liganden generiert, welche als binäre oder ternäre Komplexe mit Estrogenen (Azzi *et al.*, 1996; Breton *et al.*, 1996; Duggan *et al.*, 2008; Mazza *et al.*, 1998; Zhu *et al.*, 1993) oder Androgenen (Fournier *et al.*, 2008) vorliegen (Abbildung 9). Auch mit Inhibitoren wurde die 17βHSD1 bereits kristallisiert (Qiu *et al.*, 2002; Sam *et al.*, 1995). Die Strukturen sind in der Proteindatenbank veröffentlicht (http://www.pdb.org).

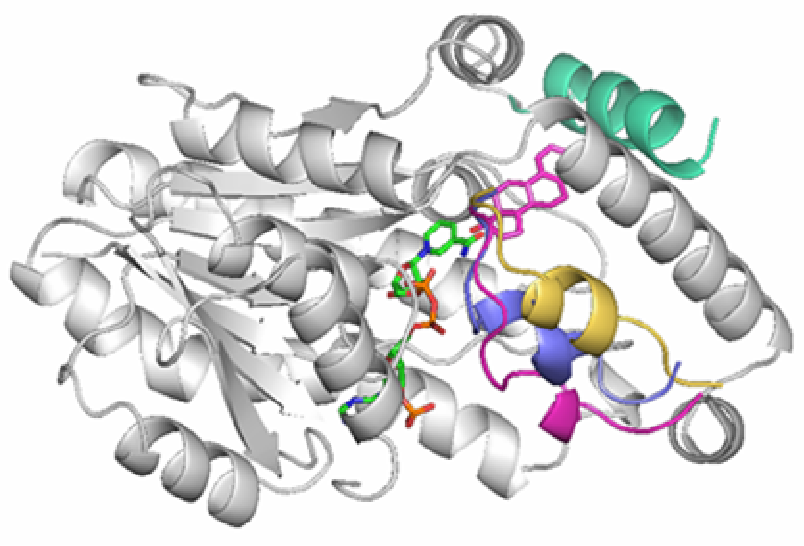


Abbildung 9: Röntgenkristallstruktur der humanen 17βHSD1 (Monomer) als Kokristallisat mit E2 (magenta) und NADP$^+$ (PDB-code 1FDT). Auflösung 2,2 Å.

Wie aus Tabelle 1 hervorgeht ist die 17βHSD1 prinzipiell dazu in der Lage, Androgene und Estrogene zu reduzieren (Gangloff *et al.*, 2003; Lin *et al.*, 2006; Luu-The *et al.*, 1995; Poutanen *et al.*, 1993). Dabei ist jedoch zu beachten, dass die Affinität zu E1 (K_m = 0,03 µM) etwa 1000fach höher ist als die zu DHEA (K_m = 33 µM) (Han *et al.*, 2000). In Mutagenesestudien konnte gezeigt werden, dass Leu149 von Bedeutung ist für diese Androgen-Estrogen-Diskriminierung (Shi und Lin, 2004). Die schwächere Affinität der Androgene lässt sich damit erklären, dass die zusätzliche Methylgruppe der Androgene (C19) durch diese Aminosäure sterisch gehindert wird. Dies bedeutet, dass die physiologische Funktion der 17βHSD1 im weiblichen Organismus die selektive Reduktion von E1 zu E2 ist.

Wie aus den Röntgenkristallstrukturen hervorgeht, lässt sich die 17βHSD1 strukturell in Substratbindungstasche, Cofaktorbindungstasche, Eintrittskanal und einen flexiblen *Loop* als wichtigste Merkmale einteilen. So kann man sich die Substratbindungstasche mit Eintrittskanal als hydrophoben Tunnel vorstellen, welcher in der Nähe des aktiven Zentrums hohe Komplementarität zum Steroid aufweist. Vor dem Eintrittskanal liegt ein *Loop*, welcher in den Kristallstrukturen aufgrund seiner Flexibilität häufig nicht aufgelöst ist. Da dieser Proteinabschnitt jedoch gleichzeitig auch die Grenze von Substrat- und Cofaktorbindungstasche repräsentiert, definiert er Volumen und Form beider Kavitäten. Das humane Enzym ist aus 327 Aminosäuren aufgebaut und liegt als Homodimer vor, dessen Untereinheiten je 34,9 kDa schwer sind (Lin *et al.*, 1992). Ein Monomer besteht aus sieben parallel angeordneten β-

Faltblättern und 11 α-Helices. Die Fläche, welche aus den Faltblättern gebildet wird, ist auf beiden Seiten von drei α-Helices umgeben, welche gleichzeitig den Rossmann fold bilden (Ghosh *et al.*, 1995).

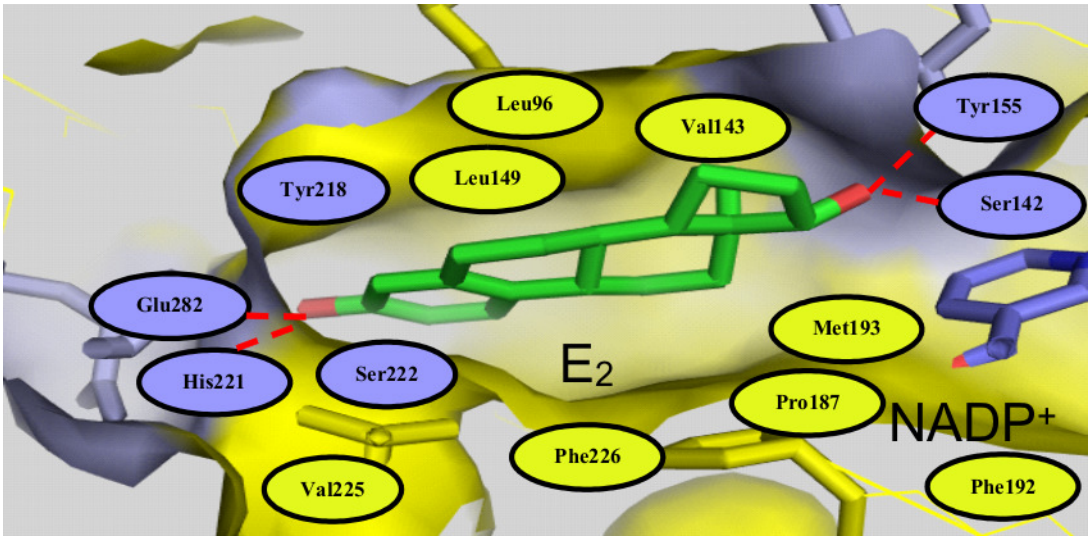


Abbildung 10: Schematische Darstellung des im aktiven Zentrum gebundenen E2 der 17βHSD1 (PDB-code 1A27). Blaue Ellipsen repräsentieren polare Aminosäuren, gelbe Ellipsen repräsentieren lipophile Aminosäuren; Wasserstoffbrückenbindungen sind in rot dargestellt.

Die katalytische Tetrade wird von den Aminosäuren Asn114, Ser142, Tyr155 und Lys159 gebildet (Filling *et al.*, 2002; Puranen *et al.*, 1997; Puranen *et al.*, 1994). Diese stabilisieren das Steroid durch Wasserstoffbrücken-Bindungen in der weitgehend hydrophoben Bindetasche (Breton *et al.*, 1996), welche an polaren Aminosäuren lediglich Tyr218 und Ser222 enthält. Diese treten jedoch offensichtlich nicht in Wechselwirkung mit dem Steroid (Abbildung 10).

1.2.4 Propagierter Katalysemechanismus

Als Katalysemechanismus für die 17βHSD1 wird ein pro-*S*-Hydridtransfer postuliert (Abbildung 11), bei dem das Hydridion vom Nicotinamidrest des Cosubstrates in Position 17 auf die α-Seite des Steroids übertragen wird (Penning, 2003). Die Protonenübertragung von Tyr155 auf die Carbonylfunktion in Position 17 erfolgt nach Ausbildung einer Wasserstoffbrückenbindung. In diesem Fall wird der Transfer durch mehrere Faktoren begünstigt. Einerseits befinden sich in direkter Nähe die protonierte Seitenkette des Lys159, sowie die 2´-OH-Gruppe der Ribose. Andererseits sind mehrere Wassermoleküle vorhanden, welche über Wasserstoffbrücken in Verbindung stehen mit der Carbonylgruppe von Asn114 im Proteinbackbone. Diese Konstellation führt zu einer Erhöhung der Acidität der phenolischen OH-Gruppe des Tyrosins und erleichtert damit die Protonenübertragung (Breton *et al.*, 1996; Ghosh *et al.*, 1995).

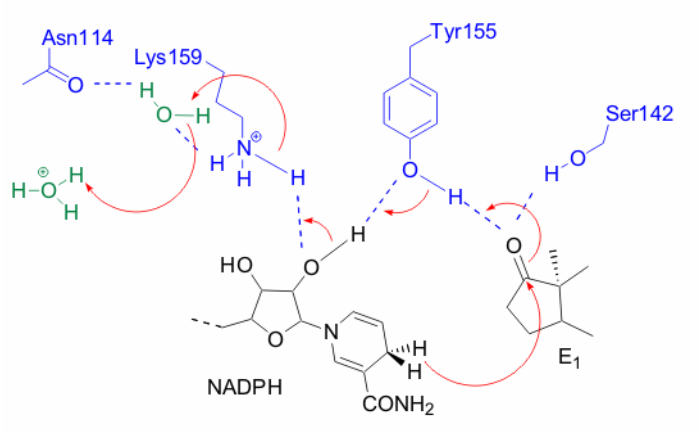


Abbildung 11: Darstellung des propagierten Katalysemechanismus der 17βHSD1. Wasserstoffbrückenbindungen sind als gestrichelte Linien dargestellt.

1.3 Estrogenabhängige Erkrankungen

1.3.1 Brustkrebs

1.3.1.1 Allgemeines

Brustkrebs ist das unkontrollierte Wachstum von Brustgewebe, welches die Fähigkeit besitzt, in andere Gewebe einzuwandern. Laut WHO starben im Jahre 2007 weltweit 548000 Frauen an Brustkrebs[1]. Das entspricht 7 % der durch Krebs verursachten Todesfälle. Damit war Brustkrebs 2007 die häufigste Krebserkrankung bei Frauen.

Die Klassifizierung bestehender Tumoren erfolgt in der Regel nach ER-Status, da die Abhängigkeit des Wachstums von Estrogenen ein Ansatz für eine mögliche Therapie sein kann. ER-exprimierende Gewebe (ER+) werden als estrogenabhängig eingestuft. Das bedeutet, dass Estrogene das weitere Fortschreiten der Erkrankung stimulieren. Tumoren, in denen kein ER nachgewiesen werden kann (ER-), werden als estrogenunabhängig angesehen (Cotterchio *et al.*, 2003). Etwa 50 – 60 % der Brusttumoren bei prämenopausalen Frauen sind ER+. Postmenopausal zeigen sogar 75 % der Tumoren estrogenabhängiges Wachstum (Keen und Davidson, 2003; Lower *et al.*, 1999).

Ein weiterer Aspekt, welcher für die Wahl der optimalen Therapie wichtig sein kann, ist die Expression des „human epidermal growth factor receptor“ (Her2/neu) (Mackey *et al.*, 2009). Dieser wird jedoch nur in 15 – 20 % der Tumoren gefunden (Martin *et al.*, 2005).

1.3.1.2 Pathophysiologie

Im allgemeinen wird die Entwicklung von Brustkrebs über mehrere Stadien hinweg angenommen (Page *et al.*, 1984). Die initiale Veränderung wird bezeichnet als „proliferative

[1] http://www.who.int/mediacentre/factsheets/fs297/en/

disease without atypia“ (PDWA), welche sich weiterentwickeln kann zur atypischen Duktalhyperplasie. Diese wird noch nicht als Krebserkrankung eingestuft. Erst das folgende Stadium, das DCIS (ductal carcinoma in situ) zählt zu den nichtinvasiven Krebserkrankungen. Hierbei handelt es sich um die Proliferation bösartiger Zellen, wobei es noch nicht zu einer Überschreitung der Basalmembran gekommen ist. Im weiteren Verlauf entsteht schließlich der invasive Brustkrebs (IBC) (Boughey *et al.*, 2007; Miki *et al.*, 2008).

Einer der wichtigsten Faktoren im frühen Stadium des Brustkrebses ist die E2-Exposition (Thomas, 1984). So ist das Brustkrebsrisiko assoziiert mit verlängerter Estrogenexposition, beispielsweise durch frühe Menarche und späte Menopause (Cauley *et al.*, 1999; Russo *et al.*, 2006). Auch im Tierversuch konnte gezeigt werden, dass Estrogene Brusttumoren sowohl hervorrufen als auch deren Wachstum stimulieren können (Nandi *et al.*, 1995; Travis und Key, 2003). Verantwortlich für die vermehrte Proliferation sind letztlich peptidische Wachstumsfaktoren, deren Expression durch Estrogene induziert wird (Garvin *et al.*, 2005; Nakamura *et al.*, 1996). Darüberhinaus sind Estrogene auch für die Upregulation verschiedener Onkogene verantwortlich (Redeuilh *et al.*, 2002).

Dieser kausale Zusammenhang zwischen Estrogenexposition und vermehrter Proliferation spiegelt sich ebenfalls in einem Mißverhältnis von E2 und E1 in malignem Gewebe wider (van Landeghem *et al.*, 1985). So konnte bei prämenopausalen Frauen und in ER+ Tumoren eine signifikante Erhöhung dieses Verhältnisses festgestellt werden (Miyoshi *et al.*, 2001).

Als Ursache für eine erhöhte Estrogenexposition des erkrankten Gewebes kommen verschiedene Enzyme in betracht. So konnte gezeigt werden, dass die Sulfatase-mRNA in malignem Gewebe erhöht ist (Utsumi *et al.*, 2000). Selbiges wurde auch für die Aromataseexpression gefunden (Brodie *et al.*, 1997).

Außerdem konnte in vielen Brustkrebsgeweben eine Überexpression der 17βHSD1 detektiert werden (Gunnarsson *et al.*, 2005; Gunnarsson *et al.*, 2001; Vihko *et al.*, 2003). Im Gegensatz dazu wurde keine Erhöhung der 17βHSD2-Expression festgestellt (Luu-The *et al.*, 1995; Vihko *et al.*, 2004; Vihko *et al.*, 2001). In mehreren Fällen wurde sogar eine Verminderung gefunden (Bulun *et al.*, 2000; Sasano *et al.*, 2000; Zeitoun *et al.*, 1998). Das daraus resultierende erhöhte Verhältnis von 17βHSD1 zu 17βHSD2 (Miyoshi *et al.*, 2001) sorgt für einen erhöhten E2-Spiegel im erkrankten Gewebe, welcher für die weitere Proliferation verantwortlich ist (Subramanian *et al.*, 2008; Suzuki *et al.*, 2000).

1.3.1.3 Therapieoptionen

In der Therapie des Brustkrebses steht zunächst die chirurgische Entfernung des Primärtumors im Vordergrund. Nach Möglichkeit werden brusterhaltende Eingriffe vorgenommen, jedoch kann auch eine Mastektomie nötig sein. Der Chirurgie schließt sich in der Regel eine Radiotherapie an, um Rezidive zu vermeiden (Fisher *et al.*, 1993; Fisher *et al.*, 2001). Diese kann auch vor der Entfernung des Primärtumors nötig sein, um diesen auf eine operable Größe

zu verkleinern. Vor allem im fortgeschrittenen Stadium oder bei Metastasierung kommt auch die Chemotherapie zur Behandlung in Frage. Daneben stehen auch Interferone, Interleukine oder Zytokine als Therapie zur Verfügung. Deren Einsatz spielt zur Zeit jedoch eine untergeordnete Rolle. Bei Expression von Her2/neu im Tumorgewebe ist der Einsatz von Trastuzumab, einem monoklonalen Antikörper eine gute Therapieoption (Boughey *et al.*, 2007; Buzdar *et al.*, 2005; Fisher *et al.*, 1999).

Für estrogenabhängigen Brustkrebs ist der endokrine Ansatz als adjuvante Therapie Mittel der Wahl (Adamo *et al.*, 2007; Miller *et al.*, 2007) (Abbildung 12). Darunter versteht man die Interferenz mit dem hormonellen System mit dem Ziel, die Estrogenwirkung zu unterdrücken (Miller *et al.*, 2007). Einerseits ist dies durch die Inhibition der Estrogenbiosynthese möglich, andererseits durch die Verhinderung der Estrogenwirkung.

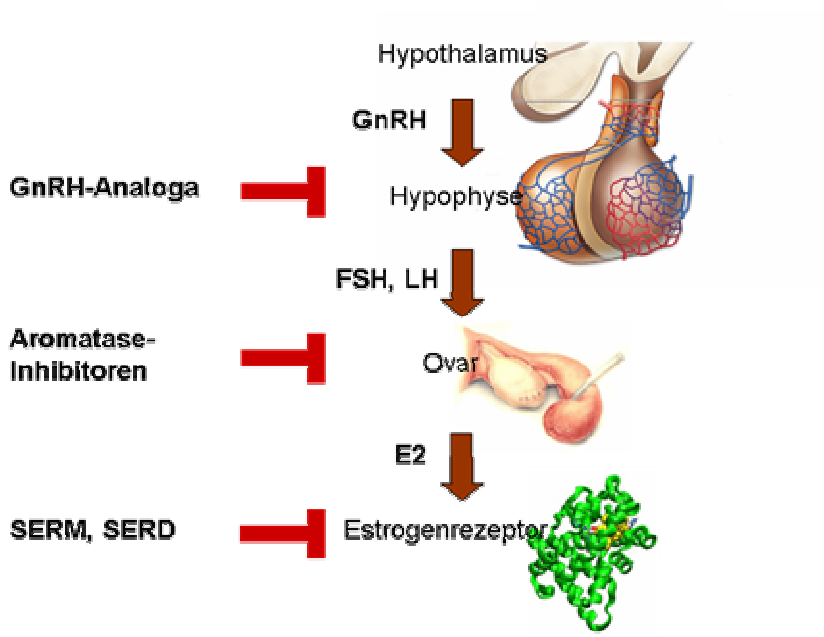


Abbildung 12: Schematische Darstellung der endokrine Therapieoptionen des Mammakarzinoms.

Die Biosynthese der Estrogene kann durch GnRH-Analoga gehemmt werden, welche den zentralen Feedbackmechanismus unterbrechen (Emons *et al.*, 2003). In diesem Fall können sowohl Agonisten wie Buserelin als auch Antagonisten wie Cetrorelix angewendet werden. Durch die völlige Unterdrückung der Estrogenbildung im gesamten Organismus, treten jedoch schwerwiegende Nebenwirkungen auf.

Peripher lässt sich die Estrogenbiosynthese durch Aromataseinhibitoren unterdrücken. Hier stehen sowohl steroidale Inhibitoren wie Exemestan als auch nicht steroidale wie Anastrozol zur Verfügung. Auch hier findet ein vollständiger Estrogenentzug statt, welcher zu Nebenwirkungen wie Osteoporose, Hitzewallungen oder Depressionen führt (Perez, 2007) (Abdulhaq und Geyer, 2008). Darüberhinaus stellt die Resistenzentwicklung ein Problem für die längerfristige Anwendung von Aromatasehemmern dar (Urruticoechea, 2007).

Die Verhinderung der Estrogenwirkung kann durch ER-Antagonisten erreicht werden. Diese Antiestrogene können unterteilt werden in selektive ER-Modulatoren (SERM) wie beispielsweise Tamoxifen und pure Antiestrogene wie Fulvestrant (Adamo *et al.*, 2007). SERM können gewebeabhängig als Agonisten oder Antagonisten am Rezeptor wirken. Daraus ergeben sich entsprechende Nebenwirkungen wie beispielsweise einem verstärkten Wachstum von Uterusgewebe bis hin zum Endometriumkarzinom (DeMichele *et al.*, 2008; Saadat *et al.*, 2007). Für Fulvestrant wurde festgestellt, dass der Abbau des Fulvestrant-ERα-Komplexes beschleunigt abläuft. Damit wird der Gehalt an ERα-Protein im erkrankten Gewebe vermindert, ohne die Expression auf mRNA-Ebene zu verändern. Deshalb wird Fulvestrant auch zu den selektiven ER-Downregulatoren (SERD) gezählt (Osborne *et al.*, 2004). Dieser Mechanismus wird auch für weitere pure Antiestrogene propagiert (Fan *et al.*, 2007). Ebenso wie für die Aromataseinhibitoren ist auch im Falle der ER-Antagonisten eine Resistenzentwicklung häufig zu beobachten (Gradishar, 2004; Hurvitz und Pietras, 2008; Jordan und O'Malley, 2007).

Eine Kombination der einzelnen Therapieoptionen ist möglich und wird auch häufig durchgeführt. Es existieren heute also effiziente Therapieoptionen in der Behandlung von Brustkrebs, welche jedoch auch eine Reihe von Nachteilen aufweisen. Deshalb besteht die Notwendigkeit, neue Ansätze zur Brustkrebstherapie zu finden.

1.3.2 Endometriose

1.3.2.1 Allgemeines

Endometriose ist definiert als histologisch nachgewiesenes ektopes endometriales Drüsen- und Stromagewebe. Meist sind viszerale und peritoneale Oberflächen betroffen. Dabei besteht jedoch keine Korrelation zwischen Ort oder Größe der Läsionen und Stärke der Symptomatik. Es handelt sich um eine nicht maligne, jedoch rezidivierende Erkrankung, die häufig assoziiert ist mit starken Schmerzen und damit einer erheblichen Beeinträchtigung der Lebensqualität. Außerdem kann eine bestehende Endometriose zu Zyklusstörungen sowie zur Infertilität führen. Endometriose wird bei 6 – 10 % der Frauen im gebärfähigen Alter diagnostiziert (Giudice und Kao, 2004). Unter den Frauen mit bestehender Infertiliät sind sogar 25 – 40 % betroffen (Ozkan *et al.*, 2008).

Nach makroskopischer Erscheinung werden drei Krankheitsbilder unterschieden: die peritoneale Endometriose, die zystische Ovarialendometriose und die retroperitoneale, tief infiltrierende Endometriose einschließlich Adenomyosis (Donnez *et al.*, 1995).

1.3.2.2 Pathophysiologie

Für die Pathogenese wurden in der Vergangenheit unterschiedliche Theorien propagiert (Donnez *et al.*, 2002). Nach der Metaplasietheorie soll pluripotentes Zölomepithel aus dem Bereich der Urogenitalfalte beim Erwachsenen ausdifferenzieren und endometriotische

Läsionen hervorrufen (Demir *et al.*, 2004; Nap *et al.*, 2004). Entsprechend der Theorie der Autotraumatisierung des Uterus soll es infolge einer Hyperperistaltik der Gebärmutter zur verstärkten Abschilferung basaler Endometriumfragmente kommen, welche dann durch den erhöhten intrauterinen Druck während der Menses in die Bauchhöhle gelangen können (Leyendecker *et al.*, 2002). Am ehesten akzeptiert ist die von Sampson postulierte Implantationstheorie, nach der normale Endometriumzellen durch retrograde Menstruation in die Bauchhöhle gelangen und sich dort nach der Implantation zu Endometrioseherden weiterentwickeln (Sampson, 1927). Zusätzlich scheinen jedoch auch andere Faktoren wie Enzymausstattung, Zytokine und Wachstumsfaktoren eine Rolle zu spielen, da nicht jeder retrograden Menstruation eine Implantation folgt. Gestützt wird diese Theorie auch durch die Beobachtung, dass bei Affen durch Spülen von Endometriumzellen durch den Uterus in den Bauchraum die Induktion einer Endometriose möglich ist (D'Hooghe und Debrock, 2002; Einspanier *et al.*, 2006).

Auf molekularer Ebene ist offenbar ein positiver Feedback-Mechanismus (Abbildung 13) zwischen E2 und Prostaglandin E_2 (PGE_2) verantwortlich für Implantation und Proliferation des ektopen Gewebes (Bulun *et al.*, 2000).

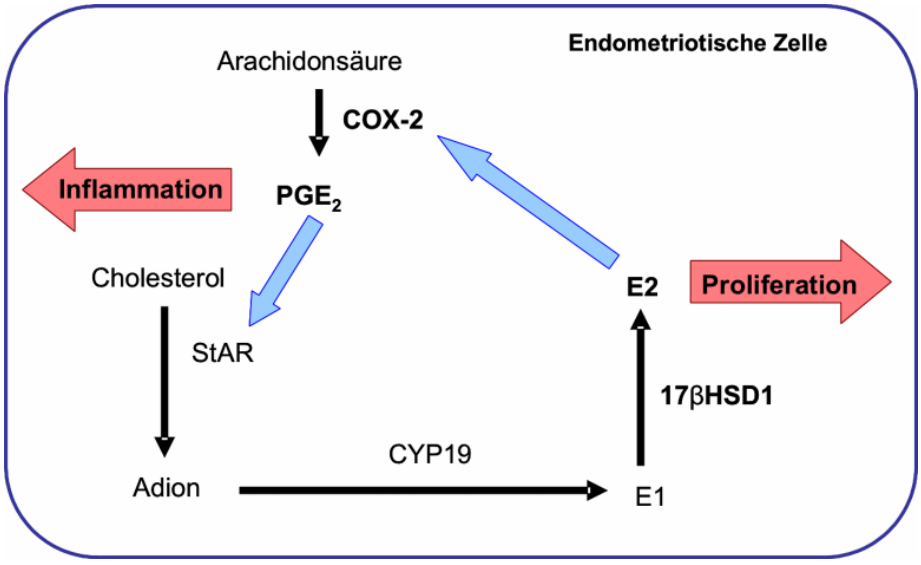


Abbildung 13: Schematische Darstellung der molekularen Ursachen der Endometriose. Blaue Pfeile repräsentieren Enzyminduktion, rote Pfeile repräsentieren Effekte. COX-2, Cyclooxygenase Typ 2; PGE2, Prostaglandin E2; StAR, steroidogenes akutes regulatorisches Enzym.

In endometriotischem Gewebe wurde eine erhöhte Expression der Cyclooxygenase (COX) Typ 2 nachgewiesen, welche für den Abbau der Arachidonsäure zu PGE_2 verantwortlich ist. PGE_2 wiederum stimuliert das steroidogene akute regulatorische Protein (StAR) (Tsai *et al.*, 2001). Infolgedessen kommt es lokal zur verstärkten Synthese von Adion aus Cholesterol, welches durch CYP19 in E1 umgewandelt wird. Für CYP19 konnte im Endometriosegewebe ebenfalls eine sehr hohe Expression festgestellt werden (Šmuc *et al.*, 2007). Das entstandene E1 wird nun durch die 17βHSD1 zum E2 reduziert, welches einerseits für das weitere Wachstum

verantwortlich ist und andererseits die COX2 induziert (Bulun *et al.*, 2005). Darüberhinaus wurden in der Peritonealflüssigkeit betroffener Frauen erhöhte Spiegel von Interleukin 1β (IL-1β) und vaskulärem endothelialen Wachstumsfaktor (VEGF) gefunden (Gazvani und Templeton, 2002; Shifren *et al.*, 1996), welche ihrerseits potente Induktoren der COX2 sind. Die erhöhte E2-Produktion im endometriotischen Gewebe (Delvoux *et al.*, 2008) wird außerdem dadurch begünstigt, dass die 17βHSD1 im erkrankten Gewebe überexprimiert zu sein scheint (Šmuc *et al.*, 2007), während die 17βHSD2-Expression vermindert ist (Zeitoun *et al.*, 1998). Ähnlich wie beim Brustkrebs liegt also auch hier ein gestörtes Verhältnis der Expression von 17βHSD1 und 17βHSD2 vor (Kitawaki *et al.*, 2002).

Zusammengefasst läuft in endometriotischen Läsionen ein Teufelskreis ab, welcher die Endometriose als Erkrankung mit zwei Komponenten charakterisiert, da sowohl ein entzündlicher Prozeß mit Zytokinen und Prostaglandinen als Mediatoren stattfindet als auch ein invasiver Vorgang, der durch die kontinuierliche E2-Produktion stimuliert wird.

1.3.2.3 Therapieoptionen

Entsprechend der beiden oben beschriebenen Charakteristika der Endometriose werden zur Zeit verschiedene Therapien angewandt. Häufig eingesetzt werden COX-Hemmer, welche das entzündliche Geschehen der Endometriose ansprechen (Laschke *et al.*, 2007) und gleichzeitig für eine Schmerzlinderung sorgen.

Um die verstärkte E2-Produktion zu bremsen wird – wie auch beim Brustkrebs – mit GnRH-Analoga, aber auch mit oralen Kontrazeptiva oder Androgenen therapiert. Antiestrogene und Aromataseinhibitoren haben aktuell noch keinen Eingang in die Behandlung der Endometriose gefunden. In ersten Studien konnten jedoch positive Resultate mit Aromataseinhibitoren erzielt werden (Bulun *et al.*, 2001).

Außerdem steht noch die Laparoskopie bzw. Laparotomie als Therapie zur Verfügung, welche in der Regel lediglich für eine vorübergehende Entfernung der endometriotischen Läsionen sorgt, da häufig Rezidive entstehen.

1.4 Neuer Therapieansatz zur Behandlung estrogenabhängiger Erkrankungen

1.4.1 17βHSD1 als Schlüsselenzym der Estradiol-Aktivierung

Wie in den Kapiteln 1.3.1.2 und 1.3.2.2 beschrieben wurde ist die kontinuierliche Produktion von Estrogenen entscheidend für Initiation und Progression von Brustkrebs und Endometriose. Für die Ausübung der estrogenen Effekte ist hauptsächlich E2 verantwortlich, da E1 etwa eine 10fach schwächere Affinität zu den ER aufweist. Die Aktivierung von E1 zu E2 wird durch die 17βHSD1 katalysiert, welche sowohl in Brusttumoren als auch in endometriotischen Läsionen

überexprimiert ist. Dadurch ist die E2-Biosynthese lokal im erkrankten Gewebe verstärkt. Somit steht mehr E2 zur Verfügung, welches die Proliferation direkt stimulieren kann. Die Bedeutung der 17βHSD1 für estrogenabhängige Erkrankungen wurde in einem transgenen Mausmodell untermauert, in dem das humane Enzym in der Nacktmaus überexprimiert wurde. Die erhöhte Enzymaktivität konnte *in vivo* und *ex vivo* nachgewiesen werden (Saloniemi *et al.*, 2007). Die so veränderten Tiere entwickelten Endometriumhyperplasie, und in ca. der Hälfte der weiblichen Versuchstiere entwickelte sich ein Mammakarzinom. Deshalb ist die Hemmung der 17βHSD1 ein interessanter Ansatz um direkt im Zielgewebe die E2-Konzentration vermindern zu können und damit das Fortschreiten der Erkrankung zu unterdrücken. Durch die Überexpression im erkrankten Gewebe würde die Enzyminhibition auch vor allem dort wirksam, wodurch weniger Nebenwirkungen erwartet werden können. Unter diesem Aspekt ist auch der Eingriff in den letzten Schritt der E2-Biosynthese zu sehen, da auf diese Art und Weise nachfolgende Reaktionen nicht beeinflusst werden.

Der Vorteil von 17βHSD1-Inhibitoren verglichen mit etablierten endokrinen Therapien besteht darin, dass nur die Biosynthese des Estrogens E2 gehemmt wird. E1 kann im Organismus weiterhin seine schwache, estrogene Wirkung entfalten und damit dem radikalen Estrogenentzug vorbeugen, welcher die typischen Nebenwirkungen wie Stimmungsschwankungen bis hin zur Depression, Osteoporose oder Hitzewallungen hervorruft. Auch bei Versagen etablierter Therapien, beispielsweise durch Resistenzentwicklung gegenüber Tamoxifen, könnten 17βHSD1-Inhibitoren als zusätzliche Option von Nutzen sein.

Deshalb ist die 17βHSD1 ein neues, vielversprechendes Target für die nebenwirkungsarme Therapie estrogenabhängiger Erkrankungen.

1.4.2 17βHSD1 zur Therapie des estrogenabhängigen Mammakarzinoms

Im Mammakarzinomgewebe würde das oben beschriebene, abnormal hohe Verhältnis der Aktivitäten von 17βHSD1 und 17βHSD2 auf ein physiologisches Niveau herabgesenkt werden. Dies ist vor allem vor dem Hintergrund von Bedeutung, dass mit einer erhöhten 17βHSD1-Expression bei Brustkrebs eine schlechte Prognose verbunden ist (Gunnarsson *et al.*, 2005; Gunnarsson *et al.*, 2001; Subramanian *et al.*, 2008).

Um entwickelte 17βHSD1-Inhibitoren auf ihre Wirkung in der Zellkultur oder in Versuchsorganismen zu testen, kommen mehrere, bereits etablierte Modelle in Betracht. Zunächst existieren zwei Zellwachstumsmodelle, in denen die Proliferation humaner Mammakarzinomzellen, welche die 17βHSD1 exprimieren, mit E1 stimuliert wird. Als Maß für die Wirksamkeit wird in diesen Experimenten die Hemmung der stimulierten Proliferation herangezogen. Hier erwiesen sich bereits steroidale 17βHSD1-Inhibitoren als wirksam. Im Modell nach Laplante et al. (Laplante *et al.*, 2008) wurde jedoch nicht deutlich, ob diese Wirksamkeit auf der Hemmung der 17βHSD1 beruht oder aufgrund potentieller antiestrogener

Wirkungen hervorgerufen wurde. Ähnlich bleibt im Modell nach Day *et al*. (Day *et al.*, 2008) unklar, ob die Wirkung möglicherweise durch einen Partialagonismus am ER entsteht. Außerdem wurden in diesen Untersuchungen relativ hohe Inhibitorkonzentrationen benötigt. Die durchgeführten Versuche zeigen zwar erste positive Ergebnisse für das Konzept der 17βHSD1-Inhibition als Brustkrebstherapie, die Entwicklung weiterer Inhibitoren, sowie die Verfeinerung der beschriebenen Modelle sind jedoch nötig.

In etablierten Tiermodellen können weiterführende Untersuchungen gemacht werden. So wurde das unter Punkt 1.4.1 beschriebene transgene Nacktmausmodell verändert, indem das humane Enzym unter dem mouse mammary tumor virus (MMTV) Promotor exprimiert wurde. In dem resultierenden Modell kann die Konversion von E1 zu E2 *in vivo* bestimmt werden. Durch Gabe eines 17βHSD1-Inhibitors wurde die Umsetzung gehemmt (Lamminen *et al.*, 2008).

Eine weitere Modifizierung des transgenen Nacktmausmodells ist die zusätzliche Exprimierung eines ERE mit Luciferase-Reportergen. Als Vorstufe zum Krankheitsmodell können hier anhand der Lumineszenzintensität Unterschiede in der Aktivität der Estrogene im Gewebe detektiert werden (Saloniemi *et al.*, 2007). In Zusammenschau mit einer Aktivitätsbestimmung der 17βHSD1 im betreffenden Gewebe lassen sich hier interessante Einblicke gewinnen.

Als Krankheitsmodelle sind vor allem zwei Xenograft-Modelle für die Indikation Brustkrebs zu erwähnen, in denen durch humane Mammakarzinomzellen Tumoren in Nacktmäusen induziert werden. Eines dieser Modelle arbeitet mit MCF-7-Zellen (Husen *et al.*, 2006a; Husen *et al.*, 2006b). Dabei handelt es sich um eine Brustkrebszelllinie, welche nur in geringem Ausmaß 17βHSD1 exprimiert (Day *et al.*, 2006; Šmuc und Rižner, 2008). Deshalb wurden die Zellen vor der Inokulation in die Flanke ovariektomierter Mäuse stabil mit 17βHSD1 transfiziert. Zur Etablierung der Tumoren wird zunächst mit E2 stimuliert. Während der Experimente wird E1 zugeführt. Als Parameter für die Wirksamkeit der getesteten Substanzen wird die Tumorgröße herangezogen. In diesem Modell wurden bereits steroidale Inhibitoren untersucht. Ihr Einsatz verhinderte das durch E1 induzierte Tumorwachstum.

Im zweiten Xenograft-Modell werden T47D-Zellen benutzt (Day *et al.*, 2008), welche selbst genügend 17βHSD1 exprimieren (Day *et al.*, 2006). Ebenso wie im MCF-7-Modell werden die Nacktmäuse vor den Versuchen ovariektomiert. Allerdings erfolgt hier keine initiale Stimulation mit E2 sondern mit E1, um die Tumoren zu etablieren. Hier können Tumorgröße, Plasmaestradiolspiegel und 17βHSD1-Aktivität im Tumor untersucht werden. Auch in diesem Modell wurden bereits steroidale Inhibitoren untersucht, welche das E1-induzierte Tumorwachstum hemmten. Außerdem konnte gezeigt werden, dass die 17βHSD1-Aktivität im Tumor ebenso wie der Plasmaspiegel an E2 abgesenkt wurden.

Die ersten positiven Ergebnisse, welche mit steroidalen Inhibitoren der 17βHSD1 erhalten wurden, untermauern die Bedeutung der 17βHSD1 in der Entwicklung, aber auch in der Therapie des Mammakarzinoms.

1.4.3 17βHSD1 zur Endometriosetherapie

In endometriotischem Gewebe würde die Hemmung der 17βHSD1 zu einer Unterbrechung des unter 1.3.2.2 beschriebenen Circulus vitiosus führen.

Auch für die Endometriose existieren mehrere interessante Tiermodelle. Über die Stufe der Nagetiere hinaus kommen hier auch Primatenmodelle in Frage. Da jedoch nur eines der Endometriosemodelle mit humanem Enzym arbeitet, sind hier entsprechende Speziesunterschiede bei der Enzymhemmung zu beachten.

Im Skinfoldchamber-Modell können unterschiedliche Nager eingesetzt werden. Hier werden den Tieren autolog Endometriumfragmente in die Nackenfalte transplantiert (Laschke *et al.*, 2005; Menger *et al.*, 2002). Diese können dann am lebenden Tier untersucht werden. Als Parameter für die Wirksamkeit einer Substanz werden die erreichte Größe des Transplantats sowie dessen vaskularisierte Zone und die Dichte der Mikrogefäße verwendet. Außerdem können VEGF und PCNA (proliferating cell nuclear antigen) als Marker für das Wachstum herangezogen werden. Bisher wurden noch keine 17βHSD1-Inhibitoren in diesem Experiment untersucht.

Das zweite Nagermodell arbeitet mit humanem Enzym. In diesem Falle werden menschliche Endometriosefragmente am Peritoneum von ovariektomierten Nacktmäusen festgenäht (Grümmer *et al.*, 2001; Scotti *et al.*, 2000). Als untersuchte Parameter dienen die Expressionsänderung E2-sensitiver Gene sowie der 17βHSD1 und der Aromatase. Als Proliferationsmarker wird Ki67 herangezogen. Außerdem wird der Blutestradiolspiegel überwacht. Die Anwendung eines 17βHSD1-Inhibitors führte zu einer Reduktion der Expression von 17βHSD1 und CYP19 (Fechner *et al.*, 2007).

Im dritten Nagetier-Versuch werden transgene Mäuse mit ubiquitärer Luciferase-Aktivität generiert. Endometriumfragmente einer Maus werden danach am Bauchfell einer zweiten, nicht transgenen Maus angenäht. Um die Wirksamkeit applizierter Substanzen zu untersuchen, kann die Änderung der Lumineszenzintensität herangezogen werden, welche im lebenden Tier gemessen werden kann. Außerdem werden Mikrogefäßdichte und Gewebeviabilität gemessen (Becker *et al.*, 2006). In diesem Modell wurde bisher kein 17βHSD1-Inhibitor untersucht.

Die weniger artifiziellen, jedoch aufwendigeren Primatenmodelle werden an Zynomolgen (*Macaca fascicularis*) oder Marmosetaffen (*Callithrix jacchus*) durchgeführt.

Im Zynomolgenmodell werden Endometriumfragmente autolog in das Abdomen transplantiert. Die Größe der entstandenen Läsionen, sowie IL-6 (Interleukin 6) und IL-6-SR (Interleukin 6 soluble receptor) als Endometriosemarker werden gemessen. Außerdem wird der Plasmaestradiolspiegel überwacht (Sillem *et al.*, 1996; Yang *et al.*, 2000).

Im Marmosetaffen dagegen verläuft die Induktion der Endometriose auf einem Weg, welcher die Entstehungstheorie nach Sampson (vgl. 1.3.2.2) aufgreift. Hier werden Endometriumzellen mit Pufferlösung durch den Uterus in den Bauchraum gespült, wo sie sich zu

Endometrioseherden entwickeln. Als Parameter für die Wirksamkeit einer Substanz werden die Größe der entstandenen Läsion sowie der Blutfluss innerhalb dieser herangezogen. Weiterhin werden die Protein- und mRNA-Expression der ER sowie von CYP19 und 17βHSD1 untersucht (Einspanier *et al.*, 2006).

Auch in Endometriosemodellen konnten bereits erste positive Resultate mit 17βHSD1-Inhibitoren erzielt werden.

Zusammenfassend unterstreichen die Ergebnisse der Tiermodelle die Bedeutung der 17βHSD1 für estrogenabhängige Erkrankungen. Die Entwicklung von 17βHSD1-Inhibitoren ist somit ein vielversprechender Ansatz zu deren Therapie.

1.4.4 Bekannte 17βHSD1-Inhibitoren

In den letzten zehn Jahren sind eine Reihe von Publikationen über 17βHSD1-Inhibitoren erschienen (Brožic *et al.*, 2008; Poirier, 2003). Die meisten Veröffentlichungen befassen sich mit steroidalen Hemmstoffen, welche sich in der Regel von E1 oder E2 ableiten (Allan *et al.*, 2006a; Allan *et al.*, 2006b; Vicker, 2006). Auch Hybridinhibitoren, in denen ein steroidales Grundgerüst über einen Linker mit einem Adenosinrest verknüpft wurde, sind beschrieben (Fournier *et al.*, 2008; Qiu *et al.*, 2002).

Weit weniger Arbeiten beschäftigen sich mit der Entwicklung nichtsteroidaler Inhibitoren. Als erste Verbindungen mit geringer Aktivität wurden Coumestrol- und Gossypol-Derivate beschrieben (Brown *et al.*, 2003; Mäkelä *et al.*, 1995). Außerdem wurden Biphenylethanon-Derivate mit einer inhibitorischen Aktivität im niedrigen micromolaren Bereich publiziert (Allan *et al.*, 2008). In der Klasse der Thienopyrimidinone wurden Verbindungen mit sehr hoher Aktivität (IC_{50} = 5 nM) beschrieben (Messinger *et al.*, 2006).

Auch im Arbeitskreis von Prof. Hartmann wurden in den letzten Jahren nichtsteroidale Inhibitoren der 17βHSD1 entwickelt. Aus der Forschungsarbeit resultieren zwei Klassen an hochaktiven und gegenüber 17βHSD2 selektiven Hemmstoffen: 1-substituierte Hydroxyphenylnaphthole (Frotscher *et al.*, 2008; Marchais-Oberwinkler *et al.*, 2008a; Marchais-Oberwinkler *et al.*, 2008b) und Bisphenyl-substituierte Arene und Heteroarene (Al-Soud *et al.*, 2009; Bey *et al.*, 2008a; Bey *et al.*, 2008b; Bey *et al.*, 2008c).

Da in der Entwicklung potentieller Arzneistoffe nicht nur eine exzellente inhibitorische Aktivität erreicht werden muss, werden in der Entwicklung im – im Unterschied zu anderen Arbeitsgruppen – zusätzliche Maßstäbe zur Beurteilung der Substanzen angelegt, welche potentielle Nebenwirkungen, pharmakokinetische Aspekte, physikochemische Eigenschaften (Lipinski´s rule of five) (Lipinski, 2000) und Speziesunterschiede adressieren (Kruchten *et al.*, 2008).

Dabei sollten nichtsteroidale Verbindungen für eine nebenwirkungsarme Therapie besser geeignet sein als steroidale, da das Risiko einer Affinität zu weiteren steroidbindenden Proteinen geringer ist.

2 ZIEL DER ARBEIT

E2 ist an der Entstehung und Progression estrogenabhängiger Erkrankungen beteiligt. Durch die Überexpression der 17βHSD1 in Brustkrebs und Endometriose wird im erkrankten Gewebe vermehrt E2 produziert, welches das Fortschreiten der Erkrankung stimuliert. Die Inhibition der 17βHSD1 eröffnet einen neuen Angriffspunkt zur sensitiven Behandlung estrogenabhängiger Erkrankungen. Durch Hemmung der 17βHSD1 sollen selektiv im erkrankten Gewebe die erhöhten E2-Spiegel abgesenkt werden. Da 17βHSD1-Inhibitoren lediglich in den letzten Schritt der E2-Biosynthese eingreifen, bleibt im gesamten Organismus das schwach aktive E1 erhalten. Damit findet kein vollständiger Estrogenentzug statt, woraus bei den etablierten Therapien starke Nebenwirkungen resultieren.

Im Arbeitskreis sollen hochpotente Inhibitoren der 17βHSD1 entwickelt werden, welche sich zur Anwendung als potentielle Therapeutika eignen. Um dies beurteilen zu können, soll zunächst ein leistungsfähiges Screeningsystem aufgebaut werden, mit dessen Hilfe sowohl pharmakodynamische als auch pharmakokinetische Aspekte evaluiert werden können (Kap.3.1). Außerdem sollen sich aus unterschiedlichen Tests Struktur-Wirkungs-Beziehungen, SARs, ableiten lassen, welche für die weitere Optimierung der Verbindungen notwendig sind (Kap.3.2 und 3.3). Relevante Eigenschaften für 17βHSD1-Inhibitoren, welche in einem Screeningsystem evaluiert werden müssen, sollen gefunden werden.

Tabelle 3: Aspekte für die Beurteilung von 17βHSD1-Inhibitoren als potentielle Therapeutika.

Pharmakodynamische Eigenschaften	*Pharmakokinetische Eigenschaften*
Inhibitorische Aktivität an 17βHSD1 (zellfrei und intrazellulär)	Intestinale Absorption
Selektivität gegenüber 17βHSD2, ERα und ERβ	Inhibition der hepatischen CYP-Enzyme (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4)
Proliferationsfördernde und -hemmende Eigenschaften	Lebermetabolismus
Toxizität	Erreichbare Plasmaspiegel
Wirksamkeit in geeigneten Modellen	

Die wichtigsten Aspekte in der Entwicklung der 17βHSD1-Inhibitoren als potentielle Therapeutika sind in Tabelle 3 zusammengefasst. Um diese Eigenschaften beurteilen zu können, sollen geeignete Tests ausgewählt und etabliert werden. Durch sinnvolle Kombination zu einem Screeningsystem soll die verlässliche und schnelle Auswahl geeigneter Verbindungen gewährleistet werden, welche für den Eintritt in die präklinische Phase in Frage kommen.

Zur Vorbereitung auf das davor anstehende *proof of principle* im Tierversuch sollen inhibitorische Aktivität und Selektivität am Enzym einer geeigneten Spezies untersucht werden. Dieser Schritt ist notwendig, da sich das humane Enzym zum Teil erheblich von den Proteinen üblicher Labortiere unterscheidet. Für die Untersuchungen wurde die Ratte als geeignetes Labortier ausgewählt, an dem auch Pharmakokinetikstudien durchgeführt werden. Im ersten Schritt soll die Aktivität des oxidativen und der reduktiven 17βHSD1s im Lebergewebe bestimmt werden (Kap. 3.4). Als nächstes sollen geeignete Präparationen angefertigt werden, um zwei getrennte Inhibitorentests aufzubauen, in welchen die Inhibition der Reduktion von E1 bzw. der Oxidation von E2 untersucht werden kann. In den etablierten Assays sollen nun die inhibitorische Wirkung der vorhandenen Verbindungen evaluiert werden (Kap. 3.5).

Um die Wirksamkeit potentieller Kandidaten für die Weiterentwicklung beurteilen zu können, soll ein weiterer Test aufgebaut werden, welcher aussagekräftig ist im Hinblick auf die Reduktion der Proliferation estrogenabhängig wachsender Zellen. In diesem Zellkulturexperiment soll die humane Brustkrebszelllinie T47D zum Einsatz kommen, welche 17βHSD1, 17βHSD2 und die ERs exprimiert. Das erhöhte Verhältnis der Expressionen von 17βHSD1 zu 17βHSD2 spiegelt die Verhältnisse im estrogenabhängigen Brustkrebs wider. Nach einer Stimulation mit E1 sollen Aussagen möglich sein über die Verminderung der Proliferation durch den Einsatz von 17βHSD1-Inhibitoren. Daneben soll der Versuch Informationen geben über proliferationsfördernde und -hemmende Wirkungen der Verbindungen selbst sowie über estrogene und antiestrogene Effekte (Kap. 3.6).

3 ERGEBNISSE

3.1 Development of a biological screening system for the evaluation of highly active and selective 17βHSD1-inhibitors as potential therapeutic agents

Patricia Kruchten, Ruth Werth, Sandrine Marchais-Oberwinkler, Martin Frotscher, Rolf W. Hartmann

Dieses Manuskript wurde als Artikel in folgendem Journal veröffentlicht:
Molecular and Cellular Endocrinology (**2009**) *301*, 154-157.

Publikation I

Abstract

17β-Hydroxysteroid dehydrogenase type 1 (17βHSD1) catalyses the intracellular conversion of oestrone (E1) to oestradiol (E2). E2 is known to be involved in the development and progression of breast cancer and endometriosis. Since 17βHSD1 is overexpressed in these oestrogen-dependent diseases, inhibition of this enzyme may be a more target-directed therapeutical approach compared to established medical treatments. For the identification of highly active and selective 17βHSD1-inhibitors that are suitable for application as potential therapeutics, there is a need for an appropriate, efficient and reliable screening system. Here, we report the development and application of our screening system using our in house library of potential 17βHSD1-inhibitors. Four potent and selective compounds with a good first pharmacokinetic profile were identified.

Introduction

17βHSD1 catalyses the NADPH dependent reduction of the weak oestrogen E1 to E2 (final step of oestrogen biosynthesis). E2 is the principal and most potent oestrogen in humans. In addition to non-genomic effects, it performs crucial functions in the endocrine system by binding to the oestrogen receptors (ERs). However, it has also been shown to stimulate the proliferation of diseases such as endometriosis (Zeitoun *et al*., 1998) and breast cancer (Thomas, 1984; Vihko *et al*., 2003). 17βHSD1 is overexpressed in endometrial tissue (Šmuc *et al*, 2007) and in many breast tumours. This leads to a high intracellular concentration of E2 which activates cell proliferation (Miyoshi *et al*., 2001). Inhibition of this enzyme is therefore an attractive novel concept for the treatment of oestrogen-dependent diseases. Compared to established endocrine therapies that systemically reduce E2 action, fewer side effects are expected.
To be suitable as potential therapeutics 17βHSD1-inhibitors must meet several requirements. High 17βHSD1 inhibitory activity and low inhibition of its physiological counterpart 17βHSD2 (Wu *et al*., 1993) are essential. Besides, the inhibitors must not activate the ERs α and β to avoid stimulation of tumour growth. Moreover, good intestinal absorption, sufficient metabolic stability and low interaction with hepatic CYP-enzymes are very important. Consequently, it is necessary to have a biological screening system on hand for the evaluation of active compounds. Here, we describe our biological test system that enabled us to identify two series of highly active non-steroidal 17βHSD1-inhibitors with good selectivities towards 17βHSD2

and both ER subtypes (Frotscher *et al*., 2008; Marchais-Oberwinkler *et al*., 2008; Bey *et al*., 2008).

Materials and methods

Cell-free competitive inhibition assay on human 17βHSD1

The assay was performed similar as described by Sam et al. (Sam *et al*., 1998). Briefly, human enzyme was partially purified from placental tissue and incubated with [2, 4, 6, 7-^{3}H]-E1 for 10 min at 37 °C in the presence of NADH. After ether extraction, substrate and product were separated by HPLC and detected with a radioflow detector (Berthold Technologies, Bad Wildbad) (Frotscher *et al*., 2008).

Cell-free competitive inhibition assay on human 17βHSD2

Human enzyme is partially purified from placental tissue and incubated with [2, 4, 6, 7-^{3}H]-E2 (500 nM) for 20 min at 37 °C in the presence of NAD^+. After ether extraction, substrate and product were separated by HPLC and detected with a radioflow detector (Frotscher *et al*., 2008).

Competitive ER binding assay

ER-binding affinity was determined similarly to a previously described method (Zimmermann *et al*, 2005). ERα or ERβ was incubated with [2, 4, 6, 7-^{3}H]-E2 and inhibitor for 1 h at room temperature. Receptor-bound and free E2 were separated by means of hydroxyapatite. The amount of bound radioactivity was measured in a liquid scintillation counter. Non-specific binding was determined with diethylstilbestrol (Bey *et al*, 2008).

Cytotoxicity assay

For evaluation of cytotoxicity, conversion of MTT was determined as described by Denizot and Lang with minor modifications (Denizot and Lang, 1986). T47D cells were incubated with the inhibitor for 3 h at 37 °C in a 5 % CO_2 humidified atmosphere. After an MTT-incubation of 3 h the cleavage of MTT to a blue formazane by mitochondrial succinate-dehydrogenase was quantified spectrophotometrically at 590 nm.

Cellular activity

T47D cells were pre-incubated with the inhibitor for 30 min. After 30 min incubation with [2, 4, 6, 7-^{3}H]-E1 at 37 °C in a 5 % CO_2 humidified atmosphere the supernatant was extracted with ether. Probe analysis was performed according to the cell-free inhibition assays.

Oestrogenicity assay

T47D cells were grown in phenolred-free medium supplemented with stripped fetal calf serum. T47D cells were incubated with the compounds for 10 days at 37 °C in a 5 % CO_2 humidified atmosphere. The medium containing the respective inhibitor was exchanged every 2 to 3 days. E2 was used as positive, ethanol as a negative control. Determination of cell number was performed by MTT-conversion as described for the cytotoxicity assay.

CaCo2-permeability assay

CaCo-2 cell culture and transport experiments are performed as described by Yee (Yee *et al*, 1997) with small modifications. Four reference compounds (atenolol, testosterone, ketoprofene, erythromycin) were used in each assay for validation. Samples were taken from the acceptor side after 0, 60, 120 and 180 min, and from the donor side after 0 and 180 min. The integrity of the monolayers is checked by measuring the transepithelial electrical resistance (TEER) before the transport experiments and by measuring lucifer yellow permeability after each assay. All samples of the CaCo-2 transport experiments are analysed by HPLC-MS/MS.

Metabolic stability assay

The assay is performed with liver microsomes from rats (Sprague Dawley, male pool; BD Gentest, USA). The incubation consists of a microsomal suspension of 0.33 mg/ml protein in phosphate buffer $NADP^+$-regenerating system ($NADP^+$, glucose-6-phosphate, glucose–6-phosphate dehydrogenase, $MgCl_2$). The reaction is initiated by the addition of test compound (final concentration 1 µM) to the pre-incubated microsomes/buffer mix at 37 °C. The samples are removed from the incubations after 0, 15, 30, and 60 min, and processed for acetonitrile precipitation. Analysis is performed by LC-MS/MS.

Inhibition of hepatic CYP-enzymes

The commercially available P450 inhibition kits from BD Gentest (Heidelberg, Germany) are used according to the manufactorer´s instructions. Compounds are tested for inhibition of the following enzymes: CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4. Inhibitory potencies are determined as IC_{50}-values.

In vivo pharmacokinetics

Male Wistar rats weighing 300-330 g (Janvier, France) are housed in a temperature-controlled room (20-22 °C) and maintained in a 12 h light/ 12 h dark cycle. Food and water are available *ad libitum*. Compounds are applied orally in a cassette dosing to four rats by using a feeding needle. The compounds are dissolved in a mixture labrasol/water (1:1) and given at a volume of 5 ml/kg. Blood samples (0.2 ml) are taken at 0, 1, 2, 3, 4, 6, 8, 10 and 24 h postdose and analysed by HPLC-MS/MS.

Results

Table 1: Requirements for inhibitors and corresponding tests.

	Necessary properties for 17βHSD1-inhibitors as potential therapeutic agents	*Relevant assays for the evaluation of the inhibitors*
Pharmacodynamic aspects	High inhibitory activity at the target enzyme	▪ Cell-free 17βHSD1 inhibition assay ▪ Cellular 17βHSD1 inhibition assay
	Selectivity towards 17βHSD2	▪ Cell-free 17βHSD2 inhibition assay
	No (anti-)oestrogenic effects	▪ Competitive ERα and ERβ binding assay ▪ T47D proliferation test
	No acute or chronic toxicity	▪ T47D MTT-cytotoxicity assay (acute) ▪ T47D proliferation test (chronic)
Pharmacokinetic aspects	Good cellular absorption	▪ Cellular inhibition assay ▪ CaCo2-permeability test
	Low interference with liver metabolism	▪ Rat liver microsome stability assay ▪ CYP-inhibition assays
	Pharmacokinetic properties	▪ *In vivo* test in rats

Requirements of 17βHSD1-inhibitors as potential therapeutic agents

For the design of a screening system that can assess relevant aspects of 17βHSD1-inhibitors as potential therapeutics it is necessary to establish the corresponding single tests for the evaluation of different properties and combine them into an efficient screening system. Important properties of the inhibitors as well as appropriate tests are given in Table 1.

Description of the developed screening system

The first and principal feature is a high inhibitory activity towards the target enzyme 17βHSD1. It can be evaluated quickly by a competitive inhibition assay using the partially purified human enzyme. With this test it is possible to deduce reliable structure-activity relationships. Besides, it is also important that inhibitors are active intracellularly. This property can be investigated by a cellular assay. Moreover, this assay provides first insights into cell permeability, intracellular metabolism and protein adsorption. To avoid side effects the physiological counterpart of the target enzyme, 17βHSD2, which catalyses the reverse reaction, should not be blocked (Fig. 1).

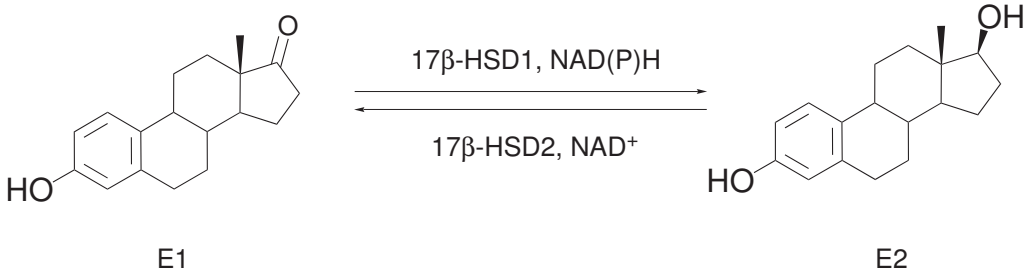


Figure 1: Interconversion of estrone (E1) and 17β-estradiol (E2) by 17βHSDs.

Therefore, a cell free competitive inhibition assay using the human 17βHSD2 must be applied at an early stage to gain additional SAR regarding selectivity. For a comparison of the inhibitors, IC_{50}-values are determined in both cell-free assays and selectivity factors are calculated. A second aspect of selectivity concerns the ERs. Neither an agonistic nor antagonistic effect is acceptable, as side effects could also be evoked via ER-antagonism. For this reason, binding affinity is evaluated for each subtype of human ER (α and β) in a competitive binding assay. Going beyond the step of ligand binding in a second functional test, the proliferation stimulating properties of the inhibitors are evaluated in a cellular system with the oestrogen-dependent breast cancer cell line T47D. For the evaluation of toxic effects, a further cellular assay has been established. Acute cytotoxicity is assessed by determining the number of living cells after a short incubation time using the MTT-test. Chronic cytotoxic effects may also be seen in the oestrogenicity assay, in which the cells are incubated with inhibitor for ten days.
In addition to these pharmacodynamic considerations pharmacokinetic aspects play also an important role in preclinical drug design. A prerequisite for the application of a compound as therapeutic is good absorption in the gastrointestinal tract. A first evidence on cell permeability is given by the cellular inhibition test mentioned before. For the determination of intestinal absorption a CaCo2-permeability assay is performed. In contrast to artificial membrane models such as PAMPA (parallel artificial membrane permeability assay) this colon carcinoma derived cell line allows for the observation of relevant metabolic steps and active transport processes in the gastrointestinal tract. To ensure a high throughput a cassette dosing procedure has been developed. Interference with liver metabolism is of special interest in drug development. On the one hand the compounds must display sufficient metabolic stability in order to reach the target. Therefore, metabolic stability is assessed by the determination of the half life time in rat liver microsomes. On the other hand the compounds should not inhibit the degradation of other endogenous or exogenous substances. To evaluate this effect inhibition of the six most important hepatic CYP-enzymes is determined in a cell-free assay. The final step in the evaluation process is an *in vivo* test in rats that is performed to assess the pharmacokintetic profile of the inhibitors.

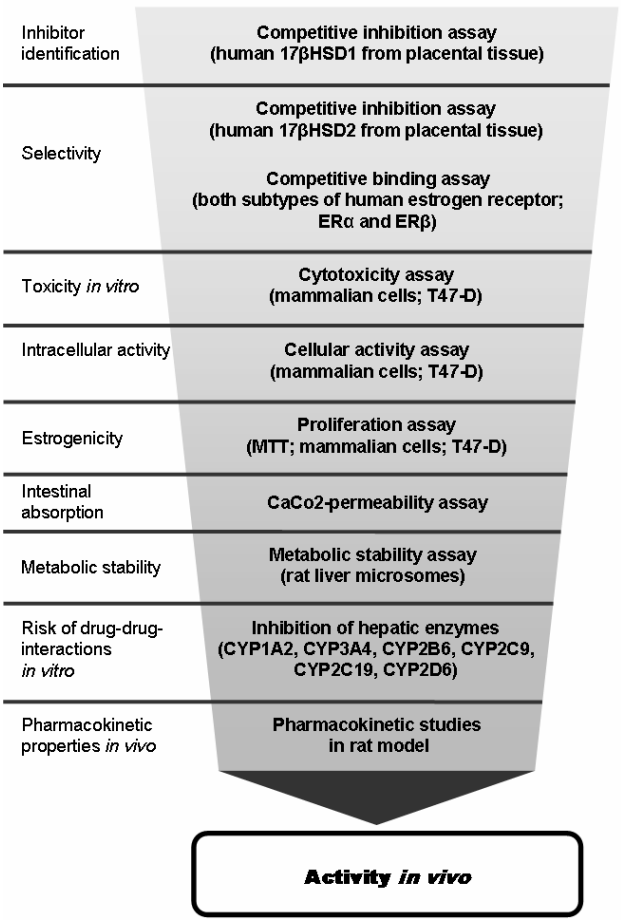


Figure 2: Combination of single assays to a powerful screening system.

For the development of a powerful screening system that provides a maximum of information it is necessary to apply the single evaluation steps in a reasonable and efficient order (Fig. 2). In every single step inhibitors undergo selection. By application of this screening sequence hit structures can be identified, the development to lead structures is supported, input in the optimisation of these lead structures is provided, and candidate substances for further evaluation can be proposed. For the fast determination of SAR three cell-free activity and selectivity assays are performed. Before applying cellular assays, cytotoxic effects are determined to make sure that the measured inhibitions are not artifactual. Subsequently, intracellular inhibition, as well as oestrogenic effects, is determined. Intestinal absorption is only determined for compounds which are intracellularly active and non oestrogenic. After that the interplay of inhibitors and hepatic enzymes is evaluated in two steps followed by pharmacokinetic studies in rats. For the determination of *in vivo* activity several models are described (Day *et al*, 2008; Einspanier *et al*, 2006; Grümmer *et al*, 2001; Husen *et al*, 2006; Laschke *et al*, 2005). Presently, the most appropriate for our screening procedure is being elaborated.

Application of the established screening system

For the evaluation of the effectiveness of the developed screening system 214 non-steroidal compounds of different structural classes were designed, synthesised and tested (Fig. 3). Eighty-two compounds were highly active 17βHSD1 inhibitors with IC_{50}-values below 1 µM, and 23 of these were also selective against 17βHSD2. The latter compounds were evaluated in the ER-affinity tests. Sixteen turned out to have a very low affinity to both ER subtypes and no cytotoxic effects on T47D cells. Five of these showed a good intracellular activity. After performing the oestrogenicity test, four compounds turned out to be candidates for extended pharmacokinetic evaluation.

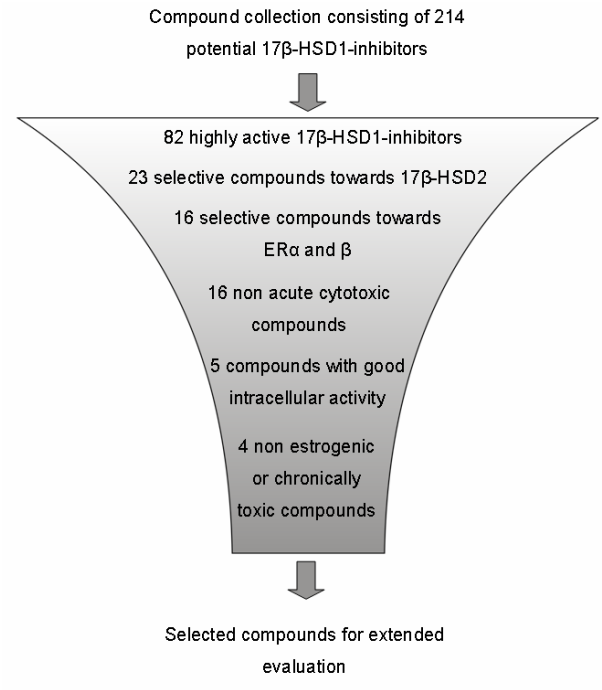


Figure 3: Efficiency of the screening system.

Discussion and Conclusion

As mentioned above, non-steroidal compounds are of major interest. Fewer side effects are expected from these compounds, since the non-steroidal scaffold should show less affinity to other steroid-binding proteins. Nevertheless, the affinity of our compounds for the ERs is of particular interest, because ERα agonism leads to further disease progression and thus impairs the therapeutic benefit. An agonistic effect on ERβ might have a benefit in the treatment of oestrogen-dependent diseases due to the putative antiproliferative effect of this receptor (Matthews and Gustaffson, 2003; Ström *et al*, 2004). However, this may lead to ERβ-derived side effects. Conversely, ERα antagonists decrease proliferation, but they also show typical anti-oestrogenic side effects. Therefore, an ERα antagonism is also not tolerable for this approach. Since a deficiency of ERβ might lead to uterine hyperproliferation, ERβ-antagonism is also undesirable (Weihua *et al*, 2000). For these reasons, we performed two ER-binding assays that enabled us to deduce SAR for the development of compounds with no or low affinity to the ERs.

For a closer investigation of the compounds there is a need for a cellular model that fulfills the following requirements. First, the cells should be of mammalian origin and be related to one of the oestrogen-dependent diseases. Secondly, they must express 17βHSD1 and 17βHSD2. Stronger expression of the former is desirable, as this is the case in the diseased tissue. Moreover, the cells should express ERα and ERβ, which leads to oestrogen dependent proliferation. For this reason, we consider that T47D cells are very appropriate for the *in vitro* studies.

After investigating the important aspects as mentioned above, the compounds must be evaluated in disease models. Using different animal models, 17βHSD1 has been validated as an appropriate target for treating breast cancer and endometriosis (Grümmer *et al*, 2001; Einspanier *et al*, 2006). For the investigation of the *in vivo* activity of our compounds we are presently examining their PK-profile and their inhibitory potency on E2-formation in the corresponding species.

In the present study we have established several single tests and combined them into a powerful screening system. In this test sequence, relevant properties of potential therapeutics were investigated at an early stage of inhibitor development. A number of non-steroidal compounds were evaluated. Using the single assays SAR were deduced and applied for the optimisation of the synthesised compounds. As a result two series of novel 17βHSD1-inhibitors with promising

features were discovered. The most interesting compounds were highly active and selective inhibitors of 17βHSD1 and showed good pharmacokinetic properties. They are candidates for a further preclinical development.

Acknowledgement

We are grateful to the Deutsche Forschungsgemeinschaft for financial support (HA1313/8-1) of this work. Patricia Kruchten is grateful for a scholarship from the European Postgraduate School (DFG) GRK532. We thank Pharmacelsus CRO for their good cooperation in terms of pharmacokinetic studies and Elexopharm GmbH for their collaboration. We thank Dr. Erika Ziegler and Emmanuel Bey for the preparation of the compounds.

References

Bey, E., Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Werth, R., Oster, A., Algül, O., Neugebauer, A., Hartmann, R.W.: Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* 16 (2008) 6423-6435.

Day, J.M., Foster, P.A., Tutill, H.J., Parsons, M.F., Newman, S.P., Chander, S.K., Allan, G.M., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J., Purohit, A.: 17beta-hydroxysteroid dehydrogenase type 1, and not type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* 122 (2008) 1931-1940.

Denizot, F., Lang, R.: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89 (1986) 271-277.

Einspanier, A., Lieder, K., Brüns, A., Husen, B., Thole, H., Simon, C.: Induction of endometriosis in the marmoset monkey (Callithrix jacchus). *Mol. Hum. Reprod.* 12 (2006) 291-299.

Frotscher, M., Ziegler, E., Marchais-Oberwinkler, S., Kruchten, P., Neugebauer, A., Fetzer, L., Scherer, C., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Design, synthesis, and biological evaluation of (hydroxyphenyl)naphthalene and -quinoline derivatives: potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* 51 (2008) 2158-2169.

Grümmer, R., Schwarzer, F., Bainczyk, K., Hess-Stumpp, H., Regidor, P.A., Schindler, A.E., Winterhager, E.: Peritoneal endometriosis: validation of an in-vivo model. *Hum. Reprod.* 16 (2001) 1736-1743.

Husen, B., Huhtinen, K., Saloniemi, T., Messinger, J., Thole, H.H., Poutanen, M.: Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* 147 (2006) 5333-5339.

Laschke, M.W., Elitzsch, A., Vollmar, B., Menger, M.D.: In vivo analysis of angiogenesis in endometriosis-like lesions by intravital fluorescence microscopy. *Fertil. Steril.* 84 Suppl 2 (2005) 1199-1209.

Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Ziegler, E., Neugebauer, A., Bhoga, U., Bey, E., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Substituted 6-phenyl-2-naphthols. Potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1): design, synthesis, biological evaluation and pharmacokinetics. *J. Med. Chem.* 51 (2008) 4685-4698.

Matthews, J., Gustafsson, J.Å.: Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol. Interv.* 3 (2003) 281-292.

Miyoshi, Y., Ando, A., Shiba, E., Taguchi, T., Tamaki, Y., Noguchi, S.: Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* 94 (2001) 685-689.

Salhab, M., Reed, M.J., Al Sarakbi, W., Jiang, W.G., Mokbel, K.: The role of aromatase and 17-beta-hydroxysteroid dehydrogenase type 1 mRNA expression in predicting the clinical outcome of human breast cancer. *Breast Cancer Res. Treat.* 99 (2006) 155-162.

Sam, K.M., Boivin, R.P., Tremblay, M.R., Auger, S., Poirier, D.: C16 and C17 derivatives of estradiol as inhibitors of 17 beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug Des. Discov.* 15 (1998) 157-180.

Šmuc, T., Pucelj, M.R., Šinkovec, J., Husen, B., Thole, H., Lanišnik Rižner, T.: Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* 23 (2007) 105-111.

Ström, A., Hartman, J., Foster, J.S., Kietz, S., Wimalasena, J., Gustafsson, J.Å.: Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc. Natl. Acad. Sci. U S A* 101 (2004) 1566-1571.

Thomas, D.B.: Do hormones cause breast cancer? *Cancer* 53 (1984) 595-604.

Vihko, P., Härkönen, P., Oduwole, O., Törn, S., Kurkela, R., Porvari, K., Pulkka, A., Isomaa, V.: 17 beta-hydroxysteroid dehydrogenases and cancers. *J. Steroid Biochem. Mol. Biol.* 83 (2003) 119-122.

Weihua, Z., Saji, S., Makinen, S., Cheng, G., Jensen, E.V., Warner, M., Gustafsson, J.Å.: Estrogen receptor (ER) beta, a modulator of ER alpha in the uterus. *Proc. Natl. Acad. Sci. U S A* 97 (2000) 5936-5941.

Wu, L., Einstein, M., Geissler, W.M., Chan, H.K., Elliston, K.O., Andersson, S.: Expression cloning and characterization of human 17 beta-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20 alpha-hydroxysteroid dehydrogenase activity. *J. Biol. Chem.* 268 (1993) 12964-12969.

Yee, S.: In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man--fact or myth. *Pharm. Res.* 14 (1997) 763-766.

Zeitoun, K., Takayama, K., Sasano, H., Suzuki, T., Moghrabi, N., Andersson, S., Johns, A., Meng, L., Putman, M., Carr, B., Bulun, S.E.: Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol. *J. Clin. Endocrinol. Metab.* 83 (1998) 4474-4480.

Zimmermann, J., Liebl, R., von Angerer, E.: 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J. Steroid Biochem. Mol. Biol.* 94 (2005) 57-66.

3.2 New insights into the SAR and binding modes of bis(hydroxyphenyl)thiophenes and benzenes: Influence of additional substituents on 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) inhibitory activity and selectivity

Emmanuel Bey, Sandrine Marchais-Oberwinkler, Matthias Negri, Patricia Kruchten, Alexander Oster, Ruth Werth, Martin Frotscher, Barbara Birk and Rolf W. Hartmann

Dieses Manuskript wurde bei folgendem Journal eingereicht:
Journal of Medicinal Chemistry (**2008**)

Publikation II

Abstract

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) is responsible for the catalytic reduction of weakly active E1 to highly potent E2. E2 stimulates the proliferation of hormone-dependent diseases via activation of the estrogen receptor α (ERα). Due to the overexpression of 17β-HSD1 in mammary tumors, this enzyme should be an attractive target for the treatment of estrogen-dependent pathologies. Recently, we have reported on a series of potent 17β-HSD1 inhibitors: bis(hydroxyphenyl) azoles, thiophenes and benzenes. In this paper, different substituents were introduced into the core structure and the biological properties of the corresponding inhibitors were evaluated. Computational methods and analysis of different X-rays of 17β-HSD1 lead to identification of two different binding modes for these inhibitors. The fluorine compound **23** exhibits an IC_{50} values of 8 nM and is the most potent non-steroidal inhibitor described so far. It also shows a high selectivity (17β-HSD2, ERα) and excellent pharmacokinetic properties after peroral application to rats.

Introduction

Estrogens are involved in the regulation of the female reproduction system. However, it is also well known that 17β-estradiol (E2), the natural ligand of the estrogen receptors (ERs) α and β, plays a critical role in the development of several estrogen-dependent pathologies like breast cancer (Travis and Key, 2003) and endometriosis (Dizerega *et al.,* 1980).
Until now, hormone-dependent breast cancers are treated using three different endocrine therapies (Bush *et al.*, 2007; Miller *et al.*, 2007): aromatase inhibitors and GnRH analogues disrupt the estrogen biosynthesis while selective estrogen receptor modulators (SERMs) or pure antiestrogens (Adamo *et al.*, 2007) prevent E2 to unfold its action at the receptor level. Besides specific disadvantages of each therapeutic approach, all of these strategies have in common a rather radical reduction of estrogen levels in the whole body leading to significant side effects.
A softer approach could be the inhibition of an enzyme of the 17β-hydroxysteroid dehydrogenase (17β-HSD) family, especially one which is responsible for the E2 formation from estrone (E1). Until now, three subtypes (1, 7 and 12) are able to catalyze this reaction, the most important being 17β-HSD1. The primary physiological role of 17β-HSD7 and 17β-HSD12 is supposed to be in the cholesterol synthesis (Ohnesorg *et al.*, 2006; Shehu *et al.*, 2008) and in the regulation of the lipid biosynthesis (Sakurai *et al.*, 2006), respectively. In addition, Day *et*

al., 2008 recently, showed that 17β-HSD12, although highly expressed in breast cancer cell lines, is inefficient in E2 formation.
17β-HSD1, which is responsible for the intracellular NAD(P)H-dependent conversion of the weak E1 into the highly potent estrogen E2, is often overexpressed in breast cancer cells (Gunnarsson *et al*., 2001; Gunnarsson *et al.*, 2005; Miyoshi *et al.*, 2001) and endometriosis. Inhibition of this enzyme is therefore regarded as a promising novel target for the treatment of estrogen-dependent diseases.
Recently, two groups (Day *et al.*, 2008; Husen *et al.*, 2006a; Husen *et al.*, 2006b) reported about the *in-vivo* efficacy of 17β-HSD1 inhibitors to reduce E1 induced tumor growth using two different mouse models and indicating that the 17β-HSD1 enzyme is a suitable target for the treatment of breast cancer.
In order to not counteract the therapeutic efficacy of 17β-HSD1 inhibitors, it is very important that the compounds are selective toward 17β-HSD2, the enzyme which catalyzes the deactivation of E2 into E1. Additionally, to avoid intrinsic estrogenic effects, the inhibitors should not show affinity to the estrogen receptors α and β.
During the last decade, several groups reported on 17β-HSD1 inhibitors, most of them having steroidal structures (Brožic *et al.*, 2008; Penning, 1996; Poirier 2003). Recently, non-steroidal cores have been published, too. Until today four classes of compounds are described: thienopyrimidinones (Karkola *et al.*, 2008; Messinger *et al.*, 2006), biphenyl ethanones (Allan *et al.*, 2008) and from our group (hydroxyphenyl)naphthalenes (Frotscher *et al.*, 2008; Marchais-Oberwinkler *et al.*, 2008; Marchais-Oberwinkler *et al.*, 2009) and bis(hydroxyphenyl)azoles, thiophenes, benzenes and aza-benzenes (Al-Soud *et al.*, 2009; Bey *et al.*, 2008a; Bey *et al.*, 2008b; Kruchten *et al.*, 2009a; Kruchten *et al.*, 2009b). The most promising compounds of the latest series are thiophenes **1**, **7** and **33**, thiazole **36** and phenylene **40** exhibiting IC_{50} values toward 17β-HSD1 in the nanomolar range and high selectivity toward 17β-HSD2 and the ERs (Chart 1).

