Received: 20 July 2021 | Revised: 22 September 2021 | Accepted: 14 October 2021

DOI: 10.1002/dta.3182

WILEY

SHORT COMMUNICATION

Are the *N*-demethylated metabolites of U-47700 more active than their parent compound? In vitro μ-opioid receptor activation of *N*-desmethyl-U-47700 and *N*,*N*-bisdesmethyl-U-47700

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Abstract

Studies on the tissue distribution of the new synthetic opioid U-47700 and its main metabolite *N*-desmethyl-U-47700 revealed about sixfold higher metabolite concentrations in pig brain as compared with the parent compound. To better assess the toxic potential of this drug, the aim of this study was to assess the in vitro μ-opioid receptor (MOR) activation potential of the main metabolites of U-47700, *N*-desmethyl-U-47700, and *N*,*N*-bisdesmethyl-U-47700, using a live cell-based reporter assay based on NanoLuc Binary Technology®. Cells stably expressing human MOR and β-arrestin 2 (βarr2), each fused via a flexible linker to two complementary inactive subunits of the nanoluciferase, were seeded on poly-D-lysine-coated 96-well plates and treated with *N*-desmethyl-U-47700, *N*,*N*-bisdesmethyl-U-47700, U-47700, or hydromorphone as reference standard. MOR activation results in functional complementation of the nanoluciferase, which can be assessed via luminescence monitoring. The potency of the metabolites is lower than that of U-47700 (EC_{50} of 186 nM for U-47700, 3770 nM for *N*-desmethyl-U-47700, and >5 μM for *N*,*N*-bisdesmethyl-U-47700). The maximal efficacy (E_{max}) observed (relative to hydromorphone, set arbitrarily at 100%) decreased from 183% to 127% and 39.2% for U-47700, *N*-desmethyl-U-47700, and *N*,*N*-bisdesmethyl-U-47700, respectively. Thus, the loss of one or two methyl groups reduced the MOR activation potential, which was more pronounced if both methyl groups were removed. It is thus anticipated that the impact on MOR exerted by the higher metabolite concentration in brain has only little—if any relevance for the strong toxic effects of U-47700.

KEYWORDS

live cell-based reporter assay, NanoLuc Binary Technology®, new synthetic opioids, U-47700, μ-opioid receptor

1 | INTRODUCTION

The misuse of highly potent new synthetic opioids (NSOs) mimicking the effect of morphine or heroine has grown into a serious health threat.[1] Although the number of new releases is declining and NSOs constitute only a relatively small subgroup of illicitly marketed substances,[2] their hazardous potential has been responsible for a dramatic rise in NSO-caused poisoning or fatal cases, especially in the United States, in the last years.[3] 3,4-Dichloro-*N*-[2-(dimethylamino) cyclohexyl]-*N*-methylbenzamide (U-47700, Figure 1) is one of the most famous non-fentanyl-derived NSO and was involved in many intoxication cases reported in literature.[4–7] The structural arrangement of the basic nitrogen and the aromatic ring system allows U-47700 to assume a spatial orientation comparable with that of morphine, resulting in a selective agonism at the μ-opioid receptor (MOR),[8] which mediates the major pharmacological effects, such as analgesia, euphoria, sedation, and respiratory depression.[9]

MOR activation can be assessed using a variety of assays, for example, a (^{35}S)GTPγS assay, G protein bioluminescence resonance energy transfer assays, or a live cell-based reporter assay based on NanoLuc Binary Technology®.[10,11] The latter assay monitors the functional complementation of a split nanoluciferase following an agonist-induced interaction of a β-arrestin 2 (βarr2) protein with MOR, which is a G protein-coupled receptor,[11] as shown in Figure 2. Both βarr2 and MOR are fused to inactive subunits of nanoluciferase, respectively the small (SmBiT) and large (LgBiT) subunit. Upon MOR activation, βarr2 is recruited to the receptor due to the phosphorylation of MOR, thus allowing for interaction between the complementary nanoluciferase subunits. Subsequently, the functionalized enzyme generates a bioluminescent signal in the presence of the substrate furimazine, which can be monitored with a standard luminometer.[11]

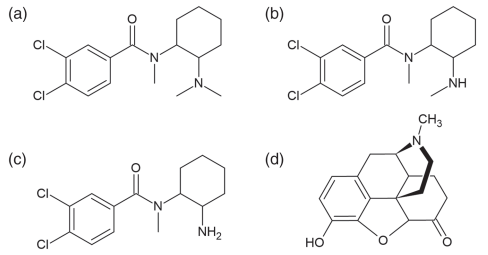

FIGURE 1 Chemical structures of (a) U-47700, (b) *N*-desmethyl-U-47700, (c) *N*,*N*-bisdesmethyl-U-47700, and (d) the reference compound hydromorphone

