DOI:10.14753/SE.2019.2219

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

Ph.D. thesis

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., Ph.D.
Ákos Zsembery, M.D., Ph.D.

Budapest
2018

DOI:10.14753/SE.2019.2219

Table of contents

DOI:10.14753/SE.2019.2219

DOI:10.14753/SE.2019.2219

List of abbreviations

14-*O*-MeM6SU: 14-*O*-methylmorphine-6-*O*-sulfate

ACC: anterior cingular cortex

ANOVA: analysis of variance

B_{max}: total density (concentration) of receptors in a sample of tissue

cAMP: cyclic adenosine monophosphate

CFA: complete Freund's Adjuvant

CNS: central nervous system

DAMGO: [D-Ala^2,N-Me-Phe^4,Gly-ol^5] enkephalin

DNP: diabetic neuropathic pain

DOR: δ-opioid receptor

DPA: Dynamic Plantar Aesthesiometer

DRG: dorsal root ganglion

EC_{50}: The concentration of an agonist that produces 50% of the maximal biological effect

ED_{30}: antinociceptive dose necessary to produce a 30% response

ED_{50}: antinociceptive dose necessary to produce a 50% response

E_{max}: maximal biological effect

f: frequency

GABA: γ-aminobutyric acid

GDP: guanosine-5'-diphosphate

GPCRs: G protein-coupled receptors

GTP: Guanosine-5'-triphosphate

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

i.pl.: intraplantar

i.v.: intravenous

IASP: International Association for the Study of Pain

ID_{50}: doses caused 50% inhibition

K_d: equilibrium dissociation constant

KOR: κ-opioid receptor

M6G: morphine-6-glucuronide

M6SU: Morphine-6-*O*-sulfate

MOR: μ-opioid receptor

MPE: maximal possible effect

MV: minute ventilation

NAL-M: naloxone methiodide

NP: neuropathic pain

NSAIDs: non-steroidal anti-inflammatory drugs

PAG: periaqueductal gray

PBS: phosphate buffered saline

PEF: peak expiratory flow

PIF: peak inspiratory time

PPT: paw pressure threshold

RT: relaxation time

RVM: rostral ventromedial medulla

s.c.: subcutaneous

SEM: standard error of mean

STZ: streptozocin (streptozotocin)

Te: time of expiration

Ti: time of inspiration

TV: tidal volume

VLO: ventrolateral orbital cortex

WBP: whole-body plethysmography

DOI:10.14753/SE.2019.2219

1. Introduction

1.1. Opioids and analgesia

Pain is described by Albert Schweitzer as a more terrible lord of mankind than even death itself. His thoughts are still apposite. The clinical practice has huge successes in pain management but still lacks the proper solution in many cases; mostly chronic painful conditions are still a huge challenge. Only in the USA approximately 100 million people are suffering from some type of chronic painful condition [1].

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 [2].

The use of opioids is going back for a long time, ancient Roman and Greek physicians described them as a powerful tool to relief pain. The start point in opioid history is the cultivation of poppy by Sumerians who described it as Hul Gil, the „joy plant" in lower Mesopotamia around 3400 B.C. Then knowledge of poppy cultivation and its euphoric effect spread to area governed by Assyrians, who passed it to Egypt and later to China. The first opioid agent morphine was isolated by Friedrich Wilhelm Sertürner in the 19th century. At this time morphine became available to treat diverse types of occasional pain (e.g. pain originated from injuries, toothache etc.). In the same century Alexander Wood invented the hypodermic syringe for medical use, which resulted in the growing use of opioids leading to an increase in the incidence of morphine addiction especially among soldiers in the USA and Europe. As an attempt to overcome the unwanted dependence causing effect of morphine, heroin (diacetylmorphine) was developed. The new compound was widely used as an anti-cough medication. Despite the goal for which heroin was introduced, its use as favorite habit was grown. This led to a strong increase of opioid addiction in society and encouraged authorities to issue the opioid control act [3–5]. Up to this date the clinical practice still lacks an opioid compound with proper efficacy but without the unwanted central nervous system (CNS) effects like addiction or respiratory depression.

Opioid analgesics are well known to exert their antinociceptive action by the activation of opioid receptors, particularly μ-opioid receptor (MOR) at spinal and suprasinal regions [6]. MOR activation by opioids results in analgesic effect as well as adverse effects. 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 [2, 6, 7, 8]. It is important to note that the abuse causing effect of opioids is less when they are prescribed for chronic pain management, especially when there is no history of previous drug abuse [9]. Still, the over-prescription in the USA led to a national opioid epidemic crisis, the use of opioids is estimated to cost over $700 million annually [10].

Beside the central opioid analgesic effect, several data support that antinociception could also be achieved by activation of functional opioid receptors in the periphery as well [11, 12]. In the late 1980s researchers started to pay attention to the peripheral analgesic effect of opioids, growing number of studies were conducted in order to investigate the possibility of peripheral antinociception [13]. The most investigated method of peripheral opioid analgesia is intra-articular injection of the MOR agonist morphine [13–15]. It has been shown that significant pain relief can be achieved after knee surgery or in chronic rheumatoid- and osteoarthritis. The effect may last even up to 7 days (similarly to local steroid or anesthetic injection). The most limiting factor of intra-articular injections is the enhanced risk of local infections [16, 17]. Locally injected morphine was effective in patients with chronic inflammatory tooth pain. Submucous injection of morphine also effectively alleviated the pain after dental surgery. These effects were absent in patients without preexisting inflammation (similarly as in animal studies) [17, 18]. Topically applied morphine attenuated pain in patients with unilateral corneal abrasions on the lesioned, but not the intact site. Importantly, morphine did not show any detrimental effect on wound healing [17, 19]. Locally injected morphine was also effective in postoperative visceral pain conditions (in the urinary bladder or after laparoscopic tubal ligation) [17]. On the other hand, several studies failed to prove peripheral analgesic effect of applied opioids. In the majority of these studies morphine was injected into non-inflamed tissues. It further supports the observation, that the peripheral analgesic effect of opioids is highly elevated under inflammatory conditions [17]. MOR, δ-opiod receptor (DOR) and also κ-opioid receptor (KOR) agonists show significantly stronger antinociceptive action in injured than in non-injured tissues of animals and also of humans [13].

Many attempts have been done in order to target the peripheral source of pain (i.e. target the peripheral opioid receptors in order to inhibit the pain transmission peripherally) to

avoid the CNS adverse effects. However, for long time the chemical modification carried out on morphine to limit its central nervous system penetration resulted in morphine analogs of weak affinity [20]. In spite the quaternary analogs that showed limited CNS penetration and lower affinity for opioid receptors, zwitterionic molecules like morphine-6-*O*-sulfate (M6SU) displayed greater antinociceptive effect than morphine [21, 22]. M6SU applied as an eye drop showed strong local antinociceptive effect in different animal models of corneal injury without any harmful effect on wound healing [23, 24].
Taken together, targeting peripheral opioid receptors offers a possible new way to treat different pain conditions, especially in the case of inflammatory pain.

1.2. Pain transmission

Nociceptors are located in the peripheral ending of primary afferents. They can be found at the end of pseudounipolar sensory neurons with cell bodies in the dorsal root-, trigeminal-, or nodose ganglia [25]. To sake of simplicity, based on axon diameter, degree of myelination and axonal conduction velocities as well as body sizes, sensory fibers are classified as Aβ, Aδ and C. Aβ fibers are stimulated by non-noxious stimuli. Most of them have low mechanical thresholds and are described as light-touch receptors. On the other hand, Aδ and C fibers carry the noxious sensory information into the spinal dorsal horn. These fibers are responsible for transmission of pain resulted from mechanical, thermal or chemical noxious stimuli in different parts in our body. Most of the Aδ fibers are associated with mechano- or thermoreceptors. C-fibers are polymodal fibers, because they are responding to multiple modalities: chemical, mechanical (touch, pressure, stretch) and thermal stimuli. Aδ and C primary sensory afferent fibers convey the pain from the site of injury into spinal cord, where they synapse with the secondary sensory neurons (spinothalamic tract), that further convey the pain to the thalamus, where third order neurons pass the information further to the somatosensory cortex. Two categories of pain transmission exist: fast and slow. Aδ fibers transmit the information relatively quickly (6 to 30 m/sec), C fibers are conducting at a lower speed (0.5 to 2 m/sec). The myelinated, large Aβ fibers conduct the information (touch, pressure, vibration) at high speed (30 to 70 m/sec) [25].
The emotional aspect of the pain is conveyed by the spinoreticular tract that terminates in the reticular information in the brainstem, where information is further processed to

thalamus and hypothalamus. Of note, sensory C fibers are responsible for conveying visceral pain and neuropathic pain from the periphery to CNS [25]. Inflammatory mediators (e.g. bradykinin, prostaglandin, serotonin, H^+, cytokines) that are released from damaged tissues surrounding the primary sensory afferents of free endings directly stimulate the nociceptors or lower their pain threshold. The later phenomenon is called primary sensitization. This will also increase the excitability of spinal neurons which can later amplify all sensory inputs including normally non-noxious stimuli conveyed by the low threshold Aβ fibers. This central sensitization strongly contributes to the pain symptoms like allodynia (non-noxious stimuli experienced as painful stimuli) and hyperalgesia (lowered pain threshold) [14, 26].

Beside the ascending pain pathway, the descending (or inhibitory) pathway also have important role in pain sensation. Areas in the brain like the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) hosting high receptor pools and containing high endogenous opioid peptide content are major points in the control of descending pain pathways. Therefore, activation of this pathway through endogenous opioid-release results in analgesia that can explain the lack of pain sensation for example during a shock condition [25].

1.2.1. Opioid receptors and their distribution

Opioid receptors belong to G-protein coupled receptors (GPCRs), they are G_i-coupled inhibitory receptors. Currently, three opioid receptor types exist: μ-opioid receptor (MOR), κ-opioid receptor (KOR) and δ-opioid receptor (DOR) named after morphine, ketocyclazocine and vas deferens, respectively [27, 28]. The International Union of Basic and Clinical Pharmacology Committee (IUPHAR) for the Receptor Nomenclature and Drug Classification issued the abbreviations MOR, KOR and DOR. Their mRNAs as well as the gene's structures were cloned and characterized. In addition, they were further subdivided into several subtypes as follows: MOR to μ1 (pain management) and μ2 (respiratory center) and μ3 (immune cells); KOR to κ1a, κ1b κ2, κ3 and DOR to δ1 and δ2 [29–34].

The activation of opioid receptors by opioid agonists results in decrease of intracellular cAMP levels through inhibition of adenylate cyclase, close of voltage-dependent Ca^{2+} channels (N type and to a lesser extent P / Q) in presynaptic nerve endings and opening

of K^+ channels at post-synaptic neurons [35]. Consequently, beside the inhibition of the release of transmitters (neurohormones and neuropeptides), such as glutamate and substance-P from the presynaptic neurons, they do inhibit the propagation of action potential by hyperpolarizing the secondary neuron cells [36].

Opioid receptors are widely distributed in the CNS and also on the periphery.

Opioid receptors in the CNS: In the brain all types of opioid receptors can be found. The most abundant opioid receptor with the widest distribution in the brain is MOR. Table 1 depicts the mRNA distribution of different opioid receptors in the human brain, which directly correlates with the receptor distribution [37]. MOR and DOR can be found in the whole area of cortical lamina whereas KOR can be found mostly in the deeper regions (lamina IV-VI.), which might contribute to the sedative effect of KOR agonists. Opioid receptors are also distributed in caudate putamen. The caudate putamen forwards information toward the ventral tegmental area and substantia nigra. This indicates the role in sensory-motor interactions, motivation and rewarding effects.

The opioid receptors can be found in other regions important in pain transmission, like the anterior cingular cortex (ACC), primary and secondary somatosensory cortex, ventrolateral orbital cortex (VLO). The ACC is in direct connection with the periaqueductal gray (PAG), which has an essential role in the descending inhibitory pathway. ACC is a key point in somatic- and also visceral pain processing. Endogenous opioids are among the neurotransmitters acting in the ACC. The role of opioids in pain transmission/inhibition was shown also in the motor cortex, rostral agranular cortex. In conclusion, opioids influence the pain transmission in the brain at every important area of pain processing [37–39].

Opioid neurons are also located at the spinal level. These inhibitory interneurons are influenced by the descending inhibitory pathway causing pre- and also post-synaptic inhibition. Opioid neurons in the midbrain inhibit GABAergic inhibitory neurons activating the descending pathway (inhibition of inhibition). Additionally, opioid receptors are also expressed in the dorsal horn and in the gray matter around brain ventricle IV and V [25, 40]. In the dorsal root ganglia (DRG) MORs, DORs and also KORs can be found. MORs are located on medium and large diameter cells, DORs mostly on large diameter neurons and KORs on small and medium neurons. These data suggest that these opioidergic neurons are important in the processing of different pain types and

they are influencing both the descending- and ascending pain pathways in the spinal cord [41].

Table 1. *Opioid receptor mRNA distribution in human brain*

Region	**δ receptor**	**μ receptor**	**κ receptor**
Prefrontal cortex			
Layers:			
I	0-low	moderate	0
II	moderate	high	moderate
III	high	low	moderate
IV	moderate	moderate	high
V	high	high	high
VI	high	high	high
Striatum			
Caudate nucleus			
interno-medial	low to moderate*	high*	moderate to high**
Putamen	low to moderate*	high*	moderate to high**

0 = undetectable; * = diffuse; ** = asymmetric cell clusters

Low, moderate, and high indicate the relative density of cells expressing receptor mRNA. Table 1. was adapted from [37].

Opioid receptors at the periphery: Beside the central opioid receptors, several data support the existence of functional opioid receptors in the periphery as well [12, 42]. These receptors are localized on peripheral terminals of sensory nerves. Pharmacological evidences – on animal models and also in humans – indicate that activation of these receptors on peripheral sensory axons also results in the mitigation of pain [20, 43–45]. Based on experimental results, all three types of opioid receptors (MOR, DOR and KOR) can be found at the periphery in functionally active state [12]. These receptors can be found on small-, medium- and large- diameter sensory neurons of animals or humans. The endogenous ligands of these receptors, opioid peptides (endorphin, enkephalin, dynorphin) were also found in immune cells infiltrating the inflamed tissues during

inflammation. Environmental stress is a strong factor in the mechanism of release of these peptides [13, 17, 42].