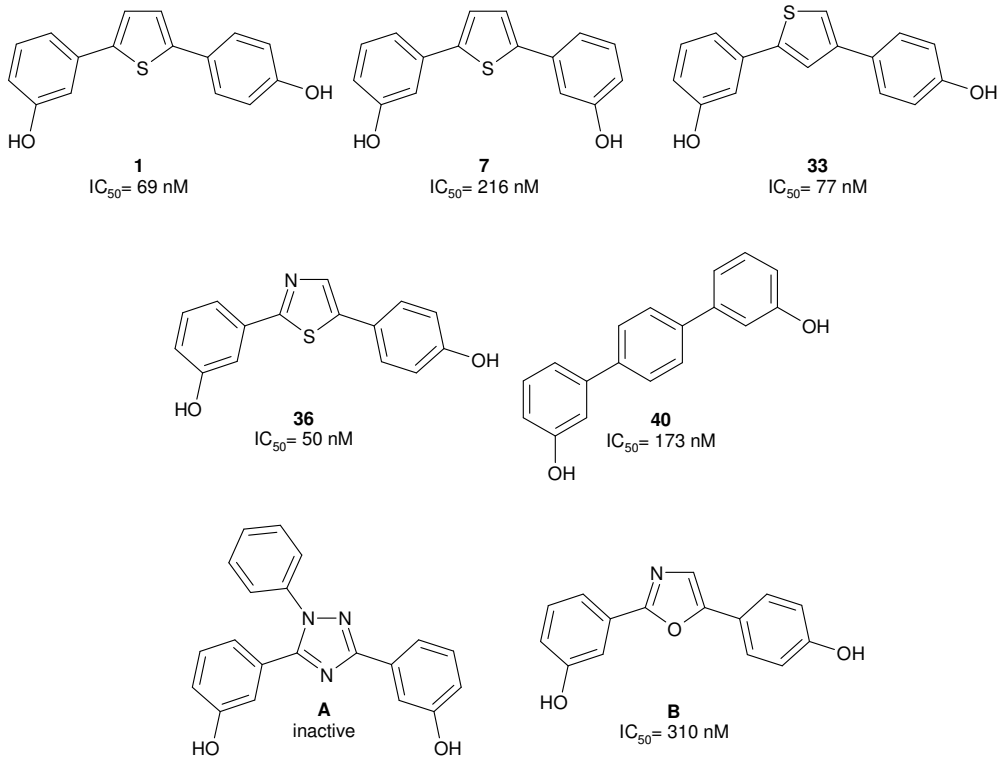


Chart 1: Described bis(hydrophenyl)azoles, thiophenes, benzenes and aza-benzenes.

In the following, we will report on structural optimizations which led to the discovery of new highly potent and selective 17β-HSD1 inhibitors.

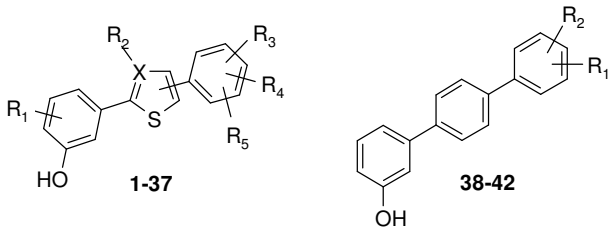


Chart 2: Title compounds.

Design

Up to now, several crystal structures of human 17β-HSD1 were resolved: as apoenzyme (i.e. PDB code: 1BHS (Ghosh *et al.*, 1995)), as binary complex (enzyme-E2, i.e. PDB code: 1IOL (Azzi *et al.*, 1996)) or as ternary complex (enzyme-E2-$NADP^+$: i.e. PDB code 1FDT (Breton *et al.*, 1996); 1A27 (Mazza, 1997); enzyme-HYC (hybride inhibitor): PDB code: 1I5R (Qiu *et al.*, 2002)).

The analysis of the ternary complexes available from 17β-HSD1 provides useful knowledge about the architecture of the enzyme and important hints for structure based drug design: a substrate binding site SUB and a cofactor binding pocket COF can be identified as well as the most important amino acids responsible for substrate and cofactor anchoring. The SUB is a narrow hydrophobic tunnel containing two polar regions at each end: His221/Glu282 on the one side and Ser142/Tyr155 on the other side, corresponding to the binding oxygens in 3- and 17-hydroxy group of E2. Additionally a flexible loop can be identified which is not well resolved in almost all the structures.

From previous results obtained in the class of bis(hydroxyphenyl)azoles, thiophenes, benzenes and aza-benzenes (Bey *et al.*, 2008a; Bey *et al.*, 2008b), a SAR study highlighted four structural features which are important for high 17β-HSD1 inhibitory activity: 1. one hydroxyphenyl moiety on the core structure is not sufficient for a high potency, 2. only the *meta-para* and *meta-meta* dihydroxy substitution pattern (O-O distance in the same range as observed for the steroid, d = 11 Å) are active, 3. the presence of the *meta*-hydroxy group is more important for inhibitory activity than the *para*-, 4. only central aromatic rings without hydrogen bond donor function like thiophene, thiazole, benzene exhibit inhibitory activity. It was also shown that a correlation seems to exist between the activity of the compounds and the electrostatic potential distribution of the molecules (Bey *et al.*, 2008a): to be active the ESP values of the different regions of the inhibitor has to be in an appropriate range.

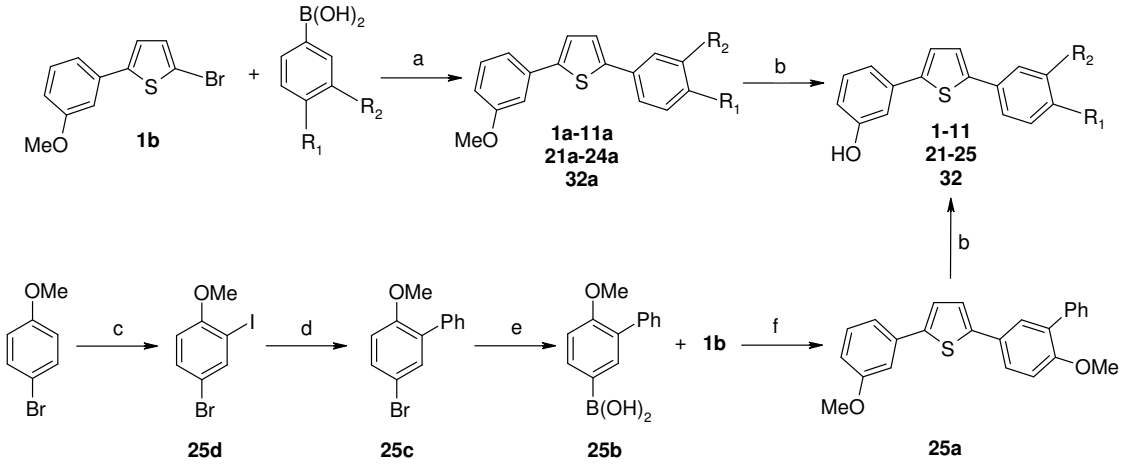
In the present report, we will present the structure optimization of this class of compounds leading to an increase in activity and in selectivity of these inhibitors. First, the influence of the bioisosteric exchange of one OH group on the enzyme activity will be determined. Secondly, the space availability around the inhibitors and the nature of the most appropriate substituent will be investigated by substitutions, either on the heterocycle, or on the hydroxyphenyl moieties. The nature of the substituent will be varied in order to investigate the possible interactions between the inhibitor and the enzyme. Thirdly, computational studies (docking studies and ESP calculations) will be performed in order to identify the most plausible binding mode for this class of compounds. Furthermore, the selectivity toward 17β-HSD2 and the ERs α and β will be determined as well as the potency of the compounds in T47D cells and inhibition of the two most important hepatic CYP enzymes. Finally, the pharmacokinetic profile of the two most promising candidates will be evaluated in rats after oral administration.

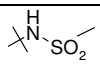
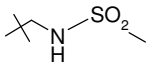
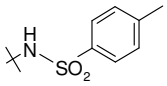
Chemistry

The synthesis of compounds **1** to **11**, **21** to **25** and **32** is presented in Scheme 1. Starting from the mono-brominated key intermediate **1b** and the appropriate commercially available boronic acids, the preparation of compounds **1a** to **11a**, **21a** to **25a** and **32a** was accomplished via Suzuki cross coupling reaction (Miyaura and Suzuki, 1995) under microwave assisted conditions (Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15

bars), 15 min). The resulting disubstituted thiophenes were subsequently submitted to ether cleavage with borontribromide (Bey *et al.*, 2008b) (Method C: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h) leading to compounds **1** to **11**, **21** to **25** and **32** (Scheme 1). In case of intermediate **25a**, the boronic acid **25b** was prepared in a three step synthesis pathway: first, an iodine substituent was selectively introduced in position 2 of the *para*-bromoanisole (compound **25d**) using (diacetoxyiodo) benzene (Togo *et al.*, 1998). Then, a selective Suzuki reaction on the iodo-position of **25d** under Method B (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h) led to the intermediate **25c** and the corresponding boronic acid **25b** was prepared using *n*-butyl lithium and triethyl borate followed by hydrolysis with diluted hydrochloric acid.

Scheme 1: Synthesis of compounds 1 to 11, 21 to 25 and 32.



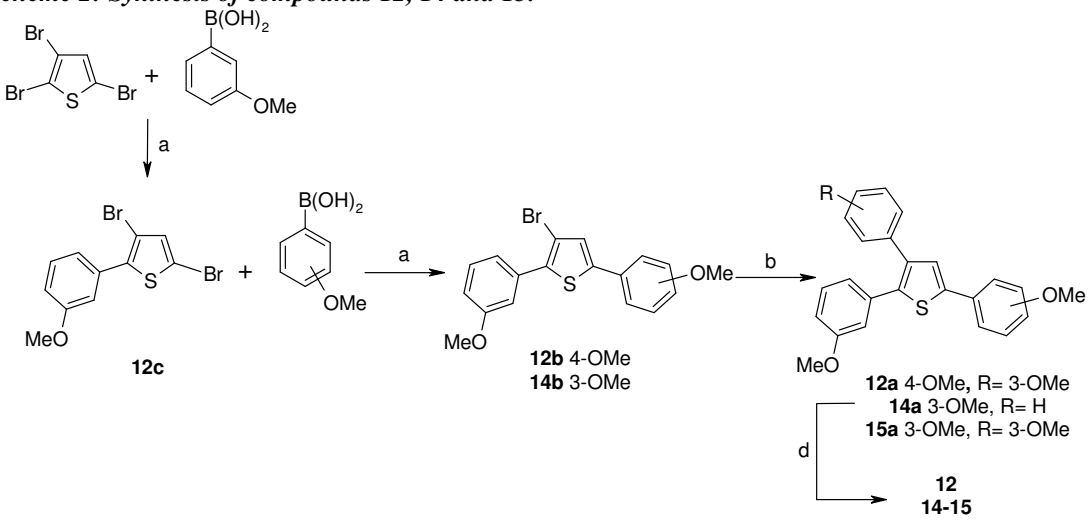
cmpd	R_1	R_2	cmpd	R_1	R_2
1	OH	H	**9**	H	
2	H	H	**10**	H	
3	F	H	**11**	H	
4	NH_2	H	**21**	OH	CH_3
5	SH	H	**22**	OH	OH
6	CN	H	**23**	OH	F
7	H	OH	**24**	OH	CF_3
8	H	F	**25**	OH	Ph
			32	F	F

Reagents and conditions: (a) Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min; (b) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h; (c) $PhI(OAc)_2$, I_2, AcOEt, 60 °C, 5 h; (d) Ph-$B(OH)_2$, Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h; (e) 1. *n*-BuLi, dry THF, 5 min, -78 °C, 2. B_3, 2 h -78°C to rt, 3. HCl 1N, rt; (f) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h.

The preparation of compounds **31** and **33** to **42** is similar to the synthetic pathway presented in Scheme 1 for compounds **1** to **11**. The first Suzuki coupling was carried out according to Method B with the corresponding dibrominated heterocycle and the methoxylated benzene boronic acid. The resulting mono substituted compounds **31b** and **33b** to **42b** were submitted to

a second cross coupling reaction under microwave assisted conditions following Method A. The compounds were subsequently demethylated with boron tribromide to yield compounds **31** and **33** to **42**.

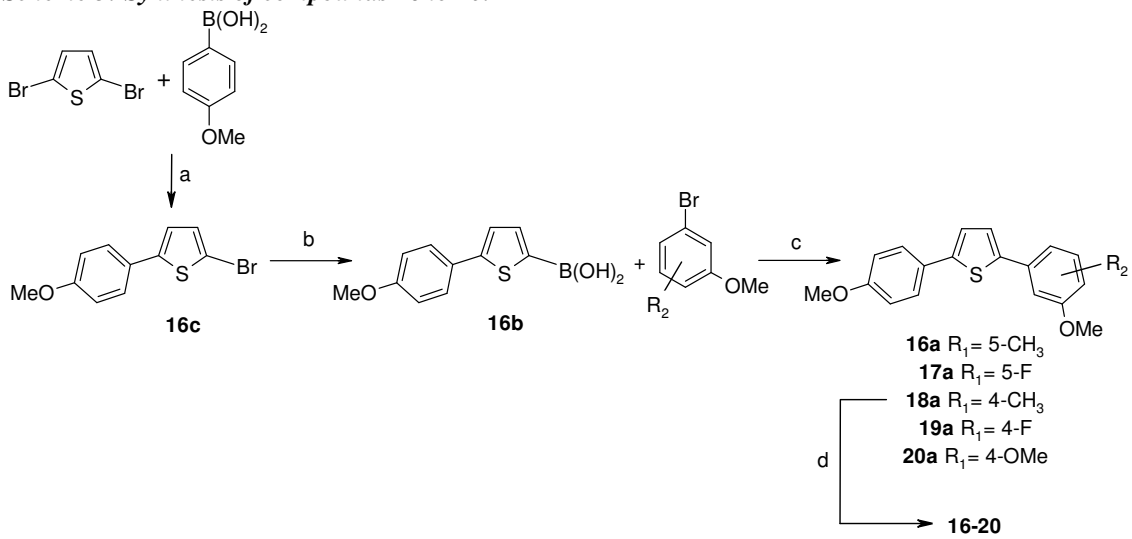
Scheme 2: Synthesis of compounds 12, 14 and 15.



Reagents and conditions: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h; (h) boronic acid, Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h; (d) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

The synthesis of compounds **12**, **14** and **15** is depicted in Scheme 2. The key intermediate mono methoxylated dibromothiophene **12b** was prepared following two successive Suzuki coupling reactions according to Method B (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h) from 2,3,5-tribromothiophene and methoxybenzene boronic acid. The reaction time of both cross couplings was carefully controlled (restricted to 4 h) in order to get a selective bromine replacement each time. Intermediates **12a**, **14a**, and **15a** were obtained via a third Suzuki coupling using Method B. The methoxy substituents were cleaved in a last step, using boron tribromide (Method C: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h).

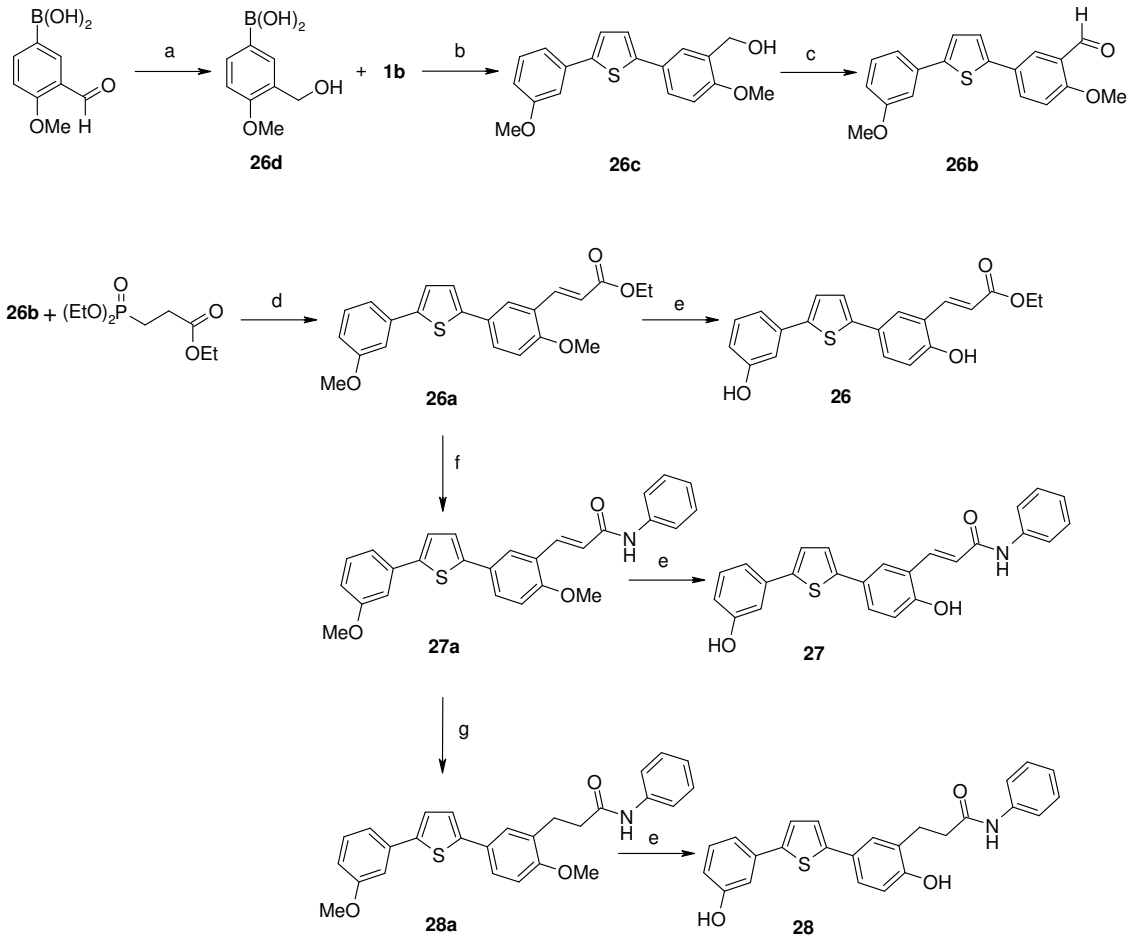
Scheme 3: Synthesis of compounds 16 to 20.



Reagents and conditions: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h; (b) 1.*n*-BuLi, anhydrous THF, -78 °C, 15 min, 2. B_3, THF, -78 °C to rt, 2 h, 3. HCl 1N; (c) Method A for **17a-20a** (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min); Method B for **16a** (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h); (d) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

Compound **13** was synthesized under microwave assisted conditions in a one pot reaction using 2,5-dibromo-3-methylthiophene and 3-hydroxyphenyl boronic acid following Method A (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars)) for 15 min.
The synthesis of the molecules bearing an additional substituent on the *meta*-hydroxyphenyl moiety of thiophene **1** (compounds **16** to **20**) is shown in Scheme 3. Intermediate **16c** was prepared via Suzuki reaction from the *para*-methoxylated benzene boronic acid and the 2,5-dibromothiophene following Method B heating the reaction 4 h instead of 20 h in order to avoid any dicoupling reaction. Treatment of **16c** with *n*-butyl lithium and triethyl borate afforded after hydrolysis with diluted hydrochloric acid the corresponding boronic acid **16b**. The resulting compound was subjected to an additional cross coupling reaction which was carried out with the appropriate bromine derivative following Method A for compounds **17a** to **20a** and Method B for compound **16a**. The hydrolysis of the methoxy groups with boron tribromide (Method C) led to compounds **16** to **20**.

Scheme 4: Synthesis of compounds 26 to 28.



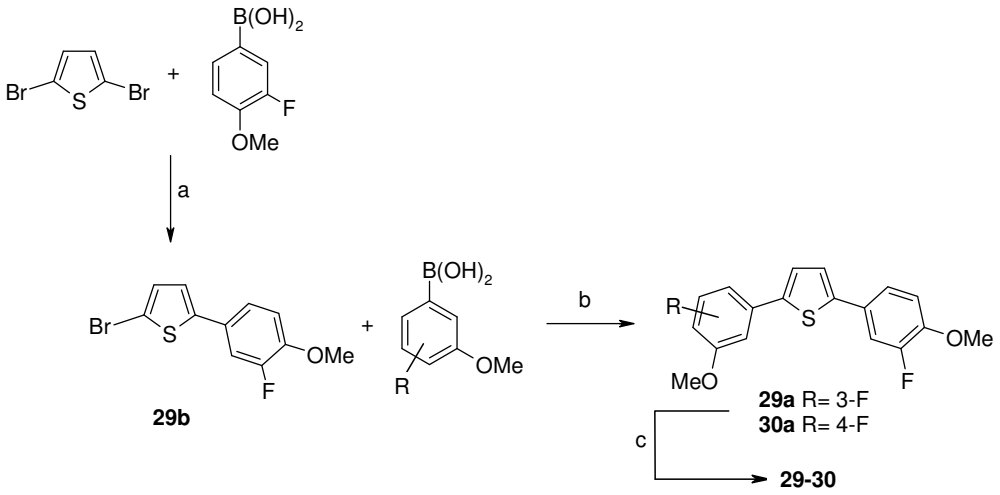
Reagents and conditions: (a) $NaBH_4$, THF/EtOH (1:1), 0 °C to rt, 2 h; (b) Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min; (c) PCC, CH_2Cl_2, rt, 30 min; (d) NaH, THF dry, rt, 4 h; (e) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h; (f) 1. LiOH, THF/H_2O (2:1), reflux, 20 h, 2. aniline, EDCI, HOBt, CH_2Cl_2, reflux, 20 h; (g) $Pd(OH)_2$, THF/EtOH (1:1), H_2, rt, 20 h.

The synthesis of compounds **26** to **28** substituted in *ortho*-position of the *para*-OH group is depicted in Scheme 4. The preparation of the key intermediate **26b** started from the commercially available 3-formyl-4-methoxyphenyl boronic acid. Reduction of the aldehyde function using sodium borohydride followed by a cross coupling reaction with **1b** under microwave irradiation according to Method A (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min) afforded the disubstituted thiophene **26c**. The alcohol function of **26c** was subsequently oxidized with pyridinium chlorochromate to yield to the key

aldehyde **26b**. It was subjected to the Horner-Wadworths-Emmons conditions (Emmons, 1961) to introduce the acrylic ester moiety (intermediate **26a**). Hydrolysis of the ester function using lithium hydroxide (Marchais-Oberwinkler *et al.*, 2008), amide bond formation with aniline, EDCI and HOBt (Pascal *et al.*, 1998) afforded compound **27a** The catalytic double bond hydrogenation of **27a** was performed using Perlman's catalyst (Hwang *et al.*, 1992). The ether functions of **26a**, **27a** and **28a** were deprotected using boron tribromide (Method C) to give the desired compounds **26** to **28**.

The synthesis of the difluorinated thiophenes **29** and **30** is presented in Scheme 5. These compounds were obtained after two successive cross coupling reactions: in a first step 2,5-dibromothiophene reacted with 3-fluoro-4-methoxyphenyl boronic acid following Method B (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h). In a second step, the resulting mono substituted thiophene **29b** was subsequently submitted to a second cross coupling reaction under microwave irradiation (Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min) to yield the intermediates **29a** and **30a**. Ether cleavage with boron tribromide led to the final compounds **29** and **30**.

Scheme 5: Synthesis of compounds 29 to 30.



Reagents and conditions: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h ; (b) Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min; (c) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

Biological Results

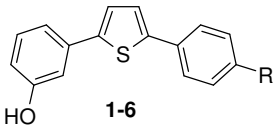
Activity: inhibition of human 17β-HSD1

Placental enzyme was partially purified following a described procedure (Bey *et al.*, 2008a; Bey *et al.*, 2008b). Tritiated E1 was incubated with 17β-HSD1, cofactor and inhibitor. After HPLC separation of substrate and product, the amount of labelled E2 formed was quantified. The inhibition values of the test compounds are shown in Tables 1 to 5. Thiophenes **1, 2**, **7** and **29**, thiazole **33** and phenylenes **35** and **37**, identified in our previous article (Bey *et al.*, 2008b), were used as reference compounds.

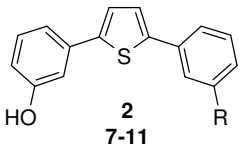
It was first investigated whether one of the two hydrophenyl moieties could be exchanged by another functional group having similar properties. Previous results (Bey *et al.*, 2008b) showed that the *meta*-hydroxy group is highly important for activity and was therefore maintained in the core structure. The exchange of the *para*-hydroxy group on the *meta-para* disubstituted thiophene (**1**, IC_{50}= 69 nM) by a bioisosteric function (F, NH_2, SH) resulted in moderate (**3**, IC_{50}= 717 nM) or weak inhibitors (**4** and **5**, IC_{50}> 5000 nM) of 17β-HSD1 (Table 1). Moving the F atom from the *para*- (compound **3**) to the *meta*-position (compound **8**) led to a small increase in activity (**8**, IC_{50}= 535 nM vs. **3**, IC_{50}= 717 nM). Replacement of the *meta*-fluorine

for a methylsulfonamide moiety (**9**) did not improve the activity (**9**, IC_{50}= 523 nM vs. **8**, IC_{50}= 535 nM), while a compound bearing a bulky substituent like tolylsulfonamide (**11**, IC_{50}= 350 nM) showed comparable activity to the mono hydroxylated thiophene (**2**, IC_{50}= 342 nM) indicating that there is some space in this region of the enzyme for substitution but it is unlikely that specific interactions between the tolylsulfonamide moiety and amino acids of the active site take place. The insertion of a C1-linker between the phenyl moiety and the methylsulfonamide group was detrimental for the activity (**9**, IC_{50}= 523 nM vs. **10**, IC_{50}> 1000 nM). It can be therefore concluded that the two hydroxy functions are necessary for high activity and the *para*-hydroxy group can not be replaced by a bioisoteric group.

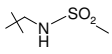
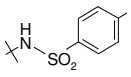
Table 1: Effect of the exchange of one OH substituent for other functional groups on human 17β-HSD1 and 17β-HSD2 inhibitory activities.



1-6



2
7-11

cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]	cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	*selectivity factor[d]*
1	OH	69	1950	28	**7**	OH	173	745	4
2	H	342	2337	7	**2**	H	342	2337	7
3	F	717	3655	5	**8**	F	535	1824	3
4	NH_2	>5000	nt		**9**		523	1575	3
5	SH	>5000	nt		**10**		>1000	nt	
6	CN	>1000	nt		**11**		350	276	1

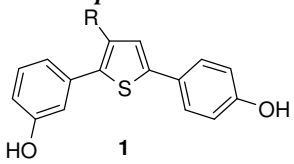
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

In order to improve the activity and the selectivity of our inhibitors, substituents capable to establish further interactions with the enzyme were added either on the heterocycle or on the hydroxyphenyl moieties. Additional functional groups were introduced in both of the *meta-para* 2,5-bis(hydroxyphenyl)thiophene **1** (IC_{50} = 69 nM) and the *meta-meta* 2,5-disubstituted derivative **7** (IC_{50} = 216 nM).

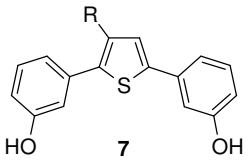
Concerning substitution on the heterocycle, two kinds of hydrophobic substituents (Me, Ph) were introduced in position 3 on the thiophene ring to investigate the space availability around the core (Table 2). The *meta-meta* thiophenes bearing a methyl (compound **13**) or phenyl (compound **14**) as well as the *meta-para* thiophene bearing a hydroxyphenyl substituent (compound **12**) showed a drop of activity compared to the reference compound **7** (IC_{50}= 216 nM vs. IC_{50}> 1000 nM, 567 nM and 493 nM for **12**, **13** and **14**, respectively). It is striking that only in case of the *meta-meta* disubstituted series the insertion of a polar *meta*-hydroxyphenyl substituents leads to an increase in activity (**15**, IC_{50}= 119 nM vs. **12**, IC_{50}> 1000 nM). This

exemplifies that there is space available for further substitution around the heterocycle only in case of the *meta-meta* bis(hydroxyphenyl) substitution pattern and that the third *meta*-OH group is certainly at an appropriate distance to establish supplementary hydrogen bond interactions with the active site.

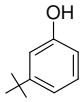
Table 2: Inhibition of human 17β-HSD1 and 17β-HSD2 by compounds bearing a supplementary substituent on the thiophene core structure.



1
12



7
13-15

cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]	cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	*selectivity factor[d]*
1	H	69	1950	28	**7**	H	173	745	4
					13	CH_3	567	856	1
					14		493[e]	n.t.	
12		>1000	n.t.		**15**		119	188	2

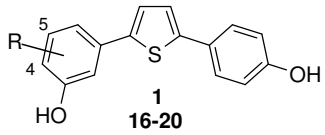
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); [e]calculated value, obtained with the LOGIT transformation; ni: no inhibition, nt: not tested

Concerning the substitution of the hydroxyphenyl rings, different groups were introduced either on the *meta*-hydroxyphenyl ring (compounds **16** to **20**) or on the *para*-hydroxyphenyl moiety (compounds **21** to **28**, Table 3). The *ortho*-position was not considered, as these compounds would not be planar any more.

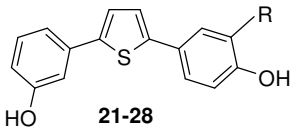
Introduction of a substituent in position 5 on the *meta*-hydroxyphenyl moiety resulted in case of a methyl group in a drop of activity (**16**, IC_{50}= 629 nM vs. **1**, IC_{50}= 69 nM). The introduction of a fluorine atom led to slight increase in activity in comparison to the unsubstituted compound **1** (**17**, IC_{50}= 42 nM vs. **1**, IC_{50}= 69 nM). Moving these functional groups to position 4 gave a highly active fluorinated compound **19** (IC_{50}= 113 nM) and a very weak methylated inhibitor **18** (IC_{50}> 5000 nM). Substituents have also been introduced in position 5 on the *para*-hydroxyphenyl ring: a polar group like a hydroxy (compound **22**) or a bulky substituent like a phenyl (compound **25**) in *ortho*- of the *para*-OH induced a decrease in activity compared to thiophene **1** (IC_{50}= 69 nM vs. IC_{50}= 402 nM and >5000 nM for **22** and **25**, respectively). The introduction of a fluorine substituent into the same position led to the highly potent compound **23** (IC_{50}= 8 nM) while substituents like methyl or trifluoromethyl showed equal or slightly better activities compared to the reference compound **1** (IC_{50}= 69 nM vs. IC_{50}= 46 nM and 38 nM for **21** and **24**, respectively). Other functional groups showing a higher flexibility like ethylacrylate (compound **26**), phenylacrylamide (compound **27**) or phenylpropaneamide (compound **28**) were also synthesized and the resulting compounds **26**, **27** and **28** turned out to have weaker inhibitory activity compared to the unsubstituted thiophene **1** (IC_{50}= 69 nM vs.

130, 427 and 620 nM for **26**, **27** and **28,** respectively). The low activity of the unconjugated compound **28** indicates that an overall distributed electronic density is an important parameter for activity. These results indicate that there is space available in this area for substituents but the nature of the substituents is probably not yet optimal (Table 3).

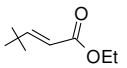
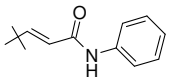
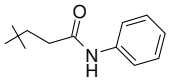
Table 3: Effect of a supplementary substituent on the hydroxyphenyl moieties on the inhibition of the human 17β-HSD1 and 17β-HSD2.



1
16-20



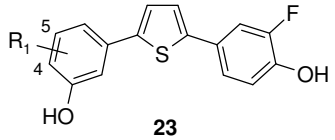
21-28

cmpd	R	IC_{50} (nM)[a]		*selectivity factor*[d]
		17β-HSD1[b]	**17β-HSD2**[c]	
1	H	69	1950	28
16	5-CH_3	629	2584	4
17	5-F	42	463	11
18	4-CH_3	>5000	nt	
19	4-F	113	183	2
20	4-OH	>5000	nt	
21	CH_3	46	1971	49
22	OH	402	1636	4
23	F	8	940	118
24	CF_3	38	97	3
25	Ph	>5000	nt	
26		130	502	4
27		427	468	1
28		620	982	2

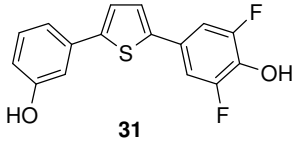
[a]Mean values of three determinations, standard deviation less than 15 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

The influence of the introduction of a second fluorine on the highly active thiophene **23** (IC_{50}= 8 nM), either one F on each hydroxyphenyl ring or two F on the same hydroxyphenyl moiety, was also examined (Table 4). When the two F were located on each hydroxyphenyl moieties, the 4-substituted fluoro derivative (compound **30**) is slightly more potent that the one with the fluorine in 5-position (**29**, IC_{50}= 29 nM, vs. **30**, IC_{50}= 17 nM). A slight decrease in activity was observed when the two fluorine substituents were present at the same hydroxyphenyl ring (compound **31**, IC_{50}= 56 nM). The exchange of the *para*-OH function of **23** by a fluorine atom (compound **32**) confirmed the essential role of this *para*-hydroxy moiety as previously observed.

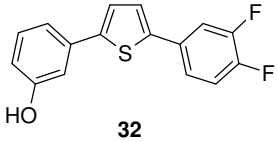
Table 4: Effect of two additional fluorine atoms on the 17β-HSD1 and 17β-HSD2 inhibitory activity.



23
29-30



31



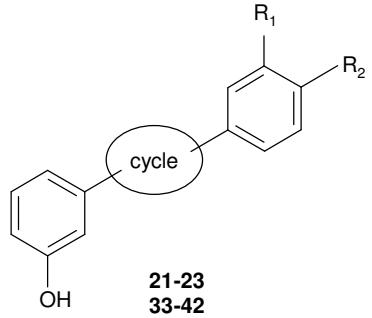
32

cmpd	R_1	IC_{50} (nM)[a]		*selectivity factor*[d]
		17β-HSD1[b]	**17β-HSD2**[c]	
23	H	8	940	118
29	5-F	29	227	8
30	4-F	17	218	13
31		56	312	6
32		780	2640	3

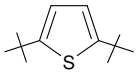
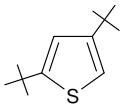
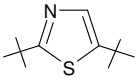
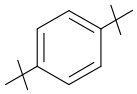
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); nt: not tested.

Methyl and fluorine substituents have been identified as functional groups able to increase the inhibitory activity of the 2,5-bis(hydroxyphenyl) thiophene **1**. Previously (Bey *et al.*, 2008b), we reported that other central core structures like 2,4-thiophene, 2,5-thiazole and 1,4-benzene lead to highly active compounds. The influence of an additional methyl or fluorine substituent at these structures was therefore also investigated (Table 5). Introduction of CH_3 or F into the *para*-hydroxyphenyl ring of **33, 36, 38** and **40** resulting in compounds **34, 35**, **37**, **39** and **42** led to equally active derivatives in case of **34** and **35** (IC_{50}= 64 nM vs. **21**, IC_{50}= 46 nM). A decrease in inhibitory activity in the thiazole and in the benzene classes of compounds was observed compared to the thiophene family (**37**, IC_{50}= 143 nM vs. **21**, IC_{50}= 46 nM; **39** and **42**, IC_{50}= 123 nM and 51 nM, respectively vs. **23**, IC_{50}= 8 nM). Amongst the investigated molecules, introduction of a methyl or fluorine substituent led only in the class of the bis(hydroxyphenyl) thiophenes to an increase in activity.

Table 5: Influence of the core and a supplementary substituent on the inhibition of the human 17β-HSD1 and 17β-HSD2.



21-23
33-42

cmpd	cycle	R_1	R_2	IC_{50} (nM)[a]		*selectivity factor*[d]
				17β-HSD1[b]	**17β-HSD2**[c]	
21		CH_3	OH	46	1971	49
23		F	OH	8	940	118
33		H	OH	77	1270	16
34		CH_3	OH	64	869	14
35		F	OH	64	510	8
36		H	OH	50	4000	80
37		CH_3	OH	143	2023	14
38		H	OH	471	4509	10
39		F	OH	123	872	7
40		OH	H	173	2259	21
41		OH	CH_3	171	1248	7
42		OH	F	51	239	5

[a]Mean values of three determinations, standard deviation less than 13 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

Selectivity: inhibition of 17β-HSD2 and affinities to the estrogen receptors α and β

In order to gain insight into the selectivity of the most active compounds, inhibition of 17β - HSD2 and the relative binding affinities to the estrogen receptors α and β were determined.
Since 17β-HSD2 catalyzes the inactivation of E2 into E1, inhibitory activity toward this enzyme must be avoided. The 17β-HSD2 inhibition was determined using an assay similar to the 17β-HSD1 test. Placental microsomes were incubated with tritiated E2 in the presence of NAD^+ and inhibitor. Separation and quantification of labelled product (E1) was performed by HPLC using radio detection. A selection of the most potent 17β-HSD1 inhibitors was tested for inhibition of 17β-HSD2. IC_{50}-values and selectivity factors (IC_{50} HSD2 / IC_{50} HSD1) are presented in Tables 1 to 5.

Mono-hydroxylated compounds (Table 1) exhibited a poor selectivity regarding 17β-HSD2, the most selective one being compound **5** with a selectivity factor of 5. This finding suggests that the *para*-OH is important for activity as well as for selectivity (selectivity of the *para-meta* derivative **1**: 28). Introduction of further substituents (Tables 2 to 5) into the highly active bis(hydroxyphenyl) scaffold induced a loss of selectivity against 17β-HSD2 except in case of compounds **21** and **23**, which exhibit excellent selectivity factors of 49 and 118, respectively toward 17β-HSD2.
A further prerequisite for 17β-HSD1 inhibitors to be used as potential drugs is that they do not show affinity for ERα and ERβ, since binding to these receptors could counteract the therapeutic concept of selective 17β-HSD1 inhibition. The binding affinities of the most selective compounds of this study were determined using recombinant human protein in a competition assay applying [^{3}H]-E2 and hydroxyapatite (Table 6). All tested compounds show very marginal to marginal affinity to the ERs except compound **23**, which binds weakly to ERβ (RBA= 1 %). Compound **21** was evaluated for estrogenic effects on the ER-positive, mammary tumor T47D cell line. No agonistic, i.e. stimulatory effect was observed after application of compound **21** even at a concentration 1000 fold higher compared to E2.

Table 6: Binding affinities for the human estrogen receptors α and β of selected compounds.

cmpd	*RBA[a] (%)*	
	ERα[b]	**ERβ[b]**
1	0.1< RBA <1	1.5
17	0.1< RBA <1	0.1< RBA <1
21	< 0.01	< 0.01
23	0.01< RBA <0.1	1
30	0.1	0.01< RBA <0.1
34	0.01< RBA <0.1	0.01< RBA <0.1
37	0.01< RBA <0.1	< 0.01

[a]RBA (relative binding affinity), E2: 100 %, mean values of three determinations, standard deviations less than 10 %; [b]Human recombinant protein, incubation with 10 nM ^{3}H-E2 and inhibitor for 1 h.

Further biological evaluations

Additionally, the intracellular potency of compounds **21** and **23** on E2 formation was evaluated using a cell line which expresses both 17β-HSD1 and 17β-HSD2 (T47D cells). Compound **21** and **23** inhibited the formation of E2 after incubation with labelled E1 showing IC_{50} values of 426 nM and 282 nM, respectively. These results indicate that both compounds are able to permeate the cell membrane and inhibit the transformation of E1 into E2.
Compounds **21** and **23** were further investigated for inhibition of the two most important human hepatic enzymes: CYP3A4 and CYP2D6, which are responsible for 75 % of drug metabolism. At a concentration of 2 µM, both compounds turned out to be equally active inhibiting the CYP3A4 by 80 (**21**) and 71 % (**23**), respectively and CYP2D6 by 55 (**21**) and 56 % (**23**), respectively. The relatively high inhibition of these enzymes has to be taken into consideration in the process of further developing these compounds but should not have an impact on the proof of concept *in vitro*.

The pharmacokinetic profiles of compound **21** and **23** were determined in rats after oral administration in a cassette dosing approach. Each group consisted of 4 male rats and the compounds were administered in doses of 10 mg/kg. Plasma samples were collected over 24 h and plasma concentrations were determined by HPLC-MS/MS. The pharmacokinetic parameters are presented in Table 7. The maximal concentration ($C_{max\ obs}$) as well as the AUC-value is higher for compound **23** (C_{max}= 1388.2 ng/mL, AUC= 19407 ng/mL) than for compound **21** (C_{max}= 905.0 ng/mL, AUC= 12275 ng/mL). The maximal plasma concentration ($t_{max\ obs}$) for compounds **21** and **23** was reached after 4.0 and 8.0 h, respectively, after oral administration. These data show that both compounds exhibit excellent pharmacokinetic properties in the rat and might therefore be good candidates for further experiments in disease-oriented rat models.

Table 7: Pharmacokinetic parameters of compounds 21 and 23 in male rats after oral application (10 mg/kg).

parameters[a]	*cmpd*	
	21	**23**
$C_{max\ obs}$ (ng/mL)	905.0	1388.2
C_z (ng/mL)	43.3	24.9
$t_{max\ obs}$ (h)	4.0	8.0
t_z (h)	24.0	24.0
$t_{1/2z}$ (h)	3.8	2.7
$AUC_{0-\infty}$ (ng/mL)	12275	19407

[a] $C_{max\ obs,}$ maximal measured concentration; C_z, last analytical quantifiable concentration; $t_{max\ obs}$, time to reach the maximum measured concentration; t_z, time of the last sample which has an analytical quantifiable concentration; $t_{1/2z}$, half-life of the terminal slope of a concentration time curve; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity.

Computational chemistry

Molecular modelling

From the biological results it became apparent that introduction of a fluorine atom in *ortho*-position to the *para*-OH phenyl thiophene structure (compound **23**) led to a significant increase in the 17β-HSD1 inhibitory activity. To get an insight into the binding mode of this compound and to better understand the favourable interactions achieved by this inhibitor in the active site, computational studies were performed by means of the docking software GOLDv3.2 and Autodock 4.1.

The choice of the 3D-structure of the enzyme, i.e. crystal structure, used for the docking studies is crucial for obtaining reliable results. It was decided to focus on X-ray structures of 17β-HSD1 having a high resolution and showing a ternary complex (to get closer to the *in vivo* 3D-enzyme structure). Three structures appeared to fulfil the criteria: 1FDT and 1A27 both describing the ternary complex: enzyme-E2-$NADP^+$ and 1I5R, describing the binary complex: enzyme-steroidal hybride inhibitor (HYC), the latter being an adenosine moiety linked to an E2 core via a C9-linker. These three crystal structures differ mainly in the location of the amino acids belonging to the flexible loop αG'βF (Pro187-Pro200). Since this loop borders both the SUB and the COF, its conformational variations strongly influence the size of both binding cavities. It is therefore important to take care of the position of this loop in the structures used for the docking studies.

In the X-ray structure 1FDT, the residues 187-200 are not well resolved, but two plausible conformations for the loop (noted 1FDT-A and 1FDT-B) have been described (Breton *et al.*, 1996) The backbones of these two loops are similar (RMSD of ~ 1 Å), while the main difference is given by the orientation of the sidechains, mainly concerning the four amino acids Phe192, Met193, Glu194 and Lys195. In 1FDT-A, Phe192 and Met193 are turned toward the outer part of the enzyme while Glu194 and Lys195 are oriented toward the substrate and the cofactor (extending the substrate binding site = open conformation). On the other hand, in 1FDT-B these two couples of residues show a reversed orientation limiting length and volume of the steroid binding site compared to 1FDT-A (= closed conformation). Although others (Karkola *et al.*, 2008) have only considered 1FDT-B, we decided to investigate both conformations of this loop.
Interestingly, the flexible loop in 1A27 shows a comparable geometry as observed in 1FDT-B, with Phe192 and Met193 oriented toward the nicotinamide moiety, also restricting the space in the substrate binding site. In case of 1I5R, the loop is shifted in direction of the cofactor, resulting in a different conformation compared to both 1FDT-A and 1FDT-B. Although, like for 1FDT-A, it extends the SUB.
Compound **23** was docked with NADPH into four different X-ray structures: 1FDT-A, 1FDT-B, 1A27 and 1I5R. Two different binding modes were observed for compound **23**: in case of 1FDT-B and 1A27, the inhibitor is located exclusively in the steroid binding site (Figure 1) adopting a similar orientation as previously described for the bis(hydroxyphenyl) oxazole **B** (Bey *et al.*, 2008a; chart 1) while for 1FDT-A and 1I5R, the inhibitor is located in between the steroid and the cofactor binding sites, interacting with the nicotinamide moiety. In the following, this binding mode will be named as alternative binding mode (Figure 2).

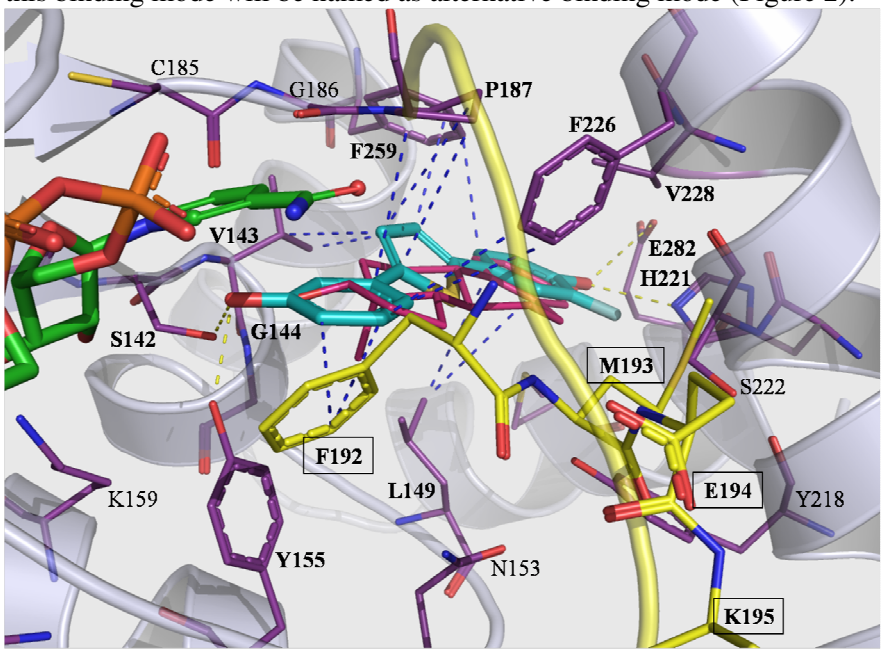


Figure 1: Docking complex between 17β-HSD1 (X-ray 1FDT-B) and compound 23 (blue; SUB binding mode). NADPH, interacting residues and ribbon rendered tertiary structure of the active site are shown. Residues of the flexible loop are rendered in sticks and colored in yellow. Hydrogen bonds and π-π stackings (and hydrophobic interactions) are drawn in yellow and blue dashed lines, respectively. For comparison, E2 is depicted in magenta lines. Figures were generated with Pymol (http://www.pymol.org).

In case of the steroidal binding mode (1FDT-B and 1A27, Figure 1) the following specific interactions can be observed: hydrogen bond interactions between the *meta*-hydroxy group of **23** and Ser142/Tyr155 ($d_{O\text{-}O}$= 2.6 Å for both amino acids) and between the *para*-OH group and His221/Glu282 ($d_{O\text{-}N}$= 2.8 Å and $d_{O\text{-}O}$= 3.8 Å, Figure 1). Additionally, hydrophobic interactions and π-π stackings (Phe226, Phe259) are also involved.

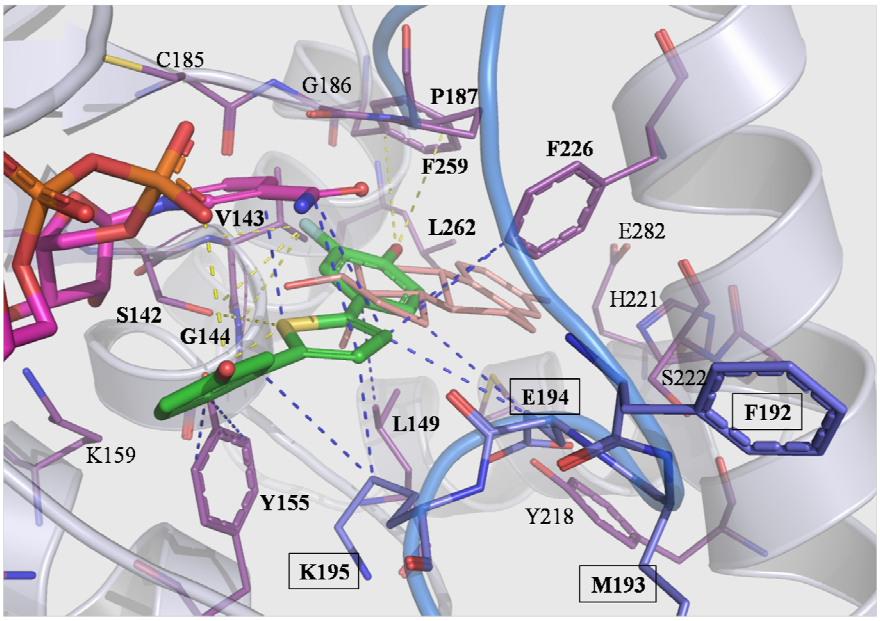


Figure 2. Docking complex between 17β-HSD1 (X-ray 1FDT-A) and compound 23 (green; alternative binding mode). NADPH, interacting residues and ribbon rendered tertiary structure of the active site are shown. Residues of the flexible loop are rendered in sticks and colored in blue. Hydrogen bonds and π-π stackings (and hydrophobic interactions) are drawn in yellow and blue dashed lines, respectively. For comparison, E2 is depicted in magenta lines.

In the alternative binding mode obtained using 1FDT-A and 1I5R (Figure 2), compound **23** is also stabilized by hydrogen bond interactions: the *meta*-OH group forms a strong H-bond with the phosphate group of the cofactor ($d_{O\text{-}O}$= 2.9 Å). The fluorine atom could establish halogen bonds with the backbone -NH- of Val143 and Gly144 ($d_{F\text{-}N}$= 3.2 Å and 3.8 Å, respectively), in addition to a halogen bond with the OH-group of Ser142 ($d_{F\text{-}O}$= 3.5 Å) which is involved in the catalytic process. Further, the *para*-OH points perpendicular toward Phe259 ($d_{O\text{-centroid}}$= 4.5 Å) indicating a possible OH-π interaction. This could explain the importance of this group observed in the SARs. Moreover, strong π-π stacking interactions seem to stabilize the inhibitor in this binding mode: between the *meta*-OHphenyl-thiophene moiety and the nicotineamide part of the cofactor (parallel-displaced configuration; distance between the two ring centers, 4.3 Å) and between the *para*-OH-phenyl-thiophene moiety and Phe226 (T-shape conformation; closest C-C contact distance 3.7 Å). Moreover, electrostatic interactions between the sulfur atom of the heterocycle with the surrounding amino acids like Tyr155 and Ser142 might also play a role as described (Bruno *et al.*, 1997).

The results presented so far suggest that both binding modes have to be considered as possible for this class of inhibitors. They depend mainly on the orientation of the flexible loop. There is only one conformation of the loop leading to a steroidal binding mode (1FDT-B/1A27). In case of 1FDT-A/1I5R the pose showing the alternative binding mode is obtained using two X-ray structures having two different conformations of the loop. Unfortunately, due to the almost identical scoring function values observed for both poses with the docking programs (Gold and Autodock), it was not possible to determine which model (1FDT-A/1I5R or 1FDT-B/1A27) is the most appropriate to describe the interactions between the inhibitor and the enzyme and therefore which is the most plausible binding mode.

Comparing both poses obtained by docking of **23** in 1FDT-A and 1FDT-B shows that there is a common area in the neighborhood of the catalytic tetrade which corresponds to the D-ring of the enzyme-substrate complex (Figure in Supporting Information).

Molecular Electrostatic Potential (MEP)

Recently, we reported on the influence of the electronic density (MEP maps, "semi-QMAR") on the potency of the inhibitors in this class of compounds (Bey *et al.*, 2008b). The 3D-structures of the inhibitors were virtually divided into three areas and a given optimal range of ESP values (in Hartree) was determined for each region (-1.7 to -1.2 × 10^{-2} for I, -1.6 to -0.9 × 10^{-2} for II

and -1.2 to -0.5 × 10^{-2} for III). The MEPs of compound **23** were calculated as shown in Figure 3. The molecular ESP distribution observed (-1.8 to -1.2 × 10^{-2} for I, -1.6 to -0.8 × 10^{-2} for II and -1.1 to -0.4 × 10^{-2} for III) fitted well to the optimal ranges identified previously, confirming the correlation between the ESP range and the potency of the compounds. The MEP maps of the natural substrate E1 and of E2 were also calculated (Figure 3) and compared to the one of **23**. The finding that the ESP distribution of **23** and E2/E1 is very different might be an indication that compound **23** does not bind in the same way as the steroid.

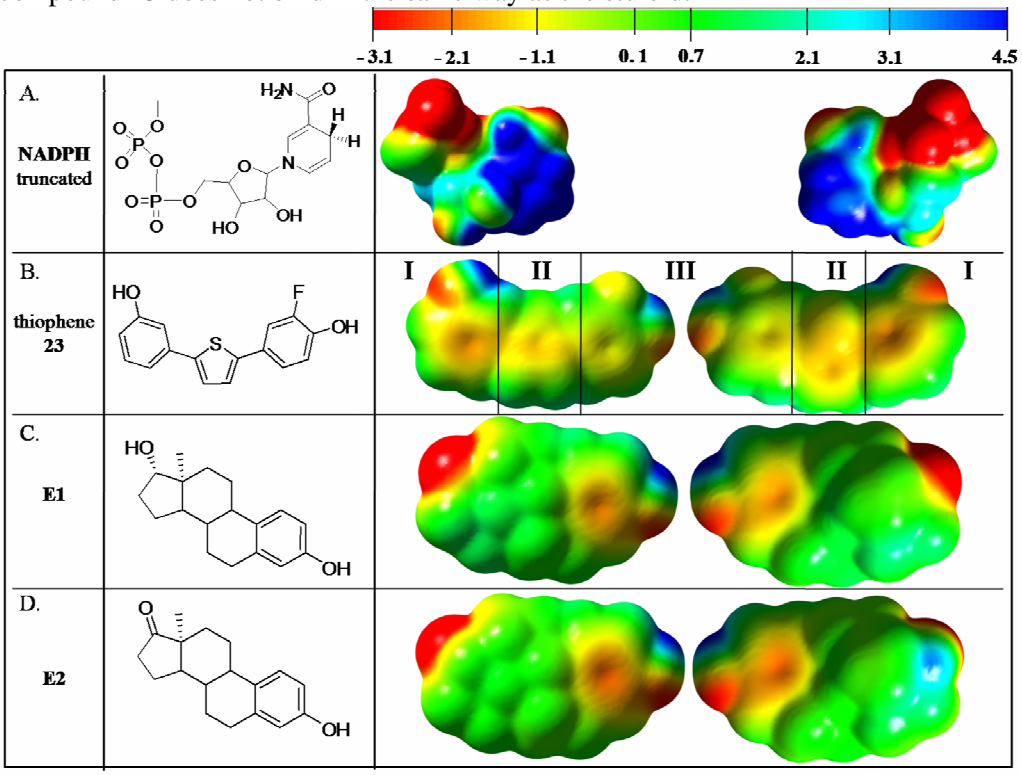


Figure 3. Structures and MEP maps of both ventral (steroidal α-side) and dorsal (steroidal β-side) views of truncated NADPH (A), thiophene 23 (B), E1 (C) and E2 (D). MEP surfaces were plotted with GaussView 3.09.

According to the alternative binding mode, the *meta*-hydroxyphenyl thiophene part of **23** overlaps with the nicotinamide part of the cofactor and forms stabilizing π-π interactions. The ESP distribution of these two entities should therefore show complementarity. To get an insight into this, the MEP map of a truncated NADPH - the counterpart of the *meta*-OHphenyl-thiophene moiety - was calculated by *ab initio* methods. As it can be seen in Figure 3 a certain complementarity was observed. The NADPH MEP maps give the explanation for the observation (Bey *et al.*, 2008b) that a strong polarization between the vertex and the base of the central ring of the inhibitors is negative for binding. Positive ESP values on the vertex side lead to repulsion effects with the nicotinamide and therefore reduce the inhibitory activity. This finding indicates that this class of compound might bind according to the alternative binding mode (Figure 2). However, this hypothesis needs to be further investigated.

Discussion and Conclusion

Structural optimizations of compound **1** led to the discovery of new substituted 2,5-bis(hydroxyphenyl)thiophene derivatives: the fluorinated **23** and the methylated **21** being the most active and selective inhibitors identified.

From a previous work in this class of compounds (Bey *et al.*, 2008b) it was demonstrated that removal of one of the two hydroxyphenyl moieties is detrimental for the activity. In this paper it was shown that replacement of the *para*-OH function by a bioisoteric group like F, NH_2, SH, CN leads to a drop of activity. The lack of hydrogen donating properties of the fluoro and cyano substituents might not be the only reason for this decrease in activity as the amino and the thiol

derivatives are also less active than the parent compound **1**. Interestingly, the omission of a C1-linker between the methylsulfonamide moiety and the phenyl ring (compound **9**) resulted in an increase of potency. Deprivation of electrons from the phenyl ring, obviously is necessary for a good inhibition. The relatively high activity observed for compound **11** (IC_{50}= 350 nM) especially compared to compound **9** (IC_{50}= 523 nM) demonstrates that in the protein there is some space available in this position for a bulky substituent. Furthermore, the tolyl group might also be involved in the stabilization of the inhibitor in the binding site, establishing π-π stacking interactions with appropriate amino acid residues present in this region.

With the aim to increase the activity and the selectivity in this class of compound, substituents were introduced on the 2,5-bis(hydroxyphenyl)thiophenes **1** and **7**. This was successful for compound **15** (IC_{50}= 119 nM vs. **7**, IC_{50}= 216 nM). Apparently, the formation of an additional hydrogen bond is responsible for this increase in inhibitory activity, while a pure π-π stacking interaction as supposed for compound **14** is not sufficient. The 2,5-disubstituted thiophenes **12** and **15** differ only in the position of one hydroxy group (*para:* compound **12,** *meta:* compound **15**). The fact that compound **15** shows a much higher activity (IC_{50}= 119 nM vs. **12,** IC_{50}> 1000 nM) indicates that only in case of **15** the geometry of the OH groups is acceptable for a reasonable interaction. It demonstrates, as observed already (Bey *et al.*, 2008a; Frotscher *et al.*, 2008), a sharp SAR and a reduced flexibility in this region of the active site.

The trisubstituted compound **14** bearing a phenyl substituent at the thiophene differs from the triazole **A** (Al-Soud *et al.*, 2009; Chart 2) only in the nature of the heterocycle. The following comparisons highlight the importance of the heterocycle for the potency of the molecules: inactive compound **A** vs. thiophene **14** (IC_{50}= 493 nM), thiophene **21** (IC_{50}= 46 nM) vs. thiazole **37** (IC_{50}= 143 nM) and thiophene **23** (IC_{50}= 8 nM) vs. benzene **39** (IC_{50}= 123 nM). It becomes apparent that the thiophene ring is the most appropriate heterocycle for high inhibitory activity. Provided that all compounds bind according to the same binding mode, there are different explanations for these results: 1. the presence of one or several nitrogens in this area of the enzyme is not well tolerated, 2. the absence of the sulfur leads to an inadequate repartition of the electron density in the molecule, 3. a reduced flexibility in the binding site is responsible that the enzyme can not adjust its geometry to the different hydroxyphenyl moieties (depending on the heterocycle, the angles between the phenyl-OHs are different).

A high increase in activity and selectivity could be reached by introduction of substituents into the hydroxyphenyl moiety, especially when the substituent is located *ortho-* of the *para*-hydroxyphenyl group (compounds **21** to **24**). Not all substituents are equally well tolerated: there is no space available for a phenyl group (compound **25**). An additional OH group (compound **22**) is obviously not able to establish specific interactions while small lipophilic substituents (methyl, compounds **21**; fluorine, compound **23** and trifluoromethyl, compound **24**) are enhancing the activity. There is enough space in this region of the enzyme to introduce a flexible chain (**26**) but conjugation seems to be necessary to achieve a higher activity as already observed with the tolylsulfonamide substituent (compound **11**).

The positive influence of the fluorine atom has often been demonstrated in medicinal chemistry (Bohm *et al.*, 2004; Muller *et al.*, 2007; Hagmann, 2008) and was also proven in this study with compound **23** (IC_{50} = 8 nM). The position of the fluorine is decisive for an increase in activity: it has to be in *meta*-position (**17** and **23**). Highest activity was achieved in *ortho-* of the hydroxy group (compound **23**). This indicates that either direct interactions of the fluorine with amino acid residues in this region of the active site or the increase of acidity of the neighboring OH groups might be responsible for this effect.

Introduction of a second fluorine atom into this fluorohydroxyphenyl ring (compound **31**) does not enhance the activity suggesting that the effects of the fluorine are not additional. A second fluorine was also added to the other hydroxyphenyl moiety (in *ortho-* and *meta-* of the *meta*-OH group) leading to compounds **29** and **30**. However, no enhancement of the activity compared to the monofluorinated **23** was observed indicating that there are no specific interactions of the second fluoro substituent.

A close look at the X-ray structures of 17β-HSD1 crystallized in presence of different steroidal ligands showed that the flexible loop (amino acids 187-200) can adopt different geometries depending on the nature of the ligand and on the absence or presence of the cofactor in the catalytic region. It indicates that some parts of the enzyme can adapt their geometry to the

molecule present in the active site in order to stabilize it. However, other parts are rigid, explaining the sharp SAR observed in this paper and previously (Bey *et al.*, 2008a; Bey *et al.*, 2008b).
Two plausible conformations of the loop in the ternary complex enzyme-E2-$NADP^+$, PDB code: 1FDT, have been described (1FDT-A, 1FDT-B). We have shown that both can be used for docking studies. In case of 1FDT-A the substrate binding site is extended, enhancing the volume of the active site. It is therefore a good model to evaluate an alternative binding mode for inhibitors which are larger than the steroid. A binding mode as observed for steroids in the X-ray structures was found when the loop closes the SUB (1FDT-B). Surprisingly, when the inhibitors were docked to the protein with the loop in the open conformation, they interact with the nicotinamide part of the cofactor. MEP calculations showed a certain complementarity between the electronic density of **23** and of the nicotinamide moiety of the cofactor indicating that this alternative binding mode is not only plausible, it might be the one which is more likely.
Up to now, designing compounds as potential 17β-HSD1 inhibitors, several groups (Bérubé *et al.*, 2004; Fournier *et al.*, 2008; Poirier *et al.*, 2005; Qiu *et al.*, 2002) tried to mimic the cofactor. Our finding of the above mentioned alternative binding mode makes another strategy very promising: the cofactor, which is likely to be present in the active site when the inhibitor is entering, could be used as partner to achieve additional interactions rather than trying to displace it.
The most potent 17β-HSD1 inhibitors **21** and **23** exhibit a higher selectivity toward 17β-HSD2 compared to parent compound, (selectivity factors 49 and 118, respectively vs. 28 for **1**). This indicates that the amino acids close to the CH_3 or F substituents must have different properties in the two 17β-HSD enzymes, which could be further exploited to increase selectivity.
The most potent inhibitors show only marginal to very little affinity to the ERα and no stimulation of cell proliferation (agonistic effect) in the ER-positive T47D cell line could be observed. The weak affinity of compound **23** for ERβ may not be critical as it is reported that ERβ exhibits anti-proliferative effects in breast cancer cells (Hartman *et al.*, 2006).
Compound **23** might be used in an appropriate animal model to prove the concept of 17β-HSD1 inhibition with non-steroidal inhibitors. This compound shows a good pharmacokinetic profile in rats.
In this paper, we described the synthesis of substituted bis(hydroxyphenyl)thiophenes, thiazoles and benzenes as inhibitors of 17β-HSD1 and the evaluation of their biological properties. The most promising compounds of this study, **21** and **23**, exhibit high selectivity toward 17β-HSD2, marginal binding to ERα and excellent pharmacokinetic profiles in rats after peroral application. These new compounds provide useful tools to validate 17β-HSD1 as a target for the treatment of estrogen-dependent diseases.

Experimental section

Chemical methods

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Lancaster, Roth, Merck or Fluka and were used without purification.
Column chromatography (CC) was performed on silica gel (70-200 µm) coated with silica, preparative thin layer chromatography (TLC) on 1 mm SIL G-100 UV_{254} glass plates (Macherey-Nagel) and reaction progress was monitored by TLC on Alugram SIL G UV_{254} (Macherey-Nagel).
IR spectra were recorded on a Bruker Vector 33 spectrometer (neat sample).
^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AM500 spectrometer (500 MHz) at 300 K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard ($CDCl_3$: δ = 7.24 ppm (^{1}H NMR) and δ = 77 ppm (^{13}C NMR), CD_3OD: δ = 3.35 ppm (^{1}H NMR) and δ = 49.3 ppm (^{13}C NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H NMR) and δ = 29.9 ppm (^{13}C NMR), CD_3SOCD_3: δ = 2.50 ppm (^{1}H NMR) and δ = 39.5 ppm (^{13}C NMR)). Signals are described as s, d, t, q, dd, m, dt

for singlet, doublet, triplet, quadruplet, doublet of doublets, multiplet and doublet of triplets, respectively. All coupling constants (*J*) are given in hertz (Hz).
Mass spectra were recorded on a TSQ Quantum (Thermofischer) instrument. Elemental analyses were performed at the Department of Instrumental Analysis and Bioanalysis, Saarland University.

Compounds 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**), 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**1a**), 3-[5-(4-hydroxyphenyl)-2-thienyl]phenol (**1**), 2-(3-methoxyphenyl)-5-phenylthiophene (**2a**), 3-(5-phenyl-2-thienyl)phenol (**2**), 2,5-bis(3-methoxyphenyl)thiophene (**7a**), 3,3'-thiene-2,5-diyldiphenol (**7**), 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (Bey *et al.*, 2008b), 4-bromo-2-iodo-1-methoxy-benzene (**25d**) (Smith *et al.*, 2006), 5-bromo-2-methoxybiphenyl (**25c**), [6-methoxy-1,1'-biphenyl-3-yl]boronic acid (**25b**) (Pfahl *et al.*, 2002), [3-(hydroxymethyl)-4-methoxyphenyl]-boronic acid (**26d**) (Duggan *et al.*, 2008), 4-bromo-2-(3-methoxyphenyl)thiophene (**33b**), 2-(3-methoxyphenyl)-4-(4-methoxyphenyl)thiophene (**33a**), 3-[4-(4-hydroxyphenyl)-2-thienyl]phenol (**29**), 5-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**36b**), 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)-1,3-thiazole (**36a**), 3-[5-(4-hydroxyphenyl)-1,3-thiazol-2-yl]phenol (**36**), 4'-bromo-3-methoxybiphenyl (**38b**), 3,4"-dimethoxy-1,1':4',1"-terphenyl (**38a**), 1,1':4',1"-terphenyl-3,4"-diol (**38**), 3,3"-dimethoxy-1,1':4',1"-terphenyl (**40a**) and 1,1':4',1"-terphenyl-3,3"-diol (**40**) (Bey *et al.*, 2008b) were prepared following described procedures.

General procedure for Suzuki coupling

Method A:
A mixture of aryl bromide (1 eq), aryl boronic acid (1.2 eq), caesium carbonate (2.2 eq) and tetrakis(triphenylphosphine) palladium (0.01 eq) was suspended in an oxygen free DME/EtOH/water (1:1:1) solution. The reaction mixture was exposed to microwave irradiation (15 min, 150 W, 150 °C, 15 bars). After cooling to rt, water was added and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by column chromatography (CC).

Method B:
A mixture of arylbromide (1 eq), aryl boronic acid (1 eq), sodium carbonate (2 eq) and tetrakis(triphenylphosphine) palladium (0.05 eq) in an oxygen free toluene/water (1:1) solution was stirred at 100 °C for 20 h under nitrogen atmosphere. The reaction mixture was cooled to rt. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC.

General procedure for ether cleavage

Method C:
To a solution of methoxyphenyl derivative (1 eq) in dry dichloromethane at -78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1 M, 3 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at rt under nitrogen atmosphere. Water was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and concentrated to dryness. The product was purified by preparative thin layer chromatography (TLC).

2-(4-Fluorophenyl)-5-(3-methoxyphenyl)thiophene (3a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 4-fluorophenylboronic acid (94 mg, 0.67 mmol), caesium carbonate (383 mg, 1.24 mmol) and tetrakis(triphenylphosphine) palladium (6.4 mg, 5.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 75 % (120 mg).

3-[5-(4-Fluorophenyl)-2-thienyl]phenol 3. The title compound was prepared by reaction of 2-(4-fluorophenyl)-5-(3-methoxyphenyl)thiophene (**3a**) (80 mg, 0.28 mmol) and boron tribromide (0.84 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 68 % (52 mg); MS, ESI: 270 $(M+H)^+$; Anal. ($C_{16}H_{11}FOS$) C, H, N.

4-[5-(3-Methoxyphenyl)-2-thienyl]aniline (4a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 4-aminophenylboronic acid (92 mg, 0.67 mmol), caesium carbonate (383 mg, 1.24 mmol) and tetrakis(triphenylphosphine) palladium (6.4 mg, 5.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 63 % (100 mg).

3-[5-(4-Aminophenyl)-2-thienyl]phenol (4). The title compound was prepared by reaction of 4-[5-(3-methoxyphenyl)-2-thienyl]aniline (**4a**) (100 mg, 0.37 mmol) and boron tribromide (1.11 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 82 % (82 mg); MS, ESI: 268 $(M+H)^+$; Anal. ($C_{16}H_{13}NOS$) C, H, N.

4-[5-(3-Methoxyphenyl)-2-thienyl]benzenethiol (5a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (250 mg, 0.93 mmol), 4-mercaptophenylboronic acid (172 mg, 1.12 mmol), caesium carbonate (636 mg, 2.05 mmol) and tetrakis(triphenylphosphine) palladium (10.8 mg, 9.3 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 61 % (160 mg).

3-[5-(4-Sulfanylphenyl)-2-thienyl]phenol (5). The title compound was prepared by reaction of 4-[5-(3-methoxyphenyl)-2-thienyl]benzenethiol (**5a**) (150 mg, 0.50 mmol) and boron tribromide (1.50 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 81 % (115 mg); MS, ESI: 285 $(M+H)^+$; Anal. ($C_{16}H_{12}OS_2$) C, H, N.

4-[5-(3-Methoxyphenyl)-2-thienyl]benzonitrile (6a). The title compound was prepared by reaction by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (200 mg, 0.74 mmol), 4-cyanophenylboronic acid (131 mg, 0.89 mmol), caesium carbonate (508 mg, 1.64 mmol) and tetrakis(triphenylphosphine) palladium (8.5 mg, 7.4 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 27 % (60 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl]benzonitrile (6). The title compound was prepared by reaction of 4-[5-(3-methoxyphenyl)-2-thienyl]benzonitrile (**6a**) (42 mg, 0.14 mmol) and boron tribromide (0.42 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 62 % (25 mg); MS (APCI): 277 $(M)^+$; Anal. ($C_{17}H_{11}NOS$) C, H, N.

2-(3-Fluorophenyl)-5-(3-methoxyphenyl)thiophene (8a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 3-fluorophenylboronic acid (94 mg, 0.67 mmol), caesium carbonate (381 mg, 1.22 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 82 % (130 mg).

3-[5-(3-Fluorophenyl)-2-thienyl]phenol (8). The title compound was prepared by reaction of 2-(3-fluorophenyl)-5-(3-methoxyphenyl)thiophene (**8a**) (130 mg, 0.45 mmol) and boron tribromide (1.35 mmol) according to method C. The product was purified by preparative TLC (dichloromethane/methanol 99:1); yield: 66 % (82 mg); MS, ESI: 271 $(M+H)^+$; Anal. ($C_{16}H_{11}FOS$) C, H, N.

***N*-(3-[5-(3-Methoxyphenyl)-2-thienyl]phenyl)methanesulfonamide (9a).** The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (160 mg, 0.60

mmol), 3-methylsulfonylaminophenylboronic acid (155 mg, 0.72 mmol), caesium carbonate (410 mg, 1.32 mmol) and tetrakis(triphenylphosphine) palladium (6.9 mg, 6.0 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 6:4); yield: 75 % (150 mg).

***N*-(3-[5-(3-Hydroxyphenyl)-2-thienyl]phenyl)methanesulfonamide (9).** The title compound was prepared by reaction of *N*-(3-[5-(3-methoxyphenyl)-2-thienyl]phenyl)methanesulfonamide (**9a**) (150 mg, 0.44 mmol) and boron tribromide (1.32 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 61 % (92 mg); MS, ESI: 346 $(M+H)^+$; Anal. ($C_{17}H_{15}NO_3S_2$) C, H, N.

***N*-(3-[5-(3-Methoxyphenyl)-2-thienyl]benzyl)methanesulfonamide (10a).** The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 3-[(methylsulphonylamino)methyl]benzeneboronic acid (153 mg, 0.67 mmol), caesium carbonate (382 mg, 1.23 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 8:2); yield: 58 % (122 mg).

***N*-(3-[5-(3-Hydroxyphenyl)-2-thienyl]benzyl)methanesulfonamide (10).** The title compound was prepared by reaction of *N*-(3-[5-(3-methoxyphenyl)-2-thienyl]benzyl)methanesulfonamide (**10a**) (122 mg, 0.37 mmol) and boron tribromide (1.11 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 33 % (44 mg); MS, ESI: 360 $(M+H)^+$; Anal. ($C_{18}H_{17}NO_3S_2$) C, H, N.

***N*-(3-[5-(3-Methoxyphenyl)-2-thienyl]phenyl)-4-methylbenzenesulfonamide (11a).** The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), [3-[[(4-methylphenyl)sulfonyl]amino]phenyl]-boronic acid (195 mg, 0.67 mmol), caesium carbonate (383 mg, 1.23 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 88 % (214 mg).

***N*-(3-[5-(3-Hydroxyphenyl)-2-thienyl]phenyl)-4-methylbenzenesulfonamide (11).** The title compound was prepared by reaction of *N*-(3-[5-(3-methoxyphenyl)-2-thienyl]phenyl)-4-methylbenzenesulfonamide (**11a**) (214 mg, 0.49 mmol) and boron tribromide (1.47 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 75 % (156 mg); MS (APCI): 421 $(M)^+$; Anal. ($C_{23}H_{19}NO_3S_2$) C, H, N.

3,5-Dibromo-2-(3-methoxyphenyl)thiophene (12c). The title compound was prepared by reaction of 2,3,5-tribromothiophene (100 mg, 0.31 mmol), 3-methoxybenzeneboronic acid (46 mg, 0.31 mmol), sodium carbonate (67 mg, 0.62 mmol) and tetrakis(triphenylphosphine) palladium (17.9 mg, 15.5 µmol) according to method B heating the reaction 4 h instead of 20 h. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 23 % (25 mg).