However, as for many other NSO, toxicokinetic (TK) and toxicodynamic parameters are only sparsely available for U-47700.[8,12] Thus, previous studies aimed to assess different TK properties of U-47700 in a comprehensive controlled animal study using a pig model following intravenous administration and with tramadol as a reference.[13] Studies on the tissue distribution revealed, as one of the most important findings, an about sixfold higher concentration of *N*-desmethyl-U-47700 in brain tissue compared with its parent compound.[13] Looking at *O*-desmethyltramadol, having a much higher binding affinity to MOR than its parent compound,[14] it should be elucidated, whether the *N*-demethylated metabolites of U-47700 (Figure 1) have any MOR activity. Together with the much higher concentration of *N*-desmethyl-U-47700 in brain, this metabolite and possibly also *N*,*N*-bisdesmethyl-U-47700 might also have a relevant impact on the toxicodynamic effects of U-47700 in vivo.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM; GlutaMAX™), Opti-MEM® I Reduced Serum Medium, Penicillin–Streptomycin (5.000 U/ml), and amphotericin B (250 μg/ml) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). Fetal bovine serum (FBS) and poly-D-lysine were supplied by Sigma-Aldrich (Overijse, Belgium). The Nano-Glo® Live Cell reagent used for the readout of the bioassay was procured from Promega (Madison, WI, USA). Hydromorphone was acquired as hydromorphone-HCl from Fagron (Nazareth, Belgium). U-47700 hydrochloride was obtained from Chiron Pharmasynth AS (Trondheim, Norway), and *N*-desmethyl-U-47700 (solid) was purchased from LGC (Wesel, Germany). *N*,*N*-Bisdesmethyl-U47700 was purchased from Cayman Chemical Company (Ann Arbor, MI, US).

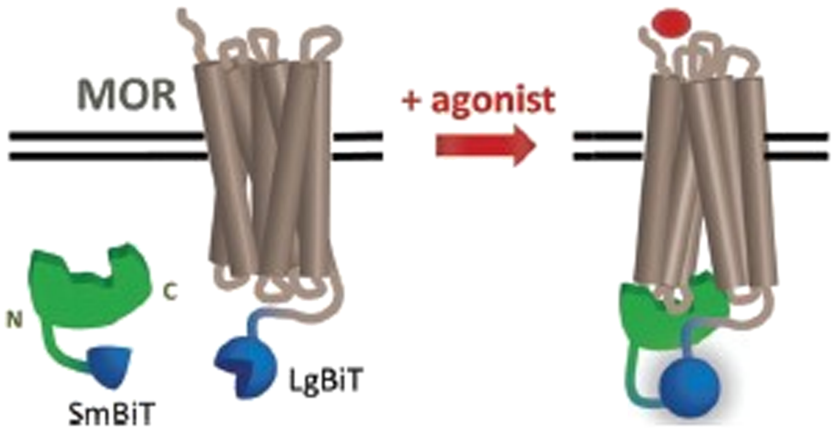

FIGURE 2 Design of the μ-opioid receptor (MOR) reporter assay with fusion of the β-arrestin 2 (βarr2) to the small subunit of nanoluciferase (SmBiT) and the G-protein coupled MOR to the large subunit of nanoluciferase (LgBiT). Figure was taken from Cannaert et al.[11] [Colour figure can be viewed at wileyonlinelibrary.com]

2.2 | Determination of the in vitro biological MOR activity

A live cell-based receptor assay was applied to assess the biological activity of the test compounds (U-47700, *N*-desmethyl-U-47700, and *N*,*N*-bisdesmethyl-U-47700) by monitoring the in vitro MOR activation. Details concerning the development of the modified human embryonic kidney (HEK) 293 T cells stable cell line used in this assay have already been described elsewhere.[11] These cells stably express human MOR or βarr2 fused via a flexible linker to the inactive subunits of the nanoluciferase (LgBiT or SmBiT), along G protein-coupled receptor kinase 2.[11,15]

