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RESEARCH ARTICLE

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Investigation of the μ- and κ-opioid receptor activation by eight new synthetic opioids using the [^{35}S]-GTPγS assay: U-47700, isopropyl U-47700, U-49900, U-47931E, *N*-methyl U-47931E, U-51754, U-48520, and U-48800

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Abstract

In 2009, new synthetic opioids appeared on the new psychoactive substances market. This class of new psychoactive substances generally poses a health risk due to the high affinity and potency of most of these compounds for the opioid receptors. It is known that overdoses can lead to respiratory depression and result in death. However, for many new synthetic opioids, data on toxicological and toxicokinetic properties are scarce. In the present study, eight U-opioids were investigated for their structure activity relationships at the μ- and κ-opioid receptors using a [^{35}S]-GTPγS assay. The potencies of the investigated U-opioids were lower than those of the reference compounds (μ-opioid receptor: hydromorphone, fentanyl; κ-opioid receptor: U-69593, U-50488). At the μ-opioid receptor, U-47700 showed the highest potency with an EC_{50} value of 111 nM, and at the κ-opioid receptor, U-51754 was found to be the most potent compound with an EC_{50} value of 120 nM. The following structural features were advantageous for activating the μ-opioid receptor: two chlorine substituents in 3,4-position at the aromatic ring, the absence of the methylene group between the amide group and the aromatic ring, a methyl group at the amide nitrogen, and/or a dimethylamine residue at the amine nitrogen of the cyclohexane ring. Further, the following structural features were beneficial for κ-opioid receptor activation: a methylene group between the amide group and the aromatic ring, a pyrrolidine residue at the amine nitrogen of the cyclohexane ring, a methyl group at the amide nitrogen, and/or a chlorine substitution at the 3,4-position of the aromatic ring.

KEYWORDS
[^{35}S]-GTPγS assay, κ-opioid receptor (KOR), μ-opioid receptor (MOR), new synthetic opioids (NSO), U-opioids

1 | INTRODUCTION

Since the first occurrence of new psychoactive substances (NPS) in 2008,[1] a rapid increase of these substances on the recreational drug market has been observed, where they are sold inter alia as "legal highs," "herbal highs," "bath salts," and "research chemicals."[2,3] According to the United Nations Office on Drugs and Crimes (UNODC), NPS refer to "new narcotic or psychotropic drugs, in pure form or in preparation, that are not listed in either the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances, but which may pose a public health threat comparable to that presented by substances listed in these conventions."[4] The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) established an NPS monitoring system, which currently monitors more than 834 different NPS.[5] The EMCDDA differentiates NPS according to their chemical structure into different classes, such as synthetic cannabinoid receptor agonists (SCRAs), synthetic cathinones, phenethylamines, tryptamines, benzodiazepines, or novel synthetic opioids (NSOs).[6]

Opioids, such as the naturally occurring morphine, have been used for thousands of years as medicines, for example, for their analgesic effects.[7,8] In the last centuries, research and investigation of opioids and their receptors have led to the development of synthetic opioids, such as fentanyl (first synthesized by Janssen[9]). Its potency is 50–100 times greater than morphine and 25–40 times greater than heroin in mice.[8,10–12] Besides the application of opioids as analgesics or anesthetics, abuse of these substances occurs. Due to their high dependence potential, this can result in serious health problems, such as respiratory depression or the development of opioid tolerance.[8] Four opioid receptor subtypes are known, the μ-opioid (MOR), the δ-opioid (DOR), the κ-opioid (KOR), and the nociceptin receptor, which are G protein-coupled receptors (GCPRs).[13,14] Compounds that were found to be MOR and DOR agonists were also shown to have mood-enhancing and euphoric properties. In addition, MOR is responsible for analgesic effects and respiratory depression.[15] Opposed to MOR and DOR, activation of the KOR results in dysphoria and anxiety[16] but also leads to analgesia.[15] KOR-agonists have a lower abuse potential, fewer effects on the gastrointestinal tract and result in less respiratory depression.[15] Ligands, which are partial MOR agonists and full KOR agonists, are of interest for pharmaceutical research, since they probably have analgesic potential with a reduced risk of respiratory depression, tolerance, and abuse.[17] Other reported adverse effects resulting from recreational opioid consumption include hypothermia, sedation, anxiety, sweating, disorientation, drowsiness, nausea, obstipation, and miosis.[18,19]

In 2009, the first NSO (o-desmethyltramadol) was reported to the EMCDDA Early Warning System.[20] Since then, 71 NSOs have been notified, with a notable rise in alerts beginning in 2013.[6,21] In 2018, NSOs represented approximately 3% of all seized NPS reported to the EMCDDA. NSOs are classified by the EMCDDA into fentanyl analogs (also known as "designer fentanyls," "fentanyl derivatives," or "fentalogs,"[8] e.g., ocfentanil) and non-fentanyl compounds (e.g., U-44700, MT-45) (see Figure 1).[6,18] Between 2012 and 2018, fentanyl derivatives formed the majority of new NSOs entering the NPS market.[22] With the fentanyl analog scheduling by the Drug Enforcement Administration (DEA) based on five structural core features in the fentanyl molecule in 2018,[23] and a new law introduced in China in 2019 controlling fentanyl analogs class-wide, a decrease in the number of new fentanyl derivatives was observed, resulting in only two of eight newly reported NSOs being based on fentanyl in 2019.[22,24] NSOs may show significantly higher potencies and efficacies compared to morphine, heroin, or fentanyl and thus pose a high risk of overdoses.[3] In many cases, little is known about their potencies and pharmacological, toxicodynamic, and toxicokinetic properties, due to the lack of medical and pharmaceutical applications.[25]

In addition to fentanyl derivatives, there have been other opioid receptor agonists developed by pharmaceutical companies in the 1970s, such as AH-opioids by Allen and Hanburys Ltd or U-opioids by Upjohn Company.[19] The reason for the development of the U-compounds was

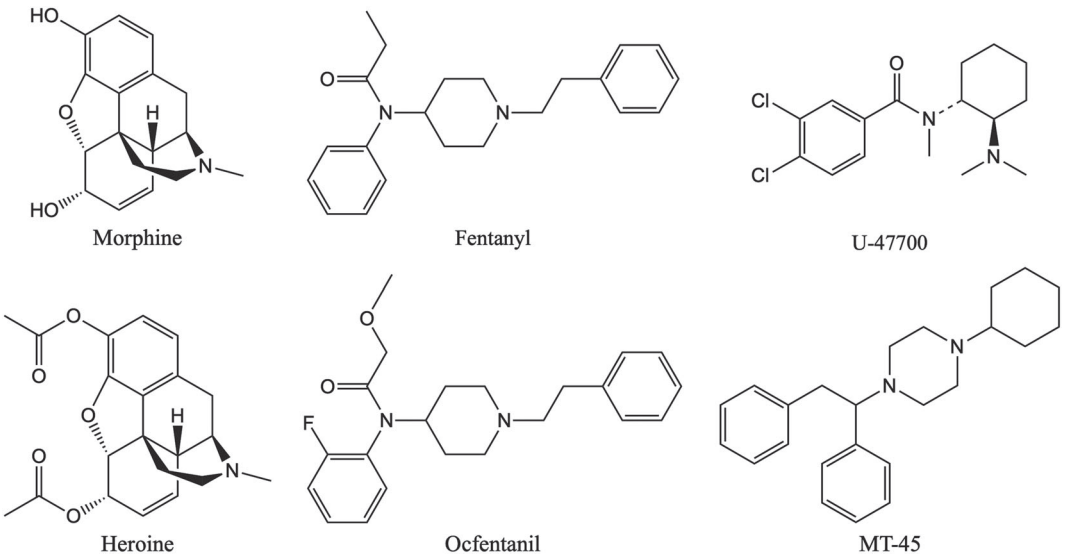

FIGURE 1 Chemical structure of common opiates, fentanyl, ocfentanil, and two non-fentanyl derived new synthetic opioids