In conclusion, the three pillars of the analgesic-antinociceptive effect of opioids are:
1) Inhibition of the nociceptive stimuli transmission from the periphery to the spinal cord
2) Activation of the descending inhibitory pathway
3) Influencing the activity of the limbic system [6, 46, 47].

1.3. Different pain disorders

1.3.1. Acute and inflammatory pain disorders

Nociceptive pain originates from tissue damage surrounding peripheral sensory neurons (e.g. postoperative pain; posttraumatic pain, as broken bone pain). Opioids are the most effective tools to treat acute pain syndromes but their side effects, especially the centrally ones, limit their clinical use. NSAIDs are usually the first choice in acute pain treatment. In severe cases they might be used in combination with opioids to achieve a synergistic antinociceptive effect, thereby reducing the opioid need [10]. Indeed, NSAIDs are good choice in the treatment of mild to moderate pain. However, limiting factors in the use of NSAIDs are their gastrointestinal damaging and cardiac adverse effects [48]. Tramadol (weak MOR agonist and serotonin- and norepinephrine reuptake inhibitor), the NMDA antagonist ketamine and in some cases the anticonvulsant gabapentinoids (gabapentin, pregabalin) are also used in the management of acute pain conditions [10].

The inevitable consequence of tissue damage is the release of inflammatory mediators such as bradykinin, serotonin, prostaglandins and cytokines as well as tissue acidification. This will lead to the direct stimulation of nociceptors and the above mentioned primary sensitization. Beside inflammatory mediators endogenous antinociceptive agents are also released at the site of inflammation like opioid peptides, somatostatin, endocannabinoids and anti-inflammatory cytokines [14].

Opioid receptors have been reported to be upregulated in inflamed tissues as well as at the spinal level. Potentiated analgesic action of opioids was also observed [49–51]. This favorable change stimulates the researchers to develop opioid agents targeting these peripheral receptors, hence the peripheral antinociception of opioids in humans has been investigated in several randomized controlled clinical trials [52, 53]. Pooled analyses of data from 19 studies suitable for meta-analysis showed only a moderate analgesic effect

after intra-articular morphine compared with placebo in patients with arthroscopic knee surgery. Morphine-6-glucuronide (M6G) has been reported to have peripheral anti-hyperalgesic effects following its systemic administration in human volunteers [45]. Due to its high hydrophilicity it has a considerable delay between peek plasma concentrations and peek central opioid effects, so peripheral antinociceptive effect can be detected within this time window [45, 54]. Although in the case of M6G central side effects, like respiratory depression was observed, design of opioids with high hydrophilicity and high efficacy may provide analgesic agents of high clinical value.

1.3.2. Neuropathic pain disorders

The definition of neuropathic pain (NP) by the International Association for the Study of Pain (IASP) is "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (IASP, 2012). Based on this definition NP is a consequence of damage to neurons in peripheral- or central nervous system or both. NP has significant undesirable impact on the economic welfare of the society [55, 56]. Therefore, to find drugs satisfactory treating NP is a major clinical goal. The management of severe acute to moderate and cancer pain by opioids is satisfactory, however opioid effectiveness in the treatment of chronic NP is controversial [2, 40, 57–61]. So far the management of NP by medication is largely based on the symptoms of disease. These medications are categorized into three groups: first, second and third line drugs. The first line medications include: tricyclic antidepressants, dual reuptake inhibitors of serotonin and norepinephrine, gabapentinoids and 5% lidocaine transdermal patch. The second line drugs are classical opioids and drugs having opioid and non-opioid actions like tramadol. Third line medications include antiepileptics, topical capsaicin, memantine and mexiletine [62]. Of note, medications considered as second line might prescribed as first line e.g. during the titration of another drug or acute NP attack.

In addition, reports have been issued on the equivocal response of NP for opioid treatments, although this responsiveness to opioid analgesics may vary across the different types of NP syndromes [2, 59, 63]. Data obtained in diabetic animals developed NP showed a significant reduction in opioid antinociceptive efficacy following systemic administration. The loss of opioid antinociceptive efficacy has been also reported following central (spinal or supraspinal) administration in diabetic animals with NP [64–

66]. It was proposed that the impaired opioid analgesia occurred as a consequence of decrease in opioid receptor reserve. In addition, the reduction in opioid receptor density in spinal and supraspinal tissues was also demonstrated in diabetic animals [67, 68].

In general, less than 50% of patients respond to treatment with all of the above mentioned drugs and 40% are inadequately treated. Therefore, 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 [69].

DOI:10.14753/SE.2019.2219

2. Objectives

Our working group recently designed and synthesized the new MOR agonist, 14-*O*-methylmorphine-6-*O*-sulfate (14-*O*-MeM6SU; Fig. 1.), which displayed high affinity for MOR and strong antinociceptive effect in acute thermal nociceptive tests. Additionally, in functional *in vitro* [^{35}S]GTPγS binding assay, 14-*O*-MeM6SU showed a full agonist character whereas morphine or M6SU showed a partial agonist nature [70].

– To assess the antinociceptive effect of test compounds in **inflammatory conditions**, three different animal models were applied: acetic acid induced writhing test in mice, formalin test in rats (acute models) and Complete Freund's Adjuvant (CFA) induced subchronic inflammatory model.

- The acetic acid-evoked writhing assay is one of the most well-established and widely used experimental models of visceral pain to assess the pain relieving actions of either NSAIDs or opioids [71, 72]. In this model chemical visceral nociception is induced by the injection of diluted acetic acid into the peritoneal cavity of the rodents.
- Formalin test is frequently applied in rats to assess the antinociceptive properties of investigated compounds. The test consists of two phases: phase I (0-10 min) and phase II (11-60 min). Phase I is a direct consequence of the irritating effect of the formalin solution injected intraplantarly (i.pl.) into the hindpaw. In the second phase inflammatory mediators (bradykinin, histamine etc.) are released, hence this test is suitable to assess the effect of test compounds on acute nociceptive and inflammatory pain as well [73].
- In case of Complete Freund's Adjuvant (CFA- a mixture of mineral oils, heat-killed mycobacteria and emulsifying agent) -evoked hyperalgesia the inflammation is induced by the application of CFA into the hind paw of animals (e.g. rats or mice). This pain model has long been used as a strategy to demonstrate the (peripheral) antinociceptive actions of opioid agonists [44, 74]. CFA induces subchronic inflammation by enhancing both cell-mediated and humoral immune responses, hence modeling the clinical conditions (e.g. rheumatoid arthritis) rather properly [75].

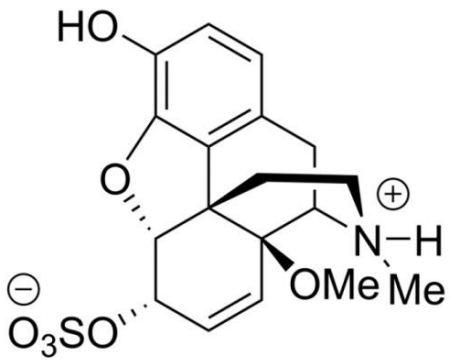

Figure 1. *The chemical structure of the novel compound 14-O-methylmorphine-6-O-sulfate (14-O-MeM6SU)*

- **Neuropathic pain** is one of the hardest painful conditions to treat. Among different NP conditions diabetic polyneuropathy deserves particular attention, since it is one of the most common NP diseases. Approximately 60-70% of diabetic patients suffer from diabetic polyneuropathy (mild to severe forms) [76]. To mimic these clinical conditions streptozocin (STZ) treated diabetic rats were investigated. STZ destroys the insulin producing β cells, hence causing type 1 diabetes with high blood glucose level.
  - First the symptoms of diabetes were confirmed: blood glucose levels, animal weights and the amount of water- and food consumption was investigated at numerous time points. Since diabetes can impair gastrointestinal motility [77], the gastric emptying of diabetic and control rats was also assessed. The elevated glucose concentration in the blood leads to neuron damage and therefore neuropathic pain, indicated by mechanical allodynia [64, 78].
  - In neuropathic conditions the effectiveness of different opioids is reduced [68]. Therefore, we also assessed and compared the degree of antinociception impairment of the novel compound (14-*O*-MeM6SU) and morphine.
  - The number of MOR was evaluated in DRG and dorsal horn tissues of diabetic and non-diabetic rats. G-protein coupled receptor activity was also measured *in vitro* to determine the efficacy of test compounds in diabetic and control rat spinal cord and brain homogenates.
- We also aimed to assess the **side effect profile** of the novel compound in comparison with other known agents. The tail-flick test is one of the most commonly used methods to assess the acute nociceptive potency and efficacy of different opioids against

thermal stimuli [43, 79]. Beside the advantages of this test, like being relatively quick and easy to perform, it might be an adequate method to predict analgesic efficacy both in humans and in other more complex pain models, as well [80]. With the tail-flick test – performed after 3 days administration of test compounds – we aimed to assess the analgesic-tolerance profiles. The effects on gastrointestinal transit and respiratory functions were also investigated, since opioid induced constipation and the respiratory depressive effects of opioids are among the main limiting factors of their use [81]. Sedative effects of test compounds were also evaluated by the assessment of their anesthesia potentiating effects.

In summary, the main aims of the thesis are:

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

- 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 14-*O*-MeM6SU and reference compounds in above mentioned pain conditions

4. To further analyze the actions of test opioid agonists at the spinal and supraspinal level under NP conditions applying biochemical assays

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

DOI:10.14753/SE.2019.2219

3. Materials and methods

3.1. Experimental 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) or Charité University Berlin, Campus Virchow Klinikum and Campus Charite Mitte, Berlin, Germany in the case of immunohistological assay. 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*.

The animal weights, phenotypes and sources are presented under appropriate method section. Each animal was used for one experiment and only once. During work researchers did the best effort to minimize the number of animals and their suffering.

3.2. Materials

The novel compound (14-*O*-MeM6SU) and the reference compound M6SU used in the present work were synthesized by Sándor Hosztafi in Department of Pharmaceutical Chemistry, Semmelweis University (Budapest, Hungary) as previously described [70]. Morphine hydrochloride was obtained from Alkaloida-ICN (Tiszavasvári, Hungary). The MOR agonist enkephalin analog Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) was obtained from Bachem Holding AG (Bubendorf, Switzerland). The radiolabeled GTP analog, [^{35}S]GTPγS (specific activity: 1000 Ci/mmol) was purchased from Hartmann Analytic (through Izotóp Intézet Kft., Budapest, Hungary). The UltimaGoldTM MV aqueous scintillation cocktail was purchased from PerkinElmer (through Per-Form Hungária Kft., Budapest, Hungary). Naloxone methiodide (NAL-M) and all other chemicals were of analytical grade and purchased from Sigma–Aldrich, Budapest, Hungary. Drugs were dissolved in 0.9% solution of NaCl with the exception of STZ,

which was dissolved in ice-cold distilled water right before injection (less than 10 min before injection). NAL-M was dissolved in saline, also right before the experiment.

Drugs or vehicle were delivered as follows: s.c. administration (under skin over the neck) 2.5 or 5 ml/kg for rats and 10 ml/kg for mice; intravenous (i.v.) injections 2.5 ml/kg for rats; intraperitoneal (i.p.) injections 2.5 ml/kg for rats; intraplantar (i.pl.) administration 100 µl/rat; intracerebroventricular (i.c.v.) injection 5 µl/animal for mice. For each dose a separate group of animals was used. All compounds were stored and handled as described in the product information sheet.

Experiments were performed in a blinded way to the drugs and doses applied. In *in vivo* tests morphine or morphine-6-*O*-sulfate (M6SU) was used as reference compound.

3.3. Acute inflammatory pain models

3.3.1. Experimental paradigm for mouse writhing test

Male NMRI mice (20-30 g) were used. The acetic acid-induced writhing test was performed as previously described [71]. Mice were injected i.p. with 0.2 ml of 0.6% acetic acid aqueous solution to induce the writhing reaction which is characterized by contractions of the abdominal musculature followed by extension of the hind limbs.

Groups of mice were injected s.c. or i.c.v. with different doses of 14-*O*-MeM6SU or M6SU followed 15 min later by an intraperitoneal (i.p.) injection of 0.6% acetic acid solution. Each mouse was then placed in individual transparent Plexiglass chambers, and 5 min after acetic acid injection the number of writhes was counted during a 10 min observation period. That is, assessments started 20 min after s.c. opioid agonist administration. For determination of writhes in control groups, animals were s.c. or i.c.v. injected with 0.9% saline solution before i.p injection of 0.6% acetic acid using a similar protocol as for the test drugs. In experiments when the antagonist action was assessed, s.c. NAL-M was co-administered with the respective agonist.

3.3.2. Experimental paradigm for rat formalin test

Male Wistar rats (200–300 g) were used. The test was performed as described previously [82]. Briefly, before the experiments the animals were daily wrapped in cloth („handling”) except their right hindlimb, which was left free, for three constitutive days in order to habituate them to the experimental conditions. On the fourth day animals were

wrapped in cloth and 2.5% formalin solution was injected into the plantar surface of the right hind paw in a volume of 50 µl/rat. Immediately after the injection, animals were placed into Plexiglass observation chambers fixed above a mirror of 45 degree angle position allowing free viewing of the paws. Then, the antinociceptive action of the investigated agents was assessed by counting the number of nociceptive behaviors (shaking, flinching, licking and elevating the painful paw) for 60 min of 5 min time periods. The observation peridod was subdivided into 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 were injected s.c. (2.5 ml/kg) 15 min prior to formalin injection. In order to assess the peripheral opioid system's contribution to the whole antinociceptive action of systemically applied opioids NAL-M was co-administered. In an other set of experiments, the test compounds were injected intraplantarly into the ipsilateral or contralateral paw (100 µl/animal) 5 min prior to the i.pl. formalin solution injection.

