

Supporting Information

Synthesis, Pharmacology and Molecular Docking Studies on 6-Desoxo-*N*-Methylmorphinans as Potent μ -Opioid Receptor Agonists

Maria Dumitrascuta,[#] Tanila Ben Haddou,[#] Elena Guerrieri, Stefan M. Noha, Lea Schläfer,
Helmut Schmidhammer, and Mariana Spetea*

Department of Pharmaceutical Chemistry, Institute of Pharmacy and Center for Molecular
Biosciences Innsbruck (CMBI), University of Innsbruck, Innrain 80-82, 6020 Innsbruck, Austria

[#]These authors contributed equally to this work.

***Corresponding author:**

Dr. Mariana Spetea

Department of Pharmaceutical Chemistry, Institute of Pharmacy and Center for Molecular
Biosciences (CMBI), University of Innsbruck, Innrain 80-82, 6020 Innsbruck, Austria

Tel.: +43 512 507 58277

Fax: +43 512 507 58299

e-mail: Mariana.Spetea@uibk.ac.at

Table of Contents

S3-S5: Pharmacological methods

S6-S7: Molecular modeling methods

S8: Figure S1

S9: Figure S2

S10: Figure S3

S11: Figure S4

S12: Figure S5

S13: References

Pharmacological Methods

In Vitro Pharmacology

Materials

Cell culture media and supplements were obtained from Sigma-Aldrich Chemicals (St. Louis, MO), or Life Technologies (Carlsbad, CA). Radioligands [³H][D-Ala²,Me-Phe⁴,Gly-ol⁵]enkephalin ([³H]DAMGO, 50 Ci/mmol), [³H]diprenorphine (37 Ci/mmol), and guanosine 5'-O-(3-[³⁵S]thio)-triphosphate ([³⁵S]GTPγS, 1250 Ci/mmol) were purchased from PerkinElmer (Boston, MA). [³H]HS665 (30.65 Ci/mmol) was prepared by Dr. Geza Toth (Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary) as described.¹ Guanosine diphosphate (GDP), GTPγS and opioid ligands, DAMGO and diprenorphine were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial sources. Compounds **1-4** and **1a-4a** were prepared as 1 mM stocks in water or 0.5% in acetic acid, respectively and further diluted to working concentrations in the appropriate medium.

Cell culture

Chinese hamster ovary (CHO) cells stably expressing human opioid receptors, MOR, DOR, or KOR (CHO-hMOR, CHO-hDOR and CHO-hKOR cell lines) were kindly provided by Dr. Lawrence Toll (SRI International, Menlo Park, CA). The CHO-hMOR and CHO-hDOR cell lines were maintained in Dulbecco's Minimal Essential Medium (DMEM)/Ham's F-12 medium supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM) and geneticin (400 μg/ml). The CHO-hKOR cell line was maintained in DMEM supplemented with FBS (10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM) and geneticin (400 μg/ml). Cell cultures were maintained at 37°C in 5% CO₂ humidified air.

Radioligand binding assays for opioid receptors

Binding assays were conducted on human opioid receptors stably transfected into CHO cells according to the published procedures.^{1,2} Cell membranes from CHO-hMOR, CHO-hDOR and CHO-hKOR cells were prepared as described previously and stored at -80 °C until use.^{1,2} Protein content of cell membrane preparations was determined by the method of Bradford using bovine serum albumin as the standard.³ Binding assays were conducted using [³H]DAMGO (1 nM), [³H]diprenorphine (0.2 nM) or [³H]HS665 (0.4 nM) for labeling MOR, DOR, and KOR, respectively. Non-specific binding was determined using 1-10 μM of the unlabeled counterpart of each radioligand. Assays were performed in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 mL. Cell membranes (15-20 μg) were incubated various concentrations of test compound and the appropriate

radioligand, [³H]DAMGO or [³H]diprenorphine for 60 min at 25 °C, or [³H]HS665 for 30 min at 0 °C. After incubation, reactions were terminated by rapid filtration through Whatman glass GF/C fiber filters. Filters were washed three times with 5 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Brandel M24R cell harvester (Gaithersburg, MD). Radioactivity retained on the filters was counted by liquid scintillation counting using a Beckman Coulter LS6500 (Beckman Coulter Inc., Fullerton, CA). All experiments were performed in duplicate and repeated at least three times. The inhibitory constant K_i values (in nM) were calculated from the competition binding curves by nonlinear regression analysis and the Cheng-Prusoff equation.⁴

