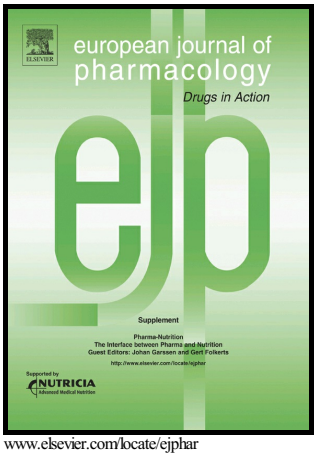

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New opioid receptor antagonist: naltrexone-14-*O*-sulfate synthesis and pharmacology

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ABSTRACT

Opioid antagonists, naloxone and naltrexone have long been used in clinical practice and research. In addition to their low selectivity, they easily pass through the blood-brain barrier. Quaternization of the amine group in these molecules, (e.g. methylnaltrexone) results in negligible CNS penetration. In addition, zwitterionic compounds have been reported to have limited CNS access. The current study, for the first time gives report on the synthesis and the *in vitro* [competition binding, G-protein activation, isolated mouse vas deferens (MVD) and mouse colon assay] pharmacology of the zwitterionic compound, naltrexone-14-*O*-sulfate. Naltrexone, naloxone, and its 14-*O*-sulfate analogue were used as reference compounds. In competition binding assays, naltrexone-14-*O*-sulfate showed lower affinity for μ, δ or κ opioid receptor than the parent molecule, naltrexone. However, the μ/κ opioid receptor selectivity ratio significantly improved, indicating better selectivity. Similar tendency was observed for naloxone-14-*O*-sulfate when compared to naloxone. Naltrexone-14-*O*-sulfate failed to activate GTPγS-binding but inhibit the activation evoked by opioid agonists (DAMGO, Ile[5,6]deltorphin II and U69593), similarly to the reference compounds. Schild plot constructed in MVD revealed that naltrexone-14-*O*-sulfate acts as a competitive antagonist. In mouse colon, naltrexone-14-*O*-sulfate antagonized the inhibitory effect of morphine with

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lower affinity compared to naltrexone and higher affinity when compared to naloxone or naloxone-14-*O*-sulfate. *In vivo* (mouse tail-flick test), subcutaneously injected naltrexone-14-*O*-sulfate antagonized morphine's antinociception in a dose-dependent manner, indicating it's CNS penetration, which was unexpected from such zwitter ionic structure. Future studies are needed to evaluate it's pharmacokinetic profile.

Keywords: *naltrexone-14-O-sulfate; opioid receptor binding; isolated organs; mouse tail-flick assay*

1 INTRODUCTION

Naltrexone and naloxone are widely used in the clinical practice as an antidote for opioid overdose (naloxone) and for the management of opioid and alcohol addiction (naltrexone) (Kiefer and Mann, 2005; Ray et al., 2010). They inhibit the binding of opioid agonists to opioid receptors (OR), that belong to G-protein coupled receptors (GPCRs). Three opioid receptor subtypes μ, δ and κ, (abbreviated here as MOR, DOR and KOR, respectively) have been pharmacologically characterized and cloned (Chen et al., 1993; Evans et al., 1992; Kieffer et al., 1992; Minami et al., 1993; Yasuda et al., 1993).

Opioid receptors are widely distributed in the central nervous system (CNS), both at supraspinal and spinal level – as well as in the periphery (Fürst, 1999; Przewłocki and Przewłocka, 2001). The activation of opioid receptors in the CNS and peripheral tissues results in appearance of both wanted (analgesia) and unwanted effects importantly respiratory depression and constipation (Holzer, 2009; Khalefa et al., 2013; Lacko et al., 2016; Mansour et al., 1995).

Naltrexone and naloxone bind to all three opioid receptors with subnanomolar-nanomolar affinity range regardless of location (Codd et al., 1995). Additionally, they are highly lipid soluble agents, thus they can easily cross the blood-brain-barrier (BBB) (Brown and Goldberg, 1985), which can be an advantage and disadvantage at the same time for therapeutic applications. Quaternary *N*-methyl derivatives of naltrexone and naloxone such as methylnaltrexone and naloxone methiodide have very limited ability to penetrate the BBB. Upon their systemic administration, they do not interfere with CNS opioid receptor-mediated effects such as withdrawal or analgesia (Brown and Goldberg, 1985; Pol et al., 1995). More importantly, naloxone methiodide showed promising results for reversing opioid-induced respiratory depression without precipitating withdrawal, while methylnaltrexone is an approved drug for the treatment of opioid-induced constipation (Lewanowitsch and Irvine, 2002; Mehta et al., 2016; Yuan et al., 1995). Sulfate ester analogues of naloxone and naltrexone were also developed as an attempt to improve their short half-life, thus enhancing

their medical effect (Lazar et al., 1994; Linder and Fishman, 1973), since these compounds are naturally occurring metabolites together with other morphinan sulfate esters (Andersson et al., 2014; Kurogi et al., 2012; Kurogi et al., 2014). Apparently, C-3 and C-6 sulfate esters may also limit BBB penetration of morphinans (Váradi et al., 2011), while morphine-6-*O*-sulfate (M6SU) displayed higher antinociceptive effect than morphine (Mori et al., 1972). Alkoxy substituted morphinans at C-14 position revealed a more potent and safer pharmacological profile (Spetea and Schmidhammer, 2012). Based on these data, our aim was to synthesize naltrexone-14-*O*-sulfate (Fig. **1**) and evaluate its pharmacological profile in biochemical (receptor binding), biological (isolated mouse *vas deferens* and colon) and *in vivo* (mouse tail-flick) assays compared to the already characterized naloxone-14-*O*-sulfate (Lazar et al., 1994; Linder and Fishman, 1973), naltrexone, and naloxone.

2 MATERIALS AND METHODS

2.1 Animals

Outbred male Wistar rats (250–300 g body weight) and male guinea pigs (~700 g body weight, LAL/HA/BR strain) were used for receptor binding assays. Rats were housed in the local animal house of BRC (Szeged, Hungary), while guinea pigs were housed in LAB-ÁLL Bt. (Budapest, Hungary). Male NMRI mice (35-45 g) for MVD assay and (20-25g) for mouse tail-flick were used. Mice were purchased from Toxi-Coop (Budapest, Hungary). Mice were housed in the local animal facility of Pharmacology and Pharmacotherapy Department (Semmelweis University, Budapest, Hungary) under conditions of animal housing and experimentation followed ethical guidelines set by the Ethical Board of Semmelweis University, based on EC Directive 86/609/EEC. Animals were kept in groups in temperature- and humidity-controlled room at a 12-h light/dark cycle, fed with standard chow and tap water ad libitum.

2.2 Chemicals

Naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate were synthesized as described in the next paragraph. The non-selective opioid receptor antagonists, naltrexone and naloxone hydrochloride were synthesized by Sándor Hosztafi or were kindly provided by the company Endo Laboratories DuPont de Nemours (Wilmington, USA). The MOR selective agonist [d-Ala^2, NMePhe^4, Gly^5-ol]enkephalin (DAMGO) was purchased from Bachem Holding AG (Bubendorf, Switzerland), the DOR selective agonist Ile^{5,6}deltorphin II (IleDelt II) was synthesized in the Isotope Laboratory of the Biological Research Center (BRC; Szeged,

Hungary), while the KOR specific agonist U69593 and [D-Pen^{2,5}]-Enkephalin (DPDPE) were purchased from Sigma–Aldrich (Budapest, Hungary). Ethylketocyclazocine (EKC) was obtained from Sterling Winthrop, Rensselaer (NY, USA). [d-Ala^2, d-Leu^5] enkephalin (DADLE) was purchased from Sigma–Aldrich (Budapest, Hungary). Morphine hydrochloride (ICN, Tiszavasvári, Hungary) and all other chemicals were of analytical grade and purchased from standard commercial sources. Drugs or saline delivered subcutaneously (s.c.) in a volume of 10 ml/kg body weight. The saline (s.c.)-injected mice acted as the control group. Drugs were dissolved in 0.9% solution of NaCl. Experiments were performed in a blinded way to the drugs and doses applied. GDP and the GTP analogue GTPγS and all other chemicals used in this study were of analytical grade were also purchased from Sigma–Aldrich (Budapest, Hungary).

The radiolabeled [^{3}H]DAMGO (specific activity: 41 Ci/mmol) [^{3}H]naloxone (specific activity: 31 Ci/mmol) and [^{3}H]IleDelt II (specific activity: 28 Ci/mmol) were radiolabeled in the Isotope Laboratory of BRC (Szeged, Hungary). The radioligands were characterized previously in the following publications (Nevin et al., 1994; Oktem et al., 1991; Szücs et al., 1987). [^{3}H]U69593 (specific activity: 30-60 Ci/mmol) was purchased from PerkinElmer (Budapest, Hungary). The radiolabeled GTP analogue, [^{35}S]GTPγS (specific activity: >1000 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary).

2.3 Chemistry

The sulfation reactions of C-3 protected naloxone and naltrexone were examined with excess pyridine-sulfur- trioxide complex in pyridine at 60 °C and the expected C-14 sulfates were isolated in high yields. Starting from 3-O-acetylnaloxone and from 3-O-acetylnaltrexone the appropriate C-14 sulfate esters were obtained, and the C-3 acetyl protecting group was hydrolyzed in 5 % methanolic sodium hydroxide. The structure of the prepared new compounds was elucidated by NMR spectroscopy. In all compounds the doublet of the C-9 proton displayed a downfield shift and observed at ~ δ 4.5 ppm. Protonated N-methyl groups can be observed at δ 2.9 – 3.0 ppm and the N-methyl shift for the zwitterionic sulfate esters are significantly higher than those of the respective salts in which the nitrogen atom is also protonated.