3.4. Subchronic inflammatory pain model: Complete Freund's Adjuvant induced inflammation in rats

Male Wistar rats (200–300 g) were used. Rats under brief isoflurane (Willy Rüsch GmbH, Böblingen, Germany) anesthesia received i.pl. injection of 0.15 ml Complete Freund's Adjuvant (CFA) (Calbiochem, San Diego, CA), 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; Ugo Basile, Comerio, Italy) as earlier described in [83]. 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. After baseline measurements separate groups of animals for each s.c. dose were used. The antinociception assessed with respect to the change in PPT of both paws after s.c. administration of drug was compared with the baseline value obtained before drug treatment in ipsilateral or contralateral paws. The doses of each drug that produced

a 60-80% antinociceptive effect in inflamed paws, without a significant effect on the contralateral non-inflamed paws, were selected for experiments designed to analyze the antagonism by NAL-M. In these experiments NAL-M was co-administered with the test compounds when the peak effect was at 30 min, or 30 min after the agonist administration, when the peak effect was achieved at 60 min. In other series of experiments, NAL-M was injected locally 5 min prior to measurement (at 25 min or 55 min, when the peak effect was achieved at 30 and 60 min, respectively).

3.5. Neuropathic pain model: experimental paradigm for streptozocin induced chronic 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 consumption of water- and food was 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, data not shown), weight match control animals were used. Weight match animals were handled and kept under the same conditions described for the diabetic (and non-diabetic control) animals. The only exception was that, weight match animals were kept only for one or two weeks prior to experiments.

3.5.1. Monitoring of diabetic and control animals

Measuring the blood glucose levels: The rat blood glucose levels were determined by Accu-Chek Active blood glucose meter (Roche Diagnostics GmbH, Germany). The animals were slightly anesthetized with 3% isoflurane in oxygen via nose cone and one drop of blood was taken from the tail veins. The maximum concentration of blood glucose that is measurable is 33.3 mmol/l therefore this value was used as a maximum. The animals were considered diabetic if the value was above 14 mmol/l [78]. The blood glucose was measured before and 72 hours after the STZ treatment and on the 1st, 2nd, 3rd, 9th and 12th week after the STZ injection. Vehicle treated group was used as absolute control.

Measuring of animal weight and food and water consumption: In the first series of experiments control (vehicle treated) and diabetic (STZ treated) animals were kept individually. The water and food consumption of the animals was measured separately for each animal before and after the STZ treatment each day for 4 weeks and at least two times a week until the 7^{th} week. The weight of the animals was checked during the 7 week period in the same way.

Gastric emptying assay: Phenol red method was performed as described earlier [84], with some minor modifications. Briefly, after 24 h of fasting, diabetic and non-diabetic rats received 1.5 ml of 1.5% methylcellulose solution containing 0.5 mg/ml phenol red (a non-absorbable marker compound) by intragastric gavage. After 20 min the rats were sacrificed, the pylorus and cardia were clamped and the stomach was removed. The content of stomach was mixed with 40 ml of 0.1 N NaOH, then 0.6 ml of this mixture was added to 1.2 ml of 7.4% trichloroacetic acid solution to precipitate proteins. Following centrifugation (15 min, 3000 g) 1.2 ml of the supernatant was added to 0.6 ml of 1 N NaOH, and the absorbance was read spectrophotometrically in triplicates at 560 nm. Gastric emptying (%) was calculated as follows: [1 – (absorbance of sample / maximal absorbance)] x 100. Maximal absorbance was measured by processing the test meal alone, as described above.

In a separate experiment, weight-matched animals were treated either with saline, or with clonidine (0.1 mg/kg) subcutaneously 30 min before the methylcellulose administration. Clonidine is an $alpha_2$ adrenoceptor agonist, which is a well-known inhibitor of gastric emptying [85].

Assessment of neuropathic pain with Dynamic Plantar Aesthesiometer (DPA): In order to determine the allodynia caused by advanced diabetes we used the Dynamic Plantar Aesthesiometer (DPA) (Ugo Basile, Italy) as described previously [86] with slight modifications based on our pilot experiments. The animals were placed in the plastic cages of the DPA once daily for 3 subsequent days ("handling"). In addition before the experiments the animals were kept in the cages also for 5 min before starting the measurement in order to habituate them. 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).

The withdrawal threshold was measured and expressed in grams, before and after the STZ treatment on every 3rd week. In the first series of experiments the animals were measured every week after STZ treatment in order to determine the peak of allodynia. Each of the hind paws were measured 3 times alternately and the average data of the 3 measurements were used for each animal. Both vehicle treated and weight match (i.e. animals with weights matching the diabetic ones) groups were used as control. After determination the time point of the peak effect further analyses were made at this time point. An animal was considered neuropathic, when the threshold value was decreased at least by 20% compared to weight match animals as prescribed previously [87]. Diabetic rats that did not develop allodynia by the 9th week were excluded from further experiments.

3.5.2. Investigation of the analgesic action of different opioids in rats with diabetic neuropathy

Data were obtained 9 and 12 weeks following STZ treatment i.e. 6 and 9 weeks after the appearance of allodynia, a major sign of painful diabetic neuropathy. The baseline of withdrawal thresholds was measured before s.c. agonist treatment. The antiallodynic action of test compounds was assessed 30, 60 and 120 min after treatment. On the 9th week after STZ treatment dose-response curves were constructed. Data (100%: mean baseline values of diabetic or non-diabetic rats) were converted to log dose units and were analyzed with linear regression. The effective doses producing 30% effect (ED_{30}; 30% elevation in the threshold after treatment in comparison with the baseline) were determined. Since DPA elevates pressure on the paws only until 50 grams to avoid tissue damage, ED_{50} values could not be accurately determined. To analyze the changes in antinociceptive potency of test compounds the calculated ED_{30} values were compared ($ED_{30diabetic}/ED_{30non\text{-}diabetic}$). Weight match animals were used as absolute control in DPA measurement. In another set of experiments the opioid antagonist, NAL-M was co-administered with the investigated agonist.

3.6. Experimental paradigms for the assessment of side effects of test compounds

3.6.1. Determination of the effect of test compounds on gastrointestinal transit

The effect of 14-*O*-MeM6SU and M6SU compared to that of morphine on gastrointestinal transit was measured *in vivo*, by using the charcoal meal method [88]. Briefly, male

NMRI mice (20-25 g) were fasted 6 h prior to the experiments, with free access to water. At the time of the experiment, a charcoal suspension (10% charcoal in 5% gum arabic) was given in a volume of 0.25 ml/mouse by an oral gavage, followed by s.c. administration of test compounds (0.1 ml/10 g). Then, 30 min 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. The doses caused 50% inhibition on gastrointestinal transit (ID_{50}) were calculated from the linear regression of dose-response curves.

3.6.2. Respiratory function tests

Respiratory function measurements were performed by unrestrained whole-body plethysmography (WBP) in conscious, spontaneously breathing male Wistar rats (200-300 g) 30 and 60 min after s.c. injection of saline, 14-*O*-MeM6SU, M6SU or morphine. Rats were placed in the chamber of a whole-body plethysmograph (PLY 3213, Buxco Europe Ltd., Winchester, UK). The flow transducers (TRD5700, Buxco Europe Ltd., Winchester, UK) were connected to the preamplifier module, which digitized the signals via an analog-to-digital converter (MAX2270 Buxco Europe Ltd., Winchester, UK). Ventilation parameters (frequency: f, tidal volume: TV, minute ventilation: MV, time of inspiration: Ti, peak inspiratory time: PIF, time of expiration: Te, peak expiratory flow: PEF and relaxation time: RT) were measured every 10 seconds during the 15-minute-long acquisition times and averaged by the BioSystem XA Software for Windows (Buxco Research Systems).

3.6.3. Determination of sedative effects of test compounds

To assess the anesthesia potentiating effect of test compounds righting reflex method was applied in male Wistar rats (200-300 g). The sleeping time (righting reflex, when the animals turned back on all four legs) was determined. To strengthen our results the novel compound's potentiating effect was investigated with two different anesthetics of different routes of administration (i.v. and inhalational) and different pharmacokinetic properties.

Thiobutabarbital-induced sleeping time: Animals received i.v. saline or thiobutabarbital (153 μmol/kg), then were placed on the left side on a pillow of 30 °C.

The sleeping time in minutes was documented. Anesthesia-potentiating effects of test drugs were studied by their s.c. administration. Thiobutabarbital was i.v. injected at the time of peak antinociceptive action of test compounds (60 min after s.c. 14-*O*-MeM6SU and 30 min after s.c. M6SU).

Isoflurane-induced sleeping time: The sleeping time was induced by inhaled 3% isoflurane in oxygen for 2 min with a 2 liters/minute flow rate via nose cone using a vaporizer (Eickemeyer Isoflo Vaporiser; Eickemeyer Veterinary Equipment Inc). The sleeping time was determined as described above. The animals were treated s.c. with 14-*O*-MeM6SU or morphine 60 or 30 min before inhaled anesthetic, respectively. Control groups were treated with saline.

3.6.4. Induction of tolerance in mice

Male NMRI mice (18–28 g; Toxicoop, Hungary) were used. Radiant heat tail flick test was used to assess antinociceptive effect of test compounds. The assay was carried out as described by Tulunay and Takemori [79] using an ITC Life Sciences equipment. Briefly, the light intensity was adjusted to set the control tail-flick latency between 1.3 and 2.8 sec. Mice failed to respond within this range were excluded from the experiments. Cut off time was set to 6 s to avoid tissue damage. A baseline latency was measured before and 30, 60, 120, 180 min after s.c. drug or saline administration. A saline treated group was used for each experiment as control. Maximal possible effect (MPE) % was calculated for each mouse as follows: [Latency after treatment–Basal Latency]/[Cut off–Basal Latency] × 100%. For each dose a separate group of animals (n = 5–12) was used. Mice were rendered tolerant to morphine hydrochloride (200 μmol/kg) or 14-*O*-MeM6SU (12 μmol/kg) by twice daily (7 AM and 7 PM) s.c. injections for 3 days. Saline injections (10 ml/kg twice daily) were used in the control animals. The degree of tolerance was determined as the ratio of the ED_{50} value of the agonist in morphine or 14-*O*-MeM6SU injected mice to that in saline injected mice. The experiment was done on the fourth day as described previously [89]. Tolerance inducing treatment dose of 14-*O*-MeM6SU was calculated by using the following equation: [morphine dose] × [14-*O*-MeM6SU naive ED_{50}]/ [morphine naive ED_{50}].

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 or 14-*O*-MeM6SU treated mice. Control ED_{50} values for morphine and 14-*O*-MeM6SU were calculated from dose response curves constructed in saline treated mice. At least three groups of 5–12 mice were used to establish dose-response curves and to estimate ED_{50} values.

MPE% was calculated in MS Excel; for the further analysis Graph-Pad Prism 5.0 software was used. ED_{50} values and confidence intervals were calculated by non-linear fit (normalized response – variable slope). Differences in ED_{50} values were considered significant when confidence intervals did not overlap.

3.7. *In vitro* receptor binding assays

Male Wistar vehicle treated or STZ treated (diabetic) rats (250-400 g) were used.

Animals were decapitated and their brains and whole spinal cords were quickly removed and prepared for receptor binding assays as previously reported [90, 91].

3.7.1. Radioligand competition binding assay

The experiments were carried out as described previously [92, 93]. Specific binding of [^{3}H]DAMGO was performed by incubating 50-100 μg of membrane protein of lumbar dorsal horn with 0.1- 4 nM [^{3}H]DAMGO in the presence or absence of 10 μM unlabelled naloxone to determine non-specific binding. Membranes were incubated for 1 hour at 22 °C in assay buffer. The reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters, followed by four washes with cold buffer (50 mM Tris–HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry (Perkin Elmer, Rodgau, Germany) at 60% counter efficiency after overnight extraction of the filters in 3 ml of scintillation fluid. Unbound radioligand was separated from the receptor bound radioligand by rapid filtration under vacuum through glass fiber filter. The radioactivity of the specifically bound radioligands was determined by liquid scintillation spectrophotometry.

All experiments were performed in duplicate and carried out at least five times. B_{max} and K_d values in saturation binding assays were determined by nonlinear regression analysis of concentration-effect curves using GraphPad Prism (GraphPad Software Inc., CA, USA).

3.7.2. Immunohistochemistry

Rats were deeply anaesthetized with isoflurane and transcardially perfused with 100 ml of phosphate buffered saline (PBS) pH 7.4, then followed by 500 ml of 4% (w/v) paraformaldehyde in phosphate buffer pH 7.4. After perfusion, DRG and spinal cord were removed from STZ treated and control animals, postfixed in the same fixatives for 90 min, and then cryo-protected overnight at 4°C in PBS containing 10% sucrose. DRGs (10 μm thick) were mounted onto gelatin coated slides. DRG mounted or spinal cord floating tissue sections were incubated with the following primary antibody rabbit polyclonal MOR antibody (1:1,000, Gramsch Laboratories, Schwabhausen, Germany). The tissue sections were washed with PBS prior to incubation with Alexa Fluor 594 donkey anti-rabbit secondary antibody (Invitrogen, Germany). Finally, the tissues were washed in PBS, mounted on vectashield (Vector Laboratories, Burlingame, CA) and viewed under a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Göttingen, Germany). To demonstrate specificity of staining, the following controls were included as mentioned in detail elsewhere [94, 95]: 1) Preabsorption of the primary antibody against MOR was verified by preabsorption with 5 μg/ml of synthetic peptide antigen for MOR (Gramsch Laboratories, Germany), for 24 hours at 4ºC; 2) Omission of either the primary antisera or the secondary antibodies.

