Pharmacological Investigations of N-Substituent Variation in Morphine and Oxymorphone: Opioid Receptor Binding, Signaling and Antinociceptive Activity

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Abstract

Morphine and structurally related derivatives are highly effective analgesics, and the mainstay in the medical management of moderate to severe pain. Pharmacological actions of opioid analgesics are primarily mediated through agonism at the μ opioid peptide (MOP) receptor, a G protein-coupled receptor. Position 17 in morphine has been one of the most manipulated sites on the scaffold and intensive research has focused on replacements of the 17-methyl group with other substituents. Structural variations at the N-17 of the morphinan skeleton led to a diversity of molecules appraised as valuable and potential therapeutics and important research probes. Discovery of therapeutically useful morphine-like drugs has also targeted the C-6 hydroxyl group, with oxymorphone as one of the clinically relevant opioid analgesics, where a carbonyl instead of a hydroxyl group is present at position 6. Herein, we describe the effect of N-substituent variation in morphine and oxymorphone in vitro and in vivo biological properties and the emerging structure-activity relationships. We show that the presence of a N-phenethyl group in position 17 is highly favorable in terms of improved affinity and selectivity at the MOP receptor, potent agonism and antinociceptive efficacy. The N-phenethyl derivatives of morphine and oxymorphone were very potent in stimulating G protein coupling and intracellular calcium release through the MOP receptor. In vivo, they were highly effective against acute thermal nociception in mice with marked increased antinociceptive potency compared to the lead molecules. It was also demonstrated that a carbonyl group at position 6 is preferable to a hydroxyl function in these N-phenethyl derivatives, enhancing MOP receptor affinity and agonist potency in vitro and in vivo. These results expand the understanding of the impact of different moieties at the morphinan nitrogen on ligand-receptor interaction, molecular mode of action and signaling, and may be instrumental to the development of new opioid therapeutics.

Introduction

The naturally occurring morphine (Figure 1), the active component of opium, has been used as an analgesic for centuries [1]. Today, effective pain control is still one of the most important therapeutic priorities [2]. Morphine and other structurally related derivatives as well as opioids with distinct structures such as fentanyl have proven to be of the utmost importance as effective analgesics for the treatment of moderate to severe pain. The pharmacological actions of clinically used opioid analgesics are primarily mediated through activation of the μ opioid peptide (MOP) receptor [3], highly expressed in the central and peripheral nervous system and various peripheral tissues. The MOP receptor together with the other members of the opioid receptor class, i.e. δ opioid peptide (DOP) and κ opioid peptide (KOP) receptors, belong to the family of G protein-coupled receptors (GPCRs), and their crystal structures are now available [4–6]. While extremely efficacious as pain relievers, opioid analgesics produce an array of side effects that can limit their clinical usefulness, including constipation, nausea, vomiting, and respiratory depression. Long-term treatment with opioids is also associated with development of tolerance to their analgesic effects, physical dependence and addiction [7].