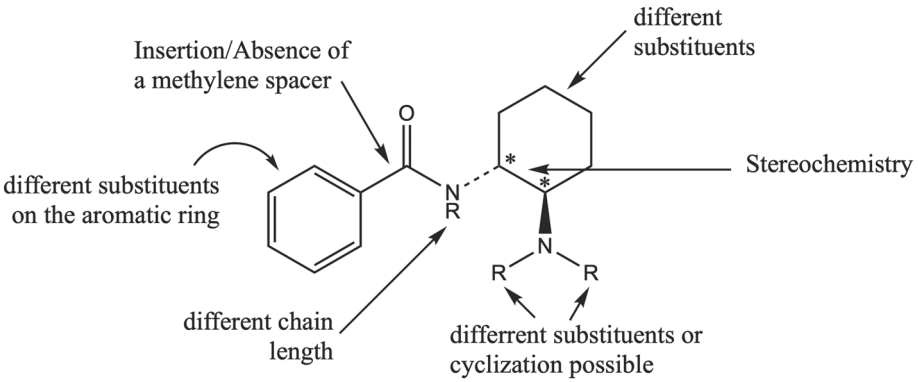

FIGURE 2 Generic chemical structure of U-opioids according to Baumann et al.[27]

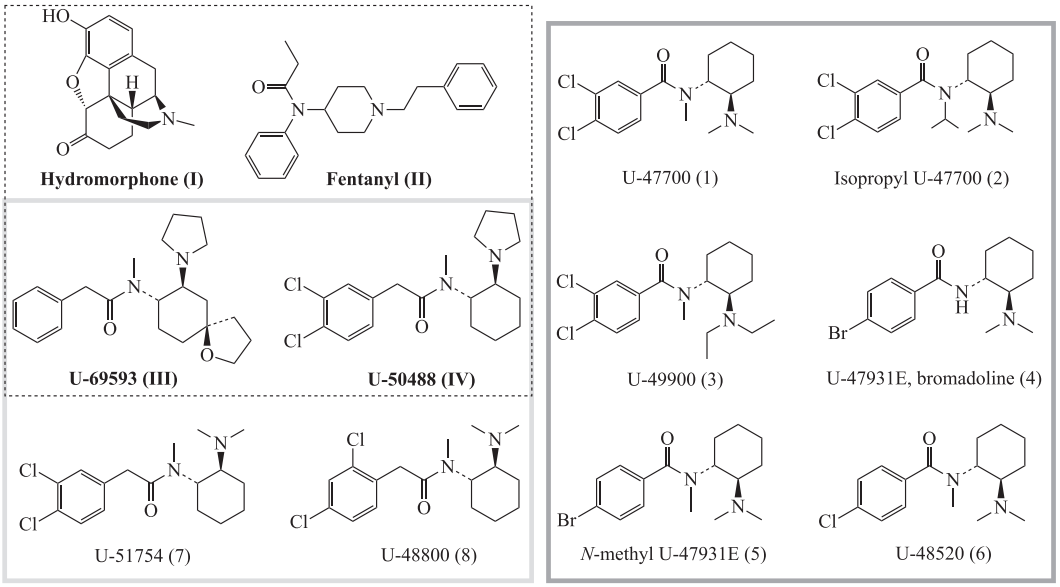

FIGURE 3 Chemical structures of the eight investigated U-opioids, the two MOR reference compounds (hydromorphone and fentanyl), and the two KOR reference compounds (U-69593 and U-50488). Dashed box: reference compounds; dashed box: reference compounds; light gray box: U-50488 group; dark gray box: U-47700 group

the search for new non-dependence producing analgesics, having potencies as high as morphine.[26] The U-opioids (for the generic chemical structure, see Figure 2) include benzamides (e.g., U-47700) and acetamides (e.g., U-50488). Few or no pharmacological properties are known for the vast majority of the U-opioids.[18] Since its first appearance on the NPS market in 2015, *trans*-3,4-dichloro-*N*-[2-(dimethylamino) cyclohexyl]-*N*-methyl-benzamide (U-47700) has been associated with several intoxications and fatalities.[28–30] In preclinical animal studies, it showed a 7.5 times greater potency than morphine.[31] U-47700 was recommended to be controlled by the DEA in 2016 in Schedule I of the Controlled Substances Act,[32] and soon after, other representatives of the U-opioids started to occur on the market,[33] such as *trans*-3,-4-dichloro-*N*-[2-(diethylamino)cyclohexyl]-*N*-methyl-benzamide (U-49900), *trans*-2-(3,4-dichlorophenyl)-*N*-2-(dimethylamino)cyclohexyl)-*N*-methylacetamide-monohydrochloride (U-51754), *trans*-2-(2,-4-dichlorophenyl)-*N*-2-(dimethylamino)cyclohexyl)-*N*-methylacetamide-monohydrochloride (U-48800), *trans*-4-bromo-*N*-2-(dimethylamino) cyclohexyl]-benzamide (U-47931E), 3,4-dibromo-*N*-methyl-*N*-[(5S,6R)-1-methyl-1-azaspiro[4.5]decan-6-yl]benzamide (U-77891), *trans*-3,-4-dichloro-*N*-methyl-*N*-[(1R,2R)-2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (U-50488), *trans*-*N*-2-(dimethylamino)cyclohexyl)-*N*-methylbenzo[d][1,3]dioxole-5-carboxamide (3,4-methylenedioxy U-47700), or *trans*-3,4-dichloro-*N*-2-(dimethylamino)cyclohexyl)-*N*-isopropylbenzamide (isopropyl U-47700). To date, no human clinical studies have been carried out for the U-opioids,[34] but various preclinical animal studies and in vitro data can be found in the literature. Szmuszkovicz and von Voigtlander[35] studied the effects of different U-opioids (U-47700, U-51754, U-47109, and U-50488 among them) in vivo in mice. Cheney et al.[36] investigated the activity of benzamide amines (e.g., U-47700 and U-48520) in mice. The only remaining sources of information on effects in humans are forensic reports (case reports, analyses of biological samples, etc.) and online user forums. On these forums, information on NPS is provided by consumers (including information on dosage, subjective reports of experience, and observed effects).[37]

Due to the relative lack of pharmacological data for the U-opioids, the aim of the present study was to investigate eight structurally closely related U-opioids (U-47700 (1), isopropyl U-47700 (2), U-49900 (3), U-47931E (4), *N*-methyl U-47931E (5), U-48520 (6), U-51754 (7), and U-48800 (8); see Figure 3) for their potential of MOR and KOR activation

using the [^{35}S]-GTPγS activity assay.* Owing to their close structural similarity, structure activity relationships (SAR) have also been derived.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

Dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$), tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), bovine serum albumin (BSA), ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), guanosine-5′-disphosphate sodium salt (GDP), and guanosine-5′-(γ-thio)triphosphate tetralithium salt (GTPγS) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Sodium chloride was purchased from Carl Roth GMbH & Co. KG (Karlsruhe, Germany). Isopropanol (Prepsolv®) and MultiScreen™ filter plates (1.2 μm) were bought from Merck (Darmstadt, Germany). Deionized water was prepared in-house using a Medica® Pro deionizer from ELGA (Celle, Germany). Human MOR and KOR membrane preparation from hamster ovary CHO-K1 cells, [^{35}S]guanosine-5′-(γ-thio)triphosphate ([^{35}S]-GTPγS, 1,250 Ci/mmol) and Ultima Gold™ were obtained from PerkinElmer (Boston, USA).