2.3.1 General procedure for the synthesis of C-14 sulfate esters

The appropriate C-14 hydroxy derivative (3 mmol) was dissolved in 10 ml dry pyridine. To the resulting solution, pyridine-SO_3 complex (1.43 g, 9.00 mmol) was added and stirred for 3.5 h at 60 °C. Cold water (10 ml) and chloroform (10 ml) were added to the suspension and was left to stand overnight in the freezer. The precipitate was collected by filtration and washed twice with cold water and crystallized from hot water. For the hydrolysis of C-3 acetyl group the crude product was dissolved in a mixture of 30 ml methanol and 5 ml 10% aqueous NaOH and stirred for 1 h at room temperature (Fig. **2**). The solution was neutralized with glacial acetic acid and the methanol was then evaporated under reduced pressure. The crude product was filtered and washed with methanol and crystallized from boiling water. The purity of naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate was at least 99% by NMR.

2.3.2 Naloxone-14-O-sulfate

The starting material 3-*O*-acetylnaloxone could be prepared quantitatively by selective acetylation of naloxone's phenolic hydroxyl group: stirring naloxone base with acetic anhydride in aqueous medium in the presence of excess $NaHCO_3$ results in 3-*O*-acetylnaloxone, which was converted to naloxone-14-O-sulfate according to the general procedure and the crude product was recrystallized from water. Yield: 56 %, m.p.: > 295 °C (dec.).

^{1}H NMR (600 MHz, DMSO-d_6): δ = 6.69 (d, *J* = 7.8 Hz, H-2, 1H), 6.66 (d, *J* = 7.8 Hz, H-1, 1H), 5.93 (d, *J* = 7.5 Hz, allyl-CH, 1H), 5.60 (d, *J* = 16.9 Hz, allyl-CH_2, 1H), 5.50 (d, *J* = 9.8 Hz, allyl-CH_2, 1H), 4.99 (s, H-5, 1H), 4.50 (s, H-9, 1H) ppm.

2.3.3 Naltrexone-14-O-sulfate

Naltrexone-14-*O*-sulfate was prepared from 3-O-acetylnaltrexone using the general procedure. Yield: 61 %, m.p.: >300 °C (dec.) (Fig. **2**).

^{1}H NMR (600 MHz, DMSO-d_6): δ = 6.65 (d, *J* = 8.1 Hz, H-2, 1H), 6.61 (d, *J* = 8.1 Hz, H-1, 1H), 4.97 (s, 1H, H-5), 4.74 (d, *J* = 5.7 Hz, H-9, 1H) ppm.

2.4 Receptor binding assays

2.4.1 Membrane preparations

Membrane fractions of rat and guinea pig brains were prepared as previously described (Benyhe et al., 1997). Briefly, the animals were decapitated and the brains were quickly removed, and homogenized on ice in 50 mM Tris-HCl buffer (pH 7.4) with a Teflon-glass

homogenizer. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4˚C and the pellet was resuspended in fresh buffer and incubated for 30 min at 37˚C. The centrifugation step was repeated, and the final pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at −80˚C until use.

2.4.2 Radioligand competition binding experiments

Aliquots of frozen rat and guinea pig membranes were centrifuged (40000 × g, 20 min, 4˚C) to remove sucrose and the pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4). The radioligand competition binding assays were performed as previously described (Lacko et al., 2012). Briefly, membranes were incubated in the presence of a given (~ 1 nM) radioligand concentration together with increasing concentrations (10^{-11} – 10^{-5} M) of unlabeled ligands such as naltrexone- and naloxone-14-*O*-sulfate, naloxone and naltrexone for comparison and the corresponding homologous opioid receptor selective ligands (DAMGO, naloxone, IleDelt II and U69593) for control. The incubation time and temperature were radioligand dependent: [^{3}H]DAMGO and [^{3}H]IleDelt II were incubated at 35˚C for 45 min., [^{3}H]naloxone 0˚C for 60 min., [^{3}H]U69593 was incubated at 30˚C for 30 min. In all circumstances the final volume was 1 ml. Total binding was measured in the presence of a radioligand in the absence of unlabeled ligands and the non-specific binding was determined in the presence of 10 μM unlabeled naloxone. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and washed three times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/C ([^{3}H]DAMGO and [^{3}H]U69593) or GF/B ([^{3}H]naloxone) glass fibers. In case of experiments performed with [^{3}H]naloxone and [^{3}H]U69593, the filters were washed in 3% polyethylenimine before filtration for 60 min to reduce non-specific binding. The radioactivity of the filters was detected in UltimaGold™ MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter. The competition binding assays were performed in duplicate and repeated at least three times.

2.4.3 Functional [^{35}S]GTPγS binding experiments

The functional [^{35}S]GTPγS binding assays were accomplished as previously described (Sim et al., 1995; Traynor and Nahorski, 1995) with slight modifications. For [^{35}S]GTPγS binding the sucrose was removed by centrifugation (40 000 *g*, 20 min, 4°C) and the pellet was suspended with ice-cold Tris-HCl, EGTA, $MgCl_2$ (TEM) buffer to obtain the appropriate protein content for the assay (~10 μg/ml). The membrane fractions were incubated at 30˚C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) and increasing

concentrations (10^{-10} – 10^{-5} M) of naltrexone- and naloxone-14-*O*-sulfate. In a different experimental setup, the incubation mixture contained increasing concentrations (10^{-10} – 10^{-5} M) of DAMGO, IleDelt II and U69593 in the presence or absence of 1 μM concentrations of naltrexone- and naloxone-14-*O*-sulfate, and naloxone and naltrexone for comparison. The experiments were performed in the presence of excess GDP (30 μM) in a final volume of 1 ml. Total binding was measured in the absence of test compounds, non-specific binding was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding. The filtration process and the detection of incorporated radioactivity were the same as described in section 2.4.2. [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

2.5 Isolated organs

2.5.1 Isolated mouse vas deferens (MVD)

Vasa deferentia were taken from NMRI mice of 35-45g weight. The preparation and the experimental procedures carried out as described previously (Rónai et al., 1977). Briefly, *vasa* were suspended between two electrodes with 0.1 g initial tension in organ baths of 5 ml volume. The upper and the lower electrodes have ring and straight form, respectively. The organ bath filled with Mg^{2+}-free Kreb's of the following composition (mM/L): NaCl, 118.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; glucose, 11.0; $CaCl_2$, 2.5., aerated with carbogen (95% O_2 + 5% CO_2) and kept at 31 ^oC. The stimulation parameters were as follows: field stimulation, pairs (100 ms pulse distance) of rectangular impulses (1 ms pulse width, 9V/cm i.e. supramaximal intensity) were repeated by 10 s.

2.5.1.1 Experimental paradigms of MVD

In the isolated organ experiments, 30–40 min equilibration was used under stimulation before adding the first dose of agonists. The concentration-effect curves for the agonists, DAMGO, DADLE, DPDPE, EKC or U69593 were constructed in cumulative manner. Preparations were then washed and allowed to regain their pre-drug twitch height. Thereafter, the *vasa deferentia* were equilibrated with naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate, naltrexone and naloxone for 20 min, and without washing a single concentration of agonist was added. In case of U69593 the antagonists were cumulatively added and allowed for 20 min equilibration time. To determine dissociation constants of the naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate, naltrexone or naloxone dose ratio (DR) values were obtained by the single-dose method described previously (Kosterlitz and Watt, 1968).

2.5.2 Isolated mouse colon

Mice were killed by cervical dislocation and distal (~ 2 cm) segment of the colon was removed and immediately placed in Petri dish containing Krebs solution of the following composition (mM/L): NaCl, 118.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; glucose, 11.0; $CaCl_2$, 2.5; $MgSO_4$, 1.2. and aerated with carbogen (mixture of 95% O_2 and 5% CO_2). Then ends of isolated colon were suspended between upper (ring) and lower (straight) electrodes in organ baths of 5 ml volume containing Kreb's solution of 36 °C bubbled with carbogen. The upper end of tissue by thread was connected to computer through amplifier.

2.5.2.1 Experimental paradigms of isolated mouse colon

After adjusting the isolated organs to 0.5 g rest tension, to induce muscle contraction field electrical stimulation of the following parameters, rectangular impulses (1 ms pulse width, 25 shock, 9 V/cm i.e. supramaximal intensity of 5 Hz, repeated every 30 seconds) were applied. Then, the isolated colons were allowed for 30-40 min equilibration period. The concentration-effect curve for morphine, DAMGO, DPDPE, U69593 were constructed in cumulative manner and without washing a single concentration of antagonists, naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate. naltrexone and naloxone was added. To determine dissociation constants of naltrexone-14-*O*-sulfate, dose ratio (DR) values were calculated as described previously (Kosterlitz and Watt, 1968).

2.6 Antinociceptive tests

2.6.1 Mouse tail-flick assay

Radiant heat tail flick test was used to assess the effects of naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate, naltrexone and naloxone on the antinociceptive effect of morphine. The assay was carried out as described by Tulunay and Takemori (Tulunay and Takemori, 1974) using IITC Life Sciences equipment. Briefly, the light intensity was adjusted to set the control tail-flick latency between 1.3 and 2.8 s. Mice failed to respond within this range were excluded from the experiments. Cut-off time was set to 6s to avoid tissue damage. A baseline latency was measured before and 30 after s.c. saline, morphine and morphine plus naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate, naltrexone or naloxone administration. A saline treated group was used for each experiment as control. For each dose a separate group of animals (n=4-8) was used.

