Synthesis and evaluation of hepatitis C antivirals: Viral E2 glycoprotein / human CD81 receptor interaction inhibitors

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Meiner Oma
† 08.03.2008

‘Die höchste Form des Glücks ist ein Leben
mit einem gewissen Grad an Verrücktheit.’
Erasmus von Rotterdam

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The present work is divided into three publications:

1. **Structural modifications of salicylates: Inhibitors of human CD81-receptor HCV-E2 interaction**

   Marcel Holzer, Sigrid Ziegler, Alexander Neugebauer, Bernd Kronenberger, Christian D. Klein, Rolf W. Hartmann

   Archiv der Pharmazie, **2008**, *341*, 478-484

2. **Microwave-assisted syntheses of amino acid ester substituted benzoic acid amides: Potential inhibitors of human CD81-receptor HCV-E2 interaction**

   Marcel Holzer, Sigrid Ziegler, Bernd Kronenberger, Christian D. Klein, Rolf W. Hartmann

   The Open Medicinal Chemistry Journal, **2008**, *2,* 21-25

3. **Identification of terfenadine as an inhibitor of human CD81-receptor HCV-E2 interaction: Synthesis and structure optimization**

   Marcel Holzer, Sigrid Ziegler, Beatrice Albrecht, Bernd Kronenberger, Artur Kaul, Ralf Bartenschlager, Lars Kattner, Christian D. Klein, Rolf W. Hartmann

   Molecules **2008**, *13*, 1081-1110

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1. List of abbreviations

AA	Amino acid
$AlCl_3$	Aluminium trichloride
AN	Antibody neutralization
Ar	Aromat
Asn	Asparagine
Bu	Buthyl
CH_2Cl_2	Dichloromethane
DCC	N,N'-dicyclohexyl carbodiimide
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DGJ	Deoxygalactonojirimycin
DMSO	Dimethyl sulfoxide
DNJ	Deoxynojirimycin
Et	Ethyl
et al	Et alii
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FC	Friedel-Crafts
FCS	Fetal calf serum
g	Gram
h	Hour
H_2O	Water
HPLC	High performance liquid chromatography
H-bond	Hydrogen-bond
HCV	Hepatitis C virus
Het	Heterocycle
iBu	Iso-butyl
IFN	Interferon
Ile	Isoleucine
IR	Infrared
IRES	Internal ribosome entry site
KI	Potassium iodide
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LDLR	Low density lipoprotein receptor
LEL	Large extracellular loop
Leu	Leucine
Lit.	Literature
L-SIGN	Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
Me	Methyl
MeOH	Methanol
MFI	Mean fluorescence intensity
MHz	Megahertz

min	Minutes
mL	Milliliter
mM	Millimolar
Mp	Melting point
$NaBH_4$	Sodium borohydride
Na_2CO_3	Sodium carbonate
NEt_3	Triethylamine
NH_4Cl	Ammonium chloride
NMR	Nuclear magnetic resonance
NS	Non-structural
PBS	Phosphate buffered saline buffer
PDB ID	Protein data bank identification
$Pd(PPh_3)_4$	Tetrakis-(triphenylphosphine) palladium (0)
PEG	Pegylated
Phe	Phenylalanine
ppm	Parts per Million
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
rt	Room temperature
SAR	Structure activity relationship
SEL	Small extracellular loop
$SOCl_2$	Thionyl chloride
SR-BI	Scavenger receptor class B type I
SVR	Sustained virologic response
tBu	Tert-butyl
VLDLR	Very low density lipoprotein receptor
WHO	World health organization
µL	Microliter
µM	Micromolar

2. Introduction

2.1 Hepatitis

Hepatitis (from Greek: hepar = liver and itis = inflammation) implies a systemic illness, in which the liver is predominantly affected. The hepatitis is associated with changes of clinical, biochemical and serological parameters and inflammatory cells in the liver tissue. Viruses, bacteria, parasites, drugs and alcohol can cause this disease. The viral hepatitis is one of the most common infectious diseases worldwide, caused by viruses A-E. Concerning the progression a differentiation between acute and chronic hepatitis is possible.[1]

2.2 Course of viral hepatitis

2.2.1 Acute viral hepatitis

Clinically, there is no possibility to differentiate between acute infections with the hepatitis viruses A-E. Concerning the gentlest form of acute hepatitis, patients show no characteristic inflammation symptoms at all, therefore the infection can only be verified by detection of increased transaminases in the blood. The incubation time varies between 2-26 weeks, depending on virus type and is free of symptoms. An uncharacteristic, flu-like prodromal stadium for 3-4 days follows. In this stadium the patients suffer from fatigue, anorexia, sickness, vomiting, abdominal pain and pression. The prodromal stadium can be followed by a 1-4 weeks lasting icteric stadium, characterized by darkened urine, decolorized feces and pruritus.[1]
The progression of an acute hepatitis has to be monitored to avoid the development of a fulminant hepatitis which occurs for example in 0.5-1 % for the hepatitis C.[1]

2.2.2 Chronic viral hepatitis

The majority of the acute viral hepatitides heals without further intricacies. However, an infection with the hepatitis viruses B, C and D may lead to a chronic course, whereas a chronic hepatitis is characterized by an inflammation of the liver lasting longer than 6 months and histopathological changes of the liver. Frequently, the patients are free from

symptoms or show unspecific ailments like abnormal fatigue and malaise. Normally, detection of increased transaminases is the only noticeable proof for a chronic hepatitis.[1]
The course of the disease ranges from an inflammation that stays below the surface of clinical detection to a rapidly proceeding hepatitis passing into cirrhosis which may lead to hepatocellular carcinoma.[1]

2.3 Hepatitis C

Hepatitis C (HC) is an inflammation of the liver caused by the hepatitis C virus (HCV) and is the major cause of liver transplantation in Europe.[2] It is estimated that 3 % of the world's population, more than 170 million people, are infected with HCV[3] (Germany: 400,000-500,000)[4]. The transmission of the disease predominantly occurs parenteral by intravenous drug abuse, sexual intercourse, piercing, tattoos and dialysis.[5] With the advent of routine blood screening for HCV antibodies (since 1991 in most countries) the risk of infection by the use of blood products has been minimized.[2]
The incubation time of HC lasts from 15 to 150 days whereas approximately 25 % of the infections heal without further complications.[1] The acute infection is associated with a symptomatic course (chapter 1.2.1) in 25 % only, for which reason the HC is often diagnosed in a late chronic stadium of the illness first.[1]
An estimated 50-80 % of the acute infections do not heal and result in a chronic HC with a spontaneous elimination of the virus in exceptional cases only. The chronic HC is not caused by the direct destruction of hepatic cells by the virus, it results from an immune response that is large enough to induce hepatic cell destruction and fibrosis but not enough to eradicate the virus.[2] After 20-30 years of chronic HC infection a fibrosis develops in 50 %, cirrhosis in 20 % of the patients. The patients with HCV induced cirrhosis do have a risk of developing a hepatocellular carcinoma of 1-4 % per year. An infection with HC causes no immunity which means a reinfection is possible at any time.[1]

2.4 Diagnosis of hepatitis C

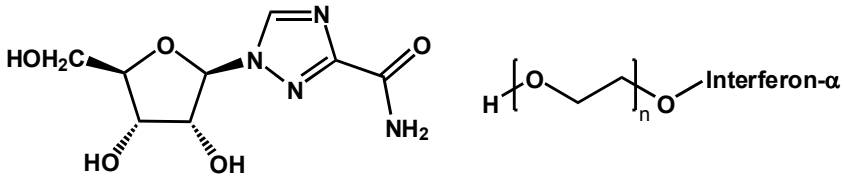
Initially the transaminases are clearly increased in 80 % of the patients which is a first hint for an acute hepatitis whereas 66 % of chronically infected patients show increased transaminases as well.[1]

Serologically, on one hand the HCV infection is diagnosed by detection of specific antibodies which is possible 6-8 weeks after infection. On the other hand an identification of the viral RNA is necessary after a positive HCV antibody diagnosis since HCV antibodies are detectable up to 20 years after healing. If no HCV antibody is found, determination of the viral RNA is necessary to exclude acute infection.[1]

Currently, an immunoblot assay is used for the identification of HCV antibodies and polymerase chain reaction for the detection of the viral RNA.[6]

2.5 Therapy of hepatitis C

Almost all patients with acute hepatitis C rapidly become HCV RNA negative on pegylated interferon-α (PEG IFN-α) therapy.[7] The current standard of treatment for chronic hepatitis C is the combination of ribavirin and PEG INF-α (figure 1) in which a large molecule of polyethylene glycol (PEG) is covalently attached to recombinant IFN-α, leading to longer half-life, better pharmacokinetic profile and better rate of virological response.