All chemical structures of the test compounds and references evaluated in this study are depicted in Figure 3. Hydromorphone and fentanyl were purchased as solutions (1 mg/ml) in methanol from Lipomed (Wheil am Rhein, Germany). *trans*-3,4-Dichloro-*N*-methyl-*N*-[(*1R*,*2R*)-2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide, monohydrochloride (U-50488), *N*-methyl-2-phenyl-*N*-(7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]decan-8-yl)acetamide (U-69593), *trans*-4-bromo-*N*-[2-(dimethylamino)cyclohexyl]-*N*-methyl-benzamide (*N*-methyl U-47931E), and *trans*-4-chloro-*N*-[2-(dimethylamino)cyclohexyl]-*N*-methyl-benzamide (U-48520) were obtained as solid powders from Cayman Chemical Company (Ann Arbor, Michigan, USA). *trans*-3,-4-Dichloro-*N*-[2-(diethylamino)cyclohexyl]-*N*-methyl-benzamide (U-49900), *trans*-2-(3,4-dichlorophenyl)-*N*-2-(dimethylamino)cyclohexyl)-*N*-methylacetamide, monohydrochloride (U-51754), and *trans*-2-(2,-4-dichlorophenyl)-*N*-2-(dimethylamino)cyclohexyl)-*N*-methylacetamide, monohydrochloride (U-48800) were acquired from LSResearchchemlab.com. *trans*-3,4-Dichloro-*N*-[2-(dimethylamino) cyclohexyl]-*N*-methyl-benzamide (U-47700) was bought from RC-King.com, *trans*-3,4-dichloro-*N*-2-(dimethylamino)cyclohexyl)-*N*-isopropylbenzamide (isopropyl U-47700) was obtained from TGC-rc.com, and *trans*-4-bromo-*N*-2-(dimethylamino)cyclohexyl]-benzamide (U-47931E) was supplied by the state office of criminal investigation in Kiel. All compounds were obtained as powders and tested for identity and purity (>95%) using GC-MS and NMR (see the supporting information), diluted in DMSO (1 mg/ml) and stored at −20°C.

2.2 | In vitro [^{35}S]-GTPγS MOR and KOR activation assay

A filtration-based [^{35}S]-GTPγS activity assay was used to examine the in vitro MOR and KOR activity of eight different U-opioids, using hydromorphone (I) and fentanyl (II) as reference compounds for MOR, and U-69593 (III) and U-50488 (IV) as reference compounds for KOR. The assay principle is based on the exchange of GDP for GTP at the Gα subunit after the activation of the GPCR with an agonist.[39] In the [^{35}S]-GTPγS assay, endogenous GTP is substituted with non-hydrolyzable [^{35}S]-labeled GTPγS, which can be measured radiometrically to determine the amount of [^{35}S]-GTPγS bound per mg of cell membrane.[40–42] The final assay volume was 200 μl, and all given concentrations are final assay concentrations referring to the free base of the respective compound. Assay preparations were performed on ice and all components were kept on ice unless specified differently. After the addition of the assay buffer (50 mM TRIS-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% BSA, 0.2 mM EDTA, pH = 7.4), 30 μM GDP and the agonist (10^{-5} M to 10^{-11} M or 10^{-4} M to 10^{-10} M in DMSO), the mixture was pre-incubated for 10 min at 37°C and 300 rpm. [^{35}S]-GTPγS (450 pM, dilutions prepared based on radioactive decay; radioligand has been frozen in aliquots after delivery to prevent thermal decomposition) was added, and the plate was further incubated for 40 min at 37°C and 300 rpm. The membrane filter plates were presoaked with 100 μl assay buffer. After transferring the samples to the filter plate, they were washed three times with washing buffer (50 mM TRIS–HCl, 0.5% BSA, pH = 7.4), and the filters were dried at 50°C. Radioactivity was determined by liquid scintillation using a liquid scintillation counter (TriCarb 2100TR, Perkin Elmer).

For basal binding, DMSO was added to the mixture instead of agonist. For determination of nonspecific binding, the highest concentration (10^{-5} M) of (I) (MOR) or (III) (KOR) and unlabeled GTPγS (100 μM) were added. Each concentration was tested in duplicate, and all experiments were repeated three times ($n = 3$).

2.3 | Data processing and analysis

Raw data were processed using Excel 365 Version 2107 for Windows. For analysis of the raw data, the percentage of the measured signal above the basal activity was determined.

Outlier testing was performed using Dixon test ($\alpha = 0.05$). All measured values were normalized to the E_{max} (top value of best fit values) of the reference compound ((I) for MOR and (III) for KOR). Curve-fitting of concentration-response curves was performed using GraphPad Prism 9 (version 9.01; GraphPad Software Inc., La Jolla, CA, USA) via nonlinear regression (three parameters, Hill slope = 1) to obtain the EC_{50} values (with 95% confidence interval, a measure of potency) and the E_{max} values (relative to the E_{max} of the reference substance, with 95% confidence interval; a measure of efficacy).

3 | RESULTS AND DISCUSSION

MOR and KOR activity of all eight test compounds was evaluated using the [^{35}S]-GTPγS assay, generating receptor activation profiles presented in Figure 4 (MOR) and Figure 5 (KOR). The derived potencies, presented as EC_{50} values, and efficacies, presented as E_{max} values (the normalized maximum response of the activation of the