[³⁵S]GTPγS functional assay for opioid receptors

Binding of [³⁵S]GTPγS to membranes from CHO cells stably expressing the human MOR was conducted according to the published procedures.^{2,5} Cell membranes were prepared in Buffer A (20 mM HEPES, 10 mM MgCl₂ and 100 mM NaCl, pH 7.4). Cell membranes (8-15 μg) in Buffer A were incubated with 0.05 nM [³⁵S]GTPγS, 10 μM GDP and various concentrations of test compound in a final volume of 1 mL, for 60 min at 25 °C. Non-specific binding was determined using 10 μM GTPγS, and the basal binding was determined in the absence of test ligand. Samples are filtered over Whatman glass GF/B fiber filters and counted as described for binding assays. All experiments were performed in duplicate and repeated at least three times. In each individual experiment, the increase in [³⁵S]GTPγS binding produced by the test compounds were normalized to the maximal stimulation of to the reference MOR full agonist DAMGO, and the nonlinear regression analysis performed on each individual curve were averaged to yield potency (EC₅₀, in nM) and efficacy (as % stim) values.

***In Vivo* Pharmacology**

Animals and Drug Administration. Male CD1 mice (30-35 g, 6-8 weeks old) were obtained from the Center of Biomodels and Experimental Medicine (CBEM) (Innsbruck, Austria), or Charles River (Sulzfeld, Germany). Mice were group-housed in a temperature controlled room with a 12 h light/dark cycle and with free access to food and water. 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. Stock solutions (1 mg/mL) of compounds **1-4** and **1a-4a** were prepared in sterile physiological 0.9% saline or in 0.5% acetic acid solutions in sterile physiological 0.9% saline, respectively, and further diluted to working doses in physiological saline. Test compounds or vehicle (saline) were administered by sc route in a volume of 10 μL/1 g of body weight. Separate groups of mice received the respective dose of compound, and individual mice were only used once for behavioral testing. Each experimental group included at least five animals. The number of

experimental animals was considered as adequate for behavioral studies, permitting statistical analysis, and suitable information of the drug effect.

Hot-plate assay

The hot-plate assay was performed as described.⁵ Each mouse was placed on a UB 35100 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. Hot-plate latencies were measured before (basal latency, BL) and 30, 60 and 120 min after drug or vehicle sc administration (test latency, TL). For establishing the dose-response effect, the antinociceptive response was expressed as percent of Maximum Possible Effect (%MPE) = $[(TL - BL)/(cut-off\ time - BL)] \times 100$ for each dose tested. The dose necessary to produce a 50% MPE (ED₅₀) and 95% confidence limits (95% CL) were calculated using the method of Litchfield and Wilcoxon.⁶

Data Analysis

Experimental data were analyzed and graphically processed using the GraphPad Prism 5.0 Software (GraphPad Prism Software Inc., San Diego, CA), and are presented as means ± SEM. Data were statistically evaluated using one-way ANOVA with Tukey's *post hoc* test or two-way ANOVA for multiple comparisons, with significance set at $P < 0.05$.

Molecular Modeling Methods

Hardware and software specifications

Molecular modeling study was performed utilizing a Fujitsu CELSIUS R940 workstation, equipped with an Intel Xeon E5-2620 v3 CPU and 16 GB of RAM, and running Microsoft Windows 8.1 operating system. For the docking study, preparation of ligands was conducted using LigandScout⁷ (version 3.1) from Inte:Ligand (<http://www.inteligand.com>), and OpenEye's conformer ensemble generator OMEGA.⁸ For the assignment of partial atomic charges the toolkit QUACPAC⁹ (version 1.7.0.2) from OpenEye (<http://www.eyesopen.com>) was employed. Molecules were docked employing GOLD^{10,11} (version 5.2) from the Cambridge Crystallographic Data Centre (<http://www.ccdc.cam.ac.uk/solutions/csd-discovery/Components/Gold/>). Evaluation of docking solutions was performed within LigandScout, which was also used for visualization purposes.