2.6.2 Dose-response relationships

Dose response curves for morphine was constructed in saline treated, naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate, naltrexone or naloxone treated mice. The applied s.c. doses

(μmol/kg) of naltrexone-14-*O*-sulfate were 0.07, 0.24, 0.71 and 2.37, naloxone-14-*O*-sulfate were 2.45, 7.36 and 24.55, naltrexone was 0.26 and naloxone was 0.75.

2.7 Data analysis

2.7.1 Binding assays

The specific binding of the radiolabeled compounds ([^{3}H]ligand, [^{35}S]GTPγS) was calculated by the subtraction of non-specific binding from total binding and was given in percentage. The data was normalized to total specific binding, which was settled 100%, which in case of [^{35}S]GTPγS also represents the basal activity of the G-protein. All experimental data were presented as means ± S.E.M. and they were presented in the function of the applied ligand concentration. Data were fitted with the professional curve fitting program, GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA), using non-linear regression.

In the competition binding assays, the inhibition of the specifically bound radioligands was given in percentage, the total specific binding and the minimum level of non-specific binding was defined as 100% and 0% respectively. Data were fitted with the 'One site competition' fitting equation to determine the concentration of the competitor ligands that displaced 50% of the radioligand (IC_{50}).

In the [^{35}S]GTPγS binding assays the 'Sigmoid dose-response' fitting was used to determine the maximal stimulation or efficacy (E_{max}) of the receptors G-protein and the potency (EC_{50}) of the stimulating ligand. Stimulation was given in percent of the specific [^{35}S]GTPγS binding observed over the basal activity, which was settled as 100%.

2.7.2 Isolated mouse vas deferens

The equilibrium dissociation constants of naltrexone-14-*O*-sulfate, naloxone-14-*O*-sulfate, naltrexone or naloxone (K_e) were calculated with the single-dose method (Kosterlitz and Watt, 1968). Test compounds affinities (K_e values) were calculated as: K_e= [antagonist concentration]/dose ratio - 1. Dose ratios (DR) were calculated by dividing the concentration values of the given opioid receptor selective agonist obtained in the presence and in the absence of the given antagonist. To determine the competitive antagonist character of naltrexone-14-*O*-sulfate compared to naltrexone on the MOR, Schild-plot was constructed as described previously (Arunlakshana and Schild, 1959). Briefly, the log of (DR-1) values obtained from the experiments performed with DAMGO were plotted against negative log (antagonist doses) in M to evaluate the slope and the X-intercept (estimated equilibrium dissociation constant for the antagonists or pA_2 value) of the Schild-plot.

2.7.3 Isolated mouse colon

The equilibrium dissociation constants (K_e) of the test compounds against morphine were determined as previously described (Kosterlitz and Watt, 1968).

2.7.4 Mouse tail-flick test

Maximal possible effect (MPE) % was calculated for each mouse as follows: [Latency after treatment – Basal Latency] / [Cut off – Basal Latency] x 100%. To determine the ED_{50} values and its confidence intervals of morphine, the MPE% values were plotted against the applied morphine doses (in the absence or presence of naltrexone- or naloxone-14-*O*-sulfate, naltrexone and naloxone) using linear regression with GraphPad Prism 5.0 software based on previous studies (Aceto et al., 1997; Garner et al., 1997; Székely et al., 1978). To assess the competitive character of naltrexone-14-*O*-sulfate or naloxone-14-*O*-sulfate, Schild-plot was constructed based on previous studies (Arunlakshana and Schild, 1959) and as described in section 2.7.2. The DRs were calculated with the ED_{50} values of morphine and the – log (antagonist doses) were given in µmol/kg. In the other part of experiments the apparent pK_B values were determined as described previously (Garner et al., 1997; Negus et al., 1993), pK_B= - Log [antagonist dose/DR-1] where 'B' is the antagonist dose (µmol/kg) and 'DR' is the dose ratio. This method is used to evaluate the antagonist potency in the presence of a single dose of a test antagonist ligand.

2.7.5 Statistical analysis

Data was statistically analyzed with One-way ANOVA, with Tukey multiple comparison post hoc test by using GraphPad Prism 5.0 program. The slopes of the Schild plots were statistically analyzed by the extra sum-of-square F test using GraphPad Prism 5.0 program based on the curve fitting guides provided by the software (http://www.graphpad.com/guides/prism/7/curve-fitting/index.htm). Significance was accepted at $P < 0.05$ level.

3 RESULTS

3.1 Examining the binding affinity of naltrexone-14-*O*-sulfate in radioligand competition binding experiments

In competition binding experiments the applied opioid radioligands functioned adequately, since their unlabeled homologous ligands displaced them with very high affinity (Table **1**, Fig. **3**). The novel opioid antagonist naltrexone-14-*O*-sulfate inhibited the

[^{3}H]naloxone specific binding with a higher K_i value compared to naltrexone and naloxone, but still with a better affinity value compared to naloxone-14-*O*-sulfate (Table **1**, Fig. **3A**). In competition binding experiments with MOR, DOR and KOR specific radioligands, naltrexone-14-*O*-sulfate displayed the following affinity rank order: $\mu > \delta > \kappa$ (Table **1**). Overall naltrexone-14-*O*-sulfate displayed lower affinity compared to naltrexone and naloxone and higher affinity than naloxone-14-*O*-sulfate for all the three opioid receptors (Table **1**, Fig. **3**). Interestingly however, the selectivity of naltrexone-14-*O*-sulfate for the MOR over the KOR dramatically increased compared to naltrexone and naloxone (Table 1, Fig. **3B-D**). Naloxone-14-*O*-sulfate showed similar higher selectivity for MOR over KOR and also over the DOR (Table **1**, Fig. **3B-D**).

3.2 Examining the antagonistic character of naltrexone-14-*O*-sulfate in functional [^{35}S]GTPγS binding experiments

In the next step we wanted to confirm the neutral antagonist character of naltrexone-14-*O*-sulfate in *in vitro* [^{35}S]GTPγS binding assay. Accordingly, in increasing concentrations naltrexone-14-*O*-sulfate did not alter $G_{i\alpha}$ basal activity (Fig. **4A**) significantly, even at the highest applied concentrations, thus it behaved as a neutral antagonist *per se*. Similar results were obtained with naloxone-14-*O*-sulfate (Fig. **4A**).

Further on we compared the antagonistic effect of naltrexone-14-*O*-sulfate to naltrexone, naloxone-14-*O*-sulfate and naloxone at a given concentration during agonist-mediated opioid receptor activation in [^{35}S]GTPγS binding assay. According to our results, 1 μM naltrexone-14-*O*-sulfate decreased the G-protein maximum efficacy of all three opioid receptors, the reduction was the most noticeable in case of MOR and DOR and less prominent with KOR (Fig. **4B-D**, Table **2**). The potency of the MOR, DOR and KOR selective agonists were 244, 45 and 70 nM, respectively (Fig. **4B-D**). The antagonist reduced the potency of all opioid agonists, among them the most prominent reduction was observed during MOR stimulation: the potency of DAMGO reduced to the micromolar range in the presence of all the antagonists (Fig. **4B**). Similar results were obtained in the presence of naltrexone-14-*O*-sulfate and naltrexone in DOR agonist potency (Fig. **4C**). The reduction of KOR agonist ligand potency was less effective in the presence of the antagonists compared to the other two opioid receptors. Naltrexone-, naloxone-14-*O*-sulfate and naltrexone only reduced U69593 potency down to high nanomolar range, on the other hand naloxone reduced agonist potency to the micromolar range (Fig. **4D**).

3.3 Opioid antagonist affinity of naltrexone-14-*O*-sulfate in *vasa deferentia* from mice.

Naltrexone-14-*O*-sulfate antagonized the inhibitory effects of agonists DAMGO, DADLE, DPDPE, U69593 and EKC. The calculated K_e value for naltrexone-14-*O*-sulfate was 3 times lower than the K_e value of naloxone-14-*O*-sulfate when they were tested against DAMGO (Table **3A** and **B**). Naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate displayed the same characteristics when tested against DPDPE or DADLE but with much higher K_e values (Table **3A** and **B**). However, their K_e values largely increased when they were determined against U69593 or EKC, thus they showed weak antagonist effect against U69593 or EKC (Table **3A** and **B**). According to the Schild regression analysis the pA_2 value was 8.15 M (95% CI: 7.86 to 8.61) and the slope was not significantly different from unity (-1.07, 95% CI: -1.38 to -0.77, F: 1.21, extra sum-of-squares F test) for naltrexone-14-*O*-sulfate against the MOR selective agonist, DAMGO indicating a competitive antagonism (Fig. **5A**). For comparison naltrexone displayed a 9.62 pA_2 value (95% CI: 9.42 to 9.9) and a Schild-slope not significantly different from unity (-0.87, 95% CI: -1.05 to -0.7, F: 79.93) in the Schild analysis (Fig. **5B**). When the Schild slope was constrained to -1 the pA_2 values were 8.22 M (95% CI: 8.06 to 8.4) and 9.48 M (95% CI: 9.33 to 9.64) for naltrexone-14-*O*-sulfate and naltrexone, respectively.

3.4 Isolated mouse colon

In the isolated colon assay naltrexone antagonized morphine-induced inhibition with a very low subnanomolar K_e value, which was significantly lower compared to naloxone, indicating a higher antagonist affinity (Table **4**). The sulfation at the C-14 position in both naltrexone and naloxone resulted in a strong decrease in their affinity compared to their parent compounds, indicated by the reduced K_e values (Table **4**). However, naltrexone-14-*O*-sulfate still displayed a lower K_e value compared to naloxone-14-*O*-sulfate, thus the observed difference in higher affinities between naltrexone and naloxone was maintained (Table **4**). DPDPE produced weak agonist action (< 20% inhibition) whereas U69593 failed to affect the colon contractions.