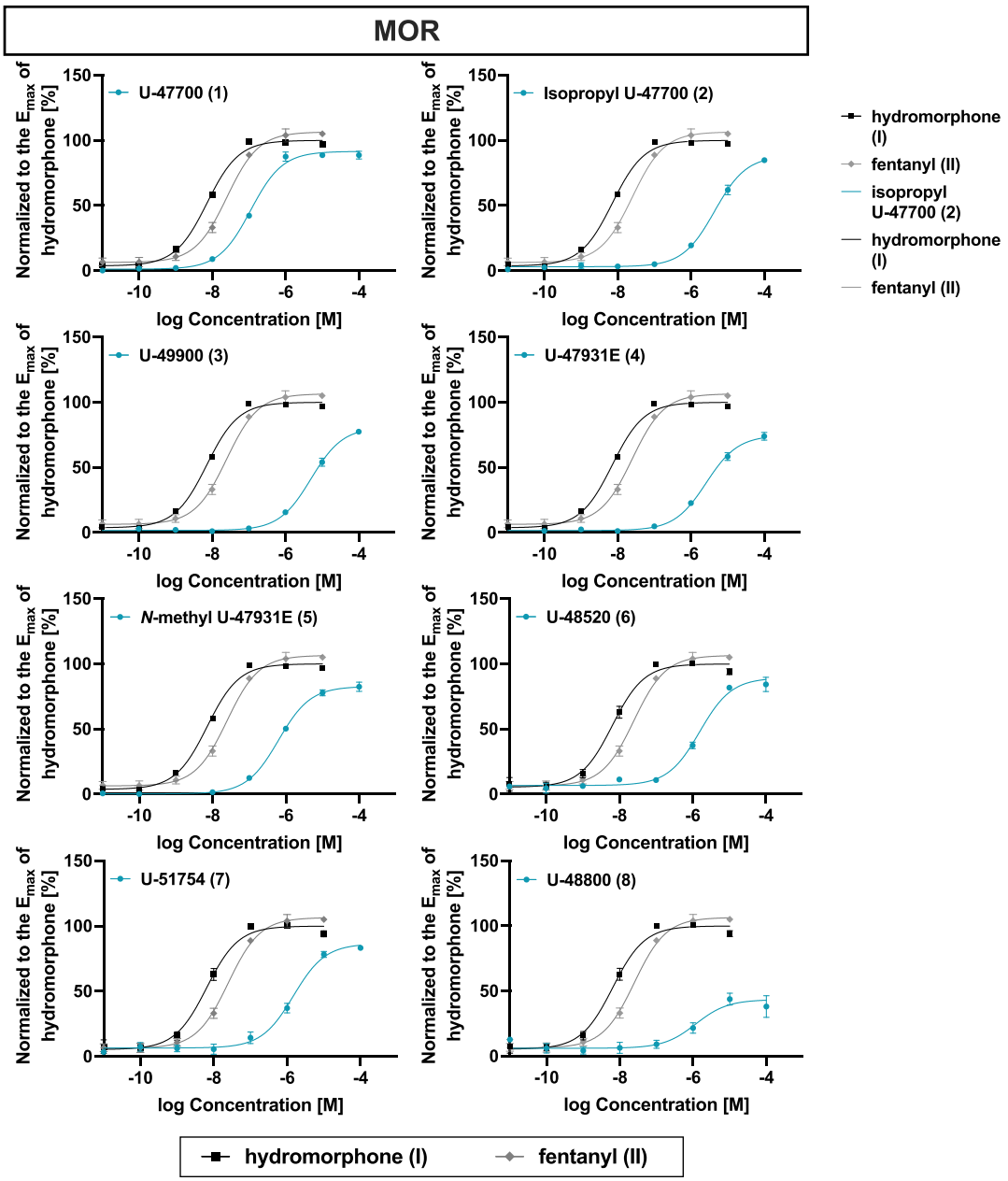

FIGURE 4 Concentration-dependent interaction of [^{35}S]-GTPγS with MOR after addition of the different U-opioids. The data are presented as the mean of the receptor activation ± standard error (SEM) expressed in percent normalized to the E_{max} value of hydromorphone (I). The experiments were performed in duplicates in three independent experiments [Colour figure can be viewed at wileyonlinelibrary.com]

receptor by the ligand), relative to the respective MOR and KOR reference compound, are presented in Table 1. Note that for (2)–(6), the maximal receptor activation at KOR could not be reached due to the relatively weak agonism displayed by these compounds at this receptor. Since the EC_{50} values of these compounds are >10,000 nM, the ratio of the EC_{50} (MOR) to the EC_{50} (KOR), which reflects the so-called potency ratio, is smaller than one.

(I) was used as a reference compound for MOR and (III) for KOR, since these compounds are well characterized and have been used previously in different studies.[17,24,38,43,44] For the same reason, (II) and (IV) were also included in this study as additional MOR and KOR reference compounds. It needs to be noted here that because different reference compounds for MOR and KOR had to be used, the comparability of the data is limited. Activation profiles could be obtained for all test compounds.

Concerning the MOR reference substances, (I) (EC_{50}: 6.75 nM, 95% CI: 5.56–8.16 nM) showed a higher potency than (II) (EC_{50}: 24.9 nM, 95% CI: 18.6–33.5 nM). In the literature, different potencies have been described for (1), even using the same assay, for example, Wentland et al.,[45]: EC_{50}: 2.6 nM ± 0.14 nM opposed to Olson et al.[46] (EC_{50}: 39 nM ± 22 nM).

Also for (II), different potencies were reported using a [^{35}S]GTPγS assay: EC_{50}: 23.0 nM ± 4.1 nM,[47] EC_{50}: 21.4 nM ± 2.3 nM,[48] and EC_{50}: 18 ± 4 nM.[49] Priyanka et al., using a [^{35}S]GTPγS assay,

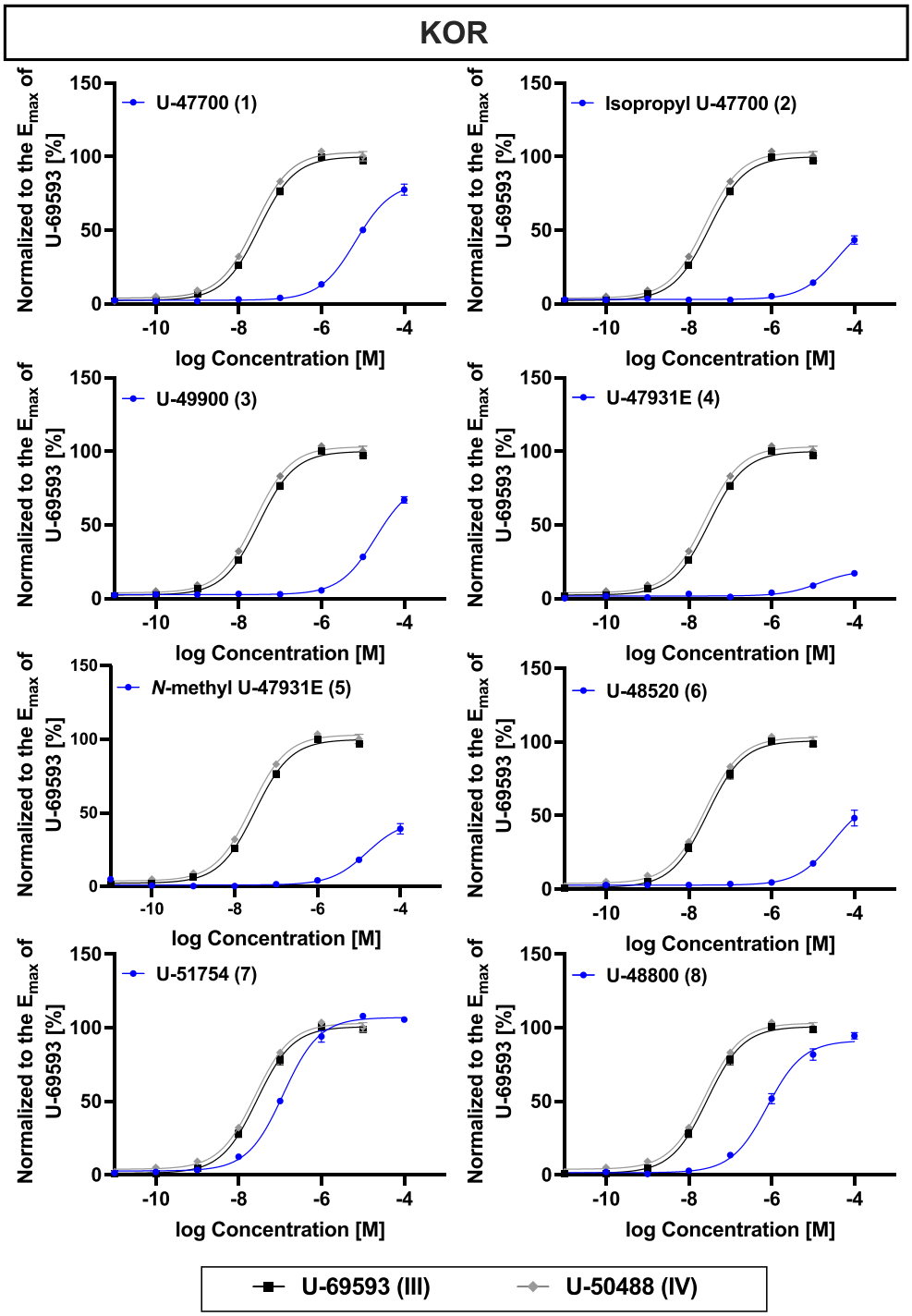

FIGURE 5 Concentration-dependent interaction of [^{35}S]-GTPγS with KOR upon stimulation with the different U-opioids. The data are presented as the mean of the receptor activation ± standard error (SEM) expressed in percent normalized to the E_{max} value of U-69593 (III). The experiments were performed in duplicates in three independent experiments [Colour figure can be viewed at wileyonlinelibrary.com]

described a higher potency for hydromorphone (39.6 nM ± 5.1 nM) than for fentanyl (51.1 nM ± 3.6 nM),[50] whereas Vandeputte et al.[51] obtained for the NanoBit® MOR-mini-G recruitment assay comparable potencies for (I) (24.1 nM [16.2–36.4 nM]) and (II) (32.7 nM [23.2–45.8 nM]). Different assays and the use of different assay condition (e.g., the concentrations of $MgCl_2$ and GTP, different incubation times, the solvent in which the test compounds were diluted, and the temperature—as we have seen when we first established our assay; data not published) might be the cause of differences in potencies reported in literature.

