Peripheral and central analgesic components of a novel opioid in the management of inflammatory and neuropathic pain

Doctoral theses

Mihály Balogh Pharm.D.

Semmelweis University
Doctoral School of Pharmaceutical Sciences

Supervisor: Mahmoud Al-Khrasani, Pharm.D., Ph.D.

Official reviewers:
Éva Keller, M.D., Ph.D.
Ildikó Világi, Ph.D.

Head of the Final Examination Committee:
Éva Szökő, Pharm.D., D.Sc.

Members of the Final Examination Committee:
Gábor Pethő, M.D., D.Sc.
Ákos Zsembery, M.D., Ph.D.

Budapest
2018

1. Introduction

Opioid analgesics are among the oldest pain medications applied by human beings, yet they are still the mainstay in the management of moderate to severe pain. On the other hand, the majority of clinically used opioid analgesics have central adverse effects such as respiratory depression, development of opioid tolerance and dependence, as well as addiction liabilities. More important, when they are misused or abused, they can cause addiction, overdose and death. These effects hamper their clinical use.

Beside the central opioid analgesic effect, several data support that antinociception could also be achieved by the activation of functional opioid receptors in the periphery as well. Opioid receptors have been reported to be upregulated in inflamed tissues as well as at the spinal level. This favorable change might offer a possibility to treat inflammatory pain by targeting peripheral opioid receptors. The clinical practice still lacks peripherally acting opioid analgesic agents that have proper efficacy, favorable side effect profiles and duration of action.

Another common pain disorder affecting patients is neuropathic pain (NP). Less than 50% of neuropathic patients respond to existing treatments and 40% are inadequately treated. Opioids are considered as second line medication for neuropathic pain. Treatment of NP so far is considered as an unmet medical need and indicates necessity for developing new therapies for these types of painful conditions. Opioid receptor number decreases in

neuropathic conditions, therefore opioid analogs of high efficacy might offer proper analgesia.

2. Objectives

1. To assess the antinociceptive efficacy and potency of the novel compound 14-*O*-methylmorphine-6-*O*-sulphate (14-*O*-MeM6SU) in comparison with known reference compounds (morphine or morphine-6-*O*-sulphate; M6SU) in:

- acute and subchronic inflammatory pain conditions: mouse writhing test, rat formalin test, rat CFA model
- neuropathic pain conditions: rat model of diabetic polyneuropathy

2. To determine the degree of antinociception impairment in advanced diabetic neuropathy in rats

3. To investigate the peripheral component in the antinociceptive action of test compounds

4. To further analyze the actions of test opioid agonists at the spinal and supraspinal level in NP conditions applying biochemical assays (G-protein activity assay)

5. To investigate the side effect profile of 14-*O*-MeM6SU in comparison with reference compounds by analyzing the:

- effects on gastrointestinal transit in mice
- effects on respiratory functions in rats
- sedative effects in rats
- tolerance profile in mice

3. Materials and methods

3.1. Animals

Animals (male Wistar rats or NMRI mice) were housed in the local animal house of the Department of Pharmacology and Pharmacotherapy, Semmelweis University (Budapest, Hungary). Housing and experiments were performed according to the European Communities Council Directives (2010/63/EU), (86/609/ECC), German science-based guidelines for laboratory animal care of the National Research Council (2003), the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§) and local animal care committee (PEI/001/276-4/2013). Animals were kept in standard cage (5 or 6 animals/cage) in a room of 20 ± 2°C temperature, 12-hour/12-hour light/dark cycle (light on at 6 A.M.). Diabetic and their control animals were kept in mash bottomed cage. Water and standard food were available *ad libitum*.

3.2. Materials

The novel compound (14-*O*-MeM6SU) and reference compound M6SU used in the present work were synthesized by Sándor Hosztafi in Department of Pharmaceutical Chemistry, Semmelweis University (Budapest, Hungary). All compounds were of analytical grade and purchased from standard commercial source. Drugs were dissolved in 0.9% solution of NaCl with the exception of streptozocin (STZ), which was dissolved in ice-cold distilled water right before injection (less than 10 min before injection). Naloxone methiodide (NAL-M) was dissolved in saline, also right before the experiment.

Experiments were performed in a blinded way to the drugs and doses applied.

3.3. Inflammatory pain models

3.3.1. Acute inflammatory models

In **mouse writhing test** male NMRI mice (20-30 g) were injected i.p. with 0.2 ml of 0.6% acetic acid aqueous solution to induce the writhing reactions (contractions of the abdominal musculature and extension of the hind limbs). 5 min after acetic acid injection the number of writhes was counted during a 10 min observation period. Groups of mice were injected subcutaneously (s.c.) or intracerebroventricularly (i.c.v.) with different doses of 14-*O*-MeM6SU or M6SU 15 min before acetic acid injection.