3.5 The antagonist action of naltrexone-14-*O*-sulfate on the antinociceptive effects of morphine in mouse tail-flick assay.

Subcutaneous naltrexone-14-*O*-sulfate or naloxone-14-*O*-sulfate treatment antagonized the antinociceptive effect of s.c. morphine with competitive characteristic in mouse tail-flick test. Morphine alone produced a 7.89 μmol/kg ED_{50} value in the assay, while in presence of

0.07, 0.24, 0.71 and 2.37 μmol/kg naltrexone-14-*O*-sulfate this value increased to 17.15, 31.79, 62.98 and 256.36 μmol/kg, respectively (Fig. **6A**). Experiments performed with naloxone-14-*O*-sulfate, morphine alone displayed a 10.22 μmol/kg ED_{50} value and 20.03, 45.77, and 123.51 μmol/kg in the presence of 2.45, 7.36 and 24.55 μmol/kg naloxone-14-*O*-sulfate, respectively (Fig. **6C**). The shifts to the right in the dose-response curves of morphine are shown (Fig. **6A** and **C**). Additionally, according to the Schild regression analysis the pA_2 value for naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate was 1.17 μmol/kg (95% CI: 0.87 to 1.76.) and -0.44 μmol/kg (95% CI: -0.78 to 1.8), respectively. The calculated Schild-slope for naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate was -0.92 (95% CI: -1.29 to -0.55) and -1.09 (95% CI: -1.98 to -0.19), respectively, which were both not significantly different from the theoretical unity (F: 0.88 and 1.58, respectively; extra sum-of-squares F test), indicating the competitive antagonist character for both compounds (Fig. **6B** and **D**). When the Schild-slope was constrained to -1 the pA_2 values of naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate were 1.11 μmol/kg (95% CI: 0.96 to 1.26) and -0.4 μmol/kg (95% CI: -0.54 to -0.26), respectively. Finally, the calculated pK_B and 95% CI (μmol/kg) values for naltrexone, naltrexone-14-*O*-sulfate, naloxone and naloxone-14-*O*-sulfate were as follows: 1.83 (1.57 to 2.09), 1.13 (1.06 to 1.21), 1.27 (0.99 to 1.55) and -0.38 (-0.51 to -0.25), respectively.

4 DISCUSSION

In this study, for the first time we presented the synthesis and *in vitro* (biochemical and biological) pharmacological analysis of a novel naltrexone analogue, naltrexone-14-*O*-sulfate. We also examined the action of the test compound on morphine antinociception *in vivo* in mouse tail-flick. In addition, naloxone and its congener, naloxone-14-*O*-sulfate (Linder and Fishman, 1973), were also pharmacologically characterized as reference compounds. The aim of the first step was to analyze the extent of opioid binding alteration of naltrexone following the chemical modification. In radioligand competition binding assays, we found the following: introduction of the sulfate group into C-14 of naltrexone and naloxone resulted in a decrease in the affinity for opioid receptor subtypes (μ, δ and κ) (Table **1**, Fig. **3**). However, naltrexone-14-*O*-sulfate had an overall higher affinity compared to naloxone-14-*O*-sulfate (Table **1**). It is worth noting that naltrexone- or naloxone-14-*O*-sulfate displayed a prominent increase in the selectivity for MOR vs KOR compared to naltrexone or naloxone (Table **1**). Our data are in agreement with previous studies on 3-*O*-sulfate and 3,14-*O*-disulfate naloxone

and naltrexone derivatives (Lazar et al., 1994), although the reduction in affinity was much more robust in these compounds compared to the 14-*O*-sulfate derivatives, especially in case of the 3,14-*O*-disulfate compounds. Next, to exclude the agonist activity of naltrexone-14-*O*-sulfate, G-protein binding and mouse vas deferens assays were applied. G-protein acts as a molecular switch to transfer the binding signal to second messenger systems, thus it holds a key function in GPCR signaling (Oldham and Hamm, 2008). In G-protein activation experiments and MVD, the sulfation of 14-hydroxyl group of naltrexone or naloxone did not alter the antagonist properties of parent agents (Fig. **4A**), because no agonist action was noted. In addition, both analogues effectively decreased opioid agonist-mediated G-protein activity and ligand potency (Table **2**, Fig. **4B-D**). The inhibition by naltrexone-14-*O*-sulfate was more prominent on MOR and DOR, and less effective on KOR, similarly to naloxone-14-*O*-sulfate (Table **2**, Fig. **4B** and **D**). Therefore, the improved MOR selectivity confirms the data obtained in the binding affinity measurements. In MVD assay, the antagonist affinity of the naltrexone-14-*O*-sulfate was examined in the presence of opioid agonist-induced smooth muscle inhibition. In these experiments both naltrexone- and naloxone-14-*O*-sulfate showed reduced affinity for all three opioid receptor subtypes compared to parent compounds (naltrexone and naloxone). However, the MOR vs KOR selectivity was improved, and naltrexone-14-*O*-sulfate displayed substantially higher selectivity ratio compared to naloxone-14-*O*-sulfate (Table **3**), all of which corresponds well with the receptor binding data. In addition, in this test model we also demonstrated the competitive antagonist property for naltrexone-14-*O*-sulfate on MOR (Fig. **5**), because Schild's plot revealed that slope value was close to unity. This character was matched that of the competitive opioid antagonist naltrexone.

Quaternary analogues of naltrexone and naloxone have been synthesized, however, the quaternary compounds have lower affinity for opioid receptors compared to zwitterionic molecules (Spetea and Schmidhammer, 2012). Sulfation was demonstrated to be a successful strategy to develop zwitterionic morphine analogues with limited BBB penetration and increased efficacy (Brown et al., 1985).

Opioid induced constipation is one of the most common unwanted effects raised during chronic opioid therapy (Edgerton and Loven, 2011). Thus, we examined how effectively naltrexone-14-*O*-sulfate can inhibit the effect of morphine on smooth muscle contraction of mouse colon, which is very crucial bowel segment affected by drug-induced constipation or diarrhea. Naltrexone-14-*O*-sulfate antagonized the inhibitory effect of morphine on colon muscle contraction similarly to naloxone-14-*O*-sulfate with nanomolar affinity. However, the

affinity of naltrexone-14-*O*-sulfate or naloxone-14-*O*-sulfate was much lower compared to that of naltrexone or naloxone (Table **4**). In accordance with our previous receptor binding and MVD studies, naltrexone-14-*O*-sulfate showed an overall higher affinity for MOR compared to naloxone-14-*O*-sulfate in the colon (Table **4**). On the other hand, we could not determine the antagonist action of naltrexone-14-*O*-sulfate or naloxone-14-*O*-sulfate against DPDPE and U69593 the selective DOR and KOR agonists, respectively, because DPDPE showed a very weak agonist effect (17%), yet U69593 failed to affect the colon contraction. In these experiments, to our best knowledge, we performed for the first time a thorough analysis of the effects of different opioid antagonists on mouse colon preparations. However, future *in vivo* experiments are warranted to fully explore the effect of these ligands on the bowel functions. The endogenous opioid system is intensely present in the gastrointestinal system (Holzer, 2009) and peripherally restricted opioid antagonist such as methylnaltrexone are among the most potent drugs for the treatment of opioid induced constipation (Zacny et al., 2015).

Finally, we extended our work to test the *in vivo* effect of subcutaneously injected naltrexone-14-*O*-sulfate compared to naloxone-14-*O*-sulfate on s.c. morphine antinociception using mouse tail-flick assay. Morphine antinociception in this test is considered for large-extent centrally based action (Fürst et al., 2005). Interestingly, both compounds in dose-dependent manner inhibited morphine-induced antinociception and showed a competitive antagonist pattern as indicated by Schild-regression slope values close to the unity (Fig. **6**.). This indicates that these compounds bearing an ionizable group that makes them more hydrophilic compared to the parent molecules were able to cross the BBB in the present work. Indeed, other polar compounds like morphine-6-glucuronide have also been reported to cross the blood brain barrier (Yoshimura et al., 1973). In addition, we have recently showed that 14-*O*-methylmorphine-6-*O*-sulfate and morphine-6-*O*-sulfate at higher doses produced CNS antinociception following systemic administration (Kiraly et al., 2015; Lacko et al., 2012; Lackó et al., 2016) in mice or rats. Hence, these data, corroborated by our recent findings, suggest that zwitter ionic structures may able to cross the BBB under specific circumstances. In the present study, we cannot judge, indeed only speculate and claim that the observed effects might be attributed to this structure of naltrexone-14-*O*-sulfate or naloxone-14-*O*-sulfate, which displays ionized and non-ionized forms that based on pH media. Therefore, further studies are necessary to answer this issue.

In order to assess the magnitude of the differences in the potency between the test antagonist (naltrexone-, naloxone-14-*O*-sulfate) and parent compounds (naltrexone and

naloxone), apparent pK_B values were evaluated as described previously (Garner et al., 1997). Previously this method has been applied by other opioid research groups (Garner et al., 1997; Negus et al., 1993) and requires a small number of animals, which can contribute to 'Reduction' within the 3Rs of ethical guidelines of animal welfare. In addition, it is worthwhile mentioning that the competitive pattern of naltrexone or naloxone is not the subject of debate, since this pharmacological profile has been reported by other research groups applied similar test and animal species (Aceto et al., 1997; Garner et al., 1997; Schmauss and Yaksh, 1984; Székely et al., 1978; Takemori and Portoghese, 1984; Walker et al., 1994). Herein, based in this analysis, the antagonist potency rank was as follows: naltrexone > naloxone > naltrexone-14-*O*-sulfate > naloxone-14-*O*-sulfate. This is consistent with competition binding, MVD and colon data on MOR in the present work. Nonetheless, there were no significant differences between naltrexone-14-*O*-sulfate and naloxone in mouse tail flick. This tendency might be contributed to the pharmacokinetic property of naloxone, which will be known to have short duration of action. Naloxone-14-*O*-sulfate was proved to be very weak antagonist indicated by the antagonist potencies, based on pK_B values (2407 nmol/kg), whereas, naltrexone, naltrexone-14-*O*-sulfate and naloxone showed antagonist potency values of 15, 78 and 55 (nmol/kg), respectively. These *in vivo* data showed the superiority (30x) of naltrexone-14-*O*-sulfate over naloxone-14-*O*-sulfate.