The best-studied substance of the test set, (1), was also the most potent and efficacious compound at MOR (EC_{50}: 111 nM, 95% CI: 89.6–137 nM, E_{max}: 91.6%, 95% CI: 88.5–94.6%).[52–54] Different values have been reported for (1) at MOR, obtained with different assays: [^{35}S]GTPγS assay: EC_{50} 214 nM ± 23 nM,[34] NanoBiT® mini-G_i: logEC_{50}−6.482 ± 0.04 (corresponding to an EC_{50} value of

TABLE 1 Assessment of in vitro MOR and KOR activation using a [^{35}S]-GTPγS assay of eight different U-opioids, represented by their EC_{50} and E_{max} values (relative to hydromorphone and fentanyl [MOR] or U-69593 and U-50488 [KOR]) as a measure of potency and efficacy, respectively

	MOR			KOR			
Compound	EC_{50} [nM] (CI: 95%)	E_{max} [%] relative to I (CI: 95%)	E_{max} [%] relative to II (CI: 95%)	EC_{50} [nM] (CI: 95%)	E_{max} [%] relative to III (CI: 95%)	E_{max} [%] relative to IV (CI: 95%)	EC_{50} (MOR) to EC_{50} (KOR) ratio
Hydromorphone (I)	6.75 (5.56–8.16)	100 (97.4–103)	93.8 (91.4–96.2)				
Fentanyl (II)	24.9 (18.6–33.5)	107 (102–111)	100 (95.8–104)				
U-69593 (III)				29.3 (26.1–32.9)	100 (98.7–102)	97.6 (96.0–99.2)	
U-50488 (IV)				24.8 (20.3–30.2)	103 (100–106)	100 (97.5–103)	
U-47700 (1)	111 (89.6–137)	91.6 (88.5–94.6)	85.9 (83.1–88.8)	6,679 (5,573–7,953)	82.4 (78.8–86.2)	80.2 (76.6–83.7)	0.02
isopropyl U-47700 (2)	4,367 (3,331–5,679)	88.2 (83.0–93.6)	82.8 (77.9–87.9)	>10,000[a]	58.9[b] (51.3–71.2)	57.3[b] (49.9–69.3)	<1
U-44990 (3)	4,987 (3,955–6,234)	80.8 (76.6–85.3)	75.9 (71.9–80.0)	>10,000[a]	80.2[b] (77.4–83.1)	78.0[b] (75.3–80.8)	<1
U-47931E (4)	2,603 (2,007–3,398)	74.6 (70.7–78.8)	70.0 (66.4–73.9)	>10,000[a]	19.2[b] (16.4–22.6)	18.7[b] (16.0–22.0)	<1
N-methyl U-47931E (5)	632 (527–755)	82.7 (80.0–85.4)	77.6 (75.1–80.1)	>10,000[a]	45.1[b] (40.6–50.2)	43.9[b] (39.5–48.8)	<1
U-48520 (6)	1,561 (1,127–2,169)	89.3 (83.7–95.1)	83.8 (78.6–89.3)	>10,000[a]	62.0[b] (54.3–73.0)	60.3[b] (52.8–71.1)	<1
U-51754 (7)	1,485 (1,000–2,214)	86.5 (80.1–93.1)	81.1 (75.2–87.4)	120 (102–141)	107 (104–110)	104 (102–107)	12.4
U-48800 (8)	1,188 (413–3,146)	43.4 (36.0–51.5)	40.7 (33.8–48.3)	786 (593–1,034)	91.3 (86.8–95.9)	88.8 (84.4–93.3)	1.51

Note: Data are given as EC_{50} (nM) and E_{max} (%) values (95% confidence interval [CI]), obtained from at least three independent experiments, performed in duplicate.

[a]Plateau was not reached, EC_{50} values to be interpreted with caution.

[b]Efficacy at highest concentration tested (100 μM). Plateau was not reached, efficacy to be interpreted with caution.

330 nM), βarr2 recruitment assay: $\log EC_{50}$–6.776 ± 0.08 (corresponding to an EC_{50} value of 168 nM),[43] and [^{35}S]GTPγS assay: EC_{50} 140 nM ± 23 nM.[55]

At KOR, the obtained EC_{50} value of (1) was 6,679 nM (95% CI: 5,573–7,953 nM), which is higher than previously reported data of 2,699 nM ± 769 nM[34] and 201 nM ± 74 nM.[55] However, for compounds with such a low potency, it is difficult to determine the exact EC_{50} value; therefore, these deviating results are not surprising.

At KOR, (7) was the most potent and efficacious compound (EC_{50}: 120 nM, 95% CI: 102–141 nM, E_{max}: 107%, 95% CI: 104–110%), out of the eight test substances.

However, for both MOR and KOR, the respective reference compounds had the highest potencies. In fact, (I) and (II) were 16 and 4 times more potent than (1) at MOR, and (III) (EC_{50}: 29.3 nM, 95% CI: 26.1–32.9 nM) and (IV) (EC_{50}: 24.8 nM, 95% CI: 20.3–30.2 nM) were 4 and 5 times more potent than (7) at KOR.

In the literature, also, different potencies for (III) and (IV) have been reported using a [^{35}S]GTPγS assay (III): (EC_{50}: 7.25 nM ± 0.9 nM,[56] EC_{50}: 22.0 nM ± 5.8 nM[57]) and (IV) (EC_{50}: 8.79 nM ± 0.74 nM,[57] EC_{50}: 16 nM ± 4 nM[46]). As stated previously, differences in the assay conditions could explain the differences with our data.

None of the test compounds showed higher efficacies than (I) (E_{max} arbitrarily set to 100%) in activating MOR; thus, they are partial agonists compared to (I). Notably, (8) (E_{max}: 43.4%, 95% CI: 36.0–51.5%) was even less than half as efficacious as (I) at MOR. (IV) (E_{max}: 103%, 95% CI: 100–106%), and (7) showed efficacies at least as high as (III) at KOR and thus is considered full agonists compared to (III) (E_{max} arbitrarily set to 100%). Apart from (3), which showed similar efficacies at MOR and KOR, compounds (1), (2), and (4)–(6) had a higher efficacy at MOR versus KOR. For (7) and (8), efficacies at KOR predominated over those at MOR. However, as previously stated, for (2)–(6), a plateau could not be reached using the tested concentrations. Therefore, as the reported E_{max} values only reflect the response at the highest tested concentration, it might be possible that the efficacies are actually higher.

For a better comparison of our results with the receptor binding affinities described in the literature and to enable a better comparability between the potencies at MOR and KOR, the ratio of the EC_{50} value at MOR to the EC_{50} value at KOR was calculated (see Table 1). If the potency ratio is greater than one, the substance shows a higher potency at KOR compared to MOR; if it is lower than one, the potency is greater at MOR.

3.1 | Structure activity relationships

The eight tested compounds are structural analogs (see Figure 3), facilitating an evaluation of the influence of minor structural changes. The SAR of several U-compounds have been evaluated in the past.[26,27,36,38,58–60]

Our results for compounds of the U-47700 group showed potency ratios below one, standing for higher potencies at MOR. Opposed to this, compounds of the U-50488 group had potency ratios greater than one. In terms of efficacy at MOR, compounds from both groups were not found in a clear order, whereas at KOR, all compounds in the U-50488 group showed higher efficacies than those in the U-47700 group.