In **rat formalin test** male Wistar rats (200-300 g) were used. 2.5% formalin solution was injected into the plantar surface of the right hind paw in a volume of 50 µl/rat. Immediately following the intraplantar (i.pl.) formalin injection, animals were placed into Plexiglass observation chambers. Then, the number of nociceptive behaviors (shaking, flinching, licking and elevating the painful paw) were counted for 60 min of 5 min time periods. The observation peridod was subdivided to two phases: Phase I: 0-10 min (caused by the irritant effect of formalin) and Phase II: 11-60 min (caused by the release of inflammatory mediators) to determine the pain events. The test compounds (14-*O*-MeM6SU and morphine) were injected s.c. (2.5 ml/kg) 15 min prior to formalin injection. In another set of experiments, the test compounds were injected intraplantarly into the ipsilateral (formalin

treated) or contralateral paw (100 µl/animal) 5 min prior to the i.pl. formalin injection.

3.3.2. Subchronic inflammatory model: CFA model

Male Wistar rats (200–300 g) were used. Animals received i.pl. injection of 0.15 ml Complete Freund's Adjuvant (CFA), a water-in-oil emulsion of killed mycobacteria, into the right hind paw. On the fourth and seventh day after i.pl. CFA-injection, baseline (pre-test compound) paw pressure thresholds (PPT) of inflamed and non-inflamed paws were assessed by paw pressure algesiometry (modified Randall-Selitto test). Then, PPTs were reevaluated at 30, 60 and 120 min after s.c. drug administration, using an arbitrary cut off weight of twice the baseline. The cut off time was considered 100% and values are expressed as percentages. In these experiments the antinociceptive effects of s.c. 14-*O*-MeM6SU and M6SU were examined.

3.4. Neuropathic pain model: advanced diabetic neuropathy in rats

Male Wistar rats of 200-300 g were used for STZ induced diabetes model. Diabetes was provoked by i.p. administration of 60 mg/kg streptozocin (STZ) in a 2.5 ml/kg volume. Vehicle treated group was used as absolute control. The blood glucose level, the weight change, the water- and food consumption of the animals were checked at numerous time points.

To justify the alteration in mechanical pain thresholds caused by the difference in the weights of diabetic (STZ treated) and non-diabetic (vehicle treated) animals (i.e.

higher weight results in higher threshold values), weight match animals were used as a control. Weight match animals were handled and kept under the same conditions described for the diabetic (and non-diabetic control) animals. The exception was that, weight match animals were kept only for one or two weeks prior to experiments. In order to determine the allodynia caused by advanced diabetes we used the Dynamic Plantar Aesthesiometer (DPA). The equipment raises a straight metal filament with a 0.5 mm diameter until it touches the paw. Then it puts pressure on the paw with an increasing force from 1 to 50 grams (cut off). In the first series of experiments the animals were measured every week after STZ treatment in order to determine the peak of allodynia. An animal was considered neuropathic, when the threshold value was decreased at least by 20% compared to weight match animals.

On the 9^{th} week after STZ treatment (peak of allodynia) dose-response curves were constructed for 14-*O*-MeM6SU and morphine. To analyze the changes in the antinociceptive potencies of test compounds the calculated ED_{30} values were compared ($ED_{30diabetic}/ED30_{non\text{-}diabetic}$).

3.5. Investigation of the peripheral component in the antinociceptive effect of test compounds

In order to assess the peripheral opioid system's contribution to the whole antinociceptive action of systemically applied opioids NAL-M was co-administered. NAL-M is a quaternary opioid antagonist that does not penetrate into the central nervous system

(CNS) in the applied dose. In rat CFA model NAL-M was applied also locally (into the inflamed paw).

3.6. *In vitro* GTP activity assay

In [^{35}S]GTPγS binding experiments the GDP→GTP exchange of the $G\alpha_{i/o}$ protein is measured by a radioactive, non-hydrolysable GTP analog, [^{35}S]GTPγS. Experiments were performed in diabetic (STZ treated) and non-diabetic (vehicle treated) brain and spinal cord homogenates 9 and 12 weeks after STZ treatment. The radioactivity of the specifically bound radioligands was determined by liquid scintillation spectrophotometry.

3.7. Assessment of side effects of test compounds

The effect of 14-*O*-MeM6SU and M6SU compared to that of morphine on **gastrointestinal transit** was measured *in vivo*, using the charcoal meal method in male NMRI mice (20-25 g). 30 minutes after drug or saline administration mice were decapitated, their small intestines were removed, and the distance travelled by the charcoal suspension was expressed as a percentage of total small intestine length.