Although our data indicate that zwitter ions might have CNS effects as well, this compound might be a valuable clinical tool in the treatment of opioid-evoked constipation. Namely, the acidic character of this antagonist and the alkaline media in the bowels might induce a pronounced polarization of this agent. Consequently, the degree of absorption will be largely slowed down and promote the local effect. Thus, this agent might be superior to the tertiary naloxone, which today in small doses is also used to treat opioid-induced constipation (Barnett et al., 2014). Therefore, future studies are needed to assess the effect of oral naltrexone-14-*O*-sulfate as well other analogues on constipation evoked by opioid analgesics.

5 CONCLUSIONS

Based on the present data sulfatation of 14-hydroxyl group of naltrexone or naloxone reduced their binding affinity to all three opioid receptors but in a much less extent as the sulfatation of 3-hydroxyl or simultaneously of 3- and 14-hydroxyl group as reported (Lazar et al., 1994). Yet the MOR vs. KOR selectivity in both compounds was improved and the modification preserved the more potent pharmacological activity of naltrexone over naloxone

in vitro. Thus, modifying naltrexone and naloxone with sulfate ester at 14-hydroxyl group is the better solution so far.

In general, medicinal chemists seek new structures to improve the therapeutic effects of known drugs. It is worth paying attention to enhance the selectivity as well as the pharmacokinetic profile of the modified drug structure. In our present work, the modification of naltrexone structure brought about decrease in the affinity but improvement in the selectivity. Although, the novel opioid antagonist displays zwitter ionic structure, it showed CNS effect. Therefore, the comprehensive study on the pharmacokinetic profile of the novel compound is a future goal.

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest in this article.

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REFERENCES

Andersson, M., Björkhem-Bergman, L., Ekström, L., Bergqvist, L., Lagercrantz, H., Rane, A., Beck, O., 2014. Detection of morphine-3-sulfate and morphine-6-sulfate in human urine and plasma, and formation in liver cytosol. Pharmacology research & perspectives 2, e00071.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14, 48-58.

Barnett, V., Twycross, R., Mihalyo, M., Wilcock, A., 2014. Opioid antagonists. J. Pain Symptom Manage. 47, 341-352.

Benyhe, S., Farkas, J., Tóth, G., Wollemann, M., 1997. Met5-enkephalin-Arg6-Phe7, an endogenous neuropeptide, binds to multiple opioid and nonopioid sites in rat brain. J. Neurosci. Res. 48, 249-258.

Brown, D.R., Goldberg, L.I., 1985. The use of quaternary narcotic antagonists in opiate research. Neuropharmacology 24, 181-191.

Chen, Y., Mestek, A., Liu, J., Hurley, J.A., Yu, L., 1993. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol. Pharmacol. 44, 8-12.

Codd, E.E., Shank, R.P., Schupsky, J.J., Raffa, R.B., 1995. Serotonin and norepinephrine uptake inhibiting activity of centrally acting analgesics: structural determinants and role in

antinociception. The Journal of pharmacology and experimental therapeutics 274, 1263-1270.

Edgerton, L., Loven, B., 2011. Clinical inquiries. What are the adverse effects of prolonged opioid use in patients with chronic pain? The Journal of family practice 60, 288-289.

Evans, C.J., Keith, D.E., Morrison, H., Magendzo, K., Edwards, R.H., 1992. Cloning of a delta opioid receptor by functional expression. Science (New York, N.Y.) 258, 1952-1955.

Fürst, S., 1999. Transmitters involved in antinociception in the spinal cord. Brain Res. Bull. 48, 129-141.

Fürst, S., Riba, P., Friedmann, T., Tímar, J., Al-Khrasani, M., Obara, I., Makuch, W., Spetea, M., Schütz, J., Przewlocki, R., Przewlocka, B., Schmidhammer, H., 2005. Peripheral versus central antinociceptive actions of 6-amino acid-substituted derivatives of 14-O-methyloxymorphone in acute and inflammatory pain in the rat. The Journal of pharmacology and experimental therapeutics 312, 609-618.

Holzer, P., 2009. Opioid receptors in the gastrointestinal tract. Regul. Pept. 155, 11-17.

Khalefa, B.I., Mousa, S.A., Shaqura, M., Lackó, E., Hosztafi, S., Riba, P., Schäfer, M., Ferdinandy, P., Fürst, S., Al-Khrasani, M., 2013. Peripheral antinociceptive efficacy and potency of a novel opioid compound 14-O-MeM6SU in comparison to known peptide and non-peptide opioid agonists in a rat model of inflammatory pain. Eur. J. Pharmacol. 713, 54-57.

Kiefer, F., Mann, K., 2005. New achievements and pharmacotherapeutic approaches in the treatment of alcohol dependence. Eur. J. Pharmacol. 526, 163-171.

Kieffer, B.L., Befort, K., Gaveriaux-Ruff, C., Hirth, C.G., 1992. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc. Natl. Acad. Sci. U. S. A. 89, 12048-12052.

Kosterlitz, H.W., Watt, A.J., 1968. Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). Br. J. Pharmacol. Chemother. 33, 266-276.

Kurogi, K., Chen, M., Lee, Y., Shi, B., Yan, T., Liu, M.-Y., Sakakibara, Y., Suiko, M., Liu, M.-C., 2012. Sulfation of buprenorphine, pentazocine, and naloxone by human cytosolic sulfotransferases. Drug metabolism letters 6, 109-115.

Kurogi, K., Chepak, A., Hanrahan, M.T., Liu, M.-Y., Sakakibara, Y., Suiko, M., Liu, M.-C., 2014. Sulfation of opioid drugs by human cytosolic sulfotransferases: metabolic labeling study and enzymatic analysis. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 62, 40-48.

Lacko, E., Riba, P., Giricz, Z., Varadi, A., Cornic, L., Balogh, M., Kiraly, K., Cseko, K., Mousa, S.A., Hosztafi, S., Schafer, M., Zadori, Z.S., Helyes, Z., Ferdinandy, P., Furst, S., Al-Khrasani, M., 2016. New Morphine Analogs Produce Peripheral Antinociception within a Certain Dose Range of Their Systemic Administration. J. Pharmacol. Exp. Ther. 359, 171-181.

Lacko, E., Varadi, A., Rapavi, R., Zador, F., Riba, P., Benyhe, S., Borsodi, A., Hosztafi, S., Timar, J., Noszal, B., Furst, S., Al-Khrasani, M., 2012. A novel μ-opioid receptor ligand with high in vitro and in vivo agonist efficacy. Curr. Med. Chem. 19, 4699-4707.

Lazar, S., Jabbouri, S., Moisand, C., Noël-Hocquet, S., Meunier, J., Ropars, C., Guillaumet, G., 1994. Synthesis and biological activity of the phosphate and sulfate esters of naloxone and naltrexone. Eur. J. Med. Chem. 29, 45-53.

Lewanowitsch, T., Irvine, R.J., 2002. Naloxone methiodide reverses opioid-induced respiratory depression and analgesia without withdrawal. Eur. J. Pharmacol. 445, 61-67.

Linder, C., Fishman, J., 1973. Narcotic antagonists. 1. Isomeric sulfate and acetate esters of naloxone (N-allylnoroxymorphone). J. Med. Chem. 16, 553-556.

Mansour, A., Fox, C., Akil, H., Watson, S., 1995. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci. 18, 22-29.

Mehta, N., O'Connell, K., Giambrone, G., Baqai, A., Diwan, S., 2016. Efficacy of Methylnaltrexone for the Treatment of Opiod-Induced Constipation: A Meta-Analysis and Systematic Review. Postgrad. Med.

Minami, M., Toya, T., Katao, Y., Maekawa, K., Nakamura, S., Onogi, T., Kaneko, S., Satoh, M., 1993. Cloning and expression of a cDNA for the rat kappa-opioid receptor. FEBS Lett. 329, 291-295.

Mori, M.-a., Oguri, K., Yoshimura, H., Shimomura, K., Kamata, O., Ueki, S., 1972. Chemical synthesis and analgesic effect of morphine ethereal sulfates. Life sciences. Pt. 1: Physiology and pharmacology 11, 525-533.

Nevin, S.T., Kabasakal, L., Otvös, F., Töth, G., Borsodi, A., 1994. Binding characteristics of the novel highly selective delta agonist, [3H]IIe5,6deltorphin II. Neuropeptides 26, 261-265.

Oktem, H.A., Moitra, J., Benyhe, S., Toth, G., Lajtha, A., Borsodi, A., 1991. Opioid receptor labeling with the chloromethyl ketone derivative of [3H]Tyr-D-Ala-Gly-(Me)Phe-Gly-ol (DAMGO) II: Covalent labeling of mu opioid binding site by 3H-Tyr-D-Ala-Gly-(Me)Phe chloromethyl ketone. Life Sci. 48, 1763-1768.

Oldham, W.M., Hamm, H.E., 2008. Heterotrimeric G protein activation by G-protein-coupled receptors. Nature reviews. Molecular cell biology 9, 60-71.