The stereochemistry of a compound has a major influence on the affinity and activity of U-compounds. Szmuszkovicz hypothesized that the *trans*-configuration is necessary for an analgesic activity of a compound. His reasoning is based on the observed lack of analgesic effects of U-54494 (a *cis*-analog of (1) with a pyrrolidine residue at the cyclohexane ring instead of an *N*-dimethyl residue) compared to its *trans* analog (1).[26] Cheney et al.[36] suggested, based on geometric analysis, that both *trans* (*R*,*R*) and (*S*,*S*) stereoisomers might be able to associate with the receptor. Szmuszkovicz[26] described that MOR agonists, such as (1), are the most potent in (*R*,*R*)-conformation, while KOR agonists, such as (IV), are the most potent in the opposite absolute configuration (*S*,*S*). This observation was confirmed by the study of several analogs of (1) in (*R*,*R*)- and (*S*,*S*)-configuration (assay based on inhibition of cAMP accumulation and hMOR internalization). For (1), both enantiomers showed a decrease in cAMP levels, while (*R*,*R*)-(1) was shown to have a higher potency than the (*S*,*S*)-enantiomer. Other (*S*,*S*)-enantiomers tested did not show a decrease in cAMP levels at the examined concentrations.[60]

In the present study, the chirality of the test compounds could not be determined using the GC-MS and NMR structure confirmation methods, because neither stereoisomer reference standards (for the GC-MS analysis) nor chiral additives to generate diastereomers (for the NMR analysis) were available. In the past, to the best of the authors' knowledge, commercially available reference compounds of U-opioids were the racemate. For example, Hsu et al.[60] reported from personal communications with Cayman Chemicals that the racemate of (1) was synthesized. Further, it is extremely unlikely that manufacturers performed the elaborate stereoselective synthesis without declaring it. Additionally, the separation of a racemate in its stereoisomers is laborious. Hence, we assumed that all used reference compounds were in fact the racemate.

Another important structural component is the absence or presence of a methylene group between the amide group and the aromatic ring, the so called "eastern methylene group." It was found that the presence of the methylene spacer leads to KOR agonists, its absence to MOR agonists.[26]

Baumann et al.[27] categorized the U-opioids into the so-called U-47700 group, that is, benzamides without a methylene spacer, and the U-50488 group, that is, acetamides with a methylene spacer. According to this, test compounds (1) to (6) belong to the U-47700 group, and (7), (8), (III), and (IV) belong to the U-50488 group. Szmuszkovicz and von Voigtlander[35] tested four U-compounds in mice (U-47109, (1), (7), and (IV)). U-47109 and (1) (both belonging to the U-47700 group) showed morphine-like behavior (straub tail, arched back, and increased locomotor activity) and analgesic properties (antagonism by naloxone), while (7) and (IV) (both belong to the U-50488 group) did not show morphine-like behavioral properties. It has been reported (tested compounds: U-47109, (1), (6), U-50211, (7), (IV), U-62066, (III)) that the compounds of the U-47700 group show higher receptor binding affinities (evaluated in guinea pig brain) for MOR and the compounds belonging to the U-50488 group for KOR; hence, the presence or absence of the methylene group has an influence on the receptor binding affinity at MOR and KOR.[38] Cheney et al.[36] argued that in absence of the methylene group, a spatial alignment of the alkaline nitrogen and aromatic carbon ring takes place, analogous to that observed in morphine,[27] which could explain the high receptor binding affinity of (1) towards MOR. If a methylene group is located between the amide group and the aromatic ring, this has an influence on the spatial alignment of the phenyl ring. Thus, the aminoamide and the aromatic ring are spatially connected. In addition, between the alkaline tertiary nitrogen atom and the amide group, the molecule is taking a torsion angle of 60° in low energy conformation, due to the methylene group and the cyclohexyl backbone, which is

considered a necessary condition for an interaction with the KOR.[27,61]

In general, our results on the receptor activation of MOR and KOR are in good alignment with reported receptor binding affinities, confirming that the U-47700 group has a higher affinity and activity at MOR and the U-50488 group at KOR.

3.2 | The U-47700 group

For the U-47700 group (compounds (1)–(6)), different structural features can be evaluated. However, as previously mentioned, with the exception of (1), the plateaus were not reached at KOR within the concentration range that has been tested for this group. Hence, influences of structural differences of this group on the potency at KOR cannot be derived.

In our experiments, the impact of substituents attached to the aromatic ring was investigated via comparison of compounds (1) (two chlorine atoms in 3,4-position), (5) (no chlorine atoms but a bromine in para position [4-position]) and (6) (one chlorine atom in para-position). (1) showed the highest potency at MOR of all tested U-compounds. Compound (5) (EC_{50}: 632 nM, 95% CI: 527–755 nM) possessed a higher potency than (6) (EC_{50}: 1,561 nM, 95% CI: 1,127–2,169 nM). In our study, (1) showed a higher potency at MOR than (6), lacking the chlorine atom in *meta*-position. Exchange of the chlorine atom of (6) with a less electronegative bromine, which results in compound (5), enhanced to some degree the potency at MOR. Efficacy at MOR was higher than at KOR for all three compounds, while (1) showed higher efficacy at KOR in comparison to (5) and (6).

It has been reported that different substituents on the phenyl ring have an influence on the binding affinity. (1) showed higher affinity for MOR (K_D: 5.3 nM) and KOR (K_D: 910 nM) than (6) (K_D (MOR): 200 nM, K_D (KOR): 2,900 nM).[38]

Using a [^{125}I]IBNtxA assay, binding affinities of 57 ± 21 nM for MOR and 653 ± 163 nM for KOR were reported for (1).[34] In another study applying the cAMP inhibition assay, (1) showed high potency at MOR (EC_{50}: 8.8 nM ± 4.9 nM), while (6) and (5) showed low potency (EC_{50} value not given).[60]

Recently, Vandeputte et al.[51] reported on the structure activity relationship of five U-compounds (*N*-ethyl-U-47700, 3,4-difluoro-U-47700, (4), 2,4-difluoro-U-48800, and U-62066) using a NanoBit® MOR-β-arr2/mini-G recruitment and AequeoScreen® assay. In an earlier publication, they investigated (1) and (3).[43] In their assay, (1) showed much higher potency (mini-G_i assay: logEC_{50}: −6.482 ± 0.04 (equal to EC_{50} of 330 nM), E_{max}: 205% ± 3.75%; β-arrestin assay: logEC_{50}: −6.776 ± 0.08 (equal to EC_{50} of 168 nM), E_{max}: 214% ± 6.35%)[43] than its difluorinated analog 3,4-difluoro-U-47700 (mini-G_i assay: EC_{50}: > 9,000 nM, E_{max}: 169% (154%–186%); β-arrestin assay: EC_{50}: >4,000 nM, E_{max}: 132% (119–147%); AequeoScreen®: EC_{50}: 1,817 nM (1,553–2,132 nM), E_{max}: 103% (101–106%)).[51]

When comparing our findings with affinity data in literature, the same trends can be observed: In line with our data, the affinity experiments of Loew et al.[38] and activity experiments of Hsu et al.[60] showed a higher affinity and potency at MOR for (1) than for (6) and (5). When exchanging chlorine atoms with bromine atoms to some degree, a higher potency and affinity can be observed (in our study: (5) and (6)).