Ribavirin PEG interferon-α

Figure 1: Structure of ribavirin and PEG interferon-α, average molecular weight 19241.10 g/mol (interferon-α: polypeptide consisting of 166 amino acids).

This combination of therapeutic agents yields overall sustained virologic response (SVR) rates of 54-56 % in which duration of the treatment varies from 24 to 48 weeks, depending on the viral genotype.[7] A transient response with relapse occurs in 10-25 % of patients despite optimal regimens whereas the cause of relapse remains unknown.[7] If the

transaminases stay normal and the viral RNA is beyond detection for 6 months after end of treatment an ongoing success of therapy is anticipated.[5] Furthermore, liver transplantation is the primary treatment option for patients with decompensated cirrhosis or hepatocellar carcinoma.[2; 8]

Concerning PEG IFN-α the antiviral activity does not result from targeting the virus directly or from affecting the viral replication cycle. Rather, it induces an IFN-stimulated non-virus-specific antiviral state within the host cells and stimulates the adaptive and innate immune system. Ribavirin, a guanosine analogue, possesses broad antiviral activity. The exact mode of action of ribavirin remains unclear so far. An attractive hypothesis to explain the effects is a lethal mutagenesis of HCV under the treatment of ribavirin.[7]

Due to the genetic heterogeneity of the virus, caused by the high replicative activity, a vaccine against hepatitis C is not available yet. However the situation is quite promising today since eradication of the virus occurs in 20-50 % of the patients, associated with specific immune responses, and clear evidence for some natural immunity has emerged recently in humans and chimpanzees. Therefore many therapeutic vaccine trials are planned or are already in progress but little information is available about their efficacy at present.[9]

Due to the severe influenza-like side effects and the limited virologic response much attention is now being focused on the development of new drugs like HCV protease, polymerase, helicase and other specific inhibitors.[10]

2.6 The hepatitis C virus

The hepatitis C virus is a positive stranded RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family. HCV was first described in 1975 as non-A, non-B hepatitis virus and identified in 1989. Despite great progress made in the past years the structure of HCV remains to be elucidated. Derived from related alphaviruses and flaviviruses it is speculated that the virion is composed of a nucleocapsid containing the genomic RNA and a double-layer lipid envelope. Furthermore, the surface glycoproteins E1 and E2 are anchored to the lipid bilayer. A schematic structure of the viral particle is given in figure 2. The HCV genome consists of 9.6 kilobases and encodes a polyprotein

precursor of about 3000 amino acids which is processed by cellular and viral proteases into the mature structural (Core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Figure 3 shows the HCV polyprotein including the biological function of the individual proteins.[11]

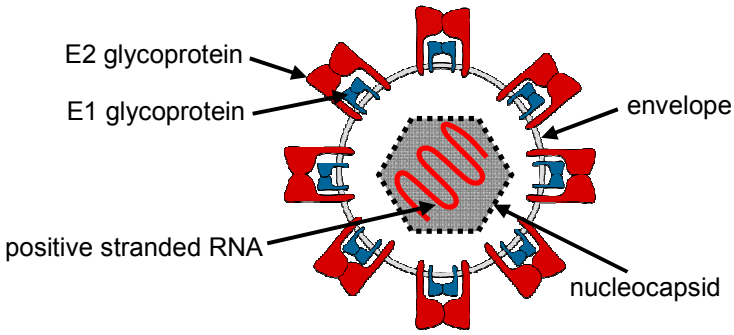


Figure 2: Schematic structure of the hepatitis C virus.

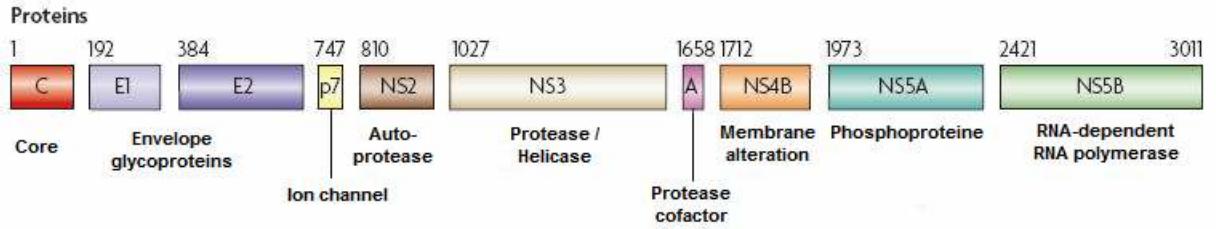


Figure 3: Hepatitis C virus proteins including their biological functions (taken from reference 11).

HCV isolates can be classified into 6 major genotypes and about 50 subtypes whereas the genotypes differ in their nucleotide sequence by 30-35 % and the subtypes by 20-25 %. This genetic heterogeneity of the population of HCV genomes (quasispecies) coexisting in an infected individual hampers the treatment of HC (see 1.5).[11; 12]

The lifecycle of HCV can be separated into 6 major steps (figure 4): Virus binding and internalization, release and uncoating, translation and polyprotein processing, RNA replication, packaging and assembly and virion release. Whereas currently little is known about the packaging, assembly and virion release.[11] In principle, each of the mentioned steps is a target for treatment of HCV.

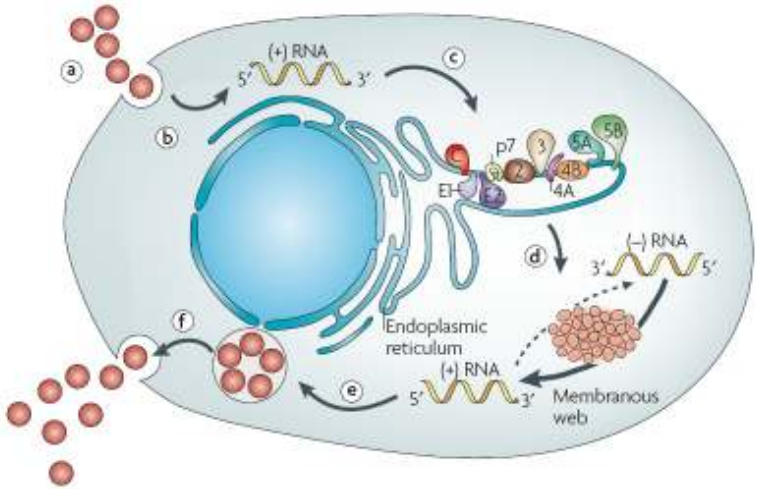


Figure 4: Lifecycle of hepatitis C virus: Virus binding and internalization (**a**), release and uncoating (**b**), translation and polyprotein processing (**c**), RNA replication (**d**), packaging and assembly (**e**), virion release (**f**) (taken from reference 11).

The targets for anti HCV therapy – presently used or discussed – are presented in the following.