Ligand preparation

First, the ligand preparation was performed with the import of the smiles codes of compounds into LigandScout, followed by checking the protonation states, along with the strain energy, as the molecules were submitted to further processing, ensuing that the three-dimensional (3D) geometry was relaxed. Next, conformational models were calculated within LigandScout (RMS distance: 0.1; maximal number of conformers: 5). Furthermore, partial atomic charges were assigned from the MMFF94,¹² by running OpenEye's toolkit QUACPAC in default mode.

Molecular docking

Molecular docking of compounds to the active structure of the MOR was conducted as recently reported,¹³ with some modifications as described. The X-ray crystal structure of the active MOR conformation was utilized (PDB accession code: 5C1M),¹⁴ accessible via the web portal of the Protein Data Bank¹⁵ (<http://www.rcsb.org/pdb/>). The amino acid residue D147 was assigned as constraint, as its relevance for the recognition of small drug-like molecules by the receptor was stressed.^{16,17} Three water molecules were included during the docking runs, i.e. HOH505, HOH526 and HOH538, accounting the results reported by Huang *et al*,¹⁴ which suggest a hydrogen-bonding network. Specifically, these water molecules are involved by mediating a polar contact from the receptor to the co-crystallized ligand, the morphinan BU72.¹⁴ Prior to docking, the preparation of the valuable and substantial 3D structure of MOR was performed within GOLD. Furthermore, the implemented consensus scoring protocol "Chemscore-GS" was employed in this study, following that the settings of the genetic algorithm (GA), a cornerstone of the docking program GOLD, were assigned to a considerably exhaustive variant (GA runs: 20; GA efficiency: 200%), as outlined elsewhere.¹⁸ In addition, the docking runs were performed with up to five conformers per ligand, and

by employing adjusted settings for the ligand flexibility, as enhanced flexibility was enabled (“flip pyramidal N”, along with “flip ring corners”). In total, five independent runs were conducted, and the three top-ranked docking solutions per molecule and conformer from each of the five runs were collected.

Evaluation

Critical non-covalent interactions between the compounds and the MOR were surmised by inferring 3D pharmacophores or pharmacophore models within LigandScout, as interesting variant to derive key findings from the retrieved poses.^{7,19}