Pol, O., Planas, E., Puig, M.M., 1995. Peripheral effects of naloxone in mice with acute diarrhea associated with intestinal inflammation. The Journal of pharmacology and experimental therapeutics 272, 1271-1276.

Przewłocki, R., Przewłocka, B., 2001. Opioids in chronic pain. Eur. J. Pharmacol. 429, 79-91.

Ray, L.A., Chin, P.F., Miotto, K., 2010. Naltrexone for the treatment of alcoholism: clinical findings, mechanisms of action, and pharmacogenetics. CNS Neurol. Disord. Drug Targets 9, 13-22.

Rónai, A.Z., Gráf, L., Székely, J.I., Dunai-Kovács, Z., Bajusz, S., 1977. Differential behaviour of LPH-(61–91)-peptide in different model systems: Comparison of the opioid activities of LPH-(61–91)-peptide and its fragments. FEBS Lett. 74, 182-184.

Sim, L.J., Selley, D.E., Childers, S.R., 1995. In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5'-[gamma-[35S]thio]-triphosphate binding. Proc Natl Acad Sci USA 92, 7242-7246.

Spetea, M., Schmidhammer, H., 2012. Recent advances in the development of 14-alkoxy substituted morphinans as potent and safer opioid analgesics. Curr. Med. Chem. 19, 2442-2457.

Szücs, M., Borsodi, A., Bogdány, A., Gaál, J., Batke, J., Tóth, G., 1987. Detailed analysis of heterogeneity of [3H]naloxone binding sites in rat brain synaptosomes. Neurochem. Res. 12, 581-587.

Traynor, J.R., Nahorski, S.R., 1995. Modulation by mu-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Mol. Pharmacol. 47, 848-854.

Tulunay, F.C., Takemori, A.E., 1974. The increased efficacy of narcotic antagonists induced by various narcotic analgesics. The Journal of pharmacology and experimental therapeutics 190, 395-400.

Váradi, A., Gergely, A., Béni, S., Jankovics, P., Noszál, B., Hosztafi, S., 2011. Sulfate esters of morphine derivatives: synthesis and characterization. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 42, 65-72.

Yasuda, K., Raynor, K., Kong, H., Breder, C.D., Takeda, J., Reisine, T., Bell, G.I., 1993. Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. Proc. Natl. Acad. Sci. U. S. A. 90, 6736-6740.

Yuan, C.S., Foss, J.F., Moss, J., 1995. Effects of methylnaltrexone on morphine-induced inhibition of contraction in isolated guinea-pig ileum and human intestine. Eur. J. Pharmacol. 276, 107-111.

Zacny, J.P., Wroblewski, K., Coalson, D.W., 2015. Methylnaltrexone: its pharmacological effects alone and effects on morphine in healthy volunteers. Psychopharmacology (Berl.) 232, 63-73.

Fig. (1). The structure of naltrexone-14-*O*-sulfate and naloxone-14-*O*-sulfate

Fig. (2). Synthesis of naltrexone-14-O-sulfate. For further details see section 2.3.

Fig. (3). The binding affinity of naltrexone-14-*O*-sulfate against [^{3}H]naloxone (**A**) and on MOR (**B**), DOR (**C**) and KOR (**D**) compared to naltrexone, naloxone-14-*O*-sulfate and naloxone in radioligand competition binding experiments. For control the affinities of the unlabeled form of the radioligands are also indicated. Data represent the specific binding of fixed concentrations of the indicated radioligands in percentage (means ± S.E.M.) in the presence of increased concentrations of the indicated unlabeled ligands. Experiments were performed in either rat (**A-C**) or guinea pig (**D**) whole brain membranes. On the X-axis "Total" represent the specific binding of the radioligands in the absence of competitor ligands. The level of total (100 %) and non-specific binding (0 %) is indicated with a dotted line on the Y-axis. K_i values are presented in Table 1. The assays were performed as described in section 2.4.2.

Fig. (4). The effect of naltrexone- and naloxone-14-*O*-sulfate on opioid receptor G-protein activity in [^{35}S]GTPγS binding experiments performed in rat **(A - C)** and guinea pig **(D)** brain membranes. (**A**) The neutral antagonistic effect of naloxone- and naltrexone-14-*O*-sulfate. (**B-D**) The effect of naloxone- and naltrexone-14-*O*-sulfate in agonist-stimulated [^{35}S]GTPγS binding experiments compared to naltrexone and naloxone on (**B**) MOR, (**C**) DOR and (**D**) KOR agonist stimulated G-protein activity. All data represents the specific binding of [^{35}S]GTPγS in percentage (means ± S.E.M.) in the presence of increasing concentration of the indicated ligands in the absence or presence of 1 μM of the indicated antagonists (**B-D**). On the X-axis "Basal" represent the specific binding of [^{35}S]GTPγS in the absence of any ligands, which also indicates basal activity (=100%, indicated by the line). E_{max} and EC_{50} values of Figure **4B-D** are presented in Table 2 and in section 3.2, respectively. The experiments were performed according to section 2.4.3.

Fig. (5). The competitive antagonist character of naltrexone-14-*O*-sulfate (**A**) compared to naltrexone (**B**) on MOR demonstrated with Schild regression analysis. The figure represents the calculated dose-ratios-1 (DR-1) of DAMGO ED_{50} values in the function of the applied naltrexone-14-*O*-sulfate or naltrexone concentrations both in logarithm form. The Schild-slope and the estimated equilibrium dissociation constant for the antagonists (pA_2 value, X-intercept) with 95% confidence intervals together with the goodness of fit (R^2) is indicated in the figure. The Schild-plot was calculated as described in section 2.7.2.

Fig. (6). The antagonist action (**A** and **C**) and competitive character (**B** and **D**) of naltrexone- and naloxone-14-*O*-sulfate on the antinociceptive action of morphine in mouse tail-flick test and in Schild regression analysis, respectively. (**A** and **C**) Figures represents the antinociceptive action in percentage (maximum possible effect) of morphine in the function of morphine concentrations in the absence and presence of the indicated naltrexone- and naloxone-14-*O*-sulfate doses. Data represents means ± S.E.M. and were analyzed as described in section 2.7.4. The experiments were performed as described in section 2.6. (**B** and **D**) Figures represents the calculated dose-ratios-1 (DR-1) of morphine ED_{50} values in the function of the applied naltrexone- or naloxone-14-*O*-sulfate doses both in logarithm form. The Schild-slope and the estimated equilibrium dissociation constant for the antagonists (pA_2 value, X-intercept) together with the goodness of fit (R^2) is indicated in the figure. The Schild-plots were calculated based on the data in Fig. 6A and C as described in section 2.7.4. Nltx-14-*O*-SU: naltrexone-14-*O*-sulfate; nx-14-*O*-SU: naloxone-14-*O*-sulfate.

Table 1. Opioid receptor affinity (K_i) values and selectivity ratios of naltrexone-14 *O*-sulfate compared with naltrexone, naloxone-14-*O*-sulfate and naloxone. The values were calculated according to the binding curves of the competition binding experiments in Fig. 3. as discussed in section 2.7.1. The affinity values of the unlabeled form of the radioligands (homologous ligand) are also indicated for positive control.

Compounds	**K_i (nM) ± S.E.M.**				**Selectivity ratio**		
	[^{3}H]naloxone[1]	**[^{3}H]DAMGO (μ)[1]**	**[^{3}H]IleDelt II (δ)[1]**	**[^{3}H]U69593 (κ)[2]**	**δ/μ**	**κ/μ**	**δ/κ**
Naltrexone-14-*O*-	15.6 ± 5.7	2.9 ± 0.5	181.2 ± 38.3	264.9 ± 80.8[b,i]	62	90	0.6

sulfate	(n=5)	(n=9)	(n=4)	(n=4)			
Naltrexone	4.8 ± 1.6 (n=3)	0.25 ± 0.04 (n=3)	20.9 ± 4.0 (n=3)	1.37 ± 0.12 (n=4)	84	5	15
Naloxone-14-*O*-sulfate	72.2 ± 17.8[c,e,g] (n=3)	7.9 ± 2.3[b,d,f,g] (n=4)	587.6 ± 192.7[b,d,f,g] (n=3)	238.8 ± 58.8[a,d,h] (n=3)	74	30	2
Naloxone	6.0 ± 1.6 (n=5)	0.9 ± 0.2 (n=3)	13.3 ± 4.2 (n=3)	11.0 ± 1.2 (n=4)	15	13	1
Homologous ligand[3]		0.9 ± 0.2 (n=3)	0.5 ± 0.2 (n=3)	3.3 ± 0.5 (n=4)		-	

[1]: Performed in rat whole brain membrane homogenate

[2]: Performed in guinea pig whole brain membrane homogenate

[3]: Refers to unlabeled naloxone, DAMGO, IleDelt II and U69593

Note: μ, δ and κ indicates the three classic opioid receptor

[a]: $P < 0.05$ compared to naltrexone, [b]: $P < 0.01$ compared to naltrexone, [c]: $P < 0.001$ compared to naltrexone, [d]: $P < 0.01$ compared to naloxone, [e]: $P < 0.001$ compared to naloxone, [f]: $P < 0.05$ compared to naltrexone-14-*O*-sulfate, [g]: $P < 0.01$ compared to naltrexone-14-*O*-sulfate, [h]: $P < 0.05$ compared to the corresponding homologues ligand, [i]: $P < 0.01$ compared to the corresponding homologues ligand (The significance level was evaluated by One-way ANOVA with Tukey multiple comparison post hoc test)

Table 2. Efficacy (E_{max}) values of opioid receptor G-protein values of opioid receptor specific stimulator ligands in the presence of 1 μM naltrexone-14-*O*-sulfate naltrexone, naloxone-14-*O*-sulfate and naloxone. The values were calculated based on agonist-stimulated [^{35}S]GTPγS binding experiments presented in Fig. 4 as discussed in section 2.7.1.