2.7 Potential targets for the treatment of hepatitis C

2.7.1 The p7 protein

It has been suggested that the p7 protein could form ion channels in the endoplasmic reticulum which might be necessary for HCV replication. This makes it a potential target for antiviral therapy. Presently some long-alkyl-chain iminosugar derivatives are under clinical evaluation (figure 5). These are derived from the glucose analogue deoxynojirimycin (DNJ) or the galactose analogue deoxygalactonojirimycin (DGJ).[13; 14]

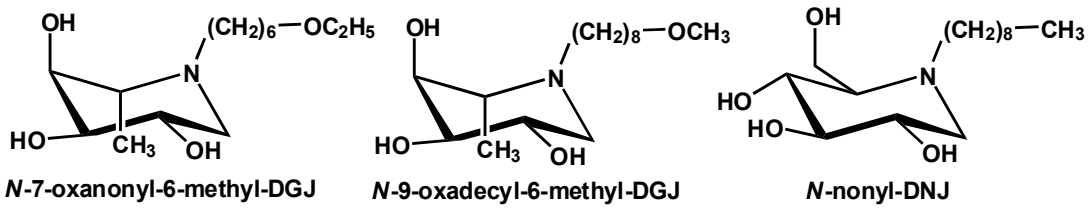


Figure 5: Structures of long-alkyl-chain iminosugars.

2.7.2 The NS2 auto-protease

The NS2 auto-protease cleaves the non-structural proteins NS2 and NS3. No NS2 inhibitors have been developed so far.[14]

2.7.3 The NS3 protease / helicase

On one hand, NS3 cleaves together with its cofactor NS4A the polyprotein at four junctions. On the other hand, NS3 holds the helicase needed to unwind the viral genomic RNA during replication. Several potential inhibitors targeting protease activity are currently at the preclinical or early clinical stage. These compounds are oligopeptides recently developed.[15] The structure of VX-950 (Telaprevir©, Vertex) is exemplarily shown in figure 6. By contrast no compound for the inhibition of the NS3 helicase has reached clinical development yet.[10; 14]

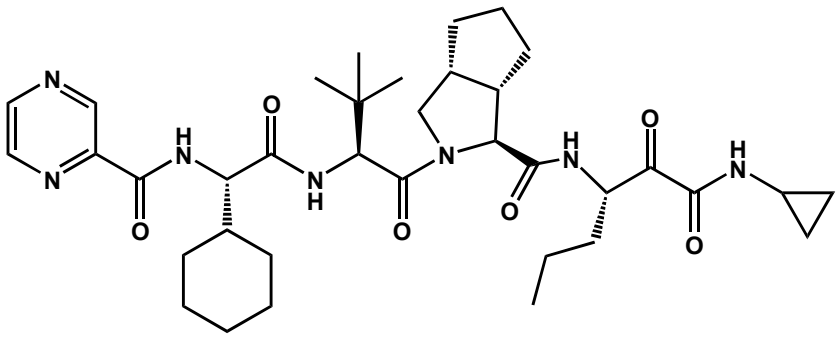


Figure 6: Structure of VX-950.

2.7.4 The NS5A phosphoprotein

The NS5A phosphoprotein plays a role in viral replication. Its exact function and three-dimensional structure is still unknown.[14] Nonetheless, recently achieved results suggest several substituted pteridines to inhibit HCV replication by interfering with NS5A. The structure of one of these compounds is shown in figure 7. Their exact mechanism of action is presently further elucidated.[16]

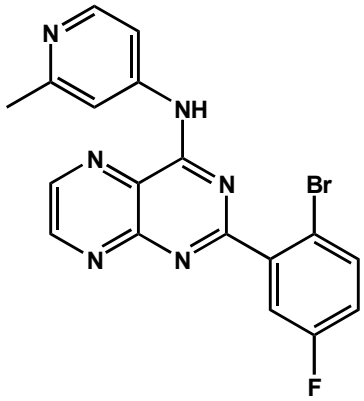


Figure 7: Substituted pteridine, an inhibitor of HCV replication.

2.7.5 The NS5B RNA-dependent RNA polymerase

The NS5B protein contains the RNA-dependent RNA polymerase (RdRp) which is the catalytic component necessary for the HCV replication process. This enzyme synthesizes RNA using an RNA template. For prevention of the RdRp activity several potential nucleoside as well as non-nucleoside inhibitors were found, currently at the preclinical or early clinical developmental stage.[10] Representative examples of them are shown in figure 8.[15]

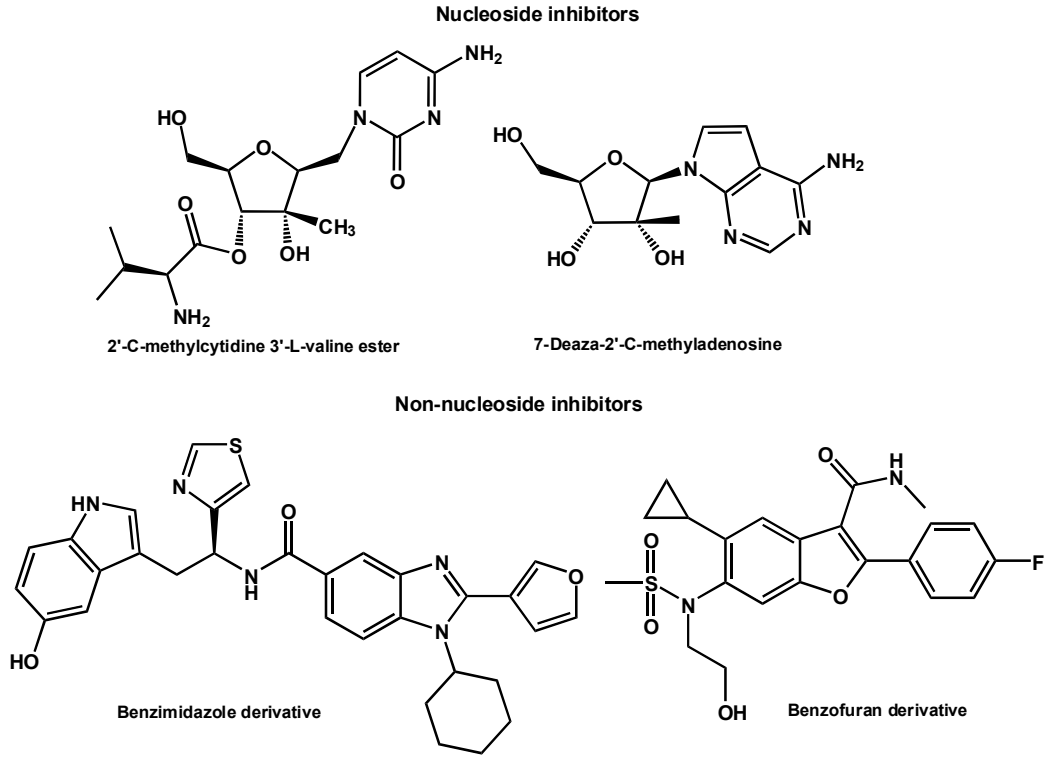


Figure 8: Structures of potential NS5B inhibitors.

2.7.6 The envelope glycoproteins E1 and E2

These glycoproteins form a non-covalent complex which is believed to be the building block for the viral envelope. Furthermore E1 and E2 are crucial for binding to human cell surface receptors and viral fusion.[17] Therefore prevention of this binding is an attractive target to protect HCV target cells against infection. This approach has been demonstrated recently by a small peptide of 14 amino acids from human CD81 large extracellular loop – with known importance in HCV-E2 binding – as well as compounds with a bis-imidazole scaffold. Both the small peptide and the bis-imidazole derivatives (general structure shown in figure 9) bind to HCV-E2 preventing CD81-HCV-E2 interaction.[18; 19]

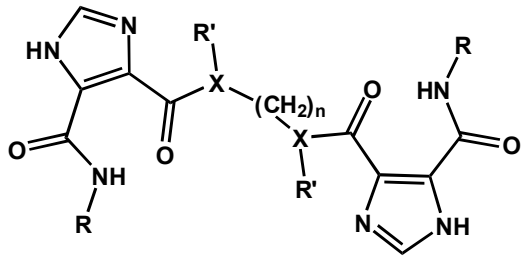


Figure 9: General structure of the bis-imidazole derivatives inhibiting CD81–HCV-E2 interaction (X = N, O, R = amino acid ester, R' = H, Me, Et, Bu, n = 2, 3, 4, 6).