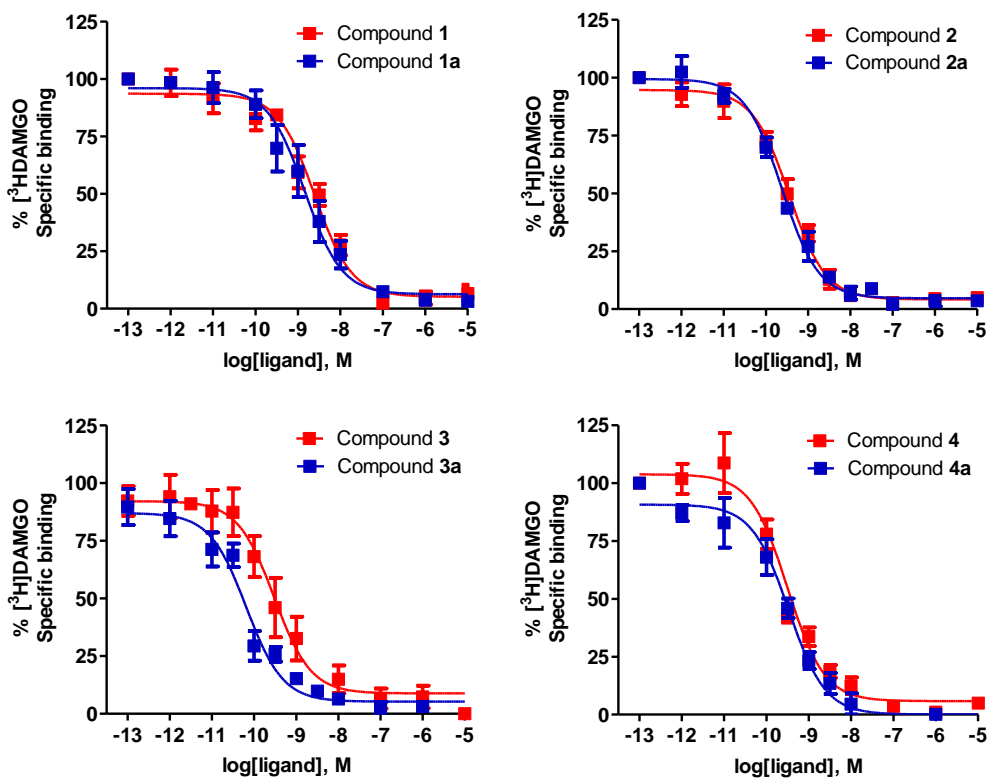


Figure S1. Binding of *N*-methylmorphinan-6-ones **1-4** and their 6-desoxy counterparts **1a-4a** to the human MOR determined using radioligand binding assays. Concentration-dependent inhibition by test compounds of $[^3\text{H}]\text{DAMGO}$ binding to CHO-hMOR cell membranes. Values are expressed as the mean \pm SEM ($n \geq 3$).

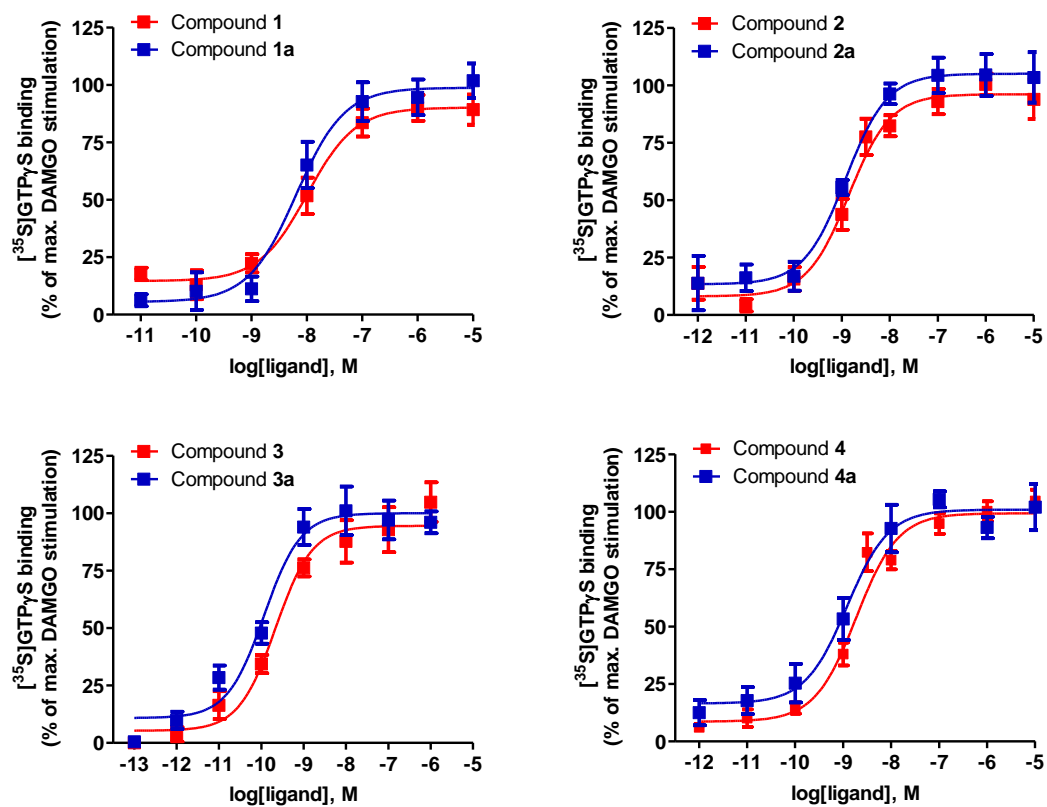


Figure S2. *In vitro* agonist activities at the human MOR of *N*-methylmorphinan-6-ones **1-4** and their 6-desoxo counterparts **1a-4a**. Stimulation of [³⁵S]GTP_γS binding by test compounds determined in the [³⁵S]GTP_γS binding assay using CHO-hMOR cell membranes. Values are expressed as the mean ± SEM (*n* ≥ 3).