Respiratory function measurements were performed by unrestrained whole-body plethysmography (WBP) in conscious, spontaneously breathing male Wistar rats (200-300 g).

To assess the **sedative (i.e. anaesthesia potentiating) effect** of test compounds righting reflex method was applied in male Wistar rats (200-300 g). Animals received i.v. saline or thiobutabarbital (153 μmol/kg), then were placed on the left side. The sleeping time in minutes was

documented. Anesthesia-potentiating effects of test drugs were studied by their s.c. administration. In another set of experiments, sleeping time was induced by inhaled 3% isoflurane. In both cases the anesthetics were administered at the time of peak effect of the investigated agonist. Animals were immediately placed sideways on a pillow of 30 °C. The sleeping time (righting reflex, when the animals turned back on all four legs) was determined.

Analgesic tolerance was induced in male NMRI mice with 3 days, twice daily agonist treatment (14-*O*-MeM6SU or morphine). Radiant heat tail flick test was used to assess antinociceptive effect of test compounds. The latencies, when mice flicked their tail, were expressed in seconds. Cut-off time was set to 6 s to avoid tissue damage. Dose response curves for each drug were determined on the fourth day, following three days of chronic s.c. saline or drug treatments. Dose response curves for morphine and 14-*O*-MeM6SU were constructed in morphine and 14-*O*-MeM6SU treated mice.

3.8. Analysis of data

All the analysis was performed with a professional statistical software: GraphPad Prism version 5.00 or 6.00 for Windows. For the comparison of more than 2 groups one- or two-way ANOVA was applied with post hoc test based on the experimental protocol. Vehicle treated groups were used as control in order to decide if the applied treatment significantly influenced the parameters. The results were considered to be statistically significant when $p < 0.05$.

4. Results

4.1. Inflammatory pain models

In both acute (mouse writhing test, rat formalin test) and subchronic (rat CFA model) models 14-*O*-MeM6SU proved to have higher analgesic potency than morphine or M6SU.

14-*O*-MeM6SU produced more potent inhibitory effect than M6SU on acetic acid-induced **writhing in mice**: 23 and 5 fold more potent than M6SU after s.c. and i.c.v. administrations, respectively. Large s.c./i.c.v. potency ratio was calculated for M6SU or 14-*O*-MeM6SU, indicating limited CNS penetration. Co-administration of the peripherally acting NAL-M antagonized the antinociceptive effect of test opioids.

Subcutaneous 14-*O*-MeM6SU or morphine attenuated the **formalin-induced pain** in a dose-dependent manner. Based on s.c. equianalgesic doses, systemic 14-*O*-MeM6SU was approx. 15 and 31 more potent than morphine in phase I and II, respectively. After local administration 14-*O*-MeM6SU was approx. 77 and 38 more potent than morphine in phase I and II, respectively. NAL-M abolished the antinociceptive effect of s.c. 506 nmol/kg 14-*O*-MeM6SU in both phases. On the other hand, NAL-M only partially affected the antinociceptive actions of morphine or the higher doses of 14-*O*-MeM6SU. In contrast to morphine, intraplantar (i.pl.) administration of 14-*O*-MeM6SU into contralateral paw failed to affect formalin-induced pain in ipsilateral paw in either phases. These data indicate, that 14-*O*-MeM6SU but

not morphine, shows peripheral antinociception in a certain dose range.

In **rat CFA model** the antinociceptive actions of lower 14-*O*-MeM6SU or M6SU doses were significantly more marked in inflamed paws compared to non-inflamed paws. Co-administered and also locally administered (into the CFA treated paw) NAL-M antagonized the antinociceptive effect of these doses. Again, these results indicate the peripheral effect of test compounds in a certain dose range.

4.2. Neuropathic pain model: advanced diabetic neuropathy in rats

Diabetic animals (blood glucose level: 14 mmol/l <) consumed significantly more water and food, yet gained significantly less weight than non-diabetic ones.

Significant reduction in paw withdrawal thresholds of diabetic animals was observed 3 weeks after STZ treatment, indicating the development of allodynia. The peak of allodynia was achieved at the 9^{th} week, therefore further experiments were performed at the 9^{th} and 12^{th} week.

Based on dose-response curves obtained in diabetic and non-diabetic rats, 7 times and no reduction in the antinociception of morphine and 14-*O*-MeM6SU, was observed, respectively. 14-*O*-MeM6SU displayed a 48 times higher potency than morphine under diabetic conditions. Co-administered NAL-M didn't affect the antiallodynic effect of test compounds, indicating the contribution of central opioid receptors. At the 12^{th} week

both compounds produced antiallodynic effects in accordance with 9th week data at the same doses.