2.7.7 The internal ribosome entry site

The HCV internal ribosome entry site (IRES) is essential for translation of the viral RNA during replication process. For this reason IRES has been considered the most attractive target for the development of RNA-based drugs. These potential inhibitors are oligonucleotides like for example ISIS-14803 which consists of 20 phosphorothioate deoxynucleotides.[20] However, development of these potential drugs was halted because of adverse side effects or because of limited efficacy.[10; 11]

2.7.8 Passive or active immunization

Passive immunization against HCV might be possible by the application of readymade antibodies whereas active immunization is achieved by administration of HCV-specific proteins to produce immunity to HCV. Following these two approaches led to proteins that are currently in preclinical or early clinical trials.[14]

2.7.9 Human receptors for HCV

The most important host receptors involved in HCV binding and internalization are, among others, the low density lipoprotein receptor (LDLR), the very low density lipoprotein receptor (VLDLR), scavenger receptor class B type I (SR-BI), claudin 1, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN) and the CD81 receptor.[11]

The LDL and VLDL receptors are known to associate with HCV particles whereas their precise role still has to be determined. The remaining four receptors mentioned above are binding partners for HCV-E2 during adhesion and viral entry.[11] Recently achieved results strengthened the possibility of potential inhibitors for the interaction of HCV with the mentioned host receptors in the future.[18; 19; 21; 22]

2.8 The CD81 receptor

CD81 is a member of the tetraspanin membrane protein superfamily, expressed on a variety of cell types, including hepatocytes. It is characterized by the presence of four transmembrane domains, three intracellular domains and two extracellular loops (figure 10). In the case of CD81 the two extracellular loops are referred to as the small and large extracellular loops (SEL and LEL).[23; 24]

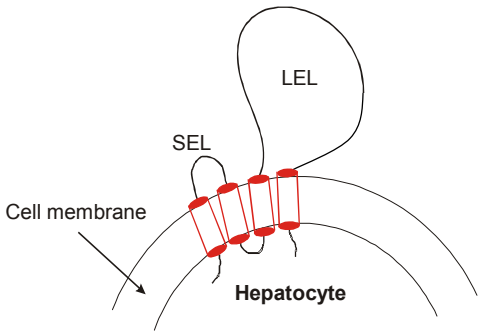


Figure 10: Schematic illustration of the CD81 receptor.

HCV-E2 binds with high affinity to the LEL of CD81 which has been demonstrated using recombinant HCV-E2, HCV E1-E2 complexes, HCV pseudoparticles as well as *bona fide* (from Latin: authentic, real) viral particles.[17; 23; 25] Therefore CD81 is very likely to be involved in mediating HCV entry as an important receptor.[17; 26] Whereas expression of

CD81 in non-hepatocyte-derived cell lines not conferring susceptibility to HCV infection, showed that additional hepatocyte-specific factors must be required for HCV entry.[11] The role of HCV-E2–CD81 binding during HCV infection is not known yet but it is assumed that this interaction may facilitate virus internalization and/or membrane fusion.[19] The elucidation of the CD81-LEL crystal structure revealed that it is a homodimer, with each monomer being composed of five α-helices (A to E).[27] Furthermore two crystallization structures of the LEL with particularly different folding of the head subdomain, the HCV-E2 binding region, are known (PDB ID 1G8Q, 1IV5) (figure 11). These different structures indicate a conformational flexibility, which is thought to be involved in binding the viral protein.[27; 28]

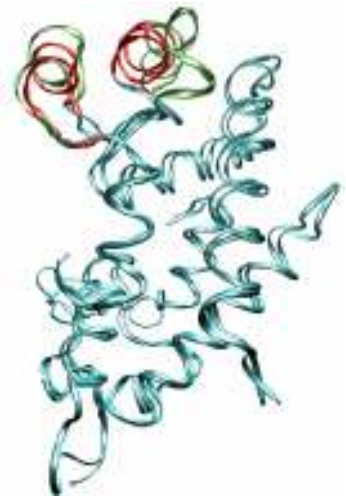


Figure 11: Superposition of the 1G8Q (green, with pronounced cleft-like motif) and the 1IV5 (red) CD81-LEL crystal structure with different crystal forms in the head subdomain (the identical LEL-structure is turquoise).

Random mutagenesis was used to determine that the E2-binding site on CD81-LEL comprises amino acids Leu162, Ile182, Asn184 and Phe186 which are essential for virus binding. The latter three amino acids are located on the D helix of the LEL, Leu162 occurs within a short helix located between the B and C helices.[24] Furthermore the HCV-E2 binding site on the LEL is highly conserved and therefore represents a potential target site for the development of small-molecule entry inhibitors.

2.9 Virtual screening approach

The important structural difference of the head subdomain between the two crystal forms of the LEL mentioned above is caused by the C and D helices. These helices form a cleft-like motif (figure 11) in the case of the 1G8Q structure which does not appear in the 1IV5 structure. Recently this motif was used by our group in a virtual high throughput screening approach aiming to identify small organic molecules fitting into this cleft. The idea was to inhibit conformational flexibility of the receptor which makes viral entry impossible.[29]

400.000 compounds obtained from different databases including the in-house substance library of our group were used for a virtual screening. Exclusion of reactive compounds as well as compounds which do not follow Lipinski's *Rule-of-Five* resulted in approximately 100.000 compounds. These compounds were then docked into the cleft of 1G8Q. This led to the identification of about 200 virtual *hits.* Compounds which were commercially available or accessible via an easy synthetic route were tested using the biological testsystem developed by Pileri et al[23] for their inhibition of the CD81-LEL–HCV-E2 interaction. As outcome of this test benzyl salicylate (figure 12) was found to be moderately active with an inhibition of 25 % at 50 µM.

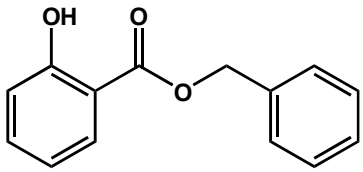
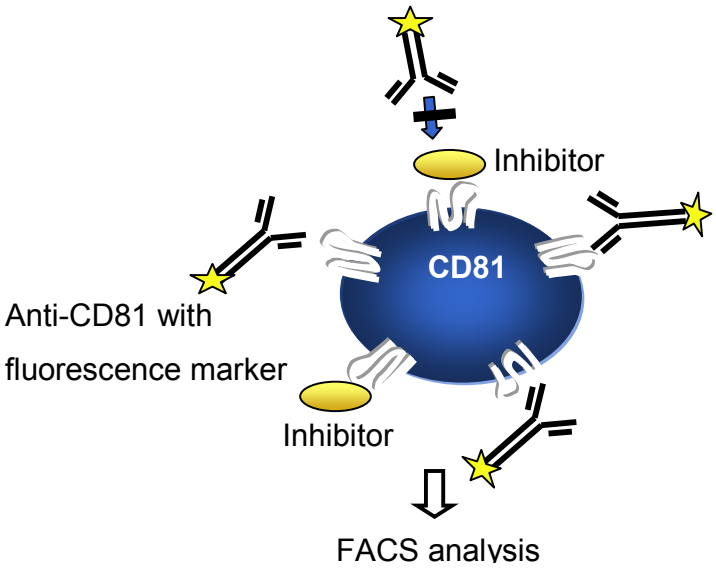


Figure 12: Benzyl salicylate.

2.10 Biological screening approach

A biological screening of natural products, current drugs including several antihistamines and our in-house substance library (approximately 350 compounds) was performed to find potential small-molecule inhibitors of the CD81-LEL–HCV-E2 interaction. Biologically active compounds should serve as experimental *hits* to increase inhibitory potency by means of further structural optimization. The biological screening was performed using a medium throughput assay developed in our group.[30] This test is based on an anti-

body neutralization assay published by Pileri et al[23] in which the compounds inhibit the binding of the fluorescence-labeled CD81 antibody JS81 to HUH7.5 cells (scheme 1). Inhibition of this interaction causes a decreased fluorescence compared to control cells without inhibitor. This was monitored by FACS measurements.