3-Bromo-2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (12b). The title compound was prepared by reaction of 3,5-dibromo-2-(3-methoxyphenyl)thiophene (**12c**) (500 mg, 1.43 mmol), 4-methoxybenzeneboronic acid (268 mg, 1.72 mmol), sodium carbonate (333 mg, 3.15 mmol) and tetrakis(triphenylphosphine) palladium (82.6 mg, 71.5 µmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 52 % (278 mg).

2,3-Bis(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (12a). The title compound was prepared by reaction of 3-bromo-2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**12b**) (250 mg, 0.67 mmol), 3-methoxybenzeneboronic acid (124 mg, 0.80 mmol), sodium carbonate (142 mg, 1.34 mmol) and tetrakis(triphenylphosphine) palladium (38.7 mg, 33.5 µmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 72 % (194 mg).

3,3'-[5-(4-Hydroxyphenyl)thiene-2,3-diyl]diphenol (12). The title compound was prepared by reaction of 2,3-bis(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**12a**) (100 mg, 0.24 mmol) and boron tribromide (2.16 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 92 % (79 mg); MS, ESI: 359 $(M-H)^+$; Anal. ($C_{22}H_{16}O_3S$) C, H, N.

3,3'-(3-Methylthiene-2,5-diyl]diphenol (13). The title compound was prepared by reaction of 2,5-dibromo-3-methylthiophene (150 mg, 0.58 mmol), 3-hydroxybenzeneboronic acid (179 mg, 1.27 mmol), caesium carbonate (868 mg, 2.79 mmol) and tetrakis(triphenylphosphine) palladium (6.7 mg, 5.8 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 4:6); yield: 45 % (73 mg); MS, ESI: 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

3-Bromo-2,5-bis(3-methoxyphenyl)thiophene (14b). The title compound was prepared by reaction of 3,5-dibromo-2-(3-methoxyphenyl)thiophene (**12c**) (250 mg, 0.72 mmol), 3-methoxybenzeneboronic acid (134 mg, 0.86 mmol), sodium carbonate (148 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (41.6 mg, 36.0 µmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 72 % (194 mg).

2,5-Bis(3-methoxyphenyl)-3-phenylthiophene (14a). The title compound was prepared by reaction of 3-bromo-2,5-bis(3-methoxyphenyl)thiophene (**14b**) (102 mg, 0.27 mmol), benzeneboronic acid (38 mg, 0.27 mmol), sodium carbonate (58 mg, 0.54 mmol) and tetrakis(triphenylphosphine) palladium (15.6 mg, 13.5 µmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 54 % (51 mg).

3,3'-(3-Phenylthiene-2,5-diyl)diphenol (14). The title compound was prepared by reaction of 2,5-bis(3-methoxyphenyl)-3-phenylthiophene (**14a**) (50 mg, 0.13 mmol) and boron tribromide (0.78 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 53 % (49 mg); MS, ESI: 345 $(M+H)^+$; Anal. ($C_{22}H_{16}O_2S$) C, H, N.

2,3,5-Tris(3-methoxyphenyl)thiophene (**15a**). The title compound was prepared by reaction of 3-bromo-2,5-bis(3-methoxyphenyl)thiophene (**14b**) (102 mg, 0.27 mmol), 3-methoxybenzene boronic acid (42 mg, 0.27 mmol), sodium carbonate (58 mg, 0.54 mmol) and tetrakis(triphenylphosphine) palladium (15.6 mg, 13.5 µmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 34 % (37 mg).

3,3',3''-Thiene-2,3,5-triyltriphenol (15). The title compound was prepared by reaction of 2,3,5-tris(3-methoxyphenyl)thiophene (**15a**) (37 mg, 0.09 mmol) and boron tribromide (0.81 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 67 % (21 mg); MS, ESI: 361 $(M+H)^+$; Anal. ($C_{22}H_{16}O_3S$) C, H, N.

5-(4-Methoxyphenyl)-2-(boronic acid)thiophene (16b). To a solution of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (100 mg, 0.37 mmol, 1 eq) in anhydrous THF cooled to -78 °C for 5 min, *n*-BuLi (1.6 M in hexane, 0.28 mL, 0.44 mmol, 1.2 eq) was added dropwise and stirred at -78 °C. After 15 min, triethyl borate (0.37 mL, 2.22 mmol, 6 eq) was added at -78°C and the mixture was stirred for 2 h. After warming to rt, the crude material was acidified with 20 mL of a 1N hydrochloric acid solution. The aqueous layer was washed with ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure. The title compound was not characterized and used without further purification.

2-(3-Methoxyphenyl-5-methylphenyl)-5-(4-methoxyphenyl)thiophene (16a). The title compound was prepared by reaction of 1-bromo-3-methoxy-5-methylbenzene (150 mg, 0.74 mmol), [5-(4-methoxyphenyl)-2-thienyl]-boronic acid (**16b**) (206 mg, 0.88 mmol), sodium carbonate (181 mg, 1.76 mmol) and tetrakis(triphenylphosphine) palladium (42.7 mg, 37.0

µmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 22 % (50 mg).

3-[5-(4-Hydroxyphenyl)-2-thienyl]-5-methylphenol (16). The title compound was prepared by reaction of 2-(3-methoxyphenyl-5-methylphenyl)-5-(4-methoxyphenyl)thiophene (**16a**) (50 mg, 0.16 mmol) and boron tribromide (0.96 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 90 % (41 mg); MS, ESI: 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

2-(3-Fluoro-5-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (17a). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (200 mg, 0.75 mmol), 3-fluoro-5-methoxybenzeneboronic acid (152 mg, 0.89 mmol), caesium carbonate (513 mg, 1.65 mmol) and tetrakis(triphenylphosphine) palladium (8.7 mg, 7.5 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 43 % (122 mg).

3-Fluoro-5-[5-(4-hydroxyphenyl)-2-thienyl]phenol (17). The title compound was prepared by reaction of 2-(3-fluoro-5-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**17a**) (100 mg, 0.32 mmol) and boron tribromide (1.92 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 88 % (80 mg); MS (APCI): 286 M^+; Anal. ($C_{16}H_{11}FO_2S$) C, H, N.

5-[5-(4-Methoxyphenyl)-2-thienyl]-2-methylphenol (18a). The title compound was prepared by reaction of 5-bromo-2-methylphenol (250 mg, 1.34 mmol), [5-(4-methoxyphenyl)-2-thienyl]-boronic acid (**16b**) (690 mg, 2.95 mmol), caesium carbonate (914 mg, 2.94 mmol) and tetrakis(triphenylphosphine) palladium (15.5 mg, 13.4 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 47 % (193 mg).

5-[5-(4-Hydroxyphenyl)-2-thienyl]-2-methylphenol (18). The title compound was prepared by reaction of 5-[5-(4-methoxyphenyl)-2-thienyl]-2-methylphenol (**18a**) (161 mg, 0.54 mmol) and boron tribromide (3.24 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 27 % (42 mg); MS, ESI: 283 $(M+H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

2-(4-Fluoro-3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (19a). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (200 mg, 0.75 mmol), 4-fluoro-3-methoxybenzeneboronic acid (152 mg, 0.89 mmol), caesium carbonate (553 mg, 1.78 mmol) and tetrakis(triphenylphosphine) palladium (8.7 mg, 7.5 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 49 % (149 mg).

2-Fluoro-5-[5-(4-hydroxyphenyl)-2-thienyl]phenol (19). The title compound was prepared by reaction of 2-(4-fluoro-3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**19a**) (100 mg, 0.32 mmol) and boron tribromide (1.92 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 88 % (80 mg); MS, ESI: 287 $(M+H)^+$; Anal. ($C_{16}H_{11}FO_2S$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-(4-methoxyphenyl)thiophene (20a). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (195 mg, 0.73 mmol), 3,4-dimethoxybenzene boronic acid (160 mg, 0.88 mmol), caesium carbonate (500 mg, 1.61 mmol) and tetrakis(triphenylphosphine) palladium (8.4 mg, 7.3 µmol) according to method A. The product was purified by CC (dichloromethane/methanol 99:1); yield: 46 % (119 mg).

4-[5-(4-Hydroxyphenyl)-2-thienyl]benzene-1,2-diol (20). The title compound was prepared by reaction of 2-(3,4-dimethoxyphenyl)-5-(4-methoxyphenyl)thiophene (**20a**) (100 mg, 0.31 mmol) and boron tribromide (2.79 mmol) according to method C. The product was purified by

preparative TLC (hexane/ethyl acetate 1:1); yield: 17 % (49 mg); MS, ESI: 285 $(M+H)^+$; Anal. ($C_{16}H_{12}O_3S$) C, H, N.

2-(4-Methoxy-3-methylphenyl)-5-(3-methoxyphenyl)thiophene (21a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (250 mg, 0.92 mmol), 3-methyl-4-methoxybenzeneboronic acid (152.8 mg, 0.92 mmol), sodium carbonate (243 mg, 2.36 mmol) and tetrakis(triphenylphosphine) palladium (53.1 mg, 46.0 µmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 54 % (154 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl)-2-methyl]phenol (21). The title compound was prepared by reaction of 2-(4-methoxy-3-methylphenyl)-5-(3-methoxyphenyl)thiophene (**21a**) (100 mg, 0.32 mmol) and boron tribromide (1.92 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 79 % (72 mg); MS, ESI: 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-(3-methoxyphenyl)thiophene (22a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (206 mg, 1.14 mmol), 3,4-dimethoxybenzeneboronic acid (247 mg, 1.36 mmol), caesium carbonate (779 mg, 2.51 mmol) and tetrakis(triphenylphosphine) palladium (13.2 mg, 11.4 µmol) according to method A. The product was purified by CC (dichloromethane/methanol 99:1); yield: 34 % (126 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl]benzene-1,2-diol (22). The title compound was prepared by reaction of 2-(3,4-dimethoxyphenyl)-5-(3-methoxyphenyl)thiophene (**22a**) (100 mg, 0.32 mmol) and boron tribromide (2.88 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 61 % (56 mg); MS, ESI: 283 $(M-H)^+$; Anal. ($C_{16}H_{12}O_3S$) C, H, N.

2-(3-Fluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (23a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (370 mg, 1.37 mmol), 3-fluoro-4-methoxybenzeneboronic acid (255 mg, 1.50 mmol), caesium carbonate (717 mg, 3.01 mmol) and tetrakis(triphenylphosphine) palladium (15.8 mg, 13.7 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 98 % (421 mg).

2-Fluoro-4-[5-(3-hydroxyphenyl)-2-thienyl]phenol (23). The title compound was prepared by reaction of 2-(3-fluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (**23a**) (240 mg, 0.76 mmol) and boron tribromide (4.56 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 90 % (195 mg); MS, ESI: 285 $(M-H)^+$; Anal. ($C_{16}H_{11}FO_2S$) C, H, N.

2-(3-Methoxyphenyl)-5-[4-methoxy-3-(trifluoromethyl)phenyl]thiophene (24a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (408 mg, 1.51 mmol), 3-trifluoromethyl-4-methoxybenzeneboronic acid (398 mg, 1.81 mmol), caesium carbonate (1033 mg, 3.32 mmol) and tetrakis(triphenylphosphine) palladium (17.5 mg, 15.1 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 75 % (412 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl]-2-(trifluoromethyl)phenol (24). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-5-[4-methoxy-3-(trifluoromethyl)phenyl]-thiophene (**24a**) (300 mg, 0.82 mmol) and boron tribromide (4.95 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 98 % (272 mg); MS, ESI: 285 $(M-H)^+$; Anal. ($C_{17}H_{11}F_3O_2S$) C, H, N.

2-(6-Methoxybiphenyl-3-yl)-5-(3-methoxyphenyl)thiophene (25a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (287 mg, 1.07 mmol), [6-methoxy-1,1'-biphenyl-3-yl]boronic acid (**25b**) (338 mg, 1.29 mmol), sodium carbonate (250

mg, 2.35 mmol) and tetrakis(triphenylphosphine) palladium (61.8 mg, 53.5 µmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 35 % (135 mg).

5-[5-(3-Hydroxyphenyl)-2-thienyl]biphenyl-2-ol (25). The title compound was prepared by reaction of 2-(6-methoxybiphenyl-3-yl)-5-(3-methoxyphenyl)thiophene (**25a**) (100 mg, 0.26 mmol) and boron tribromide (1.56 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 88 % (81 mg); MS, ESI: 343 $(M-H)^+$; Anal. ($C_{22}H_{16}O_2S$) C, H, N.

[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]methanol (26c). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (408 mg, 1.51 mmol), [3-(hydroxymethyl)-4-methoxyphenyl]-boronic acid (**26d**) (329 mg, 1.81 mmol), caesium carbonate (1032 mg, 3.32 mmol) and tetrakis(triphenylphosphine) palladium (17.5 mg, 15.1 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 8:2); yield: 12 % (59 mg).

2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]benzaldehyde (26b). To a solution of [2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]methanol (**26c**) (100 mg, 0.31 mmol, 1 eq) in dichloromethane, pyridium chlorochromate (66 mg, 0.31 mmol, 1 eq) was added in small portions over 5 min and stirred at rt. After 30 min, the reaction was quenched with water. The resulting organic layer was dried over sodium sulfate, filtered and concentrated to dryness. The title compound was not characterized and used in the next step without purification.

Ethyl-(2*E*)-3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]acrylate (26a). To a solution of sodium hydride (10.4 mg, 0.43 mmol, 1 eq) in anhydrous THF triethyl phosphonate (93 µL, 0.46 mmol, 1.1 eq) was added dropwise and stirred at rt. After 15 min, 2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]benzaldehyde (**26b**) (100 mg, 0.31 mmol, 0.6 eq) was added and stirred for 4 h at rt. To quench the reaction water was added and the resulting organic layer was washed with brine, dried over sodium sulfate, filtered, evaporated and purified by CC (hexane/ethyl acetate 7:3); yield: 98 % (120 mg).

Ethyl-(2*E*)-3-[2-hydroxy-5-[5-(3-hydroxyphenyl)-2-thienyl]phenyl]acrylate (26). The title compound was prepared by reaction of ethyl (2*E*)-3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]acrylate (**26a**) (60 mg, 0.15 mmol) and boron tribromide (0.90 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 17 % (10 mg); MS (APCI): 366 $(M)^+$; Anal. ($C_{21}H_{18}O_4S$) C, H, N.

(2*E*)-3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (27a). Ethyl-(2*E*)-3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]acrylate (**26a**) (720 mg, 2.22 mmol, 1 eq) in a solution of THF/water (2:1), was refluxed for 20 h together with lithium hydroxide (320 mg, 13.33 mmol, 6 eq). After cooling to rt, ether was added, the aqueous layer was acidified with hydrochloric acid 1N and washed with dichloromethane. The combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure. The resulting carboxylic acid was solubilized in dichloromethane (180 mg, 0.53 mmol, 1 eq) and refluxed for 20 h with EDCI (102 mg, 0.53 mmol, 1 eq) and HOBt (72 mg, 0.53 mmol, 1 eq). After cooling to rt, the organic layer was washed with a 1.5 M sodium hydrogenocarbonate solution, brine, dried over sodium sulfate, evaporated under reduced pressure and purified by CC (hexane/ethyl acetate 7:3); yield: 51 % (120 mg); MS, ESI: 442 $(M+H)^+$.

(2*E*)-3-[2-Hydroxy-5-[5-(3-hydroxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (27). The title compound was prepared by reaction of (2*E*)-3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (**27a**) (55 mg, 0.13 mmol) and boron tribromide (0.78 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 4:6); yield: 31 % (17 mg); MS, ESI: 414 $(M+H)^+$; Anal. ($C_{25}H_{19}NO_3S$) C, H, N.

3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylpropanamide (28a). (2*E*)-3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (**27a**) (50 mg, 0.11 mmol, 1 eq) was solubilized in thr mixture of THF/EtOH (1:1). After addition of palladium hydroxide (1.7 mg, 0.01 mmol, 0.1 eq) the reaction was stirred at rt under nitrogen atmosphere for 20 h. The crude mixture was filtered and the organic layer was evaporated under reduced pressure; yield: quantitative.

3-[2-Hydroxy-5-[5-(3-hydroxyphenyl)-2-thienyl]phenyl]-*N*-phenylpropanamide (28). The title compound was prepared by reaction of 3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylpropanamide (**28a**) (55 mg, 0.13 mmol) and boron tribromide (0.78 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 20 % (10 mg); MS, ESI: 416 $(M+H)^+$; Anal. ($C_{25}H_{21}NO_3S$) C, H, N.

2-Bromo-5-(3-fluoro-4-methoxyphenyl)thiophene (29b). The title compound was prepared by reaction of 2,5-dibromothiophene (500 mg, 2.10 mmol), 3-fluoro-4-methoxybenzeneboronic acid (357 mg, 2.10 mmol), sodium carbonate (432 mg, 4.20 mmol) and tetrakis(triphenylphosphine) palladium (121 mg, 1.05 mmol) according to method B. The product was purified by CC (hexane/ethyl acetate 95:5); yield: 85 % (427 mg).

2-(3-Fluoro-4-methoxyphenyl)-5-(3-fluoro-5-methoxyphenyl)thiophene (29a). The title compound was prepared by reaction of 2-bromo-5-(3-fluoro-4-methoxyphenyl)thiophene (**29b**) (100 mg, 0.41 mmol), 3-fluoro-5-methoxybenzeneboronic acid (85 mg, 0.50 mmol), caesium carbonate (280 mg, 0.90 mmol) and tetrakis(triphenylphosphine) palladium (4.7 mg, 4.1 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 82 % (113 mg).

2-Fluoro-4-[5-(3-fluoro-5-hydroxyphenyl)thien-2-yl]phenol (29). The title compound was prepared by reaction of 2-(3-fluoro-4-methoxyphenyl)-5-(3-fluoro-5-methoxyphenyl)thiophene (**30a**) (100 mg, 0.30 mmol) and boron tribromide (1.80 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 38 % (35 mg); MS, APCI: 304 $(M)^+$; Anal. ($C_{16}H_{10}F_2O_2S$) C, H, N.

2-(3-Fluoro-4-methoxyphenyl)-5-(4-fluoro-3-methoxyphenyl)thiophene (30a). The title compound was prepared by reaction of 2-bromo-5-(3-fluoro-4-methoxyphenyl)thiophene (**29b**) (100 mg, 0.41 mmol), 4-fluoro-3-methoxybenzeneboronic acid (85 mg, 0.50 mmol), caesium carbonate (279 mg, 0.90 mmol) and tetrakis(triphenylphosphine) palladium (4.7 mg, 4.1 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 72 % (100 mg).

2-Fluoro-4-[5-(4-fluoro-3-hydroxyphenyl)thien-2-yl]phenol (30). The title compound was prepared by reaction of 2-(3-fluoro-4-methoxyphenyl)-5-(3-fluoro-5-methoxyphenyl)thiophene (**30a**) (100 mg, 0.30 mmol) and boron tribromide (1.80 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 75 % (69 mg); MS, APCI: 304 $(M)^+$; Anal. ($C_{16}H_{10}F_2O_2S$) C, H, N.

2-(3,5-Difluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (31a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (430 mg, 1.60 mmol), 3,5-difluoro-4-methoxybenzeneboronic acid (357 mg, 1.92 mmol), caesium carbonate (1094 mg, 3.52 mmol) and tetrakis(triphenylphosphine) palladium (18.5 mg, 16.0 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 42 % (223 mg).

2,6-Difluoro-4-[5-(3-hydroxyphenyl)-2-thienyl]phenol (31). The title compound was prepared by reaction of 2-(3,5-difluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (**31a**)

(220 mg, 0.62 mmol) and boron tribromide (3.72 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 10 % (18 mg); MS, ESI: 305 $(M+H)^+$; Anal. ($C_{16}H_{10}F_2O_2S$) C, H, N.

2-(3,4-Difluorophenyl)-5-(3-methoxyphenyl)thiophene (32a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 3,4-difluorobenzeneboronic acid (105 mg, 0.67 mmol), caesium carbonate (383 mg, 1.23 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 90 % (152 mg).

3-[5-(3,4-Difluorophenyl)-2-thienyl]phenol (32). The title compound was prepared by reaction of 2-(3,4-difluorophenyl)-5-(3-methoxyphenyl)thiophene (**32a**) (120 mg, 0.40 mmol) and boron tribromide (1.20 mmol) according to method C. The product was purified by CC (dichloromethane/methanol 99:1); yield: 85 % (98 mg); MS, ESI: 289 $(M+H)^+$; Anal. ($C_{16}H_9F_2OS$) C, H, N.

4-(4-Methoxy-3-methylphenyl)-2-(3-methoxyphenyl)thiophene (34a). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**33b**) (400 mg, 1.49 mmol), 3-methyl-4-methoxybenzeneboronic acid (296 mg, 1.79 mmol), caesium carbonate (1019 mg, 3.27 mmol) and tetrakis(triphenylphosphine) palladium (17.2 mg, 14.9 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 69 % (320 mg).

4-[5-(3-Hydroxyphenyl)-3-thienyl]-2-methylphenol (34). The title compound was prepared by reaction of 4-(4-methoxy-3-methylphenyl)-2-(3-methoxyphenyl)thiophene (**34a**) (180 mg, 0.58 mmol) and boron tribromide (3.48 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 54 % (88 mg); MS, ESI: 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

4-(3-Fluoro-4-methoxyphenyl)-2-(3-methoxyphenyl)thiophene (35a). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**33b**) (400 mg, 1.49 mmol), 3-fluoro-4-methoxybenzeneboronic acid (303 mg, 1.78 mmol), caesium carbonate (1019 mg, 3.30 mmol) and tetrakis(triphenylphosphine) palladium (17.2 mg, 14.9 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 80 % (403 mg).

2-Fluoro-4-[5-(3-hydroxyphenyl)-3-thienyl]phenol (35). The title compound was prepared by reaction of 4-(3-fluoro-4-methoxyphenyl)-2-(3-methoxyphenyl)thiophene (**35a**) (400 mg, 1.27 mmol) and boron tribromide (7.63 mmol) according to method C. The product was purified by CC (dichloromethane/methanol 98:2); yield: 22 % (88 mg); MS, ESI: 287 $(M-H)^-$

4-(4-Methoxy-3-methylphenyl)-2-(3-methoxyphenyl)-1,3-thiazole (37a). The title compound was prepared by reaction of 5-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**36b**) (402 mg, 1.49 mmol), 3-methyl-4-methoxybenzeneboronic acid (247 mg, 1.79 mmol), caesium carbonate (1019 mg, 3.27 mmol) and tetrakis(triphenylphosphine) palladium (17.2 mg, 14.9 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 69 % (320 mg).

4-[2-(3-Hydroxyphenyl)-1,3-thiazol-5-yl]-2-methylphenol (37). The title compound was prepared by reaction of 4-(4-methoxy-3-methylphenyl)-2-(3-methoxyphenyl)-1,3-thiazole (**37a**) (80 mg, 0.26 mmol) and boron tribromide (1.56 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 3:7); yield: 16 % (11 mg), MS, ESI: 274 $(M+H)^+$; Anal. ($C_{16}H_{13}NO_2S$) C, H, N.

3-Fluoro-3'',4-dimethoxy-1,1':4',1''-terphenyl (39a). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**38b**) (175 mg, 0.67 mmol), 3-fluoro-4-

methoxybenzeneboronic acid (136.7 mg, 0.88 mmol), caesium carbonate (457 mg, 1.47 mmol) and tetrakis(triphenylphosphine) palladium (7.7 mg, 6.7 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 98:2); yield: 58 % (117 mg).

3''-Fluoro-1,1':4',1''-terphenyl-3,4''-diol (**39**). The title compound was prepared by reaction of 3-fluoro-3",4-dimethoxy-1,1':4',1"-terphenyl (**39a**) (115 mg, 0.37 mmol) and boron tribromide (2.22 mmol) according to method C. The product was purified by preparative TLC (dichloromethane/methanol 99:1); yield: 62 % (65 mg); MS, ESI: 281 $(M+H)^+$.

3,3''-Dimethoxy-4-methyl-1,1':4',1''-terphenyl (**41a**). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**38b**) (230 mg, 0.87 mmol), 4-methoxy-3-methylbenzeneboronic acid (172 mg, 1.04 mmol), caesium carbonate (595 mg, 1.91 mmol) and tetrakis(triphenylphosphine) palladium (10.1 mg, 8.7 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 98:2); yield: 53 % (140 mg).

4-Methyl-1,1':4',1''-terphenyl-3,3''-diol (**41**). The title compound was prepared by reaction of 3,3"-dimethoxy-4-methyl-1,1':4',1"-terphenyl (**41a**) (120 mg, 0.39 mmol) and boron tribromide (2.34 mmol) according to method C. The product was purified by preparative TLC (dichloromethane/methanol 97:3); yield: 38 % (42 mg); MS, ESI: 277 $(M+H)^+$.

4-Fluoro-3,3''-dimethoxy-1,1':4',1''-terphenyl (**42a**). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**38b**) (200 mg, 0.76 mmol), 4-fluoro-3-methoxybenzeneboronic acid (154 mg, 0.91 mmol), caesium carbonate (520 mg, 1.67 mmol) and tetrakis(triphenylphosphine) palladium (8.8 mg, 7.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 75 % (175 mg).

4-Fluoro-1,1':4',1''-terphenyl-3,3''-diol (42). The title compound was prepared by reaction of 4-fluoro-3,3"-dimethoxy-1,1':4',1"-terphenyl (**42a**) (175 mg, 0.57 mmol) and boron tribromide solution (3.42 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 63 % (100 mg); MS, ESI: 281 $(M+H)^+$.

Biological Methods

[2, 4, 6, 7-^{3}H]-E2 and [2, 4, 6, 7-^{3}H]-E1 were bought from Perkin Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt.
17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures (Kruchten *et al.*, 2009c; Qiu *et al.*, 2002; Zhu *et al.*, 1993). Fresh human placenta was homogenized and centrifuged. The pellet fraction contains the microsomal 17β-HSD2, while 17β-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction.

1. Inhibition of 17β-HSD1
Inhibitory activities were evaluated by a well established method with minor modifications (Lin *et al.*, 1992; Sam *et al.*, 1995; Sam *et al.*, 1998). Briefly, the enzyme preparation was incubated with NADH [500 µM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20 % of glycerol and EDTA (1mM)**.** Inhibitor stock solutions were prepared in DMSO. Final concentration of DMSO was adjusted to 1 % in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled and [2, 4, 6, 7-^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 rp chromatography column (Nucleodur C18 Gravity, 3 µm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated according to following

equation: $\%conversion = \frac{\%E2}{\%E2 + \%E1} \cdot 100$. Each value was calculated from at least three independent experiments.

2. Inhibition of 17β-HSD2

The 17β-HSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD^+ [1500 µM], test compound and a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi) for 20 min at 37 °C. Further treatment of the samples and HPLC separation was carried out as mentioned above.

3. ER affinity

The binding affinity of selected compounds to the ERα and ERβ was determined according to Zimmermann *et al.*, 2005. Briefly, 0.25 pmol of ERα or ERβ, respectively, were incubated with [2, 4, 6, 7-^{3}H]-E2 (10 nM) and test compound for 1 h at rt. The potential inhibitors were dissolved in DMSO (5 % final concentration). Non-specific-binding was performed with diethylstilbestrol (10 µM). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (5 g/ 60 mL TE-buffer). The formed complex was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). For determination of the relative binding affinity, inhibitor and E2 concentrations required to displace 50 % of the receptor bound labelled E2 were determined. RBA values were calculated according to the following equation: $RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \cdot 100$. The RBA value for E2 was arbitrarily set at 100 %.

4. Evaluation of the estrogenic activity using T47D cells

Phenol red-free medium was supplemented with sodium bicarbonate (2 g/ L), streptomycin (100 µg/ mL), insuline zinc salt (10 µg/ mL), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/ mL) and DCC-FCS 5 % (v/v). RPMI 1640 (without phenol red) was used for the experiments. Cells were grown for 48 h in phenol red-free medium. Compound **21** was added at a final concentration of 100 nM. Inhibitors and E2 were diluted in ethanol (final ethanol concentration was adjusted to 1 %). As a positive control E2 was added at a final concentration of 0.1 nM. Ethanol was used as negative control. Medium was changed every two to three days and supplemented with the respective additive. After eight days of incubation, the cell viability was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). The cleavage of MTT to a blue formazan by mitochondrial succinat-dehydrogenase was quantified spectrophotometrically at 590 nm as described by Denizot and Lang, 1986 with minor modifications. The control proliferation was arbitrarily set at 1 and the stimulation induced by the inhibitor was calculated according to following equation: $\%stimulation = \frac{[proliferation(compound-induced)-1]}{[proliferation(E2-induced)-1]} \cdot 100\%$. Each value is calculated as a mean value of at least three independent experiments

5. Inhibition of human hepatic CYPs

The commercially available P450 inhibition kits from BD Gentest™ (Heidelberg, Germany) were used according to the instructions of the manufacturer. Compounds **21** and **23** were tested for inhibition of the following enzymes: CYP2D6 and 3A4. Percentage of inhibition at 2 µM for compounds **21** and **23** were determined.

6. *In Vivo* Pharmacokinetics

Male Wistar rats weighing 300-330 g (Janvier France) were housed in a temperature-controlled room (20-22 °C) and maintained in a 12 h light/12 h dark cycle. Food and water were available *ad libitum*. They were anesthetized with a ketamine (135 mg/kg)/ xylyzine (10 mg/kg) mixture and cannulated with silicone tubing via the right jugular vein and attached to the skull with

dental cement. Prior to the first blood sampling, animals were connected to a counterbalanced system and tubing to perform blood sampling in the freely moving rat.
Compounds **21** and **23** were applied orally in a cassette dosing in 4 rats at the dose of 10 mg/kg body weight by using a feeding needle. The compounds were dissolved in a mixture labrasol/water (1:1) and given at a volume of 5mL/kg. Blood samples (0.2 mL) were taken at 0, 1, 2, 3, 4, 6, 8, 10 and 24 h postdose and collected in heparinised tubes. They were centrifuged at 3000g for 10 min, and plasma was harvested and kept at -20 °C until analyzed.
HPLC-MS/MS analysis and quantification of the samples was carried out on a Surveyor-HPLC-system coupled with a TSQ Quantum (Thermo/Fisher) triple quadrupole mass spectrometer equipped with an electrospray interface.

Computational Chemistry

1. Molecular Modelling
All molecular modelling studies were performed on Intel(R) P4 CPU 3.00 GHz running Linux CentOS 5.2. The X-ray structures of 17β-HSD1 (PDB-ID: 1A27, 1FDT and 1I5R) were obtained from the Protein Databank (Berman *et al.*, 2000) and further prepared using the BIOPOLYMER module of SYBYL v8.0 (Sybyl, Tripos Inc., St. Louis, Missouri, USA). Water molecules, E2 (or HYC for 1I5R) and sulfate ions were stripped from the PDB files and missing protein atoms were added and correct atom types set. Finally hydrogen atoms and neutral end groups were added. All basic and acidic residues were considered protonated and deprotonated, respectively. Since almost all histidines are oriented toward the outer part of the enzyme, accessible for the surface, they were considered as protonated (HIP) after a prediction run made by MolProbity (Davis *et al.*, 2007). For 1I5R the cofactor NADPH was merged into the enzyme after an accurate overlay with the hybrid inhibitor HYC and the X-rays 1A27 and 1FDT. Further, every crystal structure was minimized for 500 steps with the steepest descent minimizer as implemented in SYBYL with the backbone atoms kept at fixed positions in order to fix close contacts, followed by 2000 steps conjugate gradient minimization requested for an overall better starting structure.
Inhibitor **23** was built with SYBYL and energy-minimized in MMFF94s force-field as implemented in Sybyl. Subsequently an *ab-initio* geometry optimizations was performed gas phase at the B3LYP/6-311**G (d,p) level of density functional theory (DFT) by means of the Gaussian03 software (Bayly *et al.*, 1993; Frisch *et al.*, 2003), in order to obtain the RESP charges of compound **23**, thought to better perform in Autodock4.
Two different softwares were used for docking studies: GOLDv3.2 (Jones *et al.*, 1997) and Autodock4 (Huey *et al.*, 2007; Morris *et al.*, 1998), using the graphical user interface AutoDockTools (ADT 1.5.2). Since both allow flexible docking of ligands, no conformational search was employed to the ligand structure. For both programs the compound **23** was docked in 50 independent genetic algorithm (GA) runs.
GOLDv3.2: Active-site origin was set at the center of the steroid binding site, while the radius was set equal to 13 Å. The automatic active-site detection was switched on. Further, a slightly modified GOLDSCORE fitness function (increased scaling for hydrophobic contacts) was used and genetic algorithm default parameters were set as suggested by the GOLD authors.
Autodock4: The docking area has been defined by a box, centered on the mass center of the CD-rings of the cocrystallized E2. Grids points of 60 × 70 × 74 with 0.375 Å spacing were calculated around the docking area for all the ligand atom types using AutoGrid4. The Lamarckian genetic algorithm local search (GALS) method was used. Each docking run was performed with a population size of 200. A mutation rate of 0.02 and a crossover rate of 0.8 were used to generate new docking trials for subsequent generations. The GALS method evaluates a population of possible docking solutions and propagates the most successful individuals from each generation into the next one.
Both programs performed in a similar way, supporting the herein suggested binding modes. The quality of the docked poses was evaluated based mainly on visual inspection of the putative binding modes of the ligand, and secondly on the scoring functions, which give a good measure

to discriminate between the found binding modes for one single X-ray conformation, but do not help us to compare the poses of different X-rays.

2. MEP

For selected compounds *ab-initio* geometry optimisations were performed gas phase at the B3LYP/6-311**G (d,p) level of density functional theory (DFT) by means of the Gaussian03 software and the molecular electrostatics potential map (MEP) was plotted using GaussView3, the 3D molecular graphics package of Gaussian (Dennington *et al.*, 2003). These electrostatic potential surfaces were generated by mapping 6-311G** electrostatic potentials onto surfaces of molecular electron density (isovalue = 0.0002e/Å). The MEP maps are color coded, where red stands for negative values ($3.1*10^{-2}$ Hartree) and blue for positive ones ($4.5*10^{-2}$ Hartree).

Acknowledgments

We are grateful to the Deutsche Forschungsgemeinschaft (HA1315/8-1) for financial support. We thank Anja Palusczak, Beate Geiger and Jannine Ludwig for their help in performing the enzyme inhibition tests (17β-HSD1, 17β-HSD2, ERα and ERβ) and Dr. Ursula Müller-Vieira, Pharmacelsus CRO Saarbrücken Germany, for the hepatic CYP inhibition tests. Patricia Kruchten is grateful to the European Postgraduate School 532 (DFG) for a scholarship.

References

Adamo, V.; Iorfida, M.; Montalto, E.; Festa, V.; Garipoli, C.; Scimone, A.; Zanghi, M.; Caristi, N.: Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. *Ann. Oncol.* 18 Suppl 6 (2007) vi53-57.

Allan, G. M.; Vicker, N.; Lawrence, H. R.; Tutill, H. J.; Day, J. M.; Huchet, M.; Ferrandis, E.; Reed, M. J.; Purohit, A.; Potter, B. V.: Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem.* 16 (2008) 4438-4456.

Al-Soud, Y. A.; Bey, E.; Oster, A.; Marchais-Oberwinkler, S.; Werth, R.; Kruchten, P.; Frotscher, M.; Hartmann, R. W.: The role of the heterocycle in bis(hydroxyphenyl) triazoles for inhibition of 17beta-hydroxysteroid dehydrogenase (17beta-HSD) type 1 and type 2. *Mol. Cell. Endocrinol.* 301 (2009) 212-215.

Azzi, A.; Rehse, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S.-X.: Crystal structure of human estrogenic 17beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol. *Nat. Struct. Biol.* 3 (1996) 665-668.

Bayly, C. I.; Cieplak, P.; Cornell, W. D.; Kollman, P. A.: A well behaved electrostatic potential based method using charge restraints for determining atom-centered charges: the RESP model. *J. Phys. Chem.* 97 (1993) 10269-10280.

Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E.: The Protein Data Bank. *Nucleic Acids Res.* 28 (2000) 235-242.

Bérubé, M.; Poirier, D.: Synthesis of simplified hybrid inhibitors of type 1 17beta-hydroxysteroid dehydrogenase via cross-metathesis and sonogashira coupling Reactions. *Org. Lett.* 6 (2004) 3127-3130.

Bey, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Werth, R.; Oster, A.; Algül, O.; Neugebauer, A.; Hartmann, R. W.: Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* 16 (2008a) 6423-6435.

Bey, E.; Marchais-Oberwinkler, S.; Werth, R.; Negri, M.; Al-Soud, Y. A.; Kruchten, P.; Oster, A.; Frotscher, M.; Birk, B.; Hartmann, R. W.: Design, synthesis, biological evaluation and pharmacokinetics of bis(hydroxyphenyl) substituted azoles, thiophenes, benzenes and aza-benzenes as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1). *J. Med. Chem.* 51 (2008b) 6725-6739.

Bohm, H. J.; Banner, D.; Bendels, S.; Kansy, M.; Kuhn, B.; Muller, K.; Obst-Sander, U.; Stahl, M.: Fluorine in medicinal chemistry. *ChemBioChem* 5 (2004) 637-643.

Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C.: The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors. *Structure* 4 (1996) 905-915.

Brožic, P.; Lanišnik Rižner, T.; Gobec, S.: Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* 15 (2008) 137-150 and references therein cited.

Bruno, I. J.; Cole, J. C.; Lommerse, J. P. M.; Rowland, R. S.; Verdonk, M. L. Isostar: A library of information about non-bonded interactions. *J. Comput. Chem.* 11 (1997) 523-537.

Bush, N. J.: Advances in hormonal therapy for breast cancer. *Semin. Oncol. Nurs.* 23 (2007) 46-54.

Davis, I. W.; Leaver-Fay, A.; Chen, V. B.; Block, J. N.; Kapral, G. J.; Wang, X.; Murray, L. W.; Arendall, W. B., 3rd; Snoeyink, J.; Richardson, J. S.; Richardson, D. C.: MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res.* 35 (2007) W375-383.

Day, J. M.; Foster, P. A.; Tutill, H. J.; Parsons, M. F.; Newman, S. P.; Chander, S. K.; Allan, G. M.; Lawrence, H. R.; Vicker, N.; Potter, B. V.; Reed, M. J.; Purohit, A.: 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* 122 (2008) 1931-1940.

Denizot, F.; Lang, R.: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89 (1986) 271-277.

Dennington, I.; Roy, K. T.; Millam, J.; Eppinnett, K.; Howell, W. L.; Gilliland, R.: GaussView, 3.0; Semichem Inc., Shawnee Mission (2003).

Dizerega, G. S.; Barber, D. L.; Hodgen, G. D.: Endometriosis: role of ovarian steroids in initiation, maintenance and suppression. *Fertil. Steril.* 33 (1980) 649-653.

Duggan, H. M. E.; Leroux, F. G. M.; Malagu, K.; Martin, N. M. B.; Menear, K. A.; Smith, G. C. M.: 2-Methylmorpholine-substituted pyrido-, pyrazo- and pyrimidopyrimidine derivs. as mTOR inhibitors and their preparation, pharmaceutical compositions and use in the treatment of cancer. WO 2008023161 A1 20080228, (2008).

Emmons, W. D.: The utility of phosphonate carbanions in olefin synthesis. *J. Am. Chem. Soc.* 83 (1961) 1733-1738.

Fournier, D.; Poirier, D.; Mazumdar, M.; Lin, S.-X.: Design and synthesis of bisubstrate inhibitors of type 1 17beta-hydroxysteroid dehydrogenase: Overview and perspectives. *Eur. J. Med. Chem.* 43 (2008) 2298-2306.

Frisch, M. J.; Trucks, G. W. S., H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A. Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen,; W.; Wong, M. W. G., C.; Pople, J. A.: Gaussian, Inc.: Pittsburgh, PA: (2003).

Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W.: Design, synthesis and biological evaluation of (hydroxyphenyl)-naphthalene and quinoline derivatives: potent and selective non steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* 51 (2008) 2158-2169.

Ghosh, D.; Pletnev, V. Z.; Zhu, D. W.; Wawrzak, Z.; Duax, W. L.; Pangborn, W.; Labrie, F.; Lin, S.-X.: Structure of human estrogenic 17beta- hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* 3 (1995) 503-513.

Gunnarsson, C.; Hellqvist, E.; Stål, O.: 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* 92 (2005) 547-552.

Gunnarsson, C.; Olsson, B. M.; Stål, O.: Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* 61 (2001) 8448-8451.

Hagmann, W. K.: The many roles for fluorine in medicinal chemistry. *J. Med. Chem.* 51 (2008) 4359-4369.

Hartman, J.; Lindberg, K.; Morani, A.; Inzunza, J.; Strom, A.; Gustafsson, J. A.: Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res.* 66 (2006) 11207-11213.

Huey, R.; Morris, G. M.; Olson, A. J.; Goodsell, D. S.: A Semiempirical Free Energy Force Field with Charge-Based Desolvation. *J. Comput. Chem.* 28 (2007) 1145-1152.

Husen, B.; Huhtinen, K.; Poutanen, M.; Kangas, L.; Messinger, J.; Thole, H.: Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. *Mol. Cell. Endocrinol.* 248 (2006a) 109-113.

Husen, B.; Huhtinen, K.; Saloniemi, T.; Messinger, J.; Thole, H. H.; Poutanen, M.: Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology 147* (2006b) 5333-5339.

Hwang, K.-J.; O'Neil, J. P.; Katzenellenbogen, J. A.: 5,6,11,12-Tetrahydrochrysenes: synthesis of rigid stilbene systems designed to be fluorescent ligands for the estrogen receptor. *J. Org. Chem.* 57 (1992) 1262-1271.

Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R.: Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol* 267 (1997) 727-748.

Karkola, S.; Lilienkampf, A.; Wähälä, K.: A 3D QSAR model of 17beta-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core applying molecular dynamics simulations and ligand-protein docking. *ChemMedChem* 3 (2008) 461-472.

Kruchten, P.; Werth, R.; Bey, E.; Oster, A.; Marchais-Oberwinkler, S.; Frotscher, M.; Hartmann, R. W.: Selective inhibition of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) reduces estrogen responsive cell growth of T-47D breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 114 (2009a) 300-306.

Kruchten, P.; Werth, R.; Marchais-Oberwinkler, S.; Bey, E.; Ziegler, E.; Oster, A.; Frotscher, M.; Hartmann, R. W.: Development of biological assays for the identification of selective inhibitors of estradiol formation from estrone in rat liver preparations. *C. R. Chim.* (2009b) submitted.

Kruchten, P.; Werth, R.; Marchais-Oberwinkler, S.; Frotscher, M.; Hartmann, R. W.: Development of a biological screening system for the evaluation of highly active and selective 17beta-HSD1-inhibitors as potential therapeutic agents. *Mol. Cell. Endocrinol.* 301 (2009c) 154-157.

Lin, S.-X.; Yang, F.; Jin, J. Z.; Breton, R.; Zhu, D. W.; Luu-The, V.; Labrie, F.: Subunit identity of the dimeric 17beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem.* 267 (1992) 16182-16187.

Marchais-Oberwinkler, S.; Frotscher, M.; Ziegler, E.; Werth, R.; Kruchten, P.; Messinger, J.; Thole, H.; Hartmann, R. W.: Investigation of a new class of potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases: 6-(3'-hydroxyphenyl) naphthalenes. *Mol. Cell. Endocrinol.* 301 (2009) 205-211

Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Ziegler, E.; Neugebauer, A.; Bhoga, U. D.; Bey, E.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W.: Substituted 6-phenyl-2-naphthols. Potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation and pharmacokinetics. *J. Med. Chem.* 51 (2008) 4685-4698.

Mazza, C.: Human type I 17beta-hydroxysteroid dehydrogenase: site directed mutagenesis and X-Ray crystallography structure-function analysis. PhD Thesis, Université Joseph Fourier, Grenoble, (1997).

Messinger, J.; Hirvelä, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikäinen, O.; Saarenketo, P.; Thole, H.: New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* 248 (2006) 192-198.

Miller, W. R.; Bartlett, J. M.; Canney, P.; Verrill, M.: Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. Treat.* 103 (2007) 149-160.

Miyaura, N.; Suzuki, A.: The palladium catalysed cross-coupling reaction of phenylboronic acid with haloarenes in the presence of bases. *Synth. Commun.* 11 (1995) 513-519.

Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S.: Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* 94 (2001) 685-689.

Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J.: Automated Docking Using a Lamarckian Genetic Algorithm and Empirical Binding Free Energy Function. *J. Comput. Chem.* 19 (1998) 1639-1662.

Muller, K.; Faeh, C.; Diederich, F.: Fluorine in pharmaceuticals: looking beyond intuition. *Science* 317 (2007) 1881-1886.

Ohnesorg, T.; Keller, B.; Hrabe de Angelis, M.; Adamski, J.: Transcriptional regulation of human and murine 17beta-hydroxysteroid dehydrogenase type-7 confers its participation in cholesterol biosynthesis. *J. Mol. Endocrinol.* 37 (2006) 185-197.

Pascal, C.; Dubois, J.; Guénard, D.; Guéritte, F.: Synthesis of biphenyls mimicking the structure of the antimitotic rhazinilam. *J. Org. Chem.* 63 (1998) 6414-6420.

Penning, T. M.: 17beta-hydroxysteroid dehydrogenase: inhibitors and inhibitor design. *Endocr. Relat. Cancer* 3 (1996) 41-56.

Pfahl, M.; Al-Shamma, H. A.; Fanjul, A.; Pleynet, D. P. M.; Spruce, L. W.; Wiemann, T. R.; Ibarra, J. B.; Tachdjian, C.: Preparation of benzylidenethiazolidenediones and analogs as antitumor agents. WO 2002072009 A2 20020919 (2002).

Poirier, D.: Inhibitors of 17 beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* 10 (2003) 453-477.

Poirier, D.; Boivin, R. P.; Tremblay, M. R.; Bérubé, M.; Qiu, W.; Lin, S.-X.: Estradiol-adenosine hybrid compounds designed to inhibit type 1 17beta-hydroxysteroid dehydrogenase. *J. Med. Chem.* 48 (2005) 8134-8147.

Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D.; Lin, S.-X.: A concerted, rational design of type 1 17beta-hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity. *FASEB J.* 16 (2002) 1829-1831.

Sakurai, N.; Miki, Y.; Suzuki, T.; Watanabe, K.; Narita, T.; Ando, K.; Yung, T. M.; Aoki, D.; Sasano, H.; Handa, H.: Systemic distribution and tissue localizations of human 17beta-hydroxysteroid dehydrogenase type 12. *J. Steroid Biochem. Mol. Biol*. 99 (2006) 174-181.

Sam, K. M.; Auger, S.; Luu-The, V.; Poirier, D.: Steroidal spiro-gamma-lactones that inhibit 17beta-hydroxysteroid dehydrogenase activity in human placental microsomes. *J. Med. Chem.* 38 (1995) 4518-4528.

Sam, K. M.; Boivin, R. P.; Tremblay, M. R.; Auger, S.; Poirier, D.: C16 and C17 derivatives of estradiol as inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug. Des. Discov.* 15 (1998) 157-180.

Shehu, A.; Mao, J.; Gibori, G. B.; Halperin, J.; Le, J.; Devi, Y. S.; Merrill, B.; Kiyokawa, H.; Gibori, G.: Prolactin receptor-associated protein/17beta-hydroxysteroid dehydrogenase type 7 gene (Hsd17b7) plays a crucial role in embryonic development and fetal survival. *Mol. Endocrinol.* 22 (2008) 2268-2277.

Smith, G.; Mikkelsen, G.; Eskildsen, J.; Bundgaard, C.: The synthesis and SAR of 2-arylsulfanylphenyl-1-oxyalkylamino acids as GlyT-1 inhibitors. *Bioorg. Med. Chem.* 16 (2006) 3981-3884.

Šmuc, T.; Pucelj Ribič , M.; Šinkovec, J.; Husen, B.; Thole, H.; Lanišnik Rižner , T.: Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* 23 (2007) 105-111.

Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H.: 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* 82 (2000) 518-523.

Togo, H.; Nogami, G.; Yokoyama, M.: Synthetic application of poly[styrene(iodoso diacetate)]. *Synlett* 5 (1998) 534-536.

Travis, R. C.; Key, T. J.: Oestrogen exposure and breast cancer risk. *Breast Cancer Res*. 5 (2003) 239-247.

Zhu, D. W.; Lee, X.; Breton, R.; Ghosh, D.; Pangborn, W.; Duax, W. L.; Lin, S.-X.: Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17beta-hydroxysteroid dehydrogenase with $NADP^+$. *J. Mol. Biol.* 234 (1993) 242-244.

Zimmermann, J.; Liebl, R.; von Angerer, E.: 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J. Steroid Biochem. Mol. Biol.* 94 (2005) 57-66.

3.3 Substituted 6-phenyl-2-naphthols. Potent and selective nonsteroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17betaHSD1): design, synthesis, biological evaluation, and pharmacokinetics

Sandrine Marchais-Oberwinkler, Patricia Kruchten, Martin Frotscher, Erika Ziegler, Alexander Neugebauer, Uma D. Bhoga, Emmanuel Bey, Ursula Müller-Vieira, Josef Messinger, Hubert Thole and Rolf W. Hartmann

Dieses Manuskript wurde als Artikel in folgendem Journal veröffentlicht:
Journal of Medicinal Chemistry (**2008**) *51*, 4685-4698

Publikation III

Abstract

17β-Estradiol (E2) is implicated in the genesis and the development of estrogen-dependent diseases. Its concentration is mainly regulated by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) which catalyzes the reduction of the weak estrogen estrone (E1) to the highly potent E2. This enzyme is thus an important target for the treatment of hormone-dependent diseases. Thirty-seven novel substituted 6-phenyl-2-naphthols were synthesized and evaluated for 17β-HSD1 inhibition, selectivity toward 17β-HSD2 and the estrogen receptors (ERs) α and β as well as pharmacokinetic properties. SAR studies revealed that the compounds most likely bind according to binding mode B to the active site i.e. the 6-phenyl moiety mimicking the steroidal A-ring. While substitution at the phenyl ring decreased activity, introduction of substituents at the naphthol moiety led to highly active compounds, especially in position 1. The 1-phenyl compound **32** showed a very high inhibitory activity for 17β-HSD1 (IC_{50} = 20 nM) and good selectivity (17β-HSD2 and ERs) and pharmacokinetic properties after peroral application.

Introduction

It is well recognized that estrogens play a central role in female physiology. 17β-Estradiol (E2), the endogenous ligand of the estrogen receptors, however, is also known to be involved in the development of estrogen-dependent diseases, inducing cell proliferation in breast cancer (Travis and Key, 2003) and playing a critical role in the development and growth of endometriosis (Dizerega *et al.*, 1980).

Until today, two different strategies have been developed for the treatment of hormone-dependent breast cancer (Bush, 2007; Miller *et al.*, 2007): 1. reduction of the estrogen biosynthesis either by aromatase inhibitors, which prevent the transformation of androgens into estrogens or using GnRH analogues, which inhibit ovarian estrogen formation. 2. blockage of estrogen action at the receptor level via selective estrogen receptor modulators (SERMs).

Another approach to reduce the estrogen action could be achieved by inhibition of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1, EC1.1.1.62), the enzyme catalyzing the last step in E2 biosynthesis.

17β-HSD1 transforms the weak estrogen estrone (E1) into the most potent estrogen E2 (Chart 1). 17β-HSD1 is a cytosolic enzyme which was crystallized with different steroidal ligands (Azzi *et al.*, 1996; Breton *et al.*, 1996; Gangloff *et al.*, 2003; Ghosh *et al.*, 1995; Han *et al.*,

2000; Mazza *et al.*, 1998; Qiu *et al.*, 2002; Sawicki *et al.*, 1999; Shi and Lin, 2004). The X-ray structures provide information on the active site of the enzyme: it is an elongated hydrophobic tunnel with two polar ends (His221, Glu282 on one side and Ser142, Tyr155 on the other side, the last two amino acids are involved in the catalytic tetrad). Interestingly, another two polar amino acids (Ser222 and Tyr218) are also located in this narrow cavity, close to the B/C ring of the steroid but do not interact directly with it.

Chart 1: Interconversion of estrone (E1) to estradiol (E2).

17β-HSD1 is often over-expressed in breast cancer cells (Gunnarsson *et al.*, 2001; Gunnarsson *et al.*, 2005; Miyoshi *et al.*, 2001; Suzuki *et al.*, 2000) resulting in high intracellular E2 concentrations. Inhibition of 17β-HSD1 will prevent the transformation of E1 into E2 and thus will reduce the intracrine (Labrie, 1991) effect of E2. In contrast to aromatase inhibition the E1 level will not be affected and a basal estrogenic activity will be maintained: 17β-HSD1 inhibitors therefore might be softer therapeutics for the treatment of estrogen-dependent diseases, i.e. they should lead to less side-effects. 17β-HSD1 is therefore an attractive target for the design of new drugs for breast cancer and endometriosis.

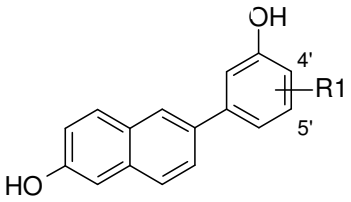
Recently, Laplante *et al.*, 2008 showed that potent estradiol type inhibitors were able to reduce the E1 induced T47D cell proliferation by 62% *in vitro.* Husen *et al.* (Husen *et al.*, 2006a; Husen *et al.*, 2006b) and Day *et al.*, 2008 have described mouse models to evaluate the efficacy of 17β-HSD1 inhibitors *in vivo*. Both groups have used ovariectomized immunodeficient mice and implanted MCF-7 cells stably transfected with human 17β-HSD1 (Husen *et al.*, 2006a; Husen *et al.*, 2006b) or T47D cells (Day *et al.*, 2008), respectively. The E1 induced tumor growth could be reduced by 17β-HSD1 inhibitors.

Regarding the therapeutic concept it is important that inhibitors of 17β-HSD1 are selective toward 17β-HSD2 – which inactivates E2 to E1, thus acting as an adversary to the type 1 enzyme – and toward the estrogen receptors α and β i.e. they should have no or little affinity to these proteins.

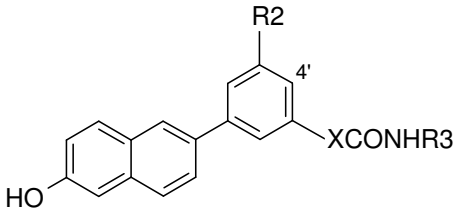
Over the last years, several groups have reported about 17β-HSD1 inhibitors, most of them having a steroidal structure (Brožic *et al.*, 2008; Laplante *et al.*, 2008; Poirier, 2003). The first non-steroidal inhibitors of 17β-HSD1, the thiophenepyrimidinones were published by Messinger *et al.*, 2006. However, most of the non-steroidal 17β-HSD1 inhibitors described so far do not appear to be drug-like. Recently, based on our experience in the design of steroid mimicking compounds (Jagusch *et al.*, 2008; Salem *et al.*, 2006; Ulmschneider *et al.*, 2005), we reported on the discovery of (hydroxyphenyl)naphthalenes as potent and selective inhibitors of 17β-HSD1 (Frotscher *et al.*, 2008). The most promising compound of this series of steroidomimetics was the 6-(3-hydroxyphenyl)-2-naphthol **1** (Chart 2).

Compound **1** can be considered as a scaffold for structure optimization. Introduction of additional substituents leading to new interactions with amino acids from the active site might increase potency and selectivity of this class of inhibitors. In this report, the rational design of novel, selective inhibitors of 17β-HSD1 – based on the analysis of possible binding modes of the scaffold in the substrate binding site of the enzyme – will be presented. Subsequently synthesis and biological evaluation of the derived substituted 6-phenyl-2-naphthols (Chart 2) will be described.

Chart 2: Synthesized compounds.



1 - 6



7 - 18

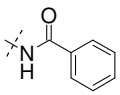
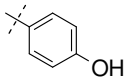
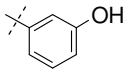
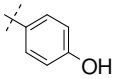
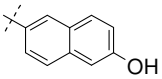
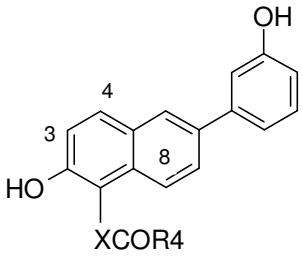
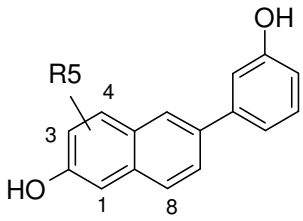
Cmpd	R1	Cmpd	R2	X	R3	Cmpd	R2	*R3*
1		**7**	-OH			**13**	$-NO_2$	
2	4'- 	**8**	-OH			**14**	$-NO_2$	
3	4'- 	**9**	-OH			**15**	$-NO_2$	
4	5'- 	**10**	-OH			**16**	$-NO_2$	
5	5'- 	**11**	-OH			**17**	$-NH_2$	
6	5'- 	**12**	-OH			**18**	$-NH_2$	

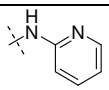
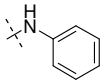
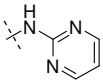
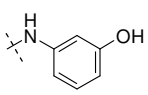
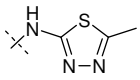
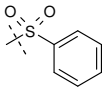
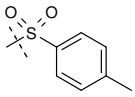
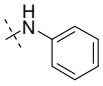
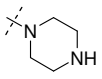
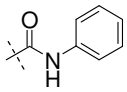
Chart 2: Synthesized compounds (continued).



19 - 30



31 - 38

Cmpd	R4	Cmpd	X	R4	Cmpd	*R5*
19		**25**			**31**	1-
20		**26**			**32**	1- 
21		**27**			**33**	1- 
22		**28**			**34**	1- 
23		**29**			**35**	3- 
24		**30**			**36**	3- 
					37	4- 
					38	8- 

Design

There are two modes for the binding of compound **1** into the substrate binding site (Figure 1). Binding mode A is characterized by interactions of the phenyl-OH with Ser142 and Tyr155 from the catalytic tetrad while the naphthol-OH interacts with His221 and Glu282. Binding mode B is characterized by the formation of hydrogen bonds between the phenyl-OH and His221, Glu282, as well as between the naphthol-OH and Ser142, Tyr155. Docking of compound **1** into the active site of 17β-HSD1 (PDB-ID: 1FDT with the amino acids of the flexible loop in the B conformation), after having removed E2, shows that the space available for additional substituents on the lipophilic core structure is different in the two binding modes. This is visualized in Figure 1 with colored arrows: green arrows indicate that there is space available for large substituents, in orange positions are shown with limited space (small substituents) and in red positions are marked where no space is available for substitution.

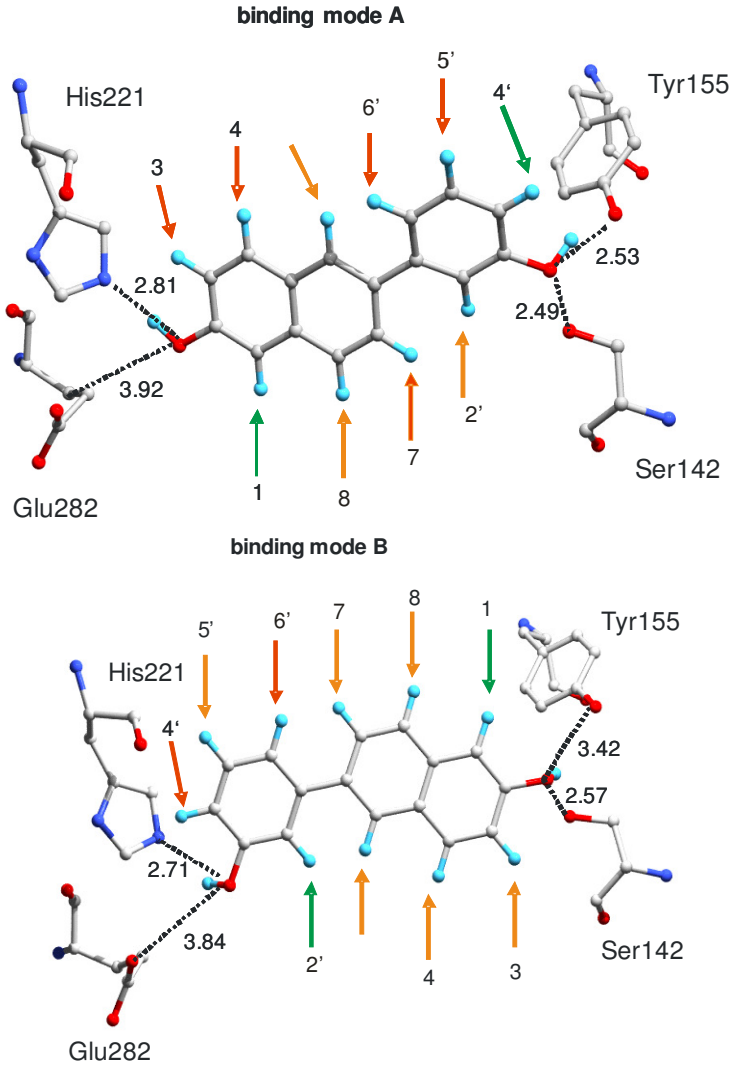


Figure 1: Two possible binding modes for compound 1. For each binding mode A and B, investigation of the space available around 1 for introduction of substituents. Green arrows indicate enough space for large substituents, orange arrows: space for smaller substituents and red arrows: no space for any substituents.

In case compound **1** binds according to mode A, space is available around position 4' of the phenyl ring and position 1 of the naphthalene moiety to introduce a substituent: a compound substituted at these positions should have some activity. On the contrary, introducing a substituent in positions 5', 6', 3, 4 or 7 should result in an inactive compound. Accordingly, in case compound **1** binds in binding mode B, a substituent in position 2' and 1 should be tolerated and lead to some activity while substitution in position 4' and 6' should result in inactive compounds. These considerations are only valid when the amino acids of the flexible loop (188-201) are in the B conformation, while there is more space available for substitution in the A conformation.
Compounds substituted at positions 4' and 5' of the phenyl ring and positions 1, 3, 4 and 8 of the naphthalene moiety were synthesized to get more insight in the binding mode of this class of compounds and to find out about the best position to gain activity.

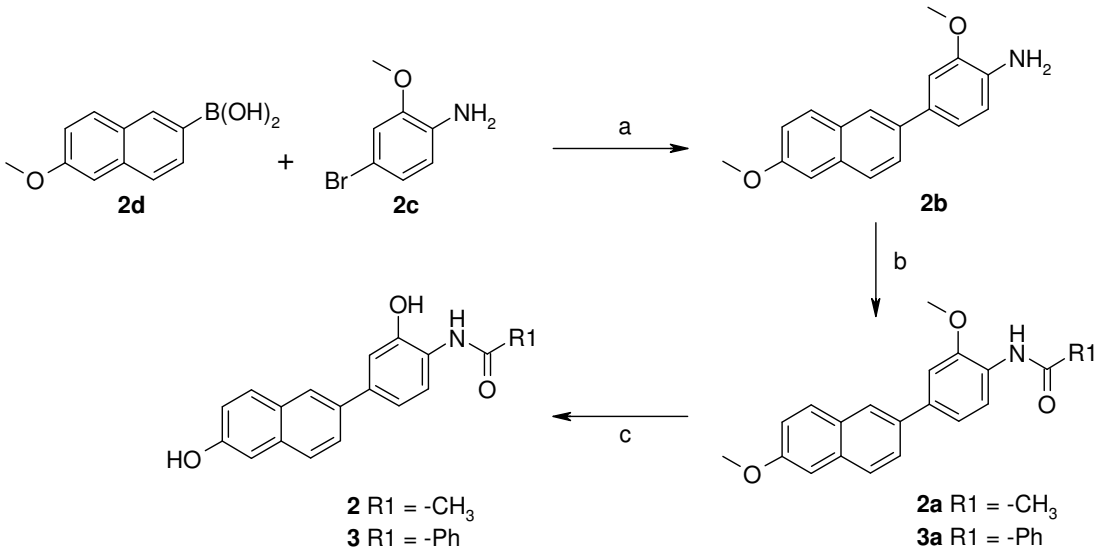
Chemistry

Substitution at the phenyl ring

The synthesis of compounds **2**-**18** substituted in position 4' and 5' of the phenyl ring is depicted in Schemes 1-4. Scheme 1 shows the synthesis of compounds **2**-**3** substituted in position 4' of

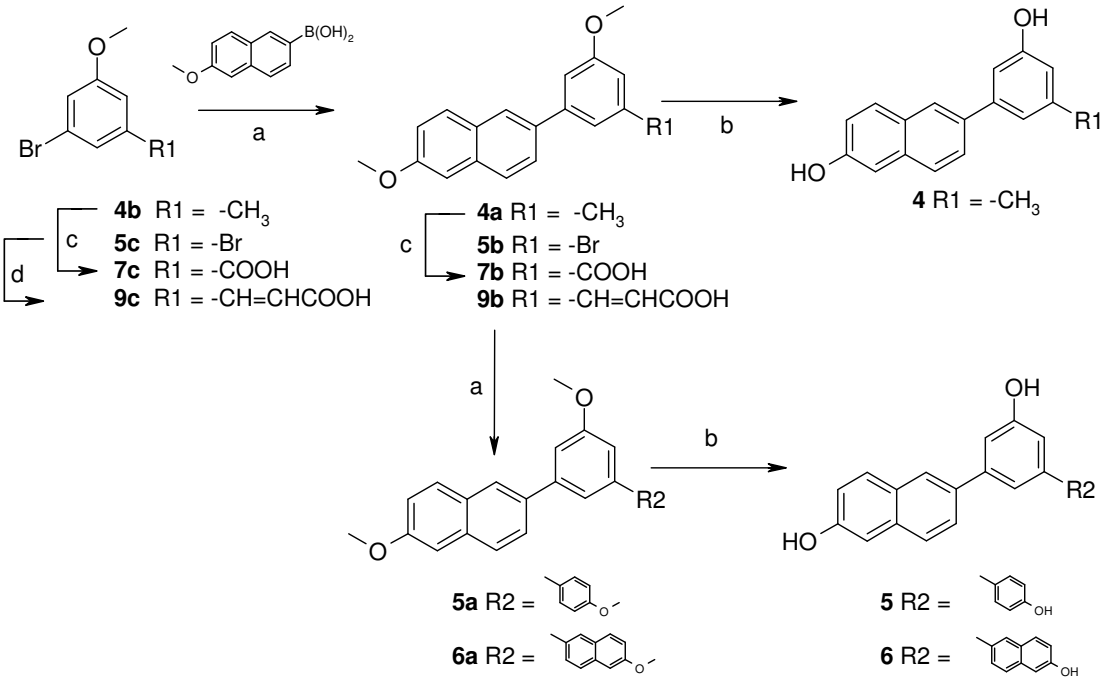
the phenyl ring with an amide moiety. These compounds were obtained from Suzuki coupling (Miyaura and Suzuki, 1995) using 4-bromo-2-methoxyaniline **2c** and 6-methoxy-2-naphthalene boronic acid **2d** as reactants. The aniline moiety was *N*-acylated with two different acid chlorides. Subsequent ether cleavage with boron tribromide (Bhatt and Kulkarni, 1983) led to **2** and **3**.

Scheme 1[a]: Synthesis of compounds 2-3.



[a] Reagents and conditions: a. $Pd(PPh_3)_4$, aq. Na_2CO_3, toluene, 80 °C, overnight; b. RCOCl, CH_2Cl_2, DMAP, RT, overnight; c. BBr_3, CH_2Cl_2, -78 °C, overnight.

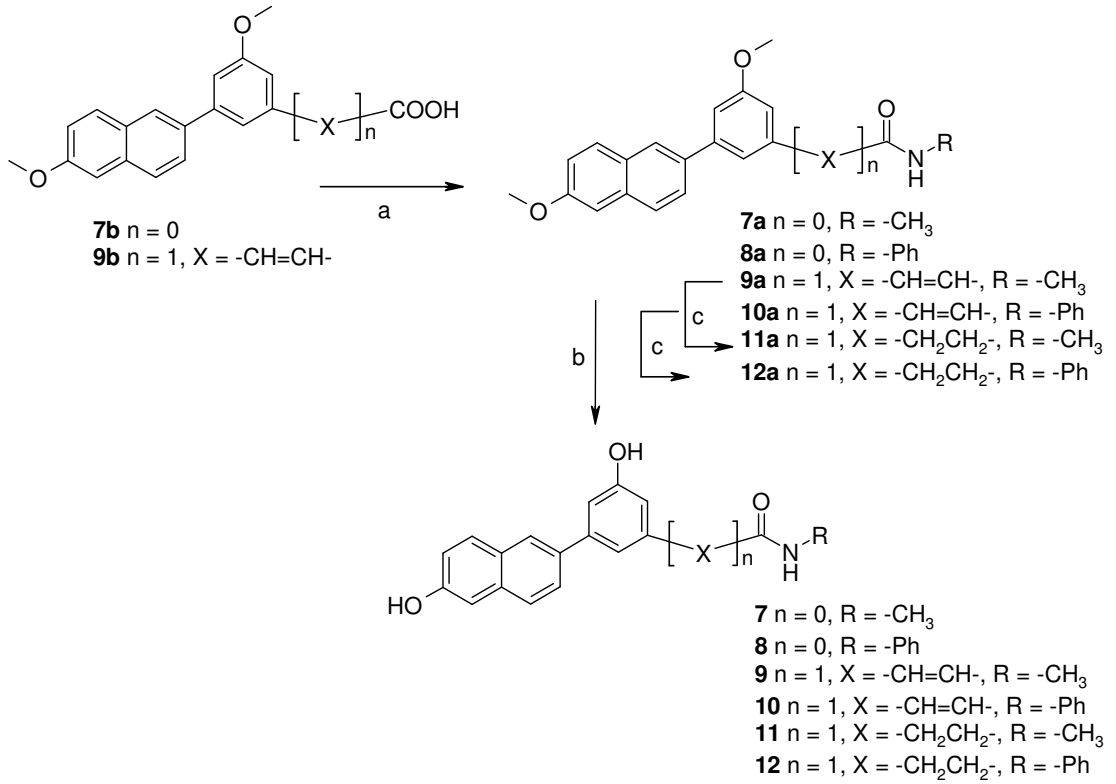
Scheme 2[a]: Synthesis of compounds 4-6.



[a] Reagents and conditions: a. $Pd(PPh_3)_4$, aq. Na_2CO_3, DME, 80 °C, 12 h to 26 h; b. BBr_3, CH_2Cl_2, -78 °C, 4 h; c. $KMnO_4$, pyridine/H_2O (2:5), 75 °C, 65 h, d. acrylic acid, PPh_3, $Pd(OAc)_2$, NEt_3, xylene, 100 °C, 11 h.

Compounds **4-6** bearing alkyl or aromatic substituents in position 5' of the phenyl ring were synthesized according to the route described in Scheme 2. Suzuki coupling between the bromo derivatives (**4b**, **5c**) and the appropriate boronic acid (one Suzuki reaction for **4a** and two successive Suzuki couplings for **5a** and **6a**), followed by a demethylation step using boron tribromide gave compounds **4**-**6**.
Compounds **7**-**8** with an amide moiety directly linked to position 5' of the phenyl ring and compounds **9**-**12** bearing the amide function linked via a C2 spacer were synthesized according to the pathway described in Scheme 3. These compounds were obtained after Suzuki coupling, acylation in presence of EDCI and HOBt (Pascal *et al.*, 1998) and ether cleavage (boron tribromide). The intermediates **7b** and **7c** were prepared by oxidation of the methyl derivatives **4a** and **4b**, respectively, with potassium permanganate (Claudi *et al.*, 2000; Scheme 2). The acrylic acid **9c**, the precursor of **9b** was prepared from the corresponding bromo derivative **5c** via Heck reaction (Patel *et al.*, 1977; Scheme 2). Subsequent catalytic hydrogenation of the double bond (**9a, 10a**) was performed using Pearlman's catalyst (Hwang *et al.*, 1992).

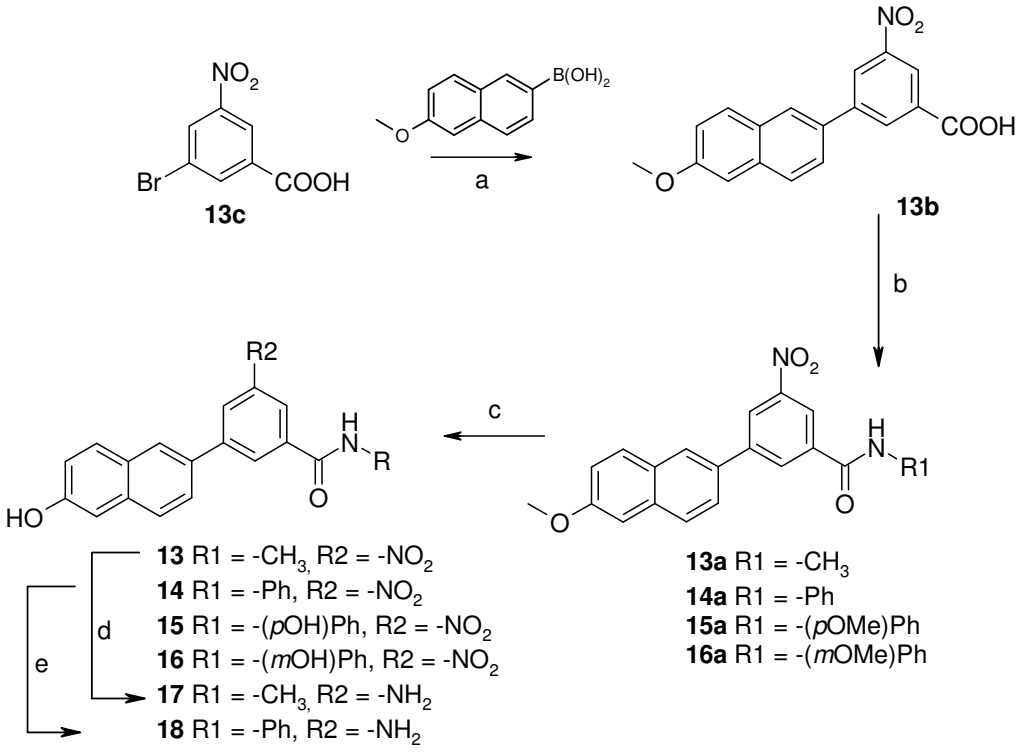
Scheme 3[a]: Synthesis of compounds 7-12.



[a] Reagents and conditions: a. RNH_2, EDCI, HOBt, CH_2Cl_2, 0 °C to RT, overnight; b. BBr_3, CH_2Cl_2, -78 °C, 4 h; c. $Pd(OH)_2$, H_2, THF, RT, 20 h.

Compounds **13**-**16** with a nitro- instead of a hydroxy-moiety in position 3' of the phenyl ring and an amide function in position 5' were also synthesized. The preparation of these compounds **13**-**16** is described in Scheme 4 and is similar to the route leading to 3'-hydroxyphenyl naphthalenes **7**-**8** mentioned above. Reduction of the nitro groups, either by hydrogenation in presence of palladium on charcoal as catalyst (Clark and Lin, 1986) or by action of tin with hydrochloric acid (Ogata *et al.*, 1987), afforded amines **17**-**18**.

Scheme 4[a]: Synthesis of compounds 13-18.