3.7.3. G-protein 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. First the brain and spinal cord were homogenized, centrifuged in ice-cold 50 mM Tris-HCl (pH 7.4) buffer and incubated at 37 ºC for 30 min in a shaking water-bath (for details see [96]). After incubation the centrifugation was repeated as described before and the final pellet was suspended in ice-cold TEM (Tris-HCl, EGTA, $MgCl_2$) buffer and stored at –80 ºC for further use. The nucleotide exchange is measured in the presence of a given ligand in increasing concentrations to measure ligand potency and the maximal efficacy. The experiments were carried out as described previously [97]. Agonist potency and maximum G-protein stimulation of reference and test compounds (see results section) were investigated in [^{35}S]GTPγS binding assays. In the diabetes induced neuropathic pain model control and STZ treated rat whole brain, spinal cord and lumbar dorsal horn

membrane homogenates were examined for MOR activity. [^{35}S]GTPγS was incubated together with the appropriate membrane homogenate together with increasing concentrations of unlabeled reference and test compounds, with excess GDP in a special buffer containing Tris, $MgCl_2$, EGTA, NaCl, and dithiothreitol.

Total binding was measured in the absence of the test compounds, while non-specific binding was determined in the presence of 10 μM unlabeled GTPγS. The bound and unbound [^{35}S]GTPγS were separated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and washed three times with 5 ml ice-cold 50 mM Tris-HCl through Whatmann GF/B glass fibers (GE Healthcare Life Sciences through Izinta Kft., Budapest, Hungary). [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times. Bound radiochemical separation and radioactivity detection were the same as discussed in radioligand competition binding assays.

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 (GraphPad Software, San Diego, California, USA, www.graphpad.com).

For the comparison of more than 2 groups one- or two-way ANOVA was applied with post hoc test based on the experimental setup (see Results section). Vehicle treated groups were used as control in order to decide if the applied treatment significantly influenced the parameters.

All data are represented as mean ± SEM. The results were considered to be statistically significant when $p < 0.05$.

DOI:10.14753/SE.2019.2219

4. Results

4.1. Acute inflammatory pain models

4.1.1. Mouse writhing test

4.1.1.1. Antinociceptive effects of 14-*O*-MeM6SU compared to M6SU

Injection of 0.6% acetic acid solution into the peritoneal cavity of mice administered s.c. or i.c.v. saline resulted in an average of 43.9 ± 1.5 (n = 43) writhes during the 10 min observation period. As shown in Fig. 2., s.c. or i.c.v. administration (20 min before testing) of both agonists produced a dose-dependent antinociceptive action. The calculated ED_{50} values with 95% confidence intervals at 20 min are shown in Table 2. 14-*O*-MeM6SU produced more potent inhibitory effect than M6SU on acetic acid-induced writhing in mice both after s.c. and i.c.v. routes (Table 2).

After s.c. administration, 14-*O*-MeM6SU was 23- fold more potent than M6SU, while after i.c.v. administration 14-*O*-MeM6SU was proved to be only 5-fold more active than M6SU in inhibition of writhing. The calculated large s.c./i.c.v. potency ratios for M6SU or 14-*O*-MeM6SU indicate limited CNS penetration (Table 2).

Table 2. *Antinociceptive potencies of 14-O-MeM6SU and M6SU against acetic acid induced nociception in mouse writhing test after 20 min of s.c. or i.c.v. administration.*

Compound	ED_{50} (nmol/kg, s.c.)	ED_{50} (pmol/mouse, i.c.v.)	s.c./i.c.v.[a]
M6SU	1993 (1282–3101)	9 (15-54)	221444
14-*O*-MeM6SU	87 (47–163)	1.7 (0.3-9.5)	51177

At least 4 animals per dose group and 3-4 doses were used for each ED_{50} determinations.

[a]s.c./i.c.v. was calculated as the ratio of ED_{50} nmol/kg, s.c./ED_{50} pmol/mouse, i.c.v

DOI:10.14753/SE.2019.2219

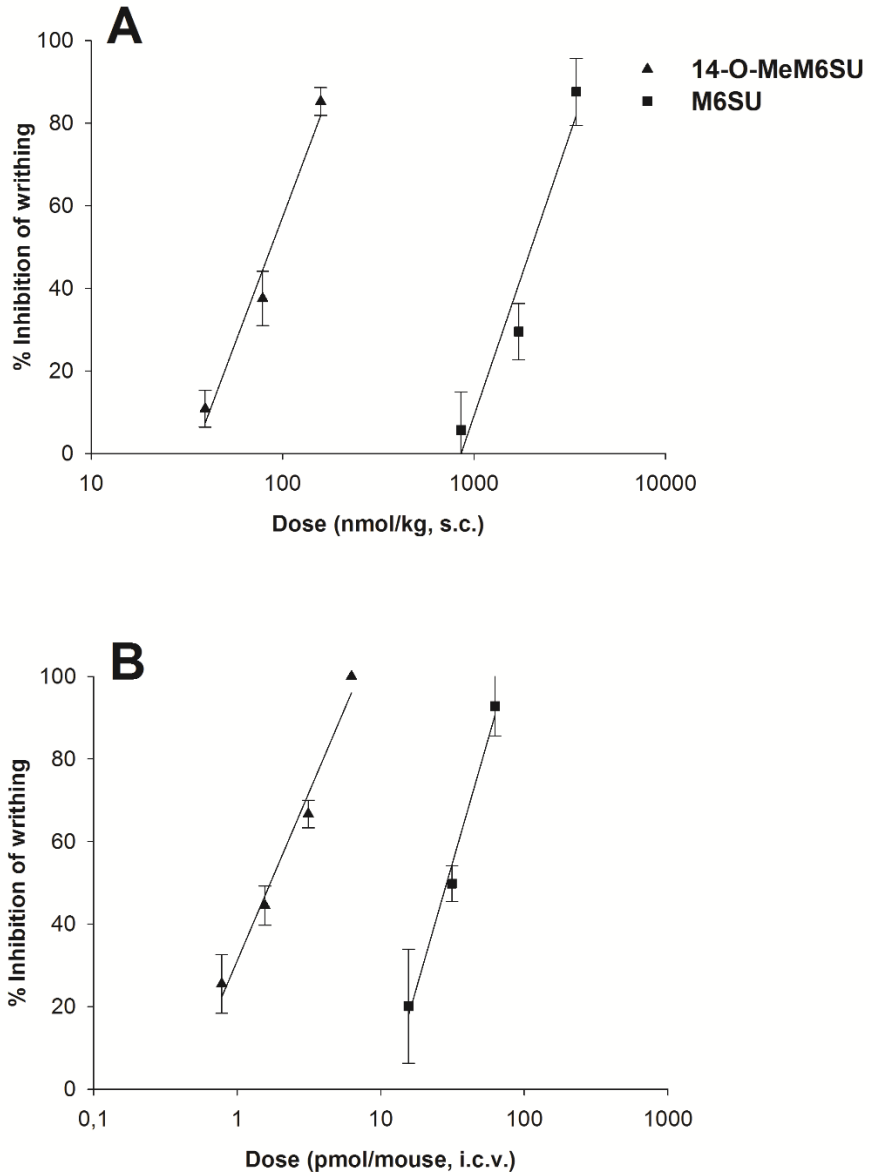

Figure 2. *Antinociceptive dose-response curves of s.c. (panel A) or i.c.v. (panel B) injected 14-O-MeM6SU and M6SU in mice writhing test.*

Points represent the mean ± S.E.M. (n= 4-5)

4.1.1.2. NAL-M antagonism on systemic 14-*O*-MeM6SU or M6SU antinociception

To evaluate the opioid specificity and the site of action of 14-*O*-MeM6SU and M6SU in acetic acid-induced writhing in mice, the effects of the agonists were assessed after systemic co-administration with the quaternary opioid antagonist NAL-M (21.3 μmol/kg, s.c). Our results show that s.c. equipotent analgesic dose of 14-*O*-MeM6SU (136 nmol/kg), and M6SU (3043 nmol/kg) significantly decreased the number of writhes at 20 min after administration. Co-administration of NAL-M antagonized the antinociceptive effect of the test opioids, as presented in Fig. 3. NAL-M treatment alone failed to affect the number of writhes (Fig. 3). Higher dose of 14-*O*-MeM6SU (272 nmol/kg) showed also NAL-M reversible antinociception (Fig. 3).

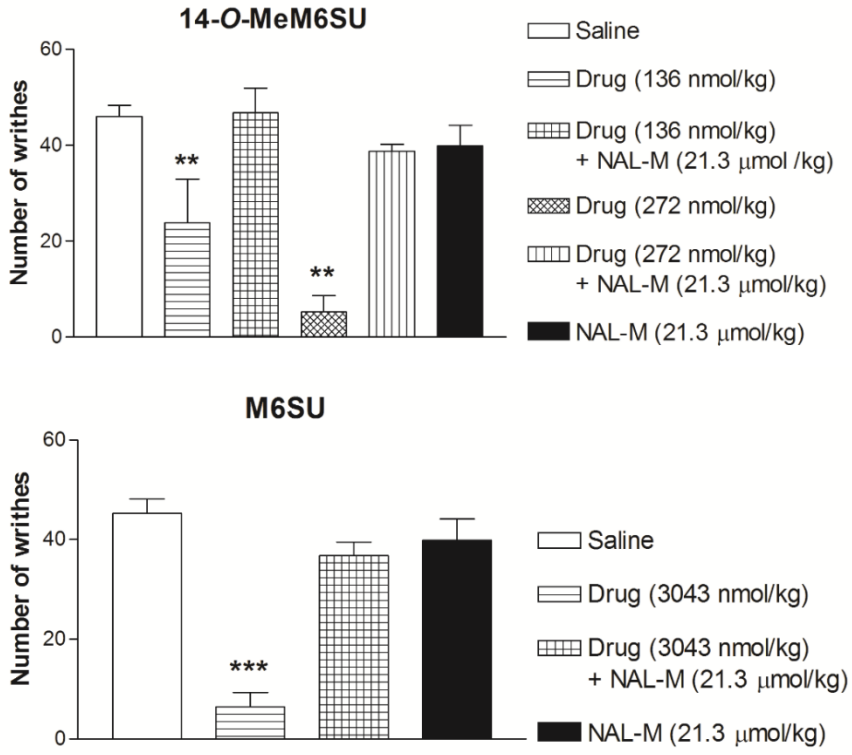

Figure 3. *The antagonist action of co-administered NAL-M (21.3 μmol/kg) on the antinociceptive effect of 14-O-MeM6SU or M6SU after 20min s.c. administration in the writhing response induced by acetic acid (i.p.) in mice.*

Data are expressed as mean ± SEM

*: significant difference versus other groups (**: p<0.01; ***: p<0.001)

(one-way ANOVA, Newman-Keuls post hoc test).

4.1.2. Rat formalin test

4.1.2.1. Antinociceptive effect of 14-*O*-MeM6SU compared to morphine

Subcutaneous 14-*O*-MeM6SU (253, 506 and 1012 nmol/kg) or morphine (3884, 7769, 15538 and 31075 nmol/kg) attenuated the formalin-induced pain in a dose-dependent manner (Fig. 4., panel A and B).

When both phases are considered, 14-*O*-MeM6SU in doses 506 and 1012 nmol/kg produced antinociception (Fig. 4., panel A), whereas 253 nmol/kg was effective only in the second phase. On the other hand, s.c. morphine caused antinociception in all tested doses in phase I and apart from the lowest dose in phase II (Fig. 4., panel B). Based on equianalgesic doses after systemic administration 14-*O*-MeM6SU was approx. 15 and 31 more potent than morphine in phase I and II, respectively.

The **locally** (i.pl.) administered (into the ipsilateral paw) 14-*O*-MeM6SU at doses of (25.3, 50.6 and 101.2 nmol/rat) or morphine (971 and 1942 nmol/rat) were also tested. The lowest dose that significantly reduced the pain in phase I was 25.3 nmol/animal for 14-*O*-MeM6SU and 1942 nmol/animal for morphine (Fig. 5.). When both phases considered, 14-*O*-MeM6SU alleviated the pain reactions in a dose of 50.6 nmol/rat and morphine only at the dose of 1942 nmol/rat (Fig. 5.). Based on equianalgesic doses after local administration 14-*O*-MeM6SU was approx. 77 and 38 more potent than morphine in phase I and II, respectively.

DOI:10.14753/SE.2019.2219

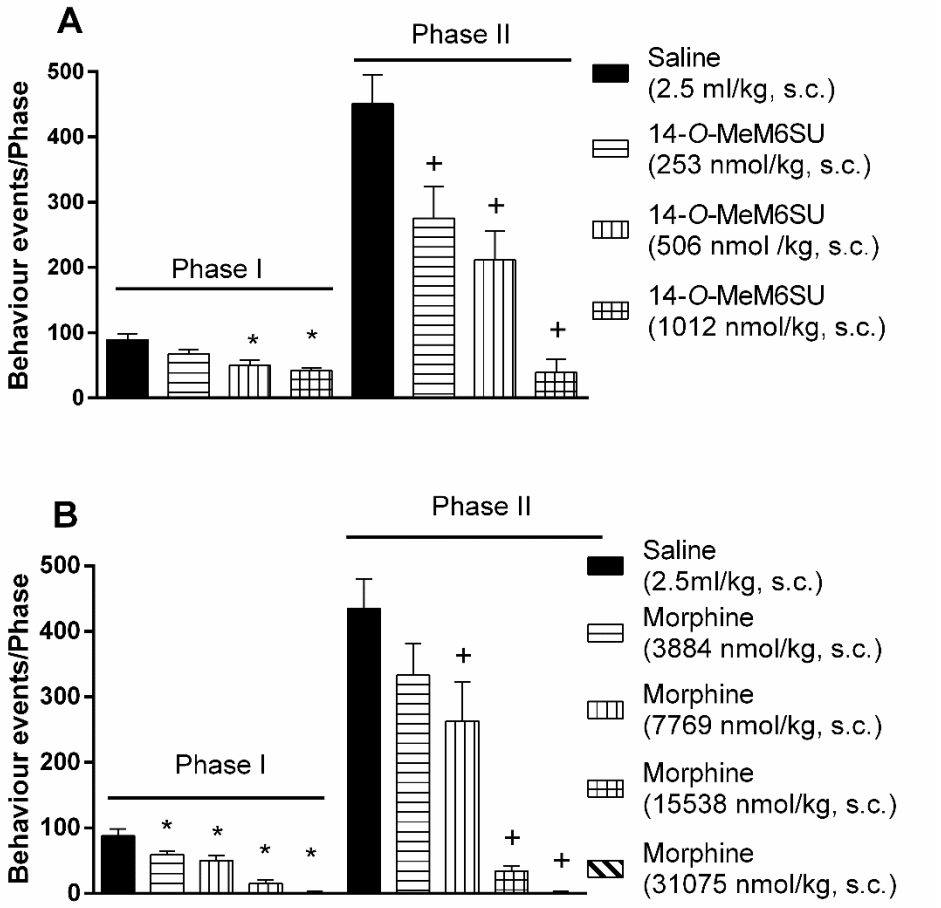

Figure 4. *The antinociceptive effect of 14-O-MeM6SU and morphine after s.c. administration in rat formalin test, in phase I (0-10 min) and phase II (11-60 min). Each column represents the cumulative data of the given phase (number of nociceptive reactions). Drugs were administered in a 2.5ml/kg volume.*

Each value represents the mean ± SEM.