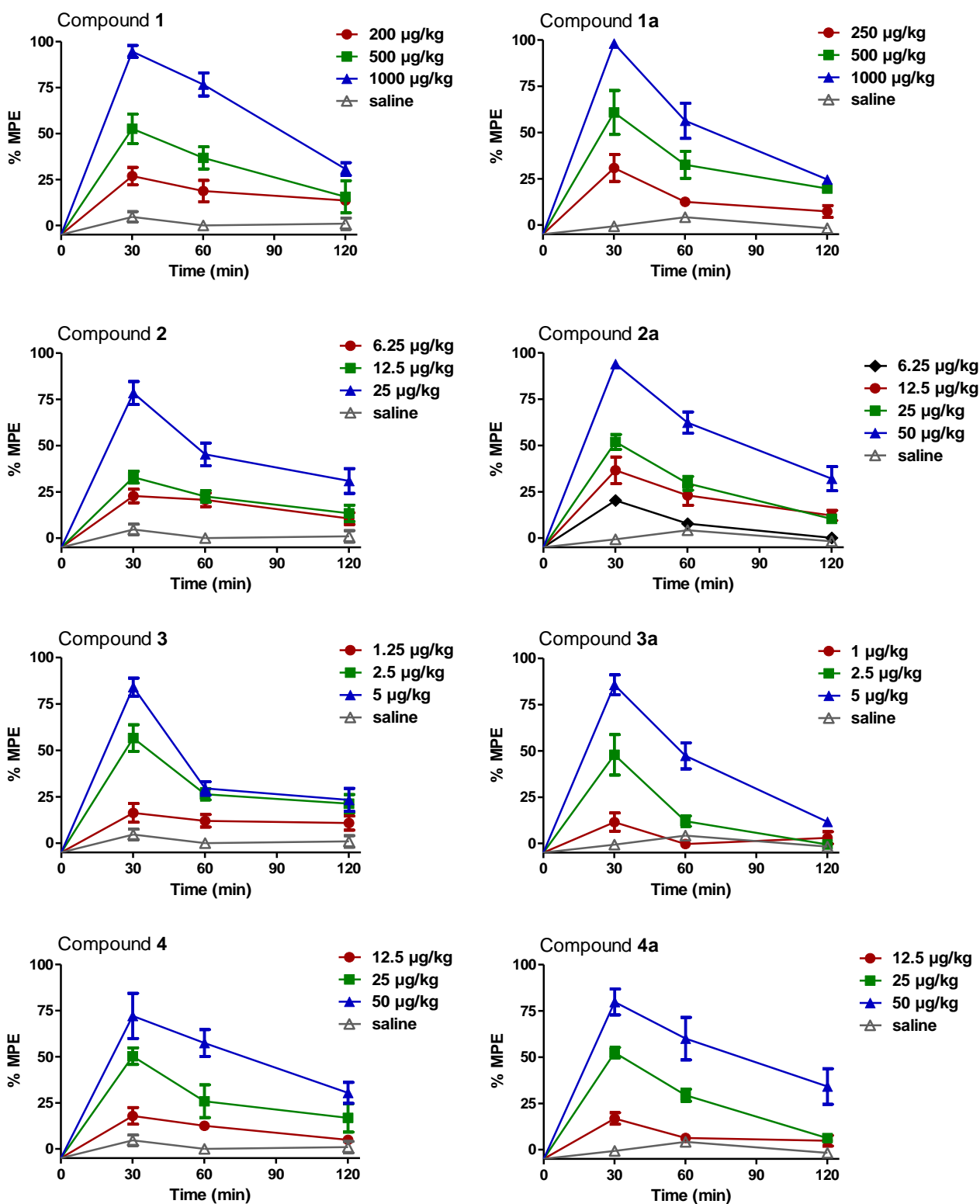


Figure S3. Time-dependent antinociceptive effects of *N*-methylmorphinan-6-ones 1-4 and their 6-desoxo counterparts 1a-4a in the hot-plate assay in mice after sc administration. Data are shown as mean %MPE \pm SEM ($n = 5-6$ mice per group).