Since the structure elucidation of morphine ninety years ago, its skeleton and its conversion to new analogues was intensively investigated. Consequently, the morphinan skeleton has been the basis of successful drug development, and several opioid drugs are available for patient use or are employed as research probes to examine opioid mechanisms at cellular and molecular levels [3,8–13]. Extensive work in the field led to innovative molecules with
new substitution patterns and more favorable pharmacological features, powerful analgesia and less undesirable effects. Established and generally accepted structure-activity relationship (SAR) models have assigned a significant role to the N-substituent in position 17 on the morphinan skeleton in defining the pharmacological behavior. Nalorphine, the N-allyl substituted analogue of morphine, was one of the first compounds to be recognized as an opioid antagonist, reversing the analgesic and respiratory depressant actions of morphine [13,14]. Further studies described that nalorphine alone can induce an antinociceptive effect, which was almost comparable to that of morphine [15,16], thus defining nalorphine as a partial agonist. Earlier reports on large series of differently N-substituted derivatives of morphine provided exciting outcomes. Exchanging the methyl group at the nitrogen of morphine by other alkyl groups reduces or abolishes analgesic activity [17]. N-Phenacyl-, N-phenoxethyl-, and N-benzhydromorphine have less than one-tenth of the analgesic potency of morphine [17]. In contrast, it was described that N-phenethil substitution resulted in a 6- to 10-fold higher analgesic potency compared to morphine in rodents, while N-cyclohexylmorphine was only one-third as effective [17]. Another targeted site on the morphine skeleton is the C-14 position, where introduction of a hydroxyl group induces an analgesic action of moderate strength [18]. Numerous highly potent morphine-like compounds are known, one of them being oxymorphone, a MOP agonist (Figure 1). Oxymorphone is used not only clinically [19], but also as a valuable scaffold for the development of new ligands interacting with the MOP receptor [9,11,20]. A representative example of the complex role played by the morphinan nitrogen in determining the pharmacological properties includes N-substituted derivatives of oxymorphone, ranging from potent agonism i.e. N-methyl, N-benzyl and N-phenethyl, to partial agonism i.e. N-dimethylallyl (naloxone) and N-cyclohexylmethyl (nalbuphene), to pure and potent antagonism i.e. N-allyl (naloxone) and N-cyclopentylmethyl (naltrexone). Substitution of the methyl with a phenylethyl group at the nitrogen in oxymorphone produces a 12-fold increase in analgesic potency [21]. Naloxone and naltrexone, the N-allyl- and N-cyclopentylmethyl analogues of oxymorphone, respectively, are two opioid antagonists clinically used for the treatment of opioid induced respiratory depression and overdose, with naltrexone being also used for the management of opioid and alcohol dependence [22,23]. In both morphine and oxymorphone series, it has been reported that the 14-hydroxy group can influence the morphine-like pharmacological profile for varying N-substituents [24,25]. In the class of agonists, the C14-hydroxyl appears to slightly reduce intrinsic in vitro potency, while increasing in vivo potency. In partial agonists, the 14-hydroxyl group considerably contributes in decreasing efficacy.

The present study was undertaken to characterize and to compare the effect of N-substituent variation in morphine and oxymorphone on in vitro (binding and functional activity) and in vivo (nociception) pharmacological properties. SAR studies were performed on a series comprising of four derivatives of morphine (1–4) and two derivatives of oxymorphone (5 and 6) (Figure 1). Although the synthesis of compounds 1 [26,27] and 3 [18] has been reported about fifty years ago, and derivative 4 was prepared twenty years ago [28], there is only spare data on their biological activities, with binding affinities and selectivities at MOP, DOP and KOP receptors not yet reported. Herein, we also describe the synthesis and biological characterization of a new N-substituted derivative of morphine, N-phenylpropoxymorphine (2). In the oxymorphone series, the N-phenethyl substituted derivative 6 was already prepared in the 1960s [29] and known as a potent opioid analgesic [21], while N-benzhydromorphone (5) was synthesized and in vitro binding and in vivo behavioral studies were first reported by May et al. [30]. To our knowledge, there are no in vivo functional activity data at opioid receptors available on any of the investigated morphine and oxymorphone derivatives. We have evaluated the ability of these compounds to stimulate G protein coupling (guanosine 5’-O-(3-[35S]thio)triphosphate, [35S]GTPγS, functional assay) in membranes of cells expressing the human recombinant opioid receptors. Moreover, in cells co-expressing opioid receptors and chimeric G proteins that force the receptor to signal through the calcium pathway, these opioid ligands were examined for their capability to promote calcium mobilization. Furthermore, in vivo efficacy in mice against acute thermal nociception (hot-plate and tail-flick tests) was examined and compared to antinociceptive potencies of the lead molecules, morphine and oxymorphone. These investigations provide valuable insights on SAR in the morphinan class of opioids, by broadening our current understanding of the impact of different moieties at the morphinan nitrogen on ligand-receptor interaction, signaling and the link between analgesic efficacy and the molecular mode of action.

![Figure 1. Structures of morphine, oxymorphone and N-substituted morphinans 1–6. Ph, phenyl. doi:10.1371/journal.pone.0099231.g001](image-url)
Materials and Methods

Ethics Statement

All animal studies were conducted in accordance with ethical guidelines and animal welfare standards according to Austrian regulations for animal research, and were approved by the Committee of Animal Care of the Austrian Federal Ministry of Science and Research. Every effort was made to minimize both the animal suffering and the number of animals used.