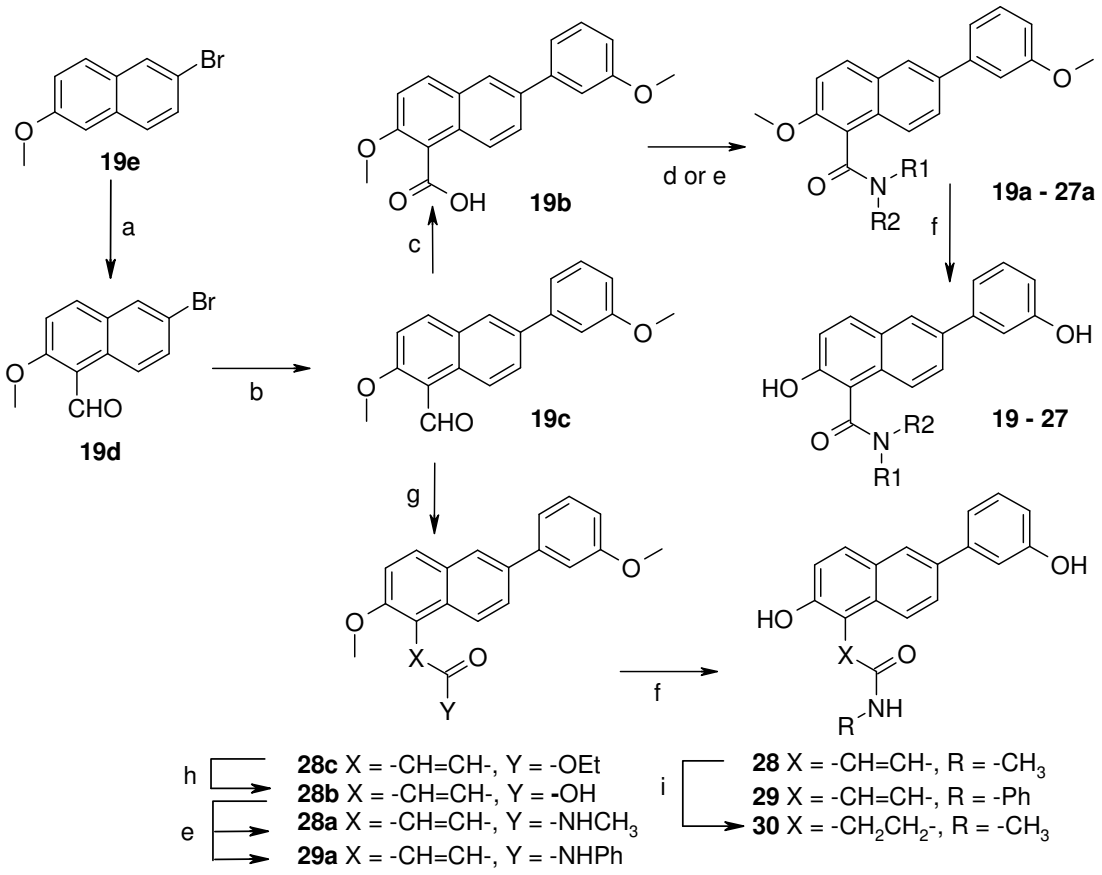
[a] Reagents and conditions: a. $Pd(PPh_3)_4$, aq. Na_2CO_3, DME, 80 °C, overnight; b. RNH_2, EDCI, HOBt, CH_2Cl_2, 0 °C to RT, 48 h; d. BBr_3, CH_2Cl_2, -78 °C, 4 h; d. H_2, Pd/C, EtOH, 0 °C to RT, overnight; e. Sn, HCl, THF, 50 °C, 1 h.

Substitution at the naphthalene ring

The synthesis of compounds **19**-**38** substituted in positions 1, 3, 4 and 8 of the naphthalene moiety is depicted in Schemes 5-9.

Substituents introduced in position 1 include carboxylic acid amides, bromine, phenyl and sulfones. Synthesis of the amides **19**-**30** is shown in Scheme 5. Formylation at position 1 of the naphthalene moiety, performed according to the procedure described by Royer and Buisson, 1980, followed by Suzuki cross-coupling led to the key intermediate, aldehyde **19c**. It was oxidized (Gao *et al.*, 2001) into the corresponding carboxylic acid **19b**. After conversion to the amides **19a**-**27a**, the ether functions were deprotected to give the desired compounds **19**-**27**. In another synthetic route, the aldehyde **19c** was subjected to the Horner-Wadsworth-Emmons conditions (Wadsworth and Emmons, 1961) to introduce an acrylic ester moiety (compound **28c**). Hydrolysis of the ester, amide formation and ether cleavage afforded compounds **28**-**29**. Reduction of the double bond by hydrogenation using Pearlman's catalyst led to **30**.

Scheme 5[a]: Synthesis of compounds 19-30.

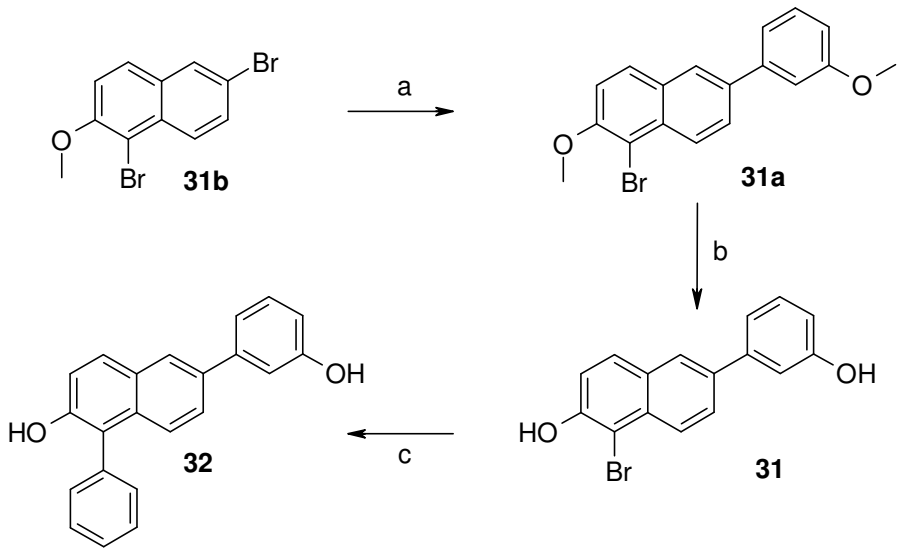


[a] Reagents and conditions: a. $TiCl_4$, dichloromethylmethylether, CH_2Cl_2, 0 °C to RT, overnight; b. 3-methoxybenzeneboronic acid, $Pd(PPh_3)_4$, toluene, aq. Na_2CO_3, 80 °C, 24 h; c. H_2NSO_3H, NaOClO, H_2O/acetone (1:2), 0 °C, 30 min; d. (i) $SOCl_2$, RT, 30 min; (ii) RNH_2, DME or CH_2Cl_2, DMAP, RT, overnight; e. RNH_2, EDCI, HOBt, CH_2Cl_2, RT, overnight; f. BBr_3, CH_2Cl_2, -78 °C to RT, overnight; g. triethylphosphonoacetate, NaH, dry DME, RT, 1 h; h. LiOH, THF/H_2O (3:1), reflux, overnight; i. $Pd(OH)_2$, H_2, ethanol/THF (2:1), RT, overnight.

Cmpd	R1	*R2*
19a / 19	-CH_3	-H
20a / 20	-Ph	-H
21a / 21	-(*m*OMe)Ph/-(*m*OH)Ph	-H
22a / 22	-piperidine-	
23a / 23	-morpholine-	
24a / 24	-piperazine-	
25a / 25	2-pyridinyl	-H
26a / 26	pyrimidin-4-yl	-H
27a / 27	5-methyl-1,3,4-thiadiazol-2-yl	-H

The synthesis of the 1-phenyl naphthalene **32** is depicted in Scheme 6. Regioselective Suzuki reaction of the 1,6-dibromonaphthalene **31b** led to the 1-bromo-6-(3'-methoxyphenyl)naphthalene **31a**. Subsequent ether cleavage led to the 1-bromonaphthol **31** which in turn was submitted to a second Suzuki coupling to give compound **32**.

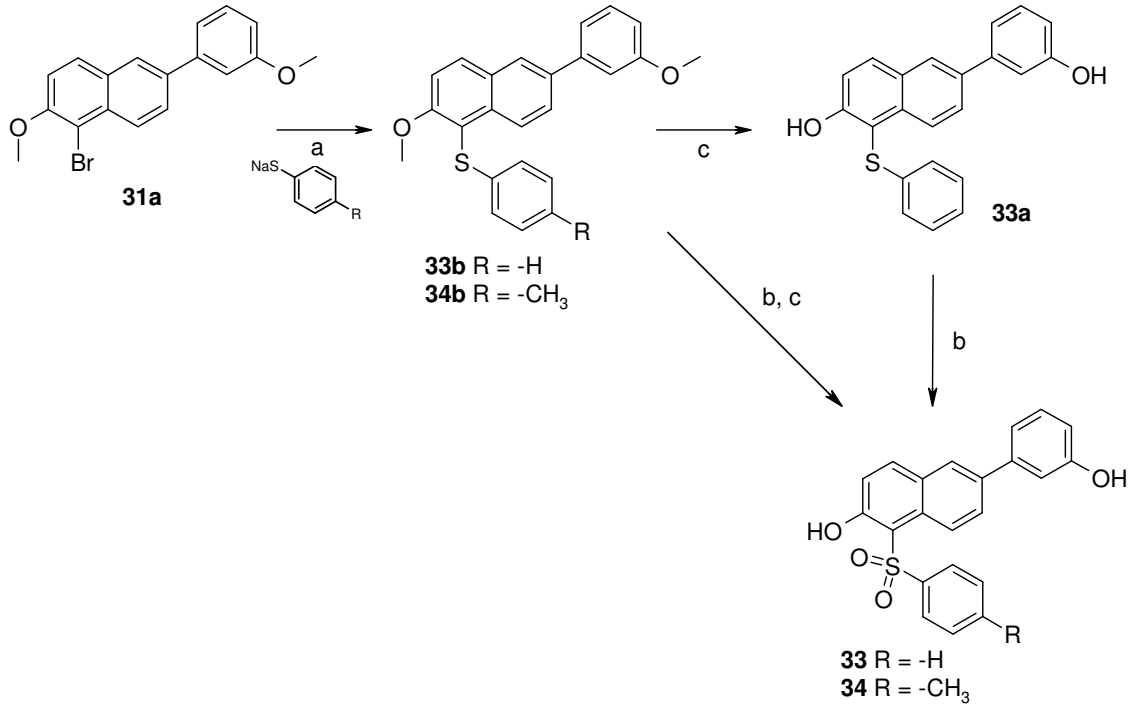
Scheme 6[a]: Synthesis of compounds 31-32.



[a] Reagents and conditions: a. 3-methoxybenzeneboronic acid, $Pd(PPh_3)_4$, toluene, aq. Na_2CO_3, 80 °C, overnight; b. BBr_3, CH_2Cl_2, -78 °C to RT, overnight; c. benzeneboronic acid, $Pd(PPh_3)_4$, toluene, aq. Na_2CO_3, 80 °C, 23 h.

The synthesis of the 1-sulfonylnaphthalenes **33**-**34** was performed according to the route described in Scheme 7 from the key intermediate **31a**. Aromatic nucleophilic substitution of the 1-bromonaphthalene **31a** by sodium benzenethiolate or 4-methylbenzenethiolate (Bahuguna *et al.*, 1982) led to the thioethers **33b** and **34b**. Hydrolysis of the methoxy groups and oxidation with *m*-CPBA (Wang *et al.*, 2006) gave the corresponding sulfones **33** and **34**.

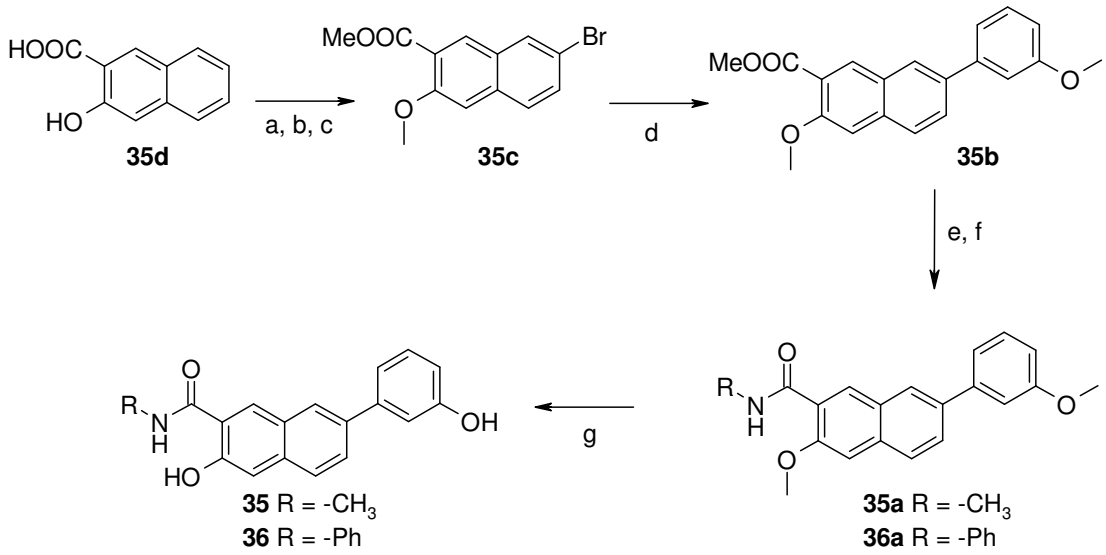
Scheme 7[a]: Synthesis of compounds 33-34.



[a] Reagents and conditions: a. DMF, reflux, 11 h; b. *m*-CPBA, CH_2Cl_2, 0 °C to RT, 12 h; c. BBr_3, CH_2Cl_2, -78 °C to RT, overnight.

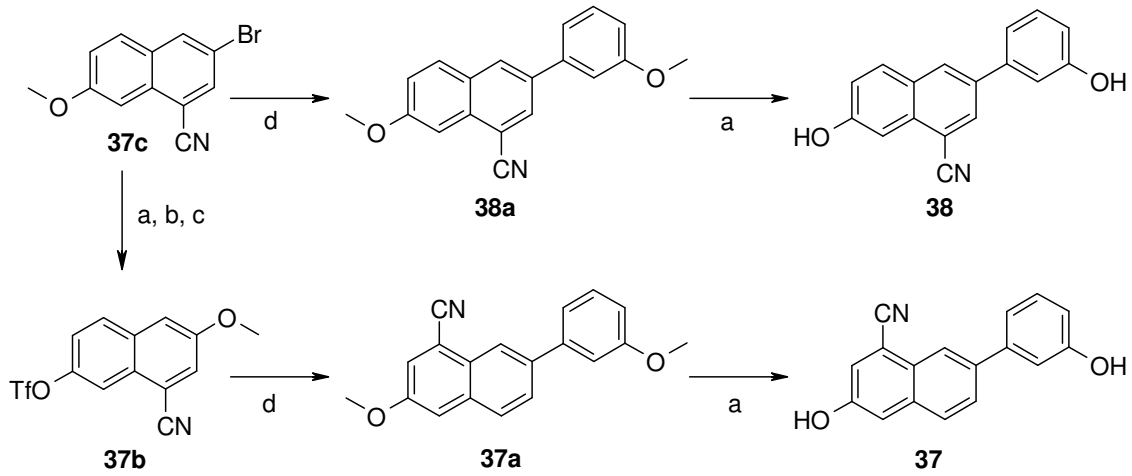
Introduction of amide moieties in position 3 of the 2-naphthol core was carried out as depicted in Scheme 8. The intermediate **35c** was synthesized using the route described by Murphy *et al.*, 1991. It was submitted to Suzuki coupling, amide formation and ether cleavage to give the final compounds **35** and **36**.

Scheme 8[a]: Synthesis of compounds 35-36.



[a] Reagents and conditions: a. Br_2, acetic acid, reflux, 2 h; b. Sn, HCl, acetic acid, reflux, 3 h; c. DMS, K_2CO_3, acetone, reflux, 3 h; d. 3-methoxybenzeneboronic acid, $Pd(PPh_3)_4$, toluene, aq. Na_2CO_3, 80 °C, overnight; e. LiOH, THF/H_2O (1:1), reflux, 90 min; f. RNH_2, EDCI, HOBt, CH_2Cl_2, 0 °C to RT, overnight; g. BBr_3, CH_2Cl_2, -78 °C to RT, overnight.

Scheme 9[a]: Synthesis of compounds 37-38.



[a] Reagents and conditions: a. pyridinium hydrochloride; 190 °C, 2 h; b. NaOMe, CuBr, reflux, 3 h; c. Tf_2O, pyridine, CH_2Cl_2, 0 °C; d. 3-methoxybenzeneboronic acid, $Pd(PPh_3)_4$, DME, aq. Na_2CO_3, 80 °C, overnight.

The synthesis of compounds substituted with a cyano moiety at position 4 (**37**) or 8 (**38**) of the 2-naphthol system started from the common intermediate **37c** (Scheme 9). The latter was obtained using the route described by Mewshaw *et al.*, 2005. Starting from **37c** the 4-cyanonaphthalene **37** was prepared in a five steps pathway according to the literature (Mewshaw *et al.*, 2005). Suzuki coupling between **37c** and 3-methoxybenzeneboronic acid

followed by a subsequent ether cleavage with pyridinium hydrochloride afforded the 8-cyanonaphthalene **38**.

Biological results

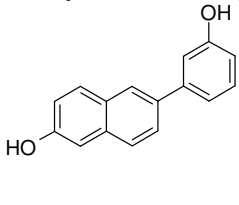
Inhibition of human 17β-HSD1

As source of enzyme, both recombinant as well as human placental enzymes were used. The incubations were run with tritiated E1, cofactor and inhibitor and led to comparable results using both enzymes. The separation between substrate and product was performed by HPLC. The percent inhibition values of all hydroxy compounds are shown in Tables 1 and 2 except for compounds **2**-**3** and **5**-**18**, which are inactive and are not reported. The IC_{50} values of selected compounds are shown in Table 3. Compounds showing less than 10% inhibition at 1 µM were considered to be inactive. All molecules with methoxy groups showed no activity (data not shown).

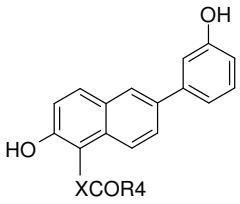
The unsubstituted compound **1** identified as lead in the previous work (Frotscher *et al.*, 2008) was used as reference compound. Compounds substituted at the position 4' and 5' of the phenyl ring **2**-**18** were all inactive except compound **4,** with a methyl group in position 5' which presented a medium activity (42% and 14% inhibition at 1 µM and 100 nM, respectively). Substituents at position 4' of the phenyl ring as well as at position 5' with the exception of methyl are not tolerated by the enzyme. A lack of space in this region of the active site might be responsible for the loss of activity compared to compound **1**. In case the compounds bind according to binding mode A, the flexible loop might reduce the space in the area of the catalytic tetrad. It could be expected that the loop adopts also other conformations, space should then be present in the region close to position 4' and 5' of the phenyl ring. The high diversity of substituents introduced at these positions, leading to inactive compounds, shows that either the loop remains located in front of the substrate binding site or that this series of compounds does not bind according to binding mode A.

A bromine and a phenyl ring were also inserted in position 1 of the naphthalene. The corresponding compounds **31** and **32** turned out to be at least as active as the reference compound **1** (Table 2). This shows that there is space available for an aromatic moiety in this region of the active site. The enhanced activity of compound **32** might be indicative of π-π stacking interactions.

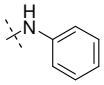
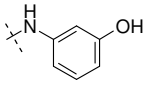
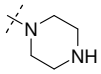
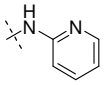
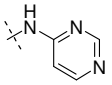
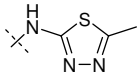
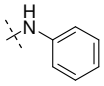
Table 1: Inhibition of human 17β-HSD1 and 17β-HSD2 by compounds 19-30.



1

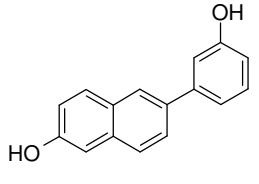


19 - 30

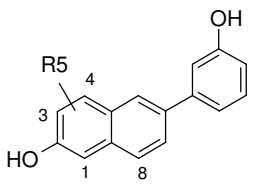
Cmpd	X	R4	Inhibition of 17β-HSD1 [%][a]		*Inhibition of 17β-HSD2 [%][c]*
			100 nM	**1 µM**	**1 µM**
1			91	94	20
19			22	76	18
20			40	80	28
21			21	70	n.d.
22			29	73	n.d.
23			28	62	n.d.
24			n.i.	45	n.d.
25			n.i.[b]	n.i.[b]	n.d.
26			n.i.[b]	n.i.[b]	n.d.
27			n.d.	73[b]	n.d.
28			19	58	n.d.
29			n.i.	60	n.d.
30			n.i.	80	36[d]

[a] Recombinant human 17β-HSD1, substrate [^{3}H]-E1 [30 nM], NADPH [1 mM] Procedure A; mean value of 2 determinations, relative standard deviation <20%, [b] Human placental 17β-HSD1, substrate [^{3}H]-E1 [500 nM], NADH [500 µM] Procedure B, mean value of 3 determinations, relative standard deviation: 10% for **27**; [c] Human placental 17β-HSD2, substrate [^{3}H]-E2 [500 nM], NAD^+ [1500 µM] Procedure D, mean value of 3 determinations, relative standard deviation: 38% for **19** and 27% for **20**; [d] Recombinant human 17β-HSD2 [^{3}H]-E2 [30 nM], NAD^+ [1 mM] Procedure C, mean value of 2 determinations, relative standard deviation <20%, n.i. = no inhibition (inhibition <10%), n.d. = not determined.

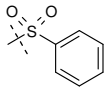
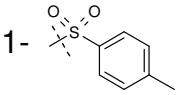
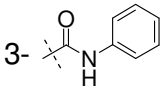
Table 2: Inhibition of human 17β-HSD1 and 17β-HSD2 by compounds 31-38.



1



31 - 38

Cmpd	R5	Inhibition of 17β-HSD1 [%][a]		*Inhibition of 17β-HSD2 [%][c]*
		100 nM	**1 µM**	**1 µM**
1		91	94	20
31	1- 	83	88	57
32	1- 	76	89	61
33	1- 	n.d.	33[b]	n.d.
34	1- 	n.d.	75[b]	n.d.
35	3- 	n.i.	18	n.d.
36	3- 	17	62	n.d.
37	4- 	73[b]	99[b]	74
38	8- 	n.i[b]	53[b]	n.d.

[a] Recombinant human 17β-HSD1, substrate [^{3}H]-E1 [30 nM], NADPH [1 mM] Procedure A; mean value of 2 determinations, relative standard deviation <20%, [b] Human placental 17β-HSD1, substrate [^{3}H]-E1 [500 nM], NADH [500 µM] Procedure B, mean value of 3 determinations, relative standard deviation <17%, [c] Human placental 17β-HSD2, substrate [^{3}H]-E2 [500 nM], NAD^+ [1500 µM] Procedure D, mean value of 3 determinations, relative standard deviation <15%; n.i. = no inhibition (inhibition <10%), n.d. = not determined.

Substitution in position 3 of the naphthalene was also investigated: the amides **35** and **36**, however, exhibited only little and moderate activity. Introduction of a cyano function in position 8 of the naphthalene ring (**38**) reduced activity while the same substituent in position 4 (**37**) was very well tolerated (Table 2).

For selected compounds IC_{50} values were determined (Table 3). Compounds **31** and **32** with an IC_{50} value of 40 nM and 20 nM, respectively, were the most potent inhibitors identified in this series (three to four times more potent than the reference compound **1**).

Table 3: IC_{50} values, selectivity factor and binding affinities for the estrogen receptors α and β for selected compounds.

Cmpd	17β-HSD1 IC_{50} [nM][a,b]	17β-HSD2 IC_{50} [nM][a,c]	Selectivity factor[d]	ERα RBA (%)[e]	*ERβ RBA (%)[e]*
1	116	5641	48	0.2	0.8
31	40	639	16	0.01<RBA<0.1	0.01<RBA<0.1
32	20	540	27	0.01<RBA<0.1	0.01<RBA<0.1

[a] Mean value of 3 determinations, relative standard deviation <20%, [b] Human placental 17β-HSD1, substrate [^{3}H]-E1 [500 nM], NADH [500 µM] Procedure B, [c] Human placental 17β-HSD2, substrate [^{3}H]-E2 [500 nM], NAD^+ [1500 µM] Procedure D, [d] IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1), [e] RBA: Relative Binding Affinity, estradiol: 100%.

Selectivity

To assess the selectivity of the most interesting compounds, inhibition of 17β-HSD2 and affinity to the estrogen receptors α and β were determined.
Since 17β-HSD2 catalyses the oxidative transformation of E2 into E1, thus "inactivating" E2, inhibition of this enzyme is contra-productive for the treatment of estrogen-dependent diseases. Briefly, human placental microsomes as source of 17β-HSD2 were incubated with [^{3}H]-E2 in the presence of NAD^+ and inhibitor. The amount of labeled E1 formed from substrate was determined after HPLC separation. Inhibition of 17β-HSD2 was measured for compounds showing more than 75% inhibition of 17β-HSD1 at 1 µM. Compounds substituted at the phenyl ring have weak to no inhibitory activity toward 17β-HSD2 (<20%, data not shown). Compounds substituted at the naphthalene ring are also weak to moderate inhibitors of 17β-HSD2 (Table 1 and 2). Using the IC_{50} values, selectivity factors (IC_{50} HSD2 / IC_{50} HSD1) were calculated (Table 3). It turned out that compounds **31** and **32** show reasonable selectivity.
Furthermore, inhibitors of 17β-HSD1 should have low or no affinity for the estrogen receptors α and β (ER α and β), since binding to these receptors could counteract the therapeutic concept of 17β-HSD1 inhibition. For the most interesting compounds, binding affinities were determined. The ER assays were performed using recombinant human protein, [^{3}H]-E2 and inhibitor. Separation of bound and free E2 was carried out using hydroxyapatite. Compounds **31** and **32** show very little affinity to the ERs (Table 3).
Since the ER binding affinity experiments do not explore the intrinsic activity at the receptor, compounds **1** and **32** were further investigated. Using the estrogen dependent mammary tumor cell line T47D, cell proliferation was monitored after incubation with the test compounds. E2 was used as positive control. At an E2 concentration of 0.1 nM, a strong stimulation of cell proliferation was observed after eight days incubation. Administered in the same concentration, compounds **1** and **32** did not show any stimulatory effect. The compounds had to be applied in a much higher concentration (100 nM, 1000 fold excess compared to E2) to see a weak stimulation (41 and 39%, respectively, of the E2 effect).

Pharmacokinetic evaluation of compound 1 and 32

To get an idea about the *in vivo* behavior of compounds **1** and **32**, their pharmacokinetics were determined in the rat. After peroral administration of **1** and **32** to male rats (10 kg/kg) (n=4) in a cassette dosing approach, plasma samples were collected over 24 h and concentrations determined by LC-MS/MS. The pharmacokinetic parameters of **1** and **32** are presented in Table 4. Each compound first showed a continuous increase in plasma concentration over time for 6 h for **1** and for 4 h for **32** (Figure 2). Maximal plasma concentration ($C_{max\ obs}$) was higher for **1** (2226 ng/ml) than for **32** (860 ng/ml), the time of maximal plasma concentration ($t_{max\ obs}$) was measured 6 h and 4 h after administration for **1** and **32**, respectively. Subsequently, plasma concentrations decrease again. Plasma levels were very low 24 h after administration. The mean

profile of the plasma concentration of **1** and **32** is depicted in Figure 2. These results indicate that both compounds exhibit a good pharmacokinetic profile in the rat and that they might be good candidates for further experiments in disease-oriented models.

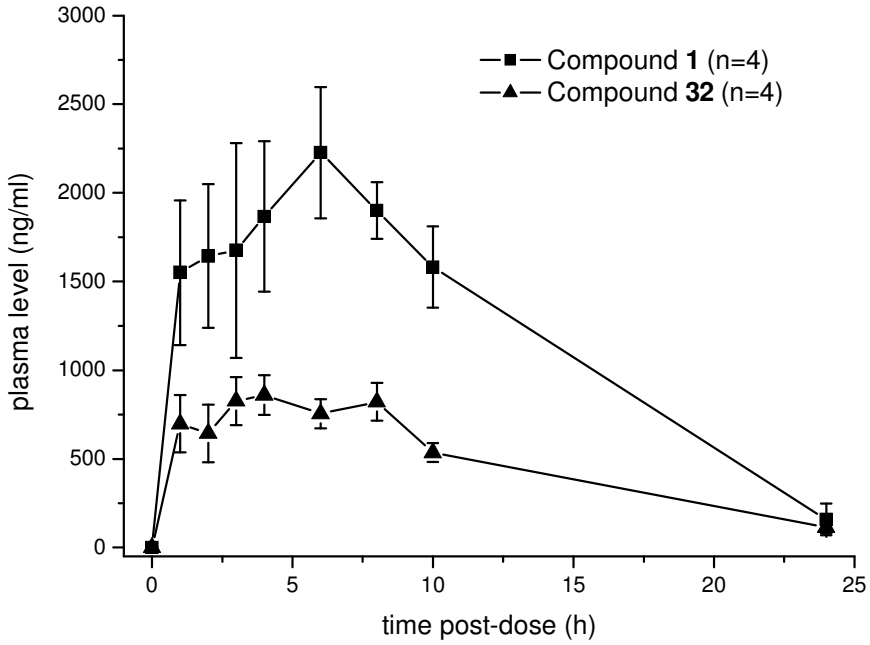


Figure 2: Mean profile (±) SEM of plasma levels (ng/ml) in rat versus time after oral application (10 mg/kg) of compounds 1 and 32 in cassette dosing.

Table 4: Pharmacokinetic parameters of compounds 1 and 32 in rats after oral application.

Cmpd	parameters[a]							
	Dose [mg/kg]	$C_{max\ obs}$ [ng/ml]	C_z [ng/ml]	$t_{max\ obs}$ [h]	t_z [h]	$t_{1/2z}$ [h]	$AUC_{0\text{-}tz}$ [ng·h/ml]	$AUC_{0\text{-}\infty}$ [ng·h/ml]
1	10	2226	159	6	24	4	29694	30698
32	10	860	115	4	24	6	11701	12669

[a] $C_{max\ obs}$: maximal measured concentration, C_z: last analytical quantifiable concentration, $t_{max\ obs}$: time to reach the maximum measured concentration, t_z: time of the last sample which has an analytical quantifiable concentration, $t_{1/2z}$: half-life of the terminal slope of a concentration – time curve, $AUC_{0\text{-}tz}$: area under the concentration-time curve up to the time t_z of the last sample, $AUC_{0\text{-}\infty}$: area under the concentration-time curve extrapolated to infinity.

Molecular Modeling

The biological results show that introduction of substituents in position 4' or 5' of the phenyl ring is detrimental for activity with the exception of a small group in 5' (medium activity). Substitution at the naphthalene ring is well tolerated in position 1 and 4, while a decrease in activity is observed in position 3 and 8. A correlation between these experimental data and the space available around each position of compound **1** (deduced from the docking poses presented in Figure 1) can be established in case of binding mode B which appears to be the most favorite binding mode of this series of substituted (3'-hydroxyphenyl)naphthalenes.

The most potent inhibitor described in this report**,** the 1-phenyl naphthol **32**, was docked in the enzyme (PDB-ID: 1FDT) to better understand the favorable interactions achieved by **32** in the active site. The obtained pose is shown in Figure 3. It is located in the substrate binding pocket according to binding mode B. It has a flat geometry, like compound **1**, only the 1-phenyl moiety is turned about 60° away from the plane of the naphthalene.
Compound **32** seems to be stabilized in the active site by hydrogen bond and hydrophobic interactions (Van der Waals and arene-arene interactions). The phenyl-OH moiety establishes hydrogen bond interactions with His221 and Glu282, the naphthalene-OH with Ser142 and Tyr155 as described for compound **1** (Frotscher *et al.*, 2008). Additionally, the phenyl-OH is stabilized via π-π interaction with Tyr218 in a parallel-displaced geometry (distance between the two ring centres: 5.09 Å). The phenylnaphthalene backbone is stabilized by hydrophobic interactions (Van der Waals). The phenyl ring in position 1 of the naphthalene seems to be involved in hydrophobic interactions with the nicotinamide part of the cofactor. The distance of the ring centres (4.72 Å) and the closest contact distance between a carbon of the phenyl ring of **32** and of the nicotinamide (3.63 Å) are in a good range as it is described for π-π interactions by McGaughey *et al.*, 1998. Furthermore, this 1-phenyl ring could additionally establish π-π interactions with Phe226 and with Tyr155 (both T-shape interaction). The distances between the ring centres are 4.23 Å and 5.42 Å, respectively. From the docking pose, it also becomes apparent that a small hydrophobic pocket is located close to position 4 of the naphthalene. The finding that the 4-cyano compound **37** is active is in agreement with the observation that there is space available for a small substituent.
Looking at the protein structure, steric hindrance and electrostatic repulsion might be the reason for the fact that amide groups in position 3 of the naphthalene (**35**-**36**) decrease activity of the parent compound **1**. However, in case of the phenyl amide **36** the aromatic moiety might be able to reach the hydrophobic area close to position 4, stabilizing the molecule and explaining a regain in activity compared to the methyl amide **35**.

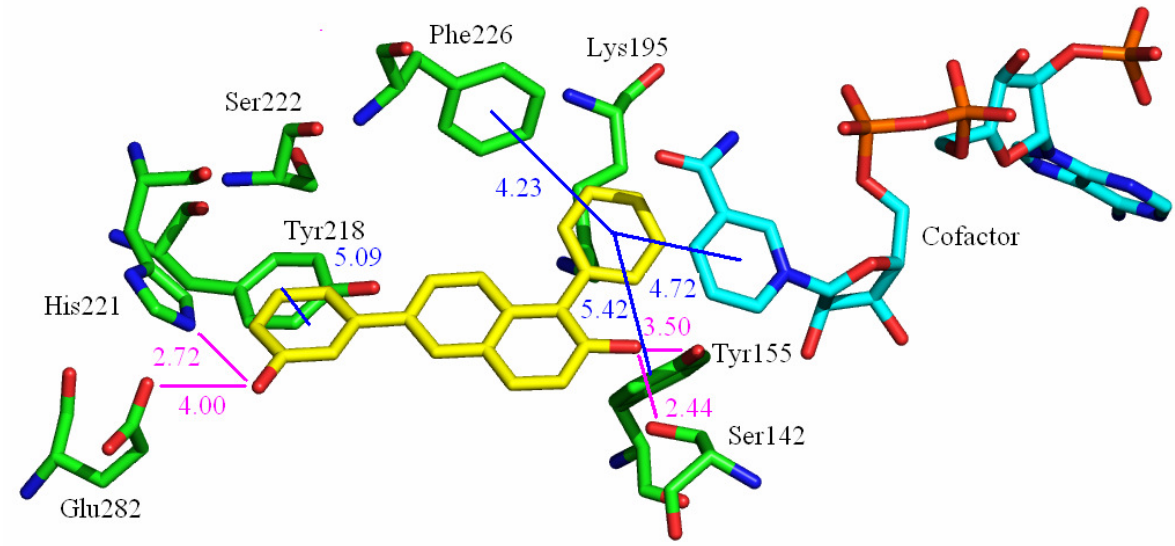


Figure 3: Structure of the 17β-HSD1 binding pocket (green amino acids) with the docked compound 32 (yellow) following binding mode B in presence of the cofactor. Hydrogen bonding interactions and π-π stacking interactions are marked by violet lines and blue lines, respectively. All distances are expressed in Å. The figure was generated with Pymol (http://www.pymol.org).

Discussion and conclusion

The biological results obtained confirm the hypothesis (Frotscher *et al.*, 2008) that substituted (3'-hydroxyphenyl)naphthalenes bind in the active site according to binding mode B, i.e. the phenyl-OH moiety of the compounds mimic the A-ring of E2. In this region, there is no or very little (5'-methylphenyl, compound **4**) space for a substituent. On the other hand, space is available around the naphthalene scaffold in positions 3, 8 and especially 1 and 4 (region

mimicking the C/D-ring of E2). These results are in agreement with the activity of substituted steroidal inhibitors of 17β-HSD1 reported in the literature: the most active compounds are substituted with large groups only at the D-ring (Poirier *et al.*, 2005; Vicker *et al.*, 2006) (position 15 or 16) and a few at the B-ring (Tremblay *et al.*, 2005) (position 6 or 7). Only a small substituent like ethyl or methoxy (Lawrence *et al.*, 2005) is tolerated in position 2 of the steroid (A-ring), but activity is often reduced. These findings are very important for drug design: the space available for substituents on the scaffold of our compounds is very limited (in the region corresponding to the A-ring of E2), while an empty cavity is present in the neighborhood of the catalytic tetrad (around the D-ring of E2).
Furthermore, our results indicate that position 1 of the naphthalene ring is appropriate for the introduction of large substituents and that aromatic groups are better tolerated than amides or sulfones. The latter substituents were introduced with the aim of establishing hydrogen bonds with Ser222 and Tyr218 which might have been adequate partners in case of binding mode A. The fact that all compounds **19**-**30** show a decrease in activity is certainly in disfavor of binding mode A, as in this mode hydrogen bond interactions should have been possible. The low activity of these compounds, however, also demonstrates that these groups are not able to interact with the nicotinamide moiety of the cofactor (binding mode B).
It remains to be elucidated whether it is possible to establish interactions with Ser222 and Tyr218 to improve activity and selectivity. It is striking that these two polar amino acids, which do not interact with the steroid, are present in the active site. However, they might be involved in the stabilization of the three dimensional structure of the protein and thus might not be free to establish another interaction.
In binding mode B, the phenyl moiety of the most potent inhibitor **32**, is located in the catalytic region where space is available. It might undergo π-π stacking interactions with the nicotinamide part of the cofactor (parallel-displaced geometry). Other π-π interactions might also occur with the aromatic amino acids Phe226 and Tyr155. It remains unclear why the 1-bromo compound **31** is more active than **1**. It might be due to the electronic effect of the bromine. However, hydrophobic interactions of the bromine with the cofactor can not be excluded.
Concerning the selectivity of the highly active 17β-HSD1 inhibitors, selected compounds were tested for inhibition of 17β-HSD2 and affinity to the estrogen receptors α and β. Compounds **31** and **32** showed a high selectivity for 17β-HSD2 exhibiting selectivity factors of 16 and 27, respectively. They also showed very little affinity to the estrogen receptors α and β (between 0.01% and 0.1% that of E2). This result is in accordance with the data reported by Mewshaw *et al.*, 2005, who found that the introduction of a phenyl group into the 1 position of 6-(4-hydroxyphenyl)-2-naphthol, reduced estrogen receptor affinity strongly. This shows that there is no or little space in the ERs to introduce a bulky substituent at the 1 position (Mewshaw *et al.*, 2005). In contrast to substitution in the 1 position, a gain in estrogen receptor affinity was described for compounds substituted at the 4 position with a cyano group (Mewshaw *et al.*, 2005). Therefore, position 1, and not position 4, is appropriate to achieve selective 17β-HSD1 inhibition. The affinity of **32** to the ERs corresponds to a very small agonistic effect. Tested in 1000 fold higher concentration than E2 using the ER-positive T47D mammary tumor cell line, it showed 39% of the stimulatory effect of E2.
In this report, we described the synthesis of substituted (3'-hydroxyphenyl)-2-naphthols as inhibitors of 17β-HSD1 and the evaluation of their biological properties. SAR studies using the 17β-HSD1 inhibition data revealed that the compounds most likely bind according to binding mode B into the active site. A new, potent and selective 17β-HSD1 inhibitor, compound **32,** was discovered. It contains a phenyl group at the 1 position of the naphthalene that may interact with binding partners in the catalytic region. It is highly selective toward 17β-HSD2 and the ERs α and β and shows a good pharmacokinetic profile after peroral application. It could be used in an *in vivo* disease-oriented model to further validate the concept that 17β-HSD1 might be a promising target for the treatment of estrogen-dependent diseases.

Experimental Section

Chemical Methods

IR spectra were measured neat on a Bruker Vector 33FT-infrared spectrometer.
^{1}H-NMR spectra were recorded on a Bruker AM500 (500 MHz) instrument at 300 K in $CDCl_3$, CD_3OD, DMSO-d_6 or acetone-d_6. Chemical shifts are reported in δ values (in ppm), the hydrogenated residues of deuteriated solvent were used as internal standard ($CDCl_3$: δ = 7.26 ppm in ^{1}H-NMR and δ = 77 ppm in ^{13}C-NMR, CD_3OD: δ = 3.35 ppm in ^{1}H-NMR and δ = 49.3 ppm in ^{13}C-NMR, DMSO-d_6: δ = 2.58 ppm in ^{1}H-NMR and δ = 39.7 ppm in ^{13}C-NMR, acetone-d_6: δ = 2.05 ppm in ^{1}H-NMR and δ = 29.8 ppm in ^{13}C-NMR). Signals are described as s, d, t, q, dd, ddd, m, b for singlet, doublet, triplet, quadruplet, doublet of doublet, doublet of doublet of doublet, multiplet and broad, respectively. All coupling constants (*J*) are given in Hz.
Mass spectra (ESI and APCI) were measured on a TSQ Quantum instrument (ThermoFisher).
Chemical names follow IUPAC nomenclature.
Starting materials (compounds **2d**, **5c, 13c, 19e**) were purchased from Aldrich, Acros, Lancaster or Fluka and were used without purification. No attempts were made to optimize yields.
Column chromatography was performed using silica gel (70-200 μm) and the reaction progress was determined by TLC analyses on ALUGRAM SIL G/UV_{254} (Macherey-Nagel). Preparative chromatography was performed on glass plate SIL G-100/UV_{254} (TLC, silica, 1 mm thick) from Macherey-Nagel.
The following compounds were prepared according to previously described procedures:
4-Bromo-2-methoxyaniline **2c** (Davis *et al.*, 1989), 1-bromo-3-methoxy-5-methylbenzene **4b** Chan *et al.*, 2001), 1,6-dibromo-2-methoxynaphthalene **31b** (Voets *et al.*, 2005), methyl 7-bromo-3-methoxy-2-naphthoate **35c** (Murphy *et al.*, 1991), 8-cyano-6-methoxy-2-naphthyl trifluoromethanesulfonate **37b** (Mewshaw *et al.*, 2005), 3-bromo-7-methoxy-1-naphthonitrile **37c** (Mewshaw *et al.*, 2005).

General procedure for Suzuki coupling:

Method A: A mixture of arylbromide (1 eq), boronic acid (1 eq), 2% aqueous solution of sodium carbonate (2 eq) and tetrakis(triphenylphosphine) palladium(0) (0.1 eq) in toluene or DME was stirred at 80 °C under nitrogen for 4 h- 26 h. The reaction mixture was cooled to room temperature, quenched by the addition of 2% HCl and extracted with dichloromethane. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated to dryness. The product was purified by chromatography.

General procedures for amide bond formation:

Method B: A mixture of carboxylic acid (1 eq) and amine (1 eq) dissolved in dichloromethane was added dropwise to a solution of EDCI (1 eq) and HOBt (1 eq) in dichloromethane at 0 °C. The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent, the residue was dissolved in ethyl acetate, washed with saturated sodium carbonate solution and brine, dried over $MgSO_4$, filtered and concentrated. The product was purified by chromatography.

Method C: A mixture of carboxylic acid (1 eq), EDCI (1 eq), HOBt (1 eq) and triethylamine (1 eq) in dichloromethane was cooled at 0 °C. The amino derivative (1 eq) was dissolved in dichloromethane and added dropwise. The reaction mixture was refluxed for 1.5 h and quenched by addition of aqueous HCl (0.1 M). The organic layer was separated, washed with sodium carbonate and brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by chromatography.

Method D: Thionyl chloride (4 eq) was added to the carboxylic acid (1 eq) under nitrogen and stirred in presence of a drop of DMF for 30 min. After evaporation of the thionyl chloride, the acid chloride (1 eq) was dissolved in dry THF or dichloromethane and added to the corresponding amine (1 eq) in solution in CH_2Cl_2, together with triethylamine (1.2 eq) and a catalytic amount of DMAP. During the addition, the temperature was kept at 0 °C. The reaction

mixture was refluxed overnight, quenched by the addition of water and extracted with ethyl acetate. The organic phase was dried over $MgSO_4$ and concentrated to dryness. The desired amide was purified by chromatography.

General procedures for ether cleavage:

Method E: To a solution of methoxy derivative (1 eq) in toluene was added aluminum chloride (3 eq to 5 eq per methoxy function) at room temperature under N_2. The reaction mixture was heated at 90 °C for 2 h and then allowed to cool to room temperature. The reaction was quenched by the addition of 2% Na_2CO_3. After extraction with ethyl acetate, the combined organic layers were washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the crude product was purified by chromatography.

Method F: To a solution of methoxy derivative (1 eq) in dichloromethane cooled at -78 °C boron tribromide (1M solution in cyclohexane, 3 eq to 5 eq per methoxy function) was slowly added under N_2. The reaction mixture was stirred at -78 °C for 1 h and then allowed to warm to room temperature. The reaction was quenched by the addition of 2% Na_2CO_3 and extracted with dichloromethane. The combined organic layers were washed with brine and dried over $MgSO_4$. After evaporation of the solvent the product was purified by chromatography.

Method G: The methoxy derivative (1 eq) and pyridinium hydrochloride (12 eq) were heated at 220 °C for 3 h. The reaction mixture was cooled at room temperature and 1 N HCl (4 ml) were added. The resulting precipitate was collected and dissolved in a small amount of ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4 and the solvent was evaporated *in vacuo*.

General procedure for reduction of the double bond:

Method H: A suspension of the olefinic compound (1 eq) and a catalytic amount of $Pd(OH)_2$ in a mixture ethanol/THF (2:1) was stirred at room temperature for 20 h under hydrogen atmosphere. After completion of the reaction, the crude was filtered and concentrated.

2-Methoxy-4-(6-methoxy-2-naphthyl)aniline hydrochloride (2b). The title compound was prepared by reaction of 4-bromo-2-methoxyaniline **2c** (400 mg, 1.98 mmol, 1 eq) with 6-methoxy-2-naphthalene boronic acid **2d** (600 mg, 2.97 mmol, 1.5 eq) according to method A. An etheral solution of hydrochloric acid (2 M) was added to the organic layer and the crystals formed were filtered off (50% yield, 312 mg). $C_{18}H_{17}NO_2 \cdot HCl$; MW 315.

***N*-[2-Methoxy-4-(6-methoxy-2-naphthyl)phenyl]acetamide (2a).** The title compound was prepared by reaction of 2-methoxy-4-(6-methoxy-2-naphthyl)aniline **2b** (140 mg, 0.50 mmol, 1 eq) with acetyl chloride (64 µl, 71 mg, 0.91 mmol, 1.8 eq) according to method D. The analytically pure compound was obtained after purification by column chromatography (gradient dichloromethane/methanol 100:0 to 98:2) in 66% yield (106 mg). $C_{20}H_{19}NO_3$; MW 321.

***N*-[2-Hydroxy-4-(6-hydroxy-2-naphthyl)phenyl]acetamide (2).** The title compound was prepared by reaction of *N*-[2-methoxy-4-(6-methoxy-2-naphthyl)phenyl]acetamide **2a** (54 mg, 0.17 mol, 1 eq) with boron tribromide (2.52 mmol, 15 eq) according to method F. The desired product was obtained after purification by preparative chromatography (dichloromethane/methanol 95:5) in 45% yield (23 mg). $C_{18}H_{15}NO_3$; MW 293; MS, ESI: 294 $(M+H)^+$.

***N*-[2-Methoxy-4-(6-methoxy-2-naphthyl)phenyl]benzamide (3a).** The title compound was prepared by reaction of 2-methoxy-4-(6-methoxy-2-naphthyl)aniline **2b** (140 mg, 0.50 mmol, 1

eq) with benzoyl chloride (0.1 ml, 123 mg, 0.88 mmol, 3 eq) according to method D. The analytically pure compound was obtained after purification by column chromatography (gradient hexane/ethyl acetate 9:1 to 5:5) in 90% yield (101 mg). $C_{25}H_{21}NO_3$; MW 383.

***N*-[2-Hydroxy-4-(6-hydroxy-2-naphthyl)phenyl]benzamide, 3.** The title compound was prepared by reaction of *N*-[2-methoxy-4-(6-methoxy-2-naphthyl)phenyl]benzamide **3a** (49 mg. 0.13 mol, 1 eq) with boron tribromide (2.25 mmol, 15 eq) according to method F. The desired product was obtained after purification by preparative chromatography (dichloromethane/methanol 95:5) in quantitative yield (46 mg). $C_{22}H_{17}NO_3$; MW 355; MS, ESI: 356 $(M+H)^+$.

2-Methoxy-6-(3-methoxy-5-methylphenyl)naphthalene (4a). The title compound was prepared by reaction of 6-methoxy-2-naphthalene boronic acid **2d** (201 mg, 1.0 mmol, 1 eq) with 1-bromo-3-methoxy-5-methylbenzene **4b** (200 mg, 1.0 mmol, 1 eq) according to method A. The product was purified by column chromatography (hexane/ethyl acetate 9:1). Yield: 60% (167 mg). $C_{19}H_{18}O_2$; MW 278; MS, ESI: 279 $(M+H)^+$.

6-(3-Hydroxy-5-methylphenyl)-2-naphthol (4). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxy-5-methylphenyl)naphthalene **4a** (26 mg, 0.10 mmol, 1 eq) with boron tribromide (1 mmol, 10 eq) according to method F. The pure compound was obtained in quantitative yield (18 mg). $C_{17}H_{14}O_2$; MW 250; MS, ESI: 249 $(M-H)^-$.

2-(3-Bromo-5-methoxyphenyl)-6-methoxynaphthalene (5b). The title compound was prepared by reaction of 6-methoxy-2-naphthalene boronic acid **2d** (510 mg, 2.54 mmol, 1.5 eq) with 1,3-dibromo-5-methoxybenzene **5c** (450 mg, 1.69 mmol, 1 eq) for 20 h according to method A. The product was purified by column chromatography (hexane/dichloromethane 8:2). Yield: 34% (199 mg). $C_{18}H_{15}BrO_2$; MW 343; MS, ESI: 343-345 $(M+H)^+$.

3,4'-Dimethoxy-5-(6-methoxy-2-naphthyl)biphenyl (5a). The title compound was prepared by reaction of 6-methoxy-2-naphthalene boronic acid **2d** (331 mg, 1.64 mmol, 1.5 eq) with 1,3-dibromo-5-methoxybenzene **5c** (290 mg, 1.09 mmol, 1 eq) for 18 h according to method A. The mono-substituted product 2-(3-bromo-5-methoxyphenyl)-6-methoxynaphthalene **5b** which was purified by column chromatography (hexane/dichloromethane 8:2) and then used in a second Suzuki coupling (182 mg, 0.53 mmol, 1 eq) with (4-methoxyphenyl)boronic acid (121 mg, 0.79 mmol, 1.5 eq) for 18 h according to method A. The final product **5a** was purified by column chromatography (hexane). Overall yield: 42% (78 mg). $C_{25}H_{22}O_3$; MW 370.

5-(6-Hydroxy-2-naphthyl)biphenyl-3,4'-diol (5). The title compound was prepared by reaction of 3,4'-dimethoxy-5-(6-methoxy-2-naphthyl)biphenyl **5a** (40 mg, 0.11 mmol, 1 eq) with boron tribromide (1.35 mmol, 12 eq) according to method F. The analytically pure product was obtained as a precipitate after the addition of water. Yield: 92% (34 mg). $C_{22}H_{16}O_3$; MW 328; MS (APCI): 329 $(M+H)^+$.

2-Methoxy-6-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]naphthalene (6a). The title compound was prepared by reaction of 6-methoxy-2-naphthalene boronic acid **2d** (472 mg, 2.34 mmol, 2.5 eq) with 2-(3-bromo-5-methoxyphenyl)-6-methoxynaphthalene **5b** (250 mg, 0.93 mmol, 1 eq) according to method A. The product was purified by column chromatography (hexane/dichloromethane 8:2). Yield: 49% (192 mg). $C_{29}H_{24}O_3$; MW 420; MS, ESI: 421 $(M+H)^+$.

6,6'-(5-Hydroxy-1,3-phenylene)di(2-naphthol) (6). The title compound was prepared by reaction of 2-methoxy-6-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]naphthalene **6a** (100 mg, 0.24 mmol, 1 eq) with boron tribromide (3.6 mmol, 15 eq) according to method F. Purification by column chromatography (hexane/ethyl acetate 9:1) afforded 90 mg of the desired product. Yield: 99%. $C_{26}H_{18}O_3$; MW: 378; MS, ESI: 379 $(M+H)^+$.

3-Bromo-5-methoxybenzoic acid (7c). To a mixture of 1-bromo-3-methoxy-5-methylbenzene **4b** (187 mg, 0.93 mmol, 1 eq) in pyridine (2 ml) and water (5 ml) was added in small portions potassium permanganate (469 mg, 2.97 mmol, 3.2 eq) at 75 °C. The reaction mixture was stirred for 65 h at 75 °C and then filtered. The aqueous layer was acidified, the precipitate was collected and dried to give the title compound in 50% yield (107 mg). $C_8H_7BrO_3$; MW 231.

3-Methoxy-5-(6-methoxy-2-naphthyl)benzoic acid (7b). To a mixture of 2-methoxy-6-(3-methoxy-5-methylphenyl)naphthalene **4a** (100 mg, 0.36 mmol, 1 eq) in pyridine (2 ml) and water (5 ml) was added in small portions potassium permanganate (182 mg, 1.15 mmol, 3.2 eq) at 75 °C. The reaction mixture was stirred for 42 h at 75 °C and then filtered. The aqueous layer was acidified, the precipitate was collected and dried to give the title compound in 27% yield (30 mg).
The title compound was also prepared by reaction of 6-methoxy-2-naphthalene boronic acid **2d** (55 mg, 0.27 mmol, 1.5 eq) with 3-bromo-5-methoxybenzoic acid **7c** (42 mg, 0.18 mmol, 1 eq) according to method A. The product was purified by preparative chromatography (hexane/ethyl acetate 7:3). Yield: 43% (23 mg). $C_{19}H_{16}O_4$; MW 308.

3-Methoxy-5-(6-methoxy-2-naphthyl)-*N*-methylbenzamide (7a). The title compound was prepared by reaction of 3-methoxy-5-(6-methoxy-2-naphthyl)benzoic acid **7b** (500 mg, 1.62 mmol, 1 eq) with a solution of methylamine (33% in ethanol, 0.22 ml, 50 mg, 1.62 mmol, 1 eq) according to Method B. Purification by column chromatography (hexane/ethyl acetate 1:1) afforded 148 mg of the desired product. Yield: 28%. $C_{20}H_{19}NO_3$; MW 321.

3-Hydroxy-5-(6-hydroxy-2-naphthyl)-*N*-methylbenzamide (7). The title compound was prepared by reaction of 3-methoxy-5-(6-methoxy-2-naphthyl)-*N*-methylbenzamide **7a** (110 mg, 0.34 mmol, 1 eq) with boron tribromide (1.7 mmol, 5 eq) according to method F. The desired compound was obtained in quantitative yield (100 mg). $C_{18}H_{15}NO_3$; MW 293; MS, ESI: 292 $(M-H)^-$.

3-Methoxy-5-(6-methoxy-2-naphthyl)-*N*-phenylbenzamide (8a). The title compound was prepared by reaction of 3-methoxy-5-(6-methoxy-2-naphthyl)benzoic acid **7b** (500 mg, 1.62 mmol, 1 eq) with aniline (151 mg, 1.62 mmol, 1 eq) according to Method B. Purification by column chromatography (hexane/dichloromethane 1:1) afforded the desired product. Yield: 33% (204 mg). $C_{25}H_{21}NO_3$; MW 383.

3-Hydroxy-5-(6-hydroxy-2-naphthyl)-*N*-phenylbenzamide (8). The title compound was prepared by reaction 3-methoxy-5-(6-methoxy-2-naphthyl)-*N*-phenylbenzamide **8a** (300 mg, 0.78 mmol, 1 eq) with boron tribromide (3.9 mmol, 5 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 99:1) afforded 32 mg of the desired product. Yield: 11%. $C_{23}H_{17}NO_3$; MW 355; MS, ESI: 356 $(M+H)^+$.

(2*E*)-3-(3-Bromo-5-methoxyphenyl)acrylic acid (9c). To a solution of 1,3-dibromo-5-methoxy-benzene (2.4 g, 9.18 mmol, 1 eq) in 2 ml xylene were successively added acrylic acid (0.63 ml, 9.18 mmol, 1 eq), $Pd(OAc)_2$ (20.7 mg, 0.01 eq), triphenylphosphine (96.2 mg, 0.37 mmol, 0.04 eq) and triethylamine (2.7 ml, 1.95 g, 19.3 mmol, 2.1 eq). The reaction mixture was stirred under nitrogen at 100 °C for 11 h. Na_2CO_3 (2 g) and water (20 ml) were added and the reaction mixture was stirred for 15 min at 100 °C. The aqueous layer was separated and acidified with conc. HCl. The resulting solid was filtered, dried and purified by column chromatography (hexane/ethyl acetate 1:1) to give 751 mg of the desired product. Yield: 32%. $C_{10}H_9Br_3O$; MW 257.

(2*E*)-3-[3-Methoxy-5-(6-methoxy-2-naphthyl)phenyl]acrylic acid (9b). The title compound was prepared by reaction of (2*E*)-3-(3-bromo-5-methoxyphenyl)acrylic acid **9c** (751 mg, 2.92 mmol, 1 eq) with 6-methoxynaphthaleneboronic acid **2d** (590 mg, 2.92 mmol, 1 eq) for 26 h according to method A. The aqueous layer was acidified with conc. HCl and the title compound precipitated. Yield: 64% (620 mg). $C_{21}H_{18}O_4$; MW 334.

(2*E*)-3-[3-Methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-methylacrylamide (9a). The title compound was prepared by reaction of (2*E*)-3-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]acrylic acid **9b** (300 mg, 0.90 mmol, 1 eq) with a solution of methylamine (33% in ethanol, 0.012 ml, 0.90 mmol, 1 eq) according to Method B. Purification by preparative chromatography (hexane/ethyl acetate 2:1) afforded 200 mg of the desired product. Yield: 64%. $C_{22}H_{21}NO_3$; MW 347.

(2*E*)-3-[3-Hydroxy-5-(6-hydroxy-2-naphthyl)phenyl]-*N*-methylacrylamide (9). The title compound was prepared by reaction of (2*E*)-3-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-methylacrylamide **9a** (200 mg, 0.58 mmol, 1 eq) with boron tribromide (29 mmol, 5 eq) according to method F. Purification by preparative chromatography (dichloromethane/methanol 9:1) afforded 25 mg of the desired product. Yield: 13%. $C_{20}H_{17}NO_3$; MW 319; MS, ESI: 320 $(M+H)^+$.

(2*E*)-3-[3-Methoxy-5-(6-methoxy-2-naphthyl)-phenyl]-*N*-phenylacrylamide (10a). The title compound was prepared by reaction of (2*E*)-3-(3-bromo-5-methoxyphenyl)acrylic acid **9c** (300 mg, 0.90 mmol, 1 eq) with aniline (83 mg, 0.90 mmol, 1 eq) according to Method B. Purification by preparative chromatography (hexane/ethyl acetate 2:1) afforded 261 mg of the desired product. Yield: 71%. $C_{27}H_{23}NO_3$; MW 409.

(2*E*)-3-[3-Hydroxy-5-(6-hydroxy-2-naphthyl)-phenyl]-*N*-phenylacrylamide (10). The title compound was prepared by reaction of (2*E*)-3-[3-methoxy-5-(6-methoxy-2-naphthyl)-phenyl]-*N*-phenylacrylamide **10a** (126 mg, 0.31 mmol, 1 eq) with boron tribromide (1.55 mmol, 5 eq) according to method F. Purification by preparative chromatography (dichloromethane/methanol 98:2) afforded 17 mg of the desired product. Yield: 14%. $C_{25}H_{19}NO_3$; MW 381; MS, ESI: 382 $(M+H)^+$.

3-[3-Methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-methylpropanamide (11a). The title compound was prepared by reaction of (2*E*)-3-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-methylacrylamide **9a** (100 mg, 0.29 mmol, 1 eq) and $Pd(OH)_2$ (2.82 mg) in ethanol (1 ml) and THF (0.5 ml) under hydrogen for 19 h according to method H. The reaction mixture was filtered and concentrated to give the desired compound in a quantitative yield (100 mg). $C_{22}H_{23}NO_3$; MW 349.

3-[3-Hydroxy-5-(6-hydroxy-2-naphthyl)phenyl]-*N*-methylpropanamide (11). The title compound was prepared by reaction of 3-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-methylpropanamide **11a** (48 mg, 0.16 mmol, 1 eq) with aluminum chloride (213 mg, 1.60 mmol, 10 eq) according to method E. Purification by preparative chromatography (dichloromethane/methanol 98:2) afforded 16 mg of the desired product. Yield: 35%. $C_{20}H_{19}NO_3$; MW 321; MS, ESI: 322 $(M+H)^+$.

3-[3-Methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-phenylpropanamide (12a). The title compound was prepared by reaction of (2*E*)-3-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-phenylacrylamide **10a** (70 mg, 0.17 mmol, 1 eq) and $Pd(OH)_2$ (2.5 mg) in ethanol (1 ml) and THF (0.5 ml) under hydrogen atmosphere for 20 h according to method H. The reaction mixture was filtered and concentrated to give the desired compound in a quantitative yield (70 mg). $C_{27}H_{25}NO_3$; MW 411.

3-[3-Hydroxy-5-(6-hydroxy-2-naphthyl)phenyl]-*N*-phenylpropanamide (12). The title compound was prepared by reaction of 3-[3-methoxy-5-(6-methoxy-2-naphthyl)phenyl]-*N*-phenylpropanamide **12a** (55 mg, 0.13 mmol, 1 eq) with aluminum chloride (291 mg, 2.19 mmol, 12 eq) according to method E. Purification by preparative chromatography (hexane/ethyl acetate 1:1) afforded 17 mg of the desired product. Yield: 23%. $C_{25}H_{21}NO_3$; MW 383.

3-(6-Methoxy-2-naphthyl)-5-nitrobenzoic acid (13b). The title compound was prepared by reaction of 6-methoxy-2-naphthalene boronic acid **2d** (0.82 g, 4.1 mmol, 1 eq) with 3-bromo-5-nitro-benzoic acid **13c** (1 g, 4.1 mmol, 1 eq) according to method A. The analytically product was obtained in quantitative yield (1.32 g) after acidification with conc. HCl and filtration. $C_{18}H_{13}NO_5$; MW: 323.

3-(6-Methoxy-2-naphthyl)-*N*-methyl-5-nitrobenzamide (13a). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-5-nitrobenzoic acid **13b** (500 mg, 1.55 mmol, 1 eq) with a solution of methylamine (33% in ethanol, 0.2 ml, 1.55 mmol, 1 eq) according to method B. Purification by column chromatography (hexane/ethyl acetate 7:3) afforded 395 mg of **13a**. Yield: 76%. $C_{19}H_{16}N_2O_4$; MW: 336.

3-(6-Hydroxy-2-naphthyl)-*N*-methyl-5-nitrobenzamide (13). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-*N*-methyl-5-nitrobenzamide **13a** (100 mg, 0.30 mmol, 1 eq) with boron tribromide (1.8 mmol, 6 eq) according to method F. Purification by preparative chromatography (dichloromethane/methanol 9:1) afforded the desired product in quantitative yield (97 mg). $C_{18}H_{14}N_2O_4$; MW: 322.

3-(6-Methoxy-2-naphthyl)-5-nitro-*N*-phenylbenzamide (14a). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-5-nitrobenzoic acid **13b** (1 g, 3.1 mmol, 1 eq) with aniline (0.3 ml, 3.1 mmol, 1 eq) according to method B. Purification by column chromatography (hexane/ethyl acetate 2:1) afforded 579 mg of the pure compound. Yield: 47%. $C_{24}H_{18}N_2O_4$; MW: 398.

3-(6-Hydroxy-2-naphthyl)-5-nitro-*N*-phenylbenzamide (14). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-5-nitro-*N*-phenylbenzamide **14a** (500 mg, 1.26 mmol, 1 eq) with boron tribromide (7.56 mmol, 6 eq) according to method F. Purification by preparative chromatography (hexane/ethyl acetate 1:1) afforded 269 mg of the desired product. Yield: 56%. $C_{23}H_{16}N_2O_4$; MW: 384.

3-(6-Methoxy-2-naphthyl)-*N*-(4-methoxyphenyl)-5-nitrobenzamide (15a). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-5-nitrobenzoic acid **13b** (500 mg, 1.55 mmol, 1 eq) with *p*-methoxyaniline (190 mg, 1.55 mmol, 1 eq) according to method B. Purification by column chromatography (hexane/ethyl acetate 9:1) afforded 128 mg of the pure compound. Yield: 19%. $C_{25}H_{20}N_2O_5$; MW: 428.

3-(6-Hydroxy-2-naphthyl)-*N*-(4-hydroxyphenyl)-5-nitrobenzamide (15). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-*N*-(4-methoxyphenyl)-5-nitrobenzamide **15a** (100 mg, 0.23 mmol, 1 eq) with boron tribromide (1.38 mmol, 6 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 99:1) afforded 48 mg of the desired product. Yield: 51%. $C_{23}H_{16}N_2O_5$; MW: 400.

3-(6-Methoxy-2-naphthyl)-*N*-(3-methoxyphenyl)-5-nitrobenzamide (16a). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-5-nitrobenzoic acid **13b** (500 mg, 1.55 mmol, 1 eq) with *m*-methoxyaniline (0.2 ml, 1.55 mmol, 1 eq) according to method B. Purification by column chromatography (hexane/ethyl acetate 9:1) afforded 404 mg of the pure compound. Yield: 61%. $C_{25}H_{20}N_2O_5$; MW: 428.

3-(6-Hydroxy-2-naphthyl)-*N*-(3-hydroxyphenyl)-5-nitrobenzamide (16). The title compound was prepared by reaction of 3-(6-methoxy-2-naphthyl)-*N*-(3-methoxyphenyl)-5-nitrobenzamide **16a** (300 mg, 0.70 mmol, 1 eq) with boron tribromide (4.2 mmol, 6 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 98:2) afforded 142 mg of the desired product. Yield: 51%. $C_{23}H_{16}N_2O_5$; MW: 400.

3-Amino-5-(6-hydroxy-2-naphthyl)-*N*-methylbenzamide hydrochloride (17). The title compound was prepared by reaction of 3-(6-hydroxy-2-naphthyl)-*N*-methyl-5-nitrobenzamide **13** (70 mg, 0.22 mmol, 1 eq) in presence of Pd/C 5% (20 mg, 0.021 mmol, 0.1 eq) in anhydrous ethanol (100 ml) under a hydrogen atmosphere overnight. After filtration over celite and evaporation of the solvent the crude was solved in acetonitrile. The expected compound **17** was obtained pure as precipitate after addition of HCl in ether (2M) in 33 % yield (21 mg). $C_{18}H_{16}N_2O_2 \cdot HCl$; MW: 328.

3-Amino-5-(6-hydroxy-2-naphthyl)-*N*-phenylbenzamide (18). A suspension of 3-(6-hydroxy-2-naphthyl)-5-nitro-*N*-phenylbenzamide **14** (200 mg, 0.52 mmol, 1 eq) in THF (5 ml) and tin pellet (124 mg, 1.04 mmol, 2 eq) and 6N HCl (1 ml) was stirred at 50 °C for 1 h. The reaction mixture was filtered and concentrated under reduced pressure. The desired compound was obtained after purification by column chromatography (hexane/ethyl acetate 1:1) in 28% yield (52 mg). $C_{23}H_{18}N_2O_2$; MW: 354.

6-Bromo-2-methoxy-1-naphthaldehyde (19d). To $TiCl_4$ (9.7 ml, 16.8 g, 0.09 mol, 2.1 eq) and dichloromethyl methyl ether (4.2 ml, 5.33 g, 0.089 mol, 1.1 eq) in solution in dichloromethane (20 ml) at 0 °C was added drop wise 2-bromo-6-methoxynaphtalene **19e** (10 g, 0.042 mol, 1 eq) in CH_2Cl_2 (200 ml), maintaining the temperature below 5 °C. After completion of the addition, the reaction mixture was stirred at room temperature overnight. Aqueous hydrochloric acid 1% (300 ml) was added to quench the reaction. The organic layer was separated. The aqueous layer was extracted with CH_2Cl_2. The organic layers were combined, washed with brine, dried over $MgSO_4$ and concentrated to give the desired compound in quantitative yield (11.1 g). $C_{12}H_9BrO_2$; MW 265.

2-Methoxy-6-(3-methoxyphenyl)-1-naphthaldehyde (19c). The title compound was prepared by reaction of 6-bromo-2-methoxy-1-naphthaldehyde **19d** (2 g, 7.55 mmol, 1 eq) with 3-methoxybenzeneboronic acid (1.3 g, 8.30 mmol, 1.1 eq) according to method A. The product was purified by column chromatography (hexane/ethyl acetate 1:1). Yield: 86% (1.89 g). $C_{19}H_{16}O_3$; MW 292.

2-Methoxy-6-(3-methoxyphenyl)-1-naphthoic acid (19b). A solution of 2-methoxy-6-(3-methoxyphenyl)-1-naphthaldehyde **19c** (200 mg, 0.68 mmol, 1 eq) in water (8 ml) and acetone (17 ml) was cooled in ice bath. Sulfamic acid (140 mg, 1.51 mmol, 2.2 eq) and sodium chlorite 80% (0.77 mmol, 1.1 eq) were added. After stirring for 30 min at 0°C, the acetone was evaporated and dichloromethane added. The organic layer was washed 3 times with brine. The title compound was obtained in 87% yield (308 mg) as yellow solid after drying over $MgSO_4$ and concentration under reduced pressure. $C_{19}H_{16}O_4$; MW 308.

2-Methoxy-6-(3-methoxyphenyl)-*N*-methyl-1-naphthamide (19a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (300 mg, 0.97 mmol, 1 eq) with a solution of methylamine (33% in ethanol, 0.12 ml, 0.97 mmol, 1 eq) according to method B. The crude product was purified by column chromatography (hexane/ethyl acetate 2:1) and **19a** was obtained in quantitative yield (311 mg). $C_{20}H_{19}NO_3$; MW 321.

2-Hydroxy-6-(3-hydroxyphenyl)-*N*-methyl-1-naphthamide (19). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-*N*-methyl-1-naphthamide **19a** (250 mg, 0.78 mmol, 1 eq) with boron tribromide (3.9 mmol, 5 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 98:2) afforded the desired product in 95 % yield (217 mg). $C_{18}H_{15}NO_3$; MW 293; MS, ESI: 292 $(M-H)^-$.

2-Methoxy-6-(3-methoxyphenyl)-*N*-phenyl-1-naphthamide (20a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (200 mg, 0.65 mmol, 1 eq) with aniline (5.8 µl, 60 mg, 0.65 mmol, 1 eq) according to method B. The title

compound was obtained after purification by column chromatography (hexane/ethyl acetate 2:1) with 85% yield (318 mg). $C_{25}H_{21}NO_3$; MW 383.

2-Hydroxy-6-(3-hydroxyphenyl)-*N*-phenyl-1-naphthamide (20). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-*N*-phenyl-1-naphthamide **20a** (217 mg, 0.57 mmol, 1 eq) with boron tribromide (3.42 mmol, 6 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 95:5) afforded the desired product in 23% yield (45 mg). $C_{23}H_{17}NO_3$; MW 355; MS, ESI: 356 $(M+H)^+$.

2-Methoxy-*N*,6-bis(3-methoxyphenyl)-1-naphthamide (21a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (300 mg, 0.97 mmol, 1 eq) with *m*-anisol (0.11 ml, 120 mg, 0.97 mmol, 1 eq) according to method B. The desired compound was obtained after purification of the crude product by column chromatography (hexane/ethyl acetate 2:1) in 41% yield (165 mg). $C_{26}H_{23}NO_4$; MW 413.

2-Hydroxy-*N*,6-bis(3-hydroxyphenyl)-1-naphthamide (21). The title compound was prepared by reaction of 2-methoxy-*N*,6-bis(3-methoxyphenyl)-1-naphthamide **21a** (150 mg, 0.36 mmol, 1 eq) with boron tribromide (2.9 mmol, 8 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 93:7) afforded the desired product in 50% yield (66 mg). $C_{23}H_{17}NO_4$; MW 371; MS, ESI: 372 $(M+H)^+$.

6-(3-Hydroxyphenyl)-1-(piperidin-1-ylcarbonyl)-2-naphthol (22). The title compound was prepared by reaction of 2-hydroxy-6-(3-hydroxyphenyl)-1-naphthoic acid (160 mg, 0.57 mmol, 1 eq) with piperidine (113 ml, 97 mg, 1.14 mmol, 2 eq) according to method D. After purification of the crude product by column chromatography (dichloromethane/methanol 95:5) compound **22** was obtained in 8% yield (16 mg). $C_{22}H_{21}NO_3$; MW 347.

4-[2-Methoxy-(6-(3-methoxyphenyl)-1-naphthyl)]-1-morpholine (23a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (200 mg, 0.65 mmol, 1 eq) with morpholine (1.1 ml, 6.5 mmol, 10 eq) according to method D. After purification of the crude product by column chromatography (dichloromethane/methanol 98:2) compound **23a** was obtained in 85% yield (194 mg). The title compound was not characterized; it was directly used for ether cleavage. $C_{23}H_{23}NO_4$; MW 377.

6-(3-Hydroxyphenyl)-1-(morpholin-4-ylcarbonyl))-2-naphthol (23). The title compound was prepared by reaction of 4-[2-methoxy-(6-(3-methoxyphenyl)-1-naphthyl)]-1-morpholine **23a** (195 mg, 0.52 mmol, 1 eq) with boron tribromide (2.6 mmol, 5 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 95:5) afforded the compound **23** in 93% yield (170 mg). $C_{21}H_{19}NO_4$; MW 349; MS, ESI: 348 $(M-H)^-$.

***tert*-Butyl 4-(2-methoxy-6-(3-methoxyphenyl)-1-naphthoyl)piperazine-1-carboxylate (24a).** The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (200 mg, 0.65 mmol, 1 eq) with Boc-piperazine (121 mg, 0.65 mmol, 1 eq) according to method B. The compound was not characterized; the crude product was directly used in the next step. $C_{28}H_{32}N_2O_5$; MW 476.

6-(3-Hydroxyphenyl)-1-(piperazin-1-ylcarbonyl))-2-naphthol (24). The title compound was prepared by reaction *tert*-butyl 4-(2-methoxy-6-(3-methoxyphenyl)-1-naphthoyl)piperazine-1-carboxylate **24a** (200 mg, 0.42 mmol, 1 eq) with boron tribromide (2.52 mmol, 6 eq) according to method F. The desired compound was obtained in 60% yield (81 mg). $C_{21}H_{20}N_2O_3$; MW 348; MS, ESI: 349 $(M+H)^+$.

2-Methoxy-6-(3-methoxyphenyl)-*N*-(2-pyridyl)-1-naphthamide (25a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (580 mg, 1.88 mmol, 1.7 eq) with 2-aminopyridine (100 mg, 1.12 mmol, 1 eq) according to method D. After purification of the crude product by column chromatography (hexane/methanol 99:1)

compound **25a** was obtained in 48% yield (206 mg). $C_{24}H_{20}N_2O_3$; MW 384; MS, ESI: 385 $(M+H)^+$.

2-Hydroxy-6-(3-hydroxyphenyl)-*N*-pyridin-2-yl-1-naphthamide (25). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-*N*-(2-pyridyl)-1-naphthamide **25a** (33 mg, 0.086 mmol, 1 eq) with boron tribromide (0.52 mmol, 6 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 99:1) afforded the desired compound in 36% yield (11 mg). $C_{22}H_{16}N_2O_3$; MW 356; MS, ESI: 357 $(M+H)^+$.

2-Methoxy-6-(3-methoxyphenyl)-*N*-(pyrimidin-4-yl)-1-naphthamide (26a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (465 mg, 1.50 mmol, 1.5 eq) with 2-aminopyrimidine (100 mg, 1.0 mmol, 1 eq) according to method D. After purification of the crude product by column chromatography (dichloromethane/methanol 98:2) compound **26a** was obtained in 45% yield (173 mg). It was not characterized, it was directly used in the next step. $C_{23}H_{19}N_3O_3$; MW 385.

2-Hydroxy-6-(3-hydroxyphenyl)-*N*-(pyrimidin-4-yl)-1-naphthamide (26). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-*N*-(pyrimidin-4-yl)-1-naphthamide **26a** (117 mg, 0.30 mmol, 1 eq) with boron tribromide (2.12 mmol, 7 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 98:2) afforded the desired compound in 96% yield (99 mg). $C_{21}H_{15}N_3O_3$; MW 357; MS, ESI: 358 $(M+H)^+$.

2-Methoxy-6-(3-methoxyphenyl)-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)-1-naphthamide (27a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-naphthoic acid **19b** (630 mg, 2 mmol, 1.2 eq) with 5-methyl-1,3,4-thiadiazole-2-amine (187 mg, 1.63 mmol, 1 eq) according to method D. After purification of the crude product by column chromatography (dichloromethane/methanol 95:5) compound **27a** was obtained in 94% yield (620 mg). The title compound was not characterized; it was directly used in the next step. $C_{22}H_{19}N_3O_3S$; MW 405.

2-Hydroxy-6-(3-hydroxyphenyl)-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)-1-naphthamide (27). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)-1-naphthamide **27a** (620 mg, 1.53 mmol, 1 eq) with boron tribromide (12.24 mmol, 8 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 98:2) afforded compound **27** in 20% yield (115 mg). $C_{20}H_{15}N_3O_3S$; MW 377; MS, ESI: 378 $(M+H)^+$.