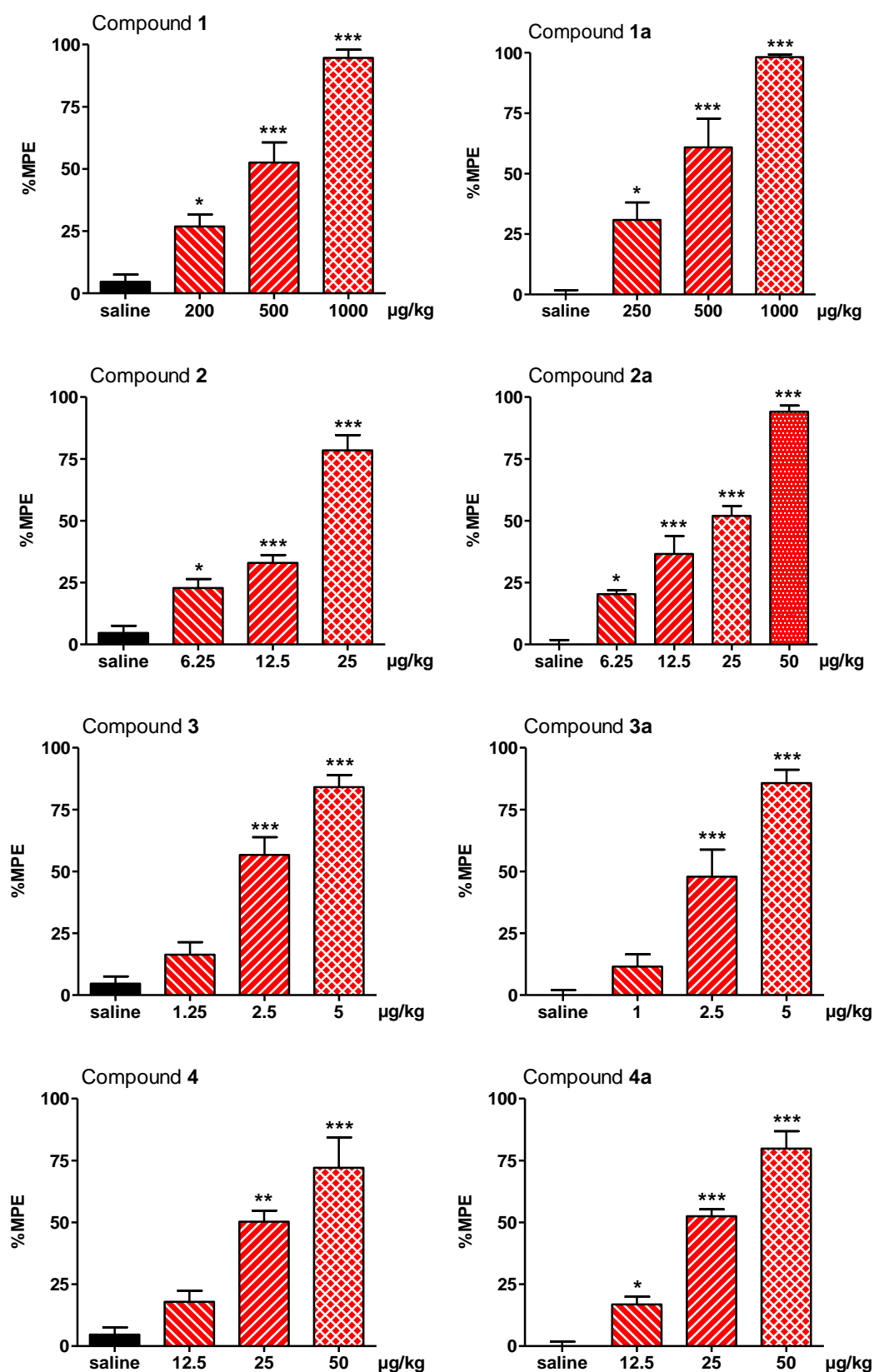


Figure S4. Dose-dependent antinociceptive effects of *N*-methylmorphinan-6-ones **1-4** and their 6-desoxo counterparts **1a-4a** in the hot-plate assay in mice at the peak of action (30 min) after sc administration. Data are shown as mean %MPE \pm SEM ($n = 5-6$ mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control (saline) group; one-way ANOVA with Tukey's *post hoc* test.