Compounds and Reagents

Opioid radioligands, [3H][D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin ([3H]DAMGO), [3H][3a,7a,8b]-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxa(4,5,10)-benzenecacetamide ([3H]U69,593) and [3H]GTPyS were purchased from PerkinElmer (Boston, USA). [3H][Ile^4,5]deltorphin II was obtained from the Institute of Isotopes Co. Ltd. (Budapest, Hungary). DAMGO, [D-Pen^2,D-Pen^5]enkephalin (DPDPE), naloxone, tris(hydroxymethyl)aminomethane (Tris), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), unlabeled GTPyS, guanosine diphosphate (GDP) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All cell culture media and supplements were from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and Invitrogen (Paisley, UK). Morphine was obtained from Gatt-Koller GmbH (Innsbruck, Austria). All other chemicals were obtained from standard commercial sources.

The synthesis of N-phenethylmorphinans (1) was performed from normorphine by alkylation with 2-phenylethyl bromide according to Clark et al. [26] using N,N-dimethylformamide (DMF) instead of ethanol as solvent, which provided higher yields. Similarly, N-phenylpropylmorphinone (2) was synthesized from normorphine using 3-phenylpropyl bromide as alkylating agent. Sodium borohydride reduction of 14-hydroxymorphinone in normorphine using 3-phenylpropyl bromide as alkylating agent. Similarly, N-phenethylmorphinans (1) were synthesized by a new route via noroxymorphone ethylene ketal. For further details see Chemistry S1.

In vitro Assays

Radioligand binding assays. Membranes were prepared from Sprague-Dawley rat or guinea pig brains as previously described [32]. All binding experiments were performed in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 ml containing 300–500 μg protein [32]. Rat brain membranes were incubated either with [3H]DAMGO (1 nM, 45 min, 35°C) or [3H][Ile^4,5]deltorphin II (0.5 nM, 45 min, 35°C). Guinea pig brain membranes were incubated with [3H]U69,593 (1 nM, 30 min, 30°C). Nonspecific binding was determined in the presence of 10 μM naloxone. Reactions were terminated by rapid filtration using a Brandel Cell Harvester (Brandel Inc., Gaithersburg, MD) and Whatman GF/B glass fiber filters pre-soaked in 0.1% polyethyleneimine for 1 h at 4°C for [3H]U69,593, or type GF/C for [3H]DAMGO and [3H][Ile^4,5]deltorphin II. Filters were washed three times with 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and bound radioactivity was measured by liquid scintillation counting. All experiments were performed in duplicate and repeated at least three times. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard [33].

[3H]GTPyS functional assays. Chinese hamster ovary (CHO) cells expressing recombinant human MOP, DOP or KOP receptors (CHO-MOP, CHO-DOP and CHO-KOP cell lines) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and Ham F-12 medium supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM) and genetin (400 μg/ml) [34]. Cell cultures were maintained at 37°C in 5% CO2 humidified air. Membranes were prepared in buffer A (20 mM HEPES, 10 mM MgCl2, and 100 mM NaCl, pH 7.4) as described [35]. Cell membranes (5 μg) were incubated with 0.05 nM [3H]GTPyS, 10 μM GDP and test compounds for 60 min at 25°C, in a total volume of 1 ml. Nonspecific binding was determined using 10 μM GTPyS, and the basal binding was determined in the absence of test ligand. Samples were filtered over Whatman GF/B glass fiber filters and counted as described for binding assays. All experiments were performed in triplicate and repeated at least three times.

Calcium mobilization assays. CHO-MOP and CHO-KOP stably expressing the C-terminally modified Gαq/11 protein, and CHO-DOP stably expressing the C-terminally modified Gαq166N5 protein were grown in DMEM/Ham F-12 medium supplemented with FBS (10%), penicillin (100 IU/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), genetin (200 μg/ml) and hygromycin B (100 μg/ml). Cell cultures kept at 37°C in 5% CO2 in humidified air were used in the calcium mobilization assays performed as previously described [36]. Cells were seeded at a density of 10^5 cells in 100 μl, into each well of a 96-well plate and incubated for 24 h at 37°C in 5% CO2 humidified air.