Scheme 1: Schematic illustration of the antibody neutralization assay.

This screening showed terfenadine (figure 13), an antihistamine, to be a moderate inhibitor of the CD81-LEL–HCV-E2 interaction (27 % at 50 µM). Other antihistamines showed no inhibitory activity.

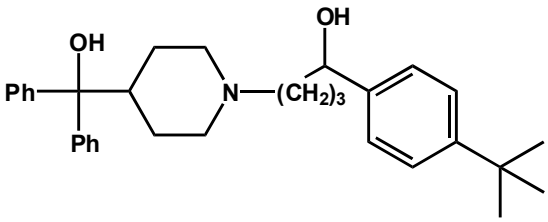


Figure 13: Terfenadine.

3. Aims of this work

Due to the absence of a vaccine against HCV and the severe side effects of the antiviral drugs currently used for the treatment of chronic HCV infection there is a need for new potent drugs. Recent results showed the interaction of the hepatitis C virus envelope glycoprotein E2 (HCV-E2) with the *large extracellular loop* (LEL) of the human CD81 cell surface receptor to be essential for viral cell entry.[26] The infection of human hepatocytes is inhibited by anti-CD81 monoclonal antibodies or by recombinant CD81-LEL.[17] Furthermore, compounds which bind to HCV-E2 are capable to block the CD81-LEL–HCV-E2 interaction.[19]

Our group chose the CD81-LEL as an innovative and promising target for the development of potential inhibitors of the CD81-LEL–HCV-E2 interaction. Concerning this approach we were currently the first and are currently the only group trying to find such inhibitors. Furthermore the crystal structure of the LEL is available facilitating virtual screening methods and a cell based assay to quantify the biological activity of the prepared compounds is established in our group.

A virtual and a biological screening performed in Prof. Hartmann's group showed benzyl salicylate as well as terfenadine to be moderate inhibitors of the CD81-LEL–HCV-E2 interaction with an inhibition of 25 % (benzyl salicylate) and 27 % (terfenadine) at 50 µM.

The aim of this thesis was the synthesis of optimized inhibitors of the CD81-LEL–HCV-E2 interaction based on the core structures of the mentioned screening *hits*. Therefore, benzyl salicylate should be derivatized by coupling the aromatic ring containing the carboxyl function to variable heterocycles and by variation of the benzyl alcohol moiety. Additionally the benzyl alcohol moiety was intended to be substituted by amino acid esters. Concerning terfenadine the aim was to structurally modify the length of the alkyl "linker" between the piperidine and the phenyl moiety, the alkyl substituent on the phenyl ring, the secondary hydroxyl group and the azacyclonol moiety. The derivatization of the original *hit* compounds was thought to increase inhibitory potency and to derive structure activity relationships.

4. Structural modifications of salicylates: Inhibitors of human CD81-receptor HCV-E2 interaction

Marcel Holzer, Sigrid Ziegler, Alexander Neugebauer, Bernd Kronenberger, Christian D. Klein, Rolf W. Hartmann

Archiv der Pharmazie, **2008**, *341*, 478-484

5. Microwave-assisted syntheses of amino acid ester substituted benzoic acid amides: Potential inhibitors of human CD81-receptor HCV-E2 interaction

Marcel Holzer, Sigrid Ziegler, Bernd Kronenberger, Christian D. Klein, Rolf W. Hartmann

The Open Medicinal Chemistry Journal, **2008***, 2*, 21-25

6. Identification of terfenadine as an inhibitor of human CD81-receptor HCV-E2 interaction: Synthesis and structure optimization

Marcel Holzer, Sigrid Ziegler, Beatrice Albrecht, Bernd Kronenberger, Artur Kaul, Ralf Bartenschlager, Lars Kattner, Christian D. Klein and Rolf W. Hartmann

Molecules ***2008****, 13,* 1081-1110

http://www.mdpi.org/molecules/papers/13051081.pdf

7. Results

The aim of the present work was the synthesis of potential inhibitors of the CD81-LEL–HCV-E2 interaction. The results of this work were published in three publications outlined below.

7.1 Structural modifications of salicylates: Inhibitors of human CD81-receptor HCV-E2 interaction

As outcome of the virtual screening procedure described in chapter 2.9 benzyl salicylate (figure 14) was found. Testing revealed this compound to be a moderate inhibitor of the CD81-LEL–HCV-E2 interaction with an inhibition of 25 % at 50 µM.

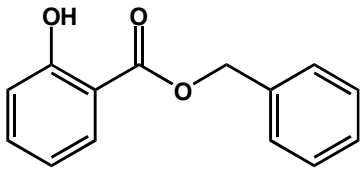


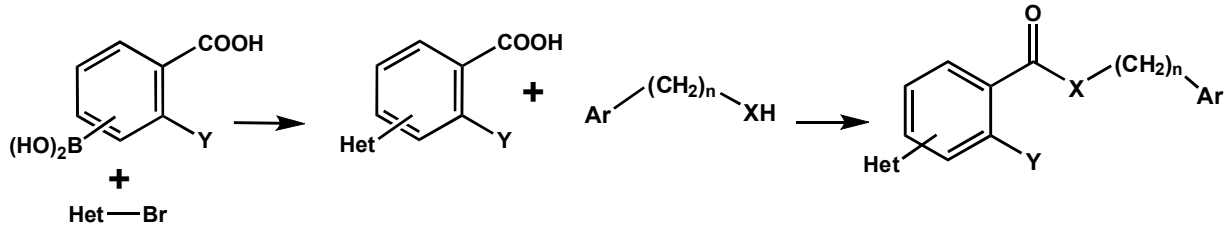
Figure 14: Benzyl salicylate (25 % inhibition at 50 µM).

This was the starting point for the synthesis of benzyl salicylates with a core structure similar to the original *hit* in which the aromatic ring containing the carboxyl function should be coupled to variable heterocycles. Furthermore a few compounds with heterocyclic substitution at the benzyl alcohol moiety were prepared. These structural modifications were performed to increase inhibition of protein-protein interaction and to improve drug-likeness.

Overall 37 compounds referring to the general structure mentioned in scheme 2 were synthesized by connecting substituted benzoic acids to alcohols and amines, respectively. The desired esters and amides were prepared by activation of the carboxylic acids using thionyl chloride and addition of the corresponding alcohols or amines (scheme 2).

The carboxylic acids needed for the formation of the target compounds were prepared using commercially available reagents. Starting from the bromo-substituted aromatic heterocycle and the corresponding boronic acid Suzuki coupling was performed with

tetrakis-(triphenylphosphine) palladium (0) as catalyst and the desired compounds (scheme 2) obtained in satisfactory yields.



Scheme 2: General structure and synthetic pathway for the preparation of the benzyl salicylates (Het = heterocycle, X = -O-, -NH-, Y = -H, -OH, Ar = aromat, n = 0, 1).

The alcohols and amines used for the formation of the desired derivatives were commercially available with the exception of the alcohol and the two amines shown in table 1. These had to be prepared starting from the bromo-substituted heterocycle and the boronic acid of the corresponding alcohol or amine via Suzuki coupling, performed as mentioned above.

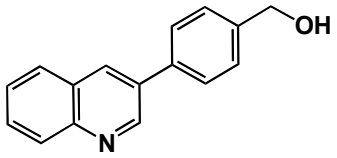
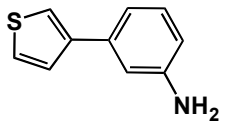
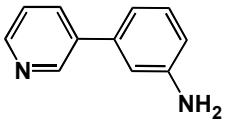
Compound




Table 1: Synthesized heterocyclic substituted alcohol and amines.