Ethyl (2*E*)-3-[2-methoxy-6-(3-methoxyphenyl)-1-naphthyl)]acrylate (28c). NaH (55-65%) (10 mg, 0.21 mmol, 1.2 eq) was suspended in 5 ml dry DME under nitrogen atmosphere together with triethylphosphonoacetate (46 mg, 0.21 mmol, 1.2 eq). The mixture was stirred for 15 min at room temperature. After the addition of 2-methoxy-6-(3-methoxyphenyl)-1-naphthaldehyde **19c** (50 mg, 0.17 mmol, 1 eq) the reaction mixture was stirred at room temperature for 1 h. Water was added and the aqueous layer extracted with ethyl acetate. The organic layers were combined and dried over $MgSO_4$. After evaporation of the solvent under reduced pressure, the title compound was obtained as yellow solid in quantitative yield (61 mg). $C_{23}H_{22}O_4$; MW 362.

(2*E*)-3-(2-Methoxy-6-(3-methoxyphenyl)-1-naphthyl)acrylic acid (28b). Lithium hydroxide (418 mg, 8.52 mmol, 5 eq) was added at 0 °C to a solution of ethyl (2*E*)-3-[2-methoxy-6-(3-methoxyphenyl)-1-naphthyl)]acrylate **28c** (600 mg, 1.66 mmol, 1 eq) in a THF/water 3:1 (20 ml). After heating the reaction mixture at reflux overnight, the solvent was removed under reduced pressure. The residue was acidified with 2N-HCl and extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The compound was pure enough to be used without purification (quantitative yield, 554 mg). $C_{21}H_{18}O_4$; MW 334.

(2*E*)-3-[2-Methoxy-6-(3-methoxyphenyl)-1-naphthyl)]-*N*-methylacrylamide (28a). The title compound was prepared by reaction of (2*E*)-3-(2-methoxy-6-(3-methoxyphenyl)-1-naphthyl)acrylic acid **28b** (400 mg, 1.20 mmol, 1 eq) with a solution of methylamine (33% in ethanol, 0.16 ml, 1.28 mmol, 1 eq) according to method B. The compound was purified by column chromatography (dichloromethane/methanol 98:2) and obtained in 44% yield (184 mg). $C_{21}H_{22}NO_3$; MW 347.

(2*E*)-3-[2-Hydroxy-6-(3-hydroxyphenyl)-1-naphthyl)]-*N*-methylacrylamide (28). The title compound was prepared by reaction of (2*E*)-3-[2-methoxy-6-(3-methoxyphenyl)-1-naphthyl)]-*N*-methylacrylamide **28a** (127 mg, 0.37 mmol, 1 eq) with boron tribromide (2.31 mmol, 8 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 95:5) afforded the desired product in 60% yield (71 mg). $C_{20}H_{17}NO_3$; MW 319; MS, ESI: 320 $(M+H)^+$.

(2*E*)-3-[2-Methoxy-6-(3-methoxyphenyl)-1-naphthyl)]-*N*-phenylacrylamide (29a). The title compound was prepared by reaction of (2*E*)-3-(2-methoxy-6-(3-methoxyphenyl)-1-naphthyl)acrylic acid **28b** (200 mg, 0.60 mmol, 1 eq) with aniline (54 µl, 56 mg, 0.60 mmol, 1 eq) according to method B. The compound was obtained in quantitative yield (245 mg). It was used in the next step without further purification. $C_{27}H_{23}NO_3$; MW 409.

(2*E*)-3-[2-Hydroxy-6-(3-hydroxyphenyl)-1-naphthyl)]-*N*-phenylacrylamide (29). The title compound was prepared by reaction of (2*E*)-3-[2-methoxy-6-(3-methoxyphenyl)-1-naphthyl)]-*N*-phenylacrylamide **29a** (70 mg, 0.17 mmol, 1 eq) with boron tribromide (1.36 mmol, 8 eq) according to method F. Purification by column chromatography (dichloromethane/methanol 95:5) afforded the product in 13% yield (8 mg). $C_{25}H_{19}NO_3$; MW 381; MS, ESI: 382 $(M+H)^+$.

3-[2-Hydroxy-6-(3-hydroxyphenyl)-1-naphthyl)]-*N*-methylpropanamide (30). The title compound was prepared by hydrogenation of (2*E*)-3-[2-hydroxy-6-(3-hydroxyphenyl)-1-naphthyl)]-*N*-methylacrylamide **28** (40 mg, 0.13 mmol, 1 eq) with $Pd(OH)_2$ according to method H. The compound **30** was obtained in quantitative yield (40 mg). $C_{20}H_{19}NO_3$; MW 321; MS, ESI: 322 $(M+H)^+$.

1-Bromo-2-methoxy-6-(3-methoxyphenyl)naphthalene (31a). The title compound was prepared by reaction of 1,6-dibromo-2-methoxynaphthalene **31b** (5.75 g, 18.20 mmol, 1 eq) with 3-methoxybenzeneboronic acid (2.77 g, 18.20 mmol, 1 eq) according to method A. Purification by column chromatography (hexane) afforded the desired compound in 81% yield (5.06 g). $C_{18}H_{15}BrO_2$; MW 343.

1-Bromo-6-(3-hydroxyphenyl)-2-naphthol (31). The title compound was prepared by reaction of 1-bromo-2-methoxy-6-(3-methoxyphenyl)naphthalene **31a** (500 mg, 1.46 mmol, 1 eq) with boron tribromide (7.3 mmol, 5 eq) according to method F. It was obtained in quantitative yield (460 mg). $C_{16}H_{11}BrO_2$; MW 315; MS, ESI: 313-315 $(M-H)^-$.

6-(3-Hydroxyphenyl)-1-phenyl-2-naphthol (32). The title compound was prepared by reaction of 1-bromo-6-(3-hydroxyphenyl)-2-naphthol **31** (50 mg, 0.16 mmol, 1 eq) with benzeneboronic acid (19.4 mg, 0.16 mmol, 1 eq) according to method A. The crude product was purified by column chromatography (hexane/ethyl acetate 7:3) to give **32** in 30% yield (15 mg). $C_{22}H_{16}O_2$; MW 312; MS, ESI: 311 $(M-H)^-$.

2-Methoxy-6-(3-methoxyphenyl)-1-(phenylsulfanyl)naphthalene (33b). A mixture of 1-bromo-2-methoxy-6-(3-methoxyphenyl)naphthalene **31a** (850 mg, 2.47 mmol, 1 eq) and sodium benzenethiolate (391 mg, 2.96 mmol, 1.2 eq) in dry DMF (10 ml) was refluxed for 11 h under nitrogen atmosphere. The reaction mixture was poured in ice, the precipitate was collected, washed with water and dried in a desiccator (42% yield, 385 mg). The compound was pure enough to be used in the next step without further purification. $C_{24}H_{20}O_2S$; MW 372.

6-(3-Hydroxyphenyl)-1-(phenylsulfanyl)-2-naphthol (33a). The title compound was prepared by reaction of 2-methoxy-6-(3-methoxyphenyl)-1-(phenylsulfanyl)naphthalene **33b** (376 mg, 1.01 mmol, 1 eq) with boron tribromide (8 mmol, 8 eq) according to method F. The compound was obtained in 94% yield (325 mg). $C_{22}H_{16}O_2S$; MW 344; MS, ESI: 343 $(M-H)^-$.

6-(3-Hydroxyphenyl)-1-(phenylsulfonyl)-2-naphthol (33). To a solution of 6-(3-hydroxyphenyl)-1-(phenylsulfanyl)-2-naphthol **33a** (71 mg, 0.21 mmol, 1 eq) in anhydrous dichloromethane (10 ml) at 0 °C, was added *m*-CPBA (192 mg, 0.82 mmol, 3.9 eq) in CH_2Cl_2 (10 ml). The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 12 h. The reaction was poured into ice water and the crude was extracted with ethyl acetate. The combined organic layers were washed with $NaHCO_3$ and brine and dried over Na_2SO_4. After purification of the crude product by column chromatography compound **33** was obtained in 50% yield (38 mg). $C_{22}H_{16}O_4S$; MW 376; MS, ESI: 375$(M-H)^-$.

2-Methoxy-6-(3-methoxyphenyl)-1-[(4-methylphenyl)sulfanyl]naphthalene (34b). A mixture of 1-bromo-2-methoxy-6-(3-methoxyphenyl)naphthalene **31a** (100 mg, 0.29 mmol, 1 eq) and sodium 4-methylbenzenethiolate (51 mg, 0.34 mmol, 1.2 eq) in dry DMF (10 ml) was refluxed for 11 h under nitrogen atmosphere. The reaction mixture was poured in ice. The precipitate formed was collected, washed with water and dried in a desiccator (73% yield, 80 mg). The compound was pure enough to be used in the next step without further purification. $C_{25}H_{22}O_2S$; MW 386.

6-(3-Hydroxyphenyl)-1-(4-methylphenyl)sulfonyl)-2-naphthol (34). To a solution of 2-methoxy-6-(3-methoxyphenyl)-1-[(4-methylphenyl)sulfanyl]naphthalene **34b** (385 mg, 1.03 mmol, 1 eq) in anhydrous dichloromethane (10 ml) at 0 °C was added *m*-CPBA (1.42 g, 8.24 mmol, 8.2 eq) in CH_2Cl_2 (10 ml). The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 12 h, poured into ice water and extracted with ethyl acetate. The combined organic layers were washed with $NaHCO_3$ and brine and dried over Na_2SO_4. A monodemethylation occurred during the oxidation step. The second methoxy group of the product (105 mg, 0.26 mmol, 1 eq) was then removed by reaction with boron tribromide (2.1 mmol, 8 eq) according to method F. The product was obtained in 79% yield (80 mg). $C_{23}H_{18}O_4S$; MW 390; MS, ESI: 389$(M-H)^-$.

Methyl 3-methoxy-7-(3-methoxyphenyl)-2-naphthoate (35b). The title compound was prepared by reaction of methyl 7-bromo-3-methoxy-2-naphthoate **35c** (2.2 g, 7.45 mmol, 1 eq) with 3-methoxyphenyl boronic acid (1.37 g, 8.95 mmol, 1.5 eq) according to method A. The reaction mixture was purified by column chromatography (dichloromethane/hexane, gradient 5:5 to 7:3, dichloromethane/methanol 95:5) to give 1.58 g of compound **35b**. Yield: 66%. $C_{20}H_{18}O_4$; MW 322.

3-Methoxy-7-(3-methoxyphenyl)-*N*-methyl-2-naphthamide (35a). The title compound was prepared by reaction of 3-methoxy-7-(3-methoxyphenyl)-2-naphthoic acid (323 mg, 1.05 mmol, 1 eq) with a solution of methylamine (33% in ethanol, 0.13 ml, 1.05 mmol, 1 eq) according to method C. Purification by column chromatography (hexane/ethyl acetate 8:2) gave 247 mg of the amide **35a**. Yield: 74%. $C_{20}H_{19}NO_3$; MW 321; MS, ESI: 322 $(M+H)^+$.

3-Hydroxy-7-(3-hydroxyphenyl)-*N*-methyl-2-naphthamide (35). The title compound was prepared by reaction of 3-methoxy-7-(3-methoxyphenyl)-*N*-methyl-2-naphthamide **35a** (93 mg. 0.29 mol, 1 eq) with boron tribromide (5.5 mmol, 19 eq) according to method F. The product was obtained in a quantitative yield (87 mg). $C_{18}H_{15}NO_3$; MW 293; MS, ESI: 294 $(M+H)^+$.

3-Methoxy-7-(3-methoxyphenyl)-*N*-phenyl-2-naphthamide (36a). The title compound was prepared by reaction of 3-methoxy-7-(3-methoxyphenyl)-2-naphthoic acid (323 mg, 1.05 mmol, 1 eq) with aniline (95 µl, 1.05 mmol, 1 eq) according to method C. Purification by column

chromatography (hexane/ethyl acetate 8:2 and 6:4) gave 281 mg of the amide **36a**. Yield: 70%. $C_{25}H_{21}NO_3$; MW 383; MS, ESI: 384 $(M+H)^+$.

3-Hydroxy-7-(3-hydroxyphenyl)-*N*-phenyl-2-naphthamide (36). The title compound was prepared by reaction of 3-methoxy-7-(3-methoxyphenyl)-*N*-phenyl-2-naphthamide **36a** (86 mg, 0.22 mol, 1 eq) with boron tribromide (4.45 mmol, 20 eq) according to method F. After purification of the crude product by column chromatography (hexane/ethyl acetate 6:4) compound **36** was obtained in 50% yield (40 mg). $C_{23}H_{17}NO_3$; MW 355; MS, APCI: 355 $(M)^+$.

3-Methoxy-7-(3-methoxyphenyl)-1-naphthonitrile (37a). The title compound was prepared by reaction of 8-cyano-6-methoxy-2-naphthyl trifluoromethanesulfonate **37b** (466 mg, 1.4 mmol, 1 eq) with 3-methoxybenzene boronic acid (260 mg, 1.7 mmol, 1.2 eq) according to method A. The reaction mixture was purified by column chromatography (hexane/ethyl acetate 7: 3) to give **37a** as a yellow solid. Yield: 25% (101 mg). $C_{19}H_{15}NO_2$; MW 289.

3-Hydroxy-7-(3-hydroxyphenyl)-1-naphthonitrile (37). The title compound was prepared by reaction of 3-methoxy-7-(3-methoxyphenyl)-1-naphthonitrile **37a** (100 mg, 0.35 mmol, 1 eq) with pyridinium hydrochloride (492 mg, 4.3 mmol, 12 eq) according to method G. The desired compound was obtained in 64% yield (58 mg). $C_{17}H_{11}NO_2$; MW 261; MS, ESI: 260 $(M-H)^-$.

7-Methoxy-3-(3-methoxyphenyl)-1-naphthonitrile (38a). The title compound was prepared by reaction of 3-bromo-7-methoxy-1-naphthonitrile **37c** (1.4 g, 1.76 mmol, 1 eq) with 3-methoxybenzene boronic acid (320 mg, 2.11 mmol, 1.2 eq) according to method A. The reaction mixture was purified by column chromatography (hexane/ethyl acetate 7:3) to give **38a** as a yellow solid. Yield: 53% (270 mg). $C_{19}H_{15}NO_2$; MW 289.

7-Hydroxy-3-(3-hydroxyphenyl)-1-naphthonitrile (38). The title compound was prepared by reaction of 7-methoxy-3-(3-methoxyphenyl)-1-naphthonitrile **38a** (110 mg, 0.39 mmol, 1 eq) with pyridinium hydrochloride (547 mg, 4.76 mmol, 12.2 eq) according to method G. The compound was obtained in 62% yield (63 mg). $C_{17}H_{11}NO_2$; MW 261; MS, ESI: 260 $(M-H)^-$.

Biological Methods

[2,4,6,7-^{3}H]-E1 and [2,4,6,7-^{3}H]-E2 were purchased from Perkin Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt. T47D cells (passage 9) were obtained from ECACC, Salisbury. FCS was purchased from Sigma, Taufkirchen. Cell culture media and dextran coated charcoal stripped FCS (DCC-FCS) were bought from CCPRO, Oberdorla. Other chemicals were purchased from Sigma, Roth or Merck.

1. 17β-HSD1 and 17β-HSD2 Enzyme preparation

Recombinant human enzyme (Procedure A and C):
Recombinant baculovirus was produced by the "Bac to Bac Expression System" (Invitrogen). Recombinant bacmid was transfected to Sf9 insect cells using "Cellfectin Reagent" (Invitrogen). Sixty hours later cells were harvested; the microsomal fraction was isolated as described by Puranen *et al.*, 1994. Aliquots containing 17β-HSD1 or 17β-HSD2 were stored frozen until determination of enzymatic activity.

Human enzyme enriched from placental tissue (Procedure B and D):
17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures (Ghosh *et al.*, 1995; Zhu *et al.*, 1993). Fresh human placenta was homogenized and the enzymes were separated by subcellular fractionation (centrifugation method). For the purification of 17β-HSD1, the cytosolic fraction was precipitated with ammonium sulfate. 17β-HSD2 was obtained from the microsomal fraction. Aliquots containing 17β-HSD1 or 17β-HSD2 were stored frozen.

2. Inhibition of 17β-HSD1

The synthesized compounds were tested for their ability to inhibit 17β-HSD1 according to Procedure A (recombinant human enzyme, percentage of inhibition determination). For select compounds, IC_{50} values were determined according to Procedure B (human placental enzyme). Procedure A and B differ from enzyme source and substrate concentration. The two procedures have been compared and give similar results.

Procedure A using recombinant human enzyme

Assay: Recombinant human protein (0.1 µg/mL) was incubated in 20 mM KH_2PO_4 pH 7.4 with 30 nM [^{3}H]-estrone and 1mM NADPH for 30 min at room temperature, in the presence of potential inhibitors at concentrations of 1 µM or 100 nM. Inhibitor stock solutions were prepared in DMSO. Final concentration of DMSO was adjusted to 1% in all samples. The enzyme reaction was stopped by addition of 10% trichloroacetic acid (final concentration). Samples were centrifuged in a microtiter plate at 4000 rpm for 10 min. Supernatants were applied to reverse phase HPLC on a Waters Symmetry C18 column, equipped with a Waters Sentry Guard column. Isocratic HPLC runs were performed at room temperature at a flow rate of 1 mL/min of acetonitrile/water (48:52) as running solvent. Radioactivity of the eluate was monitored by a Packard Flow Scintillation Analyzer. Total radioactivities for estrone and estradiol were determined in each sample. The conversion rate was calculated according to the following formula: % conversion = 100 * [(cpm E2 in sample with inhibitor)/(cpm E1 in sample with inhibitor + cpm E2 in sample with inhibitor)]/[(cpm E2 in sample without inhibitor)/(cpm E1 in sample without inhibitor + cpm E2 in sample without inhibitor)]. Percentage of inhibition was calculated according to the following equation: % inhibition = 100 - % conversion. Each value was calculated from two independent experiments.

Procedure B using human placental enzyme

Assay: Inhibitory activities were evaluated by an established method with minor modifications (Lin *et al.*, 1992; Sam *et al.*, 1995; Sam *et al.*, 1998). Briefly, the enzyme preparation was incubated with NADH [500µM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20 % of glycerol and EDTA 1mM. Inhibitor stock solutions were prepared in DMSO. Final concentration of DMSO was adjusted to 1% in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 rp chromatography column (Nucleodur C18 Gravity, 3µm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated according to following equation: $\%conversion = \frac{\%E2}{\%E2 + \%E1} \bullet 100$. Each value was calculated from at least three independent experiments.

2. Inhibition of 17β-HSD2

The synthesized compounds were tested for their ability to inhibit 17β-HSD2 according to Procedure C (recombinant human enzyme) or D (human placental enzyme). For select compounds, IC_{50} values were determined according to Procedure D (human placental enzyme). Procedure C and D differ from enzyme source and substrate concentration. The two procedures have been compared and give similar results.

Procedure C using recombinant human enzyme

The 17β-HSD2 inhibition assay was performed as previously described for 17β-HSD1 according to procedure A from the recombinant human protein, using [^{3}H]-E2 as substrate [30 nM] and NAD^+ [1 mM] as cofactor.

Procedure D using human placental enzyme
The 17β-HSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD^+ [1500 µM], test compound and a mixture of unlabelled- and [^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi) for 20 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above for17β-HSD1.

3. ER affinity
The binding affinity of select compounds to the ERα and ERβ was determined according to Zimmermann *et al.*, 2005 using recombinant human proteins. Briefly, 0.25 pmoles of ERα or ERβ, respectively, were incubated with [^{3}H]-E2 (10 nM) and test compound for 1 h at room temperature. The potential inhibitors were dissolved in DMSO (5% final concentration). Non-specific-binding was performed with diethylstilbestrol (10 µM). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (5 g/ 60 ml TE-buffer). The formed complex was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). For determination of the relative binding affinity, RBA, inhibitor and E2 concentrations required to displace 50% of the receptor bound labelled E2 were determined. RBA values were calculated according to the following equation: $RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \bullet 100$. The RBA value for E2 was arbitrarily set at 100%.

4. Proliferation assay
Cell culture: Stock culture of T47D cells (ecacc, United Kingdom) was maintained in RPMI-1640 supplemented with sodium bicarbonate (2 g/l), streptomycin (100 µg/ml), insuline zinc salt (10 µg/ml), sodium pyruvate (1 mM), penicillin (100 U/ml) and FCS 10% (vol/vol). Cells were cultured at 37 °C under 5% CO_2 humidified atmosphere. Medium was changed every two to three days, cells were subcultured every four to five days.

Evaluation of estrogenic effects on the estrogen dependent human breast cancer cell line T47D: Phenol red-free medium was supplemented with sodium bicarbonate (2 g/l), streptomycin (100 µg/ml), insuline zinc salt (10 µg/ml), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/ml) and DCC-FCS 5% (vol/vol). RPMI 1640 (without phenol red) was used for the experiments. Cells (7500 cells/96-wellplate) were grown for 48 h in phenol red-free medium. The compounds tested were added at a final concentration of 100 nM. Inhibitors and E2 were diluted in ethanol (final ethanol concentration was adjusted to 1%). As a positive control E2 was added at a final concentration of 0.1 nM. Ethanol was used as negative control. Medium was changed every two to three days and supplemented with the respective additive. After eight days of incubation, the cell viability was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). The cleavage of MTT to a blue formazan by mitochondrial succinat-dehydrogenase was quantified spectrophotometrically at 590 nm as described by Denizot and Lang, 1986 with minor modifications. The control proliferation was arbitrarily set at 1 and the stimulation induced by the inhibitor was calculated according to following equation: $\%stimulation = \frac{[proliferation(compound-induced)-1]}{[proliferation(E2-induced)-1]} \bullet 100\%$. Each value is calculated as a mean value of at least three independent experiments.

5. Evaluation of plasma concentrations of compounds 1 and 32 after peroral application to adult male rats in cassette dosing.
Four adult male Wistar rats (Janvier, France) were used. Animals were housed in a temperature-controlled room (20-22 °C) and maintained in a 12 h light/12 h dark cycle. Food and water were available *ad libitum*. Rats were anaesthetized with a ketamine (135 mg/kg)/ xylazine (10 mg/kg)

mixture and cannulated with silicone tubing via the right jugular vein. Prior to the first blood sampling, animals were connected to a counterbalanced system and tubing, to perform blood sampling in the freely moving rat. Compounds **1** and **32,** dissolved in labrasol/water (1:1) as vehicle, were administered perorally at doses of 10 mg/kg in a cassette dosing approach. Each application group consisted of 4 rats. At Time 0, compounds **1** and **32** were applied and blood samples (200 µl) were taken at 1, 2, 3, 4, 6, 8, 10 and 24 h post-dose, collected in heparinised tubes and stored on ice. Plasma was harvested and kept at –20 °C until being assayed. HPLC-MS/MS analysis and quantification of the samples was carried out on a Surveyor-HPLC-system coupled with a TSQ Quantum (ThermoFisher) triple quadrupole mass spectrometer equipped with an electrospray ioninterface. This test was performed according to the Laws on Animal Care and Use and had been approved by the local Animal Care Committee.

Molecular modeling

All molecular modelling studies were performed on Intel(R) P4 CPU 3.00GHz running Linux Suse 9.3. X-ray structure of 17β-HSD1 (PDB-ID: 1FDT, www.pdb.org) was prepared using the BIOPOLYMER module of SYBYL v7.0 (Sybyl, Tripos Inc., St. Louis, Missouri, USA). Water molecules, E2 and sulfate ions were stripped from the PDB file and missing protein atoms were added and correct atom types set. Finally hydrogen atoms and neutral end groups were added. All basic and acidic residues were considered protonated and deprotonated, respectively. Further, the crystal structure was minimized for 200 steps with the steepest descent minimizer as implemented in SYBYL with the backbone atoms kept at fixed positions in order to fix close contacts (Arg37).
Docking of selected inhibitors, built with SYBYL and energy-minimized in MMFF94s force-field as implemented in Sybyl, into the substrate binding site was performed by the docking program GOLD v.3.0.1 (Jones *et al.*, 1997). Since GOLD docking program allows flexible docking of ligands, no conformational search was employed to the ligand structures. Ligands were docked in 50 independent genetic algorithm (GA) runs. Active-site origin was set at the center of the steroid binding site, while the radius was set equal to 13 Å. The automatic active-site detection was switched on. Further, the CHEMSCORE fitness function was used and genetic algorithm default parameters were set as suggested by the GOLD authors.
The quality of the docked poses was evaluated based on the scoring function, which give a good measure to discriminate between the found binding modes, and mainly on visual inspection of the putative binding modes of the ligands.

Acknowledgment.

We thank Ms Anja Palusczak and Ms Beate Geiger for their help in performing the *in vitro* tests (17β-HSD1 Procedure B, 17β-HSD2 Procedure C, ERα and ERβ). We are grateful to Hormos Medical Corp. (Finland) for performing the inhibition test on 17β-HSD1 (Procedure A) and 17β-HSD2 (Procedure C). We thank Matthias Negri for useful discussion concerning the modeling section and for his help with Figure 3. P.K. is grateful to the European Postgraduate School 532 (DFG) for a scholarship.

Supporting information

Spectroscopic data (^{1}H-NMR, ^{13}C-NMR, IR) and purity data of compounds **2-3, 5, 7-8, 10-11, 19-24, 26, 28-32, 35, 37-38.**
This material is available free of charge via internet at http://pubs.acs.org.

References

Azzi, A.; Rehse, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S.-X.: Crystal structure of human estrogenic 17beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol. *Nat. Struct. Biol.* 3 (1996) 665-668.

Bahuguna, R. P.; Joshi, Y. C.; Dobhal, M. P.; Pande, R. K.; Joshi, B. C.: Spectral studies of some substituted arylthiobenzo[f]quinolines. *J. Heterocyclic Chem.* 19 (1982) 957-960.
Bhatt, V. M.; Kulkarni, S. U.: Cleavage of ethers. *Synthesis* 4 (1983) 249-282.
Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C.: The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors. *Structure* 4 (1996) 905-915.
Brožic, P.; Lanišnik Rižner, T.; Gobec S.: Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* 15 (2008) 137-150 and references therein cited.
Bush, N. J.: Advances in hormonal therapy for breast cancer. *Semin. Oncol. Nurs.* 23 (2007) 46-54.
Chan, J. H.; Hong J. S.; Hunter III, R. N.; Orr, G. F.; Cowan, J. R.; Sherman, D. B.; Sparks, S. M.; Reitter, B. E.; Andrews III, C. W.; Hazen, R. J.; St. Clair, M.; Boone, L. R.; Ferris, R. G.; Creech, K. L.; Roberts, G. B.; Short, S. A.; Weaver, K.; Ott, R. J.; Ren, J.; Hopkins, A.; Stuart, D. I.; Stammers, D. K.: 2-Amino-6-arylsulfonylbenzonitriles as non-nucleoside reverse transcriptase inhibitors of HIV-1. *J. Med. Chem.* 44 (2001) 1866-1882.
Clark, C. R.; Lin, C.-M.; Sansom R. T.: Anticonvulsant activity of 2- and 3-aminobenzanilides. *J. Med. Chem.* 29 (1986) 1534-1537.
Claudi, F.; Di Stefano, A.; Napolitani, F., Gian, M.; Giorgioni, G.; Fontenla, J. A.; Montenegro, G. Y.; Rivas, M. E.; Rosa, E.; Michelotto, B.; Orlando, G.; Brunetti, L.: Binding and preliminary evaluation of 5-hydroxy- and 10-hydroxy-2,3,12,12a-tetrahydro-1H-[1]benzoxepino[2,3,4-ij]isoquinolines as dopamine receptor ligands. *J. Med. Chem.* 43 (2000) 599-608.
Davis, S. E.; Rauckman, B. S.; Chan, J. H.; Roth B.: 2,4-Diamino-5-benzylpyrimidines and analogs as antibacterial agents. 11. Quinolylmethyl analogs with basic substituents conveying specificity. *J. Med. Chem.* 32 (1989) 1936-1942.
Day, J. M.; Foster, P. A.; Tutill, H. J.; Parsons, M. F. C.; Newman, S. P.; Chander, S. K.; Allan, G. M.; Lawrence, H. R.; Vicker, N.; Potter, B. V. L.; Reed, M. J.; Purohit, A.: 17beta-hydroxysteroid dehydrogenase type 1 and not type 12 is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* 122 (2008) 1931-1940.
Denizot, F.; Lang, R.: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89 (1986) 271-277.
Dizerega, G. S.; Barber, D. L.; Hodgen, G. D.: Endometriosis: Role of ovarian steroids in initiation, maintenance and suppression. *Fert. Steril.* 1980, 33, 649-653.
Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W.: Design, synthesis and biological evaluation of (hydroxyphenyl)-naphthalene and quinoline derivatives: potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* (2008) DOI: 10.1021/jm701447v.
Gangloff, A.; Shi, R.; Nahoum, V.; Lin, S.-X.: Pseudo-symmetry of C19 steroids, alternative binding orientations and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase. *Faseb J.* 17 (2003) 274-276.
Gao, Y.; Voigt, J.; Zhao, H.; Pais, G. C. G.; Zhang, X.; Wu, L.; Zhang, Z.-Y.; Burke, T. R. Jr.: Utilization of a peptide lead for the discovery of a novel PTP1B-binding motif. *J. Med. Chem.* 44 (2001) 2869-2878.
Ghosh, D.; Pletnev, V. Z.; Zhu, D. W.; Wawrzak, Z.; Duax, W. L.; Pangborn, W.; Labrie, F.; Lin, S.-X.: Structure of human estrogenic 17beta-hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* 3 (1995) 503-513.
Gunnarsson, C.; Hellqvist, E.; Stål, O.: 17beta-Hydroxysteroid dehydrogenases involved in local estrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* 92 (2005) 547-552.
Gunnarsson, C.; Olsson, B.; Stål, O.: Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* 61 (2001) 8448-8451.
Han, Q.; Campbell, R. L.; Gangloff, A.; Huang, Y. W.; Lin, S.-X.: Dehydroepiandrosterone and dihydrotestosterone recognition by human estrogenic 17beta-hydroxysteroid

dehydrogenase. C-18/C-19 steroid discrimination and enzyme-induced strain. *J. Biol. Chem.* 275 (2000) 1105-1111.

Husen, B.; Huhtinen, K.; Poutanen, M.; Kangas, L.; Messinger, J.; Thole, H.: Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. *Mol. Cell. Endocrinol.* 248 (2006a) 109-113.

Husen, B.; Huhtinen, K.; Saloniemi, T.; Messinger, J.; Thole, H.; Poutanen, M.: Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* 147 (2006b) 5333-5339.

Hwang. K.-J.; O'Neil, J. P.; Katzenellenbogen J. A.: 5,6,11,12-Tetrahydrochrysenes: synthesis of rigid stilbene systems designed to be fluorescent ligands for the estrogen receptor. *J. Org. Chem.* 57 (1992) 1262-1271.

Jagusch, C.; Negri, M.; Hille, U. E.; Hu, Q.; Bartels, M.; Jahn-Hoffmann, K.; Pinto-Bazurco Mendieta, M. A.; Rodenwaldt, B.; Müller-Vieira, U.; Schmidt, D.; Lauterbach, T., Recanatini, M.; Cavalli, A.; Hartmann, R.W.: Synthesis, biological evaluation and molecular modelling studies of methyleneimidazole substituted biaryls as inhibitors of human 17alpha-hydroxylase-17,20-lyase (CYP17). Part I: Heterocyclic modifications of the core structure. *Bioorg. Med. Chem.* 16 (2008) 1992-2010.

Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R.: Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* 267 (1997) 727-748.

Labrie, F.: Intracrinology. *Mol. Cell. Endocrinol.* 78 (1991) C113-C118.

Laplante, Y.; Cadot, C.; Fournier, M.-A.; Poirier, D.: Estradiol and estrone C-16 derivatives as inhibitors of type 1 17beta-hydroxysteroid dehydrogenase: blocking of ER+ breast cancer cell proliferation induced by estrone. *Bioorg. Med. Chem.* 16 (2008) 1849-1860.

Lawrence, H. R.; Vicker, N.; Allan, G. M.; Smith, A.; Mahon, M. F.; Tutill, H. J.; Purohit, A.; Reed, M. J.; Potter, B. V.: Novel and potent 17beta-hydroxysteroid dehydrogenase type 1 inhibitors. *J. Med. Chem.* 48 (2005) 2759-2762.

Lin, S.-X.; Yang, F.; Jin, J. Z.; Breton, R.; Zhu, D. W.; Luu-The, V.; Labrie, F.: Subunit identity of the dimeric 17beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem.* 267 (1992) 16182-16187.

Mazza, C.; Breton, R.; Housset, D.; Fontecilla-Camps, J. C.: Unusual charge stabilization of $NADP^+$ in 17beta-hydroxysteroid dehydrogenase. *J. Biol. Chem.* 273 (1998) 8145-8152.

McGaughey, G. B.; Gagné, M.; Rappé, A.: K. π-Stacking Interactions. *J. Biol. Chem.* 273 (1998) 15458-15463.

Messinger, J.; Hirvelä, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikainen, O.; Saarenketo, P.; Thole, H.: New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* 248 (2006) 192-198.

Mewshaw, R. E.; Edsall, R. J. Jr.; Yang, C.; Manas, E. S.; Xu, Z. B.; Henderson, R. A.; Keith, J. C. Jr.; Harris, H. A.: ERβ ligands. 3. Exploiting two binding orientations of the 2-phenylnaphthalene scaffold to achieve ER beta selectivity. *J. Med. Chem.* 48 (2005) 3953-3979.

Miller, W. R.; Bartlett, J. M. S.; Canney, P.; Verrill, M.: Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. Treat.* 103 (2007) 149-160.

Miyaura, N.; Suzuki, A.: Palladium-catalyzed cross-coupling reaction of organoboron compounds. *Chem. Rev.* 95 (1995) 2457-2483.

Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S.: Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* 94 (2001) 685-689.

Murphy, R. A.; Kung, H. F.; Kung, M.-P.; Billings, J.: Synthesis and characterisation of iodobenzamide analogues: potential D-2 dopamine receptor imaging agents. *J. Med. Chem.* 33 (1991) 171-178.

Ogata, M.; Sato, K.; Konishi, T.: Sulfonamido-benzamide derivatives. EP 84-810006 (1987).

Pascal, C.; Dubois, J.; Guénard, D.; Guéritte, F.: Synthesis of biphenyls mimicking the structure of the antimitotic rhazinilam. *J. Org. Chem.* 63 (1998) 6414-6420.

Patel, B. A.; Ziegler, C. B.; Cortese, N. A.; Plevyak, J. E.; Zebovitz, T. C.; Terpko, M.; Heck, R. F.: Palladium-catalyzed vinylic substitution reactions with carboxylic acid derivatives. *J. Org. Chem.* 42 (1977) 3903-3907.

Poirier, D. Inhibitors of 17beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* 10 (2003) 453-477.

Poirier, D.; Boivin, R. P.; Tremblay, M. R.; Bérubé, M.; Qiu, W.; Lin, S.-X.: Estradiol-adenosine hybrid compounds designed to inhibit type 1 17beta-hydroxysteroid dehydrogenase. *J. Med. Chem.* 48 (2005) 8134-8147.

Puranen, T. J.; Poutanen, M. H.; Peltoketo, H. E.; Vihko, P. T.; Vihko, R. K.: Site-directed mutagenesis of the putative active site of human 17beta-hydroxysteroid dehydrogenase type 1. *Biochem. J.* 304 (1994) 289-293.

Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D.; Lin, S.-X.: A concerted, rational design of type 1 17beta-hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity. *Faseb J.* 16 (2002) 1829-1831.

Royer, R.; Buisson, J.-P.: Recherches sur les dérivés nitrés d'intérêt biologique. XX. Modalités de synthèse des dérivés méthoxylés ou halogénés de nitro-2 naphtofurannes. *Eur. J. Med. Chem. Chimica Therapeutica* 15 (1980) 275-278.

Salem, O. I.; Frotscher, M.; Scherer, C.; Neugebauer, A.; Biemel, K.; Streiber, M.; Maas, R.; Hartmann, R.W.: Novel 5alpha-reductase inhibitors: synthesis, structure-activity studies, and pharmacokinetic profile of phenoxybenzoylphenyl acetic acids. *J. Med. Chem.* 49 (2006) 748-759.

Sam, K. M.; Auger, S.; Luu-The, V.; Poirier, D.: Steroidal spiro-gamma-lactones that inhibit 17beta-hydroxysteroid dehydrogenase activity in human placental microsomes. *J. Med. Chem.* 38 (1995) 4518-4528.

Sam, K. M.; Boivin, R. P.; Tremblay, M. R.; Auger, S.; Poirier, D.: C16 and C17 derivatives of estradiol as inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug. Des. Discov.* 15 (1998) 157-180.

Sawicki, M. W.; Erman, M.; Puranen, T.; Vihko, P.; Ghosh, D.: Structure of the ternary complex of human 17beta-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and $NADP^+$. *Proc. Natl. Acad. Sci. USA* 96 (1999) 840-845.

Shi, R.; Lin, S.-X.: Cofactor hydrogen bonding onto the protein main chain is conserved in the short chain dehydrogenase/reductase family and contributes to nicotinamide orientation. J. *Biol. Chem.* 279 (2004) 16778-16785.

Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H.: 17beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* 82 (2000) 518-523.

Travis, R. C.; Key, T. J.: Estrogen exposure and breast cancer risk. *Breast Cancer Res.* 5 (2003) 239-247.

Tremblay, M. R.; Boivin, R. P.; Luu-The, V.; Poirier, D.: Inhibitors of type 1 17beta-hydroxysteroid dehydrogenase with reduced estrogenic activity: modifications of the positions 3 and 6 of estradiol. *J. Enzyme Inhib. Med. Chem.* 20 (2005) 153-163.

Ulmschneider, S.; Müller-Vieira, U.; Mitrenga, M.; Hartmann, R.W.; Marchais-Oberwinkler, S.; Klein, C. D.; Bureik, M.; Bernhardt, R.; Antes, I.; Lengauer, T.: Synthesis and evaluation of imidazolylmethylenetetrahydronaphthalenes and imidazolylmethyleneindanes: potent inhibitors of aldosterone synthase. *J. Med. Chem.* 48 (2005) 1796-1805.

Vicker, N.; Lawrence, H. R.; Allan, G. M.; Bubert, C.; Smith, A.; Tutill, H. J.; Purohit, A.; Day, J. M.; Mahon, M. F.; Reed, M. J.; Potter, B. V.: Focused libraries of 16-substituted estrone derivatives and modified E-ring steroids: inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *ChemMedChem.* 1 (2006) 464-481.

Voets, M.; Antes, I.; Scherer, C.; Müller-Vieira, U.; Biemel, K.; Barassin, C.; Marchais-Oberwinkler, S.; Hartmann R. W.: Heteroaryl-substituted naphthalenes and structurally modified derivatives: selective inhibitors of CYP11B2 for the treatment of congestive heart failure and myocardial fibrosis. *J. Med. Chem.* 48 (2005) 6632-6642.

Wadsworth, W. S. Jr.; Emmons, W. D.: The utility of phosphonate carbanions in olefin synthesis. *J. Am. Chem. Soc.* 83 (1961) 1733-1738.

Wang, G.; Nikolovska-Coleska, Z.; Yang, C.-Y.; Wang, R.; Tang, G.; Guo, J.; Shangary, S.; Qiu, S.; Gao, W.; Yang, D.; Meagher, J.; Stuckey, J.; Krajewski, K.; Jiang, S.; Roller, P. P.; Abaan, H. O.; Tomita, Y.; Wang, S.: Structure-based design of potent small-molecule inhibitors of anti-apoptotic Bcl-2 proteins. *J. Med. Chem.* 49 (2006) 6139-6142.

Zhu, D. W.; Lee, X.; Breton, R.; Ghosh, D.; Pangborn, W.; Daux, W. L.; Lin, S.-X. J.: Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17 beta-hydroxysteroid dehydrogenase with $NADP^+$. *Mol. Biol.* 234 (1993) 242-244.

Zimmermann, J.; Liebl, R.; von Angerer, E.: 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor α. *J. Steroid. Biochem. Mol. Biol.* 94 (2005) 57-66.

3.4 Aging reduces the efficacy of estrogen substitution to attenuate cardiac hypertrophy in female spontaneously hypertensive rats.

Virginija Jazbutyte, Kai Hu, Patricia Kruchten, Emmanuel Bey, Sebastian Maier, Karl-Heinrich Fritzemeier, Katja Prelle, Christa Hegele-Hartung, Rolf W. Hartmann, Ludwig Neyses, Georg Ertl, Theo Pelzer

Dieses Manuskript wurde als Artikel in folgendem Journal veröffentlicht:
Hypertension (**2006**) *48*, 579-586.

Publikation IV

Abstract

Clinical trials failed to show a beneficial effect of postmenopausal hormone replacement therapy, whereas experimental studies in young animals reported a protective function of estrogen replacement in cardiovascular disease. Because these diverging results could in part be explained by aging effects, we compared the efficacy of estrogen substitution to modulate cardiac hypertrophy and cardiac gene expression among young (age 3 months) and senescent (age 24 months) spontaneously hypertensive rats (SHRs), which were sham operated or ovariectomized and injected with placebo or identical doses of 17β-estradiol (E2; 2 µg/kg body weight per day) for 6 weeks (n = 10/group). Blood pressure was comparable among sham-operated senescent and young SHRs and not altered by ovariectomy or E2 treatment among young or among senescent rats. Estrogen substitution inhibited uterus atrophy and gain of body weight in young and senescent ovariectomized SHRs, but cardiac hypertrophy was attenuated only in young rats. Cardiac estrogen receptor-α expression was lower in intact and in ovariectomized senescent compared with young SHRs and increased with estradiol substitution in aged rats. Plasma estradiol and estrone levels were lower not only in sham-operated but surprisingly also in E2-substituted senescent SHRs and associated with a reduction of hepatic 17β-hydroxysteroid dehydrogenase type 1 enzyme activity, which converts weak (ie, estrone) into potent estrogens, such as E2. Aging attenuates the antihypertrophic effect of estradiol in female SHRs and is associated with profound alterations in cardiac estrogen receptor-α expression and estradiol metabolism. These observations contribute to explain the lower efficiency of estrogen substitution in senescent SHRs.

Introduction

The development of cardiovascular disease in postmenopausal women follows declining estrogen plasma levels with a delay of several years (August and Oparil, 1999). The underlying mechanisms are still largely unknown. Observational studies initially suggested a protective function of postmenopausal hormone replacement therapy (HRT) against heart disease with risk reductions of ≤35% to 50% (Hillard *et al*., 1991). But controlled clinical end point studies, such as Heart and Estrogen/progestin Replacement Study (HERS) and the recently terminated Women's Health Initiative trial, reported no protective function of HRT consisting of conjugated equine estrogens plus medroxy-progesterone-acetate for the primary or secondary prevention of coronary artery disease (Rossouw *et al.*, 2002). In contrast, estrogen supplementation in animal models of human heart disease was mostly associated with beneficial

hormone effects. But the majority of these studies were conducted in young animals and, thus, do not take the possibility of aging effects into account, which might, in part, explain controversial results between animal and human studies (van Eickels *et al.*, 2005; Wallen *et al.*, 2000; Williams *et al.*, 2004). Until recently, these shortcomings could be attributed to the lack of animalmodels that mimic postmenopausal hypertension. Meanwhile, senescent spontaneously hypertensive rats (SHRs) and aged Dahl salt-sensitive rats have been identified and characterized as suitable models to study postmenopausal hypertension. As reported recently, female SHRs stop cycling at the age of 10 to 12 months and develop further increases in blood pressure thereafter (Reckelhoff and Fortepiani, 2004). Similar studies have been conducted in Dahl salt-sensitive rats (Hinojosa-Laborde *et al.*, 2004). Increased blood pressure in postmenopausal SHRs is associated with the natural cessation of ovarian function and decreasing estrogen plasma levels, which closely resemble observations in postmenopausal women (Burt *et al.*, 1995). These studies provided substantial and new insight into gender and aging aspects of hypertension. In contrast, aging aspects in the development of cardiac hypertrophy, gene expression, and function are less well documented, although it seems conceivable that estrogen substitution of young and postmenopausal female SHRs might result in different cardiac phenotypes. Therefore, and because most previous studies including our own were conducted in young animals, we determined cardiovascular function, cardiac gene expression, as well as plasma sex hormone profiles in sham-operated and ovariectomized young and senescent female SHRs on long-term treatment with placebo or identical, body weight-adjusted doses of 17β-estradiol (E2). In particular, we speculated whether the efficacy of estrogen substitution to modulate cardiac hypertrophy and gene expression might be blunted in aged SHRs.

Methods

Animal Model and Treatment

Female SHRs (SHR/Ncr1Ico) obtained from Charles River Laboratories (IFFA/CREDO, Lyon, France) at the age of 12 weeks were kept under standard conditions until reaching postmenopause at 24 months of age (senescent SHRs, n=30). A second batch of animals was purchased from the same supplier at the age of 12 weeks for parallel studies in young SHRs (n=30). Young and senescent SHRs were repartitioned into 6 study groups including sham operation (young: n=10; old: n=10 per group), ovariectomy ([ovx] young: n=10; old: n=10), and ovariectomy plus estradiol supplementation ([ovx+E2] young: n=10; old: n=10). All of the animals were kept under standard conditions including a 12 hours on/off light cycle, commercial diet, and water *ad libitum*. Ovarectomies and sham operations were performed under isoflurane anesthesia (isoflurane 1.5 volume [vol] percent supplemented by 0.5 L of oxygen per minute) after pretreatment with tribromoethanol/amylene hydrate ([Avertin] 2.5% weight/vol, 6 µL/g body weight IP). Ovariectomized rats were injected either with placebo (95 µL of peanut oil + 5 µL of ethanol SC) or a body weight-adjusted dose of E2 (95 µL peanut oil plus 2 µg/kg BW per day of E2 dissolved in 5 µL of EtOH) on a daily basis. All of the animals were treated for 6 weeks before hemodynamic and morphometric measurements. A separate set of young (n=10) and senescent (n=7) SHRs was ovariectomized and treated with E2 under exactly identical conditions to assess hepatic enzyme activity levels of 17β-hydroxysteroid dehydrogenases (17βHSDs) type 1 (17βHSD1) and type 2 (17βHSD2). All of the protocols were reviewed and accepted by the local ethics committee and performed in accordance with the current National Institutes of Health guide for the care and use of laboratory animals.

Hemodynamic Analysis

Hemodynamic measurements were performed according to published protocols after 6 weeks of continuous treatment under light isoflurane anesthesia and spontaneous respiration (isoflurane 1.5 vol percent supplemented by 0.5 L of oxygen per minute) (Pelzer *et al.*, 2005). Pressure curves were measured via polyethylene tubing and a microtip manometer (Millar Instruments) calibrated to midchest level. Systolic and diastolic blood pressure measurements were obtained

on catheter placement in the thoracic aorta. A calibrated flowmeter (2.5 mm; Statham) was placed around the ascending aorta for continuous measurement of aortic blood flow (cardiac output). Measurements were performed by a trained observer blinded for treatment groups. Animals with nonphysiological heart rate <250 bpm were excluded from hemodynamic analysis (senescent rats: sham: n=3; ovx: n=2; ovx+E2: n=2). Blood pressure in senescent SHRs was also monitored by telemetry (online supplement II, please see http://hyper. ahajournals.org).

Morphometry

Body weight, heart weight, uterus weight, and tibia length were determined after hemodynamic analysis. Relative heart weight was determined by normalizing absolute heart weight for tibia length.

Plasma Hormone Level

Plasma samples obtained after hemodynamic analysis and within identical time frames after hormone injection were used for radioimmunoassay (RIA) measurements of E2, estrone (E1), total testosterone, free-testosterone, and 4-androstendione according to the manufacturer's instructions (Diagnostic Systems Laboratories, Inc). Cross-reactivity of the third-generation estradiol RIA was 6.9% with E1 and with other hormones was not detectable. Cross-reactivity of the E1 RIA was 1.25% with estradiol and with other hormones was not detectable. Cross-reactivity for androstenedione RIAs with dihydrotestosterone was 0.08%, with E1 0.03%, and with E2 and testosterone not detectable. Cross-reactivity for testosterone RIAs with dihydrotestosterone was 6.6%, with androstenedione 0.9%, and with E2 not detectable. Information on RIA cross-reactivities were provided by the manufacturer.

Cardiac Protein Expression

Cardiac α and β myosin heavy chain protein (α- and β-MHC) expression was analyzed by SDS-PAGE electrophoresis with subsequent visualization of protein bands by silver staining according to published protocols (Pelzer *et al.*, 2005). Left ventricular samples were homogenized in ice-cold sample buffer containing a protease inhibitor mixture, centrifuged, and 500 ng of the extract were subjected to SDS-PAGE analysis using a 6% polyacrylamide gel containing 5% glycerol for 18 hours at 4°C before band visualization by silver staining (Silver Stain Plus, Bio Rad). Band intensities for α- and β-MHC were determined by densitometry using the ScanPack 3.0 software (Biometra).
Cardiac protein expression was also analyzed by Western blots using unfractionated ventricular protein extracts, which were separated on 7% to 10% polyacrylamide sodium dodecyl sulfate gels followed by electrophoretic transfer on nitrocellulose membranes. Nonspecific background was blocked using 5% nonfat milk powder in PBS/Tween 20 (1 hour, room temperature). The following primary antibodies were used: anti-ERα (ER21, rabbit polyclonal; generous gift of Dr G. Greene, University of Chicago, Chicago, IL), anti-ERβ (ER3919, rabbit polyclonal; raised against rat ERβ ligand binding domain; see online supplement I for details), anti-phospholamban (mouse monoclonal, Alexis), anti-phospho-phospholamban (rabbit polyclonal, Upstate Biotechnology), anti-SERCA 2 (rabbit polyclonal, Abcam), and anti-αB crystallin (rabbit polyclonal, StressGen). Antirabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase (anti-mouse, anti-rabbit; Amersham, 1:5000) and the ECL detection system (Amersham) were used to visualize immunoreactive proteins. Gel loading was normalized to α-B-crystallin expression.

17βHSD1 and 17βHSD2 Activity Assays

Frozen liver samples were homogenized and fractionated into cytosolic extracts for measurements of 17βHSD1 activity and microsomal extracts to assess 17βHSD2 activity (Qiu *et al.*, 2002; Zhu *et al.*, 1993). Enzymatic 17βHSD activities were measured according to published protocols at 37°C in a phosphate buffer supplemented with 20% glycerol (vol/vol) and 1 mmol/L of EDTA the presence of oxidized nicotinamide-adenine dinucleotide and [^{3}H]-estradiol for 20´ in 17βHSD2 assays or in the presence of reduced nicotinamideadenine

dinucleotide and [^{3}H]-E1 for 10´ in 17βHSD1 assays followed by steroid extraction with ether (Lin *et al.*, 1992; Sam *et al.*, 1997; Sam *et al.*, 1995). [^{3}H]-Estradiol and [^{3}H]-E1 were obtained from Perkin Elmer LAS. Substrates and products were separated using acetonitrile/water (47.5:52.5 vol/vol) as a mobile phase on a C18 rp chromatography column (Nucleodur C18 Gravity, 3 µm, Macherey-Nagel) connected to a high performance liquid chromatography system (Agilent 1100 Series, Agilent Technologies). Steroid detection and quantification was performed using a radioflow detector (Berthold Technologies). Substrate conversion rates were calculated, and the formation of 1 µmol of product per minute was defined as 1 U of enzyme activity. Specific activities are given in units per milligram of protein.

Statistics

Statistical significance was calculated by 1-way ANOVA followed by Student-Newman-Keuls post hoc testing in all of the experiments except 17βHSD activities, in which statistical significance was determined by 2-sided t tests. Values are mean ± SEM, and $P<0.05$ was considered significant.

Results

Morphometry

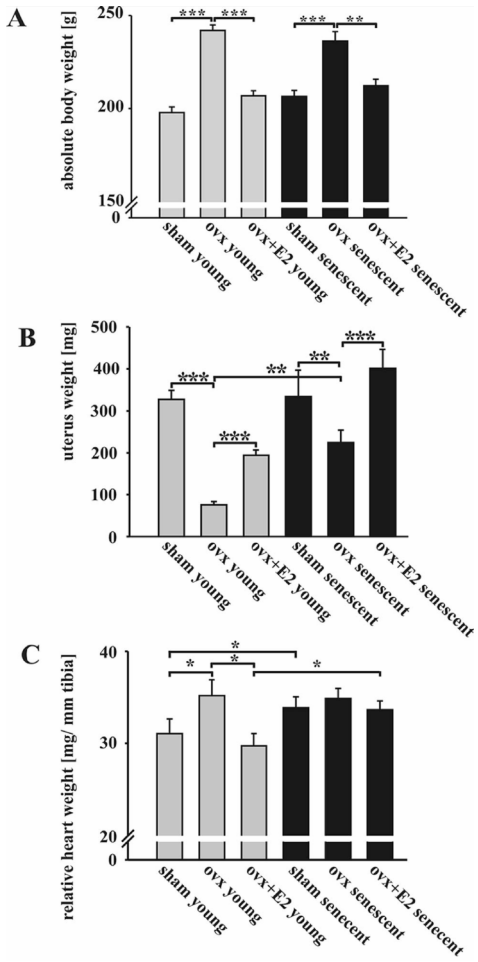


Figure 1: A, Body weight of young and senescent SHRs after sham operation (sham), ovarectomy plus placebo treatment (ovx), and ovarectomy plus substitution with E2 (ovx+E2). B, Uterus weight of young and senescent SHRs after sham operation, ovarectomy plus placebo treatment, and ovarectomy plus substitution with E2. C, Relative heart weight of young and senescent SHRs after sham operation ovarectomy plus placebo treatment and ovarectomy plus substitution with E2. Bars indicate mean±SEM (n=10 animals per group; ***P<0.001, **P<0.01, *P<0.05).

Body weight was comparable among sham-operated senescent and young rats (Figure 1A). The gain of body weight in ovariectomized animals was attenuated by E2 treatment in young and aged SHRs. Uterus weight, which was not different between sham-operated young and senescent rats, decreased with ovariectomy, but residual uterus weight was higher in senescent SHRs (Figure 1B). Estradiol treatment resulted in increased uterus mass in ovariectomized young and senescent SHRs. Relative heart weight, which was higher in sham-operated senescent compared with young SHRs, increased in young but not in senescent rats on ovariectomy (Figure 1C). Estradiol substitution attenuated cardiac hypertrophy in young but not in senescent rats. Cardiac mass was higher in estrogen-substituted senescent compared with estrogen substituted young SHRs.

Hemodynamics and Telemetric BloodPressure Analysis

Ovariectomy and E2 substitution did not affect blood pressure among the groups of aged and among the groups of young SHRs (Table). However, mean blood pressure levels were higher in sham-operated and estrogen-substituted senescent compared with young SHRs receiving estrogen substitution. Left ventricular stroke volume and cardiac output were comparable among all of the groups of senescent and young rats. Telemetric blood pressure analysis was in good agreement with invasive measurements and did not reveal a blood pressure-lowering effect of E2 substitution in senescent SHRs (online supplement II).

Table: Invasive Hemodynamic Measurements. Hemodynamic measurements in young and senescent SHRs after sham operation (sham), ovariectomy plus placebo treatment (ovx), and ovariectomy plus substitution with E2 (ovx+E2). HR indicates heart rate; CO, cardiac output; SAP, systolic blood pressure; MP, mean blood pressure; SV, stroke volume. *P<0.05 vs ovariectomized young; †P<0.05 vs ovariectomized E2 young.

Treatment Group	n	HR, bpm	CO, ml/min	SAP mm Hg	MP, mm Hg	*SV, µl/beat*
Sham young	10	341 ± 5	58 ± 2.1	221 ± 5.8	174 ± 5.3	170 ± 7.8
Ovx young	10	355 ± 7	56 ± 1.5	219 ± 5.7	171 ± 4.8	157 ± 4.7
Ovx+E2 young	10	363 ± 8	61 ± 2.1	210 ± 6.5	162 ± 5.4	170 ± 6.5
Sham senescent	7	312 ± 13*	48 ± 4.0	244 ± 13.5	192 ± 7.4†	148 ± 7.4
Ovx senescent	8	319 ± 7*	50 ± 3.4	236 ± 14.2	181 ± 8.8	159 ± 14.6
Ovx+E2 senescent	8	325 ± 8	52 ± 3.4	243 ± 9.1	192 ± 4.3†	159 ± 8.2

Plasma Sex Hormone Levels

Plasma E2, E1, total, and free testosterone, as well as androstendione plasma levels, were significantly higher in sham-operated young compared with senescent SHRs (Figure 2). Ovariectomy decreased plasma levels of E2 and E1 in young rats but had no such effect in senescent SHRs. Plasma androgen levels were lower in ovariectomized SHRs of both age groups. Estradiol, as well as E1, levels were higher in young SHRs treated with E2 compared with placebo-treated ovariectomized animals. In contrast, plasma E2 and E1 levels in E2-substituted senescent SHRs were not different from placebo-treated aged rats. E1 and estradiol levels were closely correlated among all of the samples ($P<0.001$; $R^2=0.71$).

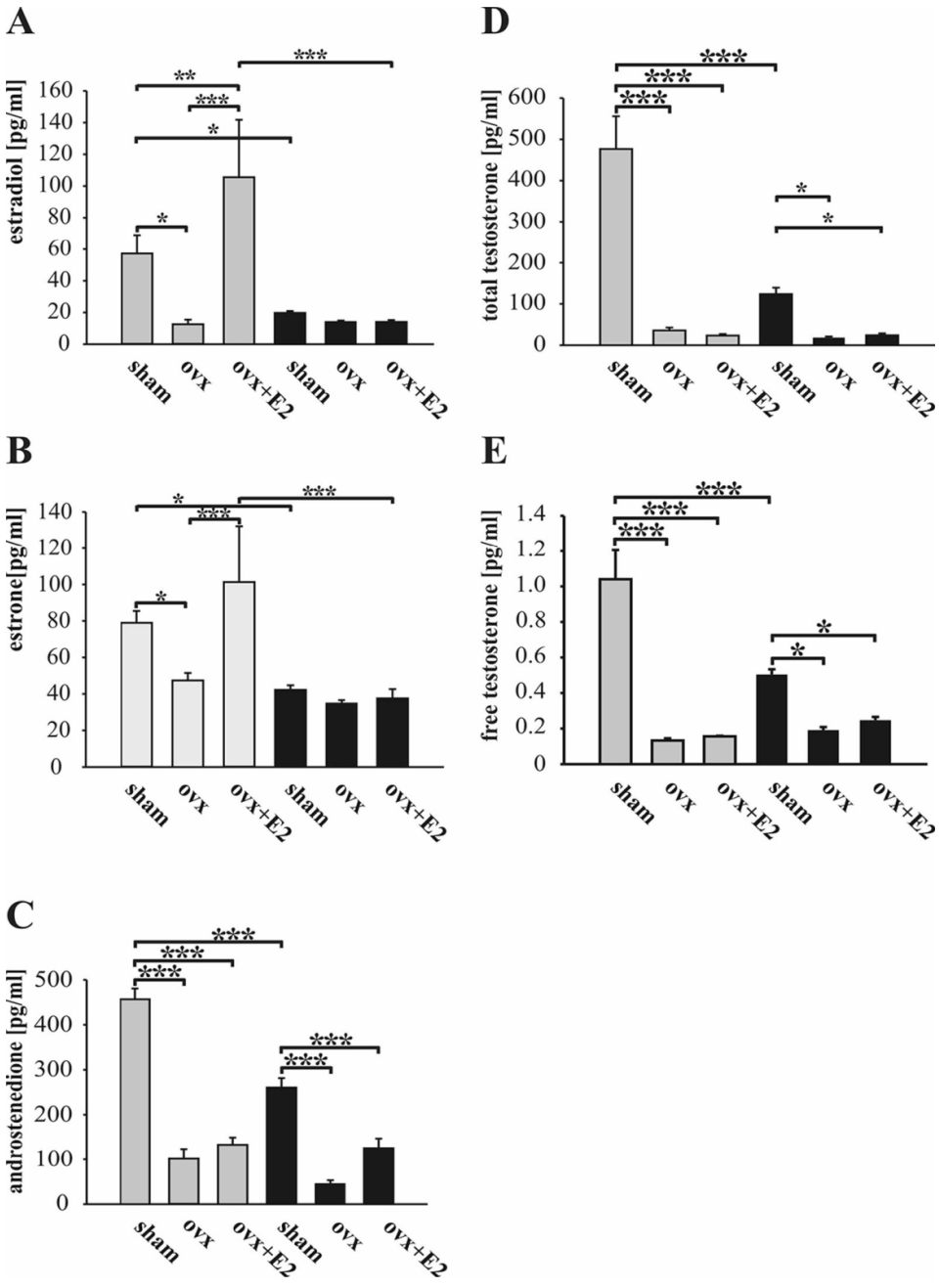


Figure 2: Sex hormone plasma levels of young (grey) and senescent SHRs (black) after sham operation (sham), ovarectomy plus placebo treatment (ovx), and ovarectomy plus substitution with E2 (ovx+E2). All of the measurements were performed using plasma samples obtained within identical time frames (15 to 19 hours) after hormone or placebo injection. Bars indicate mean±SEM (n=10 animals per group; ***P<0.001, **P<0.01, *P<0.05).

Cardiac MHC Expression

The α-MHC protein was the predominant cardiac isomyosin in sham-operated young SHRs, whereas senescent hearts contained both myosin subtypes at approximately equal amounts (Figure 3). Ovariectomy decreased cardiac α-MHC expression in young SHRs and shifted the MHC ratio toward α-MHC accumulation (sham: 1.74±0.12 versus ovx 1.07±0.07; P<0.01; n=10 per group). E2 substitution of ovariectomized young SHRs resulted in increased amounts of α-MHC protein (ovx+E2: 1.52±0.13; P<0.01; versus ovx; n=10 per group). In contrast, ovariectomy and E2 treatment conferred only insignificant effects on cardiac MHC expression in senescent SHRs.

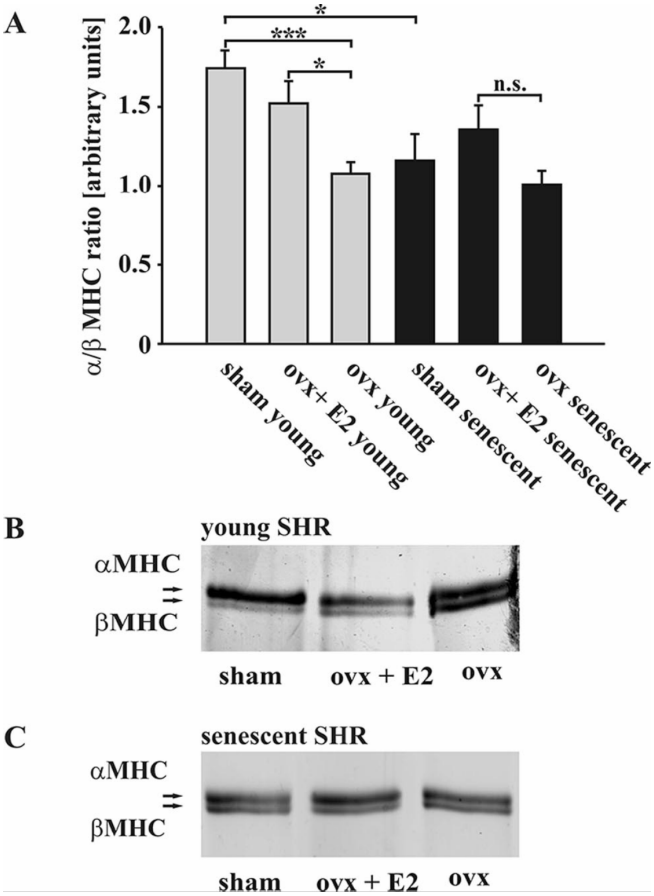


Figure 3: Cardiac α/β MHC protein ratios of young and senescent SHRs after sham operation (sham), ovarectomy plus placebo treatment (ovx), and ovarectomy plus substitution with E2 (ovx+E2) including representative silver stained sodium dodecyl sulfate gels from young and senescent SHRs. Bars indicate mean±SEM (n=10 animals per group; ***P<0.001, *P<0.05).

Cardiac Protein Expression Analysis

Cardiac estrogen receptor α (ERα) expression was lower in sham-operated and ovariectomized senescent compared with young SHRs (Figure 4A). E2 substitution increased cardiac ERα expression in senescent rats to levels that were no longer different from those observed among young animals. In contrast, cardiac ERβ expression was uniform and not different among all of the treatment groups (Figure 4B). The expression levels of cardiac calcium handling proteins such as SERCA2, phospholamban, and phosphorylated phospholamban were not different among all of the animals (data not shown).

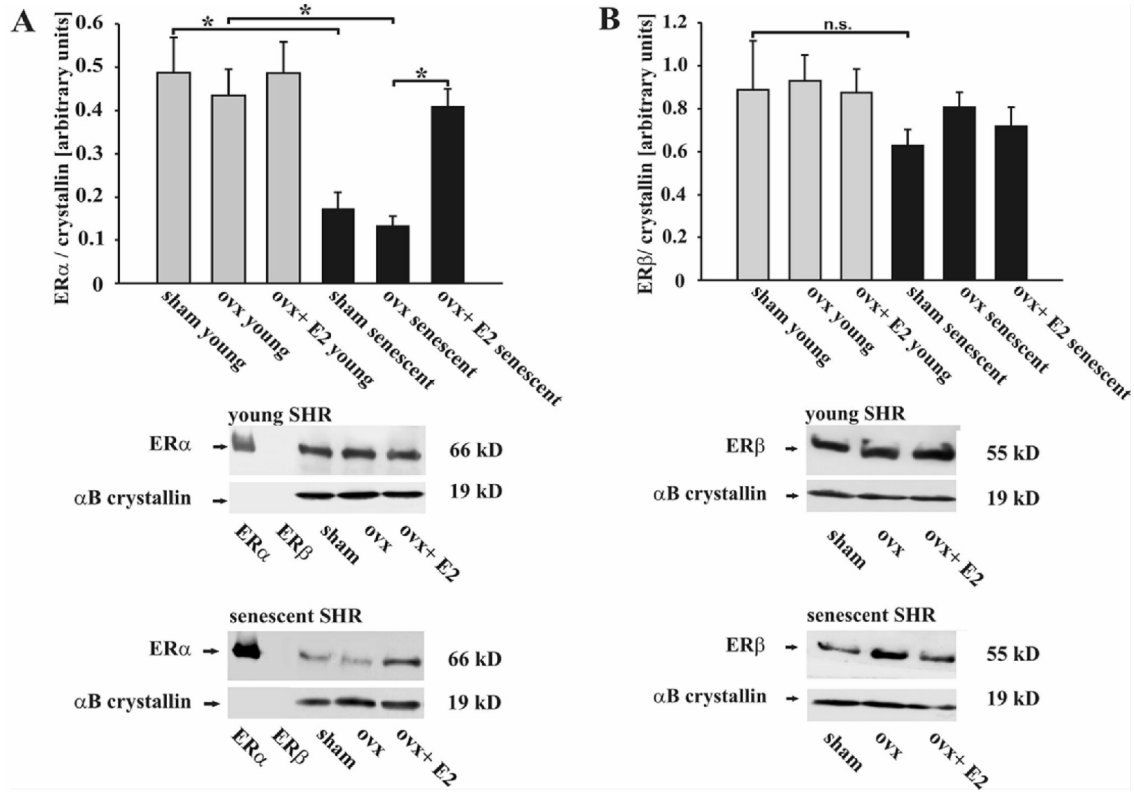


Figure 4: Cardiac ERα (A) and ERβ (B) expression in young and senescent SHRs after sham operation (sham), ovarectomy plus placebo treatment (ovx), and ovarectomy plus substitution with E2 (ovx+E2). Representative Western blot experiments shown below include positive and negative controls (recombinant human ERα and ERβ) for the ERα antibody. Control experiments showing specificity for the ERβ antibody are available from online supplement I. Bars indicate mean±SEM (n=10; *P<0.05).

17βHSD1 and 17βHSD2 Activity

Hepatic 17βHSD1 activity, which catalyzes the reduction of weak estrogens, such as E1, to potent estrogens, such as E2, was significantly lower in senescent compared with young SHRs receiving estrogen substitution (Figure 5A). Activity levels of 17βHSD2, which metabolizes E2 to less potent estrogens, such as E1, was comparable among estrogen-treated young and senescent SHRs (Figure 5B).

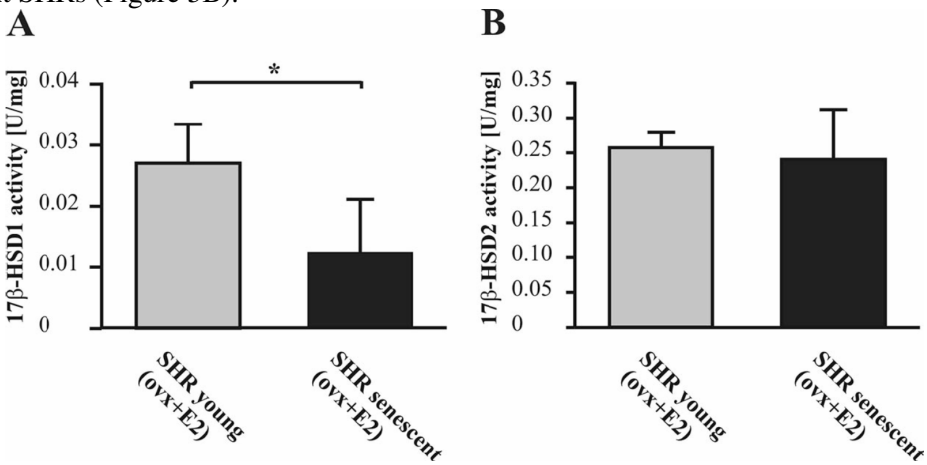


Figure 5. Hepatic 17βHSD1 (A) and 17βHSD2 (B) activities in ovarectomized (ovx) and E2-substituted young and senescent SHRs. 17βHSD activities were measured in quadruplicate in each individual animal. Bars indicate mean 17βHSD1 and 17βHSD2 activities in units per milligram of protein±SEM (young SHR: n=10; senescent SHR: n=7; *P<0.05).

Discussion

The primary result of this study is the observation that estrogen substitution is significantly less efficient to attenuate cardiac hypertrophy in senescent compared with young female SHRs. Although several biomarkers, such as body weight, uterus weight, and cardiac ERα expression, were responsive to E2 supplementation of senescent SHRs, heart weight and cardiac isomyosin expression were not. Lower E2 plasma levels in sham-operated and in estradiol-substituted senescent rats together with lower 17βHSD1 activity indicate that aging in SHRs is associated not only with reduced E2 synthesis but also with altered E2 metabolism. These observations could at least in part explain the blunted effect of E2 on cardiac hypertrophy in aged SHRs.

Aging is associated with increased blood pressure and cardiac hypertrophy in postmenopausal women (August and Oparil, 1999; Burt *et al.*, 1995). Blood pressure levels were comparable and not different among all 3 of the groups of young rats and among the different groups of senescent SHRs, which is in line with previous findings, including our own (Pelzer *et al.*, 2005; Sharkey *et al.*, 1999). Telemetric blood pressure measurements in senescent SHRs were in good agreement with invasive blood pressure levels and again did not reveal a blood pressure-lowering effect of estradiol. In line with previous observations, blood pressure differed, however, between some groups of aged and young SHRs, because systolic and mean blood pressure was higher in intact and in estrogen-treated senescent SHRs compared with young rats receiving hormone substitution (Fortepiani *et al.*, 2003).

Cardiac hypertrophy, which is frequently associated with hypertension, represents an independent predictor of cardiovascular mortality (Levy *et al.*, 1990). Increased cardiac mass in sham-operated senescent compared with young SHRs could result from higher blood pressure levels in aged rats, although statistical significance was not reached in comparisons between treatment groups receiving identical treatment (ie, intact young versus intact senescent, etc). In line with previous observations, E2 efficiently attenuated cardiac hypertrophy in ovariectomized young SHRs, but blood pressure levels were not significantly different among all of the groups of young SHRs (Pelzer *et al.*, 2005; Widder *et al.*, 2003). These findings indicate that estrogens are able to attenuate cardiac growth also by direct and blood pressure-independent mechanisms, such as cardiac ANP expression and mitogen-activated protein kinase activation, as we and others have reported before (Babiker *et al.,* 2004). But in contrast to young animals, estrogen substitution failed to attenuate cardiac hypertrophy in aged SHRs. These observations are supported by measurements of cardiac isomyosin expression. Cardiac hypertrophy in rodents is associated with a prototypical switch in cardiac isomyosin expression from predominant α- to β-MHC expression. As one would predict from measurements of cardiac mass, estrogen treatment reversed the downregulation of α-MHC in ovariectomized young but not in aged SHRs.

Estrogen effects on cardiac hypertrophy in senescent rats are at present not fully understood. Xu *et al*. (2000) reported on increased left ventricular remodeling in ovariectomized adult Sprague-Dawley rats that was prevented by E2 substitution. However, the aim of these studies, which were conducted in normotensive rats, was clearly different from the present study and not designed to compare the efficiency of E2 substitution between young and adult rats (Fortepiani *et al.*, 2003). The role of estrogens in hypertension and cardiac hypertrophy in aged rats has also been evaluated by Hinojosa-Laborde *et al.* (2004), who provided solid evidence for increased blood pressure in ovariectomized compared with sham-operated Dahl salt-sensitive rats that was attenuated by estrogen substitution. But cardiac hypertrophy has so far not been assessed in E2-treated Dahl rats (Hinojosa-Laborde *et al*., 2000). Sharkey *et al.* (1999) reported that absolute heart weight was not affected by E2 treatment of aged, heart failure-prone hypertensive rats (SHHF/Mcc-fa^{cp}). However the focus of these studies, which were conducted in intact and heart failure-prone rats, was on young animals. Moreover, the lack of complete hemodynamic data, estrogen plasma levels, and estrogen receptor expression did not allow for a more comprehensive analysis and a direct comparison between E2-supplemented young and senescent rats.

General inefficiency of estrogen treatment does not exist in aged rats, because established biomarkers of estrogen activity, such as uterine weight and body weight, responded to hormone treatment in young and aged SHRs as observed here and reported before (Xu *et al*., 2003). Although uterus weight decreased with ovariectomy in young and in senescent SHRs, the loss

of uterine mass was less pronounced in senescent SHRs, which supports the hypothesis that uterus mass in sham-operated aged rats is less representative for estrogen action.
Aging is associated with profound alterations of male and female sex hormone plasma levels in humans and in rodents. Moreover, heart weight and body weight, as well as blood pressure, are subjected to regulation via estrogens and androgens, including their pharmacologically active metabolites (Burger *et al*., 2002; Burger, 2002). As expected, plasma estradiol levels were lower in ovariectomized SHRs and restored to physiological levels in E2-treated young SHRs. Lower plasma E2 levels in intact senescent compared with young SHRs match with previous reports and with decreased plasma levels of E1. E1 is generated by different 17β-steroid dehydrogenases that catalyze the conversion of potent into less potent estrogens in an initial step of E2 metabolism (Adamski and Jakob, 2001; Vihko *et al.*, 2004) Surprisingly, plasma samples from estrogen-substituted senescent SHRs, which were taken within identical time frames after hormone injection in young and aged animals, revealed low E2 levels that were indistinguishable from ovariectomized and placebo-treated senescent SHRs. These results, which are supported also by low E1 plasma levels in E2-substituted senescent SHRs, suggest that estrogen metabolism differs between young and senescent SHRs. Interestingly, E2 plasma levels were also lower in aged compared with young Dahl salt-sensitive rats receiving E2 supplementation via silastic implants (30±2 versus 20±3 ng/mL; young versus old; $P<0.05$). But these findings in 12-month compared with 4-month-old rats were not explicitly interpreted as an indicator of increased E2 metabolism (Hinojosa-Laborde *et al*., 2004). The present study, thus, substantiates previous findings and provides completely independent evidence for aging-associated alteration in estrogen metabolism.
Estrogen metabolism is initiated by 17βHSDs, which are expressed in multiple isoforms in multiple tissues and catalyze not only the conversion of potent into less potent estrogens but also opposite reactions yielding high affinity from low-affinity ER ligands. Oxidative 17βHSDs, which include isoforms 2, 4, 8, 10, and 11, convert high-affinity to low-affinity estrogen receptor ligands (Adamski and Jakob, 2001; Penning, 1997; Peltoketo *et al*., 1999). Comparable hepatic 17βHSD2 activity levels among E2-substituted young and senescent SHRs suggest that 17βHSD2 is most likely not responsible for lower E2 plasma levels in E2-substituted senescent SHRs. However, we cannot rule out that oxidative 17βHSD isoforms other than 17βHSD2 might show increased activities in senescent SHRs. In contrast to oxidative enzymes, reductive 17βHSDs, such as the type 1 enzyme, catalyze the synthesis of potent, high-affinity estrogen receptor ligands, such as E2, from low-affinity precursors, such as E1. E1 plasma levels in senescent SHRs provide a suitable substrate pool for 17βHSD1 to catalyze the formation of E2, and it is therefore conceivable that lower hepatic 17βHSD1 activities in senescent compared with young SHRs might have contributed to lower E2 plasma levels. Although the extent to which 17βHSD1 regulates E2 plasma levels has up to now not been determined, different hepatic 17βHSD1 activity profiles in young and senescent SHRs further support the idea that aging is associated with functionally important alterations in estrogen metabolism.
The biological function of estrogens is mediated by 2 different estrogen receptor subtypes, ERα and estrogen receptor β (ERβ), which are functionally expressed in numerous tissues and cell types, including cardiac myocytes and vascular cells (Green *et al*., 1986; Mendelsohn and Karas, 1999). In contrast to previous studies, which failed to detect measurable amounts of ERα and ERβ protein in the mouse heart, several independent studies reported on robust cardiac expression levels of both ER subtypes in rats and humans (Pelzer *et al*., 2005; Forster *et al*., 2004; Nordmeyer *et al*., 2004). However, cardiac expression levels of ERα and ERβ may not only vary between different species but also among young and senescent SHRs. Therefore, it is interesting to note that cardiac ERα expression was significantly lower in sham-operated and ovariectomized aged rats, whereas ERβ was detected at comparable amounts in the heart of young and senescent rats. The observation that estradiol upregulates ERα expression in vascular cells suggests that lower plasma E2 levels might result in a downregulation of cardiac ERα expression (Ihionkhan *et al*., 2002) This hypothesis is supported by increased cardiac ERα content in E2 supplemented senescent SHRs; although plasma E2 levels remained low in these rats, ERα-mediated signal transduction seems, at least in part, to be functional in aged rats.