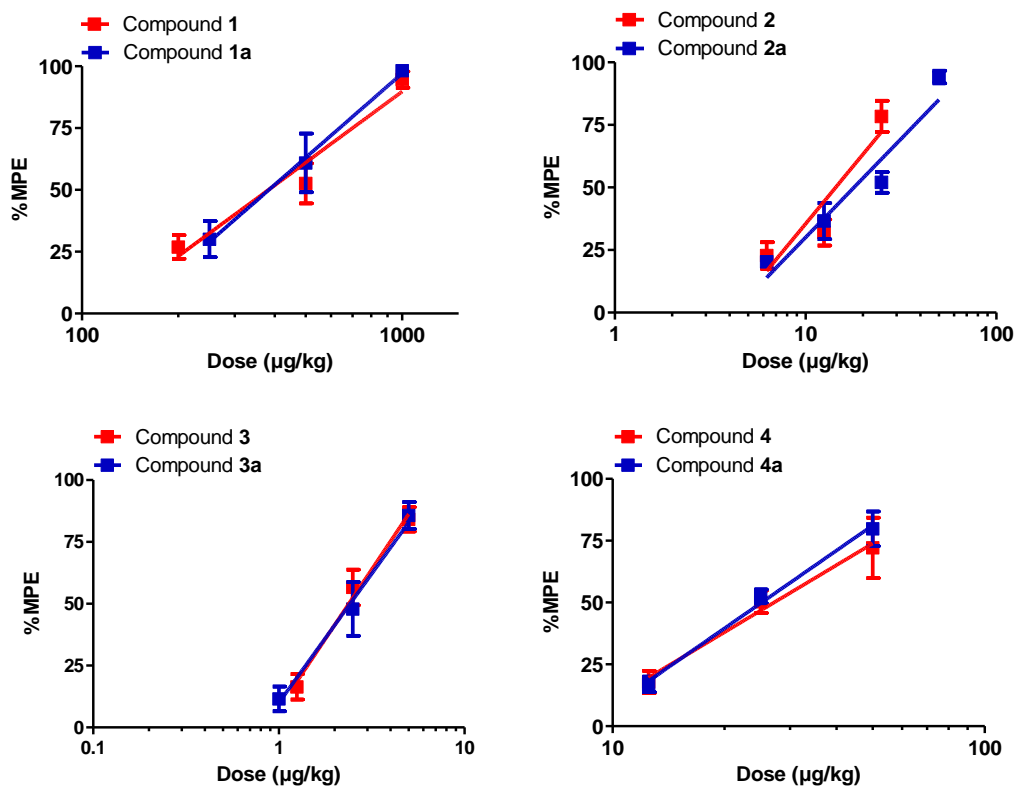


Figure S5. Comparison of dose-dependent antinociceptive effects of *N*-methylmorphinan-6-ones **1-4** and their 6-desoxo counterparts **1a-4a** in the hot-plate assay in mice at 30 min after sc administration. Data are shown as mean %MPE \pm SEM ($n = 5-6$ mice per group). $P > 0.05$; $F_{(2,24)} = 47.01$ for **1** vs **1a**, $F_{(2,31)} = 77.78$ for **2** vs **2a**, $F_{(2,28)} = 44.19$ for **3** vs **3a**, and $F_{(2,24)} = 40.46$ for **4** vs **4a**, two-way ANOVA.