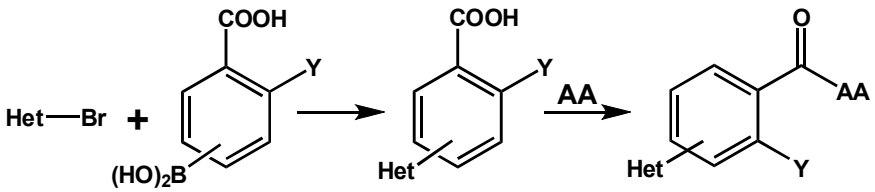
Biological testing of the target compounds was performed by means of the medium-throughput assay mentioned in chapter 2.10. No synthesized compound showed an increase concerning the inhibition of the CD81-LEL–HCV-E2 interaction compared to the original hit benzyl salicylate.

A possible explanation for the low activity of the compounds might be an unfavorable substitution pattern of the core structure that diminishes binding affinity to the LEL compared to the original hit.

7.2 Microwave-assisted syntheses of amino acid ester substituted benzoic acid amides: Potential inhibitors of human CD81-receptor HCV-E2 interaction

The results gained in 7.1 indicated that a different substitution pattern of benzyl salicylate is required to increase inhibitory potency. Therefore several heterocyclic substituted benzoic acid amides were synthesized. The idea was to maximize more specific hydrogen-bonding and electrostatic interactions with the presumed binding site to increase inhibition.

We decided to retain the heterocyclic substituent at the benzoic acid moiety mentioned in 7.1. Furthermore the benzyl ester of benzyl salicylate was replaced by amino acid esters which led to 11 derivatives of the general structure shown in scheme 3.



Scheme 3: General structure and synthetic pathway for the preparation of the desired benzoic acid amides (Het = Heterocycle, Y = -H, -OH, AA = L-alanine ethyl ester, L-phenylalanine ethyl ester, L-tryptophane methyl ester).

For the preparation of the desired benzoic acid amides microwave-assisted N, N'-dicyclohexyl carbodiimide (DCC) coupling was used to get the compounds in satisfying yields. Eleven derivatives were synthesized according to the pathway given in scheme 3.

Before we decided to apply microwave-assisted amide syntheses, activation of the carboxylic acids by means of thionyl chloride followed by addition of the corresponding amino acid esters was tried. Since this did not lead to the desired compounds DCC coupling reaction under standard conditions was performed next leading to the desired am-

ides in very poor yields. Therefore microwave-assisted DCC coupling reaction was tried to increase the yield of the desired products. This attempt finally led to the target compounds in satisfying yields.

The prepared amides were tested for their inhibition of the CD81-LEL–HCV-E2 interaction using the medium throughput assay mentioned in chapter 2.10. The synthesized benzoic acid amides showed no increased activity compared to the original *hit* compound benzyl salicylate.

7.3 Identification of terfenadine as an inhibitor of human CD81-receptor HCV-E2 interaction: Synthesis and structure optimization

The biological screening mentioned in chapter 2.10 showed terfenadine (figure 15) to be moderately active with an inhibition of 27 % of the CD81-LEL–HCV-E2 interaction at 50 µM. Consequently series of terfenadine derivatives were prepared to increase inhibitory activity and to derive structure activity relationships.

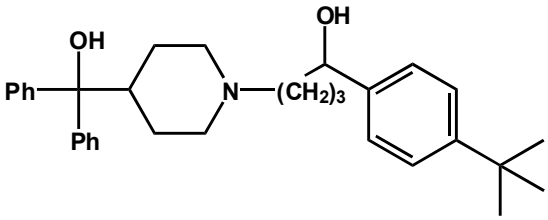


Figure 15: Terfenadine (27 % inhibition at 50 µM).

The following structural features were modified: length of the alkyl "linker" between the piperidine and the phenyl moiety, alkyl substituent at the phenyl ring, secondary hydroxy group and the azacyclonol moiety (figure 16).

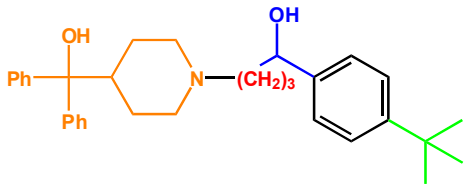
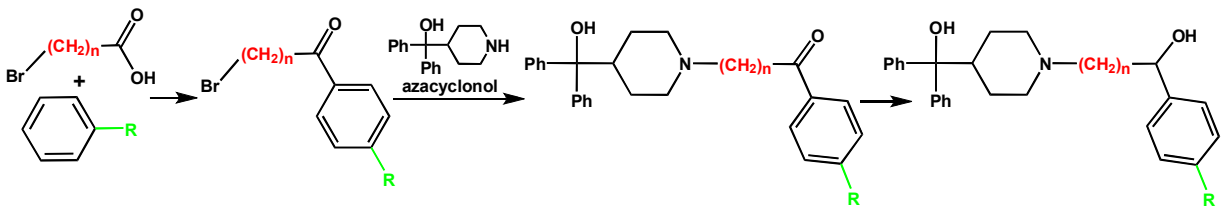


Figure 16: Performed derivatizations of terfenadine (coloured as mentioned in the text).

Concerning the derivatization of the alkyl linker length and the alkyl substituent at the phenyl ring a general structure and the synthetic pathway of the prepared 47 compounds is shown in scheme 4.

The desired derivatives were synthesized starting from commercially available compounds. The first step of the preparation was a Friedel-Crafts (FC) acylation of the benzene derivative with aluminium chloride as catalyst and the carboxylic acid chloride of the corresponding ω-bromo-carboxylic acid. In the next reaction step the obtained 1-aryl-ω-bromo ketones were coupled nucleophilically to azacyclonol via microwave assisted syntheses leading to terfenadine precursors which were reduced using sodium borohydride. This led to the corresponding terfenadine derivatives as racemates (scheme 4).



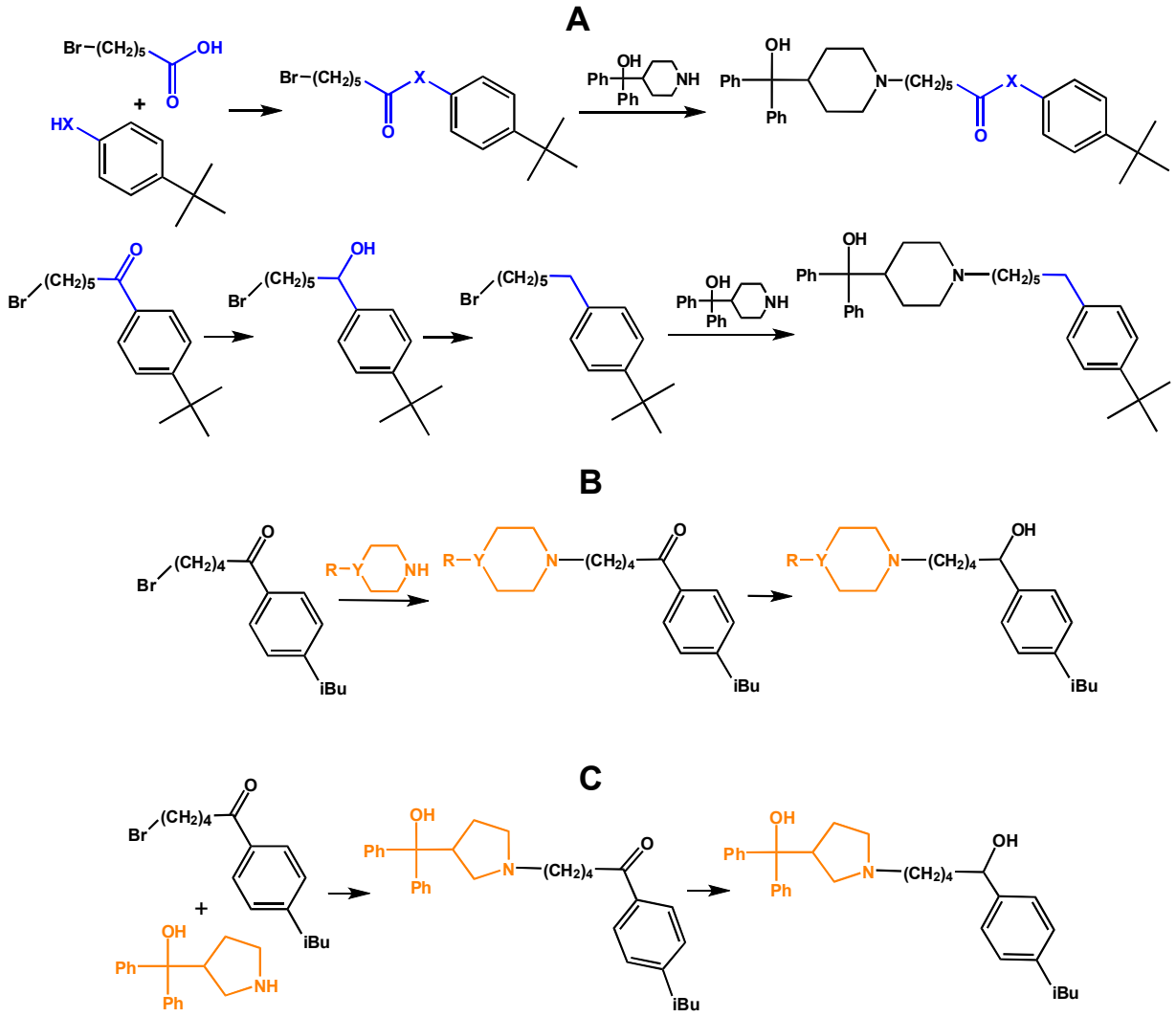
Scheme 4: Synthesized terfenadine derivatives with variation of the alkyl linker length and the substituent at the phenyl ring (n = 3-5; R = H; C_1-C_4).