In the present study, influences of *N*-substituents were evaluated comparing (1) with (2), and (4) with (5). Both (1) and (2) have chlorine atoms in 3,4-position on the aromatic ring, whereas (4) and (5) have a bromine in 4-position. Compound (4) is (5)'s *N*-demethylated analog, (2) possesses an isopropyl residue instead of (1)'s methyl group at *N*-amide. Compound (1) showed a higher potency at MOR compared to (2) (EC_{50}: 4367 nM, 95% CI: 3,331–5,679 nM). However, the efficacies of (1) and (2) (E_{max}: 88.2%, 95% CI: 83.0–93.6%) were in the same range at MOR. At KOR, (1) (E_{max}: 82.4%, 95% CI: 78.8–86.2%) was more efficacious than (2) (E_{max}: 58.9%, 95% CI: 51.3–71.2%) in the highest concentration tested. At MOR, (5) (E_{max}: 82.7%, 95% CI: 80.0–85.4%) showed a higher potency and efficacy than (4) (EC_{50}: 2,603 nM, 95% CI: 2,007–3,398 nM, E_{max}: 74.6%, 95% CI: 70.7–78.8%). At KOR, (5) (E_{max}: 45.1%, 95% CI: 40.6–50.2%) showed a higher efficacy than (4) (E_{max}: 19.2%, 95% CI: 16.4–22.6%) at the highest concentration tested.

The influence of substituents attached to the amide nitrogen has been evaluated previously using a cAMP inhibition assay.[60] Different U-compounds related to (1) but differing in their residues attached to the aromatic ring and their *N*-demethylated analogs were investigated (among them (1), its *N*-demethylated analog U-47109, (4) and (5)). U-47109 showed a slightly higher potency than (1) (EC_{50} (1): 8.8 nM ± 4.9 nM, EC_{50} (U-47109): 3.0 ± 0.3 nM), the EC_{50} values of (4) and (5) were not given.[60] However, in studies in mice, U-47109 (ED_{50} (tail flick): 11 mg/kg sc, ED_{50} (tail pinch): 11 mg/kg sc, ED_{50} (inclined screen): >100 mg/kg sc, ED_{50} (HCl writhing): 9 mg/kg sc) showed lower potency than (1) (ED_{50} (tail flick): 0.2 mg/kg sc, ED_{50} (tail pinch): 0.2 mg/kg sc, ED_{50} (inclined screen): 9 mg/kg sc, ED_{50} (HCl writhing): 0.2 mg/kg sc).[35] Vandeputte et al.[51] examined *N*-ethyl-U-47700 (differing from (1) in an ethyl residue attached to the nitrogen), using NanoBit® MOR-β-arr2 and mini-G recruitment assays and the AequeoScreen® assay. *N*-ethyl-U-47700 showed lower potency (mini-G_i assay: EC_{50}: 767 nM (599–975 nM), E_{max}: 247% (237–258%); β-arrestin assay: EC_{50}: 451 nM (239–821 nM), E_{max}: 161% (144–178%); AequeoScreen®: EC_{50}: 241 nM (148–405 nM), E_{max}: 139% (131–147%)),[51] than the previously reported data for (1).[43] Compound (4) also was examined (mini-G_i assay: EC_{50}: 1703 nM (1,072–2,786 nM), E_{max}: 64.1% (58.6–70.2%); β-arrestin assay: EC_{50}: 2,856 nM (1,953–4,226 nM), E_{max}: 52.8% (49.3–56.5%); AequeoScreen®: EC_{50}: 554 nM (423–723 nM), E_{max}: 85.9% (82.4–89.4%)), showing lower potency than (1) and N-ethyl-U-47700.[51]

Hence, based on our observation, a methyl group at the amide nitrogen seems to be superior to a hydrogen atom or a bulkier residue, such as an isopropyl group, for the activation of MOR. While this is in contrast with the reported slightly higher potency of the U-47700 *N*-amide demethylated analog (U-47109) compared to (1),[60] our findings are in line with a higher activity observed in animal studies[35] and the reported higher potencies for (1) in comparison to *N*-ethyl-U-47700

and (4).[43,51] Since the potencies of (4) and (5) were not evaluated by Hsu et al.,[60] their assay differed from ours and we did not examine U-47109 in our study, further investigation is necessary.

The final structural feature that can be evaluated in the U-47700 group is the influence of the substituents on the amine residue of the cyclohexane ring by the comparison of (1), which has an *N*-dimethylamino group, and (3), which has an *N*-diethylamino group.

Our data showed that (1) was about 45 times more potent than (3) at MOR (EC_{50}: 4,987 nM, 95% CI: 3,955–6,234 nM). Compound (1) also was also more efficacious at MOR than (3) (MOR: E_{max}: 80.8%, 95% CI: 76.6–85.3%; KOR: E_{max}: 80.2%, 95% CI: 77.4–83.1%), but at KOR, their efficacy was comparable.

Since (3) is a completely new substance and not part of the patented U-compounds by Upjohn Company,[36] published data on it are scarce. Vasudevan et al.[43] examined the potencies of different NSO at MOR (among these (1) and (3) (mini-G_i assay: logEC_{50}: −5.424 ± 0.2 (equal to EC_{50} of 3,767 nM), E_{max}: 72.6% ± 10.85%; β-arrestin assay: logEC_{50}: −5.197 ± 0.03 (equal to EC_{50} of 6,353 nM), E_{max}: 128% ± 3.19%) in a mini-G_i and βarr2 recruitment assay using NanoBiT®, resulting in a much higher potency of (1) over (3).

Based on our observations and supported by the higher potency of (1) compared to (3), as reported in literature,[43] the dimethylamine nitrogen group seems to be advantageous over the diethylamine nitrogen group for activity at MOR.

3.3 | The U-50488 group

Three different structural features could be evaluated within the U-50488 group.

The first feature investigated is the influence of amino nitrogen substituents on the cyclohexane ring, comparing U-51754 (7), which has a dimethylamine residue and U-50488 (IV), which has a pyrrolidine residue.

In our experiments, (IV) (EC_{50}: 24.8 nM, 95% CI: 20.3–30.2 nM) was almost five times more potent at KOR than (7), whereas their efficacy was in the same range. No statement on MOR activation can be made here since (IV) was not tested at this receptor.

Both substances have been previously investigated for their MOR and KOR affinity in guinea pig brain. It was found that the amino nitrogen substituents only had a small influence on MOR affinity but a major impact on the KOR affinity ((7): K_D (MOR): 220 nM, K_D (KOR): 71 nM, ratio MOR/KOR: 3.1; (IV): K_D (MOR): 430 nM, K_D (KOR): 2.2 nM, ratio MOR/KOR: 195).[38] Also, in animal studies using the tail pinch test, (IV) showed a higher potency (ED_{50}: 2.5 mg/kg sc) compared to (7) (ED_{50}: 7.0 mg/kg sc). However, using the tail flick test, (IV) (ED_{50}: 2.5 mg/kg sc) and (7) (ED_{50}: 2.8 mg/kg sc) showed comparable results. Further, both compounds had analgesic but no morphine-like behavioral properties.[35]

Hence, our observations concerning the activity of both substances are in alignment with reported higher binding affinities of (IV) for the KOR[38] and higher potency in animal studies.[35] However, since (IV) was not tested on MOR, it remains unclear whether there is a full agreement of the reported data with our study. Results indicate that the pyrrolidine ring of (IV) is advantageous over the dimethylamine residue in (7) for the activation of KOR. Further investigation might be necessary.

Second, different substituents on the aromatic ring were investigated, comparing (7), which possesses two chlorine atoms in 3,4-position, and (8), which has two chlorine atoms in ortho and para position (2,4-position) (see Figure 3).