Table 1. Opioid receptor binding affinities and selectivities at MOP, DOP and KOP receptors.

| Compound | K_i (nM) | Selectivity ratios |
|----------|----------|--------------------|
| Morphine | 6.55±0.74 | 217±19             | 113±9 | 33 | 17 |
| Oxymorphone | 0.97±0.05 | 80.5±5.5            | 61.6±1.2 | 83 | 51 |
| 1        | 0.93±0.14 | 370±5.5             | 107±18 | 40 | 115 |
| 2        | 79.5±1.1  | 869±171             | 565±24 | 11 | 7  |
| 3        | 16.4±1.1  | 1,081±271           | 789±77 | 66 | 48 |
| 4        | 4.60±0.01 | 163±17              | 513±66 | 35 | 112 |
| 5        | 359±31    | 1,078±35            | 75.0±8.0 | 3 | 0.2 |
| 6        | 0.54±0.03 | 128±0.2             | 84.2±7.2 | 24 | 156 |

Binding assays were performed with membranes from rat brain (MOP and DOP receptors) and guinea pig brain (KOP receptors). Values represent the mean ± SEM of at least three experiments each performed in duplicate. doi:10.1371/journal.pone.0099231.t001
Figure 2. In vitro agonist activities at the MOP receptor of morphine, oxymorphone and N-methylmorphinans 1, 4 and 6. Concentration-response curves in (A) [35S]GTPγS functional assay with membranes from CHO expressing human MOP receptor and (B) calcium mobilization experiments performed with CHO cells co-expressing the human MOP receptor and the Gαq protein. Activity is calculated as percentage of maximal stimulation produced by DAMGO. Data are shown as the mean ± SEM (n=3).

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**Results and Discussion**

Opioid receptor binding affinities and selectivities of the four derivatives of morphine (1–4) and two derivatives of oxymorphone (5 and 6) were determined by in vitro competition binding assays using membranes from rat brain (MOP and DOP opioid receptors) or guinea pig brain (KOP opioid receptors) [32]. Receptor type-specific radioligands were used i.e. [3H]DAMGO (MOP), [3H]DPDPE (DOP) and [3H]U69,593 (KOP) were calculated using nonlinear curve fitting analysis. Data are represented as the mean ± SEM. For in vitro assays, the effective dose ED50 and 95% confidence limits (95% CL) were calculated using the method of Litchfield and Wilcoxon [38].

**In vivo Testing**

**Animals.** Sprague-Dawley rat and guinea pig brains used in in vitro assays were obtained from the Institut für Labortierkunde und Laborgenetik, Medizinische Universität Wien (Himberg, Austria). Male CD1 mice (25–30 g) were used in in vivo studies. Mice were housed in groups of five and were kept in a temperature-regulated environment under a controlled 12 h light/dark cycle with free access to food and water at all times except during testing.

**Drug administration.** Vehicle or solutions of test compounds prepared in sterile physiological saline (0.9%) were administered subcutaneously (s.c.) to mice in a volume of 10 μl per 1 g body weight. At least three doses were tested, and 5–6 animals per dose were used. The dose ranges for the investigated opioids were: morphine (1.25–5 mg/kg), oxymorphone (0.2–1 mg/kg), and compounds 1 (0.05–0.5 mg/kg), 4 (0.5–5 mg/kg), and 6 (0.1–0.5 mg/kg).

**Nociceptive assessments.** The hot-plate test was performed as described [37]. Each mouse was placed on a UB 33100 hot/cold plate (Ugo Basile s.r.l., Varese, Italy) kept at 55°C, and the occurrence of a nociceptive response (licking or shaking a paw, jumping) was observed. To confine the mice to a certain observation area, a colourless plastic cylinder of 20 cm diameter was placed on the hot plate. In order to avoid possible tissue injury, a cut-off time of 12 s was used. The tail-flick test was performed using an UB 37360 Ugo Basile analgesiometer (Ugo Basile s.r.l., Varese, Italy) as previously described [37]. The reaction time required by the mouse to remove its tail due to the radiant heat was measured and defined as the tail-flick latency. A cut-off time of 10 s was used in order to minimize tissue damage.