*: significant difference vs. vehicle treated group in Phase I ($p<0.05$)

+: significant difference vs. vehicle treated group in Phase II ($p<0.05$)

(one-way ANOVA followed by Fisher's LSD post hoc test, n= 4-11)

DOI:10.14753/SE.2019.2219

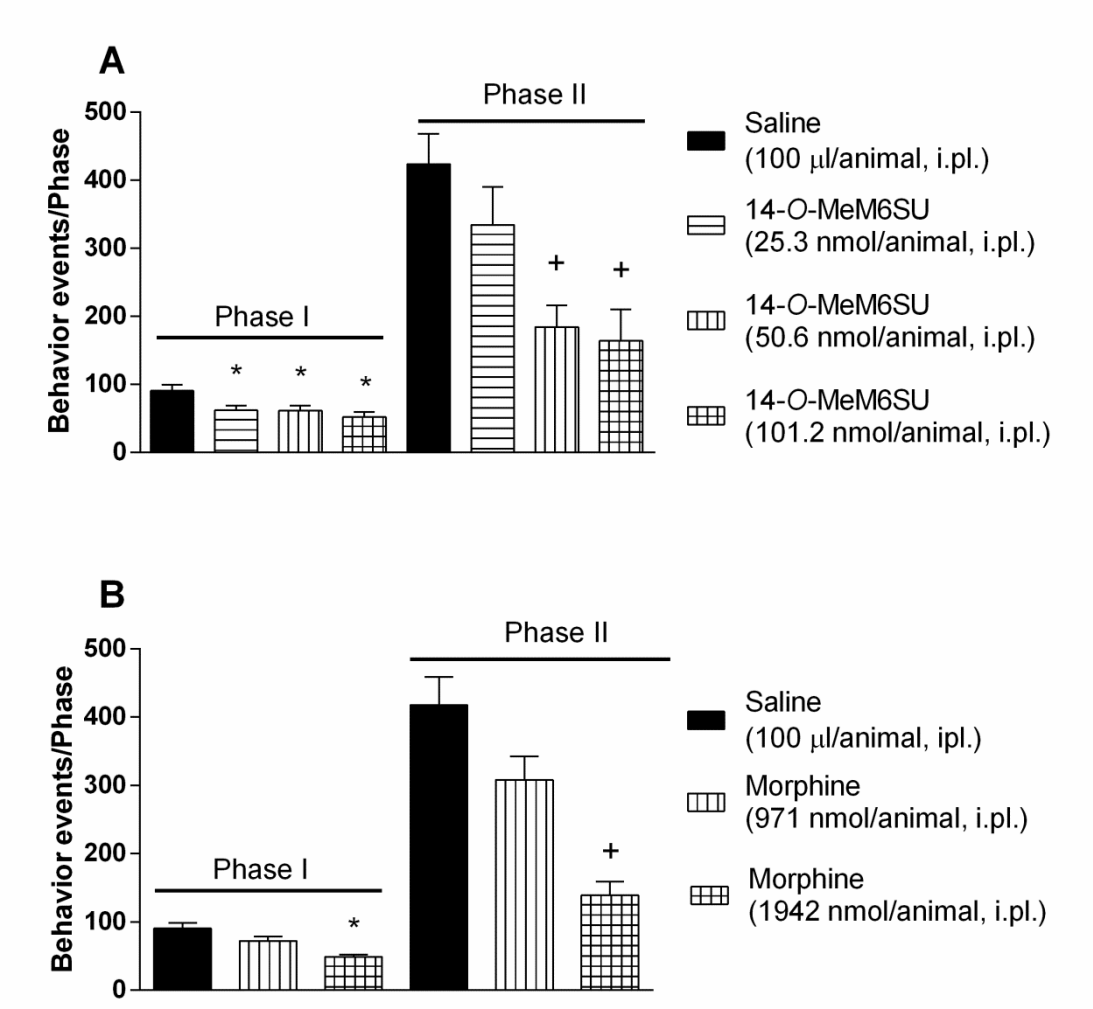

Figure 5. *The antinociceptive effect of 14-O-MeM6SU and morphine after local (i.pl.) administration in rat formalin test applying 50μl 2.5% formalin into the right hind paw, in phase I (0-10 min) and phase II (11-60 min). Each column represents the cumulative data of the given phase (number of nociceptive reactions). Drugs were administered in a 100 μl/animal volume.*

Each value represents the mean ± SEM. (n=4-11)

*: significant difference vs. vehicle treated group in Phase I ($p<0.05$)

+: significant difference vs. vehicle treated group in Phase II ($p<0.05$)

(one-way ANOVA followed by Fisher's LSD post hoc test, n= 4-11)

DOI:10.14753/SE.2019.2219

4.1.2.2. NAL-M antagonism on systemic 14-*O*-MeM6SU or morphine antinociception

Co-administered opioid antagonist NAL-M (10.6 μmol/kg, s.c.) abolished the antinociceptive effect of s.c. 506 nmol/kg 14-*O*-MeM6SU in both phases (Fig. 6., panel A). On the other hand, NAL-M failed to antagonize the effect of 1012 nmol/kg 14-*O*-MeM6SU in phase I, yet partially affected the antinociceptive effect in phase II (Fig. 4, panel A).

In case of morphine, NAL-M antagonized its antinociceptive effect of (15 538 nmol/kg, s.c.) morphine in phase I (Fig. 6., panel B). In phase II NAL-M only partially antagonized the antinociceptive action of the same dose of morphine.

4.1.2.3. The antinociceptive effects of 14-*O*-MeM6SU or morphine after administration into the contralateral paw

Intraplantar (i.pl.) administration of 50.6 nmol/rat 14-*O*-MeM6SU into contralateral paw failed to affect formalin-induced pain in ipsilateral paw in either phases (Fig. 7., panel A), though it was effective when administered into ipsilateral (formalin treated) paw (Fig. 5.). At a higher dose (101.2 nmol/rat) 14-*O*-MeM6SU showed antinociception only in phase I. However, 1942 nmol/animal morphine injected into the contralateral paw (i.pl.) produced antinociceptive effect on both phases (Fig. 7., panel B). This effect is in accordance with that obtained following s.c. 7769 nmol/kg (Fig. 4., panel B).

The antagonist effect of NAL-M (section 4.1.2.2.) and these data indicate the presence of peripheral antinociceptive component of both compounds, but only 14-*O*-MeM6SU showed action that was of completely peripheral origin at the dose of 506 nmol/kg.

DOI:10.14753/SE.2019.2219

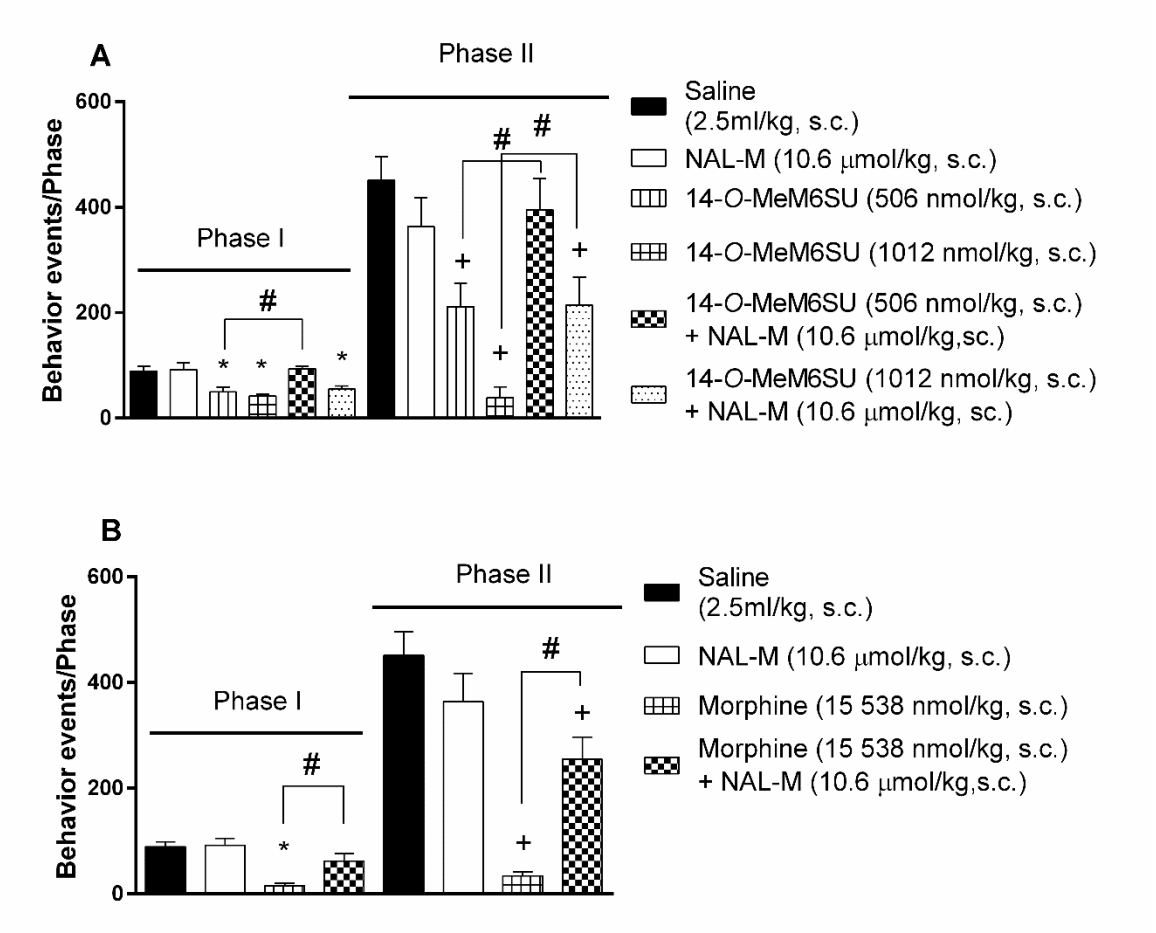

Figure 6. *The antagonist effect of s.c. co-administered NAL-M (10.6 μmol/kg) on the antinociceptive effect of s.c. 14-O-MeM6SU (panel A) and morphine (panel B) in rat formalin test. Each column represents the cumulative data of the given phase (number of nociceptive reactions). Drugs were administered in a 2.5 ml/kg volume.*

Each value represents the mean ± SEM.

*: significant difference vs. vehicle treated group in Phase I ($p<0.05$)

+: significant difference vs. vehicle treated group in Phase II ($p<0.05$)

#: significant difference between the signed groups ($p<0.05$)

(one-way ANOVA followed by Fisher's LSD post hoc test, n= 5-11)

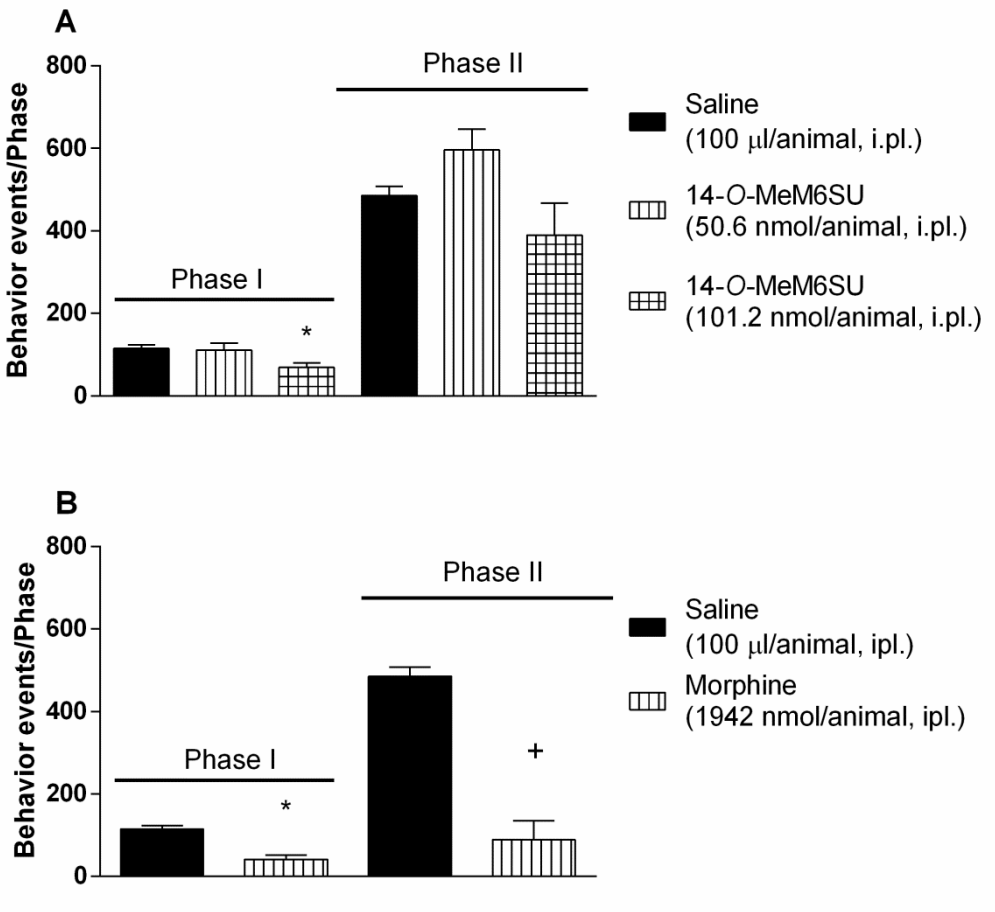

Figure 7. *The antinociceptive effect of 14-O-MeM6SU (panel A) and morphine (panel B) after administration into the contralateral paw. Drugs were administered in a 100 μl/animal volume. Each column represents the cumulative data of the given phase (number of nociceptive reactions).* Each value represents the mean ± SEM.