At MOR, the potencies of (7) (EC_{50}: 1485 nM, 95% CI: 1,000–2,214 nM) and (8) (EC_{50}: 1188 nM, 95% CI: 413–3,146 nM) were comparable; however, (7) (E_{max}: 86.5% 95% CI: 80.1–93.1%) was twice as efficacious as (8). At KOR, (7) was 6.5 times more potent than (8) (EC_{50}: 786 nM, 95% CI: 593–1,034 nM; E_{max}: 91.3%, 95% CI: 86.8–95.9%) and was also more efficacious. For (8), no affinity or activity data have been published yet, but its fluorinated analog, 2,4-difluoro-U-48800, has been investigated in a reason study, revealing very low potencies at MOR (β-arrestin assay: EC_{50}: >22,000 nM, E_{max}: 3.85% (maximum response at highest concentration); AequeoScreen®: EC_{50}: >28,000, E_{max}: 45.9% (maximum response at highest concentration)).[51] Possessing the chlorine atoms in 3,4-position, rather than 2,4-position, seems more advantageous for a higher potency at KOR. Likewise, our results show that the 2,4-position might be disadvantageous for the efficacy at MOR. The higher potency of (8) in our study compared to the published potencies of 2,4-difluoro-U-48800 at MOR suggests that difluorination might lead to less potent compounds at MOR (as described earlier when comparing (1) and its difluorinated analog). However, since different assays have been used, the activity of 2,4-difluoro-U-48800 was not evaluated on KOR, and no structural analog of the U-47700 group with chlorine atoms in 2,4-position has been examined, further investigations are necessary.

Comparing (III) and (IV), there are two structural differences: the presence or absence of an oxaspiro[4.5]decyl system and the absence (III) or presence (IV) of chlorine substituents on the aromatic ring. In our experiments, both compounds had potencies and efficacies in a similar range at KOR (III: EC_{50}: 29.3 nM, 95% CI: 26.1–32.9 nM). However, the activity at MOR was not evaluated.

U-62066 (not tested in our study) possesses a oxaspiro[4.5]decyl system (as (III)) but also has two chlorine atoms in 3,4-position of the aromatic ring (as (IV)).

In guinea pig brain receptor binding studies by Lahti et al.,[58] (IV) showed following affinities: IC_{50} (MOR): 1,900 nM, IC_{50} (KOR): 7.4 nM, IC_{50} (MOR)/IC_{50} (KOR): 256. The insertion of an oxaspiro [4.5]decyl system, resulting in U-62066, showed a slightly increase of the affinity for KOR but had a major effect on the affinity for MOR (U-62066: IC_{50} (MOR): 210.0 nM, IC_{50} (KOR): 2.5 nM, IC_{50}(MOR)/IC_{50}(KOR): 84.0). The absence of the chlorine atoms, resulting in (III), slightly decreased the affinity for KOR, whereas the affinity for MOR strongly decreased ((III): IC_{50} (MOR): 4,600 nM, IC_{50} (KOR): 9.5 nM, IC_{50} (MOR)/IC_{50} (KOR): 484). In a mouse tail flick test, (III) showed an ED_{50} of 3.6 mg/kg, while (IV) showed ED_{50} of 2.7 mg/kg.[58] Supported by data reported by Loew et al.,[38] the absence or presence of an oxaspiro[4.5]decyl system and/or chlorine atoms on the aromatic ring

does not suggest a major impact on KOR activity, but on KOR selectivity due to much higher affinity of (IV) and U-62066 for MOR ((IV): K_i (MOR): 430 nM, K_i (KOR): 2.2 nM, ratio (MOR/KOR): 195; (III): K_i (MOR): 1700 nM, K_i (KOR): 7.2 nM, ratio (MOR/KOR): 236). U-62066 also has been investigated in activity assays (mini-G_i assay: EC_{50}: 1788 nM (1,294–2,503 nM), E_{max}: 126% (118–133%); β-arrestin assay: EC_{50}: 1,904 nM (1,369–2,678 nM), E_{max}: 97.0% (90.3–104%); AequeoScreen®: EC_{50}: 598 nM (420–846 nM), E_{max}: 134% (128–141%)) [Vandeputte, 2022], showing low (but in comparison with other U-compounds as, e.g., (4) higher) potencies at MOR, being in line with its reported low MOR affinity.

Our results are in agreement with reported low difference of affinity data for (IV) and (III) at KOR.[38,58] Since both substances were not examined at MOR, no statement can be made about their potency ratios.

In general, results of in vitro affinity and activity assays cannot be directly translated into resulting in vivo effects, since other important aspects must be considered. First, the availability depending on the route of administration needs to be evaluated.[62] Next, the compounds need to enter the target tissue, which, in case of opioids, includes crossing the blood–brain barrier.[63] Also, the rate of biotransformation and potential activity of metabolites can have a major influence on the activity in vivo.[32] In addition, the investigated mechanism of activation can have an impact on the determined receptor activation (e.g., β-arrestin recruitment vs. G protein signaling).[24,36] Therefore, it must be pointed out that a direct extrapolation of the effects observed in vitro to effects anticipated in vivo is not possible. Cheney et al.[36] attributed the observation of higher in vivo potencies in combination with lower in vitro binding affinities to a higher lipophilicity of the U-opioids compared to morphine, which is associated with an improved permeation of the blood–brain barrier. Considering the reported intoxications for U-47700,[52–54] higher in vivo potencies than the herein presented in vitro potencies are likely.

4 | CONCLUSION

The present study investigated the potential of eight different U-opioids to activate MOR and KOR. Furthermore, we discussed in detail the effect of structural alterations on MOR and KOR activities. The potencies and efficacies were determined using a [^{35}S]-GTPγS activity assay optimized for MOR and KOR. For all compounds, concentration-response curves could be obtained. The most prevalent compound of this set, (1), was also the most potent compound at MOR, and (7) was the most potent substance at KOR. Nevertheless, the two U-opioids (III) and (IV), which have been used as KOR reference compounds, were four and five times more potent than (7) at KOR.

The following structural features were identified to be advantageous for a high activity at MOR: two chlorine atoms in 3,4-position on the aromatic ring, the absence of a methylene group between the amide group and aromatic ring, a methyl group on the amide nitrogen, and/or a dimethylamine residue on the amine nitrogen of the cyclohexane ring. Additionally, several publications reported the requirement of a *trans* (*1R*,*2R*) configuration for high binding affinity at MOR.[26,27,36,37,60] For a potent KOR agonist, the following structural features were found to be beneficial: a methylene group between the amide and the aromatic ring, a pyrrolidine residue on the cyclohexane ring, a methyl group on the amide nitrogen, and/or 3,4-position of the chlorine atoms of the aromatic ring. Further, it has been reported that a *trans* (*1S*,*2S*) configuration is a requirement for a high binding affinity at KOR.[26,27,36,37,60] It should be noted that in vitro results cannot be directly extrapolated to in vivo effects and that the presented results primarily reflect the intrinsic potential of the test compounds to activate MOR or KOR. In the human body, additional factors must be considered such as bioavailability, metabolic stability, the formation of active metabolites, blood–brain barrier penetration, and off-target effects. Further studies (e.g., investigation of receptor activation via β-arrestin recruitment, evaluation of potency at other receptors, and investigation of the pharmacological parameters of metabolites) are therefore necessary. In addition, there are other U-opioids that should be assayed for their receptor activation potential to gain further insight into the structure activity correlations of U-opioids at MOR and KOR.

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CONFLICT OF INTEREST

The authors declare no 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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ENDNOTE

* Because of the reported low affinity of (1) for DOR (K_i (MOR): 0.91 ± 0.11 nM, K_i (KOR): 110 nM ± 11 nM, K_i (DOR): 480 ± 110 nM;[38] K_i (MOR): 57 ± 21 nM, K_i (KOR): 653 ± 163 nM, K_i (DOR): 1105 ± 223 nM[34] and low activity at DOR (EC_{50} (MOR): 140 nM, EC_{50} (KOR): 201 nM, EC_{50} (DOR): 4540 nM;[38] EC_{50} (MOR): 214 ± 23 nM, EC_{50} (KOR): 2699 ± 769 nM, EC_{50} (DOR): 5161 ± 1,357 nM,[34] we decided not to examine the activity of U-compounds at DOR.

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