Perspectives

The current study provides first evidence for lower efficacy of estrogen substitution and altered E2 metabolism in senescent SHRs. Estrogen substitution in postmenopausal women is frequently initiated during the transition phase to menopause. Because SHRs stop cycling at ≈12 months of age, estrogen substitution in ovariectomized 24-month-old senescent SHRs does not resemble estrogen replacement at an early postmenopausal stage. Further studies will, thus, be required to determine whether initiation of estrogen replacement in SHRs during the decline of endogenous estrogen plasma levels is more efficacious than in senescent SHRs. Further studies will also be required to determine the functional relevance of individual 17βHSD isoforms that have not yet been studied in young and in senescent SHRs.

Sources of Funding

T.P. has received support from the Interdisciplinary Center for Clinical Research Würzburg ([IZKF] TP F-11); V.J. received support from the German Academic Exchange Service (DAAD); S.K.G.M. received support from the Deutsche Forschungsgemeinschaft "DFG" (Ma2252); L.N. has received support from the Medical Research Council (United Kingdom), the British Heart Foundation, and the EUMORPHIA consortium (European Union framework program 5); and G.E. has received support from the Deutsche Forschungsgemeinschaft "DFG" (SFB 688).

Disclosures

T.P. and L.N. have received financial support from the Schering AG Berlin that relates to the evaluation of novel steroid hormone receptor ligands.

References

Adamski J., Jakob F.J.: A guide to 17beta-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol*. 171 (2001) 1-4.

August P., Oparil S.: Hypertension in women. *J. Clin. Endocrinol. Metab*. 84 (1999) 1862-1866.

Babiker F.A., De Windt L.J., van Eickels M., Thijssen V., Bronsaer R.J., Grohe C., van Bilsen M., Doevendans P.A.: 17Beta-estradiol antagonizes cardiomyocyte hypertrophy by autocrine/paracrine stimulation of a guanylyl cyclase A receptor-cyclic guanosine monophosphate-dependent protein kinase pathway. *Circulation* 109 (2004) 269-276.

Burger H.G., Dudley E.C., Robertson D.M., Dennerstein L.: Hormonal changes in the menopause transition. *Recent Prog. Horm. Res*. 57 (2002a) 257-275.

Burger H.G.: Androgen production in women. *Fertil. Steril*. 77 Suppl 4 (2002b) S3-5.

Burt V.L., Whelton P., Roccella E.J., Brown C., Cutler J.A., Higgins M., Horan M.J., Labarthe D.: Prevalence of hypertension in the US adult population. Results from the Third National Health and Nutrition Examination Survey, 1988–1991. *Hypertension* 25 (1995) 305-313.

Forster C., Kietz S., Hultenby K., Warner M., Gustafsson J.Å.: Characterization of the ERbeta -/-mouse heart. *Proc. Natl. Acad. Sci. U S A* 101 (2004) 14234-14239.

Fortepiani L.A., Zhang H., Racusen L., Roberts L.J., 2nd, Reckelhoff J.F.: Characterization of an animal model of postmenopausal hypertension in spontaneously hypertensive rats. *Hypertension* 41 (2003) 640-645.

Green S., Walter P., Greene G., Krust A., Goffin C., Jensen E., Scrace G., Waterfield M., Chambon P.: Cloning of the human oestrogen receptor cDNA. *J. Steroid Biochem.* 24 (1986) 77-83.

Hillard T.C., Whitcroft S., Ellerington M.C., Whitehead M.I.: The long-term risks and benefits of hormone replacement therapy. *J. Clin. Pharm. Ther*. 16 (1991) 231-245.

Hinojosa-Laborde C., Craig T., Zheng W., Ji H., Haywood J.R., Sandberg K.: Ovariectomy augments hypertension in aging female Dahl salt-sensitive rats. *Hypertension* 44 (2004) 405-409.

Hinojosa-Laborde C., Lange D.L., Haywood J.R.: Role of female sex hormones in the development and reversal of dahl hypertension. *Hypertension* 35 (2000) 484-489.

Ihionkhan C.E., Chambliss K.L., Gibson L.L., Hahner L.D., Mendelsohn M.E., Shaul P.W.: Estrogen causes dynamic alterations in endothelial estrogen receptor expression. *Circ. Res*. 91 (2002) 814-820.

Levy D., Garrison R.J., Savage D.D., Kannel W.B., Castelli W.P.: Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N. Engl. J. Med*. 322 (1990) 1561-1566.

Lin S.X., Yang F., Jin J.Z., Breton R., Zhu D.W., Luu-The V., Labrie F.: Subunit identity of the dimeric 17 beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem*. 267 (1992) 16182-16187.

Mendelsohn M.E., Karas R.H.: The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med*. 340 (1999) 1801-1811.

Nordmeyer J., Eder S., Mahmoodzadeh S., Martus P., Fielitz J., Bass J., Bethke N., Zurbrugg H.R., Pregla R., Hetzer R., Regitz-Zagrosek V.: Upregulation of myocardial estrogen receptors in human aortic stenosis. *Circulation* 110 (2004) 3270-3275.

Peltoketo H., Luu-The V., Simard J., Adamski J.: 17Beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; Nomenclature and main characteristics of the 17HSD/KSR enzymes. *J. Mol. Endocrinol*. 23 (1999) 1-11.

Pelzer T., Jazbutyte V., Hu K., Segerer S., Nahrendorf M., Nordbeck P., Bonz A.W., Muck J., Fritzemeier K.H., Hegele-Hartung C., Ertl G., Neyses L.: The estrogen receptor-alpha agonist 16alpha-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. *Cardiovasc. Res*. 67 (2005) 604-612.

Penning T.M.: Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Rev*. 18 (1997) 281-305.

Qiu W., Campbell R.L., Gangloff A., Dupuis P., Boivin R.P., Tremblay M.R., Poirier D., Lin S.X.: A concerted, rational design of type 1 17betahydroxysteroid dehydrogenase inhibitors: Estradiol-adenosine hybrids with high affinity. *Faseb J*. 16 (2002) 1829-1831.

Reckelhoff J.F. Fortepiani L.A.: Novel mechanisms responsible for postmenopausal hypertension. *Hypertension* 43 (2004) 918-923.

Rossouw J.E., Anderson G.L., Prentice R.L., LaCroix A.Z., Kooperberg C., Stefanick M.L., Jackson R.D., Beresford S.A., Howard B.V., Johnson K.C., Kotchen J.M., Ockene J.: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA*. 288 (2002) 321-333.

Sam K.M., Auger S., Luu-The V., Poirier D.: Steroidal spiro-gammalactones that inhibit 17 beta-hydroxysteroid dehydrogenase activity in human placental microsomes. *J. Med. Chem*. 38 (1995) 4518-4528.

Sam K.M., Boivin R.P., Tremblay M.R., Auger S., Poirier D.: C16 and C17 derivatives of estradiol as inhibitors of 17 beta-hydroxysteroid dehydrogenase type 1: Chemical synthesis and structure-activity relationships. *Drug Des. Discov*. 15 (1997) 157-180.

Sharkey L.C., Holycross B.J., Park S., Shiry L.J., Hoepf T.M., McCune S.A., Radin M.J.: Effect of ovariectomy and estrogen replacement on cardiovascular disease in heart failure-prone SHHF/Mcc-fa cp rats. *J. Mol. Cell. Cardiol*. 31 (1999) 1527-1537.

van Eickels M., Schreckenberg R., Doevendans P.A. Meyer R., Grohe C., Schluter K.D.: The influence of oestrogen-deficiency and ACE inhibition on the progression of myocardial hypertrophy in spontaneously hypertensive rats. *Eur. J. Heart Fail*. 7 (2005) 1079-1084.

Vihko P., Härkönen P., Soronen P., Törn S., Herrala A., Kurkela R., Pulkka A. Oduwole O., Isomaa V.: 17 Beta-hydroxysteroid dehydrogenases–their role in pathophysiology. *Mol. Cell. Endocrinol*. 215 (2004) 83-88.

Wallen W.J., Cserti C., Bélanger M.P., Wittnich C.: Gender-differences in myocardial adaptation to afterload in normotensive and hypertensive rats. *Hypertension* 36 (2000) 774-779.

Widder J., Pelzer T., von Poser-Klein C., Hu K., Jazbutyte V., Fritzemeier K.H., Hegele-Hartung C., Neyses L., Bauersachs J.: Improvement of endothelial dysfunction by selective estrogen receptor-alpha stimulation in ovariectomized SHR. *Hypertension* 42 (2003) 991-996.
Williams J.K., Suparto I.: Hormone replacement therapy and cardiovascular disease: lessons from a monkey model of postmenopausal women. *Ilar J*. 45 (2004) 139-146.
Xu Y., Arenas I.A., Armstrong S.J., Davidge S.T.: Estrogen modulation of left ventricular remodeling in the aged heart. *Cardiovasc. Res*. 57 (2003) 388-394.
Zhu D.W., Lee X., Breton R., Ghosh D., Pangborn W., Daux W.L., Lin S.X.: Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17 beta-hydroxysteroid dehydrogenase with $NADP^+$. *J. Mol. Biol*. 234 (1993) 242-244.

3.5 Development of biological assays for the identification of selective inhibitors of estradiol formation from estrone in rat liver preparations

Développement de tests d´activités pour l´identification d´inhibiteurs sélectifs de la transformation d´estrone en estradiol chez le rat

Patricia Kruchten, Ruth Werth, Sandrine Marchais-Oberwinkler, Emmanuel Bey, Erika Ziegler, Alexander Oster, Martin Frotscher and Rolf W. Hartmann

Dieser Artikel wird in folgendem Journal veröffentlicht
Comptes Rendus Chimie, Manuskript akzeptiert.

Publikation V

Abstract

Estradiol (E2) is the most important estrogen in humans. Besides its physiological effects it is involved in the initiation and progression of estrogen dependent diseases like breast cancer and endometriosis. Common endocrine therapies have a strong influence on systemic E2 action and thus cause the corresponding side effects. For an optimisation of therapy in estrogen dependent diseases there is need for new innovative approaches. 17β-Hydroxysteroid dehydrogenase type 1 (17βHSD1) is considered as a promising target in therapy, because it catalyses the reduction of estrone (E1) to E2 (last step of E2-biosynthesis) and is often overexpressed in diseased tissue. Highly active and selective inhibitors of the human 17βHSD1 with promising properties for further preclinical development have been identified in our group. Further investigations regard the demonstration of efficacy in an appropriate animal experiment. For this reason, adequate tests for the assessment of the inhibitors in other species have to be established. Here we describe the development of two assays for determination of E2-activation and -inactivation, respectively, as well as the identification of inhibitors of E2-formation in rat liver preparations and their evaluation for selectivity.

Résumé

Estradiol (E2) est le plus important des estrogènes chez l'homme. En plus de son rôle physiologique, il est impliqué dans l'initiation et la progression de maladies liées aux estrogènes comme le cancer du sein et l'endométriose. Les thérapies couramment utilisées influencent l'action systémique de E2 ce qui cause de sévères effets secondaires. Il est donc nécessaire de trouver une nouvelle stratégie pour le traitement de ces maladies. L'enzyme 17βHSD1 est considérée comme une cible prometteuse pour les thérapies du cancer du sein et de l'endométriose car elle catalyse la dernière étape dans la cascade de synthèse de E2 (réduction de estrone (E1) en E2) et car elle est souvent surexprimée dans les tissues atteints. De nouveaux inhibiteurs de l'enzyme humaine 17βHSD1 hautement actifs, sélectifs et présentant un profil prometteur pour des essais précliniques ont été synthétisés, évalués et identifiés par notre groupe. De plus amples investigations seraient nécessaires pour montrer l'efficacité de ces produits dans un modèle animal approprié. Pour cette raison, de nouveaux tests doivent être mis

au point pour évaluer l'activité de ces inhibiteurs dans d'autres espèces animales. Nous décrivons dans ce rapport la mise au point de deux tests d'activité chez le rat permettant de déterminer l'activation ou l'inactivation de E2 par ces inhibiteurs, d'identifier les composés actifs et d'évaluer leurs capacités à inhiber la formation de E2.

Introduction

Breast carcinoma is the leading cause of cancer mortality among women in the Western hemisphere (Jemal *et al.*, 2002). Endometriosis is diagnosed in about 10 % of women in the reproductive age (Giudice and Kao, 2004). It has been shown that estrogens play an important role in both diseases (Thomas, 1984; Dizerega *et al.*, 1980; Zeitoun *et al.*, 1998). Common therapies target systemic action of estradiol (E2) and consequently cause severe side effects. Thus, there is need for novel therapeutical approaches. 17β-Hydroxysteroid dehydrogenase type 1 (17βHSD1), which catalyses the NADPH-dependent reduction of the weakly potent estrogen estrone (E1) to the highly active E2 (Fig. 1), is often overexpressed in the diseased tissue (Gunnarsson *et al.*, 2005; Gunnarsson *et al.*, 2001; Miyoshi *et al.*, 2001; Suzuki *et al.*, 2000; Šmuc *et al.*, 2007). Hence, a selective inhibition of this enzyme would lead to a reduction of active estrogen especially in the concerned tissue. Therefore, 17βHSD1 is considered as an appropriate new target for the therapy of estrogen dependent diseases.

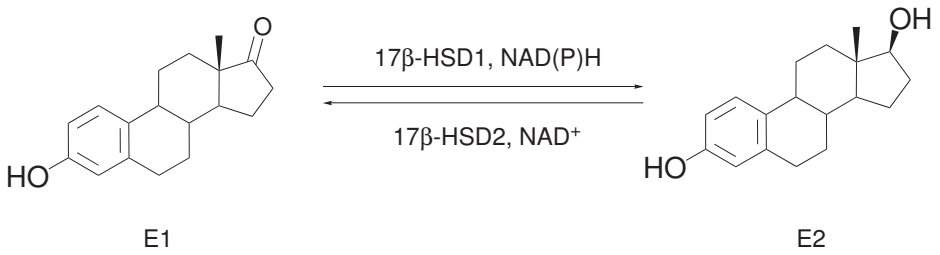


Figure 1: Estrogen interconversion by 17βHSDs.

Several requirements must be fulfilled for 17βHSD1-inhibitors to be applied in therapy. The major pharmacodynamic demands are high inhibitory activity at the target enzyme, selectivity towards its physiological counterpart 17βHSD2, which inactivates E2 to E1 (Fig. 1), and towards the estrogen receptors (ER) α and β, which mediate estrogenic effects. Moreover, the most important hepatic CYP-enzymes should not be inhibited to avoid interference with metabolism of other endogenous or exogenous compounds. Besides, the inhibitors must meet pharmacokinetic prerequisites such as cell permeability, metabolic stability, and a sufficient intestinal absorption. For this reason, a biological screening system was developed and applied to our compound collection of potential h17βHSD1-inhibitors (Kruchten *et al.*, 2009b). Using this test sequence a number of highly active and selective inhibitors of the human target enzyme were identified to have adequate pharmacokinetic properties and hence to be suitable for further evaluations in a disease oriented animal model (Day *et al.*, 2008; Einspanier *et al.*, 2006; Grümmer *et al.*, 2001; Husen *et al.*, 2006; Laschke *et al.*, 2005).
The efficacy of selected compounds was demonstrated in T47D cells. Thus, a first proof of concept for the indication of breast cancer was performed (Kruchten *et al.*, 2009a). With respect on endometriosis therapy, the efficacy should be proven in an appropriate animal model like the rat. Before performing *in vivo* experiments it must be shown, that the inhibitors of the human enzyme are active in the rat enzyme as well. For the selection of suitable compounds to investigate in the animal model, there is need for appropriate tests.
Here we report the establishment of two assays for the determination of selective inhibition of E2-production from E1 in rat tissue preparations. Using these two assays 100 potential 17βHSD1-inhibitors were evaluated for inhibitory activity of estrogen conversion.

Results

Ammonium sulphate precipitate of rat liver cytoplasm was used for the evaluation of the reduction of E1 to E2. Rat liver microsomes were applied for the reverse oxidative reaction. For

tissue preparation fresh rat livers were homogenised and fractionally centrifuged. Ammonium sulphate precipitate was prepared and immediately tested for specific conversion of E1 into E2. Accordingly, the microsomal fraction was obtained and evaluated for activity. In both cases a specific reaction was detectable with a reasonable conversion. The amount of product formed was dependent on the dilution factor of the respective tissue preparation. The microsomal fraction showed a higher specific enzymatic activity (0.26 U/mg protein) than the ammonium sulphate precipitate (0.03 U/mg protein). Protein content was determined according to Bradford (Bradford, 1976).
For an easy and reproducible test procedure it is desirable to preserve activity of the obtained fractions during storage. Thus, fractions were frozen in liquid nitrogen and retested for activity after they had been thawed. No activity was detected (data not shown). In a second procedure freezing buffer was supplemented with 20 % of glycerol. Samples were frozen in liquid nitrogen and thawed repeatedly and after each cycle tested for activity. Under the applied conditions, enzymatic activity was maintained even after two freeze-thaw-cycles (Fig. 2).

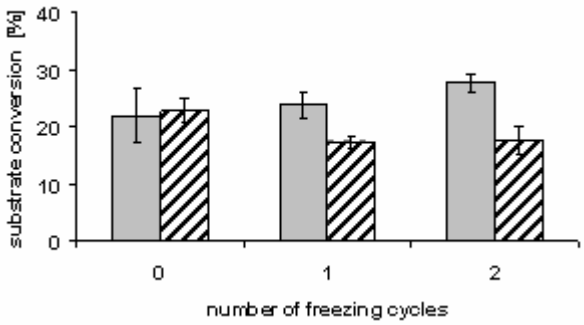


Figure 2: Maintenance of enzymatic activity after repeated freezing. Grey bars represent ammonium sulphate precipitate from cytosolic fraction, striped bars microsomal fraction. Conditions of E1- and E2-conversion were identical for each enzyme preparation.

Since both enzymes in principle are able to catalyse the respective back-reaction in a non cellular environment after addition of the corresponding cofactor (Sherbet *et al.*, 2007), the reverse conditions were applied to each fraction. In both cases the respective back-reaction was observed running to a smaller extent indicating reasonable purity of the preparations (Fig. 3).

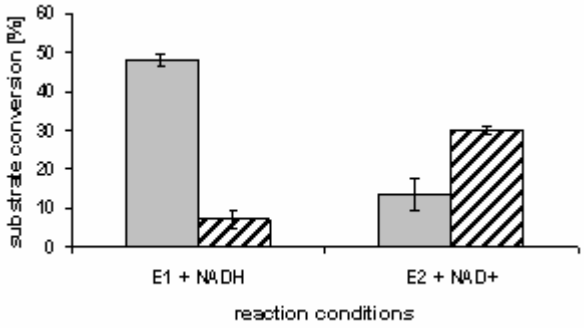


Figure 3: Direction of reaction under different conditions. Grey bars represent ammonium sulphate precipitate from cytosolic fraction, striped bars microsomal fraction. Conditions of E1- and E2-conversion were identical for each enzyme preparation. Concentration of E1 or E2, respectively: 500 nM, concentration of NADH: 500 µM, concentration of NAD^+: 1500 µM, 37 °C.

For comparison of the results of the rat enzymes with the human enzymes, assay conditions have to be similar. Therefore, substrate concentrations (500 nM, each) were adopted from the established test procedures and respective cosubstrates were added in excess (Bey *et al.*, 2008a). For reproducible inhibitor evaluation, the enzymatic conversion must be linear over time. Hence, time-dependent conversion was determined. In the cytosolic precipitate conversion was linear for 15 min whereas reaction ran linearly for 30 min in the microsomal fraction.

After definition of the respective reaction conditions 100 compounds were tested for inhibition of E2-formation. The compounds can be assigned to the structure classes depicted in Figure 4, which were shown to contain selective inhibitors of h17βHSD1 (Bey *et al.*, 2008a; Bey *et al.*, 2008b; Frotscher *et al.*, 2008; Marchais-Oberwinkler *et al.*, 2008). For a comparison of human and rat data compounds with high, medium and without inhibitory activity at the human enzyme were chosen comprising a high structural diversity.

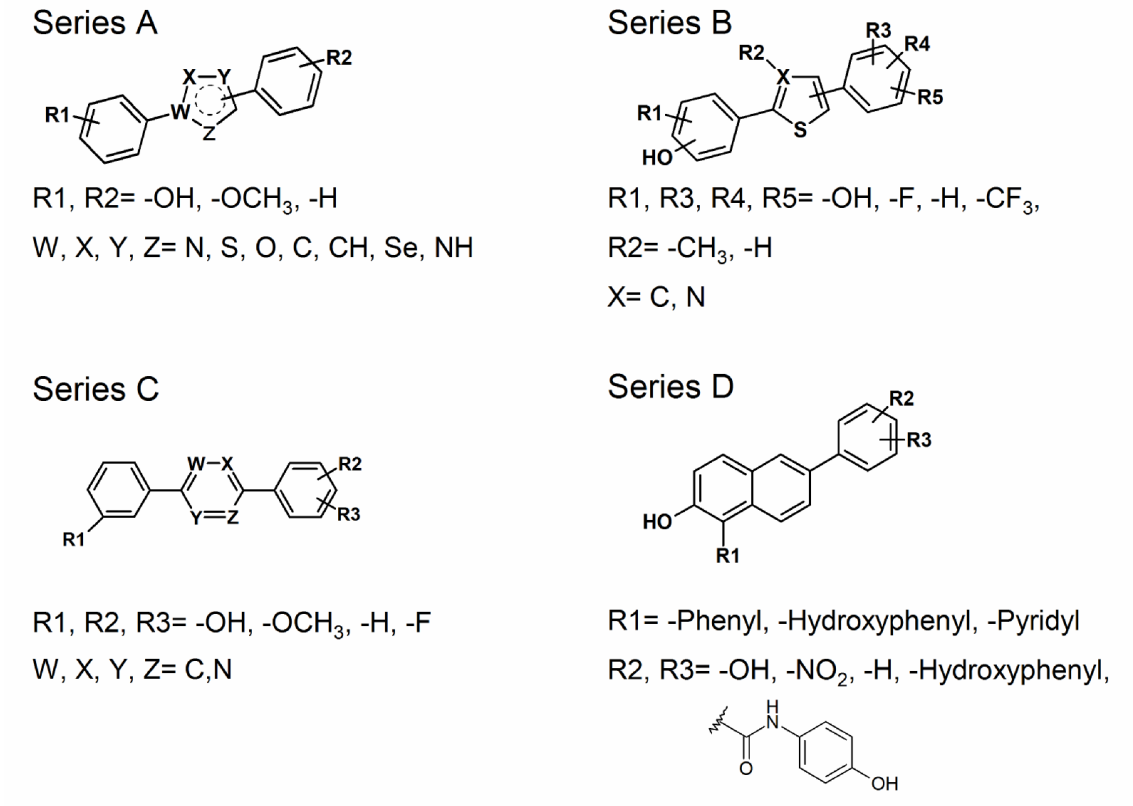


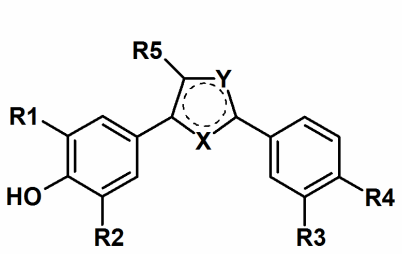
Figure 4: Structures of evaluated compounds.

For the identification of inhibitors of the rat enzyme, percent inhibition of the compounds at a concentration of 1 µM was determined. Although most of the screened compounds were inactive at the rat enzyme (data not shown), inhibitors could be identified both in the class of bis(hydroxyphenyl)heteroarenes and -arenes and 6-aryl-substituted 2-hydroxynaphthalenes (Tab. 1).

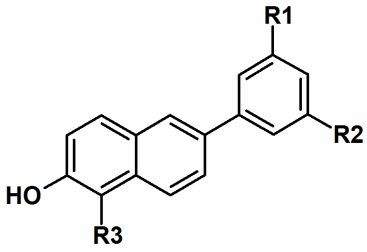
Ten compounds turned out to be moderate or good inhibitors of the rat E2-production (% inhibition > 25 %). For these compounds, IC_{50}-values were determined. Four compounds showed IC_{50}-values below 1 µM (Tab. 2).

Interestingly, in case of the bis(hydroxyphenyl)heteroarenes only sulphur-containing heterocycles as core showed inhibition of the enzymatic reaction, the 2,5-disubstitution of the heterocycle in general leading to a higher activity than the 2,4-disubstitution. The combination of both substitution pattern, as implemented in the 2,4,5-tris(hydroxyphenyl)-thiophene (**6**), does not increase inhibitory potency but leads to medium activity. Moreover, it is striking that the highly active inhibitors of this series are fluorine-substituted, the most potent being compound **4**.

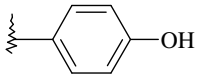
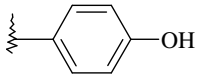
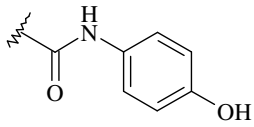
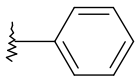
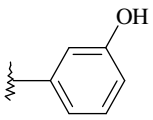
Table 1: Structure of selected compounds tested for inhibition of E2-formation from E1 in rats.



1 - 6



7 - 11

Cmpd	X	Y	R1	R2	R3	R4	*R5*
1	C	S	CH_3	H	OH	H	H
2	C	S	F	H	OH	H	H
3	S	C	F	H	OH	H	H
4	S	C	F	F	OH	H	H
5	S	C	F	H	OH	F	H
6	S	C	H	H	H	OH	
7			OH		H		
8			NO_2		H		
9			OH	H			
10			OH	H			
11			H	H	H		

Regarding compounds with a naphthalene system most of the identified inhibitors were 6-hydroxyphenyl-substituted 2-naphthols (**7**, **9**, and **10**). Obviously, the hydroxyl-substituent of the phenyl moiety is needed for activity in the rat enzyme (**11**). But also a 6-nitrophenyl-substituted 2-naphthol (**8**) showed moderate inhibition of E2-production. Generally, two substitution sites in addition to the hydroxy-groups seem to be tolerated, the 5´-position of the hydroxy- or nitrophenyl-moiety and the 1-position of the naphthalene core, leading to comparable inhibitory activities (**7** and **9**). In 1-position of the hydroxynaphthalene core a phenyl-moiety increased inhibitory activity compared to the unsubstituted compound (11 % inhibition at 1 µM vs. of 69 % for **9**). However, further substitution of this phenyl-ring decreased activity again (data not shown).

Table 2: Inhibition of rat E2- and E1-formation by selected compounds.

compound	h17βHSD1[a]	E2-formation[b]		*E1-formation*[c]	
	IC_{50} [µM]	Inhibition [%][d,e]	IC_{50} [µM][d]	Inhibition [%][d,e]	IC_{50} [µM][d]
1	< 0.1	27	2.60	51	0.98
2	< 0.1	55	0.85	83	0.24
3	< 0.1	50	1.08	68	0.42
4	< 0.1	81	0.27	95	0.08
5	< 0.1	47	0.96	69	0.45
6	n.i.[f]	28	2.28	65	0.37
7	n.i.[f]	49	1.08	66	0.56
8	n.i.[f]	37	1.60	56	0.68
9	< 0.1	69	0.56	48	1.06
10	< 0.1	26	5.37	< 10	6.25
11	> 10	< 10	n.d.[g]	n.d.[g]	n.d.[g]

[a]human placenta, ammonium sulphate precipitate from cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 µM], data given for comparison; [b]Rat liver, ammonium sulphate precipitate from cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 µM]; [c]Rat liver, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM]; [d]Mean values of three determinations standard deviation less than 17%, [e]inhibitor concentration 1 µM; [f]n.i., no inhibition; [g]n.d., not determined

Comparing inhibition of human and rat E2-synthesis it is noticeable that highly active h17βHSD1-inhibitors show also moderate to good inhibition of rat E2-formation. On the other hand, there are also compounds among the ten best inhibitors in the rat which lack inhibitory activity at h17βHSD1. Remarkably, in 5´-position even a large substituent is tolerated by the rat enzyme while this compound is inactive at the human protein (Tab. 2, compounds **7** and **8**). Nevertheless as a rule highly active h17βHSD1-inhibitors were observed to show inhibition of E2-activation in rat liver preparations.

Discussion and conclusion

Using our screening system we had been able to identify a number of h17βHSD1-inhibitors with suitable properties for further preclinical studies (Bey *et al.*, 2008a; Bey *et al.*, 2008b; Frotscher *et al.*, 2008; Marchais-Oberwinkler *et al.*, 2008). As a next step, their efficacy should be demonstrated in a disease oriented animal model. Currently, several animal models are available using different species and addressing the two estrogen dependent diseases, breast cancer and endometriosis.

Regarding breast cancer two models can be considered for application, both using nude mice as host for human breast cancer cells (Husen *et al.*, 2006a; Husen *et al.*, 2006b; Day *et al.*, 2008). However, due to the artificial environment of the established tumour cell line the prediction of side effects of the inhibitors is limited.

Concerning endometriosis several models are potentially usable for the evaluation of inhibitors. One of them is similar to the breast cancer experiments in nude mice and therefore bears similar advantages and drawbacks. It uses human endometrial fragments, which are introduced into the peritoneal cavity of nude mice (Grümmer *et al.*, 2001; Fechner *et al.*, 2007). Furthermore, there are two monkey models available, in which an endometriosis is established by introducing

autografts of endometrial tissue into the abdomen of the animals. One model is working with marmoset monkeys, non menstruating primates, the other with menstruating cynomolgus monkeys (Einspanier *et al.*, 2006; Yang *et al.*, 2000). Both give a good insight into endometriosis and its potential treatments. However, major disadvantage of these models is the fact that they are very labour-intensive and require monkey specific 17βHSD1-inhibitors. A further described model is applicable to different rodent species such as hamster, mouse, and rat. In this experiment, endometrial autografts are implanted into the dorsal skinfold (Menger *et al.*, 2002; Laschke *et al.*, 2005). This is a relatively artificial model, but on the other hand has the advantage of host-derived disease tissue. Moreover, aminoacid-sequences of rat and mouse proteins are known and can possibly be used for the identification of inhibitors.
However, before performing an animal experiment in a certain species pharmacokinetic data of the active inhibitors have to be determined for the respective animal. First routine pharmacokinetic-studies in rats are promising, and we decided to screen for inhibitors of the rat E2-formation first aiming at a potential application in the mentioned endometriosis model.
For this purpose, rat liver was used as enzyme source, because 17βHSD1-activity was described for this tissue (Martel *et al.*, 1992). As it is the case in humans, rat 17βHSD1 is a cytosolic enzyme, whereas 17βHSD2 is membrane associated (Akinola *et al.*, 1996; Akinola *et al.*, 1998). Consequently, tissue was homogenised and fractionated by centrifugation. In both obtained fractions (cytosol and microsomes) the expected conversions were found to run specifically. The finding that there is obviously a pronounced E2-inactivation might be related to the discovery that enzymatic activity in case of E2-oxidation is age dependent as was shown before (Jazbutyte *et al.*, 2006).
Screening of our substance library containing of highly active 17βHSD1-inhibitors for selective inhibiton of E2-formation in the rat led to ten compounds with moderate to good activity. Keeping in mind that the compounds were designed as substrate mimetics a higher number of compounds active in the rat enzyme were expected. On the other hand, those compounds with high inhibitory activity towards the human enzyme were also moderate to good inhibitors of rat E2-formation. Structure-activity-relationships are similar for both species although there is only an amino acid identity of 68 % between the two proteins (Ghersevich *et al.*, 1994). Inhibitors of the rodent E1-reduction which were not active in the human enzyme (**6**, **7**, and **8**) have large substituents in positions which differ from those of the highly active h17βHSD1-inhibitors. This finding indicates that there is additional space available in the rat enzyme which is not present in the human.
In conclusion, we successfully established assays for the inhibition of E2-formation from E1 and vice versa in rats. Using these tests, we are able to determine the potency of a compound to selectively inhibit E2-formation in rats. Applying the developed assays a screening for rat 17βHSD1-inhibitors was performed and compounds with a satisfying inhibitory activity were identified.

Experimental section

[^{3}H]-E1 and [^{3}H]-E2 were purchased from Perkin Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt. Other chemicals were purchased from Sigma, Roth or Merck.

Tissue preparation and determination of inhibition

Cytosol and microsomal fraction from rat liver tissue were separated according to a procedure described previously (Jazbutyte *et al.*, 2006; Sam *et al.*, 1997). Ammonium sulphate precipitate from cytosolic fraction was used for evaluation of E2-formation. Microsomal fraction was purified and used for the determination of E2-inactivation.
For determination of enzymatic activity, the preparations were incubated with NADH and [^{3}H]-E1 (precipitate) or NAD^+ and [^{3}H]-E2 (microsomes), respectively, at 37 °C in a phosphate buffer (pH 7.4) supplemented with 20 % of glycerol and EDTA 1 mM. Reaction was stopped with $HgCl_2$ and steroids were extracted into ether. Substrates and products were separated using an acetonitrile/water (45/55 v/v) mixture as mobile phase in a C18 rp chromatography column

(Nucleodur C18, 3 µm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad).

Specific enzymatic activity

Protein amount of the preparations was determined as described by Bradford (Bradford, 1976). Specific activities were given in U/mg protein.
Conversion rates were evaluated and the formation of one micromole of product per minute was defined as representing 1U enzyme activity.

Acknowledgement

We are grateful to the Deutsche Forschungsgemeinschaft for financial support (HA1313/8-1) of this work. P.K. is grateful for a scholarship of European Postgraduate School (DFG) GRK532. We thank Beate Geiger for her help in evaluation of inhibitors and Pharmacelsus CRO for providing us with rat livers.

References

Akinola, L.A., Poutanen, M., Peltoketo, H., Vihko, R., Vihko, P.: Characterization of rat 17 beta-hydroxysteroid dehydrogenase type 1 gene and mRNA transcripts. *Gene* 208 (1998) 229-238.

Akinola, L.A., Poutanen, M., Vihko, R.: Cloning of rat 17 beta-hydroxysteroid dehydrogenase type 2 and characterization of tissue distribution and catalytic activity of rat type 1 and type 2 enzymes. *Endocrinology* 137 (1996) 1572-1579.

Bey, E., Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Werth, R., Oster, A., Algül, O., Neugebauer, A., Hartmann, R.W.: Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* 16 (2008a) 6423-6435.

Bey, E., Marchais-Oberwinkler, S., Werth, R., Negri, M., Al-Soud, Y., Kruchten, P., Oster, A., Frotscher, M., Birk, B., Hartmann, R.W.: Design, Synthesis, Biological Evaluation and Pharmacokineticsof Bis(hydroxyphenyl)substituted Azoles, Thiophenes, Benzenes and Azabenzenes as Potent and Selective non-steroidal Inhibitors of 17β-Hydroxysteroid-Dehydrogenase Type 1 (17β-HSD1). *J. Med. Chem.* 51 (2008b) 6725-6739.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 (1976) 248-254.

Day, J.M., Foster, P.A., Tutill, H.J., Parsons, M.F., Newman, S.P., Chander, S.K., Allan, G.M., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J., Purohit, A.: 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* 122 (2008) 1931-1940.

Dizerega, G.S., Barber, D.L., Hodgen, G.D.: Endometriosis: role of ovarian steroids in initiation, maintenance, and suppression. *Fertil. Steril.* 33 (1980) 649-653.

Einspanier, A., Lieder, K., Brüns, A., Husen, B., Thole, H., Simon, C.: Induction of endometriosis in the marmoset monkey (Callithrix jacchus). *Mol. Hum. Reprod.* 12 (2006) 291-299.

Fechner, S., Husen, B., Thole, H., Schmidt, M., Gashaw, I., Kimmig, R., Winterhager, E., Grümmer, R.: Expression and regulation of estrogen-converting enzymes in ectopic human endometrial tissue. *Fertil Steril* 88 (2007) 1029-1038.

Frotscher, M., Ziegler, E., Marchais-Oberwinkler, S., Kruchten, P., Neugebauer, A., Fetzer, L., Scherer, C., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Design, synthesis, and biological evaluation of (hydroxyphenyl)naphthalene and -quinoline derivatives: potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* 51 (2008) 2158-2169.

Ghersevich, S., Nokelainen, P., Poutanen, M., Orava, M., Autio-Harmainen, H., Rajaniemi, H., Vihko, R.: Rat 17 beta-hydroxysteroid dehydrogenase type 1: primary structure and regulation of enzyme expression in rat ovary by diethylstilbestrol and gonadotropins in vivo. *Endocrinology* 135 (1994) 1477-1487.

Giudice, L.C., Kao, L.C.: Endometriosis. *Lancet* 364 (2004) 1789-1799.

Grümmer, R., Schwarzer, F., Bainczyk, K., Hess-Stumpp, H., Regidor, P.A., Schindler, A.E., Winterhager, E.: Peritoneal endometriosis: validation of an in-vivo model. *Hum. Reprod.* 16 (2001) 1736-1743.

Gunnarsson, C., Hellqvist, E., Stål, O.: 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* 92 (2005) 547-552.

Gunnarsson, C., Olsson, B.M., Stål, O.: Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* 61 (2001) 8448-8451.

Husen, B., Huhtinen, K., Poutanen, M., Kangas, L., Messinger, J., Thole, H.: Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. *Mol. Cell. Endocrinol.* 248 (2006a) 109-113.

Husen, B., Huhtinen, K., Saloniemi, T., Messinger, J., Thole, H.H., Poutanen, M.: Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* 147 (2006b) 5333-5339.

Jazbutyte, V., Hu, K., Kruchten, P., Bey, E., Maier, S.K., Fritzemeier, K.H., Prelle, K., Hegele-Hartung, C., Hartmann, R.W., Neyses, L., Ertl, G., Pelzer, T.: Aging reduces the efficacy of estrogen substitution to attenuate cardiac hypertrophy in female spontaneously hypertensive rats. *Hypertension* 48 (2006) 579-586.

Jemal, A., Thomas, A., Murray, T., Thun, M.: Cancer statistics, 2002. *CA Cancer J. Clin.* 52 (2002) 23-47.

Kruchten, P., Werth, R., Bey, E., Oster, A., Marchais-Oberwinkler, S., Frotscher, M., Hartmann, R.W.: Selective inhibition of 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) reduces estrogen responsive cell growth of T47-D breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 114 (2009a) 200-206.

Kruchten, P., Werth, R., Marchais-Oberwinkler, S., Frotscher, M., Hartmann, R.W.: Development of a biological screening system for the evaluation of highly active and selective 17β-HSD1-inhibitors as potential therapeutics. *Mol. Cell. Endocrinol.* 301 (2009b) 154.

Laschke, M.W., Elitzsch, A., Vollmar, B., Menger, M.D.: In vivo analysis of angiogenesis in endometriosis-like lesions by intravital fluorescence microscopy. *Fertil. Steril.* 84 Suppl. 2 (2005) 1199-1209.

Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Ziegler, E., Neugebauer, A., Bhoga, U., Bey, E., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Substituted 6-phenylnaphthols. Potent and selective nonsteroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1): Design, synthesis, biological evaluation, and pharmacokinetics. *J. Med. Chem.* 51 (2008) 4685.

Martel, C., Rhéaume, E., Takahashi, M., Trudel, C., Couet, J., Luu-The, V., Simard, J., Labrie, F.: Distribution of 17 beta-hydroxysteroid dehydrogenase gene expression and activity in rat and human tissues. *J. Steroid Biochem. Mol. Biol.* 41 (1992) 597-603.

Menger, M.D., Laschke, M.W., Vollmar, B.: Viewing the microcirculation through the window: some twenty years experience with the hamster dorsal skinfold chamber. *Eur. Surg. Res.* 34 (2002) 83-91.

Miyoshi, Y., Ando, A., Shiba, E., Taguchi, T., Tamaki, Y., Noguchi, S.: Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* 94 (2001) 685-689.

Sam, K.M., Boivin, R.P., Tremblay, M.R., Auger, S., Poirier, D.: C16 and C17 derivatives of estradiol as inhibitors of 17 beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug Des. Discov.* 15 (1997) 157-180.

Sherbet, D.P., Papari-Zareei, M., Khan, N., Sharma, K.K., Brandmaier, A., Rambally, S., Chattopadhyay, A., Andersson, S., Agarwal, A.K., Auchus, R.J.: Cofactors, redox state, and

directional preferences of hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* 265-266 (2007) 83-88.

Šmuc, T., Pucelj, M.R., Šinkovec, J., Husen, B., Thole, H., Lanišnik Rižner, T.: Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* 23 (2007) 105-111.

Suzuki, T., Moriya, T., Ariga, N., Kaneko, C., Kanazawa, M., Sasano, H.: 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* 82 (2000) 518-523.

Thomas, D.B.: Do hormones cause breast cancer? *Cancer* 53 (1984) 595-604.

Yang, J.Z., Agarwal, S.K., Foster, W.G.: Subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin modulates the pathophysiology of endometriosis in the cynomolgus monkey. *Toxicol. Sci.* 56 (2000) 374-381.

Zeitoun, K., Takayama, K., Sasano, H., Suzuki, T., Moghrabi, N., Andersson, S., Johns, A., Meng, L., Putman, M., Carr, B., Bulun, S.E.: Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol. *J. Clin. Endocrinol. Metab.* 83 (1998) 4474-4480.

3.6 Selective inhibition of 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) reduces estrogen responsive cell growth of T47D breast cancer cells

Patricia Kruchten, Ruth Werth, Emmanuel Bey, Alexander Oster, Sandrine Marchais-Oberwinkler, Martin Frotscher, Rolf W. Hartmann

Dieses Manuskript wurde als Artikel in folgendem Journal veröffentlicht:
Journal of Steroid Biochemistry and Molecular Biology (**2009**) *114*, 200-206.

Publikation VI

Abstract

The most potent estrogen estradiol (E2) plays a pivotal role in the initiation and progression of estrogen dependent diseases. 17β-Hydroxysteroid dehydrogenase type 1 (17βHSD1) catalyses the NADPH-dependent E2-formation from estrone (E1). It is often overexpressed in breast cancer and endometriosis. For this reason, inhibition of 17βHSD1 is a promising strategy for the treatment of these diseases. In the present paper, we investigate the estrogen responsive cell growth of T47D breast cancer cells, the intracellular inhibitory activity of non-steroidal 17βHSD1-inhibitors and their effects on estrogen dependent cell growth *in vitro*. At equal concentrations the estrogens E1 and E2 induced the same extent of growth stimulation indicating fast intracellular conversion of E1 into E2. Application of inhibitors selectively prevented stimulation of proliferation evoked by E1-treatment whereas E2-mediated stimulation was not affected. Furthermore, intracellular E2-formation from E1 was significantly inhibited with IC_{50}-values in the nanomolar range. In conclusion, our findings strongly support suitability of non-steroidal 17βHSD1-inhibitors for the treatment of estrogen dependent diseases.

Introduction

17β-Hydroxysteroid dehydrogenase type 1 (17βHSD1) catalyses intracellularly the NADPH-dependent reduction of the weakly active estrone (E1) to the highly potent estradiol (E2). Besides its physiological effects in the development and differentiation of estrogen-sensitive tissues, E2 is involved in the initiation and progression of estrogen dependent diseases like breast cancer (Thomas, 1984; Cauley *et al*., 1999; Russo *et al*., 2006) and endometriosis (Dizerega *et al*., 1980; Zeitoun *et al*., 1998).
In addition to the type 1 enzyme, until recently 17βHSD7 and 12 were supposed to primarily catalyse intracellular conversion of E1 into E2 (Krazeisen *et al*., 1999; Luu-The *et al*., 2006). But 17βHSD7 was found to be mainly involved in cholesterol synthesis (Marijanovic *et al*., 2003; Ohnesorg and Adamski, 2006), and 17βHSD12 was observed to be inefficient in intracellular E2-production compared to 17βHSD1 even at high expression levels as was demonstrated using T47D cells (Day *et al*., 2008).
In many breast cancer tissues (Gunnarsson *et al*., 2001; Gunnarsson *et al*., 2005; Vihko *et al*., 2003) and endometriotic lesions (Šmuc *et al*., 2007) overexpression of 17βHSD1 could be detected. In contrast, expression of 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2), which catalyses the deactivation of E2 and therefore plays a protective role (Luu-The *et al*., 1995; Vihko *et al*., 2001; Vihko *et al*., 2004), was not found to be increased, in several cases even decreased (Zeitoun *et al*., 1998; Bulun *et al*., 2000; Sasano *et al*., 2006). The resulting

enhancement of 17βHSD1 to 17βHSD2 expression ratio (Miyoshi *et al*., 2001) maintains the supply of tissue with E2 which is needed for further proliferation (Suzuki *et al*., 2000). For this reason, the importance of 17βHSD1 in estrogen dependent diseases was recognised (Oduwole *et al*., 2004), and its inhibition is considered as a promising strategy for their treatment.
Until now different endocrine therapies have been administered in breast cancer (Miller *et al*., 2007). SERMs (Selective Estrogen Receptor Modulators) and pure antiestrogens like fulvestrant (Osborne *et al*., 1976) block the estrogen action at the receptor level while aromatase inhibitors and GnRH-analogues restrain the formation of estrogens. This strong reduction of systemic estrogen action is a rather radical approach resulting in the well-known side effects of these strategies like osteoporosis, hot flushes, or depressive mood. A softer therapy could be the inhibition of 17βHSD1 catalysing the last step of the E2 biosynthesis. Compared to established endocrine breast cancer therapies, which systemically reduce E2-action (Miller *et al*., 2007), fewer side effects are expected, because mainly tissue overexpressing 17βHSD1 will be affected.
Over the last decade, several steroidal (Brožic *et al*., 2008) and few non-steroidal (Messinger *et al*., 2006; Karkola *et al*., 2008; Allan *et al*., 2008) classes of 17βHSD1-inhibitors were discovered. Recently, we described two series of non-steroidal compound classes (Frotscher *et al*., 2008; Marchais-Oberwinkler *et al*., 2008; Bey *et al*., 2008a; Bey *et al*., 2008b), which were evaluated by means of our screening system (Kruchten *et al*., 2009). Since inhibitors show high selectivities towards 17βHSD2 and both subtypes (α and β) of the estrogen receptor (ER) as well as promising pharmacokinetic properties, they are appropriate for further investigations.
Before application in a suitable animal model can be envisaged, efficacy should be shown in cellular experiments. For this purpose, estrogen dependent breast cancer cell lines as T47D or MCF-7 are available. T47D cells were chosen, because they express 17βHSD1 and 17βHSD2 in sufficient amount in contrast to MCF-7 (Day *et al*., 2006). Additionally, they show a high 17βHSD1 to 17βHSD2 expression ratio (Duncan and Reed, 1995), which is similar as in the diseased tissue. The main objective of this work is to examine whether selective 17βHSD1-inhibition is an appropriate way to reduce estrogen dependent cell proliferation.

Materials and Methods

Chemicals

E1, E2 and MTT were obtained from Sigma, Seelze. Radioactive labeled [2, 4, 6, 7-^{3}H]-E1 (50-100 Ci/mmol) and [2, 4, 6, 7-^{3}H]-E2 (70-115 Ci/mmol) were purchased from Perkin Elmer, Boston. Quickszint Flow 302 and Quickszint 212 scintillator fluids were bought from Zinsser Analytic, Frankfurt. Recombinant ERα and ERβ were purchased from Invitrogen, Carlsbad. Other chemicals were received from Sigma, Roth or Merck.

Cell culture

T47-D cells were obtained from ECACC, Salisbury. Stripped FCS and cell culture media were purchased from CCpro, Oberdorla. Stock culture of cells was routinely cultivated in RPMI 1640 medium supplemented with 10 % FCS (Sigma), L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), insulin zinc salt (10 µg/mL) and sodium pyruvate (1 mM) at 37 °C under 5 % CO_2 humidified atmosphere. Subculture was carried out every two to three days.

Cell free inhibition assays of 17βHSD1 and 17βHSD2

For the purification of 17βHSD1, the cytosolic fraction of human placenta was precipitated with ammonium sulphate following a well established procedure (Sam *et al*., 1997). 17βHSD2 was obtained from the microsomal fraction.
Inhibitory activities were evaluated as described before (Bey *et al*., 2008a; Bey *et al*., 2008b). Briefly, for determination of 17βHSD1-inhibition the enzyme preparation was incubated with NADH [500 µM] in presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20 % of glycerol and EDTA 1 mM. The enzymatic reaction was started by

addition of [2, 4, 6, 7-^{3}H]-E1 (500 nM, 0.15 µCi). After 10 min, the incubation was stopped with $HgCl_2$.
The 17βHSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD^+ [1500 µM], test compound and [2, 4, 6, 7-^{3}H]-E2 (500 nM, 0.11 µCi) for 20 min at 37°C. Steroids were extracted into diethylether. Substrate and product were separated using acetonitrile/water (45:55) as mobile phase in a C18 reversed phase chromatography column (Nucleodur C18, 3 µm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad; Quickszint Flow 302, efficiency 50 %).

ER-affinity assay

Binding affinity of inhibitors to ERα or ERβ was determined as described before (Bey *et al*., 2008a) similarly to Zimmermann et al. (Zimmermann *et al*., 2005). Briefly, 0.25 pmol of ERα or ERβ, respectively, were incubated with [2, 4, 6, 7-^{3}H]-E2 (10 nM, 0.07 µCi) and test compound for one hour at room temperature. Non-specific-binding was performed with diethylstilbestrol (10 µM). After incubation, ligand-receptor complexes were bound to hydroxyapatite (HAP; 5 g/ 60 mL TE-buffer). After centrifugation the HAP-fraction was separated, washed and resuspended in ethanol. For radiodetection, scintillation cocktail (Quickszint 212) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). The relative binding affinity was calculated after inhibitor and E2 concentrations required to displace 50 % of the receptor bound labelled E2 were determined, using the following equation:

$$RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \cdot 100$$

The RBA value for E2 was arbitrarily set at 100 %.

MTT-Cytotoxicity assay

The number of living cells was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). Experiments were performed in 96-well cell culture plates in DMEM supplemented with 10 % FCS. Cells were incubated for three hours with 2.5 µM of test compound at 37 °C in a humidified atmosphere at 5 % CO_2. For cleavage reaction MTT-solution (5 mg/mL in PBS) was added and incubation was continued for another three hours. Reaction stop and cell lysis were carried out by addition of sodium dodecyl sulphate (SDS) in 0.01N HCl (10 %). The produced blue formazan was quantified spectrophotometrically at 590 nm as described by Denizot and Lang with minor modifications (Denizot and Lang, 1986).

Cellular inhibition assay of 17βHSD1

Experiments were performed in 24-well plates in DMEM supplemented with 10 % FCS (Pan Biotech GmbH, Aidenbach), L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), insulin-zinc-salt (10 µg/mL) and sodium pyruvate (1 mM). After an adaption phase of 24 hours the medium was changed for fresh serum free DMEM and a solution of test compound in DMSO was added. The final concentration of DMSO was adjusted to 1 % in all samples. After a pre-incubation of 30 min, incubation was started by addition of [2, 4, 6, 7-^{3}H]-E1 (50 nM, 0.15 µCi). After 30 min, the conversion reaction was stopped by removing the supernatant medium. The steroids contained in the aspirated medium were extracted into diethylether. Further treatment of the samples was carried out as described above.

Proliferation assay

RPMI 1640 (without phenol red) was used for the experiments and was supplemented with streptomycin (100 µg/mL), insuline zinc salt (10 µg/mL), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/mL) and charcoal-stripped FCS 5 % (v/v). Cells were

grown for two days in phenol red-free medium before washing and addition of estrogens and/ or compounds. Inhibitors, 4-hydroxytamoxifen, E1 and E2 were diluted in ethanol. The final ethanol concentration was adjusted to 1 %. The medium was changed every two to three days and supplemented with the respective additive. After eight days of culture without passage in the presence of the respective additives, the cell viability was evaluated measuring the reduction of MTT as described above. Proliferation in presence of vehicle was set at 100 %.

Statistics

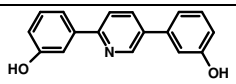
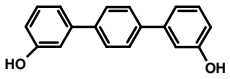
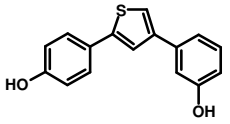
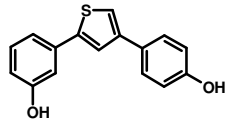
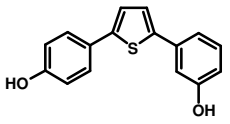
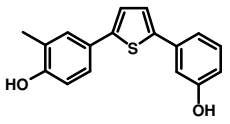
Statistical significance was calculated by two-sided t-tests. Values are mean ± SEM. $P < 0.05$ was considered as significant, $P < 0.003$ as highly significant.

Results

Selection of appropriate candidate compounds for extended investigation in T47-D cells

Following our screening procedure several compounds had been identified for further evaluation (Bey *et al*., 2008a; Bey *et al*., 2008b). Table 1 shows data of the most promising 17βHSD1-inhibitors. They all are bis(hydroxyphenyl)substituted six or five membered aryls, the latter consisting of 2,5- and 2,4-disubstituted thiophenes.
For comparison of the compounds, their inhibitory activities at 17βHSD1 and 17βHSD2 were determined in cell free procedures. All compounds showed IC_{50}-values below 200 nM for inhibition of 17βHSD1 and selectivity factors of more than 10 towards 17βHSD2. Affinities to the ERs were marginal with RBA-values below 1.0 % (E2: RBA = 100 %) except for **5** with slightly enhanced ERβ-affinity. Nevertheless, binding affinities did never exceed the RBA of E1 (RBA(ERα) = 5.5 %; RBA(ERβ) = 3.1 %). Furthermore, survival rate of the cells after three hours in presence of 2.5 µM of 17βHSD1-inhibitor was evaluated to ensure that intracellular inhibitory activities were not caused by unspecific cytotoxic effects. No significant reduction of cell number was observed indicating, that the compounds do not exert cytotoxic effects at the given concentration. Evaluation of the compounds in a cellular 17βHSD1-inhibition assay using T47-D cells showed inhibitory activities, which were less pronounced compared to the cell free assay. Nevertheless, all inhibitors showed IC_{50}-values below 500 nM in intact cells.
Based on the data presented in Table 1, candidates for the investigation of the influence of selective 17βHSD1-inhibitors on estrogen-responsive cell growth were chosen. For selection, different aspects were taken into account. Aside from inhibitory activity and selectivity towards 17βHSD2, affinities to the ERs were an important criterion. Since estrogenic or antiestrogenic effects would interfere with the investigation of estrogen dependent cell proliferation, inhibitors with lower RBA-values were preferred to those with higher binding affinity. Structural diversity was an additional aspect for selection. Thus, one representative of each core structure was chosen: **2** because of its very low ERα-affinity, **4** for its higher inhibitory activity and slightly better selectivity compared to **3**, and **6**, the most potent inhibitor in the cell free assay with promising data in the other tests (Tab. 1).

Table 1: Inhibitory activity of compounds 1-6 at 17βHSD1 and 17βHSD2, binding affinities to ERα and ERβ and cell survival rate after compound application.

cmpd	structure	cell-free assays; IC_{50} [nM][a,b]			RBA [%][a,b,f]		survival rate[a,h]	cellular assay[a,i]
		17βHSD1[c]	17βHSD2[d]	selectivity factor[e]	ERα[g]	ERβ[g]	[%]	IC_{50} [nM]
1		101	3399	34	<0.1	<0.01	90	382
2		173	2259	13	<0.001	<0.1	95	316
3		151	1690	11	<0.1	<1.0	105	404
4		77	1271	17	<0.1	<1.0	102	413
5		69	1953	28	<1.0	<10	99	469
6		46	1971	43	<0.01	<0.01	109	425

[a]Mean values of three determinations, standard deviation less than 16 %; [b]data given for comparison, [c]Human placenta, ammonium sulphate precipitate from cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 µM]; [d]Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM]; [e]IC_{50}(17βHSD2)/ IC_{50}(17βHSD1); [f]RBA (relative binding affinity), E2: 100 %, <0.01 = 0.001 < RBA < 0.01, <0.1 = 0.01 < RBA < 0.1, <1.0 = 0.1 < RBA < 1.0, <10 = 1.0 < RBA < 10; [g]human recombinant protein, incubation with 10 nM [^{3}H]-E2 and inhibitor for 1 h; [h]survival rate of T47D cells after incubation with compound at 2.5 µM for 3 hours, amount of living cells determined by MTT-conversion, control: 100%; [i]intact T47D cells substrate [^{3}H]-E1 + E1 [50 nM].

Investigation of estrogen stimulation of cell proliferation in T47D cells

Extended evaluations of 17βHSD1-inhibitors were carried out in the estrogen dependent breast cancer cell line T47D. For experiments, phenol red-free culture medium supplemented with 5 % of stripped FCS was used. Prior to inhibitor application, estrogen responsiveness of the cells was confirmed. For this purpose, E1 or E2 was added to the culture medium at different concentrations ranging from 0.1 pM to 10 nM, and the number of living cells was determined after eight days of incubation without passage (Fig. 1A). E1 stimulated cell proliferation to the same extent as E2, although E1 is known to be the weaker estrogen. A significant stimulation could be observed at a concentration as low as 0.5 pM. Maximum stimulation was reached at an estrogen concentration of 0.1 nM. Consequently, an estrogen-concentration of 0.1 nM was chosen for further experiments.

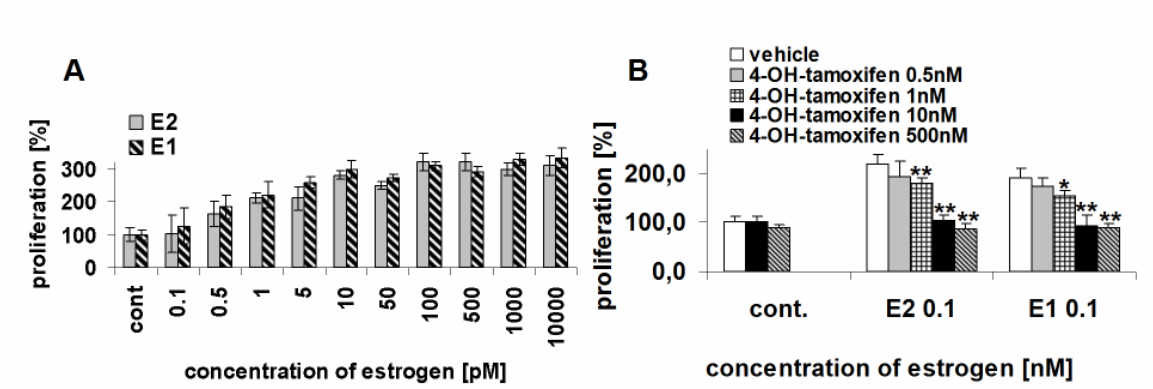


Figure 1: A. Concentration dependent stimulation of cell proliferation (T47D). Grey bars represent E2-stimulation, striped bars E1-stimulation. The control was arbitrarily set at 100 %. First significant stimulation was observed at 0.5 pM ($p < 0.05$) B. Effect of 4-hydroxytamoxifen on estrogen-stimulated cell proliferation in vitro. T47D cells were treated with 4-hydroxytamoxifen at 10 or 500 nM in presence (0.1 nM) or absence of E2 or E1, respectively and at 0.5 nM or 1 nM (0.1 nM) in presence of E2 or E2. *P < 0.05, ** p < 0.003.

In order to confirm that estrogen stimulation is receptor-mediated, cells were treated with 4-hydroxytamoxifen, the active metabolite of tamoxifen, in presence or absence of 0.1 nM of E1 or E2, respectively (Fig. 1B). The number of living cells was determined after eight days without passage. 4-Hydroxytamoxifen was added in four concentrations in presence of estrogen ranging from 0.5 nM to 500 nM and in two concentrations (10 nM and 500 nM) in absence of estrogen. Even at a concentration of 500 nM, 4-hydroxytamoxifen did not stimulate or reduce cell proliferation. The stimulation, which was evoked by 0.1 nM of E1 or E2, respectively, was dose dependent and fully prevented by simultaneous addition of 4-hydroxytamoxifen at 10 nM. This finding demonstrates, that E1 and E2 unfold their growth stimulatory effect via ER-activation.

Effects of selective 17βHSD1-inhibitors on estrogen stimulated cell proliferation

Compounds **2** and **6** were tested at higher concentrations. They were able to reduce E1-stimulation to control-level when added at a concentration of 1 µM. An example of the dose dependent reduction of stimulated cell growth is given in figure 2. First significant reduction could be observed at an inhibitor concentration of 0.1 nM. At 1 nM highly significant growth inhibition was seen. Therefore, a concentration of 1 nM was chosen for further experiments.

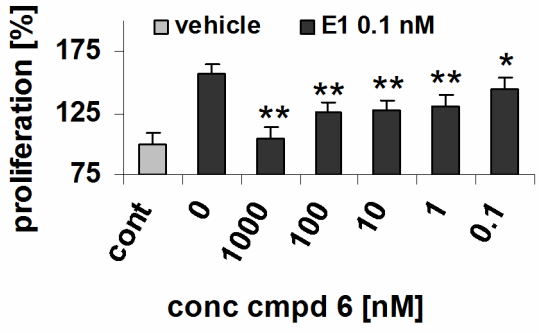


Figure 2: Concentration dependent inhibition of E1-stimulated cell growth for compound 6 in T47D cells. Grey bar represents the vehicle treated control. Black bars represent E1-treated cells. 6 was added at different concentrations ranging from 0.1 nM to 1000 nM. Cells were incubated with the respective additives for eight days without passage. Medium was changed every two to three days. Vehicle = ethanol, *p < 0.05, ** p < 0.003.

The ability of the inhibitors to reduce estrogen stimulated cell growth was evaluated in a proliferation assay using T47D cells (Fig. 3). The compounds should not stimulate or reduce cell proliferation *per se*, because this finding would indicate estrogenic or toxic effects. In

contrast, selective 17βHSD1-inhibitors should reduce stimulation when added simultaneously with E1, as they inhibit E1-conversion to E2. Consequently, the smaller amount of highly active E2 in the cells should result in a decrease of cell growth stimulation. In case of simultaneous addition of E2 and 17βHSD1-inhibitor, E2-stimulated cell proliferation should not be prevented, provided that the inhibitor does not exert antiestrogenic effects at the receptor level.

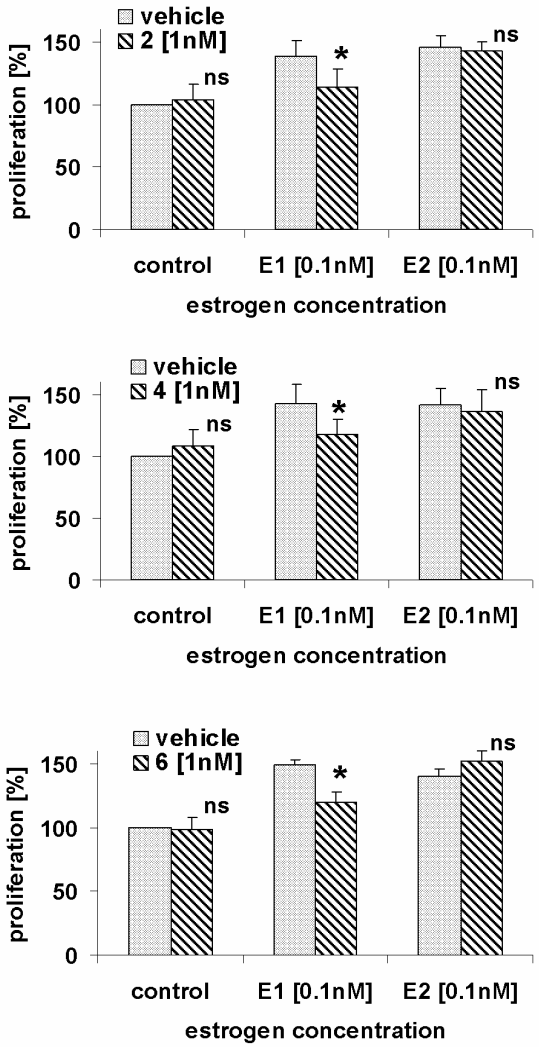


Figure 3: Effects of compounds 2, 4, and 6 on estrogen-stimulated cell proliferation in T47D. Cells were grown in phenol red-free RPMI 1640 medium supplemented with 5% stripped FCS before and during treatment. Proliferation was stimulated with E1 or E2 at a dose of 0.1 nM, respectively. Compounds 2, 4, and 6 were added at a concentration of 1 nM in presence or absence of E1 or E2. Cells were incubated with the respective additives for eight days without passage. Medium was changed every two to three days. Vehicle = ethanol, *p < 0.05, ns p > 0.05. Each panel represents the mean of at least three independent experiments.

Compounds **2**, **4**, and **6** were evaluated in the proliferation assay as representatives of 1,4-disubstituted arenes (**2**), 2,4-disubstituted thiophenes (**4**), and 2,5-disubstituted thiophenes (**6**). Experiments were conducted in phenol red-free medium supplemented with 5 % of stripped FCS. E1 or E2 was added in combination with 17βHSD1-inhibitor or alone. Inhibitors were added at a concentration of 1 nM in presence or absence of 0.1 nM of E1 or E2, respectively.
E1 and E2 stimulated cell proliferation to the same extent. None of the inhibitors caused any significant alteration of cell proliferation when added at 1 nM without estrogen. Even at concentrations of 100 nM, inhibitors did not show any significant reduction or stimulation of basal cell growth (data not shown). This result was expected regarding the determined RBA-values and shows that the inhibitors do not act as estrogens at the applied concentrations.
Simultaneous treatment of the cells with E1 and 17βHSD1-inhibitor led to significant reduction of cell proliferation compared to the treatment with E1 alone. The finding, that all inhibitors

were effective at the same concentration is in accordance with the comparable IC_{50}-values determined in the cellular inhibition assay. In contrast, none of the three inhibitors influenced E2-stimulated cell growth. The finding that 17βHSD1-inhibitors are able to selectively reduce E1-mediated cell proliferation without influencing E2-stimulation clearly demonstrates that this effect is caused by 17βHSD1-inhibiton and not by receptor blockade.

Discussion and Conclusion

The present paper shows that selective 17βHSD1-inhibitors are able to reduce the stimulation of proliferation induced by E1-addition to T47D cells.
This cell line was chosen because intracellular enzyme concentrations especially 17βHSD1 and 17βHSD2 are similar to those found in estrogen dependent diseases (Day *et al*., 2006). T47D cells express ERs and show estrogen dependent proliferation (Horwitz, 1981; Malet *et al*., 1991). The enhanced ratio of 17βHSD1 to 17βHSD2 as it is observed in T47D cells is very similar to that seen in diseased tissue. This elevated proportion leads to a pronounced intracellular E2-production from E1.
Estrogen responsive growth of the obtained cells was confirmed by addition of E1 or E2 at several concentrations. The stimulation of cell proliferation was dose dependent with both estrogens. The finding that the weaker estrogen E1 stimulates cell proliferation to the same extent as does E2 when given at the same concentration can be explained by a rapid intracellular conversion of E1 into E2. The high ratio of 17βHSD1/ 17βHSD2 leads to an estrogen balance in the cells which is characterised by an excess of E2. Hence, the formed E2 is responsible for ER-activation and consequently for enhanced cell growth in the E1-treated cells confirming the relevance of T47D cells as a model for estrogen dependent diseases.
Since cell proliferation was not altered by application of 17βHSD1-inhibitors alone even at a concentration of 100 nM, we conclude that our compounds do neither activate the ER nor show unspecific reduction of proliferation by for example toxic effects.
As found in case of 4-hydroxytamoxifen, blockade of the ERs would reduce E2-mediated cell proliferation. When added in combination with E2, the inhibitors did not reduce hormone-mediated cell proliferation. Thus, it can be excluded that the compounds reduce cell proliferation via ER-blockade at the given concentration. Considering these findings, the tested 17βHSD1-inhibitors do not interfere with the ERs at the concentrations applied in the proliferation assay as was expected from the receptor assays.
The stimulation achieved by E1-addition could be reduced by simultaneous application of different non-steroidal 17βHSD1-inhibitors. This observation is in agreement with experiments of Day *et al*. (2008) and Laplante *et al.* (2008), who describe significant reduction of E1-induced proliferation by steroidal 17βHSD1-inhibitors in a cell culture experiment. Both groups use higher inhibitor concentrations than 1 nM as used in our experiment: 500 nM and 100 nM, respectively. Moreover, in case of Laplante *et al.,* the inhibitor caused a stimulation of cell proliferation when added at a concentration of 10 nM to the cells, suggesting estrogenic activity. In the experiment of Day *et al.,* the used inhibitor reduced the E2-stimulated cell proliferation at the applied concentration. To our knowledge we are the first to show selective inhibition of E1-stimulated proliferation with non-steroidal inhibitors at very low concentrations without affecting the ER.
Inhibition of 17βHSD1 is an innovative, novel concept which might be superior or at least adequate to existing endocrine treatment modalities. One current therapy option is the application of SERMs like e.g. tamoxifen, which block the ER. It is successfully administered in the majority of ER+ tumors. But SERMs have several drawbacks. At first they show estrogenic effects in the uterus and therefore can evoke endometrial carcinomas (Saadat *et al*., 2007). Secondly, they only antagonize estrogenic effects. The proliferation-stimulating agent E2 is still present in the diseased tissue. By antagonizing the estrogenic effects unselectively in the whole body, SERMs cause side effects (Abdulhaq and Geyer, 2008). Moreover, in long term treatment resistance development occurs often. Therefore, there is need for a therapy change after a certain time (Gradishar, 2004). Consequently, SERMs are an effective but not an optimal treatment of breast cancer.

For the reduction of E2 in the diseased tissue there are different possibilties. One can use GnRH-analogues, which suppress estrogen production at ovarian level. Since they totally reduce the estrogens in the whole body, they cause strong side effects.
Another possibility is administration of aromatase inhibitors, which is normally applied in postmenopausal women. In reproductive age, usually the central feedback mechanism has to be suppressed by additional application of GnRH-analogues. The advantages of aromatase inhibitors are effectiveness, low risk of estrogenic effects and inhibition of the estrogen synthesis, which is a more causal approach than SERM-treatment. But the total blockage of estrogen synthesis is a reason for severe side effects (Abdulhaq and Geyer, 2008; Perez, 2007). As described for the SERMs there is also the risk of resistance development, which is an additional drawback.
We conclude that nowadays, we have effective medical treatments in hands but they are not the optimal therapy. Therefore, we think that new therapeutical approaches have to be considered. For this purpose, we propose 17βHSD1-inhibitors. In first animal studies, they show promising results in breast cancer treatment. 17βHSD1-inhibitors selectively suppress the biosynthesis of the proliferation-stimulating E2. Since the enzyme shows a tissue specific expression pattern and in case of breast cancer even an overexpression, a better selectivity of E2-reduction will be reached. Therefore, 17βHSD1-inhibitors should show fewer side effects than current therapeutic agents and may be less problematic in treatment of premenopausal women. For these reasons, we consider 17βHSD1-inhibitors as potential further option in the treatment of breast cancer.
In the present paper, we focus on the indication of breast cancer but 17βHSD1-inhibitors may also have a benefit in endometriosis, which is also an estrogen dependent disease. In endometriosis 17βHSD1 was also found to be overexpressed (Šmuc *et al*., 2006) while expression of 17βHSD2 seems to be reduced (Bulun *et al*., 2006). Therefore, in endometriosis there is an environment present which favours E2-production over E2-inactivation. This enhanced ratio of 17βHSD1/ 17βHSD2 may be regulated by 17βHSD1-inhibitors. Therefore, 17βHSD1-inhibitors may also be valuable for the treatment of endometriosis.
In summary, we were able to show, that selective non-steroidal 17βHSD1-inhibitors are appropriate to reduce E1-mediated T47D cell proliferation, while E2-induced stimulation was not affected. This finding validates 17βHSD1-inhibition as an effective strategy in inhibition of uncontrolled cell proliferation in estrogen dependent diseases. Consequently, 17βHSD1-inhibitors should be considered as an additional therapy option in breast cancer and endometriosis.

Acknowledgement

We are grateful to the Deutsche Forschungsgemeinschaft (HA1315/8-1) for financial support. We thank Beate Geiger for her help in performing the enzyme inhibition tests (17βHSD1, 17βHSD2, ERα and ERβ) and Diana Schäfer for performing cytotoxicity assays. Patricia Kruchten is grateful to the European Postgraduate School 532 (DFG) for a scholarship.

References

Abdulhaq, H., Geyer, C.: Safety of adjuvant endocrine therapy in postmenopausal women with breast cancer. *Am. J. Clin. Onc.* 31 (2008) 595-605.
Allan, G.M., Vicker, N., Lawrence, H.R., Tutill, H.J., Day, J.M., Huchet, M., Ferrandis, E., Reed, M.J., Purohit, A., Potter, B.V.: Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem.* 16 (2008) 4438-4456.
Bey, E., Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Werth, R., Oster, A., Algül, O., Neugebauer, A., Hartmann, R.W.: Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* 16 (2008a) 6423-6435.
Bey, E., Marchais-Oberwinkler, S., Werth, R., Negri, M., Al-Soud, Y., Kruchten, P., Oster, A., Frotscher, M., Birk, B., Hartmann, R.W.: Design, synthesis, biological evaluation and pharmacokinetics of bis(hydroxyphenyl)substituted azoles, thiophenes, benzenes and

azabenzenes as potent and selective non-steroidal inhibitors of 17β-hydroxysteroid-dehydrogenase type 1 (17β-HSD1). *J. Med. Chem.* 51 (2008b) 6725-6739.

Brožic, P., Lanišnik Rižner, T., Gobec, S.: Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* 15 (2008) 137-150 and references cited therein.

Bulun, S.E., Cheng, Y.H., Yin, P., Imir, G., Utsunomiya, H., Attar, E., Innes, J., Julie Kim, J.: Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Mol. Cell. Endocrinol.* 248 (2006) 94-103.

Bulun, S.E., Zeitoun, K.M., Takayama, K., Sasano, H.: Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance. *J. Mol. Endocrinol.* 25 (2000) 35-42.

Cauley, J.A., Lucas, F.L., Kuller, L.H., Stone, K., Browner, W., Cummings, S.R.: Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group. *Ann. Intern. Med.* 130 (1999) 270-277.

Day, J.M., Foster, P.A., Tutill, H.J., Parsons, M.F., Newman, S.P., Chander, S.K., Allan, G.M., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J., Purohit, A.: 17beta-hydroxysteroid dehydrogenase type 1, and not type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* 122 (2008) 1931-1940.