Hot-plate and tail-flick latencies were measured before (basal latency, BL) and 30, 60 and 120 min after drug or vehicle s.c. administration (test latency, TL). For establishing the dose-response effect, the antinociceptive response was expressed as percent of Maximum Possible Effect (%MPE) = [(TL – BL)/(cut-off time – BL)]×100 for each dose tested.

**Data Analysis**

Binding and functional data were analyzed with the GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Concentration-response curves were constructed and inhibition constant (Ki, nM), agonist potency (EC50, nM) and efficacy (Emax, % of maximum stimulation with respect to the maximal stimulation produced by DAMGO). Data are represented as the mean ± SEM (n=3).

**Opioid Activities of N-phenethylmorphinans**

Density of 50,000 cells per well into 96-well black, clear-bottom plates. After 24 h, the cells were loaded with medium supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37°C. The loading solution was replaced by Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black, for 10 min at 37°C. After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA), fluorescence changes were recorded. All experiments were performed in duplicate and repeated at least three times.
In vitro agonist potency and efficacy.

| Compound | MOP | DOP | KOP |
|----------|-----|-----|-----|
|          | E<sub>C50</sub> (nM) | E<sub>max</sub> (%)<sup>c</sup> | E<sub>C50</sub> (nM) | E<sub>max</sub> (%)<sup>c</sup> | E<sub>C50</sub> (nM) | E<sub>max</sub> (%)<sup>c</sup> |
| Morphine | 344±5.1 | 109±14 | 1.09±3 | 712±36 | 12±11 | 0.07±0.05 |
| Oxymorphone | 3.38±0.76 | 89±1 / 7 | 68±6 | 11±2 | 1±1 | 0.05±0.01 |
| Compound 1 | 4.33±0.7 | 11±1 | 12±17 | 1.09±3 | 0.07±0.05 |
| Compound 2 | 6.23±0.66 | 97±3 | 110±1 | 22±34 | 0.01±0.01 |

<sup>a</sup> Membranes from CHO cells stably transfected with human MOP, DOP or KOP receptors were used.

<sup>b</sup> CHO cells co-expressing chimeric G proteins and recombinant human MOP, DOP or KOP receptors were used.

<sup>c</sup> E<sub>max</sub> is expressed in percentage relative to maximal stimulation produced by DAMGO (MOP), DPDPE (DOP) or U69,593 (KOP).

<sup>d</sup> inactive

<sup>**</sup>d inactived

<sup>c</sup>m

<sup>**</sup>m

<sup>f</sup>ND, not determined due to very low binding affinity at the KOP receptor.

<sup>e</sup> crc incomplete

<sup>**</sup> crc incomplete

<sup>**</sup> crc incomplete

<sup>**</sup> crc incomplete

In this study, in order to assess initially MOP receptor-evaluated using a [35S]GTP<sup>S</sup> binding response (E<sub>max</sub>) to the reference opioid agonists, DAMGO (MOP), DPDPE (DOP) and U69,593 (KOP). Potencies as EC<sub>50</sub> values and efficacies as maximum response (E<sub>max</sub>) to the reference opioid agonists are presented in Table 2. Opioid receptor-mediated G protein signaling was evaluated using a [35S]GTP<sup>S</sup> binding assay in membranes from CHO cells stably expressing either the human MOP, DOP or KOP receptors [35]. In CHO<sub>MOP</sub> cell membranes, all three compounds 1, 4 and 6 produced concentration-dependent increase in [35S]GTP<sup>S</sup> binding (Figure 2A). Oxymorphone derivative 6 was the most potent MOP agonist with an EC<sub>50</sub> value of 2.63 nM, roughly equivalent to that of oxymorphone, while also showing similar efficacies. This N-phenethyl substituted 6 also proved to be more potent than DAMGO (EC<sub>50</sub> = 19.3 nM) in stimulating G protein signaling. High agonist potency was also...
depicted by the morphine derivative 1, being about 3-fold more potent as MOP agonist than morphine, and 2-fold than DAMGO. In contrast, the N-phenethyl-14-hydroxynormorphine (4) exhibited the lowest potency having an EC50 value similar to morphine (Table 2). The rank order of agonist potencies to promote MOP receptor mediated-G protein coupling correlates well with binding affinities at the MOP receptor observed in the radioligand binding studies (Table 1).