*: significant difference vs. vehicle treated group in Phase I ($p<0.05$)

+: significant difference vs. vehicle treated group in Phase II ($p<0.05$)

(one-way ANOVA followed by Fisher's LSD hoc test in the case of 14-*O*-MeM6SU and unpaired t-test with two-tailed p value in the case of morphine; n= 4-5)

DOI:10.14753/SE.2019.2219

4.2. Subchronic inflammatory pain model: CFA induced inflammation in rats

4.2.1. Antinociceptive effects of 14-*O*-MeM6SU and M6SU after systemic administration in CFA model in Randall-Selitto test

CFA treatment reduced the PPT to approximately 65 ± 2 % (n=70) and 70 ± 2 % (n=55) of baseline response on the 4th and 7th days after the treatment, respectively. The s.c. doses of 14-*O*-MeM6SU (32 - 1012 nmol/kg) and M6SU (137 – 8758 nmol/kg) were tested for their analgesic actions in CFA-induced inflammatory pain (Fig. 8). M6SU produced peak analgesic effect at 30 min, while 14-*O*-MeM6SU did so at 60 min (Table 3, Fig. 8.). The antinociceptive action of the test compounds was significantly more marked in inflamed paws compared to non-inflamed paws in doses presented in Fig. 9. The analgesic effects of s.c. 14-*O*-MeM6SU and M6SU did not differ between inflamed and non-inflamed paws at doses over 506 nmol/kg and 4379 nmol/kg, respectively. The antinociceptive actions of s.c. 14-*O*-MeM6SU (126, 253 and 506 nmol/kg) and M6SU (547, 1095 and 2189 nmol/kg) were further tested for their peripheral analgesic actions in inflamed paws in separate experiments (see chapter 4.2.2.).

Table. 3. *Antinociceptive potencies of 14-O-MeM6SU and M6SU in inflamed and non-inflamed paws in the Randall-Selitto test in rats after s.c. administration.*

Compound	ED_{50} (95% confidence limits)					
	30 min		60 min		120 min	
	L	R	L	R	L	R
14-*O*-MeM6SU	305 (155-601)	86 (33-220)	388 (201-751)	45[a] (22-92)	792 (388-1620)	110 (47-258)
M6SU	2313 (1226-4364)	292[a] (114-749)	2931 (1434-5998)	520 (222-1216)	8920 (1100-72318)	1651 (583-4673)

ED_{50} (nmol/kg, s.c.); [a]Peak of effect; L: left paw (non-inflamed paw); R: right paw (inflamed paw)

DOI:10.14753/SE.2019.2219

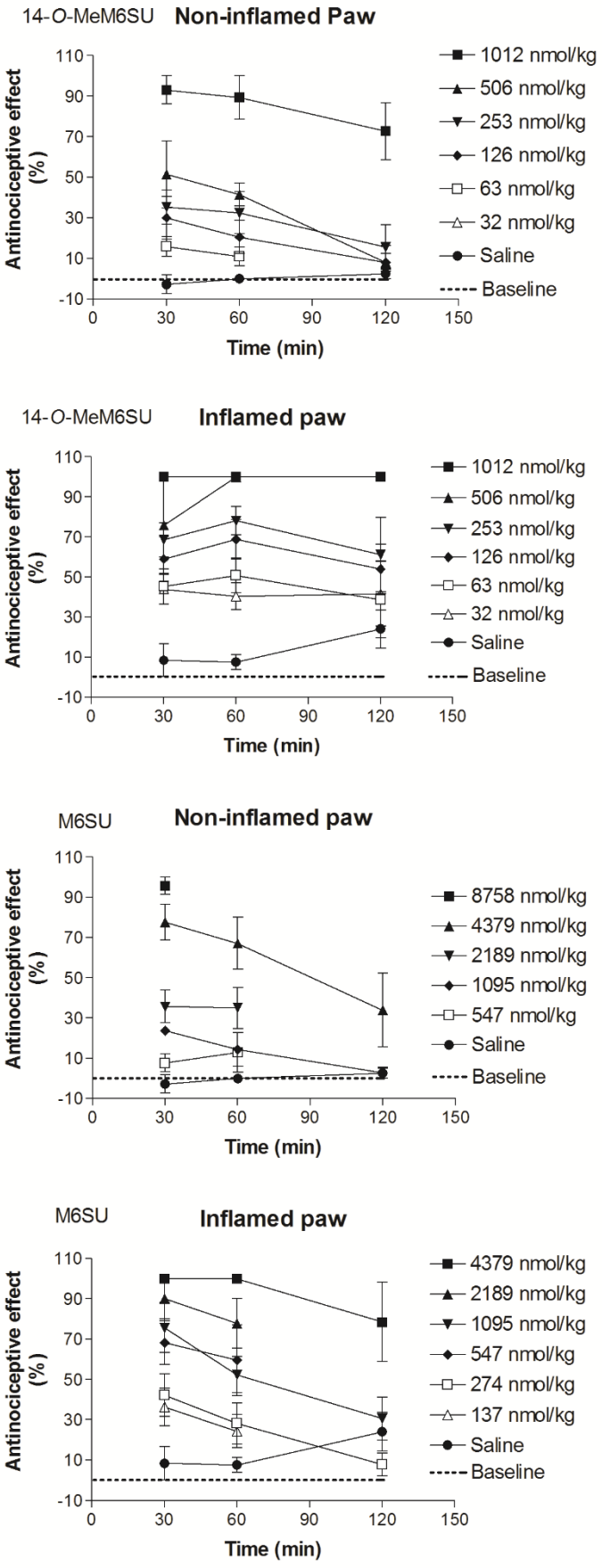

Figure 8.

Time-course of the antinociceptive effect of s.c. administered 14-O-MeM6SU and M6SU in non-inflamed and inflamed rat hind paws. Drugs were delivered in a volume of 5ml/kg body weight.

Each point represents the mean ± SEM (n= at least 4).

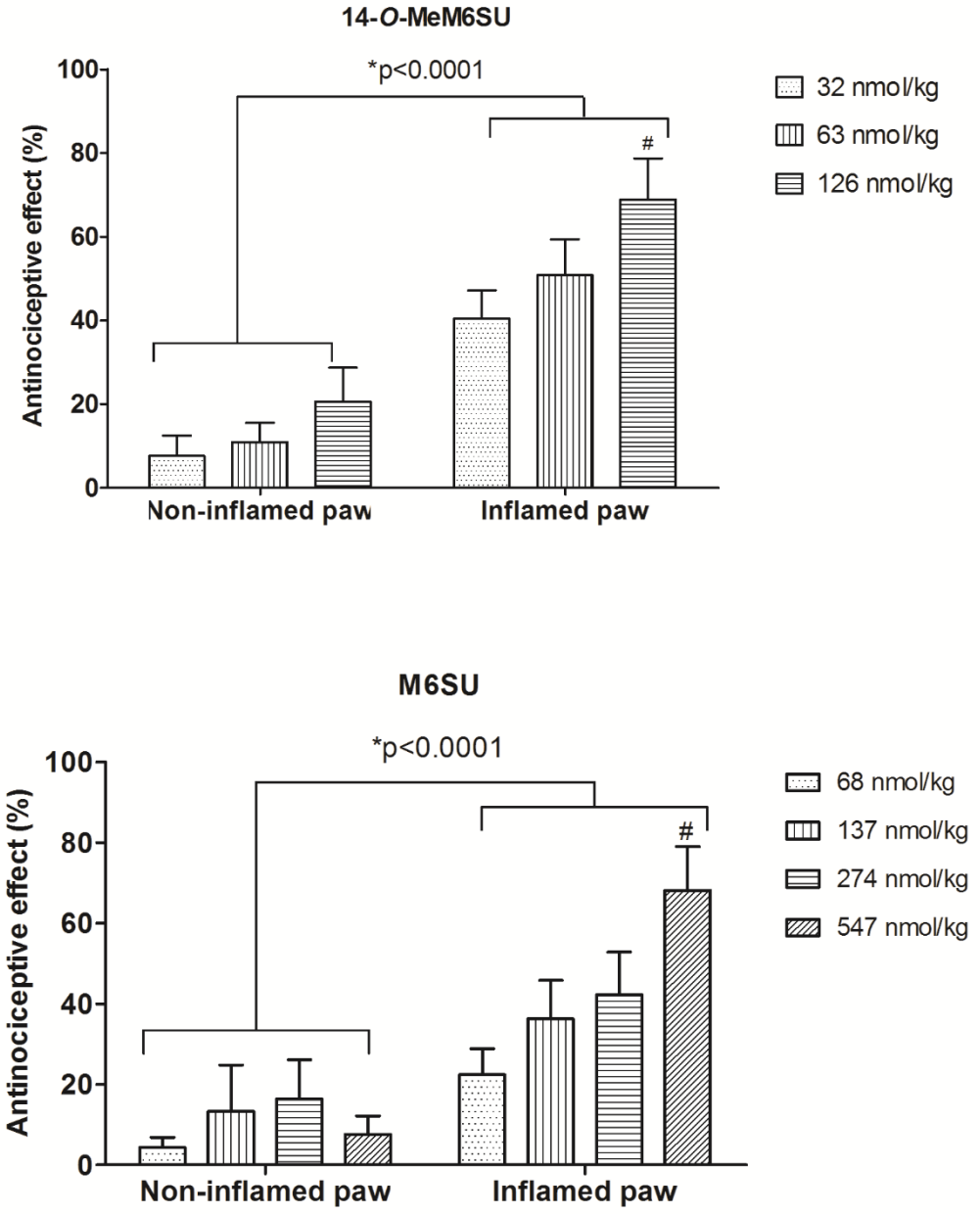

Figure 9. *The antinociceptive effects of s.c. administered 14-O-MeM6SU and M6SU. Drugs were delivered in a volume of 5 ml/kg body weight. Data were obtained 30 min after injection of M6SU and 60 min after injection of 14-O-MeM6SU. Each value represents the mean ± SEM.*

#: significant differences between the lowest and highest doses. ($p<0.05$)

(Two-way ANOVA with Bonferroni's post hoc test, n= 5-12)

DOI:10.14753/SE.2019.2219

4.2.2. Antagonist effects of s.c. and i.pl. NAL-M on the antinociceptive actions of s.c. 14-*O*-MeM6SU or M6SU

The analgesic effects produced by s.c. 126 nmol/kg 14-*O*-MeM6SU and 547 nmol/kg M6SU were antagonized both by s.c (21.3 μmol/kg) or i.pl. (0.43 μmol/rat) administration of NAL-M. No differences in PPTs were observed between saline and NAL-M injected animals (Fig. 10.). In an other experiment set we also tested the antagonist effect of s.c. NAL-M (21.3 μmol/kg) on the antinociceptive effects produced by s.c. 253 or 507 nmol/kg 14-*O*-MeM6SU and 1095 or 2189 nmol/kg M6SU (Fig. 11.). In these experiments, NAL-M partially reversed the antinociceptive effect of 14-*O*-MeM6SU and totally the antinociceptive effect of 1095 nmol/kg M6SU. However, NAL-M failed to reverse the antinociceptive effect of 2189 nmol/kg M6SU (Fig. 11.).

These results indicate the contribution of CNS to the total antinociception of systemically administered test compounds at higher doses.

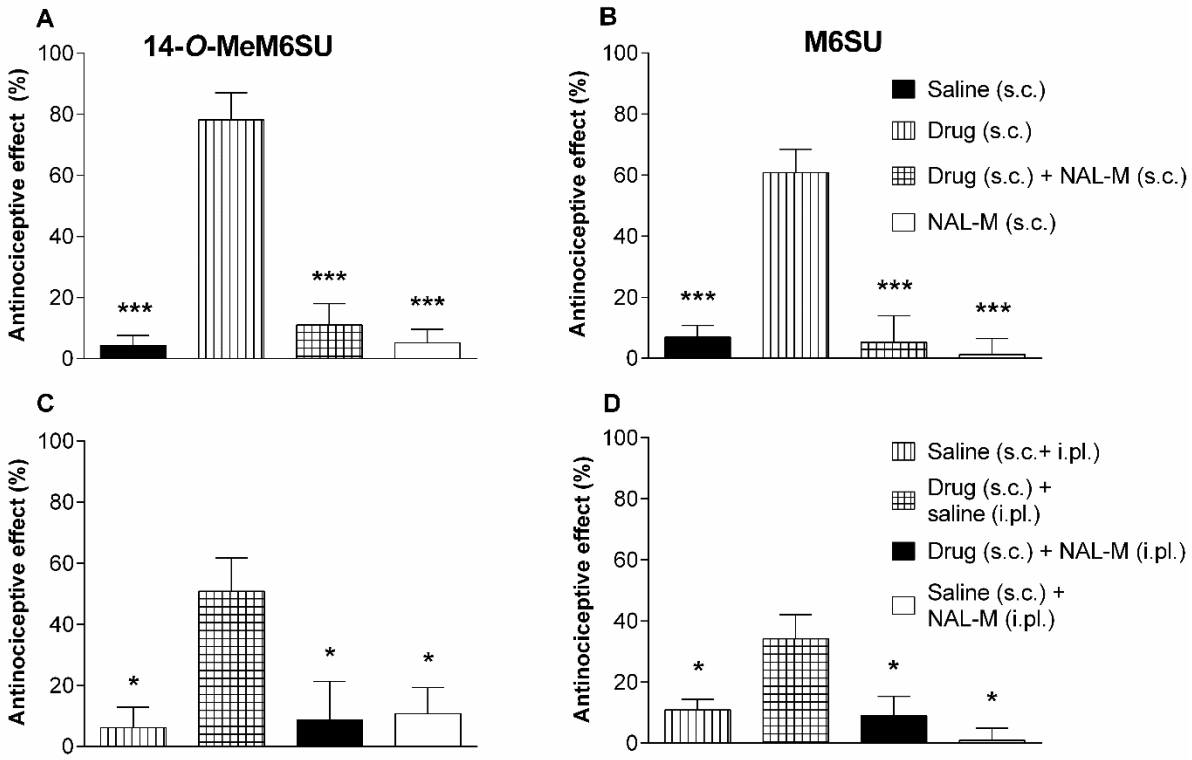

Figure 10. *The antagonist effect of (21.3 µmol/kg, s.c.) (panel A, B) or (0.43 µmol/rat, i.pl.) (panel C, D) naloxone methiodide (NAL-M) against s.c. antinociceptive effects of 14-O-MeM6SU (126 nmol/kg) and M6SU (547 nmol/kg) in rat inflamed paws. Drugs were delivered in a volume of 5 ml/kg body weight and 100 µl/rat for s.c. and i.pl. administration, respectively. Each data point was obtained 30 min after injection of M6SU, saline or NAL-M and 60 min after injection of 14-O-MeM6SU, saline or NAL-M.*