Compounds	**E_{max} ± S.E.M. (%)**		
	DAMGO (μ)[1]	**IleDelt II (δ)[1]**	**U69593 (κ)[2]**
Ligand alone	136.5 ± 2 (n=3)	143.5 ± 2.7 (n=3)	143.6 ± 2.7 (n=5)
+ naltrexone-14-*O*-sulfate	120.3 ± 12.1 (n=3)	119.9 ± 4.4[a] (n=4)	128.8 ± 4.4 (n=3)
+ naltrexone	110.6 ± 3.3 (n=3)	117 ± 1.9[b] (n=4)	118 ± 2 (n=3)
+ naloxone-14-*O*-sulfate	119.1 ± 4.2 (n=3)	116.7 ± 2.8[a] (n=3)	129.4 ± 3.9 (n=3)
+ naloxone	114.3 ± 6.6 (n=3)	116.9 ± 3.2[a] (n=3)	123.1 ± 10 (n=3)

[1]: Performed in rat whole brain membrane homogenate

[2]: Performed in guinea pig whole brain membrane homogenate

Note: μ, δ and κ indicates the three classic opioid receptors

[a]: P < 0.01 compared to ligand alone, [b]: P < 0.001 compared to ligand alone (The significance level was evaluated by One-way ANOVA with Tukey multiple comparison post hoc test)

Table 3A and B. The affinity values (K_e) and selectivity ratios of naltrexone-14-*O*-sulfate compared to naltrexone, naloxone-14-*O*-sulfate and naloxone on MOR, DOR and KOR in agonist induced inhibition in isolated mouse *vasa deferentia* biological assays. The experiments were performed and analyzed as described in section 2.5.1 and 2.7.2, respectively.

Table 3A

Compounds	K_e (nM) ± S.E.M.			Selectivity ratio		
	DAMGO (μ)	DADLE (δ)	EKC (κ)	δ/μ	κ/μ	δ/κ
Naltrexone-14-*O*-sulfate	5.9 ± 0.5 (n=11)	77.5 ± 7.8 (n=3)	755.0 ± 7.3[a,d] (n=3)	13	127	0.1
Naltrexone	0.3 ± 0.1 (n=4)	23.7 ± 2.2 (n=4)	16.4 ± 3.4 (n=3)	79	54	1.4
Naloxone-14-*O*-sulfate	16.8 ± 1.9[c,f,n] (n=11)	216.9 ± 51.0[b,e,g] (n=4)	913.6 ± 218.2[b,e] (n=4)	13	54	0.2
Naloxone	1.7 ± 0.3 (n=4)	22.5 ± 3.2 (n=4)	18.4 ± 1.5 (n=4)	14	11	1.2

Table 3B

Compounds	K_e (nM) ± S.E.M.			Selectivity ratio		
	DAMGO (μ)	DPDPE (δ)	U69593 (κ)	δ/μ	κ/μ	δ/κ
Naltrexone-14-*O*-sulfate	8.5 ± 2.1 (n=3)	37.6 ± 5.1[a,e] (n=4)	367.9 ± 78.0[c,e] (n=3)	4.4	43.3	0.1
Naltrexone	0.5 ± 0.1 (n=4)	7.9 ± 0.9 (n=3)	12.1 ± 4.1 (n=4)	15.8	24.2	0.7
Naloxone-14-*O*-sulfate	18.1 ± 0.7[c,e,g] (n=4)	54.7 ± 12.0[b,f] (n=4)	542.3 ± 35.5[c,f,g] (n=4)	3	29.9	0.1
Naloxone	1.5 ± 0.1 (n=4)	4.6 ± 1.3 (n=6)	5.8 ± 1.3 (n=4)	3	3.8	0.8

Note: μ, δ and κ indicates the three classic opioid receptors

[a]: P < 0.05 compared to naltrexone, [b]: P < 0.01 compared to naltrexone, [c]: P < 0.001 compared to naltrexone, [d]: P < 0.05 compared to naloxone, [e]: P < 0.01 compared to naloxone, [f]: P < 0.001 compared to naloxone, [g]: P < 0.05 compared to naltrexone-14-*O*-sulfate, [h]: P < 0.001 compared to naltrexone-14-*O*-

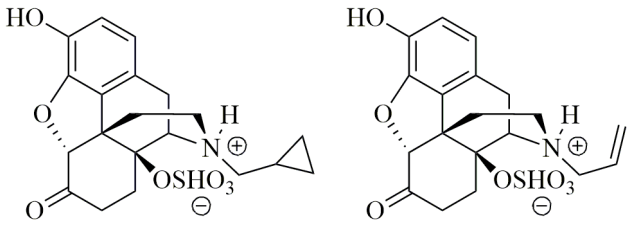
sulfate (The significance level was evaluated by One-way ANOVA with Tukey multiple comparison post hoc test)

Table 4. The affinity values (K_e) of naltrexone-14-*O*-sulfate compared to naltrexone, naloxone-14-*O*-sulfate and naloxone in morphine induced inhibition in isolated mouse colon assay. The experiments were performed and analyzed as described in section 2.5.2 and 2.7.3, respectively.

Compounds	**K_e ± S.E.M. (nM) against morphine**	**N**
Naltrexone-14-*O*-sulfate	10.4 ± 5.0	4
Naltrexone	0.7 ± 0.4	3
Naloxone-14-*O*-sulfate	25.4 ± 8.2[a,b]	4
Naloxone	2.1 ± 0.4	5

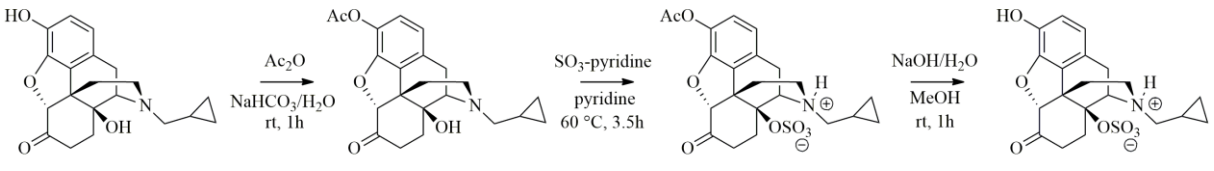
[a]: P < 0.05 compared to naltrexone, [b]: P < 0.05 compared to naloxone

Fig. 1



naltrexone-14-*O*-sulfate naloxone-14-*O*-sulfate

Fig. 2



naltrexone 3-*O*-acetylnatrexone 3-*O*-acetylnaltrexone-14-*O*-sulfate naltrexone-14-*O*-sulfate

Fig. 3

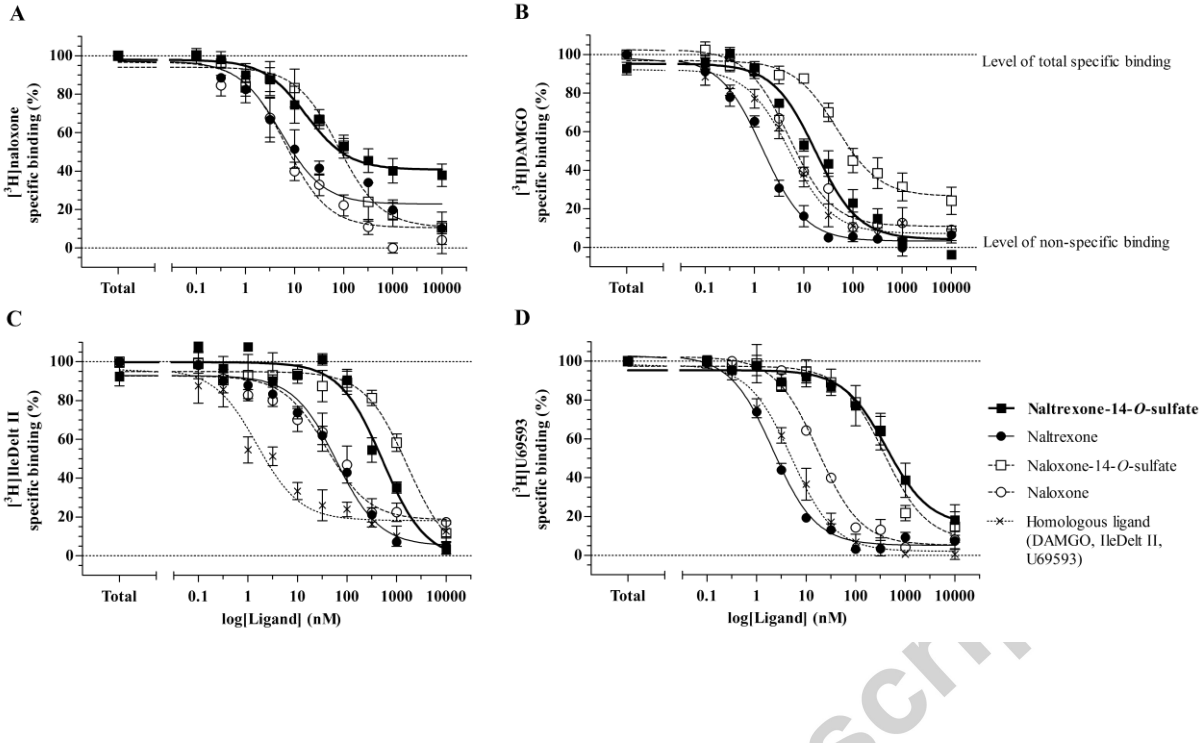

A
B
C
D
[3H]naloxone specific binding (%)
[3H]DAMGO specific binding (%)
[3H]IleDelt II specific binding (%)
[3H]U69593 specific binding (%)
Total
0.1
1
10
100
1000
10000
log[Ligand] (nM)
Level of total specific binding
Level of non-specific binding
Naltrexone-14-O-sulfate
Naltrexone
Naloxone-14-O-sulfate
Naloxone
Homologous ligand (DAMGO, IleDelt II, U69593)