By comparing the agonist potency at the hMOP receptor expressing CHO cells, potencies of derivatives 1, 4 and 6, morphine and oxymorphone to stimulate [35S]GTPγS binding were decreased considerably in hDOP (EC50 = 3.0 nM for DPDPE) and hKOP receptors (EC50 = 42.7 nM for U69,593) expressing cells (Table 2). While in CHO-DOR cell membranes, they showed high efficacies, much reduced to no stimulation was measured at the KOP receptor. Due to very low binding affinity at the KOP receptor, we did not investigate the activity at the KOP receptor of compound 4 in the [35S]GTPγS binding.

In this study, we have also examined the potency and efficacy of derivatives 1, 4 and 6 to evoke changes in intracellular calcium concentration using a whole cell fluorescence-based assay [36]. In CHO-DOR cells stably expressing the Gαo,q566Di5 chimeric protein, and in CHO-KOP cells expressing the Gαo,q566Di5 chimeric protein, the N-phenethylnoroxymorphone (6) showed the highest potency. Compared to DAMGO (EC50 = 42.7 nM) compound 6 was about 2-fold more potent. Among the two morphine derivatives, N-phenethylmorphine (1) was about 3-fold more potent than morphine and equipotent to DAMGO, and about 3-fold more active than its 14-hydroxy analogue 4 in evoking calcium mobilization (Table 2). In CHO-DOR cells expressing the Gαo,q566Di5 chimeric protein, and in CHO-KOP cells expressing the Gαo,q566Di5 chimeric protein, the investigated derivatives stimulated calcium release with considerably lower potencies or were even found inactive, which is in line with the findings from [35S]GTPγS functional assays (Table 2).

The findings from our in vitro studies including binding affinity and potency at the MOP receptor together with earlier reports on the analgesic effects of compounds 1 [17] and 6 [21] and preliminary experiments were used to establish the appropriate dose range for in vivo investigations. Antinociceptive properties of morphine derivatives 1 and 4, and oxymorphone analogue 6 were assessed in mice after s.c. administration using two nociceptive tests, hot-plate and tail-flick [37]. All three MOP agonists produced time- and dose-dependent effects in both nociceptive assays (Figures 3 and 4) with compounds 1 and 6 being the most effective against acute thermal nociception. The peak antinociception occurred generally 30 min after drug s.c. administration (Figure 3). Antinociceptive potencies expressed as ED50 values with 95% confidence limits are listed in Table 3, and were compared with those of the reference opioids drugs, morphine and oxymorphone. In agreement with the earlier observations of Winter et al. [17], morphine derivative 1 was also shown in our study to be a more potent antinociceptive than morphine. In the hot-plate and tail-flick tests, it was 22- and 28-fold, respectively, more effective than morphine. First data on the antinociceptive effect of N-phenethyl-14-hydroxynormorphine (4) are described herein, revealing this MOP agonist as a potent antinociceptive agent with a 2- to 3-fold increased potency than morphine. Compound 6, the N-phenethyl analogue of oxymorphone, was found to be highly active with about 2-fold higher potency than oxymorphone, and comparable potency to 1. It was up to 8-fold more potent than its 6-hydroxy counterpart 4 in inducing an antinociceptive response, indicating a 6-keto substitution to be preferable toward improved analgesic properties. Besides analgesia, MOP agonists are well-known to induce other behavioral changes. While in this study, generally, no major alterations in locomotor activity and no sedative effects were observed at any of the tested doses of compounds 1, 4 and 6, representing about 3- to 4-fold the analgesic ED50 dose, further investigations will be needed to establish the therapeutic index of these compounds.