Each value represents the mean ± SEM

*: significant difference versus the effect of agonist in inflamed paw

(*: $p<0.05$; ***: $p< 0.001$)

(one-way ANOVA, Newman-Keuls post hoc test, n= 6-35)

DOI:10.14753/SE.2019.2219

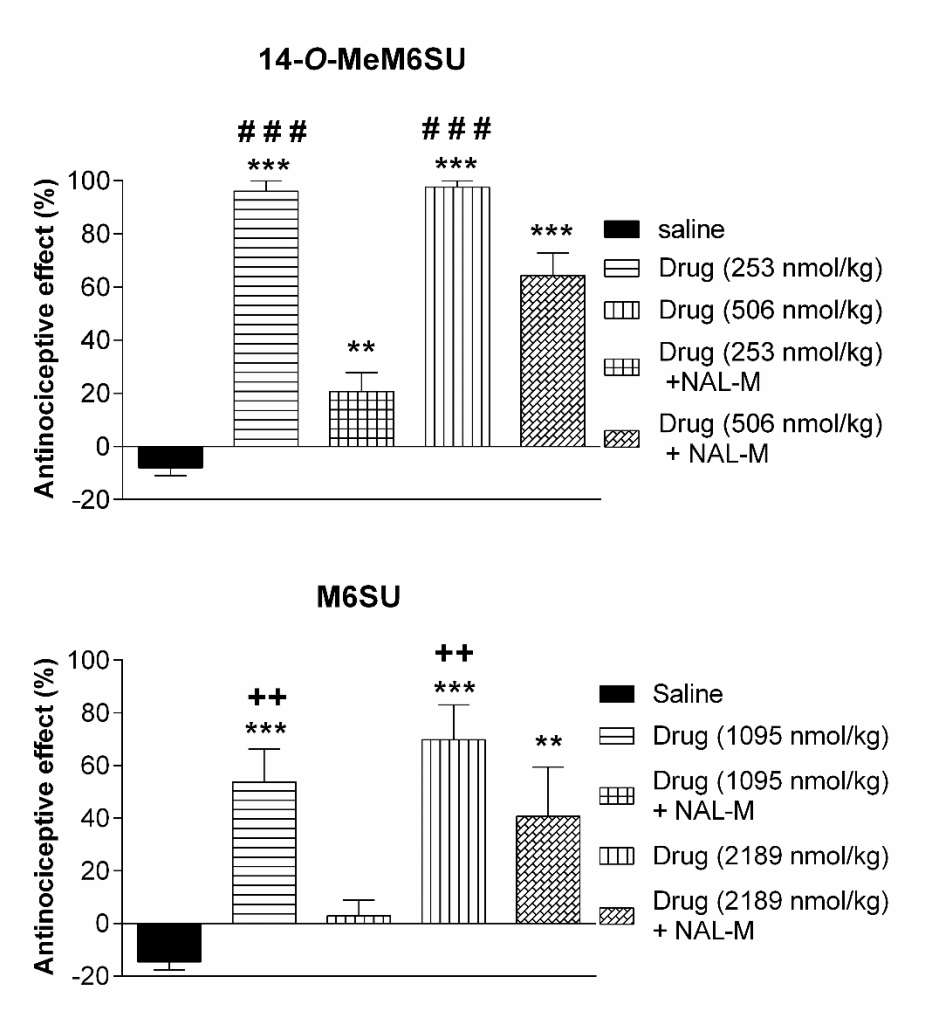

Figure 11. *The antagonist effect of (21.3 µmol/kg, s.c.) naloxone methiodide (NAL-M) against s.c. antinociceptive effects of 14-O-MeM6SU (253 and 506 nmol/kg) and M6SU (1095 and 2189 nmol/kg) in rat inflamed paws. Drugs were delivered in a volume of 5 ml/kg body weight. Each data point was obtained 30 min after injection of M6SU or saline and 60 min after injection of 14-O-MeM6SU or saline.*

Each value represents the mean ± SEM.

*: significant difference versus saline in inflamed paw (**: p<0.01; ***: p< 0.001)

#: significant difference versus 14-*O*-MeM6SU + NAL-M (###: p< 0.001)

+: significant difference versus M6SU (1095 nmol) + NAL-M (++: p<0.01)

(one-way ANOVA, Newman-Keuls post hoc test, n= 4-9)

4.3. Neuropathic pain model: diabetic polineuropathy

4.3.1. The development of diabetic symptoms and neuropathic pain (allodynia) in STZ treated rats

Significant increase in blood glucose concentration of STZ-treated rats compared to vehicle treated animals was achieved 72 h following intraperitoneal STZ injections. This hyperglycaemia was maintained during the entire experimental period indicating the development of diabetes (Fig. 12, panel A).

Water intake of STZ treated rats was significantly increased in comparison with the vehicle treated group 48 h following treatment (Fig. 12., panel B). The food consumption of rats with hyperglycaemia reached a significant increase 5 days after treatment and this increase was kept during the experimental period (data not shown). Significant decrease in body weights of diabetic rats compared to age matched rats was also observed. Therefore, weight matched non-diabetic rats were used for a comparison when the antinociceptive effects of test compounds were measured.

No differences were found between the rates of gastric emptying in 12-weeks diabetic (80 ± 2%, n=23) and non-diabetic weight matched rats (82 ± 2%, n=20), whereas 0.1 mg/kg clonidine, an alpha$_2$-adrenoceptor agonist used as a positive control, markedly delayed the emptying in weight-matched control animals (55 ± 2%, n=7, $p<0.01$ vs. saline-treated rats).

Figure 12 panel C depicts significant decreases in paw withdrawal thresholds in the DPA test 3 weeks following STZ injection indicating the development of mechanical allodynia, which is a key symptom in the diagnosis of neuropathic pain. At the 9th or 12th week, the nociceptive thresholds of diabetic animals were significantly lower compared to the baseline measured prior to STZ-treatment, indicating the peak of allodynia. No significant difference in developed allodynia was observed between the 9th and 12th week following STZ-treatment. Therefore, in our subsequent studies the analgesic action of test compounds, as well as MOR functioning, were analysed in the 9th and 12th weeks after induction of diabetes.

DOI:10.14753/SE.2019.2219

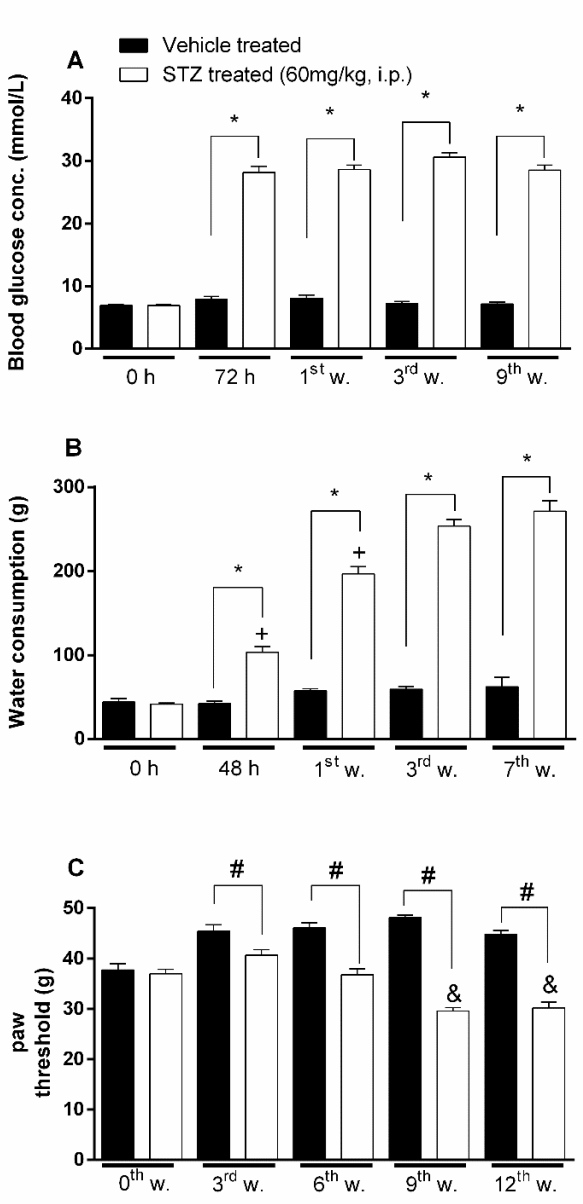

Figure 12. *The changes in blood glucose levels in mmol/ml (panel A, n=10-81), water consumption in grams (panel B, n=11-32) and hind paw withdrawal thresholds (panel C, n=11-67) in grams prior to and after STZ- or vehicle treatments.*

Each value represents the mean ± SEM. w: week

*: significant difference between the signed groups ($p<0.05$)

#: significant difference between the signed groups ($p<0.05$)

+: significant difference vs. all other groups ($p<0.05$)

&: significant difference vs. all other groups except the 12th or 9th week diabetic animals (in the case of the 9th and 12th week, respectively; $p<0.05$)

(one way ANOVA followed by Newman-Keuls post hoc test)

DOI:10.14753/SE.2019.2219

4.3.2. The impairment of the antinociceptive effect of systemic 14-*O*-MeM6SU and morphine in advanced diabetes in rats

Prior to detailed analysis of the antinociceptive and anti-allodynic effects, the peak antinociceptive effects of 14-*O*-MeM6SU and morphine were established (60 min for 14-*O*-MeM6SU and 30 min for morphine). Thus, these times of peak effects were chosen for further analysis in the entire pain study by DPA.

The calculated ED_{30} values of 14-*O*-MeM6SU were 434 nmol/kg and 335 nmol/kg for diabetic and non-diabetic animals, respectively. In the case of morphine the ED_{30} values were 20692 nmol/kg and 6589 nmol/kg for diabetic and non-diabetic animals, respectively. Based on the calculated ED_{30} values ($ED_{30diabetic}/ED_{30non\text{-}diabetic}$) there was no significant change in the antinociceptive effect of 14-*O*-MeM6SU in diabetic animals. On the other hand, morphine was 7 times less effective in diabetic, than in non-diabetic ones. In addition, these data demonstrate, that 14-*O*-MeM6SU displayed a 48 times higher potency than morphine in diabetic conditions based on the compared ED_{30} values (Fig. 13).

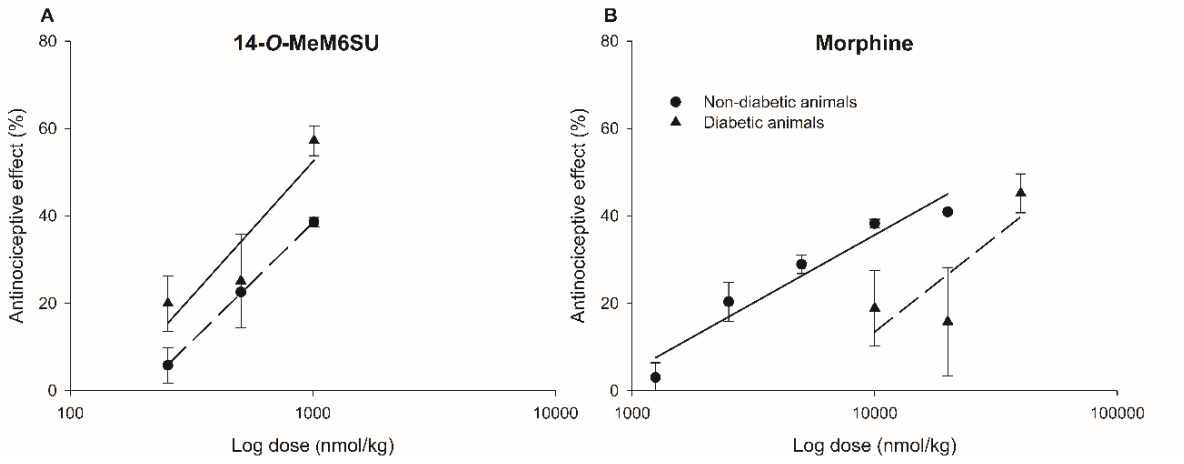

Figure 13. *Dose-response curves of 14-O-MeM6SU and morphine in diabetic and non-diabetic animals obtained with DPA.* Data is represented as mean ± SEM (n= 5-10).

DOI:10.14753/SE.2019.2219

4.3.3. The antiallodynic effects of systemic 14-*O*-MeM6SU and morphine in diabetic rats

The following data were obtained on the 9th and 12th weeks after STZ treatment that is, 6 and 9 weeks after the significant appearance of allodynia, a major sign of painful diabetic neuropathy.