As already described in previous studies,[10,11,16,17] the engineered cells were routinely maintained at 37°C and 5% CO_2 under humidified atmosphere in DMEM (GlutaMAX™) with different supplements. The day prior to the experiments, cells were seeded on white poly-D-lysine-coated 96-well plates at 5×10^4 cells/well and incubated overnight. Afterwards, 90-µl of Opti-MEM® I and 25 µl of the Nano-Glo® Live Cell reagent (a non-lytic detection reagent containing the cell permeable furimazine substrate) were added to each well. Subsequently, the plate was placed into a TriStar^2 LB 942 multimode microplate reader (Berthold Technologies GmbH & Co., Germany). Luminescence was recorded during the equilibration period until the signal stabilized (±15 min). Subsequently, 20 µl/well of concentrated (6.75-fold, as 20 µl was added to generate a final volume of 135 µl/well) stock solutions of the test compounds (U-47700, *N*-desmethyl-U-47700, *N*,*N*-bisdesmethyl-U-47700; Figure 1) or hydromorphone (as a reference compound; Figure 1) in Opti-MEM® I/methanol were added. Hydromorphone was used as a reference agonist for normalization, given its extensive use in prior work, thus allowing maximal comparability with other data, generated with the same assay.[17,18] All compounds were tested in concentrations ranging between 10 pM and 100 µM (in-well). The luminescence was constantly monitored for 120 min. In all experiments, solvent controls were included.

Statistical analysis and curve fitting were performed using GraphPad Prism 5.00 software (San Diego, CA, USA). The concentration–response curves were generated using data points obtained in three independent experiments, with duplicates run for each concentration within an experiment. The results are represented as mean area under the curve (AUC) ± standard error of mean (SEM). All results were normalized to the maximal activity (E_{max}) of the reference compound hydromorphone (= 100%). Curve fitting of concentration–response curves via nonlinear regression (four-parametric logistic fit) was utilized to determine the potency (EC_{50}) and the efficacy (E_{max}) of the investigated compounds.

3 | RESULTS AND DISCUSSION

The results of the MOR activation assay, including mean receptor activation (E_{max}) and the potency (EC_{50}) of all substances, are presented in Figure 3 and in Table 1. The in vitro MOR activation analysis of U-47700 and its metabolites demonstrates a lower potency for the metabolites than for U-47700, with EC_{50} values of 186 nM for U-47700, 3770 nM for *N*-desmethyl-U-47700 and >5 µM for *N*,*N*-bisdesmethyl-U-47700. In addition, the maximal effect observed (relative to the 100% MOR activation of the reference compound hydromorphone) declined from 183% to 127% and 39.2% for U47700, *N*-desmethyl-U47700, and *N*,*N*-bisdesmethyl-U-47700, respectively (Figure 3). For the latter, no E_{max} value could be determined as the maximal plateau phase was not reached, but the maximal achieved effect (at the highest concentration tested) is given. The EC_{50} and E_{max} obtained for U-47700 are well in line with those obtained previously, that is, 167 nM and 214%, respectively.[18]

Previous studies examining the tissue distribution of U-47700 and its main metabolite *N*-desmethyl-U-47700 in pigs revealed that in

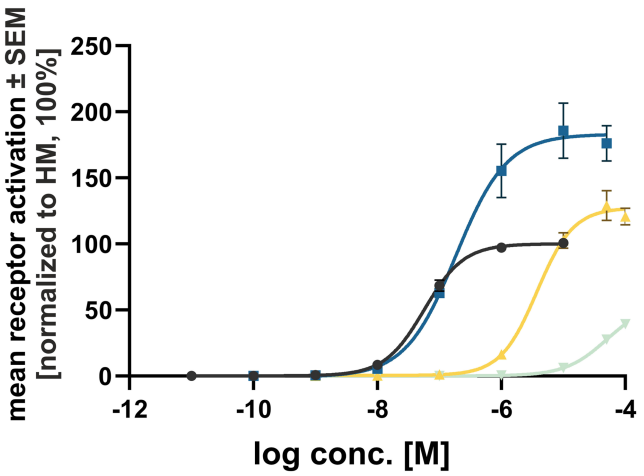

FIGURE 3 Concentration-dependent interaction of the µ-opioid receptor (MOR) with β-arrestin 2 protein upon stimulation with hydromorphone (HM, black line/dot), U47700 (blue line/square), *N*-desmethyl-U47700 (yellow line/triangle up), and *N*,*N*-bisdesmethyl-U47700 (green line/triangle down). Data are given in a semi-logarithmic plot as mean receptor activation ± standard error of mean (SEM) ($n = 3$), normalized to the maximal activity (E_{max}) of HM (= 100%) and plotted against the logarithmic concentration of the compound [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 The EC_{50} and E_{max} values are presented as a measure of potency and efficacy, respectively (95% CI profile likelihood)