**Conclusions**

Position 17 in morphine has been one of the most manipulated sites on the scaffold and intensive research has focused on replacements of the 17-methyl group with other substituents. Structural variations at the N-17 of the morphinan skeleton have

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**Figure 3.** Time-course of antinociceptive effects produced by morphine, oxymorphone and N-methylmorphinans 1, 4 and 6. The effect of morphine (1.25–5 mg/kg), oxymorphone (0.2–1 mg/kg), and compounds 1 (0.05–0.5 mg/kg), 4 (0.5–3 mg/kg), and 6 (0.1–0.5 mg/kg) in the hot-plate test (A, left panel) and in the tail-flick test (B, right panel). Hot-plate and tail-flick latencies (in seconds) were determined in mice before (0 min) and after s.c. drug administration (30, 60 and 120 min). Data are shown as the mean ± SEM (n = 5–6 mice per group).

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**Figure 4.** Dose-dependent antinociceptive effects produced by morphine, oxymorphone and N-methylmorphinans 1, 4 and 6. (A) Hot-plate test. (B) Tail-flick test. Hot-plate and tail-flick latencies (as %MPE) are shown at 30 min (peak of action) after s.c. drug administration to mice. Data are shown as the mean ± SEM (n = 5–6 mice per group).

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Table 3. Antinoceptive activities.

|        | Hot-plate test | Tail-flick test |
|--------|----------------|-----------------|
|        | ED<sub>50</sub> (mg/kg, s.c.) (95% CL)* | ED<sub>50</sub> (mg/kg, s.c.) (95% CL)* |
| Morphine | 2.43 (1.38–4.27) | 2.43 (1.38–4.27) |
| Oxymorphone | 0.38 (0.19–0.78) | 0.38 (0.19–0.78) |
| 1        | 0.11 (0.045–0.26) | 0.11 (0.045–0.26) |
| 4        | 1.12 (0.46–2.69) | 1.12 (0.46–2.69) |
| 6        | 0.18 (0.074–0.46) | 0.18 (0.074–0.46) |

*Antinociceptive potencies determined 30 min after s.c. drug administration in mice shown as ED<sub>50</sub> values with 95% confidence limits (95% CL) (n=5–6 mice per group).

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resulted in a diversity of compounds appraised as valuable and therapeutic agents and important research tools [3,9,11,12]. Furthermore, discovery of therapeutically useful morphine-like drugs has also targeted the C-6 hydroxyl group, with oxymorphone as one example of the clinically relevant opioid analogues, where a carbonyl instead of a hydroxyl group is present at position 6 [9,59]. Taken together, in the present study we highlight on the significant outcomes of N-substituent variation in morphine and oxymorphone on <i>in vitro</i> and <i>in vivo</i> biological properties and the emerging SAR. The presented data clearly reflect that a N-phenethyl moiety in position 17 is highly favorable regarding enhanced affinity and selectivity at the MOP receptor, potent agonism and antinoceptive action. The increased lipophilicity of the N-phenethyl derivatives compared to the parent compounds may also contribute to the increased potency. Besides, it was also demonstrated that a carbonyl group at position 6 is preferable to a hydroxyl function in the N-phenethyl substituted molecules, augmenting MOP receptor affinity and agonist potency <i>in vitro</i> and <i>in vivo</i>. Though morphine derivatives, N-phenethylmorphine (1) and N-phenethyl-14-hydroxynormorphone (4), and the oxymorphone analogue N-phenethylnormorphone (6) have been developed many years ago, this is the first report on their opioid receptor binding and signaling, and antinociceptive efficacy. This report clarifies the activities of these molecules at the opioid receptors for the first time, serving as a systematic study of understanding their mode of action and the link between agonist-induced G protein signaling events leading to the high analgesic efficacy. Moreover, these results reveal that targeting position 17 is a viable approach toward improving the pharmacological properties, and may be instrumental to the development of new opioids for therapeutic use in the clinic. Considering the interesting functional profile of these MOP agonists and their high efficacy as antinociceptive agents, it is of interest to investigate other intracellular signaling pathways (i.e., interactions with regulatory proteins such as β-arrestins) and their side-effect profile in future studies.

Supporting Information

Chemistry S1

(DOCX)

Author Contributions

Conceived and designed the experiments: MS HS SB SH GC. Performed the experiments: TBH SB SH DM. Analyzed the data: TBH MS SB SH DM GC. Contributed reagents/materials/analysis tools: MS HS SB SH GC. Wrote the paper: TBH MS SB SH DM GC.
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