Day, J.M., Tutill, H.J., Newman, S.P., Purohit, A., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J.: 17Beta-hydroxysteroid dehydrogenase Type 1 and Type 2: association between mRNA expression and activity in cell lines. *Mol. Cell. Endocrinol.* 248 (2006) 246-249.

Denizot, F., Lang, R.: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89 (1986) 271-277.

Dizerega, G.S., Barber, D.L., Hodgen, G.D.: Endometriosis: role of ovarian steroids in initiation, maintenance, and suppression. *Fertil. Steril.* 33 (1980) 649-653.

Duncan, L.J., Reed, M.J.: The role and proposed mechanism by which oestradiol 17 beta-hydroxysteroid dehydrogenase regulates breast tumour oestrogen concentrations. *J. Steroid Biochem. Mol. Biol.* 55 (1995) 565-572.

Frotscher, M., Ziegler, E., Marchais-Oberwinkler, S., Kruchten, P., Neugebauer, A., Fetzer, L., Scherer, C., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Design, synthesis, and biological evaluation of (hydroxyphenyl)naphthalene and -quinoline derivatives: Potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* 51 (2008) 2158-2169.

Gradishar, W.J.: Tamoxifen--what next? *The oncologist* 9 (2004) 378-384.

Gunnarsson, C., Hellqvist, E., Stål, O.: 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* 92 (2005) 547-552.

Gunnarsson, C., Olsson, B.M., Stål, O.: Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* 61 (2001) 8448-8451.

Horwitz, K.B.: Is a functional estrogen receptor always required for progesterone receptor induction in breast cancer? *J. Steroid Biochem.* 15 (1981) 209-217.

Karkola, S., Lilienkampf, A., Wähälä, K.: A 3D QSAR model of 17beta-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core applying molecular dynamics simulations and ligand-protein docking. *ChemMedChem* 3 (2008) 461-472.

Krazeisen, A., Breitling, R., Imai, K., Fritz, S., Möller, G., Adamski, J.: Determination of cDNA, gene structure and chromosomal localization of the novel human 17beta-hydroxysteroid dehydrogenase type 7(1). *FEBS Lett.* 460 (1999) 373-379.

Kruchten, P., Werth, R., Marchais-Oberwinkler, S., Frotscher, M., Hartmann, R.W.: Development of a biological screening system for the evaluation of highly active and selective 17β-HSD1-inhibitors as potential therapeutics. *Mol. Cell. Endocrinol.* (2008) doi:10.1016/j.mce.2008.1009.1035.

Laplante, Y., Cadot, C., Fournier, M.A., Poirier, D.: Estradiol and estrone C-16 derivatives as inhibitors of type 1 17beta-hydroxysteroid dehydrogenase: blocking of ER+ breast cancer cell proliferation induced by estrone. *Bioorg. Med. Chem.* 16 (2008) 1849-1860.

Luu-The, V., Tremblay, P., Labrie, F.: Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women. *Mol. Endocrinol.* (Baltimore, Md 20 (2006) 437-443.
Luu-The, V., Zhang, Y., Poirier, D., Labrie, F.: Characteristics of human types 1, 2 and 3 17 beta-hydroxysteroid dehydrogenase activities: oxidation/reduction and inhibition. *J. Steroid Biochem. Mol. Biol.* 55 (1995) 581-587.
Malet, C., Vacca, A., Kuttenn, F., Mauvais-Jarvis, P.: 17 beta-estradiol dehydrogenase (E2DH) activity in T47D cells. *J. Steroid Biochem. Mol. Biol.* 39 (1991) 769-775.
Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Ziegler, E., Neugebauer, A., Bhoga, U., Bey, E., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Substituted 6-phenyl-2-naphthols. Potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation, and pharmacokinetics. *J. Med. Chem.* 51 (2008) 4685-4698.
Marijanovic, Z., Laubner, D., Möller, G., Gege, C., Husen, B., Adamski, J., Breitling, R.: Closing the gap: identification of human 3-ketosteroid reductase, the last unknown enzyme of mammalian cholesterol biosynthesis. *Mol. Endocrinol.* Baltimore, Md 17 (2003) 1715-1725.
Messinger, J., Hirvelä, L., Husen, B., Kangas, L., Koskimies, P., Pentikäinen, O., Saarenketo, P., Thole, H.: New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* 248 (2006) 192-198.
Miller, W.R., Bartlett, J.M., Canney, P., Verrill, M.: Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. and Treat.* 103 (2007) 149-160.
Miyoshi, Y., Ando, A., Shiba, E., Taguchi, T., Tamaki, Y., Noguchi, S.: Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* 94 (2001) 685-689.
Oduwole, O.O., Li, Y., Isomaa, V.V., Mäntyniemi, A., Pulkka, A.E., Soini, Y., Vihko, P.T.: 17beta-hydroxysteroid dehydrogenase type 1 is an independent prognostic marker in breast cancer. *Cancer Res.* 64 (2004) 7604-7609.
Ohnesorg, T., Adamski, J.: Analysis of the 5' flanking regions of human and murine HSD17B7: identification of a cholesterol dependent enhancer region. *Mol. Cell. Endocrinol.* 248 (2006) 164-167.
Osborne, C.K., Bolan, G., Monaco, M.E., Lippman, M.E.: Hormone responsive human breast cancer in long-term tissue culture: effect of insulin. *Proc. Natl. Acad. Sci. U S A* 73 (1976) 4536-4540.
Perez, E.A.: Safety of aromatase inhibitors in the adjuvant setting. *Breast Cancer Res. Treat.* 105 Suppl 1 (2007) 75-89.
Russo, J., Fernandez, S.V., Russo, P.A., Fernbaugh, R., Sheriff, F.S., Lareef, H.M., Garber, J., Russo, I.H.: 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB J.* 20 (2006) 1622-1634.
Saadat, M., Truong, P.T., Kader, H.A., Speers, C.H., Berthelet, E., McMurtrie, E., Olivotto, I.A.: Outcomes in patients with primary breast cancer and a subsequent diagnosis of endometrial cancer: comparison of cohorts treated with and without tamoxifen. *Cancer* 110 (2007) 31-37.
Sam, K.M., Boivin, R.P., Tremblay, M.R., Auger, S., Poirier, D.: C16 and C17 derivatives of estradiol as inhibitors of 17 beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug Des. Discov.* 15 (1997) 157-180.
Sasano, H., Suzuki, T., Nakata, T., Moriya, T.: New development in intracrinology of breast carcinoma. *Breast Cancer* 13 (2006) 129-136.
Šmuc, T., Pucelj, M.R., Šinkovec, J., Husen, B., Thole, H., Lanišnik Rižner, T.: Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* 23 (2007) 105-111.
Šmuc, T., Rupreht, R., Šinkovec, J., Adamski, J., Rižner, T.L.: Expression analysis of estrogen-metabolizing enzymes in human endometrial cancer. *Mol. Cell. Endocrinol.* 248 (2006) 114-117.
Suzuki, T., Moriya, T., Ariga, N., Kaneko, C., Kanazawa, M., Sasano, H.: 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* 82 (2000) 518-523.

Thomas, D.B.: Do hormones cause breast cancer? *Cancer* 53 (1984) 595-604.
Vihko, P., Härkönen, P., Oduwole, O., Törn, S., Kurkela, R., Porvari, K., Pulkka, A., Isomaa, V.: 17 beta-hydroxysteroid dehydrogenases and cancers. *J. Steroid Biochem. Mol. Biol.* 83 (2003) 119-122.
Vihko, P., Härkönen, P., Soronen, P., Törn, S., Herrala, A., Kurkela, R., Pulkka, A., Oduwole, O., Isomaa, V.: 17 beta-hydroxysteroid dehydrogenases--their role in pathophysiology. *Mol. Cell. Endocrinol.* 215 (2004) 83-88.
Vihko, P., Isomaa, V., Ghosh, D.: Structure and function of 17beta-hydroxysteroid dehydrogenase type 1 and type 2. *Mol. Cell. Endocrinol.* 171 (2001) 71-76.
Zeitoun, K., Takayama, K., Sasano, H., Suzuki, T., Moghrabi, N., Andersson, S., Johns, A., Meng, L., Putman, M., Carr, B., Bulun, S.E.: Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol. *J. Clin. Endocrinol. Metab.* 83 (1998) 4474-4480.
Zimmermann, J., Liebl, R., von Angerer, E.: 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J. Steroid Biochem. Mol. Biol.* 94 (2005) 57-66.

4 DISKUSSION UND AUSBLICK

4.1 Vergleichende Bewertung innovativer Ansätze zur Therapie estrogenabhängiger Erkrankungen

Wie in Kapitel 1.3 dargelegt, besteht nach wie vor die Notwendigkeit, neue Ansätze in der Therapie estrogenabhängiger Erkrankungen zu verfolgen, da die etablierten endokrinen Therapeutika zwar wirksam sind, jedoch systemisch die Estrogenbiosynthese bzw. -wirkung unterdrücken und somit nicht die optimale Behandlung darstellen. Unter diesem Aspekt werden nicht nur Inhibitoren der 17βHSD1 sondern auch der Sulfatase entwickelt. Auch ERβ, 17βHSD7 und 12 werden als potentielle *Targets* in der Behandlung estrogenabhängiger Erkrankungen propagiert.

4.1.1 Sulfataseinhibitoren

Die Sulfatase (Estronsulfatase, Steroidsulfatase, Arylsulfatase C) ist ein Enzym, welches Steroidhormone aus ihrer inaktiven Transportform, die im Blut größtenteils vorliegt, wieder freisetzt. Durch Abspaltung des in der Transportform vorhandenen 3-Sulfatrestes entstehen erneut die ursprünglichen Steroide (Pasqualini *et al.*, 1989). Dabei steht die Freisetzung von E1 aus E1-sulfat (E1-S) und die von DHEA aus DHEA-sulfat (DHEA-S) im Vordergrund, da im Blut vorwiegend E1-S und DHEA-S vorhanden sind (Pasqualini *et al.*, 1996).

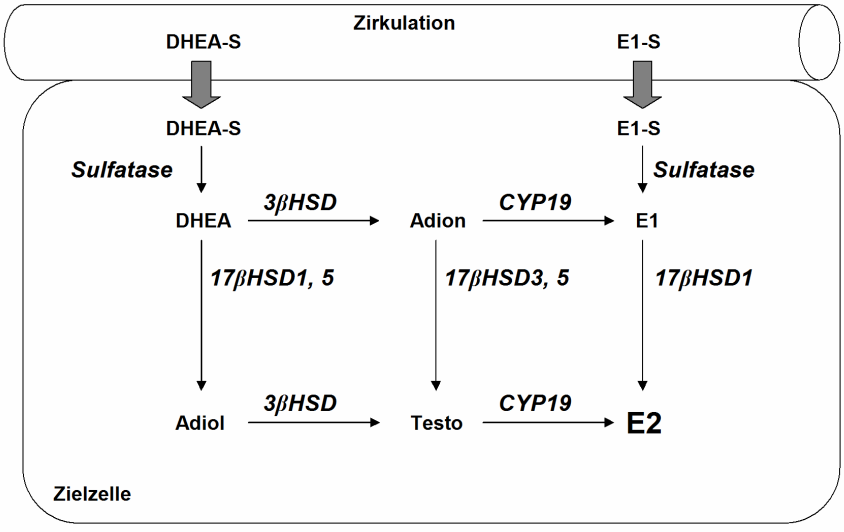


Abbildung 14: Lokale Darstellung von E2 aus den Transportformen DHEA-S und E1-S.

DHEA kann durch die 3βHSD zum Adion und durch CYP19 weiterhin zu E1 umgesetzt werden. Alternativ kann DHEA auch durch die 17βHSD1 bzw. 5 zum Adiol und erst

nachfolgend durch 3βHSD und CYP19 zum E2 umgesetzt werden. E1 kann im Zielgewebe unter Einfluß der 17βHSD1 direkt zu E2 reduziert werden (Abbildung 14).

Das bedeutet, dass zur Darstellung des aktiven Hormons E2 im Zielgewebe in jedem Falle der Einfluß einer reduktiven 17βHSD benötigt wird. Da in estrogenabhängigen Erkrankungen die 17βHSD1 häufig stark überexprimiert ist (Gunnarsson *et al.*, 2001; Vihko *et al.*, 2003), dürfte ihr Einfluß auf die E2-Darstellung entscheidend sein.

Die Bedeutung der Sulfatase für die Entstehung und Progression von Brustkrebs wird durch unterschiedliche Studien deutlich. Die Sulfatase-mRNA-Expression korreliert negativ mit dem krankheitsfreien Überlebensintervall nach der Diagnose Brustkrebs (Miyoshi *et al.*, 2003; Utsumi *et al.*, 1999). Sie kann also als prognostischer Marker angesehen werden. Diese Feststellung legt den Schluß nahe, dass eine erhöhte Sulfataseexpression zu einem hohen Estrogenspiegel im Tumor führt, welcher das Wachstum stimuliert. Darüberhinaus wurde gefunden, dass die Sulfatase in Brustkrebsgewebe überexprimiert ist (Utsumi *et al.*, 2000) und 50 bis 200fach aktiver ist als die Aromatase (Pasqualini *et al.*, 1996). Deshalb wird propagiert, dass das im Brustkrebsgewebe vorhandene E1 größtenteils durch die Sulfatase bereitgestellt wird. Dem gegenüber steht jedoch die Tatsache, dass die CYP19-Aktivität zur Biosynthese der Estrogene unerlässlich ist, da nur durch die Aromatisierung des A-Rings der Androgene Estrogene gebildet werden können. Die Sulfatase moduliert also lediglich die Aktivität der bereits gebildeten Estrogene. Durch Hemmung der Sulfatase erhofft man sich jedoch, speziell das überexprimierte hochaktive Enzym im erkrankten Gewebe zu beeinflussen und damit eine nebenwirkungsärmere Therapieoption bieten zu können als es Aromataseinhibitoren sind.

Ein sich vom Coumarin ableitender Inhibitor der Sulfatase (Reed *et al.*, 2005) STX64 (667COUMATE) wurde bereits erfolgreich im Krankheitsmodell für Brustkrebs eingesetzt (Purohit *et al.*, 2000). Hierbei wurde in Ratten ein Brusttumor induziert, dessen Wachstum nach Ovariektomie mit E1-S stimuliert wurde. Nach einer peroralen Applikation des Inhibitors (10mg/kg/d) wurde die Lebersulfatase-Aktivität um 93 % gehemmt. Das Tumorvolumen wurde im Vergleich zur Kontrolle signifikant vermindert.

Auch in einer klinischen Studie der Phase I wurde STX64 erfolgreich eingesetzt (Stanway *et al.*, 2006). Am Ende der Behandlungsperiode war die Sulfataseaktivität im Brustkrebsgewebe zu 99 % gehemmt. In vier Patienten wurde eine Stabilisierung der Erkrankung für 2,75 bis 7 Monate erreicht. Ebenfalls wurde eine Absenkung der Plasmaspiegel an E1, E2, Adiol, DHEA, Adion und Testosteron festgestellt. Eine mögliche Akkumulation von E1-S im Gewebe, welche insbesondere vor dem Hintergrund möglich ist, dass die Sulfotransferase ebenfalls im Mammakarzinomgewebe exprimiert ist (Pasqualini *et al.*, 1992), wurde nicht untersucht. Es wurde jedoch deutlich, dass nicht nur Estrogene, sondern auch Androgene beeinflusst werden. Daraus lassen sich auch einige der in der Studie beobachteten Nebenwirkungen wie Hitzewallungen, Stimmungsschwankungen oder Kopfschmerzen erklären. Dies zeigt das

Nebenwirkungspotential der Sulfataseinhibitoren auf, welches durch die alternative Hemmung der 17βHSD1 möglicherweise umgangen werden kann, da hier selektiv in den letzten Schritt der E2-Biosynthese eingegriffen wird.

Zusammengefasst stellt die Inhibition des Sulfataseweges einen vielversprechenden Ansatz zur endokrinen Therapie von Brustkrebs dar. Auch die Anwendung bei Endometriose (Purohit *et al.*, 2008) oder beim Endometriumkarzinom (Foster *et al.*, 2008) wurde kürzlich vorgeschlagen. Um nebenwirkungsärmere Therapieoptionen zu entwickeln, sollte der Eingriff in die Darstellung des E2 jedoch möglichst spät im Syntheseweg erfolgen. Somit sollten Inhibitoren der Sulfatase nebenwirkungsärmer sein als Aromatasehemmstoffe. Optimal wäre jedoch der Eingriff in den letzten Schritt der E2-Synthese durch Hemmung der 17βHSD1.

4.1.2 ERβ-Agonisten

Eine etablierte Therapie des estrogenabhängigen Brustkrebses sind die ERα-Antagonisten. Da sie effektiv in der Behandlung sind, entstand die Idee, auch den ERβ als *Target* zur Behandluing estrogenabhängiger Erkrankungen zu nutzen. Aufgrund der beobachteten antiproliferativen Effekte von ERβ (Ström *et al.*, 2004; Weihua *et al.*, 2000) wurde propagiert, dass ERβ-Agonisten möglicherweise positive Effekte bei estrogenabhängigen Erkrankungen haben könnten. Durch Heterodimerisierung mit dem ERα soll dessen proliferationsfördernde Wirkung abgefangen werden (Lindberg *et al.*, 2003; Matthews und Gustafsson, 2003).

In Zellkulturexperimenten wurde gefunden, dass der ERβ bei Koexpression mit ERα antiproliferativ wirkt. So wurde in der ERα-exprimierenden Brustkrebszelllinie T47D tetrazyklinabhängig ERβ exprimiert. In Zellen, in denen die ERβ-Expression unterdrückt war, induzierte E2 eine Zellproliferation, während in ERβ exprimierenden Zellen keine Wachstumsstimulation durch E2 beobachtet wurde (Covaleda *et al.*, 2008). Darüberhinaus führt ein erhöhtes ERα/ ERβ-Verhältnis, wie es in vielen Brusttumoren gefunden wurde, zur verstärkten Proliferation des Gewebes unter Einfluß eines ERα-Agonisten (Covaleda *et al.*, 2008). Deshalb wurde vorgeschlagen, dieses erhöhte Wachstum durch ERβ-Agonisten zu unterdrücken (Saji *et al.*, 2005).

Weiterhin wurde gefunden, dass ein erhöhtes Verhältnis von ERβ zu ERα offenbar mit einer niedrigeren Wahrscheinlichkeit zur malignen Transformation im Brustgewebe assoziiert ist (Shaaban *et al.*, 2003). Damit könnte der ERβ nicht nur bei der Progression, sondern auch bei der Initiation von Brustkrebs eine Rolle spielen. In beiden Fällen scheint jedoch die relative Expression zum ERα entscheidend zu sein.

Weniger gut kann die Wirkung von ERβ-Agonisten in Geweben beurteilt werden, welche ERα nicht exprimieren. Hier ist die genaue Funktion von ERβ weiterhin unklar. Damit können die Folgen einer ERβ-Aktivierung, welche potentielle Nebenwirkungen darstellen, nur schwer vorhergesagt werden.

Experimente an knockout-Mäusen zeigten, dass eine verringerte Expression von ERβ vor Osteoporose schützt (Ke *et al.*, 2002). Dies deutet auf den Einfluß des Rezeptors auf das Knochenwachstum hin und lässt vermuten, dass eine potentielle Nebenwirkung von ERβ-Agonisten die Osteoporose ist.

Außerdem wird durch den Agonismus am ERβ nicht die eigentliche pathophysiologische Veränderung angesprochen, sondern lediglich die verstärkte E2-Wirkung auf hohem Niveau abgefangen.

Zusammengefasst bieten ERβ-Agonisten einen interessanten Ansatz zur Therapie estrogenabhängiger Erkrankungen. Es sind jedoch weitere Untersuchungen nötig, um beurteilen zu können, ob dieser Ansatz effizient und hinsichtlich der Nebenwirkungen vertretbar ist.

4.1.3 17βHSD7 und 12 als potentielle Targets bei estrogenabhängigen Erkrankungen

Nach der Identifikation der beiden 17βHSD-Subtypen 7 (Krazeisen *et al.*, 1999) und 12 (Moon und Horton, 2003) wurde in ersten Experimenten zur Charakterisierungen gefunden, dass beide Enzyme zu den reduktiven 17βHSDs gehören. Durch die Untersuchung potentieller steroidaler Substrate wurde gezeigt, dass beide Subtypen in der Lage sind, die Reduktion von E1 zu E2 zu katalysieren (Liu *et al.*, 2005; Luu-The *et al.*, 2006; Peltoketo *et al.*, 1999b). Auch das Expressionsmuster wurde evaluiert. 17βHSD7 und 12 wurden in estrogenabhängigen Geweben detektiert (Liu *et al.*, 2007; Törn *et al.*, 2003). Aus diesen Befunden wurde geschlossen, dass beide Proteine eine wesentliche Rolle spielen bei der E2-Aktivierung.

Weitere Studien an Brustkrebsgewebe zeigten, dass die 17βHSD7 in 47 % der Proben und Typ 12 sogar in 83 % der Proben detektiert wurden. Außerdem war die Expression der 17βHSD12 im Tumorgewebe signifikant erhöht verglichen mit gesundem Gewebe (Song *et al.*, 2006). Deshalb wurde für beide Proteine propagiert, dass sie involviert sind in Entstehung und Fortschreiten estrogenabhängiger Erkrankungen (Liu *et al.*, 2005).

Untersuchungen mit einem breiteren Spektrum an potentiellen Substraten führten dazu, dass auch andere Funktionen der 17βHSD7 und 12 in Betracht gezogen wurden (Blanchard und Luu-The, 2007; Marijanovic *et al.*, 2003). So wurde festgestellt, dass die 17βHSD7 die Umsetzung von Zymosteron zu Zymosterol effizient katalysiert. Darüberhinaus wurde eine intensive Expression in Lebergewebe gefunden (Breitling *et al.*, 2001; Liu *et al.*, 2005). Damit wurde das Enzym der Cholesterolbiosynthese zugeordnet (Ohnesorg und Adamski, 2006; Ohnesorg *et al.*, 2006). Für die 17βHSD12 konnte eine Funktion in der Fettsäuresynthese gefunden werden (Moon und Horton, 2003).

Darüberhinaus wurde durch Untersuchungen an Brustkrebszellen festgestellt, dass die Subtypen 7 und 12 intrazellulär nicht in der Lage sind, E1 effizient zu E2 zu reduzieren (Day *et al.*, 2008). Die gefundene Expression in Brustkrebsgewebe konnte zwar bestätigt werden, jedoch wurde keine Korrelation zur Überlebenszeit der Patienten oder prognostischen Faktoren gefunden

(Jansson, 2008). Kürzlich wurde die 17βHSD12 selbst als prognostischer Faktor in Brustkrebs vorgeschlagen. Hier wurde jedoch ein Zusammenhang mit einer Dysregulation des Fettsäurestoffwechsels gesehen, welche das Tumorwachstum stimuliert (Nagasaki *et al.*, 2009). Zusammengefasst haben die beiden 17βHSD Subtypen 7 und 12 offenbar keinen relevanten Einfluß auf die E2-Produktion in estrogenabhängigen Erkrankungen. Sie können der Cholesterol- bzw. Fettsäuresynthese zugeordnet werden. Ob eine Beeinflussung dieser Stoffwechselwege via Inhibition der 17βHSD7 oder 12 für die Therapie estrogenabhängiger Erkrankungen geeignet ist, muß in weitern Studien untersucht werden.

4.2 Bewertung des Testsystems

4.2.1 Zellfreier Screeningassay für 17βHSD1-Inhibitoren

Zur Evaluierung der inhibtorischen Aktivität der potentiellen 17βHSD1-Hemmstoffe wird im etablierten Screeningsystem Enzym aus humaner Plazenta aufgereinigt. Damit entfallen eine aufwendige Transfektion geeigneter Zellen sowie deren zeit- und kostenintensive Kultur. Die Stabilität der erhaltenen Präparation ist überraschend hoch, so dass die Enzymaktivität auch nach einem Jahr noch gewährleistet ist.

Durch Aufarbeitung aus humanem Gewebe wird natives Enzym erhalten, welches jedoch im Gemisch mit weiteren cytosolischen Enzymen vorliegt. Dies ist insbesondere dann problematisch, wenn weitere Enzyme enthalten sein können, welche dieselbe Reaktion katalysieren können. Im Falle der Plazentapräparation sind also die 17βHSD-Subtypen 7 und 12 von Interesse, welche hier in geringerem Ausmaß exprimiert werden. Wie aus Tabelle 1 hervorgeht, ist die 17βHSD7 ein zellmembranständiges Enzym, während die 17βHSD12 microsomal lokalisiert ist. Demgegenüber ist die 17βHSD1 löslich und liegt damit im Cytosol vor. Durch fraktionierte Zentrifugation werden während der Aufarbeitung zunächst Zelldebris und schwere Zellbestandteile abgetrennt. Damit wird die 17βHSD7 aus dem Gemisch entfernt. In einem Ultrazentrifugationsschritt werden nachfolgend Cytosol und Microsomen voneinander getrennt. Da nur mit dem Überstand weitergearbeitet wird, stellt auch die 17βHSD12, welche sich in den Microsomen befindet, kein Problem im etablierten Test dar.

Die Messung der 17βHSD1-Aktivität erfolgt mittels Radiodetektion. Anders als im von Langer und Engel beschriebenen Test (Langer und Engel, 1958), in welchem der Cofaktorumsatz photometrisch gemessen wird, ist somit sichergestellt, dass spezifisch die Reduktion von E1 zu E2 betrachtet wird. Damit spielen Enzyme, welche andere NADPH-abhängige Reaktionen katalysieren, keine Rolle.

Die etablierte Methode ist geeignet, schnell eine große Zahl an Substanzen auf ihre inhibitorische Aktivität an 17βHSD1 zu prüfen, da die Quantifizierung mittels HPLC-Separation mit anschließender Radiodetektion erfolgt. Durch die mögliche Determinierung von

IC_{50}-Werten können zuverlässig Struktur-Wirkungs-Beziehungen abgeleitet werden. Über die intrazelluläre Wirksamkeit können jedoch keine Aussagen getroffen werden. Um beispielsweise Membrangängigkeit, Adsorption oder intrazellulären Metabolismus zu bestimmen sind weitere Tests nötig.

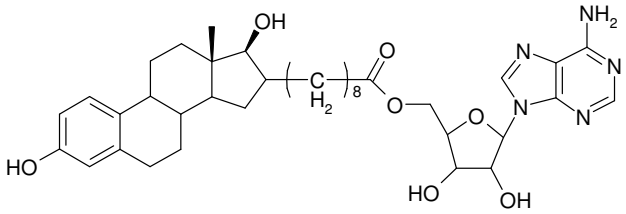


Abbildung 15: Referenzverbindung für den 17βHSD1-Screeningassay.

Die Evaluierung eines bereits publizierten 17βHSD1-Inhibitors (EM-1745, Abbildung 15) aus der Arbeitsgruppe von Prof. Poirier lieferte einen IC_{50}-Wert von 31 nM (Literaturwert: IC_{50} = 52 nM) (Poirier, 2003; Qiu *et al.*, 2002). Somit wurde auch durch Validierung mit externen Proben gezeigt, dass die zuverlässige Identifikation von 17βHSD1-Inhibitoren mit Hilfe des Screeningassays möglich ist.

4.2.2 Relevanz der Selektivitätstestung gegenüber 17βHSD2

Ein wichtiger Aspekt der 17βHSD1-Inhibtioren ist die Selektivität gegenüber Enzymen, welche in der Lage sind, E2 zu inaktivieren. Auch unter der Voraussetzung, dass die E2-Biosynthese im erkrankten Gewebe durch die Anwendung der Hemmstoffe stark vermindert ist, muss gewährleistet sein, dass auch von außen eindringendes E2 (z.B. Sulfataseweg, vgl. 4.1.1) inaktiviert werden kann. Zudem würde eine Hemmung der 17βHSD2 zusätzliche unerwünschte Wirkungen in anderen Geweben hervorrufen. Daher ist es wichtig, über einen zellfreien Inhibitionstest zu verfügen, um auch hier SARs ableiten zu können, welche in das weitere Inhibitordesign eingehen.

Auch für diesen Test wurde eine Gewebepräparation aus humaner Plazenta verwendet, da die 17βHSD2 hier stark exprimiert ist. Eine Trennung vom Subtyp 1 durch Ultrazentrifugation ist möglich, da die 17βHSD2 microsomal lokalisiert ist, während die 17βHSD1 ein lösliches Enzym ist (Tabelle 2).

Problematischer ist die Expression der 17βHSD4 im plazentaren Gewebe (Adamski *et al.*, 1995). Aufgrund ihrer peroxisomalen Expression (Normand *et al.*, 1995; van Veldhoven *et al.*, 1996) ist eine vollständige Trennung der Subtypen durch Zentrifugation nicht möglich. Daher stellt sich die Frage, inwieweit der zu etablierende Test durch die Präsenz der 17βHSD4 beeinflusst wird. Da die 17βHSD2 in stärkerem Ausmaß als die 17βHSD4 in plazentarem Gewebe exprimiert wird und außerdem mit einem K_m-Wert von 0,21 µM (Wu *et al.*, 1993) eine höhere Affinität zu E2 hat als die 17βHSD4 mit K_m = 0,81 µM (Adamski *et al.*, 1995), wurde zunächst die erhaltene microsomale Fraktion für die weiteren Versuche verwendet. In den

durchgeführten kinetischen Untersuchungen konnten keine Hinweise auf die Interferenz der 17βHSD4 gefunden werden: Der erhaltene K_m-Wert von 403 nM ± 80 nM stimmt gut mit dem von Puranen beschriebenen Wert (390 nM ± 90 nM) überein, welcher an in HEK293-Zellen exprimiertem Enzym bestimmt wurde (Puranen *et al.*, 1999). Desweitern konnte die Reduktion von E2 in der Microsomenfraktion vollständig durch Testosteron gehemmt werden. Da die 17βHSD4 keine Androgene als Substrate akzeptiert (Adamski *et al.*, 1995), sollte sich deren Umsatz auch nicht durch die Zugabe von Testosteron hemmen lassen. Daher ist die aus plazentarem Gewebe aufgereinigte Microsomenfraktion geeignet, um einen Test zur Evaluierung der 17βHSD2-Inhibition aufzubauen.

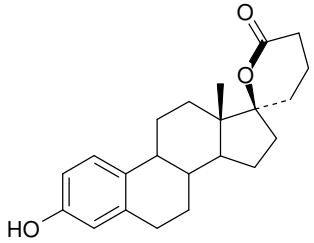


Abbildung 16: Referenzverbindung für den Inhibitionstest an 17βHSD2.

Der etablierte Test wurde nochmals validiert, indem ein bereits publizierter 17βHSD2-Inhibitor getestet wurde (Abbildung 16), dessen Hemmaktivität am Homogenat transfizierter HEK293-Zellen gemessen wurde (Poirier *et al.*, 2001). Mit einem IC_{50}-Wert von 11 nM ± 0,1 nM wurde die Verbindung als hochaktiver Hemmstoff identifiziert. Ein direkter Vergleich mit dem von Poirier erhaltenen Wert (IC_{50} = 90nM) ist jedoch schlecht möglich, da in diesem Fall nicht E1, sondern Adion als Substrat verwendet wurde. Der im etablierten Test erhalten Wert liegt jedoch in derselben Größenordnung wie der Literaturwert, welcher an in HEK-Zellen exprimiertem Enzym bestimmt wurde. Somit kann geschlossen werden, dass der etablierte Test sich eignet, um Hemmstoffe der 17βHSD2 zu identifizieren und damit auch, um die Selektivität der entwickelten 17βHSD1-Inhibitoren zu untersuchen.

4.2.3 Auswahl geeigneter Zellen für die zelluläre Evaluierung der 17βHSD1-Inhibitoren

Nach der Identifizierung hochaktiver 17βHSD1-Inhibitoren ist es entscheidend, zu untersuchen, ob diese Verbindungen auch intrazellulär in der Lage sind, ihre Wirkung zu entfalten. Ein Test an intakten Zellen schließt über die bloße Hemmaktivität hinausgehend auch Aspekte der Pharmakokinetik ein, beispielsweise Permeation durch die Zellmembran, Proteinadsorption oder intrazelluläre Metabolisierung der Substanzen. Um einen solchen Test zu etablieren, muß eine geeignete Zelllinie ausgewählt werden.

Tabelle 4 zeigt eine Auswahl verschiedener Zelllinien, welche prinzipiell für die Evaluation der 17βHSD1-Inhibitoren in Betracht kommen. Im Hinblick auf weiterführende Untersuchungen sollten die Zellen nicht nur die 17βHSD1 exprimieren, sondern auch verschiedene andere

Anforderungen erfüllen. Zunächst sollten die verwendeten Zellen in Relation stehen zur propagierten Indikation. Das heißt idealerweise sollte es sich um Brustkrebs- oder Endometriosezellen handeln. Da zur Zeit keine Endometriosezelllinien kommerziell erhältlich sind, wurden Zervix- und Endometriumkarzinomzellen stattdessen in Betracht gezogen. Außerdem ist eine Reihe von Brustkrebszelllinien zu erwerben, welche ebenfalls in die nähere Auswahl kamen.

Weiterhin ist es wichtig, dass die 17βHSD1 in einem Ausmaß exprimiert wird, welches die verlässliche Detektion der Estrogenkonversion gestattet. Daher wurde neben dem Expressionslevel auch auf die intrazelluläre Aktivität des Enzyms geachtet. Zellen ohne oder mit nur schwacher 17βHSD1-Aktivität kommen zur Etablierung zellulärere Assays also nicht in Frage. Darüberhinaus sollen die 17βHSD-Subtypen 1 und 2 in einem Verhältnis exprimiert werden, welches die Gegebenheiten im erkrankten Gewebe widerspiegelt. Also sollte die 17βHSD1 dominant gegenüber der 17βHSD2 sein.

Um mögliche Auswirkungen der 17βHSD1-Inhibition auf die Zellproliferation untersuchen zu können, wurden ER-Expression sowie estrogenabhängiges Wachstum als weitere Voraussetzungen angesehen.

Tabelle 4: Übersicht der Zelllinien welche potentiell für die Evaluierung von 17βHSD1-Inhibitoren geeignet sind. Dominante 17βHSD-Subtypen sind fett gedruckt. 17βHSD-Subtypen mit schwacher Aktivität sind kursiv gedruckt. Die Expression nicht aufgeführter 17βHSD-Subtypen ist nicht beschrieben.

Karzinom-zelllinie	**Herkunft**	**ER**	*Expression der 17βHSD-Subtypen*	
			Reduktiv	**Oxidativ**
HeLa	Zervix	-	17βHSD1, 17βHSD5, 17βHSD7, 17βHSD12	
Ishikawa	Endometrium	+ kein estrogen-abhängiges Wachstum	Keine 17βHSD1, 17βHSD7, **17βHSD12**	keine 17βHSD2, 17βHSD4, 17βHSD8,
MDA-MB-231	Mamma	-		**17βHSD2**
JEG-3	Mamma	-	**17βHSD1,** keine 17βHSD3, 17βHSD5, 17βHSD7, 17βHSD12	keine 17βHSD2, 17βHSD4, 17βHSD8, 17βHSD10, 17βHSD11
ZR-75	Mamma	+	*17βHSD1*, 17βHSD3, 17βHSD5, 17βHSD7, 17βHSD12	keine 17βHSD2, 17βHSD4, 17βHSD8, **17βHSD10,** 17βHSD11
MCF-7	Mamma	+	*17βHSD1*, 17βHSD7, **17βHSD12**	*17βHSD2*, 17βHSD4, 17βHSD8
T47D	Mamma	+	**17βHSD1,** keine 17βHSD3, 17βHSD5, 17βHSD7, 17βHSD12	17βHSD2, 17βHSD4, 17βHSD8, 17βHSD10, keine 17βHSD11

Betrachtet man Tabelle 4 unter diesen Aspekten, so stellt sich die humane Brustkrebszelllinie T47D als bestes der aufgeführten Modelle für die Evaluation von 17βHSD1-Inhibitoren heraus. Sie exprimiert 17βHSD1 und 17βHSD2 in einem Verhältnis, welches der pathophysiologischen Situation im Mammakarzinom ähnelt (Day *et al.*, 2006). Die ER werden exprimiert, und die Zellen zeigen estrogenabhängiges Wachstum (Camby und Kiss, 1993). Deshalb wurde diese Zelllinie für die weiteren zellulären Untersuchungen ausgewählt.

4.2.4 Bedeutung der Untersuchung ER-vermittelter Effekte der 17βHSD1-Inhibitoren

Da 17βHSD1-Inhibitoren auf Grundlage der Röntgenkristallstruktur des Enzyms sowie der chemischen Struktur des E2 entwickelt wurden (Bey *et al.*, 2008a; Marchais-Oberwinkler *et al.*, 2008b) und somit potentielle Steroidomimetika darstellen, besteht die Gefahr, dass sie Affinität zu den ER besitzen und so selbst möglicherweise estrogene Effekte ausüben. Wie in Kapitel 1.1.3 beschrieben haben die Rezeptor-Subtypen unterschiedliche Funktionen im Organismus. Die Inhibitoren wiederum könnten bei vorhandener Affinität als Agonisten oder als Antagonisten wirken. Vereinfacht können also vier Fälle betrachtet werden.

Agonisten am ERα würden klassische estrogene Wirkungen ausüben und somit das Wachstum des erkrankten Gewebes fördern. Deshalb sind 17βHSD1-Inhibitoren, welche gleichzeitig agonistisch an diesem Subtyp wirken für die weitere Entwicklung ungeeignet. Dagegen würden ERα-Antagonisten als Antiestrogene positiven Einfluß auf die Erkrankung nehmen. Dennoch würden sich aus einem dualen Wirkprinzip aus 17βHSD1-Hemmung und ERα-Blockade auch die typischen Nebenwirkungen der Antiestrogene ergeben. Damit sind solche Inhibitoren zwar grundsätzlich für die weitere Entwicklung geeignet, müssen aber unter dem Aspekt der Nebenwirkungen kritisch beurteilt werden.

Agonisten am ERβ wirken möglicherweise antiproliferativ. Dies würde sich positiv auf den Verlauf der bestehenden Krankheit auswirken. Dennoch ist zu beachten, dass ein zusätzliches *Target* der Inhibitoren auch weitere Nebenwirkungen hervorrufen wird. Da über die genaue Funktionsweise des ERβ weiterhin diskutiert wird, muß auch ein Agonismus an diesem Rezeptorsubtyp mit Vorsicht behandelt werden. Antagonisten des ERβ wären durch ihre potentiellen proliferationsfördernden Wirkungen ungeeignet zur Therapie von Brustkrebs und Endometriose.

Zusammenfassend hat also jedes Wirkprinzip an den Rezeptoren bestimmte Nachteile, welche sich entweder auf den Verlauf der Erkrankung beziehen oder aber auf die Induzierung zusätzlicher Nebenwirkungen. Deshalb wäre es wünschenswert, 17βHSD1-Inhibitoren zu entwickeln, welche keine Affinität zu den ER besitzen.

Um die Gefahr der Interferenz der Hemmstoffe mit den Rezeptoren beurteilen zu können, wird zunächst ein Test benötigt, welcher Aussagen zulässt über deren Bindung an die ER. Auch in diesem Fall wurde zellfreien Assays den Vorzug gegeben, um SARs ableiten zu können, welche ggf. zu einer Verringerung der Rezeptoraffinität im Designprozeß beitragen können.

Für die weitere Entwicklung ist damit ein wichtiger Punkt, welche Affinität zu den Rezeptoren für 17βHSD1-Inhibitoren akzeptabel ist. Diese Fragenstellung wird durch verschiedene Faktoren beeinflusst.

Zunächst verhindern 17βHSD1-Inhibitoren lediglich die Bildung von E2 im Gewebe. Mit der Präsenz von E1 muß weiterhin gerechnet werden. In den ER-Bindungstests wurden für E1

folgende relativen Bindungsaffinitäten, (RBA-Werte) ermittelt: RBA(E1, ERα) = 5,5%, RBA(E1, ERβ) = 3,1%. Um also gewährleisten zu können, dass die entwickelten Inhibitoren keine rezeptorvermittelten Effekte ausüben, muß ihre ER-Bindung in der benötigten Gewebskonzentration unter der des E1 liegen.

Da jedoch im zellulären Aktivitätstest IC_{50}-Werte ermittelt wurden, welche 10-20mal höher liegen als die Substratkonzentration (E1), muß davon ausgegangen werden, dass die Inhibitoren im erkrankten Gewebe entsprechend höher konzentriert sein müssen als E1. Folglich muß ihre Affinität auch um den Faktor 10-20geringer sein als die des E1. Damit scheint ein RBA-Wert von 0,1% oder kleiner angemessen für die Weiterentwicklung der Inhibitoren.

RBA-Werte über 0,1% müssen unter dem Aspekt der intrinsischen Aktivität kritisch beurteilt werden. Um diese beurteilen zu können, wurde ein weiterer zellulärer Test entwickelt, in dem untersucht wird, ob die 17βHSD1-Inhibitoren das Zellwachstum der estrogenabhängigen Zelllinie T47D fördern. Dabei werden sowohl agonistische Effekte an ERα erfasst als auch Antagonismus an ERβ sowie die unspezifische Proliferationsförderung. Diese können im etablierten Test zwar nicht unterschieden werden, die Weiterentwicklung der Substanz wäre aber in jedem der möglichen Fälle nicht vertretbar. Somit genügt die Aussage, welche mit Hilfe dieses Tests getroffen werden kann zur Beurteilung der 17βHSD1-Inhibitoren.

Ein weiterer Vorteil dieses Versuchs besteht darin, dass auch proliferationshemmende Wirkungen erkannt werden können. Gegebenenfalls können entsprechende Verbindungen in dieser Hinsicht weiteruntersucht werden.

Damit wurden zwei Tests ins Screeningsystem integriert, welche geeignet sind, um die (anti)estrogenen Eigenschaften der 17βHSD1-Inhibitoren zu beurteilen. Entsprechend können Verbindungen, welche nicht den Anforderungen entsprechen, bereits in einem frühen Stadium aus der weiteren Entwicklung ausgeschlossen werden.

4.2.5 Stellenwert des MTT-Assays zur Ermittelung der zellulären Überlebensrate

Ein übliches Verfahren zur Beurteilung der Zytotoxizität neuer Verbindungen ist die Evaluierung in einem MTT-Assay, welcher Aufschluß gibt über die Überlebensrate der Zellen nach Inkubation mit der betreffenden Verbindung. Die Auswertung dieses Testes beruht darauf, dass der Tetrazoliumring des eingesetzten MTT nur in lebenden Zellen zum Formazan reduziert wird. Durch Lyse der Zellen mit dem Detergenz SDS werden die entstandenen, dunkelblauen Kristalle in Lösung gebracht, und die Farbintensität kann photometrisch vermessen werden. Dabei ist die Menge an gebildetem Farbstoff direkt proportional zur Zahl der lebenden Zellen (Denizot und Lang, 1986). Die Lebendzellzahl kann damit anhand einer Kalibriergeraden bestimmt werden. Dieser Test soll Hinweise geben auf mögliche toxische Wirkungen der Verbindungen. In der Regel werden dafür unterschiedliche Konzentrationen der Substanz an mehreren Zelllinien über eine Inkubationszeit von 48h untersucht.

Auch im etablierten Screeningsystem ist ein MTT-Test eingeschlossen, welcher jedoch lediglich über eine Inkubationszeit von 3h läuft. Außerdem ist die Konzentration der Inhibitoren mit 2,5µM moderat. Damit ist der etablierte Test nicht als Ersatz für die Evaluation der Zytotoxizität zu sehen. Durch diesen Assay sollen lediglich stark toxische Verbindungen identifiziert und ausgefiltert werden.

Die Inkubationszeit wurde auf 3h festgelegt, da die Kontaktzeit der 17βHSD1-Inhibitoren im zellulären Aktivitätstest 1h beträgt. Durch die dreifach verlängerte Inkubation im MTT-Test können toxische Einflüsse der Substanzen im Zelltest sicher ausgeschlossen werden. Mit 2,5µM wurde die Konzentration der Inhibitoren doppelt so hoch angesetzt wie die im Zelltest verwendete. Somit kann bei einer Überlebensrate von 100% im MTT-Test eine Beeinflussung des zellulären Inhibitionstests durch zytotoxische Effekte sicher ausgeschlossen werden.

4.2.6 Bedeutung von PK-Parametern für die Entwicklung von 17βHSD1-Inhibitoren

Da 17βHSD1-Inhibitoren als potentielle Therapeutika entwickelt werden, ist es notwendig, die Verbindungen auch auf ihre PK-Eigenschaften hin zu untersuchen. Um auch diesen Aspekt in die Entwicklung einfließen lassen zu können, sollten entsprechende Evaluierungen bereits in einem frühen Stadium erfolgen.

Zunächst spielt die intestinale Aufnahme in den Blutkreislauf, die Absorption, eine wichtige Rolle, damit der Wirkstoff zum Zielgewebe gelangen kann. Dieser Vorgang wird im aufgebauten Screeningsystem an CaCo2-Zellen untersucht. Im Gegensatz zu einfacheren Membranmodellen wie PAMPA (parallel artificial membrane permeation assay) bietet die zelluläre Zusammensetzung des CaCo2-Monolayers eine bessere Simulation der Permeation durch das Dünndarmepithel. Auch können bestimmte Transport- und Effluxprozesse bereits berücksichtigt werden. Durch die Klassifiezierung der Substanzen im System nach Yee (Yee, 1997) können die Permeabilitäten mit denen bereits zugelassener Arzneistoffe verglichen werden, deren Absorptionsmechanismus bekannt ist. Damit lässt der CaCo2-Assay eine verlässliche Einschätzung der intestinalen Absorption zu.

Ein weiterer, wichtiger Gesichtspunkt der PK ist der Metabolismus in der Leber. Dieser betrifft sowohl die Wirksamkeit der Substanzen, welche durch eine schnelle Metabolisierung beeinträchtigt wird, als auch die Nebenwirkungen der Inhibtioren. Durch Hemmung der hepatischen CYP-Enzyme können sich nämlich andere Verbindungen, welche Substrate dieser Enzyme sind, anreichern und somit toxische Effekte ausüben. Daher wurden in das Screeningsystem zwei Tests integriert, welche den Metabolismus betreffen. Einerseits wird die metabolische Stabilität der 17βHSD1-Inhibitoren mit Hilfe von Rattenlebermikrosomen untersucht, da nur Substanzen mit genügend langer Halbwertszeit geeignet sind, um als Arzneimittel für diese Indikation eingesetzt zu werden. Andererseits wird die Hemmung der hepatischen CYP-Enzyme durch die entwickelten 17βHSD1-Inhibitoren untersucht.

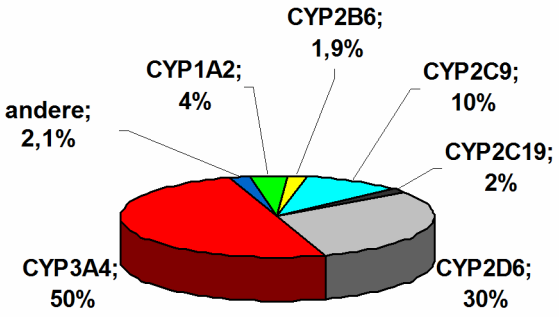


Abbildung 17: humaner Arzneistoffmetabolismus in der Leber. Angegeben ist der prozentuale Anteil unterschiedlicher hepatischer CYP-Enzyme am Arzneistoffmetabolismus.

Aus Abbildung 17 geht hervor, dass über 95% des Arzneistoffmetabolismus durch die hepatischen CYP-Enzyme 1A2, 2B6, 2C9, 2C19, 2D6 und 3A4 erfolgen (Smith *et al.*, 1997b). Deshalb wird die Hemmung dieser sechs wichtigsten (Anzenbacher und Anzenbacherova, 2001; Bertz und Granneman, 1997), humanen Isoenzyme durch 17βHSD1-Inhibitoren in sechs einzelnen Assays untersucht. Damit kann das Nebenwirkungspotential, welches aufgrund der Beeinflussung des Metabolismus entsteht, weitgehend eingeschätzt werden.

Distribution und Elimination als weitere Parameter sind erst in *in vivo* PK-Studien eingeschlossen. Dabei wird die Ratte als Versuchstier bevorzugt gewählt. Entsprechend der Fragestellung kann jedoch auch die Untersuchung in einer anderen Spezies nötig sein.

4.2.7 Gesamtbewertung des Screeningsystems

Die serielle Anwendung verschiedener Testverfahren (Abbildung 18) zeigte, dass es möglich ist, hochaktive 17βHSD1-Inhibitoren zu entwickeln, welche gleichzeitig auch Selektivität aufweisen gegenüber 17βHSD2 sowie den ER. Dabei konnte durch die sinnvolle Aufeinanderfolge der einzelnen Tests die verlässliche Ableitung von SARs gewährleistet werden, welche in das weitere Design der Inhibitoren eingingen. Auch eine gute intrazelluläre Hemmung des Zielenzyms sowie gute PK-Eigenschaften wurden erreicht (Bey *et al.*, 2008c; Marchais-Oberwinkler *et al.*, 2008b). Ein weiterer Vorteil der seriellen Testanwendung ist, dass Verbindungen mit unzureichenden Eigenschaften frühzeitig ausgefiltert werden können. Damit kann deren weitere, kostspielige Evaluation verhindert werden (Kruchten *et al.*, 2009c).

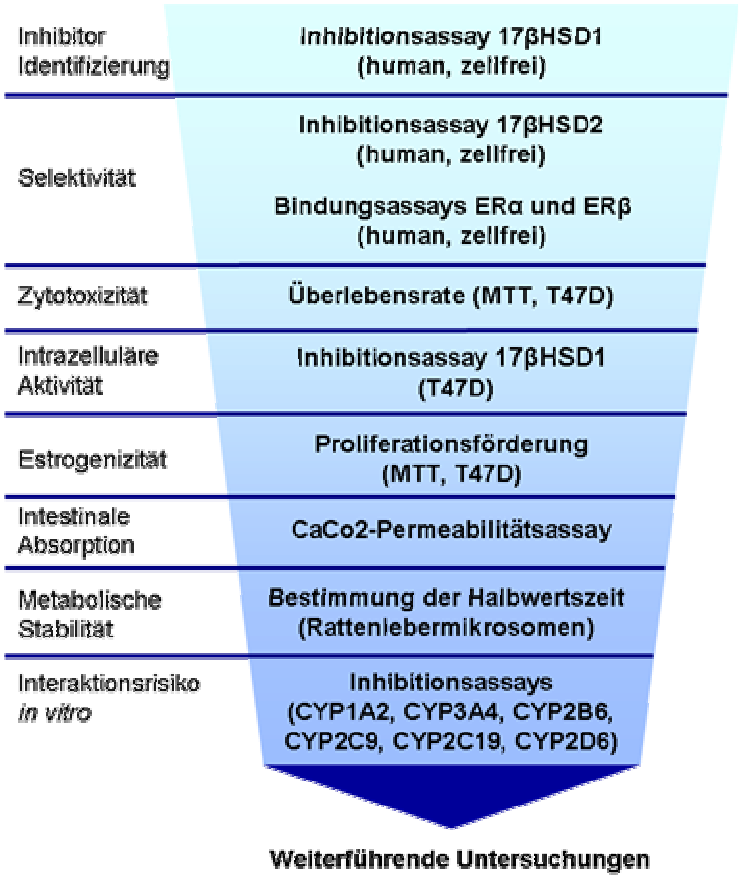


Abbildung 18: Screeninsystem zur Auffindung hochaktiver, selektiver 17βHSD1-Inhibitoren als potentielle Therapeutika

Im Screeningsystem werden die entwickelten Verbindungen auf die für 17βHSD1-Inhibitoren als Therapeutika relevanten Eigenschaften hin geprüft. Als weiterführende Untersuchungen werden Experimente betrachtet, welche das *proof of concept* zum Ziel haben, oder direkt damit in Zusammenhang stehen. Beispielsweise kann es sich um die Testung ausgewählter Inhibitoren am Enzym anderer Spezies oder um PK-Studien handeln.

Verbindungen, welche das Screeningsystem passiert haben und ein akzeptables Profil aufwiesen, wurden im Rahmen weiterführender Untersuchungen auf ihre Wirksamkeit in der Zellkultur geprüft (*in vitro proof of concept*). Ein positives Ergebnis dieses Experiments kann nicht nur als Validierung des Konzepts der 17βHSD1-Hemmung zur Therapie estrogenabhängiger Erkrankungen gesehen werde. Da anhand dreier Verbindungen die Wirksamkeit in der Zellkultur belegt werden konnte, darf man ebenfalls schließen, dass die Anwendung des Screeningsytems zur Auswahl geeigneter Inhibitoren für die weiteren Untersuchungen führt.

4.2.8 *In vitro proof of concept*

Zur ersten Validierung der 17βHSD1 als *Target* wurde zunächst ein Experiment in der Zellkultur durchgeführt (Kap. 3.6). Dabei wurde die Modulation des estrogenabhängigen Zellwachstums durch 17βHSD1-Inhibitoren untersucht. Das Experiment beruht darauf, dass das

Zellwachstum von T47D Zellen durch Aktivierung des ER stimulierbar ist. Durch eine Reduktion an aktivem Estrogen in der Zelle oder durch Verminderung der ER-Aktivierung kann diese Stimulation abgeschwächt oder sogar verhindert werden. Um zeigen zu können, dass die 17βHSD1-Inhibition ein adäquates Konzept zur Normalisierung des estrogenstimulierten Zellwachstums ist, müssen beide Wirkprinzipien in entsprechenden Versuchen differenziert werden können. Deshalb wurde eine entsprechende experimentelle Anordnung gewählt, welche sich eignet, um beide Wirkmechanismen zu unterscheiden. Außerdem wurden Verbindungen benutzt, von denen bereits bekannt war, dass sie intrazellulär die 17βHSD1 hemmen können und damit in der Lage sind, die Konzentration an E2 in der Zelle zu vermindern. Abbildung 19 zeigt beispielhaft das Ergebnis entsprechender Untersuchungen.

Aus den erhaltenen Resultaten lassen sich Aussagen ableiten, welche die Wirkungsweise der Inhibitoren betreffen. Der Vergleich des Hemmstoffs alleine mit der unbehandelten Kontrolle zeigt, dass die Verbindung selbst in der eingesetzten Konzentration weder estrogene noch anders vermittelte proliferationsfördernde Effekte ausübt. Ebenso bleibt das basale Zellwachstum unbeeinträchtigt. Die Inhibitoren vermitteln also in der angewendeten Konzentration auch keine proliferationshemmenden Effekte, deren Mechanismus nicht bekannt ist.

Durch die Zugabe von Estrogenen wird die Proliferation stimuliert. Dabei fällt diese Stimulation in den E1-stimulierten Proben ebenso stark aus wie in den E2-behandelten, obwohl E1 ein schwächeres Estrogen ist. Dies deutet darauf hin, dass E1 durch die 17βHSD1 intrazellulär zu E2 umgewandelt wird, welches dann den stimulierenden Effekt auslöst.

Durch gleichzeitige Behandlung der Zellen mit 17βHSD1-Inhibitor und E1 wird die Stimulation des Zellwachstums unterdrückt, da durch die Hemmung der 17βHSD1 in der Zelle weniger E2 gebildet wird, welches das Wachstum anregen kann. Die simultane Anwendung von E2 und 17βHSD1-Inhibitor dagegen führt zu einer unverminderten Proliferationsförderung. Daraus lässt sich schließen, dass die Inhibitoren in der angewandten Konzentration keine antiestrogenen Wirkung entfalten.

Durch die vollständige Interpretation der Ergebnisse konnte gezeigt werden, dass die selektive Hemmung der 17βHSD1 zu einer Wachstumsnormalisierung im Zellkulturversuch führt, welche nicht durch antagonistische Wirkung an den ER vermittelt wird (Kruchten *et al.*, 2009a).

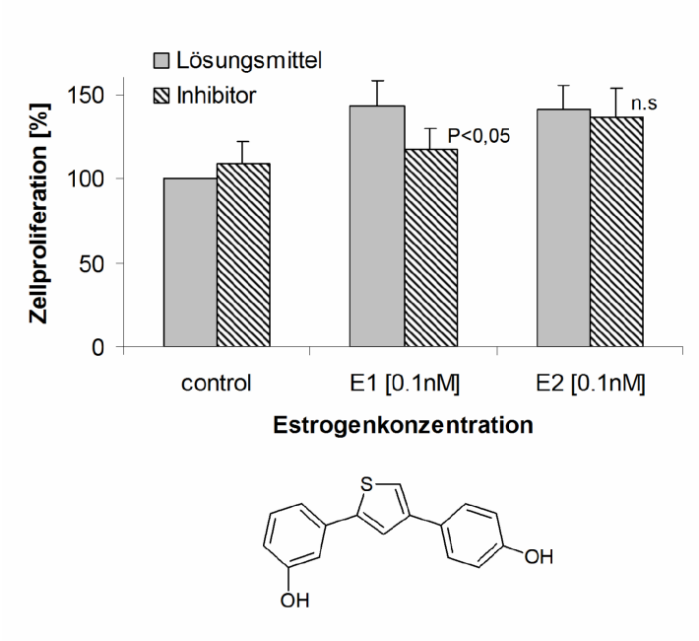


Abbildung 19: Modulation des estrogenabhängigen Zellwachstums durch einen nichtsteroidalen 17βHSD1-Inhibitor.

Ähnliche Experimente mit steroidalen Verbindungen wurden auch von Laplante *et al*. und Day *et al*. durchgeführt. Beide Gruppen benötigen jedoch höhere Inhibitorkonzentrationen, um die E1-Stimulation zu vermindern. Im Falle von Laplante wurde darüberhinaus das Zellwachstum durch den Inhibitor selbst gefördert, was auf eine estrogene Wirkung der Verbindung hindeutet. Ein Test auf antiestrogene Wirkung im Sinne eines Partialagonismus wurde hier nicht an der Zellkultur durchgeführt (Laplante *et al.*, 2008). In der Gruppe von Day wurde dieser Teil des Versuchs ausgeführt. Da die E2-vermittelte Stimulation vermindert wurde, scheint es sich in diesem Fall um einen 17βHSD1-Inhibitor mit antiestrogener Wirkkomponente zu handeln. Damit kann nicht eindeutig unterschieden werden, ob die Reduktion des stimulierten Zellwachstums auf die Inhibition der 17βHSD1 oder auf die Rezeptorblockade zurückzuführen ist (Day *et al.*, 2008). Die offensichtliche Rezeptoraffinität der Substanzen beider Arbeitsgruppen belegt wiederum das erhöhte Nebenwirkungspotential, welches mit dem steroidalen Grundgerüst einhergeht.

Zusammenfassend wurde im Zellkulturversuch gezeigt, dass die Hemmung der 17βHSD1 eine geeignete Strategie ist, um das estrogenstimulierte Wachstum von Brustkrebszellen zu normalisieren. Weiterhin wurde belegt, dass nichtsteroidale 17βHSD1-Inhibitoren entwickelt und durch das bestehende Screeningsystem selektiert werden konnten, welche in der Lage sind, das estrogenabhängige Zellwachstum zu modulieren.

4.3 Ausblick

4.3.1 Auswahl eines geeigneten Tiermodells zur Evaluierung der 17βHSD1-Inhibitoren

Da der durchgeführte Test auf Wirksamkeit in der Zellkultur vielversprechende Resultate ergeben hat, ist es im nächsten Schritt notwendig, diese Ergebnisse an einem relevanten Krankheitsmodell zu bestätigen. Wie in den Kapiteln 1.4.2 und 1.4.3 beschrieben ist, stehen prinzipiell mehrere Modelle für die Indikationen Brustkrebs und Endometriose zur Verfügung. Um zu einem aussagekräftigen Ergebnis zu kommen, müssen die durchgeführten Versuche mehrere Anforderungen erfüllen. Im Vorangehenden wurden deshalb bereits Modelle selektiert, welche in Bezug auf estrogenabhängige Erkrankungen relevant sind und in denen die Expression der 17βHSD1 gezeigt wurde.

Die so ausgewählten Brustkrebsmodelle (Day *et al.*, 2008; Husen *et al.*, 2006a) bieten den Vorteil, dass das jeweilige Experiment an humanem Enzym durchgeführt wird. Die eingesetzten Nacktmäuse fungieren hier lediglich als Träger für die aus humanen Brustkrebszellen etablierten Tumoren. Damit ist sichergestellt, dass die entwickelten Inhibitoren Aktivität am Zielenzym besitzen. Im Falle des MCF-7-Xenograftmodells wurden die Tumorzellen vor der Inokulation mit 17βHSD1 transfiziert. Damit ist dieses Modell artifizieller als das zweite ausgewählte T47D-Xenograft-Experiment, da T47D-Zellen die 17βHSD1 natürlich in ausreichendem Maße exprimieren (Day *et al.*, 2006). Obwohl beide Modelle relativ artifiziell sind, bieten sie dennoch die Möglichkeit, Aussagen zu treffen über die Beeinflussung von Tumoren im lebenden Organismus. Aufgrund der passenden Eigenschaften der T47D-Zellen als Brustkrebsmodell zur Evaluierung von 17βHSD1-Inhbitioren (Kapitel 4.2.3) ist das T47D-Xenograftmodell gut geeignet, um das *proof of concept* zu erbringen.

Im Bereich der Endometriose arbeitet nur eines der relevanten Modelle mit humanem Enzym (Grümmer *et al.*, 2001). In diesem Modell ist es möglich, die Auswirkung von 17βHSD1-Inhibitoren auf humanes, endometriotisches Gewebe zu untersuchen, welches im Bauchraum von Nacktmäusen angenäht wurde. Diese Tatsache wäre von Vorteil für die Evaluation der vorhandenen 17βHSD1-Inhibitoren, da auch hier sichergestellt ist, dass die eingesetzten Verbindungen das Zielenzym prinzipiell hemmen können. Ein Nachteil dieses Modells ist jedoch, dass die Induktion der endometriotischen Läsionen in der Maus artifiziell ist.

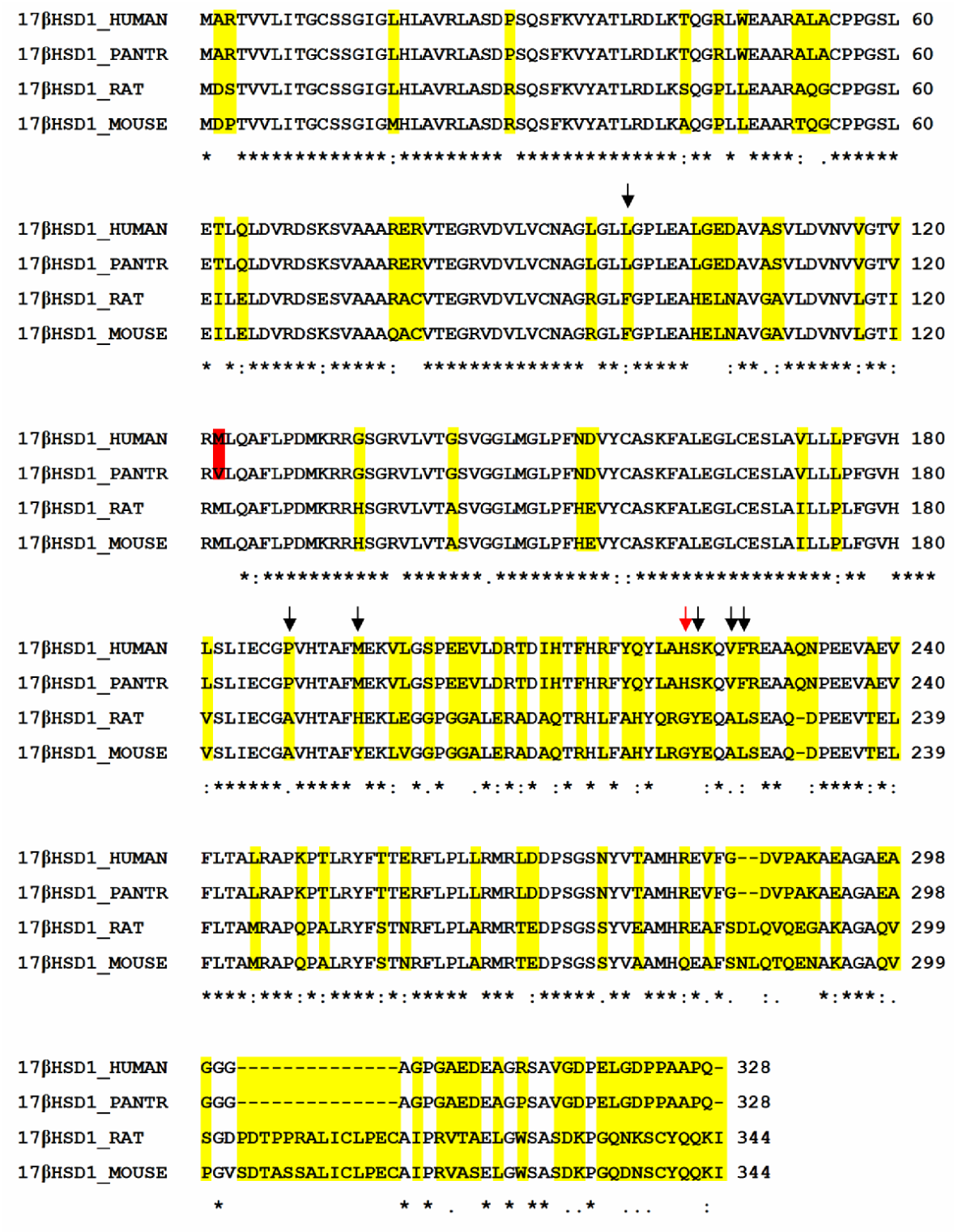


Abbildung 20: CLUSTAL 2.0.10 Multiples Sequenzalignment der 17βHSD1-Sequenzen der Spezies Mensch (HUMAN), Schimpanse (PANTR), Ratte (RAT) und Maus (MOUSE); gelbe Markierung: unterschiedliche Aminosäuren in humanem und Nagerenzymen; rote Markierung: unterschiedliche Aminosäuren in menschlichem und Schimpansenenzym; schwarze Pfeile: unterschiedliche Aminosäuren der Substratbindungstaschen von menschlichem und Nagerenzym; roter Pfeil: unterschiedliche Aminosäure in der katalytischen Tetrade in menschlichem und Nagerenzym.

In den übrigen Experimenten sind Speziesunterschiede hinsichtlich der inhibitorischen Aktivität der entwickelten Inhibitoren in Betracht zu ziehen. Sowohl das Skinfoldchamber-Modell (Menger *et al.*, 2002), welches an unterschiedlichen Nagetieren durchgeführt werden kann, als auch die beiden Primatenmodelle (Einspanier *et al.*, 2006; Yang *et al.*, 2000) sind interessant. Die Vorteile eines Skinfoldchamber-Versuchs an der Ratte wären einerseits die Möglichkeit der intravitalen Kontrolle der bestehenden Läsionen (Laschke *et al.*, 2005), die geringeren Kosten und andererseits die Tatsache, dass erste PK-Untersuchungen in der Regel an der Ratte durchgeführt werden. Die Primatenmodelle sind dagegen wegen ihres naturnahen experimentellen *Designs* attraktiv. Um ein geeignetes Modell auswählen zu können, muß jedoch geprüft werden, ob die zu testenden Inhibitoren am Enzym der zur Auswahl stehenden Spezies aktiv sind. Dies wirft zunächst die Frage nach Aminosäureidentität und -homologie der 17βHSD1 der potentiellen Versuchstiere mit dem humanen Protein auf.

Abbildung 20 zeigt das *Alignment* des humanen Enzyms mit denen der üblichen Labortiere Ratte (*Rattus norvegicus*), Maus (*Mus musculus*) sowie mit dem des Schimpansen (*Pan troglodytes*) als Vertreter der Primaten. Dabei fällt auf, dass humanes und Schimpansenenzym sich lediglich in der Aminosäure 122 unterscheiden (rote Markierung), wo das Methionin im humanen Enzym gegen Valin ausgetauscht ist. Da diese Aminosäure nicht direkt in der Substratbindetasche liegt (vgl. Abbildung 10), ist die Wahrscheinlichkeit hoch, dass Verbindungen, welche das humane Enzym kompetitiv hemmen, auch am Schimpansenprotein Aktivität zeigen. Vergleicht man dagegen Mensch- und Nagerenzyme, so findet man wesentlich größere Unterschiede in den Aminosäuresequenzen (gelbe Markierungen).

Tabelle 5: Aminosäurenhomologien und -identitäten der 17βHSD1 unterschiedlicher Spezies verglichen mit dem humanen Enzym (gesamte Sequenz).

Spezies	**Homologie [%]**	*Identität [%]*
Schimpanse	98,5	98,5
Ratte	87	70,8
Maus	77	69,1

Die aus dem *Alignment* resultierenden Proteinhomologien und -identitäten sind in Tabelle 5 zusammengefasst. Dabei ergibt sich für den Primaten eine Aminosäureidentität von 98,5 %. Dagegen sind die die Identitäten zwischen menschlichem und Nagetierprotein mit 70,8 % für die Ratte und 69,1 % für die Maus nur mäßig. Vergleicht man die Aminosäuren der Substratbindungstaschen von humanem und Nagerenzym, so findet man ebenfalls gravierende Unterschiede. Von 14 relevanten Aminosäuren in der Substratbindungstasche (vgl. Kapitel 1.2.3) unterscheiden sich sieben (schwarze Pfeile), von denen sogar eine zur katalytischen Tetrade gehört (roter Pfeil). Dies gilt sowohl für das Maus- als auch für das Rattenenzym.

Damit besteht das Risiko, dass die Inhibitoren der humanen 17βHSD1 am Nagerenzym weniger oder sogar inaktiv sind. Um dies zu überprüfen, wurden zwei Tests entwickelt, welche geeignet sind, um die Hemmung der E1-Reduktion bzw. der E2-Oxidation durch Enzympräparationen aus Rattenlebergewebe zu beurteilen (Kruchten *et al.*, 2009b). Insbesondere im Hinblick auf die Testung der Hemmstoffe im Skinfoldchamber-Modell sind diese Untersuchungen von hohem Interesse.

Im Ergebnis konnten Verbindungen gefunden werden, welche die reduktive Reaktion in einem akzeptablen Ausmaß zu hemmen vermögen. Jedoch wurde in diesen Fällen auch die Oxidation des E2 inhibiert. Somit stehen am Rattenenzym aktive aber nicht selektiver Verbindungen zur Verfügung.

Entsprechende Untersuchungen am Maus- oder Affenenzym wurden bisher nicht durchgeführt. Da aus dem *Alignment* jedoch abzuleiten ist, dass das Primatenenzym dem humanen wesentlich ähnlicher ist als die Nagetierproteine, wäre es interessant, die entwickelten Verbindungen auf ihre Wirksamkeit am Affenenzym zu testen. Dabei ist zu beachten, welche Primaten für ein Endometriosemodell in Frage kommen. Da bisher in relevanten Primatenexperimenten lediglich Weißbüschelaffen oder Javaneraffen als Modellorganismus beschrieben wurden, scheint es sinnvoll, ähnliche Untersuchungen an diesen Spezies durchzuführen. Entsprechend der Ergebnisse, kann dann entschieden werden, ob ein *proof of concept* in einem der Primatenmodelle anzustreben ist.

4.3.2 17βHSD2 – ein neues Target in der Osteoporosetherapie?

Osteoporose ist eine Erkrankung des Knochens, welche gekennzeichnet ist durch eine Verringerung der Knochensubstanz. Der Abbau der Knochensubstanz entsteht durch ein Ungleichgewicht der Aktivität der Knochenzellen, durch welches die Osteolyse beschleunigt abläuft. Durch die verringerte Knochenmasse steigt die Wahrscheinlichkeit von Krankenhausaufenthalten, welche durch Frakturen verursacht werden. Die durch Hüftfrakturen hervorgerufenen Krankenhausaufenthalte enden laut WHO in ca. 20 % der Fälle letal[2].

Die höchste Abnahme der Knochendichte findet bei Frauen im Alter zwischen 50 und 55 Jahren statt (Berger *et al.*, 2008). In ca. 80 % der Osteoporosefälle handelt es sich um postmenopausale Frauen und ca. 30 % der Frauen nach der Menopause entwickeln diese Krankheit. Diese Tatsache ist damit zu erklären, dass postmenopausal der E2-Spiegel im Blut stark absinkt. Das prämenopausal in höherer Konzentration vorhandene E2 wirkt sowohl auf die Osteoblasten als auch auf die Osteoclasten. In den Osteoblasten übt E2 mitogene und proliferationsfördernde Effekte aus, indem es die Expression des Wachstumshormonrezeptors erhöht. Damit wird der Knochenaufbau durch die Osteoblasten gefördert. Dagegen wird die Aktivität der Osteoclasten

[2] WHO Technical Report Series No. 921: Prevention and Management of Osteoporosis, ISBN-13: 9789241209212

durch E2 vermindert und ihre Apoptose gefördert (Turner *et al.*, 1994). Somit bremst E2 die Osteolyse (Abbildung 21A).

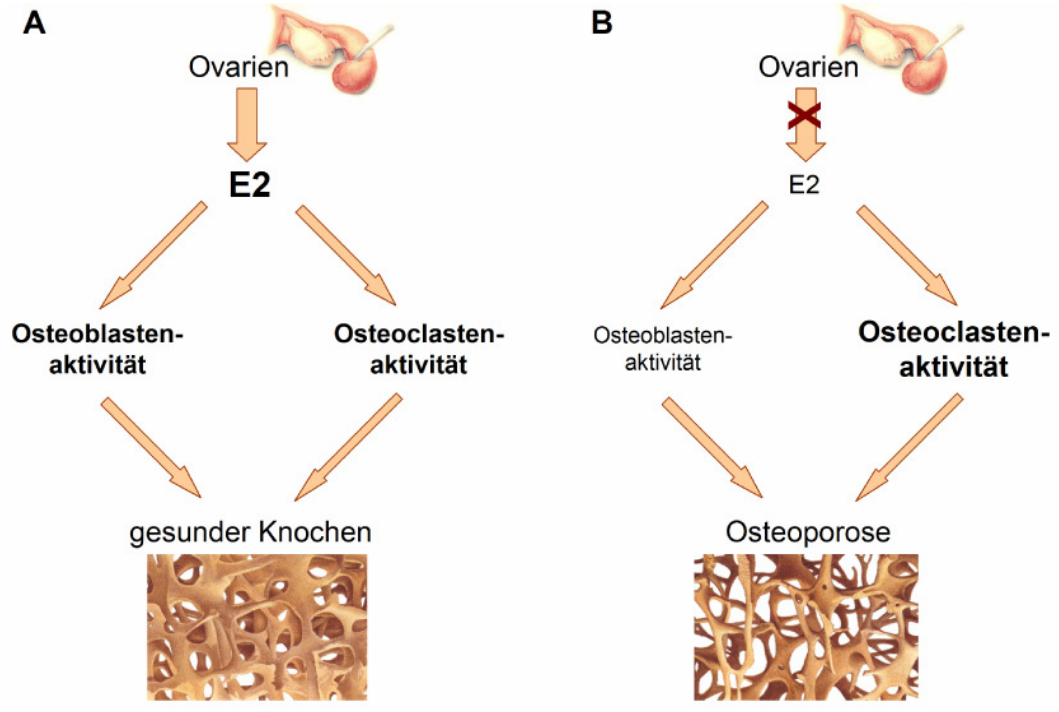


Abbildung 21: Einfluß von E2 auf die Aktivität von Osteoblasten und Osteoclasten. A. prämenopausales Gleichgewicht der Knochenzellaktivitäten unter dem Einfluß hoher E2-Blutspiegel. B. postmenopausal gestörtes Gleichgewicht der Knochenzellaktivitäten durch verringerte E2-Spiegel.

Die Abnahme der E2-Plasmaspiegel in der Menopause trägt maßgeblich zur Pathogenese der Erkrankung bei. Durch die verminderte Verfügbarkeit des E2 sind die positiven Effekte auf die Osteoblastenaktivität und die negative Wirkung auf die Osteoclastenaktivität herabgesetzt (Abbildung 21B). Daneben wird durch die geringere Konzentration von E2 im Knochen die Bildung von IL-1, IL-6 und Tumornekrosefaktor-α (TNF-α) erhöht. TNF-α wiederum regt die Osteoklastendifferenzierung an, was zu einer weiterhin erhöhten Osteolyse führt (Compston, 2001; Jerome *et al.*, 1997). Dies wird auch dadurch bestätigt, dass bei Frauen über 65 Jahren der E2-Spiegel mit der Knochendichte korreliert. Außerdem kann dem verstärkten, postmenopausalen Knochenabbau durch Estrogenersatz vorgebeugt werden (Harris *et al.*, 2002).