This synthetic procedure facilitated the preparation of a large variety of compounds in a minimum of time since azacyclonol, the second component for the coupling reaction, was commercially available.

For further modification the secondary hydroxyl group was substituted by an ester and an amide function. Furthermore it was reduced to the corresponding alkane. The general structure and the synthetic pathway are shown in scheme 5.

The exchange of the azacyclonol component by smaller piperidine moieties led to 11 terfenadine derivatives which were synthesized via microwave assisted nucleophilic substitution followed by reduction of the ketone function leading to the alcohols as racemates (scheme 5).

The piperidine of the azacyclonol moiety was exchanged by a pyrrolidine following the synthetic pathway mentioned in scheme 5. The prepared alcohol was obtained as racemate.



Scheme 5: Synthesized compounds: **A:** Amide, ester and the corresponding alkane (X = -O-, -NH-); **B:** Derivatives with small piperidine residues (R = -H, -CH_3, -OH, =O, benzyl, Y = -CH_2-, -O-); **C:** Derivatives with modified azacyclonol moiety.

The synthesized 63 terfenadine derivatives were tested for their inhibitory potency using the antibody neutralization (AN) assay mentioned above (chapter 2.10).

This showed that the alkyl substituent R (scheme 4) at the phenyl ring has a major influence on the activity. Another important feature is obviously the length of the linker, whereas the activities of the ketones and the corresponding alcohols did not differ significantly. Furthermore, reduction of the ketones to the corresponding alcohols and terfenadine led to a decreased activity for compounds with small substituents at the phenyl ring whereas a reduction of bulky substituted compounds led to derivatives with compa-

rable inhibition. In general compounds with n = 3 showed a lower inhibition than those with n = 4 or 5.

Enlargement of R from hydrogen to bulkier alkyl substituents increases inhibition. A maximum of inhibition, not depending on the linker length, could be reached generally by the use of *n*-propyl for the ketones and *iso*-butyl for the alcohols (scheme 4). The reduction of the ketones with R = *iso*-propyl to the corresponding alcohols led to a nearly complete loss of biological activity. The most active compound in this series of terfenadine derivatives (figure 17) shows that the hexanol linker combined with an *iso*-butyl group at the phenyl ring is the most favorable substitution pattern in this class of compounds.

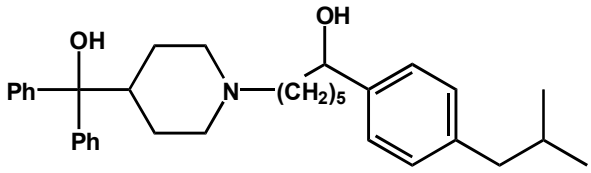


Figure 17: Most active compound 69 % inhibition at 50 µM.

Reduction of the secondary hydroxyl group to the corresponding alkane (scheme 5) led to a strong decrease of inhibition. Exchange of the ketone by an ester function did not influence the inhibitory activity, whereas the amide group increased the inhibition. These SAR results indicate that the functional group X (scheme 5) might act as an H-bond acceptor interacting with the CD81 protein.

Replacement of the azacyclonol moiety by smaller piperidine residues led to a loss of inhibitory activity. Substitution of the piperidine by a 4-benzyl group increased the inhibition to a moderate level (scheme 5). Again bulky substituents are essential for activity at this part of the molecule.

Exchange of the piperidine of the azacyclonol moiety (scheme 5) by pyrrolidine did not lead to a significant change of the inhibitory activity. Obviously due to the flexibility of the chain, for both heterocycles appropriate conformations can be found.

Selected compounds with high, moderate and low inhibition in the AN assay were tested for their inhibition in an infectivity assay showing that compounds with high biological activity in the neutralization assay showed good inhibition in the infectivity assay as well. This clearly indicates that inhibition of the protein-protein interaction leads to a reduction

of infectivity. On the other hand there are compounds which reduced infectivity without having been active in the AN assay. One plausible reason for this phenomenon could be an interaction of these compounds with an additional target which is involved in viral infection.

8. Discussion

Today, the search for new lead structures for future treatment of certain illnesses is based on biological experiments or on virtual screening of compound libraries. In the present work both approaches were used to find lead structures for the development of inhibitors of the CD81-LEL–HCV-E2 interaction. This interaction represents an attractive target for the synthesis of potential inhibitors since binding of HCV-E2 to the human CD81 receptor is essential for viral entry into host cells.[17; 27] In more detail blocking the binding of HCV-E2 to CD81 by for example anti-E2 monoclonal antibodies or recombinant human CD81-LEL prevents infection.[17] On the other hand the CD81 receptor is not sufficient for viral fusion since expression on non-hepatocyte-derived cell lines does not facilitate infection with HCV indicating the necessity of additional hepatocyte-specific factors.[11]

Choosing CD81-LEL as a target represents a completely new approach for the development of potential inhibitors for the treatment of HCV infection. Pharmaceutical companies follow 'classical' protein targets like HCV protease or polymerase. Additionally the LEL crystal structure and a biological test system for the potential inhibitors are available. Together with VanCompernolle[19] and Dhanasekaran[18] targeting the E2 glycoprotein with a small peptide or organic molecules, our group is the first trying to inhibit the CD81-LEL–HCV-E2 interaction.

In general prevention of protein-protein interactions is hard to realize by small molecular inhibitors. The challenge of such an approach is to find compounds that have sufficient affinity toward shallow or superficial binding sites that offer only limited chemical functionalities. This stands in marked contrast to active sites or ligand binding pockets with high affinity to substrates and consequently to substrate analogs or other small molecules.[31] General reviews on this topic are given by Yin and Hamilton and Fry.[32; 33] Therefore our approach might be a 'high risk' but nonetheless promising advancement since we do not target a superficial protein-protein interaction but a cleft within the LEL as binding site for our compounds. This cleft is formed by two α-helices of the LEL with conformational flexibility which is thought to be involved in virus binding and internalization. The synthesized molecules fitting into this cleft are thought to inhibit this conformational flexibility and therefore prevent CD81-LEL–HCV-E2 interaction (chapter 2.9).