Fig. 4

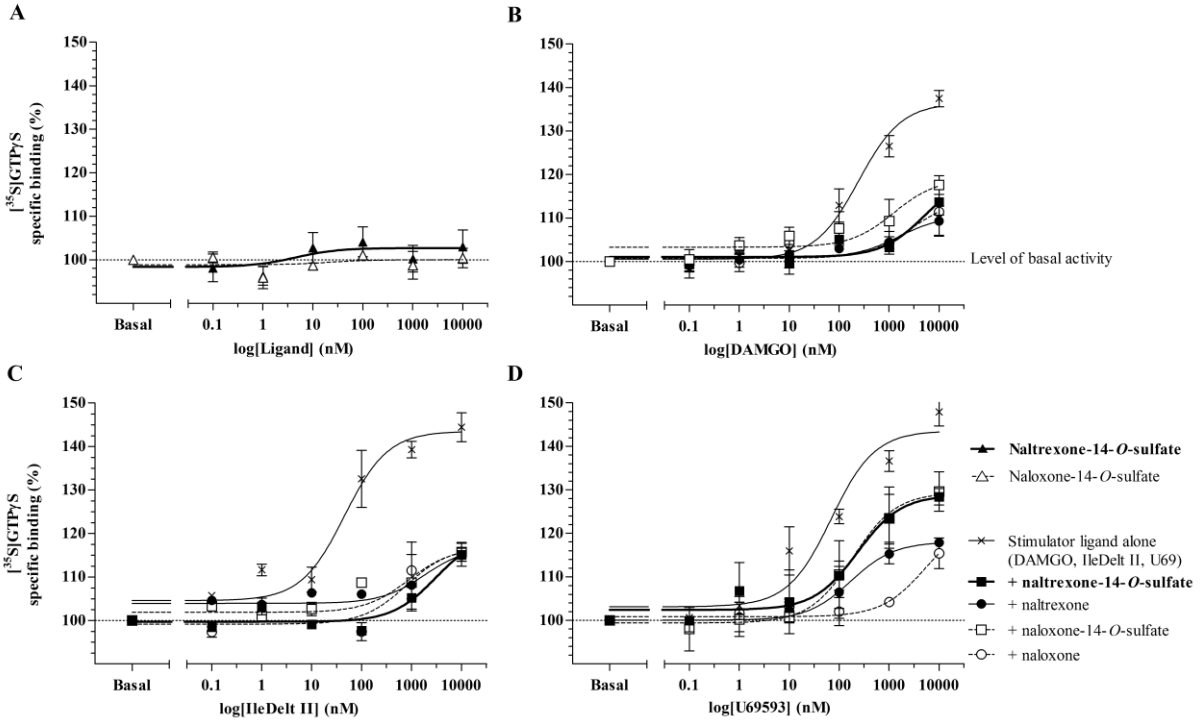

A
B
C
D
[35S]GTPγS specific binding (%)
Basal
log[Ligand] (nM)
log[DAMGO] (nM)
log[IleDelt II] (nM)
log[U69593] (nM)
Level of basal activity
Naltrexone-14-O-sulfate
Naloxone-14-O-sulfate
Stimulator ligand alone (DAMGO, IleDelt II, U69)
+ naltrexone-14-O-sulfate
+ naltrexone
+ naloxone-14-O-sulfate
+ naloxone

Fig. 5

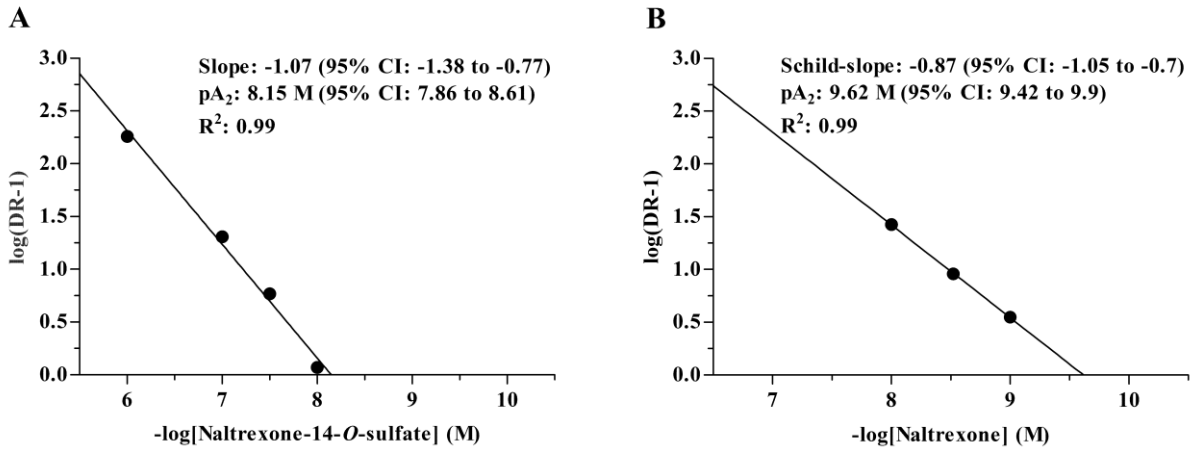
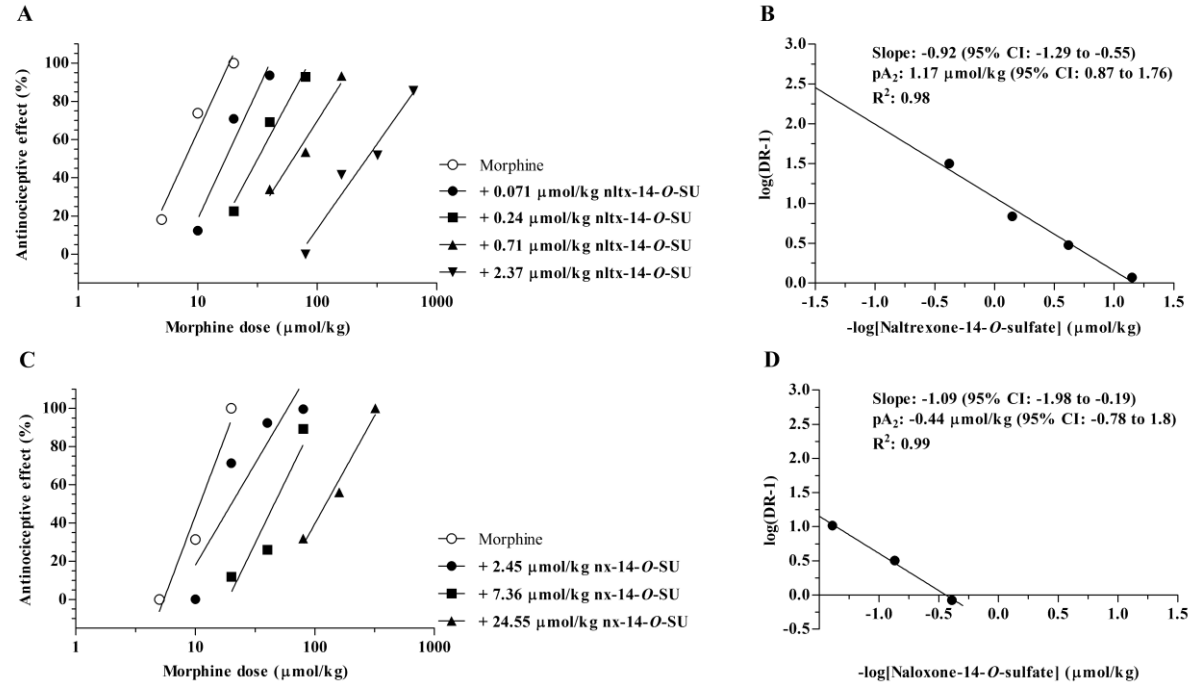

A
Slope: -1.07 (95% CI: -1.38 to -0.77)
pA2: 8.15 M (95% CI: 7.86 to 8.61)
R2: 0.99
log(DR-1)
-log[Naltrexone-14-O-sulfate] (M)
B
Schild-slope: -0.87 (95% CI: -1.05 to -0.7)
pA2: 9.62 M (95% CI: 9.42 to 9.9)
R2: 0.99
log(DR-1)
-log[Naltrexone] (M)

Fig. 6


A
Antinociceptive effect (%)
Morphine dose (µmol/kg)
Morphine
+ 0.071 µmol/kg nltx-14-O-SU
+ 0.24 µmol/kg nltx-14-O-SU
+ 0.71 µmol/kg nltx-14-O-SU
+ 2.37 µmol/kg nltx-14-O-SU
B
Slope: -0.92 (95% CI: -1.29 to -0.55)
pA2: 1.17 µmol/kg (95% CI: 0.87 to 1.76)
R2: 0.98
log(DR-1)
-log[Naltrexone-14-O-sulfate] (µmol/kg)
C
Antinociceptive effect (%)
Morphine dose (µmol/kg)
Morphine
+ 2.45 µmol/kg nx-14-O-SU
+ 7.36 µmol/kg nx-14-O-SU
+ 24.55 µmol/kg nx-14-O-SU
D
Slope: -1.09 (95% CI: -1.98 to -0.19)
pA2: -0.44 µmol/kg (95% CI: -0.78 to 1.8)
R2: 0.99
log(DR-1)
-log[Naloxone-14-O-sulfate] (µmol/kg)