Nicht nur die Ovarien sind Quelle des im Knochen vorhandenen E2. Auch die 17βHSD1 sowie CYP19 tragen in den Knochenzellen selbst zur Aufrechterhaltung der E2-Konzentration in den Osteoblasten bei. Ebenso wird die 17βHSD2, welche E2 zu E1 inaktiviert, in Osteoblasten exprimiert (Dong *et al.*, 1998; Janssen *et al.*, 1999; Purohit *et al.*, 1992). Damit wird der intrazelluläre E2-Spiegel in diesen Knochenzellen intrakrin moduliert. Ein neuer, schonender Ansatz zur Erhöhung der E2-Konzentration im Knochen wäre deshalb die Inhibition der 17βHSD2 (Abbildung 22). Die Bedeutung der 17βHSD2 in der Pathogenese der Osteoporose wird auch dadurch untermauert, dass transgene Mäuse, in denen die 17βHSD2 überexprimiert

ist, charakterisiert sind durch ein niedrigeres Gewicht, verringerte Knochenmineraldichte und retardiertes Wachstum (Shen *et al.*, 2008).

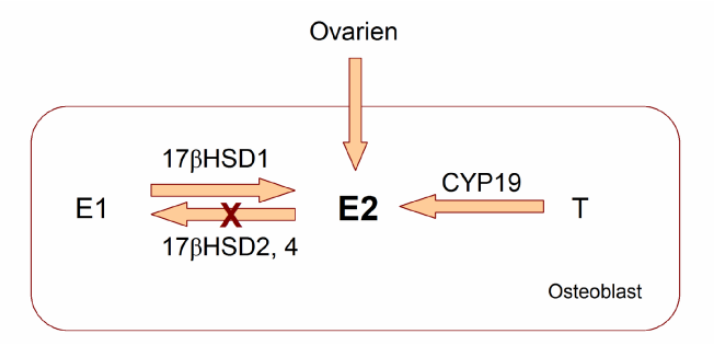


Abbildung 22: Konzept der 17βHSD2-Inhibition als möglicher Ansatz zur Prävention und/ oder Therapie der postmenopausalen Osteoporose.

Durch die Hemmung der Inaktivierung des Hormons bei gleichzeitiger ungebremster Synthese durch CYP19 und die 17βHSD1 ist eine Anreicherung zu erwarten. Dadurch könnten die positiven Effekte des lokal erhöhten E2-Spiegels ausgenutzt werden, während die Nebenwirkungen einer Hormonersatztherapie möglicherweise umgangen werden können.

Im Rahmen der Dissertation wurden auch bereits Strukturen identifiziert, welche die 17βHSD2 inhibieren und sogar moderate Selektivität gegenüber der 17βHSD1 aufweisen. Diese könnten als Basis für die Entwicklung hochaktiver und selektiver Inhibitoren der 17βHSD2 genutzt werden. Auch die bereits etablierten Tests bezüglich ER-Affinität, Estrogenizität, CYP-Inhibition könnten zur Auffindung und Beurteilung geeigneter Inhibitoren genutzt werden.

5 ZUSAMMENFASSUNG

Im Rahmen der Entwicklung selektiver Inhibitoren der 17β-Hydroxysteroid-Dehydrogenase Typ 1, wurde ein komplexes Screeningsystem aufgebaut, welches sich zur Selektion vielversprechender Verbindungen sowie zur Ableitung von Struktur-Wirkungsbeziehungen eignet. Dadurch wurden zwei Leitstrukturen gefunden und hinsichtlich Selektivität und Pharmakokinetik optimiert. Die sequentielle Kombination der einzelnen Tests führte zur rationellen Identifizierung von Verbindungen, deren Eigenschaften einen Versuch zur Targetvalidierung erlauben.

Deshalb wurden Aktivität und Selektivität ausgewählter Verbindungen am Rattenenzym evaluiert. Dazu wurden Leberpräparationen auf ihre altersabhängige Fähigkeit hin untersucht, E1 und E2 ineinander umzuwandeln. Es wurde eine signifikante Abnahme der E2-Produktion mit steigendem Alter festgestellt. Die Präparationen wurden zur Etablierung zweier Tests auf Inhibtion der Estrogenkonversion genutzt, durch welche Hemmstoffe der E2-Produktion identifiziert wurden.

In einem Zellkulturversuch wurden selektierte Verbindungen auf ihre Fähigkeit zur Modulierung der estrogenabhängigen Proliferation hin untersucht. Die gefundene, selektive Inhibition der durch E1 stimulierten Proliferation bei gleichzeitig unbeeinflusstem, E2-induzierten Wachstum untermauert den Ansatz der 17βHSD1-Inhibiton zur Therapie des Mammakarzinoms. Damit wurden eine erste Targetvalidierung erbracht, wirksame Inhibitoren identifiziert und die Effizienz des Screeningsystems gezeigt.

6 SUMMARY

A complex screening system was developed for the identification and optimisation of selective 17β-Hydroxysteroid-Dehydrogenase type 1 inhibitors. Compounds were designed, synthesised, and after testing active structures were identified. Elucidation of the structure-activity-relationships resulted in the identification of two lead structures which were further optimised. The rational combination and sequential application of the single tests led to an efficient identification of compounds suitable for target validation.

Persuing this issue, activity and selectivity evaluation in rats were performed. Therefore, the age dependent ability of rat liver preparations to convert E1 into E2 or *vice versa* was investigated. E2-production was found to be reduced in the aged rats. Having shown both activities the liver preparations were used for the development of two inhibition tests. Applying these assays active inhibitors of E2-production were identified.

For target validation, inhibitors selected in the screening assay were investigated in a cell culture experiment. Their ability to modulate estrogen-dependent growth was shown. E1-induced proliferation was selectively reduced by simultaneous inhibitor-addition. In contrast, E2-mediated growth was not affected. This supports the approach of 17βHSD1-inhibition as therapy option for breast cancer. Thus, a first target validation was performed, effective inhibitors were identified and the efficiency of the screening system was shown.

7 ANHANG

7.1 Abkürzungsverzeichnis

17βHSD	17β-Hydroxysteroid-Dehydrogenase
3βHSD	3β-Hydroxysteroid-Dehydrogenase
Adion	Androstendion
AKR	Aldo-Keto Reduktase
AUC	area under the curve
CC	Säulenchromatographie
CD_3OD	deuteriertes Methanol
$CDCl_3$	deuteriertes Chloroform
CO	cardiac output
COX	Cyclooxygenase
CYP	Cytochrom P450
CYP11A1	side chain cleaving enzyme, Desmolase
CYP17	17α-Hydroxylase-C17/20-Lyase
CYP19	Aromatase
DCIS	ductal carcinoma *in situ*
DFT	density function theory
DHEA	Dehydroepiandrosteron
DHEA-S	DHEA-sulfat
DHT	Dihydrotestosteron
DMEM	Dulbecco´s modified Eagle Medium
DMF	Dimethylformamid
DMSO	Dimethylsulfoxid
E1	Estron
E1-S	E1-sulfat
E2	Estradiol
E2-S	E2-sulfat
E3	Estriol
EDTA	Ethylendiamintetraessigsäure
EGF	epidermal growth factor
Eq	Äquivalent
ER	Estrogenrezeptor
ERE	estrogen-responsive element
ESP	elektrostatisches Potential
FCS	fetales Kälberserum
FSH	Follikelstimuliernders Hormon, Follitropin
GA	genetischer Algorithmus
GnRH	Gonadotropin Releasing Hormon
HAP	Hydroxyapatit
Her2/neu	human epidermal growth factor receptor
HERS	Heart and Estrogen/ Progestin Replacemant Study
HPLC	high performance liquid chromatography
HR	heart rate
HRT	Hormonersatztherapie
HSD	Hydroxysteroid-Dehydrogenase
IBC	invasiver Brustkrebs
IGF-1	insulin-like growth factor

IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-6-SR	Interleukin 6 soluble receptor
LH	Luteinisierendes Hormon, Lutropin
MAP	mitogen-activated protein
MEP	molecular electrostatic potential
MHC	myosin heavy chain protein
MISS	membrane-initiated steroid signalling
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
NAD^+	Nicotinsäureamid-Adenin-Dinukleotid
NADH	Nicotinsöureamid-Adenin-Dinukleotid (reduzierte Form)
$NADP^+$	Nicotinsäureamid-Adenin-Dinucleotidphosphat
NADPH	Nicotinsäureamid-Adenin-Dinucleotidphosphat (reduzierte Form)
Ovx	ovariektomiert
PAGE	Polyacrylamid-Gelelektrophorese
PAMPA	parallel artificial membrane permeability assay
PCNA	proliferating cell nuclear antigen
PDB	Proteindatenbank
PDWA	proliferative disease without atypia
PGE_2	Prostaglandine E_2
PK	Pharmakokinetik
RBA	relative Bindungsaffinität
RIA	Radioimmunoassay
SAP	systolic blood pressure
SAR	Struktur-Wirkungsbeziehung
SDR	Short-Chain Dehydrogenase/ Reduktase
SDS	Natriumdodecylsulfat
SERD	selektiver Estrogenrezeptor Downregulator
SERM	selektiver Estrogenrezeptor Modulator
SHR	spontaneously hypertensive rats
StAR	steroidogenes akutes regulatorisches Protein
SV	stroke volume
T	Testosteron
TE	Tris-EDTA
TEER	transepithelialer elektrischer Widerstand
TLC	Dünnschichtchromatographie
TNF-α	Tumornekrosefaktor-α
VEGF	vaskulärer endothelialer Wachstumsfaktor
WHI	Women´s health initiative
WHO	Weltgesundheitsorganisation

7.2 Abbildungsverzeichnis

7.3 Tabellenverzeichnis

7.4 Literaturverzeichnis

Abdulhaq, H., Geyer, C.: Safety of adjuvant endocrine therapy in postmenopausal women with breast cancer. *Am. J. Clin. Oncol.* 31 (2008) 595-605.

Adamo, V., Iorfida, M., Montalto, E., Festa, V., Garipoli, C., Scimone, A., Zanghi, M., Caristi, N.: Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. *Ann. Oncol.* 18 Suppl 6 (2007) vi53-57.

Adamski, J., Husen, B., Marks, F., Jungblut, P.W.: Purification and properties of oestradiol 17 beta-dehydrogenase extracted from cytoplasmic vesicles of porcine endometrial cells. *Biochem. J.* 288 (Pt 2) (1992) 375-381.

Adamski, J., Normand, T., Leenders, F., Monte, D., Begue, A., Stehelin, D., Jungblut, P.W., de Launoit, Y.: Molecular cloning of a novel widely expressed human 80 kDa 17 beta-hydroxysteroid dehydrogenase IV. *Biochem. J.* 311 (Pt 2) (1995) 437-443.

Agarwal, A.K., Auchus, R.J.: Minireview: cellular redox state regulates hydroxysteroid dehydrogenase activity and intracellular hormone potency. *Endocrinology* 146 (2005) 2531-2538.

Al-Soud, Y.A., Bey, E., Oster, A., Marchais-Oberwinkler, S., Werth, R., Kruchten, P., Frotscher, M., Hartmann, R.W.: The role of the heterocycle in bis(hydroxyphenyl)triazoles for inhibition of 17beta-Hydroxysteroid Dehydrogenase (17beta-HSD) type 1 and type 2. *Mol. Cell. Endocrinol.* 301 (2009) 212-215.

Albrecht, E.D., Aberdeen, G.W., Pepe, G.J.: The role of estrogen in the maintenance of primate pregnancy. *Am. J. Obstet. Gynecol.* 182 (2000) 432-438.

Allan, G.M., Bubert, C., Vicker, N., Smith, A., Tutill, H.J., Purohit, A., Reed, M.J., Potter, B.V.: Novel, potent inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* 248 (2006a) 204-207.

Allan, G.M., Lawrence, H.R., Cornet, J., Bubert, C., Fischer, D.S., Vicker, N., Smith, A., Tutill, H.J., Purohit, A., Day, J.M., Mahon, M.F., Reed, M.J., Potter, B.V.: Modification of estrone at the 6, 16, and 17 positions: novel potent inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *J. Med. Chem.* 49 (2006b) 1325-1345.

Allan, G.M., Vicker, N., Lawrence, H.R., Tutill, H.J., Day, J.M., Huchet, M., Ferrandis, E., Reed, M.J., Purohit, A., Potter, B.V.: Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem.* 16 (2008) 4438-4456.

Anzenbacher, P., Anzenbacherova, E.: Cytochromes P450 and metabolism of xenobiotics. *Cell Mol. Life Sci.* 58 (2001) 737-747.

Azzi, A., Rehse, P.H., Zhu, D.W., Campbell, R.L., Labrie, F., Lin, S.X.: Crystal structure of human estrogenic 17 beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol. *Nat. Struct. Biol.* 3 (1996) 665-668.

Becker, C.M., Wright, R.D., Satchi-Fainaro, R., Funakoshi, T., Folkman, J., Kung, A.L., D'Amato, R.J.: A novel noninvasive model of endometriosis for monitoring the efficacy of antiangiogenic therapy. *Am. J. Pathol.* 168 (2006) 2074-2084.

Berger, C., Langsetmo, L., Joseph, L., Hanley, D.A., Davison, K.S., Josse, R., Kreiger, N., Tenenhouse, A., Goltzman, D.: Change in bone mineral density as a function of age in women and men and association with the use of antiresorptive agents. *Cmaj* 178 (2008) 1660-1668.

Bertz, R.J., Granneman, G.R.: Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin. Pharmacokinet.* 32 (1997) 210-258.

Bey, E., Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Werth, R., Oster, A., Algül, O., Neugebauer, A., Hartmann, R.W.: Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* 16 (2008a) 6423-6435.

Bey, E., Marchais-Oberwinkler, S., Negri, M., Kruchten, P., Oster, A., Werth, R., Frotscher, M., Birk, B., Hartmann, R.W.: New Insights into the SAR and Binding Modes of Bis(hydroxyphenyl)thiophenes and Benzenes: Influence of Additional

Substituents on 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) Inhibitory Activity and Selectivity. *J. Med. Chem.* (2008b) submitted.

Bey, E., Marchais-Oberwinkler, S., Werth, R., Negri, M., Al-Soud, Y.A., Kruchten, P., Oster, A., Frotscher, M., Birk, B., Hartmann, R.W.: Design, synthesis, biological evaluation and pharmacokinetics of bis(hydroxyphenyl) substituted azoles, thiophenes, benzenes, and aza-benzenes as potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1). *J. Med. Chem.* 51 (2008c) 6725-6739.

Biswas, M.G., Russell, D.W.: Expression cloning and characterization of oxidative 17beta- and 3alpha-hydroxysteroid dehydrogenases from rat and human prostate. *J. Biol. Chem.* 272 (1997) 15959-15966.

Blanchard, P.G., Luu-The, V.: Differential androgen and estrogen substrates specificity in the mouse and primates type 12 17beta-hydroxysteroid dehydrogenase. *J. Endocrinol.* 194 (2007) 449-455.

Blomquist, C.H., Lindemann, N.J., Hakanson, E.Y.: 17 beta-hydroxysteroid and 20 alpha-hydroxysteroid dehydrogenase activities of human placental microsomes: kinetic evidence for two enzymes differing in substrate specificity. *Arch. Biochem. Biophys.* 239 (1985) 206-215.

Boughey, J.C., Gonzalez, R.J., Bonner, E., Kuerer, H.M.: Current treatment and clinical trial developments for ductal carcinoma *in situ* of the breast. *Oncologist* 12 (2007) 1276-1287.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 (1976) 248-254.

Breitling, R., Krazeisen, A., Möller, G., Adamski, J.: 17beta-hydroxysteroid dehydrogenase type 7--an ancient 3-ketosteroid reductase of cholesterogenesis. *Mol. Cell. Endocrinol.* 171 (2001) 199-204.

Breton, R., Housset, D., Mazza, C., Fontecilla-Camps, J.C.: The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors. *Structure* 4 (1996) 905-915.

Brodie, A., Lu, Q., Nakamura, J.: Aromatase in the normal breast and breast cancer. *J. Steroid Biochem. Mol. Biol.* 61 (1997) 281-286.

Brown, W.M., Metzger, L.E., Barlow, J.P., Hunsaker, L.A., Deck, L.M., Royer, R.E., Vander Jagt, D.L.: 17-beta-Hydroxysteroid dehydrogenase type 1: computational design of active site inhibitors targeted to the Rossmann fold. *Chem. Biol. Interact.* 143-144 (2003) 481-491.

Brožic, P., Lanišnik Rižner, T., Gobec, S.: Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* 15 (2008) 137-150 und zitierte Literatur. .

Brueggemeier, R.W.: Aromatase, aromatase inhibitors, and breast cancer. *Am. J. Ther.* 8 (2001) 333-344.

Bulun, S.E., Lin, Z., Imir, G., Amin, S., Demura, M., Yilmaz, B., Martin, R., Utsunomiya, H., Thung, S., Gurates, B., Tamura, M., Langoi, D., Deb, S.: Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. *Pharmacol. Rev.* 57 (2005) 359-383.

Bulun, S.E., Yang, S., Fang, Z., Gurates, B., Tamura, M., Zhou, J., Sebastian, S.: Role of aromatase in endometrial disease. *J. Steroid Biochem. Mol. Biol.* 79 (2001) 19-25.

Bulun, S.E., Zeitoun, K.M., Takayama, K., Sasano, H.: Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance. *J. Mol. Endocrinol.* 25 (2000) 35-42.

Buzdar, A.U., Ibrahim, N.K., Francis, D., Booser, D.J., Thomas, E.S., Theriault, R.L., Pusztai, L., Green, M.C., Arun, B.K., Giordano, S.H., Cristofanilli, M., Frye, D.K., Smith, T.L., Hunt, K.K., Singletary, S.E., Sahin, A.A., Ewer, M.S., Buchholz, T.A., Berry, D., Hortobagyi, G.N.: Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. *J. Clin. Oncol.* 23 (2005) 3676-3685.

Camby, I., Kiss, R.: In vitro estradiol-sensitivity characterization of the MCF-7, ZR-75, MDA-MB-231 and T47-D human breast neoplastic cell lines. *Anticancer Res.* 13 (1993) 2355-2360.

Cauley, J.A., Lucas, F.L., Kuller, L.H., Stone, K., Browner, W., Cummings, S.R.: Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group. *Ann. Intern. Med.* 130 (1999) 270-277.

Chai, Z., Brereton, P., Suzuki, T., Sasano, H., Obeyesekere, V., Escher, G., Saffery, R., Fuller, P., Enriquez, C., Krozowski, Z.: 17 beta-hydroxysteroid dehydrogenase type XI localizes to human steroidogenic cells. *Endocrinology* 144 (2003) 2084-2091.

Chen, Z., Lee, W.R., Chang, S.H.: Role of aspartic acid 38 in the cofactor specificity of Drosophila alcohol dehydrogenase. *Eur. J. Biochem.* 202 (1991) 263-267.

Chen, Z., Yuhanna, I.S., Galcheva-Gargova, Z., Karas, R.H., Mendelsohn, M.E., Shaul, P.W.: Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J. Clin. Invest.* 103 (1999) 401-406.

Compston, J.E.: Sex steroids and bone. *Physiol. Rev.* 81 (2001) 419-447.

Cotterchio, M., Kreiger, N., Theis, B., Sloan, M., Bahl, S.: Hormonal factors and the risk of breast cancer according to estrogen- and progesterone-receptor subgroup. *Cancer Epidemiol. Biomarkers Prev.* 12 (2003) 1053-1060.

Coutts, A.S., Murphy, L.C.: Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. *Cancer Res.* 58 (1998) 4071-4074.

Covaleda, A.M., van den Berg, J.H., Vervoort, J.J., van der Saag, P., Ström, A., Gustafsson, J.A., Rietjens, I.M., Murk, A.J.: Influence of cellular ER{alpha}/ER{beta} Ratio on the ER{alpha}-Agonist Induced Proliferation of Human T47D Breast Cancer Cells. *Toxicol. Sci.* (2008).

D'Hooghe, T.M., Debrock, S.: Endometriosis, retrograde menstruation and peritoneal inflammation in women and in baboons. *Hum. Reprod. Update* 8 (2002) 84-88.

Daufeldt, S., Lanz, R., Allera, A.: Membrane-initiated steroid signaling (MISS): genomic steroid action starts at the plasma membrane. *J. Steroid Biochem. Mol. Biol.* 85 (2003) 9-23.

Day, J.M., Foster, P.A., Tutill, H.J., Parsons, M.F., Newman, S.P., Chander, S.K., Allan, G.M., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J., Purohit, A.: 17beta-hydroxysteroid dehydrogenase type 1, and not type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* 122 (2008) 1931-1940.

Day, J.M., Tutill, H.J., Newman, S.P., Purohit, A., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J.: 17Beta-hydroxysteroid dehydrogenase Type 1 and Type 2: association between mRNA expression and activity in cell lines. *Mol. Cell. Endocrinol.* 248 (2006) 246-249.

Delvoux, B., Groothuis, P., D'Hooghe, T., Kyama, C., Dunselman, G., Romano, A.: Increased production of 17{beta}-Estradiol in Endometriosis Lesions is the Result of impaired Metabolism. *J. Clin. Endocrinol. Metab.* (2008) doi:10.1210/j.c.2008-2218.

DeMichele, A., Troxel, A.B., Berlin, J.A., Weber, A.L., Bunin, G.R., Turzo, E., Schinnar, R., Burgh, D., Berlin, M., Rubin, S.C., Rebbeck, T.R., Strom, B.L.: Impact of raloxifene or tamoxifen use on endometrial cancer risk: a population-based case-control study. *J. Clin. Oncol.* 26 (2008) 4151-4159.

Demir, A.Y., Groothuis, P.G., Nap, A.W., Punyadeera, C., de Goeij, A.F., Evers, J.L., Dunselman, G.A.: Menstrual effluent induces epithelial-mesenchymal transitions in mesothelial cells. *Hum. Reprod.* 19 (2004) 21-29.

Denizot, F., Lang, R.: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89 (1986) 271-277.

Deroo, B.J., Korach, K.S.: Estrogen receptors and human disease. *J. Clin. Invest.* 116 (2006) 561-570.

Dong, Y., Qiu, Q.Q., Debear, J., Lathrop, W.F., Bertolini, D.R., Tamburini, P.P.: 17Beta-hydroxysteroid dehydrogenases in human bone cells. *J. Bone Miner. Res.* 13 (1998) 1539-1546.

Donnez, J., Nisolle, M., Casanas-Roux, F., Brion, P., N., D.C.F.: Stereometric evaluation of peritoneal endometriosis and endometriotic nodules of the rectovaginal septum. *Hum. Reprod.* 11 (1995) 224-228.

Donnez, J., Van Langendonckt, A., Casanas-Roux, F., Van Gossum, J.P., Pirard, C., Jadoul, P., Squifflet, J., Smets, M.: Current thinking on the pathogenesis of endometriosis. *Gynecol. Obstet. Invest.* 54 Suppl 1 (2002) 52-58; discussion 59-62.

Dotzlaw, H., Leygue, E., Watson, P.H., Murphy, L.C.: Expression of estrogen receptor-beta in human breast tumors. *J. Clin. Endocrinol. Metab.* 82 (1997) 2371-2374.

Duax, W.L., Pletnev, V., Addlagatta, A., Bruenn, J., Weeks, C.M.: Rational proteomics I. Fingerprint identification and cofactor specificity in the short-chain oxidoreductase (SCOR) enzyme family. *Proteins* 53 (2003) 931-943.

Dufort, I., Rheault, P., Huang, X.F., Soucy, P., Luu-The, V.: Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase. *Endocrinology* 140 (1999) 568-574.

Duggan, H., Malagu, K., Leroux, F., Martin, N., Menear, K., Smith, G.: 2-Methylmorpholine Pyrido-, Pyrazo-, and Pyrimido-Pyrimidine Derivatives as mTOR Inhibitors. WO 2008023161 (A1) (2008).

Einspanier, A., Lieder, K., Brüns, A., Husen, B., Thole, H., Simon, C.: Induction of endometriosis in the marmoset monkey (*Callithrix jacchus*). *Mol. Hum. Reprod.* 12 (2006) 291-299.

Emons, G., Gründker, C., Günthert, A.R., Westphalen, S., Kavanagh, J., Verschraegen, C.: GnRH antagonists in the treatment of gynecological and breast cancers. *Endocr. Relat. Cancer* 10 (2003) 291-299.

Fan, M., Rickert, E.L., Chen, L., Aftab, S.A., Nephew, K.P., Weatherman, R.V.: Characterization of molecular and structural determinants of selective estrogen receptor downregulators. *Breast Cancer Res. Treat.* 103 (2007) 37-44.

Fechner, S., Husen, B., Thole, H., Schmidt, M., Gashaw, I., Kimmig, R., Winterhager, E., Grümmer, R.: Expression and regulation of estrogen-converting enzymes in ectopic human endometrial tissue. *Fertil. Steril.* 88 (2007) 1029-1038.

Filling, C., Berndt, K.D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jornvall, H., Oppermann, U.: Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *J. Biol. Chem.* 277 (2002) 25677-25684.

Fisher, B., Costantino, J., Redmond, C., Fisher, E., Margolese, R., Dimitrov, N., Wolmark, N., Wickerham, D.L., Deutsch, M., Ore, L., et al.: Lumpectomy compared with lumpectomy and radiation therapy for the treatment of intraductal breast cancer. *N. Engl. J. Med.* 328 (1993) 1581-1586.

Fisher, B., Dignam, J., Wolmark, N., Wickerham, D.L., Fisher, E.R., Mamounas, E., Smith, R., Begovic, M., Dimitrov, N.V., Margolese, R.G., Kardinal, C.G., Kavanah, M.T., Fehrenbacher, L., Oishi, R.H.: Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. *Lancet* 353 (1999) 1993-2000.

Fisher, B., Land, S., Mamounas, E., Dignam, J., Fisher, E.R., Wolmark, N.: Prevention of invasive breast cancer in women with ductal carcinoma in situ: an update of the national surgical adjuvant breast and bowel project experience. *Semin. Oncol.* 28 (2001) 400-418.

Fomitcheva, J., Baker, M.E., Anderson, E., Lee, G.Y., Aziz, N.: Characterization of Ke 6, a new 17beta-hydroxysteroid dehydrogenase, and its expression in gonadal tissues. *J. Biol. Chem.* 273 (1998) 22664-22671.

Foster, P.A., Woo, L.W., Potter, B.V., Reed, M.J., Purohit, A.: The use of steroid sulfatase inhibitors as a novel therapeutic strategy against hormone-dependent endometrial cancer. *Endocrinology* 149 (2008) 4035-4042.

Fournier, D., Poirier, D., Mazumdar, M., Lin, S.X.: Design and synthesis of bisubstrate inhibitors of type 1 17beta-hydroxysteroid dehydrogenase: Overview and perspectives. *Eur. J. Med. Chem.* (2008).

Frotscher, M., Ziegler, E., Marchais-Oberwinkler, S., Kruchten, P., Neugebauer, A., Fetzer, L., Scherer, C., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann,

R.W.: Design, synthesis, and biological evaluation of (hydroxyphenyl)naphthalene and -quinoline derivatives: Potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* 51 (2008) 2158-2169.

Gangloff, A., Garneau, A., Huang, Y.W., Yang, F., Lin, S.X.: Human oestrogenic 17beta-hydroxysteroid dehydrogenase specificity: enzyme regulation through an NADPH-dependent substrate inhibition towards the highly specific oestrone reduction. *Biochem. J.* 356 (2001) 269-276.

Gangloff, A., Shi, R., Nahoum, V., Lin, S.X.: Pseudo-symmetry of C19 steroids, alternative binding orientations, and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase. *Faseb J.* 17 (2003) 274-276.

Garvin, S., Nilsson, U.W., Dabrosin, C.: Effects of oestradiol and tamoxifen on VEGF, soluble VEGFR-1, and VEGFR-2 in breast cancer and endothelial cells. *Br. J. Cancer* 93 (2005) 1005-1010.

Gazvani, R., Templeton, A.: Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. *Reproduction* 123 (2002) 217-226.

Geissler, W.M., Davis, D.L., Wu, L., Bradshaw, K.D., Patel, S., Mendonca, B.B., Elliston, K.O., Wilson, J.D., Russell, D.W., Andersson, S.: Male pseudohermaphroditism caused by mutations of testicular 17 beta-hydroxysteroid dehydrogenase 3. *Nat. Genet.* 7 (1994) 34-39.

Ghosh, D., Pletnev, V.Z., Zhu, D.W., Wawrzak, Z., Duax, W.L., Pangborn, W., Labrie, F., Lin, S.X.: Structure of human estrogenic 17 beta-hydroxysteroid dehydrogenase at 2.20 A resolution. *Structure* 3 (1995) 503-513.

Giudice, L.C., Kao, L.C.: Endometriosis. *Lancet* 364 (2004) 1789-1799.

Gradishar, W.J.: Tamoxifen--what next? *Oncologist* 9 (2004) 378-384.

Groothuis, P.G., Dassen, H.H., Romano, A., Punyadeera, C.: Estrogen and the endometrium: lessons learned from gene expression profiling in rodents and human. *Hum. Reprod. Update* 13 (2007) 405-417.

Grümmer, R., Schwarzer, F., Bainczyk, K., Hess-Stumpp, H., Regidor, P.A., Schindler, A.E., Winterhager, E.: Peritoneal endometriosis: validation of an *in-vivo* model. *Hum. Reprod.* 16 (2001) 1736-1743.

Gunnarsson, C., Hellqvist, E., Stål, O.: 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* 92 (2005) 547-552.

Gunnarsson, C., Olsson, B.M., Stål, O.: Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* 61 (2001) 8448-8451.

Gustafsson, J.Å.: Estrogen receptor beta--a new dimension in estrogen mechanism of action. *J. Endocrinol.* 163 (1999) 379-383.

Hall, J.M., McDonnell, D.P.: The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 140 (1999) 5566-5578.

Hall, P.F.: Cytochrome P-450 C21scc: one enzyme with two actions: hydroxylase and lyase. *J. Steroid Biochem. Mol. Biol.* 40 (1991) 527-532.

Ham, J., Parker, M.G.: Regulation of gene expression by nuclear hormone receptors. *Curr. Opin. Cell Biol.* 1 (1989) 503-511.

Han, Q., Campbell, R.L., Gangloff, A., Huang, Y.W., Lin, S.X.: Dehydroepiandrosterone and dihydrotestosterone recognition by human estrogenic 17beta-hydroxysteroid dehydrogenase. C-18/c-19 steroid discrimination and enzyme-induced strain. *J. Biol. Chem.* 275 (2000) 1105-1111.

Harris, H.A., Katzenellenbogen, J.A., Katzenellenbogen, B.S.: Characterization of the biological roles of the estrogen receptors, ERalpha and ERbeta, in estrogen target tissues in vivo through the use of an ERalpha-selective ligand. *Endocrinology* 143 (2002) 4172-4177.

He, X.Y., Merz, G., Mehta, P., Schulz, H., Yang, S.Y.: Human brain short chain L-3-hydroxyacyl coenzyme A dehydrogenase is a single-domain multifunctional enzyme. Characterization of a novel 17beta-hydroxysteroid dehydrogenase. *J. Biol. Chem.* 274 (1999) 15014-15019.

He, X.Y., Merz, G., Yang, Y.Z., Mehta, P., Schulz, H., Yang, S.Y.: Characterization and localization of human type 10 17beta-hydroxysteroid dehydrogenase. *Eur. J. Biochem.* 268 (2001) 4899-4907.

He, X.Y., Schulz, H., Yang, S.Y.: A human brain L-3-hydroxyacyl-coenzyme A dehydrogenase is identical to an amyloid beta-peptide-binding protein involved in Alzheimer's disease. *J. Biol. Chem.* 273 (1998) 10741-10746.

Heldring, N., Pawson, T., McDonnell, D., Treuter, E., Gustafsson, J.Å., Pike, A.C.: Structural insights into corepressor recognition by antagonist-bound estrogen receptors. *J. Biol. Chem.* 282 (2007) 10449-10455.

Huang, Y.W., Pineau, I., Chang, H.J., Azzi, A., Bellemare, V., Laberge, S., Lin, S.X.: Critical residues for the specificity of cofactors and substrates in human estrogenic 17beta-hydroxysteroid dehydrogenase 1: variants designed from the three-dimensional structure of the enzyme. *Mol. Endocrinol.* 15 (2001) 2010-2020.

Hurvitz, S.A., Pietras, R.J.: Rational management of endocrine resistance in breast cancer: a comprehensive review of estrogen receptor biology, treatment options, and future directions. *Cancer* 113 (2008) 2385-2397.

Husen, B., Huhtinen, K., Poutanen, M., Kangas, L., Messinger, J., Thole, H.: Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. *Mol. Cell. Endocrinol* 248 (2006a) 109-113.

Husen, B., Huhtinen, K., Saloniemi, T., Messinger, J., Thole, H.H., Poutanen, M.: Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* 147 (2006b) 5333-5339.

Janssen, J.M., Bland, R., Hewison, M., Coughtrie, M.W., Sharp, S., Arts, J., Pols, H.A., van Leeuwen, J.P.: Estradiol formation by human osteoblasts via multiple pathways: relation with osteoblast function. *J. Cell. Biochem.* 75 (1999) 528-537.

Jansson, A.: 17beta-hydroxysteroid dehydrogenase enzymes and breast cancer. *J. Steroid Biochem. Mol. Biol.* (2008) doi:10.1016/j.jsbmb.2008.1012.1012.

Jansson, A.K., Gunnarsson, C., Cohen, M., Sivik, T., Stål, O.: 17beta-hydroxysteroid dehydrogenase 14 affects estradiol levels in breast cancer cells and is a prognostic marker in estrogen receptor-positive breast cancer. *Cancer Res.* 66 (2006) 11471-11477.

Jensen, E.V., Cheng, G., Palmieri, C., Saji, S., Mäkelä, S., Van Noorden, S., Wahlström, T., Warner, M., Coombes, R.C., Gustafsson, J.Å.: Estrogen receptors and proliferation markers in primary and recurrent breast cancer. *Proc. Natl. Acad. Sci. USA* 98 (2001) 15197-15202.

Jerome, C.P., Turner, C.H., Lees, C.J.: Decreased bone mass and strength in ovariectomized cynomolgus monkeys (Macaca fascicularis). *Calcif Tissue Int* 60 (1997) 265-270.

Jez, J.M., Bennett, M.J., Schlegel, B.P., Lewis, M., Penning, T.M.: Comparative anatomy of the aldo-keto reductase superfamily. *Biochem. J.* 326 (Pt 3) (1997) 625-636.

Jin, J.Z., Lin, S.X.: Human estrogenic 17beta-hydroxysteroid dehydrogenase: predominance of estrone reduction and its induction by NADPH. *Biochem. Biophys. Res. Commun.* 259 (1999) 489-493.

Jordan, V.C., O'Malley, B.W.: Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J. Clin. Oncol.* 25 (2007) 5815-5824.

Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., Ghosh, D.: Short-chain dehydrogenases/reductases (SDR). *Biochemistry* 34 (1995) 6003-6013.

Katzenellenbogen, B.S.: Estrogen receptors: bioactivities and interactions with cell signaling pathways. *Biol. Reprod.* 54 (1996) 287-293.

Katzenellenbogen, B.S., Bhardwaj, B., Fang, H., Ince, B.A., Pakdel, F., Reese, J.C., Schodin, D., Wrenn, C.K.: Hormone binding and transcription activation by estrogen

receptors: analyses using mammalian and yeast systems. *J. Steroid Biochem. Mol. Biol.* 47 (1993) 39-48.

Ke, H.Z., Brown, T.A., Qi, H., Crawford, D.T., Simmons, H.A., Petersen, D.N., Allen, M.R., McNeish, J.D., Thompson, D.D.: The role of estrogen receptor-beta, in the early age-related bone gain and later age-related bone loss in female mice. *J. Musculoskelet. Neuronal Interact.* 2 (2002) 479-488.

Keen, J.C., Davidson, N.E.: The biology of breast carcinoma. *Cancer* 97 (2003) 825-833.

Keller, B., Grote, K., Adamski, J.: *In silico* Northern blot, an automated method to determine expression patterns from EST databases, reveals tissue specificity of murine 17beta-hydroxysteroid dehydrogenase type 11. *Mol. Cell. Endocrinol.* 248 (2006) 242-245.

Khanna, M., Qin, K.N., Wang, R.W., Cheng, K.C.: Substrate specificity, gene structure, and tissue-specific distribution of multiple human 3 alpha-hydroxysteroid dehydrogenases. *J. Biol. Chem.* 270 (1995) 20162-20168.

Kitawaki, J., Kado, N., Ishihara, H., Koshiba, H., Kitaoka, Y., Honjo, H.: Endometriosis: the pathophysiology as an estrogen-dependent disease. *J. Steroid Biochem. Mol. Biol.* 83 (2002) 149-155.

Krazeisen, A., Breitling, R., Imai, K., Fritz, S., Möller, G., Adamski, J.: Determination of cDNA, gene structure and chromosomal localization of the novel human 17beta-hydroxysteroid dehydrogenase type 7(1). *FEBS Lett.* 460 (1999) 373-379.

Kruchten, P., Werth, R., Bey, E., Oster, A., Marchais-Oberwinkler, S., Frotscher, M., Hartmann, R.W.: Selective inhibition of 17beta-hydroxysteroid dehydrogenase type 1 (17betaHSD1) reduces estrogen responsive cell growth of T47-D breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 114 (2009a) 200-206.

Kruchten, P., Werth, R., Marchais-Oberwinkler, S., Bey, E., Ziegler, E., Oster, A., Frotscher, M., Hartmann, R.W.: Development of biological assays for the identification of selective 1 inhibitors of estradiol formation from estrone in rat liver preparations. *C. R. Chimie* (2009b) submitted.

Kruchten, P., Werth, R., Marchais-Oberwinkler, S., Frotscher, M., Hartmann, R.W.: Development of a biological screening system for the evaluation of highly active and selective 17β-HSD1-inhibitors as potential therapeutics. *Mol. Cell. Endocrinol.* 301 (2008) 154-157.

Kruchten, P., Werth, R., Marchais-Oberwinkler, S., Frotscher, M., Hartmann, R.W.: Development of a biological screening system for the evaluation of highly active and selective 17β-HSD1-inhibitors as potential therapeutics. *Mol. Cell. Endocrinol.* 301 (2009c) 154-157.

Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J.Å.: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138 (1997) 863-870.

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J.Å.: Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* 93 (1996) 5925-5930.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.R., Chambon, P.: Functional domains of the human estrogen receptor. *Cell* 51 (1987) 941-951.

Labrie, F.: Intracrinology. *Mol. Cell. Endocrinol.* 78 (1991) C113-118.

Labrie, F., Luu-The, V., Lin, S.X., Simard, J., Labrie, C., El-Alfy, M., Pelletier, G., Bélanger, A.: Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease. *J. Mol. Endocrinol.* 25 (2000) 1-16.

Lamminen, T., Saloniemi, T., Huhtinen, K., Koskimies, P., Messinger, J., Husen, B., Thole, H., Poutanen, M.: *In vivo* mouse model for analysis of hydroxysteroid (17beta) dehydrogenase 1 inhibitors. *Mol. Cell. Endocrinol.* (2008).

Langer, L.J., Engel, L.L.: Human placental estradiol-17 beta dehydrogenase. I. Concentration, characterization and assay. *J. Biol. Chem.* 233 (1958) 583-588.

Laplante, Y., Cadot, C., Fournier, M.A., Poirier, D.: Estradiol and estrone C-16 derivatives as inhibitors of type 1 17beta-hydroxysteroid dehydrogenase: blocking of ER+ breast cancer cell proliferation induced by estrone. *Bioorg. Med. Chem.* 16 (2008) 1849-1860.

Laschke, M.W., Elitzsch, A., Scheuer, C., Vollmar, B., Menger, M.D.: Selective cyclo-oxygenase-2 inhibition induces regression of autologous endometrial grafts by down-regulation of vascular endothelial growth factor-mediated angiogenesis and stimulation of caspase-3-dependent apoptosis. *Fertil. Steril.* 87 (2007) 163-171.

Laschke, M.W., Elitzsch, A., Vollmar, B., Menger, M.D.: In vivo analysis of angiogenesis in endometriosis-like lesions by intravital fluorescence microscopy. *Fertil. Steril.* 84 Suppl 2 (2005) 1199-1209.

Leenders, F., Adamski, J., Husen, B., Thole, H.H., Jungblut, P.W.: Molecular cloning and amino acid sequence of the porcine 17 beta-estradiol dehydrogenase. *Eur. J. Biochem.* 222 (1994) 221-227.

Leenders, F., Tesdorpf, J.G., Markus, M., Engel, T., Seedorf, U., Adamski, J.: Porcine 80-kDa protein reveals intrinsic 17 beta-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities. *J. Biol. Chem.* 271 (1996) 5438-5442.

Leyendecker, G., Herbertz, M., Kunz, G., Mall, G.: Endometriosis results from the dislocation of basal endometrium. *Hum. Reprod.* 17 (2002) 2725-2736.

Lin, S.X., Shi, R., Qiu, W., Azzi, A., Zhu, D.W., Dabbagh, H.A., Zhou, M.: Structural basis of the multispecificity demonstrated by 17beta-hydroxysteroid dehydrogenase types 1 and 5. *Mol. Cell. Endocrinol.* 248 (2006) 38-46.

Lin, S.X., Yang, F., Jin, J.Z., Breton, R., Zhu, D.W., Luu-The, V., Labrie, F.: Subunit identity of the dimeric 17 beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem.* 267 (1992) 16182-16187.

Lindberg, M.K., Moverare, S., Skrtic, S., Gao, H., Dahlman-Wright, K., Gustafsson, J.Å., Ohlsson, C.: Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. *Mol. Endocrinol.* 17 (2003) 203-208.

Lipinski, C.A.: Drug-like properties and the causes of poor solubility and poor permeability. *J. Pharmacol. Toxicol. Methods* 44 (2000) 235-249.

Liu, H., Robert, A., Luu-The, V.: Cloning and characterization of human form 2 type 7 17beta-hydroxysteroid dehydrogenase, a primarily 3beta-keto reductase and estrogen activating and androgen inactivating enzyme. *J. Steroid Biochem. Mol. Biol.* 94 (2005) 173-179.

Liu, H., Zheng, S., Bellemare, V., Pelletier, G., Labrie, F., Luu-The, V.: Expression and localization of estrogenic type 12 17beta-hydroxysteroid dehydrogenase in the cynomolgus monkey. *BMC Biochem.* 8 (2007) 2.

Lower, E.E., Blau, R., Gazder, P., Stahl, D.L.: The effect of estrogen usage on the subsequent hormone receptor status of primary breast cancer. *Breast Cancer Res. Treat.* 58 (1999) 205-211.

Lukacik, P., Kavanagh, K.L., Oppermann, U.: Structure and function of human 17beta-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* 248 (2006) 61-71.

Luu-The, V.: Analysis and characteristics of multiple types of human 17beta-hydroxysteroid dehydrogenase. *J. Steroid Biochem. Mol. Biol.* 76 (2001) 143-151.

Luu-The, V., Bélanger, A., Labrie, F.: Androgen biosynthetic pathways in the human prostate. *Best. Pract. Res. Clin. Endocrinol. Metab.* 22 (2008) 207-221.

Luu-The, V., Labrie, C., Simard, J., Lachance, Y., Zhao, H.F., Couet, J., Leblanc, G., Labrie, F.: Structure of two in tandem human 17 beta-hydroxysteroid dehydrogenase genes. *Mol. Endocrinol.* 4 (1990) 268-275.

Luu-The, V., Tremblay, P., Labrie, F.: Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women. *Mol. Endocrinol.* 20 (2006) 437-443.

Luu-The, V., Zhang, Y., Poirier, D., Labrie, F.: Characteristics of human types 1, 2 and 3 17 beta-hydroxysteroid dehydrogenase activities: oxidation/reduction and inhibition. *J. Steroid Biochem. Mol. Biol.* 55 (1995) 581-587.

Luu The, V., Labrie, C., Zhao, H.F., Couet, J., Lachance, Y., Simard, J., Leblanc, G., Cote, J., Bérubé, D., Gagne, R., et al.: Characterization of cDNAs for human estradiol 17 beta-dehydrogenase and assignment of the gene to chromosome 17: evidence of two

mRNA species with distinct 5'-termini in human placenta. *Mol. Endocrinol.* 3 (1989) 1301-1309.

Maaroufi, Y., Lacroix, M., Lespagnard, L., Journe, F., Larsimont, D., Leclercq, G.: Estrogen receptor of primary breast cancers: evidence for intracellular proteolysis. *Breast Cancer Res.* 2 (2000) 444-454.

Mackey, J., McLeod, D., Ragaz, J., Gelmon, K., Verma, S., Pritchard, K., Laing, K., Provencher, L., Charbonneau, L.F.: Adjuvant targeted therapy in early breast cancer. *Cancer* (2009).

Mäkelä, S., Poutanen, M., Lehtimäki, J., Kostian, M.L., Santti, R., Vihko, R.: Estrogen-specific 17 beta-hydroxysteroid oxidoreductase type 1 (E.C. 1.1.1.62) as a possible target for the action of phytoestrogens. *Proc. Soc. Exp. Biol. Med.* 208 (1995) 51-59.

Marchais-Oberwinkler, S., Frotscher, M., Ziegler, E., Werth, R., Kruchten, P., Messinger, J., Thole, H., Hartmann, R.W.: Structure-activity study in the class of 6-(3'-hydroxyphenyl)naphthalenes leading to an optimization of a pharmacophore model for 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) inhibitors. *Mol. Cell. Endocrinol.* 301 (2008a) 205-211.

Marchais-Oberwinkler, S., Kruchten, P., Frotscher, M., Ziegler, E., Neugebauer, A., Bhoga, U., Bey, E., Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W.: Substituted 6-phenyl-2-naphthols. Potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation, and pharmacokinetics. *J. Med. Chem.* 51 (2008b) 4685-4698.

Marijanovic, Z., Laubner, D., Möller, G., Gege, C., Husen, B., Adamski, J., Breitling, R.: Closing the gap: identification of human 3-ketosteroid reductase, the last unknown enzyme of mammalian cholesterol biosynthesis. *Mol. Endocrinol.* 17 (2003) 1715-1725.

Márquez, D.C., Pietras, R.J.: Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* 20 (2001) 5420-5430.

Martel, C., Rhéaume, E., Takahashi, M., Trudel, C., Couet, J., Luu-The, V., Simard, J., Labrie, F.: Distribution of 17 beta-hydroxysteroid dehydrogenase gene expression and activity in rat and human tissues. *J. Steroid Biochem. Mol. Biol.* 41 (1992) 597-603.

Martin, M., Pienkowski, T., Mackey, J., Pawlicki, M., Guastalla, J.P., Weaver, C., Tomiak, E., Al-Tweigeri, T., Chap, L., Juhos, E., Guevin, R., Howell, A., Fornander, T., Hainsworth, J., Coleman, R., Vinholes, J., Modiano, M., Pinter, T., Tang, S.C., Colwell, B., Prady, C., Provencher, L., Walde, D., Rodriguez-Lescure, A., Hugh, J., Loret, C., Rupin, M., Blitz, S., Jacobs, P., Murawsky, M., Riva, A., Vogel, C.: Adjuvant docetaxel for node-positive breast cancer. *N. Engl. J. Med.* 352 (2005) 2302-2313.

Matthews, J., Gustafsson, J.Å.: Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol. Interv.* 3 (2003) 281-292.

Mazza, C., Breton, R., Housset, D., Fontecilla-Camps, J.C.: Unusual charge stabilization of $NADP^+$ in 17beta-hydroxysteroid dehydrogenase. *J. Biol. Chem.* 273 (1998) 8145-8152.

Mendelsohn, M.E., Karas, R.H.: The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* 340 (1999) 1801-1811.

Menger, M.D., Laschke, M.W., Vollmar, B.: Viewing the microcirculation through the window: some twenty years experience with the hamster dorsal skinfold chamber. *Eur. Surg. Res.* 34 (2002) 83-91.

Messinger, J., Hirvelä, L., Husen, B., Kangas, L., Koskimies, P., Pentikäinen, O., Saarenketo, P., Thole, H.: New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* 248 (2006) 192-198.

Miettinen, M.M., Mustonen, M.V., Poutanen, M.H., Isomaa, V.V., Vihko, R.K.: Human 17 beta-hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured cells and characteristic cell- and tissue-specific expression. *Biochem. J.* 314 (Pt 3) (1996) 839-845.

Miki, Y., Suzuki, T., Sasano, H.: Intracrinology of sex-steroids in ductal carcinoma *in situ* (DCIS) of human breast: Comparison to invasive ductal carcinoma (IDC) and non-

neoplastic breast. *J. Steroid Biochem. Mol. Biol.* doi:10.1016/j.jsbmb.2008.12.021 (2008).

Miller, W.R., Bartlett, J.M., Canney, P., Verrill, M.: Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. Treat.* 103 (2007) 149-160.

Miller, W.R., O'Neill, J.: The importance of local synthesis of estrogen within the breast. *Steroids* 50 (1987) 537-548.

Miyoshi, Y., Ando, A., Hasegawa, S., Ishitobi, M., Taguchi, T., Tamaki, Y., Noguchi, S.: High expression of steroid sulfatase mRNA predicts poor prognosis in patients with estrogen receptor-positive breast cancer. *Clin. Cancer Res.* 9 (2003) 2288-2293.

Miyoshi, Y., Ando, A., Shiba, E., Taguchi, T., Tamaki, Y., Noguchi, S.: Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* 94 (2001) 685-689.

Moghrabi, N.A., S.: 17ß-Hydroxysteroid Dehydrogenases: Physiological Roles in Health and Disease. *TEM* Vol. 9 (1998) 265-270.

Möller, G., Adamski, J.: Multifunctionality of human 17beta-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* 248 (2006) 47-55.

Montano, M.M., Ekena, K., Krueger, K.D., Keller, A.L., Katzenellenbogen, B.S.: Human estrogen receptor ligand activity inversion mutants: receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens. *Mol. Endocrinol.* 10 (1996) 230-242.

Moon, Y.A., Horton, J.D.: Identification of two mammalian reductases involved in the two-carbon fatty acyl elongation cascade. *J. Biol. Chem.* 278 (2003) 7335-7343.

Morani, A., Warner, M., Gustafsson, J.A.: Biological functions and clinical implications of oestrogen receptors alfa and beta in epithelial tissues. *J. Intern. Med.* 264 (2008) 128-142.

Moreau, F., Mittre, H., Benhaim, A., Bois, C., Bertherat, J., Carreau, S., Reznik, Y.: Aromatase expression in the normal human adult adrenal and in adrenocortical tumors: biochemical, immunohistochemical, and molecular studies. *Eur. J. Endocrinol.* 160 (2008) 93-99.

Mosselman, S., Polman, J., Dijkema, R.: ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* 392 (1996) 49-53.

Murdoch, F.E., Gorski, J.: The role of ligand in estrogen receptor regulation of gene expression. *Mol. Cell. Endocrinol.* 78 (1991) C103-108.

Nagasaki, S., Suzuki, T., Miki, Y., Akahira, J.I., Kitada, K., Ishida, T., Handa, H., Ohuchi, N., Sasano, H.: 17{beta}-Hydroxysteroid Dehydrogenase Type 12 in Human Breast Carcinoma: A Prognostic Factor via Potential Regulation of Fatty Acid Synthesis. *Cancer Res.* (2009).

Nakamura, J., Savinov, A., Lu, Q., Brodie, A.: Estrogen regulates vascular endothelial growth/permeability factor expression in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors. *Endocrinology* 137 (1996) 5589-5596.

Nandi, S., Guzman, R.C., Yang, J.: Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc. Natl. Acad. Sci. USA* 92 (1995) 3650-3657.

Nap, A.W., Groothuis, P.G., Demir, A.Y., Evers, J.L., Dunselman, G.A.: Pathogenesis of endometriosis. *Best. Pract. Res. Clin. Obstet. Gynaecol.* 18 (2004) 233-244.

Navot, D., Bergh, P.: Preparation of the human endometrium for implantation. *Ann. N Y Acad. Sci.* 622 (1991) 212-219.

Nelson, L.R., Bulun, S.E.: Estrogen production and action. *J. Am. Acad. Dermatol.* 45 (2001) S116-124.

Nemere, I., Pietras, R.J., Blackmore, P.F.: Membrane receptors for steroid hormones: signal transduction and physiological significance. *J. Cell. Biochem.* 88 (2003) 438-445.

Nilsson, S., Gustafsson, J.Å.: Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. *Breast Cancer Res.* 2 (2000) 360-366.

Normand, T., Husen, B., Leenders, F., Pelczar, H., Baert, J.L., Begue, A., Flourens, A.C., Adamski, J., de Launoit, Y.: Molecular characterization of mouse 17 beta-hydroxysteroid dehydrogenase IV. *J. Steroid Biochem. Mol. Biol.* 55 (1995) 541-548.

Ohnesorg, T., Adamski, J.: Analysis of the 5' flanking regions of human and murine HSD17B7: identification of a cholesterol dependent enhancer region. *Mol. Cell. Endocrinol.* 248 (2006) 164-167.

Ohnesorg, T., Keller, B., Hrabe de Angelis, M., Adamski, J.: Transcriptional regulation of human and murine 17beta-hydroxysteroid dehydrogenase type-7 confers its participation in cholesterol biosynthesis. *J. Mol. Endocrinol.* 37 (2006) 185-197.

Ohno, S., Nishikawa, K., Honda, Y., Nakajin, S.: Expression in *E. coli* and tissue distribution of the human homologue of the mouse Ke 6 gene, 17beta-hydroxysteroid dehydrogenase type 8. *Mol. Cell. Biochem.* 309 (2008) 209-215.

Osborne, C.K., Wakeling, A., Nicholson, R.I.: Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br. J. Cancer* 90 Suppl 1 (2004) S2-6.

Ozers, M.S., Ervin, K.M., Steffen, C.L., Fronczak, J.A., Lebakken, C.S., Carnahan, K.A., Lowery, R.G., Burke, T.J.: Analysis of ligand-dependent recruitment of coactivator peptides to estrogen receptor using fluorescence polarization. *Mol. Endocrinol.* 19 (2005) 25-34.

Ozkan, S., Murk, W., Arici, A.: Endometriosis and infertility: epidemiology and evidence-based treatments. *Ann. N Y Acad. Sci.* 1127 (2008) 92-100.

Page, D.L., Dupont W.D., Rogers L.W., al., e.: Atypical Hyperplastic Lesions of the Female Breast

A Long-Term Follow-Up Study. *Cancer* 55 (1984) 2698-2708.

Palmieri, C., Cheng, G.J., Saji, S., Zelada-Hedman, M., Warri, A., Weihua, Z., Van Noorden, S., Wahlström, T., Coombes, R.C., Warner, M., Gustafsson, J.Å.: Estrogen receptor beta in breast cancer. *Endocr. Relat. Cancer* 9 (2002) 1-13.

Pasqualini, J.R., Chetrite, G., Blacker, C., Feinstein, M.C., Delalonde, L., Talbi, M., Maloche, C.: Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *J. Clin. Endocrinol. Metab.* 81 (1996) 1460-1464.

Pasqualini, J.R., Gelly, C., Nguyen, B.L., Vella, C.: Importance of estrogen sulfates in breast cancer. *J. Steroid Biochem.* 34 (1989) 155-163.

Pasqualini, J.R., Schatz, B., Varin, C., Nguyen, B.L.: Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer. *J. Steroid Biochem. Mol. Biol.* 41 (1992) 323-329.

Pelletier, G., Luu-The, V., Li, S., Labrie, F.: Localization of type 8 17beta-hydroxysteroid dehydrogenase mRNA in mouse tissues as studied by in situ hybridization. *J. Histochem. Cytochem.* 53 (2005) 1257-1271.

Peltoketo, H., Isomaa, V., Maentausta, O., Vihko, R.: Complete amino acid sequence of human placental 17 beta-hydroxysteroid dehydrogenase deduced from cDNA. *FEBS Lett.* 239 (1988) 73-77.

Peltoketo, H., Luu-The, V., Simard, J., Adamski, J.: 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J. Mol. Endocrinol.* 23 (1999a) 1-11.

Peltoketo, H., Nokelainen, P., Piao, Y.S., Vihko, R., Vihko, P.: Two 17beta-hydroxysteroid dehydrogenases (17HSDs) of estradiol biosynthesis: 17HSD type 1 and type 7. *J. Steroid Biochem. Mol. Biol.* 69 (1999b) 431-439.

Penning, T.M.: 17ß-Hydroxysteroid dehydrogenase: inhibitors and inhibitor design. *Endocr. Relat. Cancer* 3 (1996) 41-56.

Penning, T.M.: Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Rev.* 18 (1997) 281-305.

Penning, T.M.: Hydroxysteroid dehydrogenases and pre-receptor regulation of steroid hormone action. *Hum. Reprod. Update* 9 (2003) 193-205.

Penning, T.M., Burczynski, M.E., Jez, J.M., Hung, C.F., Lin, H.K., Ma, H., Moore, M., Palackal, N., Ratnam, K.: Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and

tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem. J.* 351 (2000) 67-77.

Pepe, G.J., Albrecht, E.D.: Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr. Rev.* 16 (1995) 608-648.

Perez, E.A.: Safety of aromatase inhibitors in the adjuvant setting. *Breast. Cancer Res. Treat.* 105 Suppl 1 (2007) 75-89.

Picard, D., Yamamoto, K.R.: Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.* 6 (1987) 3333-3340.

Pletnev, V.Z., Weeks, C.M., Duax, W.L.: Rational proteomics II: electrostatic nature of cofactor preference in the short-chain oxidoreductase (SCOR) enzyme family. *Proteins* 57 (2004) 294-301.

Poirier, D.: Inhibitors of 17 beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* 10 (2003) 453-477.

Poirier, D., Bydal, P., Tremblay, M.R., Sam, K.M., Luu-The, V.: Inhibitors of type II 17beta-hydroxysteroid dehydrogenase. *Mol. Cell. Endocrinol.* 171 (2001) 119-128.

Poutanen, M., Miettinen, M., Vihko, R.: Differential estrogen substrate specificities for transiently expressed human placental 17 beta-hydroxysteroid dehydrogenase and an endogenous enzyme expressed in cultured COS-m6 cells. *Endocrinology* 133 (1993) 2639-2644.

Pratt, W.B., Jolly, D.J., Pratt, D.V., Hollenberg, S.M., Giguere, V., Cadepond, F.M., Schweizer-Groyer, G., Catelli, M.G., Evans, R.M., Baulieu, E.E.: A region in the steroid binding domain determines formation of the non-DNA-binding, 9 S glucocorticoid receptor complex. *J. Biol. Chem.* 263 (1988) 267-273.

Prehn, C., Möller, G., Adamski, J.: Recent advances in 17beta-hydroxysteroid dehydrogenases. *J. Steroid Biochem. Mol. Biol.* doi:10.1016/j.jsbmb.2008.12.024 (2008).

Puranen, T., Poutanen, M., Ghosh, D., Vihko, P., Vihko, R.: Characterization of structural and functional properties of human 17 beta-hydroxysteroid dehydrogenase type 1 using recombinant enzymes and site-directed mutagenesis. *Mol. Endocrinol.* 11 (1997) 77-86.

Puranen, T.J., Kurkela, R.M., Lakkakorpi, J.T., Poutanen, M.H., Itäranta, P.V., Melis, J.P., Ghosh, D., Vihko, R.K., Vihko, P.T.: Characterization of molecular and catalytic properties of intact and truncated human 17beta-hydroxysteroid dehydrogenase type 2 enzymes: intracellular localization of the wild-type enzyme in the endoplasmic reticulum. *Endocrinology* 140 (1999) 3334-3341.

Puranen, T.J., Poutanen, M.H., Peltoketo, H.E., Vihko, P.T., Vihko, R.K.: Site-directed mutagenesis of the putative active site of human 17 beta-hydroxysteroid dehydrogenase type 1. *Biochem. J.* 304 (Pt 1) (1994) 289-293.

Purohit, A., Flanagan, A.M., Reed, M.J.: Estrogen synthesis by osteoblast cell lines. *Endocrinology* 131 (1992) 2027-2029.

Purohit, A., Fusi, L., Brosens, J., Woo, L.W., Potter, B.V., Reed, M.J.: Inhibition of steroid sulphatase activity in endometriotic implants by 667 COUMATE: a potential new therapy. *Hum. Reprod.* 23 (2008) 290-297.

Purohit, A., Woo, L.W., Potter, B.V., Reed, M.J.: *In vivo* inhibition of estrone sulfatase activity and growth of nitrosomethylurea-induced mammary tumors by 667 COUMATE. *Cancer Res.* 60 (2000) 3394-3396.

Qiu, W., Campbell, R.L., Gangloff, A., Dupuis, P., Boivin, R.P., Tremblay, M.R., Poirier, D., Lin, S.X.: A concerted, rational design of type 1 17beta-hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity. *Faseb J.* 16 (2002) 1829-1831.

Rao, M.C., Midgley, A.R., Jr., Richards, J.S.: Hormonal regulation of ovarian cellular proliferation. *Cell* 14 (1978) 71-78.

Redeuilh, G., Attia, A., Mester, J., Sabbah, M.: Transcriptional activation by the oestrogen receptor alpha is modulated through inhibition of cyclin-dependent kinases. *Oncogene* 21 (2002) 5773-5782.

Reed, M.J., Purohit, A., Woo, L.W., Newman, S.P., Potter, B.V.: Steroid sulfatase: molecular biology, regulation, and inhibition. *Endocr Rev* 26 (2005) 171-202.

Richards, J.S.: Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiol. Rev.* 60 (1980) 51-89.

Richards, J.S., Russell, D.L., Robker, R.L., Dajee, M., Alliston, T.N.: Molecular mechanisms of ovulation and luteinization. *Mol. Cell. Endocrinol.* 145 (1998) 47-54.

Riesewijk, A., Martín, J., van Os, R., Horcajadas, J.A., Polman, J., Pellicer, A., Mosselman, S., Simón, C.: Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol. Hum. Reprod.* 9 (2003) 253-264.

Robker, R.L., Richards, J.S.: Hormonal control of the cell cycle in ovarian cells: proliferation versus differentiation. *Biol. Reprod.* 59 (1998) 476-482.

Russo, J., Fernandez, S.V., Russo, P.A., Fernbaugh, R., Sheriff, F.S., Lareef, H.M., Garber, J., Russo, I.H.: 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB J.* 20 (2006) 1622-1634.

Saadat, M., Truong, P.T., Kader, H.A., Speers, C.H., Berthelet, E., McMurtrie, E., Olivotto, I.A.: Outcomes in patients with primary breast cancer and a subsequent diagnosis of endometrial cancer: comparison of cohorts treated with and without tamoxifen. *Cancer* 110 (2007) 31-37.

Saji, S., Hirose, M., Toi, M.: Clinical significance of estrogen receptor beta in breast cancer. *Cancer Chemother. Pharmacol.* 56 Suppl 1 (2005) 21-26.

Saloniemi, T., Lamminen, T., Huhtinen, K., Welsh, M., Saunders, P., Kujari, H., Poutanen, M.: Activation of androgens by hydroxysteroid (17beta) dehydrogenase 1 *in vivo* as a cause of prenatal masculinization and ovarian benign serous cystadenomas. *Mol. Endocrinol.* 21 (2007) 2627-2636.

Sam, K.M., Auger, S., Luu-The, V., Poirier, D.: Steroidal spiro-gamma-lactones that inhibit 17 beta-hydroxysteroid dehydrogenase activity in human placental microsomes. *J. Med. Chem.* 38 (1995) 4518-4528.

Sampson, J.A.: Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am. J. Obstet. Gynecol.* 14 (1927) 422-469.

Sasano, H., Okamoto, M., Mason, J.I., Simpson, E.R., Mendelson, C.R., Sasano, N., Silverberg, S.G.: Immunolocalization of aromatase, 17 alpha-hydroxylase and side-chain-cleavage cytochromes P-450 in the human ovary. *J. Reprod. Fertil.* 85 (1989) 163-169.

Sasano, H., Suzuki, T., Takeyama, J., Utsunomiya, H., Ito, K., Ariga, N., Moriya, T.: 17-beta-hydroxysteroid dehydrogenase in human breast and endometrial carcinoma. A new development in intracrinology. *Oncology* 59 Suppl 1 (2000) 5-12.

Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J.Å., Safe, S.: Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *J. Biol. Chem.* 275 (2000) 5379-5387.

Sawicki, M.W., Erman, M., Puranen, T., Vihko, P., Ghosh, D.: Structure of the ternary complex of human 17beta-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and $NADP^+$. *Proc. Natl. Acad. Sci. USA* 96 (1999) 840-845.

Schlegel, B.P., Jez, J.M., Penning, T.M.: Mutagenesis of 3 alpha-hydroxysteroid dehydrogenase reveals a "push-pull" mechanism for proton transfer in aldo-keto reductases. *Biochemistry* 37 (1998) 3538-3548.

Scotti, S., Regidor, P.A., Schindler, A.E., Winterhager, E.: Reduced proliferation and cell adhesion in endometriosis. *Mol. Hum. Reprod.* 6 (2000) 610-617.

Shaaban, A.M., O'Neill, P.A., Davies, M.P., Sibson, R., West, C.R., Smith, P.H., Foster, C.S.: Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. *Am. J. Surg. Pathol.* 27 (2003) 1502-1512.

Shafqat, N., Marschall, H.U., Filling, C., Nordling, E., Wu, X.Q., Björk, L., Thyberg, J., Mårtensson, E., Salim, S., Jörnvall, H., Oppermann, U.: Expanded substrate screenings of human and Drosophila type 10 17beta-hydroxysteroid dehydrogenases (HSDs) reveal multiple specificities in bile acid and steroid hormone metabolism: characterization of multifunctional 3alpha/7alpha/7beta/17beta/20beta/21-HSD. *Biochem. J.* 376 (2003) 49-60.

Shen, Z., Peng, Z., Sun, Y., Vaananen, H.K., Poutanen, M.: Overexpression of human hydroxysteroid (17beta) dehydrogenase 2 induces disturbance in skeletal development in young male mice. *J. Bone Miner. Res.* 23 (2008) 1217-1226.

Sherbet, D.P., Papari-Zareei, M., Khan, N., Sharma, K.K., Brandmaier, A., Rambally, S., Chattopadhyay, A., Andersson, S., Agarwal, A.K., Auchus, R.J.: Cofactors, redox state, and directional preferences of hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* 265-266 (2007) 83-88.

Shi, R., Lin, S.X.: Cofactor hydrogen bonding onto the protein main chain is conserved in the short chain dehydrogenase/reductase family and contributes to nicotinamide orientation. *J. Biol. Chem.* 279 (2004) 16778-16785.

Shifren, J.L., Tseng, J.F., Zaloudek, C.J., Ryan, I.P., Meng, Y.G., Ferrara, N., Jaffe, R.B., Taylor, R.N.: Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J. Clin. Endocrinol. Metab.* 81 (1996) 3112-3118.

Shim, W.S., Conaway, M., Masamura, S., Yue, W., Wang, J.P., Kmar, R., Santen, R.J.: Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells *in vivo*. *Endocrinology* 141 (2000) 396-405.

Sillem, M., Hahn, U., Coddington, C.C., 3rd, Gordon, K., Runnebaum, B., Hodgen, G.D.: Ectopic growth of endometrium depends on its structural integrity and proteolytic activity in the cynomolgus monkey (*Macaca fascicularis*) model of endometriosis. *Fertil. Steril.* 66 (1996) 468-473.

Simpson, E.R.: Sources of estrogen and their importance. *J. Steroid Biochem. Mol. Biol.* 86 (2003) 225-230.

Smith, C.L., Nawaz, Z., O'Malley, B.W.: Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol. Endocrinol.* 11 (1997a) 657-666.

Smith, D., Ackland, M., Jones, B.: Properties of cytochrome P450 isoenzymes and their substrates Part 2: properties of cytochrome P450 substrates. *Drug Discov. Today* 2 (1997b) 479-486.

Šmuc, T., Pucelj, M.R., Šinkovec, J., Husen, B., Thole, H., Lanišnik Rižner, T.: Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* 23 (2007) 105-111.

Šmuc, T., Rižner, T.L.: Expression of 17β-hydroxysteroid dehydrogenases and other estrogen-metabolizing enzymes in different cancer cells. *Chem. Biol. Interact.* (2008) doi:10.1016/j.cbi.2008.1010.1038.

Song, D., Liu, G., Luu-The, V., Zhao, D., Wang, L., Zhang, H., Xueling, G., Li, S., Desy, L., Labrie, F., Pelletier, G.: Expression of aromatase and 17beta-hydroxysteroid dehydrogenase types 1, 7 and 12 in breast cancer. An immunocytochemical study. *J. Steroid Biochem. Mol. Biol.* 101 (2006) 136-144.

Song, R.X.: Membrane-initiated steroid signaling action of estrogen and breast cancer. *Semin. Reprod. Med.* 25 (2007) 187-197.

Stanway, S.J., Purohit, A., Woo, L.W., Sufi, S., Vigushin, D., Ward, R., Wilson, R.H., Stanczyk, F.Z., Dobbs, N., Kulinskaya, E., Elliott, M., Potter, B.V., Reed, M.J., Coombes, R.C.: Phase I study of STX 64 (667 Coumate) in breast cancer patients: the first study of a steroid sulfatase inhibitor. *Clin. Cancer Res.* 12 (2006) 1585-1592.

Ström, A., Hartman, J., Foster, J.S., Kietz, S., Wimalasena, J., Gustafsson, J.Å.: Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc. Natl. Acad. Sci. USA* 101 (2004) 1566-1571.

Su, E.J., Cheng, Y.H., Chatterton, R.T., Lin, Z.H., Yin, P., Reierstad, S., Innes, J., Bulun, S.E.: Regulation of 17-beta Hydroxysteroid Dehydrogenase Type 2 in Human Placental Endothelial Cells. *Biol. Reprod.* (2007).

Subramanian, A., Salhab, M., Mokbel, K.: Oestrogen producing enzymes and mammary carcinogenesis: a review. *Breast Cancer Res. Treat.* 111 (2008) 191-202.

Suzuki, T., Miki, Y., Nakamura, Y., Moriya, T., Ito, K., Ohuchi, N., Sasano, H.: Sex steroid-producing enzymes in human breast cancer. *Endocr. Relat. Cancer* 12 (2005) 701-720.

Suzuki, T., Moriya, T., Ariga, N., Kaneko, C., Kanazawa, M., Sasano, H.: 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* 82 (2000) 518-523.

Thomas, D.B.: Do hormones cause breast cancer? *Cancer* 53 (1984) 595-604.

Thompson, E.A., Jr., Siiteri, P.K.: The involvement of human placental microsomal cytochrome P-450 in aromatization. *J. Biol. Chem.* 249 (1974) 5373-5378.

Törn, S., Nokelainen, P., Kurkela, R., Pulkka, A., Menjivar, M., Ghosh, S., Coca-Prados, M., Peltoketo, H., Isomaa, V., Vihko, P.: Production, purification, and functional analysis of recombinant human and mouse 17beta-hydroxysteroid dehydrogenase type 7. *Biochem. Biophys. Res. Commun.* 305 (2003) 37-45.

Travis, R.C., Key, T.J.: Oestrogen exposure and breast cancer risk. *Breast Cancer Res.* 5 (2003) 239-247.

Tsai, S.J., Wu, M.H., Lin, C.C., Sun, H.S., Chen, H.M.: Regulation of steroidogenic acute regulatory protein expression and progesterone production in endometriotic stromal cells. *J. Clin. Endocrinol. Metab.* 86 (2001) 5765-5773.

Tseng, L., Mazella, J.: Kinetic studies of human endometrial hydroxysteroid dehydrogenase. *J. Steroid Biochem.* 14 (1981) 437-442.

Turner, R.T., Riggs, B.L., Spelsberg, T.C.: Skeletal effects of estrogen. *Endocr. Rev.* 15 (1994) 275-300.

Urruticoechea, A.: The oestrogen-dependent biology of breast cancer. Sensitivity and resistance to aromatase inhibitors revisited: a molecular perspective. *Clin. Transl. Oncol.* 9 (2007) 752-759.

Utsumi, T., Yoshimura, N., Takeuchi, S., Ando, J., Maruta, M., Maeda, K., Harada, N.: Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer. *Cancer Res.* 59 (1999) 377-381.

Utsumi, T., Yoshimura, N., Takeuchi, S., Maruta, M., Maeda, K., Harada, N.: Elevated steroid sulfatase expression in breast cancers. *J. Steroid Biochem. Mol. Biol.* 73 (2000) 141-145.

van Landeghem, A.A., Poortman, J., Nabuurs, M., Thijssen, J.H.: Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Res.* 45 (1985) 2900-2906.

van Veldhoven, P.P., Croes, K., Asselberghs, S., Herdewijn, P., Mannaerts, G.P.: Peroxisomal beta-oxidation of 2-methyl-branched acyl-CoA esters: stereospecific recognition of the 2S-methyl compounds by trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase. *FEBS Lett.* 388 (1996) 80-84.

Vegeto, E., Allan, G.F., Schrader, W.T., Tsai, M.J., McDonnell, D.P., O'Malley, B.W.: The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* 69 (1992) 703-713.

Vicker, N., Lawrence, H., Allan, G., et al.: Focused Libraries of 16-Substituted Estrone Derivatives and Modified E-Ring Steroids: Inhibitors of 17ß-Hydroxysteroid-Dehydrogenase Type 1. *Chem. Med. Chem.* (2006) 464-481.

Vihko, P., Härkönen, P., Oduwole, O., Törn, S., Kurkela, R., Porvari, K., Pulkka, A., Isomaa, V.: 17 beta-hydroxysteroid dehydrogenases and cancers. *J. Steroid Biochem. Mol. Biol.* 83 (2003) 119-122.

Vihko, P., Härkönen, P., Soronen, P., Törn, S., Herrala, A., Kurkela, R., Pulkka, A., Oduwole, O., Isomaa, V.: 17 beta-hydroxysteroid dehydrogenases--their role in pathophysiology. *Mol. Cell. Endocrinol.* 215 (2004) 83-88.

Vihko, P., Isomaa, V., Ghosh, D.: Structure and function of 17beta-hydroxysteroid dehydrogenase type 1 and type 2. *Mol. Cell. Endocrinol.* 171 (2001) 71-76.

Walter, P., Green, S., Greene, G., Krust, A., Bornert, J.M., Jeltsch, J.M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., et al.: Cloning of the human estrogen receptor cDNA. *Proc. Natl. Acad. Sci. USA* 82 (1985) 7889-7893.

Wasada, T., Akamine, Y., Kato, K., Ibayashi, H., Nomura, Y.: Adrenal contribution to circulating estrogens in woman. *Endocrinol. Jpn.* 25 (1978) 123-128.

Webster, N.J., Green, S., Jin, J.R., Chambon, P.: The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* 54 (1988) 199-207.

Weihua, Z., Saji, S., Makinen, S., Cheng, G., Jensen, E.V., Warner, M., Gustafsson, J.Å.: Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. *Proc. Natl. Acad. Sci. USA* 97 (2000) 5936-5941.

Williamson, D.H., Lund, P., Krebs, H.A.: The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* 103 (1967) 514-527.

Wu, L., Einstein, M., Geissler, W.M., Chan, H.K., Elliston, K.O., Andersson, S.: Expression cloning and characterization of human 17 beta-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20 alpha-hydroxysteroid dehydrogenase activity. *J. Biol. Chem.* 268 (1993) 12964-12969.

Wu, X., Lukacik, P., Kavanagh, K.L., Oppermann, U.: SDR-type human hydroxysteroid dehydrogenases involved in steroid hormone activation. *Mol. Cell. Endocrinol.* 265-266 (2007) 71-76.

Yang, J.Z., Agarwal, S.K., Foster, W.G.: Subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin modulates the pathophysiology of endometriosis in the cynomolgus monkey. *Toxicol. Sci.* 56 (2000) 374-381.

Yee, S.: In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man--fact or myth. *Pharm. Res.* 14 (1997) 763-766.

Zeitoun, K., Takayama, K., Sasano, H., Suzuki, T., Moghrabi, N., Andersson, S., Johns, A., Meng, L., Putman, M., Carr, B., Bulun, S.E.: Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol. *J. Clin. Endocrinol. Metab.* 83 (1998) 4474-4480.

Zhu, D.W., Lee, X., Breton, R., Ghosh, D., Pangborn, W., Daux, W.L., Lin, S.X.: Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17 beta-hydroxysteroid dehydrogenase with $NADP^+$. *J. Mol. Biol.* 234 (1993) 242-244.