4.3. *In vitro* GTP activity assay

14-*O*-MeM6SU produced similar G-protein coupling in spinal cord tissues obtained from diabetic and non-diabetic rats after 9 or 12 weeks of STZ and vehicle treatment, respectively. On the other hand, morphine showed significantly reduced efficacy (E_{max}) of G-protein coupling in spinal cord tissues of diabetic rats.

Neither compounds showed significant differences in maximal efficacy (E_{max}) and ligand potency (EC_{50}) between brain samples of diabetic and non-diabetic rats.

4.4. Side effects of test compounds

S.c. administered 14-*O*-MeM6SU, M6SU and morphine inhibited the **gastrointestinal transit** of charcoal in a dose-dependent manner.

None of the **respiratory parameters** determined by unrestrained WBP showed significant differences between the saline-treated control or drug-treated groups in investigated doses.

NAL-M reversible antinociceptive doses of 14-*O*-MeM6SU or M6SU failed to potentiate the **sleeping time** induced by thiobutabarbital. However, at higher doses both compounds lengthened the sleeping time. 14-*O*-MeM6SU in contrast to morphine in some analgesic doses also failed to prolong the sleeping time of isoflurane.

Tolerance profile of 14-*O*-MeM6SU and morphine were investigated with mouse tail-flick assay following 3 days twice daily treatment. In this test 14-*O*-MeM6SU was 17

times more potent in analgesic action than morphine. Treatment for 3 days with morphine resulted in a 3.41− and a 2.02-fold decrease of the antinociceptive effect of morphine and 14-*O*-MeM6SU, respectively. Treatment with s.c. 14-*O*-MeM6SU resulted in a 5.86- and 3.34-fold decrease in the antinociceptive effect of morphine and 14-*O*-MeM6SU, respectively. 14-*O*-MeM6SU showed promising analgesia either in morphine or 14-*O*-MeM6SU chronically treated mice compared to morphine.

Summarizing, in terms of sedative- and tolerance inducing effects 14-*O*-MeM6SU showed a more favorable profile, whereas in the case of gastrointestinal and respiratory effects no significant differences were shown in comparison with reference compounds.

5. Conclusions

1. The novel compound, **14-*O*-MeM6SU proved to have higher potency and efficacy** in acute and subchronic inflammatory pain models and also in the model of advanced diabetic neuropathy.

2. Advanced **diabetic neuropathy** results in a significant reduction in the antiallodynic effects of partial agonists like morphine in contrast to 14-*O*-MeM6SU, the opioid agonist with high efficacy.

3. **The peripheral opioid antinociception is achievable** when the pain associated with acute or subchronic **inflammation,** even after systemic administration. However, proper dose titration is fundamental. Systemic administration could offer a future

tool to avoid the risk of infections and physical damages following local injection of opioids.
In **neuropathic conditions** the role of CNS seems to be essential.

4. Significant attenuation of **G-protein activation** by morphine but not 14-*O*-MeM6SU at the level of spinal cord, key traffic point in the pain transmission was observed. This tendency might mirror further advantage for the novel compound over morphine. Finally, the reduction of opioid analgesia in the management of advanced painful diabetes may be circumvent by using high efficacy opioids, such as 14-*O*-MeM6SU, which provide superior analgesic effect over morphine.

5. In terms of **side effects** 14-*O*-MeM6SU showed a more favorable profile compared to reference compounds regarding sedative (anesthesia potentiating) and tolerance inducing effects.

In different pain conditions different opioids and different treatment protocols are necessary to be applied since the efficacy of different opioids is not just physicochemical property- but also pain type dependent.
Our results indicate that 14-*O*-MeM6SU and similar compounds might be of high clinical value.

6. List of own publications

6.1. Own publications involved in the present thesis

1. Kiraly, K., Caputi, F., Hanuska, A., Kató, E., **Balogh, M.**, Köles, L., Palmisano, M., Riba, P., Hosztafi, S., Romualdi, P., Candeletti, S., Ferdinandy, P., Fürst, S., Al-Khrasani, M., 2015. A new potent analgesic agent with reduced liability to produce morphine tolerance. Brain Res. Bull. 117, 32–38. doi:10.1016/j.brainresbull.2015.07.005 (IF: 2.572)