Compound	EC_{50} (95% CI)	E_{max} (95% CI)
Hydromorphone (HM)	57.0 nM (47.3–67.8 nM)	100% (99.7%–104%)
U47700	186 nM (106–351 nM)	183% (164%–206%)
N-Desmethyl-U47700	3770 nM (2524–5495 nM)	127% (118%–138%)
N,*N*-Bisdesdimethyl-U47700	>5 µM	39.2%

Note. As the maximal plateau was not reached for *N*,*N*-bisdesmethyl-U47700, the maximal achieved effect is given as no E_{max} values could be determined.

most organs, the concentration of *N*-desmethyl-U-47700 was twofold to eightfold higher than that of the parent compound.[13] U-47700 itself is a potent MOR agonist with an in vivo activity 7.5–12-fold greater than morphine.[7] Nevertheless, functional in vitro activity assays showed a lower in vitro potency (EC_{50} of 140 nM) at MOR than that of morphine (EC_{50} of 31 nM), and radioligand-binding assays also showed a lower in vitro affinity to MOR (K_i of 57 nM) than morphine (K_i of 5 nM).[7] Thus, concerning brain tissue with a high density of opioid receptors,[19] the elucidation of MOR activity of the *N*-demethylated metabolites of U-47700 is essential to assess their potential toxicity. However, our in vitro MOR activation analysis of U-47700 and its metabolites revealed a negative impact of the loss of the methyl group(s) on the MOR activation potential. The metabolites were only capable of activating the MOR to a minor degree when compared with the parent compound. This effect was even more pronounced when both methyl groups were removed. Regarding U-47700 and *N*-desmethyl-U-47700, the EC_{50} value increased (thus, potency decreased) by a factor of 20 through loss of one methyl group.

These results are in line with those obtained by Truver et al.,[20] who, using a radioligand-binding assay, assessed competition with [^{3}H]DAMGO binding to rat brain tissue to examine the binding affinity of both U-47700 metabolites, expressed as inhibitory constant (K_i). In that assay, the affinity at MOR was higher for U-47700 ($K_i = 11$ nM) than for *N*-desmethyl-U-47700 ($K_i = 206$ nM) and *N*,*N*-bisdesmethyl-U-47700 ($K_i = 4080$ nM). Thus, a negative effect on the affinity was observed, which was also more pronounced upon loss of both methyl groups. In general, different binding studies of various U-compounds have already shown that the spatial relationship between the aryl moiety and the aminoamide is decisive for the MOR versus the κ-opioid receptor-binding affinity. In this constellation, the sterically unhindered *N*,*N*-dimethylamino group enhanced the MOR affinity.[8] The results of our study are consistent with the study by Truver et al.[20] and additionally indicate that the presence of both methyl groups at the tertiary amino group itself is necessary for the higher MOR activation potential.

Thus, in contrast to the pharmacodynamic relationship between tramadol and its main metabolite *O*-desmethyltramadol, the findings of this study indicated that the high *N*-desmethyl-U-47700 concentration in brain likely only has little—if any—impact on the strong and unpredictable toxic effects of U-47700 at MOR. In conclusion, although the complexity of interactions taking place in a living organism complicates straightforward translation of in vitro data to the in vivo situation, our data support the hypothesis that the strong toxic effects of U-47700 and its higher in vivo activity than morphine are primarily owing to U-47700 itself, which, as a result of its high lipophilicity (logP 4.09),[7] shows an improved penetration of the blood–brain-barrier.

4 | CONCLUSION

This study revealed that single or twofold *N*-demethylation of U-47700 resulted in a decrease in MOR activation potential, with the effect being much more pronounced upon loss of both methyl groups. Insofar, *N*-demethylated metabolites of U-47700 only showed a weak intrinsic MOR activation potential when compared with the parent compound and the reference compound hydromorphone. Thus, despite a high metabolite concentration in porcine brain tissue, human in vitro MOR activation data suggest that MOR activation by the *N*-demethylated metabolites is unlikely to be a relevant contributory factor to the toxic effects of U-47700, assuming a comparable tissue distribution of U-47700 and its metabolites in humans and pigs.

ACKNOWLEDGEMENT

The authors thank Marthe Vandeputte for her support and reading the manuscript.

CONFLICT OF INTEREST

The authors declare that there are no financial or other relations that could lead to a conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Nordmeier F, Cannaert A, Stove CP, Schmidt PH, Meyer MR, Schaefer N. Are the *N*-demethylated metabolites of U-47700 more active than their parent compound? In vitro μ-opioid receptor activation of *N*-desmethyl-U-47700 and *N*,*N*-bisdesmethyl-U-47700. *Drug Test Anal*. 2022;14(4):713-717. doi:10.1002/dta.3182