References

1. Guerrieri, E.; Mallareddy, J. R.; Tóth, G.; Schmidhammer, H.; Spetea, M. Synthesis and Pharmacological Evaluation of [³H]HS665, a Novel, Highly Selective Radioligand for the Kappa Opioid Receptor. *ACS Chem. Neurosci.* **2015**, *6*, 456–463.
2. Spetea, M.; Berzetei-Gurske, I. P.; Guerrieri, E.; Schmidhammer, H. Discovery and Pharmacological Evaluation of a Diphenethylamine Derivative (HS665), a Highly Potent and Selective κ Opioid Receptor Agonist. *J. Med. Chem.* **2012**, *55*, 10302–10306.
3. Bradford, M. M. A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254.
4. Cheng, Y.; Prusoff, W. H. Relationship Between the Inhibition Constant (K₁) and the Concentration of Inhibitor Which Causes 50 Per Cent Inhibition (I₅₀) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.
5. Ben Haddou, T.; Malfacini, D.; Calo, G.; Aceto, M. D.; Harris, L. S.; Traynor, J. R.; Coop, A.; Schmidhammer, H.; Spetea, M. Exploring Pharmacological Activities and Signaling of Morphinans Substituted in Position 6 as Potent Agonists Interacting with the μ Opioid Receptor. *Mol. Pain* **2014**, *10*, 48.
6. Litchfield, J. T. Jr.; Wilcoxon, F. A Simplified Method of Evaluating Dose-effect Experiments. *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99–113.
7. Wolber, G.; Langer, T. LigandScout: 3-D Pharmacophores Derived from Protein-Bound Ligands and Their Use as Virtual Screening Filters. *J. Chem. Inf. Model.* **2005**, *45*, 160–169.
8. Hawkins, P. C.; Skillman, A. G.; Warren, G. L.; Ellingson, B. A.; Stahl, M. T. Conformer Generation with OMEGA: Algorithm and Validation using High Quality Structures from the Protein Databank and Cambridge Structural Database. *J. Chem. Inf. Model.* **2010**, *50*, 572–584.
9. QUACPAC 1.7.0.2: OpenEye Scientific Software, Santa Fe, NM. <http://www.eyesopen.com>.
10. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and Validation of a Genetic Algorithm for Flexible Docking. *J. Mol. Biol.* **1997**, *267*, 727–748.
11. Verdonk, M. L.; Chessari, G.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Nissink, J. W.; Taylor, R. D.; Taylor, R. Modeling Water Molecules in Protein-Ligand Docking using GOLD. *J. Med. Chem.* **2005**, *48*, 6504–6515.
12. Halgren, T. A. Merck Molecular Force Field. II. MMFF94 van der Waals and Electrostatic Parameters for Intermolecular Interactions. *J. Comput. Chem.* **1996**, *17*, 520–552.
13. Noha, S. M.; Schmidhammer, H.; Spetea, M. Molecular Docking, Molecular Dynamics and Structure-Activity Relationship Explorations of 14-Oxygenated *N*-Methylmorphinan-6-ones as Potent μ -Opioid Receptor Agonists. *ACS Chem. Neurosci.* **2017**, *8*, 1327–1337.
14. Huang, W.; Manglik, A.; Venkatakrisnan, A. J.; Laeremans, T.; Feinberg, E. N.; Sanborn, A. L.; Kato, H. E.; Livingston, K. E.; Thorsen, T. S.; Kling, R. C.; Granier, S.; Gmeiner, P.; Husbands, S. M.; Traynor, J. R.; Weis, W. I.; Steyaert, J.; Dror, R. O.; Kobilka, B. K. Structural Insights into μ -Opioid Receptor Activation. *Nature* **2015**, *524*, 315–321.

15. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235–242.
16. Henriksen, G.; Platzer, S.; Marton, J.; Hauser, A.; Berthele, A.; Schwaiger, M.; Marinelli, L.; Lavecchia, A.; Novellino, E.; Wester, H. J. Syntheses, Biological Evaluation, and Molecular Modeling of ¹⁸F-Labeled 4-Anilidopiperidines as μ -Opioid Receptor Imaging Agents. *J. Med. Chem.* **2005**, *48*, 7720–7732.
17. Dosen-Micovic, L.; Ivanovic, M.; Micovic, V. Steric Interactions and the Activity of Fentanyl Analogs at the μ -Opioid Receptor. *Bioorg. Med. Chem.* **2006**, *14*, 2887–2895.
18. Verdonk, M. L.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Taylor, R. D. Improved Protein-Ligand Docking using GOLD. *Proteins* **2003**, *52*, 609–623.
19. Wallach, I. Pharmacophore Inference and its Application to Computational Drug Discovery. *Drug Develop. Res.* **2011**, *72*, 17–25.