With the use of both above mentioned approaches – the virtual and the biological screening – we maximized the possibilities to find lead structures for further structural optimization. As outcome of the biological screening terfenadine, an antihistamine was found to be a moderate inhibitor of the CD81-LEL–HCV-E2 interaction. In the course of this biological screening several antihistamines besides terfenadine were tested to exclude the activity of terfenadine referring to antihistaminic mechanisms. Other antihistamines showed no inhibition. Furthermore benzyl salicylate showed a moderate inhibition in the virtual screening followed by biological validation as well. Both *hits* were structural modified to increase the inhibitory activity and to derive structure activity relationships. This led to a large variety of compounds. In the course of the derivatization the planarity of the original *hit* compounds was retained to maximize the possible interactions with the cleft-like region which is relatively small but quite long.

Structural modification of benzyl salicylate did not afford compounds with an increased inhibitory potency. On one hand a plausible reason for this might be an unfavorable substitution pattern of the core structure that diminishes binding affinity to the LEL compared to the original *hit*. On the other hand the reliability of the virtual screening methods is still limited but under sustained development. This makes the gained virtual results possibly uncertain leading to conclusions which have to be scrutinized. Otherwise the derivatization of terfenadine led to compounds with a clearly increased inhibitory potency compared to the original *hit*. Furthermore the modifications of terfenadine facilitated significant structure activity relationships. Briefly, elongated molecules with bulky substituents show the highest biological activity. In addition an H-bond acceptor at the alkyl linker between the phenyl and the azacyclonol moiety is essential for activity. The most active compound within this series of terfenadine derivatives decreases the CD81-LEL–HCV-E2 interaction by 69 % inhibition at 50 µM. This concentration is compared to the organic compounds prepared by VanCompernolle et al – with an inhibition of 90 % at 500 µM – and the small peptide developed by Dhanasekaran et al – with an inhibition of 50 % at 3.5 mM – a promising result. An explanation for the difference concerning the percental inhibition might be the differing approaches that were followed. The synthesized terfenadine derivatives are thought to target the cleft-like region which appears as a more promising binding site for small-molecular ligands. Purely superficial regions – targeted by the groups of VanCompernolle and Dhanasekaran – offer only limited

chemical functionalities for interaction with the potential inhibitors hardening their development and the improvement of the inhibitory potency.

The biological activity achieved in the antibody neutralization (AN) assay was confirmed in an infectivity test system using viral particles. This clearly indicates that inhibition of the protein-protein interaction leads to a reduction of infectivity. Furthermore this test showed compounds to be moderate inhibitors in the infectivity assay which were not active in the (AN) assay. One plausible reason for this phenomenon could be an interaction of these compounds with an additional target which is involved in viral infection. Along with this, it can not be excluded that our compounds in addition also interact with the E2 mimicking epitope of the antibody. These possible mechanisms remain to be elucidated.

Concerning further optimization of the inhibitory potency additional structural modifications are possible. The most active compound found in this work could serve as new lead structure. This may facilitate further structure activity relationships and improve biological activity in which the essential structural features mentioned above should be kept.

9. Abstract / Zusammenfassung

The aim of the present work was to prepare compounds which inhibit the CD81-LEL–HCV-E2 interaction by binding to CD81-LEL.

Starting point was a virtual screening using the open conformation of the LEL followed by synthesis and biological validation by means of an antibody neutralization assay.[23] This showed benzyl salicylate (figure 18) to be moderately active with an inhibition of 25 % at 50 µM. Furthermore a biological screening revealed terfenadine (figure 18) to be a moderate inhibitor of the CD81-LEL–HCV-E2 interaction (27 % at 50 µM) as well.

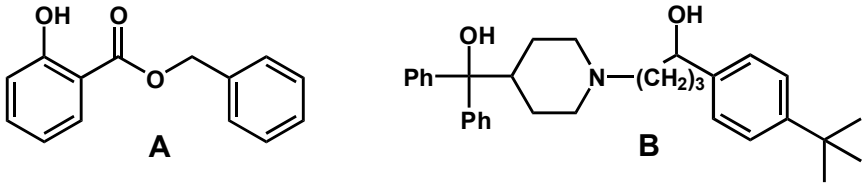


Figure 18: Benzyl salicylate (**A**) and terfenadine (**B**).

These two *hits* served as lead structures for further syntheses to increase inhibitory potency. Concerning benzyl salicylate 48 compounds with diverse substitution patterns were prepared. The synthesized compounds showed no increased inhibitory potency compared to benzyl salicylate.

For the derivatization of terfenadine 63 compounds with different structural modifications were synthesized. Their biological activity clearly demonstrates that a bulky substitution pattern at both parts of the molecule is necessary for activity as well as an H-bond acceptor at the alkyl linker. The most active compound in this series of derivatives is shown in figure 19.

Additional experiments with the terfenadine derivatives using viral particles revealed that there might be additional HCV infection reducing mechanisms which remain to be elucidated.

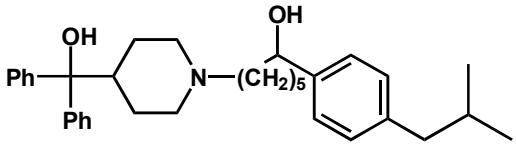


Figure 19: Most active terfenadine derivative (69 % inhibition at 50 µM).

Das Ziel dieser Arbeit war die Darstellung von Verbindungen, die die CD81-LEL–HCV-E2 Interaktion durch Bindung an CD81-LEL verhindern.

Der Ausgangspunkt hierfür war ein virtuelles Screening unter Zuhilfenahme der offenen Konformation der LEL, gefolgt von Synthese und biologischer Validierung mittels eines Antikörper-Neutralisationstests.[23] Hierbei wurde Benzylsalicylat (Abbildung 20) als mäßig aktive Verbindung mit einer Hemmung von 25 % bei 50 µM gefunden. Weiterhin zeigte ein biologisches Screening, daß Terfenadin (Abbildung 20) ebenfalls ein mäßiger Inhibitor (27 % bei 50 µM) der CD81-LEL–HCV-E2 Interaktion ist.

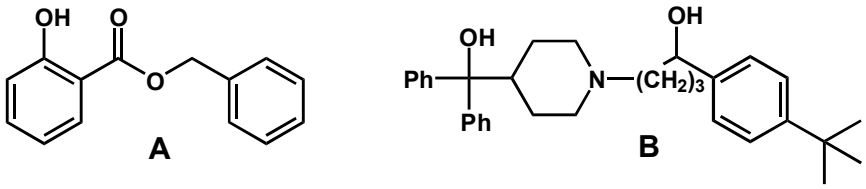


Abbildung 20: Benzylsalicylat (**A**) und Terfenadin (**B**).

Diese beiden *Hits* dienten als Leitstrukturen für die weitere Synthese zur Erhöhung der Hemmung. Von Benzylsalicylat wurden 48 Derivate mit verschiedenartigem Substitutionsmuster dargestellt. Die dargestellten Verbindungen zeigten keine gesteigerte Hemmung im Vergleich zu Benzylsalicylat.

Zur Derivatisierung des Terfenadin wurden 63 Verbindungen mit unterschiedlichen strukturellen Modifikationen hergestellt. Die biologische Aktivität dieser Verbindungen zeigte deutlich, daß ein voluminöser Substituent an beiden Seiten des Moleküls, sowie ein Wasserstoffbrückenakzeptor an dem Alkyllinker nötig sind. Die Verbindung mit dem höchsten Hemmwert dieser Derivate ist in Abbildung 21 dargestellt.

Ergänzende Experimente mit viralen Partikeln zeigten, daß bei den Terfenadinderivaten weitere, die Infektiosität von HCV reduzierende Mechanismen eine Rolle spielen könnten. Die Aufklärung dieser Mechanismen bedarf weiterer Untersuchungen.

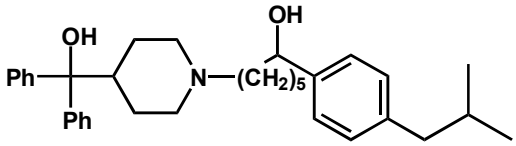


Abbildung 21: Aktivstes Terfenadinderivat (69 % Hemmung bei 50 µM).

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