2. Lacko, E., Riba, P., Giricz, Z., Varadi, A., Cornic, L., **Balogh, M**., Kiraly, K., Csekő, K., Mousa, S.A., Hosztafi, S., Schafer, M., Zadori, Z.S., Helyes, Z., Ferdinandy, P., Fürst, 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. doi:10.1124/jpet.116.233551 (IF: 3.867)

3. **Balogh, M.**, Zoltán, Zádori S., Lázár, B., Karádi, D., László, S., Shaaban, Mousa, A., Hosztafi, S., Zádor, F., Riba, P., Schäfer, M., Fürst, S., Al-Khrasani, M., 2018. The Peripheral Versus Central Antinociception of a Novel Opioid Agonist: Acute Inflammatory Pain in Rats. Neurochem. Res. 0. doi:10.1007/s11064-018-2542-7 (IF: 2.581)

6.2. Own publications not involved in the present thesis

1. Zádor, F., **Balogh, M.**, Váradi, A., Zádori, Z.S., Király, K., Szűcs, E., Varga, B., Lázár, B., Hosztafi, S., Riba, P., Benyhe, S., Fürst, S., Al-Khrasani, M., 2017. 14-O-

Methylmorphine: A Novel Selective Mu-Opioid Receptor Agonist with High Efficacy and Affinity. Eur. J. Pharmacol. 814, 264–273. doi:10.1016/j.ejphar.2017.08.034 (IF: 2.896)

2. Végh, D., Somogyi, A., Bányai, D., Lakatos, M., **Balogh, M.**, Al-Khrasani, M., Fürst, S., Vizi, E.S., Hermann, P., 2017. Effects of articaine on [3H]noradrenaline release from cortical and spinal cord slices prepared from normal and streptozotocin-induced diabetic rats and compared to lidocaine. Brain Res. Bull. 135, 157–162. doi:10.1016/j.brainresbull.2017.10.011 (IF: 3.033)

3. Fehér, Á., Tóth, V.E., Al-Khrasani, M., **Balogh, M**., Lázár, B., Helyes, Z., Gyires, K., Zádori, Z.S., 2017. Analysing the effect of I1 imidazoline receptor ligands on DSS-induced acute colitis in mice. Inflammopharmacology 25, 107–118. doi:10.1007/s10787-016-0299-7 (IF: 2.59)

4. Erdei, A.I., Borbély, A., Magyar, A., Taricska, N., Perczel, A., Zsíros, O., Garab, G., Szűcs, E., Ötvös, F., Zádor, F., **Balogh, M**., Al-Khrasani, M., Benyhe, S., 2018. Biochemical and pharmacological characterization of three opioid-nociceptin hybrid peptide ligands reveals substantially differing modes of their actions. Peptides 99, 205–216. doi:10.1016/j.peptides.2017.10.005 (IF: 2.778)

5. Zádor, F., Király, K., Váradi, A., **Balogh, M**., Fehér, Á., Kocsis, D., Erdei, A.I., Lackó, E., Zádori, Z.S., Hosztafi, S., Noszál, B., Riba, P., Benyhe, S., Fürst, S., Al-Khrasani, M., 2017. New opioid receptor antagonist: Naltrexone-14-O-sulfate synthesis and pharmacology. Eur. J. Pharmacol. 809, 111–121. doi:10.1016/j.ejphar.2017.05.024 (IF: 2.896)

6. **Balogh, M.,** Varga, B. K., Karádi, D. Á., Riba, P., Puskár, Z., Kozsurek, Al-Khrasani, M Király, K. (2019). Similarity and dissimilarity in antinociceptive effects of dipeptidyl-peptidase 4 inhibitors, Diprotin A and vildagliptin in rat inflammatory pain models following spinal administration. Brain Res. Bull., 147, 78-85. (IF: 3.44)

7. Lázár, B., Brenner, G. B., Makkos, A., **Balogh, M.,** László, S. B., Al-Khrasani, M., , László, T., Tiszlavicz L., Bihari, Z., Giricz, Z., Szabó, D., Helyes, Z., Ferdinandy, P., Gyires, K., Zádori, Z. S. (2019). Lack of Small Intestinal Dysbiosis Following Long-Term Selective Inhibition of Cyclooxygenase-2 by Rofecoxib in the Rat. Cells, 8(3), 251. (IF: 4.829)

8. **Balogh, M.**, Zádor, F., Zádori, Z. S., Mohammed, S., Király, K., Mohammadzadeh, A., Riba, P., Benyhe, S., Gyires, K., Schäfer, M., Fürst, S., Al-Khrasani, M. (2019). Efficacy-based perspective to overcome reduced opioid analgesia of advanced painful diabetic neuropathy in rats. Frontiers in Pharmacology, 10, 347. (IF: 3.831)