Subcutaneous 14-*O*-MeM6SU (253, 506 and 1012 nmol/kg) and morphine (10000, 20000 and 40000 nmol/kg) were tested for their antiallodynic actions in diabetic rats with allodynia (Fig. 14.). 14-*O*-MeM6SU in all tested doses significantly ameliorated the allodynia (Fig. 14., panel A), whereas morphine only at the highest dose (40000 nmol/kg) attenuated it significantly (Fig. 14., panel B).

When we compared the effects of 14-*O*-MeM6SU and morphine doses in diabetic and non-diabetic rats, morphine in lower doses (from 1250 nmol/kg) induced significant antinociceptive actions in weight match rats. 14-*O*-MeM6SU at the 253 nmol/kg dose, which already produced antiallodynic effects in diabetic rats, failed to show any significant antinociceptive action in weight match animals. However, at higher doses (506 nmol/kg) it produced antinociception in weight match rats. This means that 14-*O*-MeM6SU, but not morphine did produce antiallodynic effects in a dose devoid of antinociception in naïve rats. This effect might be attributed to the decrease in number of opioid receptors, which in turn affects the action of morphine but not that of 14-*O*-MeM6SU.

We analysed the lowest antiallodynic dose of 14-*O*-MeM6SU and morphine (253 and 40000 nmol/kg, respectively) at 12th weeks advanced diabetic rats, also. Both compounds produced antiallodynic effects in accordance with 9th week data at the same doses (data not shown).

DOI:10.14753/SE.2019.2219

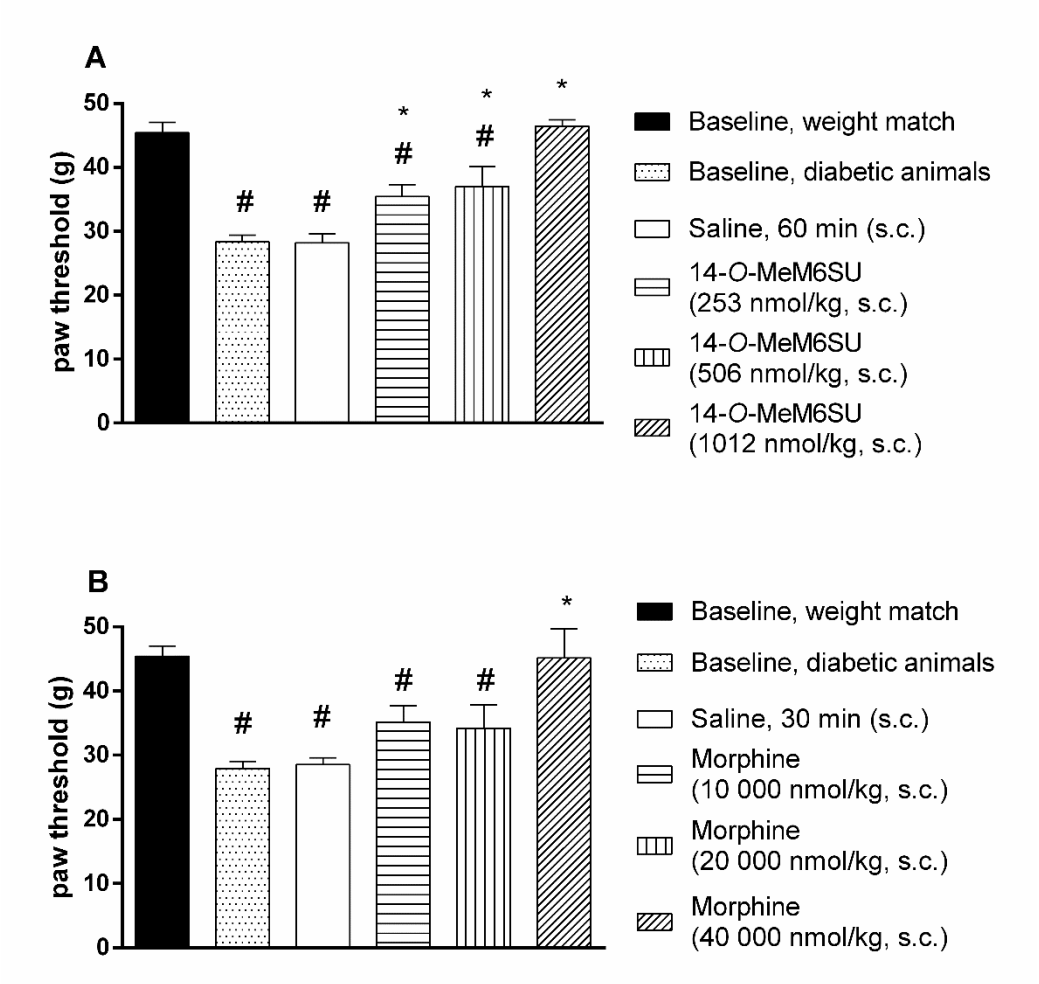

Figure 14. *The systemic analgesic effects of 14-O-MeM6SU (panel A, n=5-29) and morphine (panel B, n=5-34) in STZ treated diabetic rats with neuropathy on DPA test following systemic (s.c.) administration at 9th week. Data were obtained 60 min after the injection of 14-O-MeM6SU and 30 min in the case of morphine injection (time of peak effect). Drugs were administered in a 2.5ml/kg volume.*

Each value represents the mean in grams ± SEM.

*: significant difference vs. diabetic baseline or saline treated group ($p<0.05$)

#: significant difference vs. weight match control group ($p<0.05$)

(one way ANOVA followed by Newman-Keuls post hoc test)

4.3.4. The antagonist effect of co-administered NAL-M on the systemic analgesic effect of 14-*O*-MeM6SU or morphine in diabetic rats

The antagonist action of NAL-M (10.6 μmol/kg, s.c.) was tested against s.c. 14-*O*-MeM6SU and morphine doses producing antiallodynic effect. In these experiments NAL-M failed to alter the antiallodynic action of test compounds (Fig. 15), indicating the contribution of the central nervous system. NAL-M alone had no effect (n= 5, data not shown).

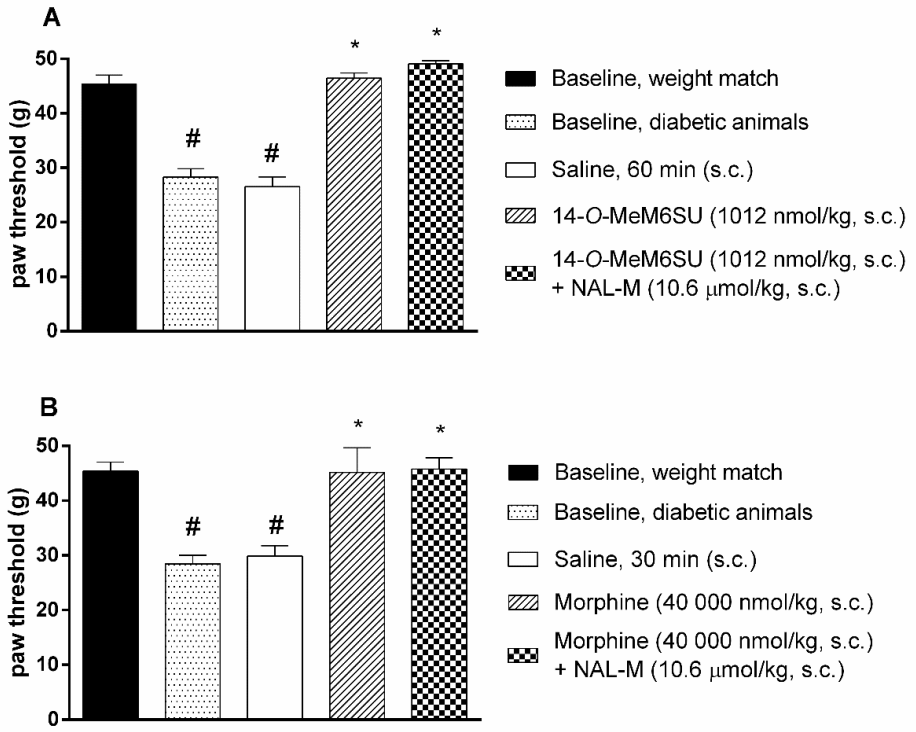

Figure 15. *The antagonist effect of s.c. co-administered NAL-M (10.6 μmol/kg) on the analgesic effect of s.c. 14-O-MeM6SU (panel A, n=5-20) and morphine (panel B, n= 5-20) in STZ treated neuropathic animals in doses that reversed the allodynia and elevated PPT on diabetic and weight match animals. Data were obtained 60 min after 14-O-MeM6SU and 30 min after morphine injection. Drugs were administered in a 2.5ml/kg volume. NAL-M failed to antagonize the effect of the compounds.*

Each value represents the mean in grams ± SEM.

*: significant difference vs. saline treated diabetic group ($p<0.05$)

#: significant difference vs. weight match control group ($p<0.05$)

(one way ANOVA followed by Newman-Keuls post hoc test)

4.4. Side effect profiles of test products

4.4.1. Inhibitory effect of systemic 14-*O*-MeM6SU, M6SU and morphine on gastrointestinal transit in mice

S.c. administered 14-*O*-MeM6SU, M6SU and morphine in dose-dependent manner inhibited the gastrointestinal transit of charcoal. The calculated ID_{50} (nmol/kg) and confidence intervals were 250 (205-305), 325 (70-1517) and 2228 (666-7455) for 14-*O*-MeM6SU, M6SU and morphine, respectively. These results indicate that the test compounds inhibit the gastrointestinal transit in antinociceptive doses.

4.4.2. Respiratory effects of 14-*O*-MeM6SU and M6SU compared to morphine in awake unrestrained rats

The effects of 14-*O*-MeM6SU (253 nmol/kg), M6SU (1095 nmol/kg) and morphine (7776 nmol/kg) on rat pulmonary parameters were analyzed. None of the respiratory parameters determined by unrestrained WBP (f, MV, TV, Ti, Te, PIF, PEF, RV) showed significant differences between the saline-treated control or drug-treated groups 30 and 60 minutes following their s.c. injection. None of the drugs caused any sedative effect, the animals were at rest by the end of the measurements, but when the WBP chambers were opened they became vivid.

4.4.3. Sedative effects of test compounds

The effect of systemic 14-*O*-MeM6SU and M6SU on thiobutabarbital-induced sleeping: Thiobutabarbital (153 μmol/kg, i.v.) produced a sleeping time of 10 ± 3, 10 ± 5 and 8 ± 4 min in the presence of s.c. saline, 14-*O*-MeM6SU (126 nmol/ kg) and M6SU (547 nmol/ kg), respectively (Fig. 16.). At higher agonist doses the sleeping time was longer than that of saline (Fig. 16).

The effect of systemic 14-*O*-MeM6SU and morphine on isoflurane induced sleeping: The impact of 14-*O*-MeM6SU and morphine on rat sleeping time initiated by inhaled isoflurane was investigated. Subcutaneous 506 nmol/kg but not 1012 nmol/kg of 14-*O*-MeM6SU failed to affect the sleeping time in rats evoked by inhaled isoflurane (Fig. 17.). Morphine significantly prolonged the sleeping time in doses of 7769 nmol/kg and 15538 nmol/kg (Fig. 17.). Longer sleeping time evoked by test compounds compared to saline indicates the CNS effects (sedation).

DOI:10.14753/SE.2019.2219

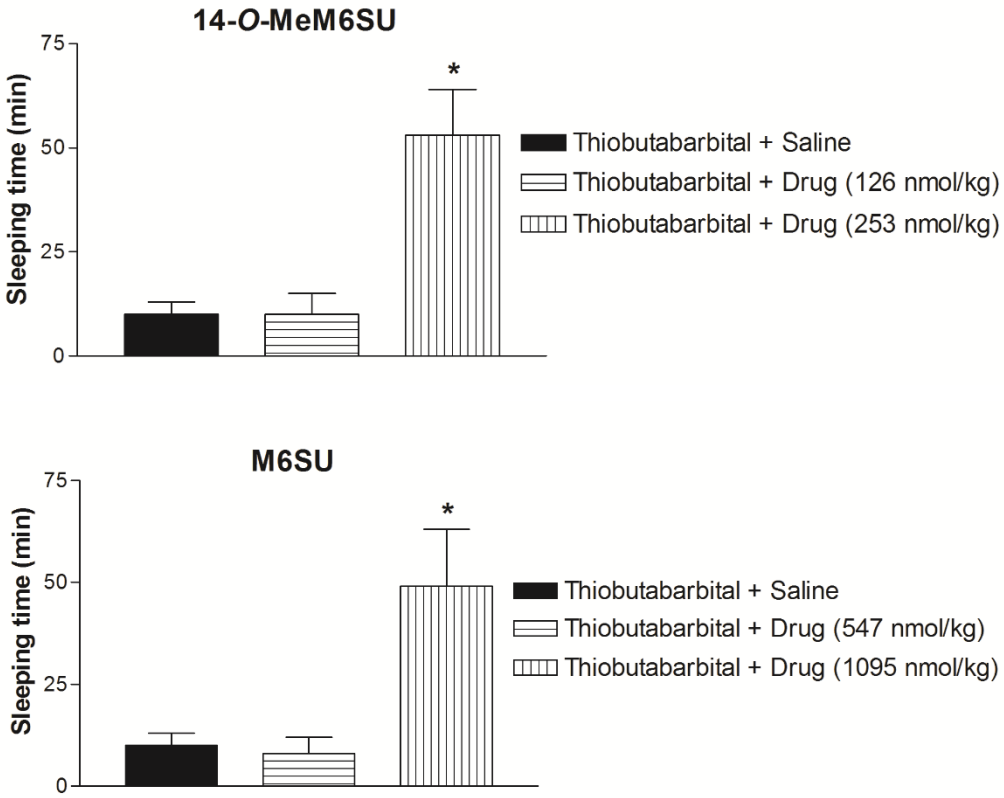

Figure 16. *The effect of s.c. 14-O-MeM6SU and M6SU on thiobutabarbital (153 µmol/kg, i.v.) induced sleeping time*

Each value is represented as mean ± SEM,

*: significant difference versus saline ($p<0.05$)

(one-way ANOVA followed by Fisher's LSD post hoc test, n=5-10)

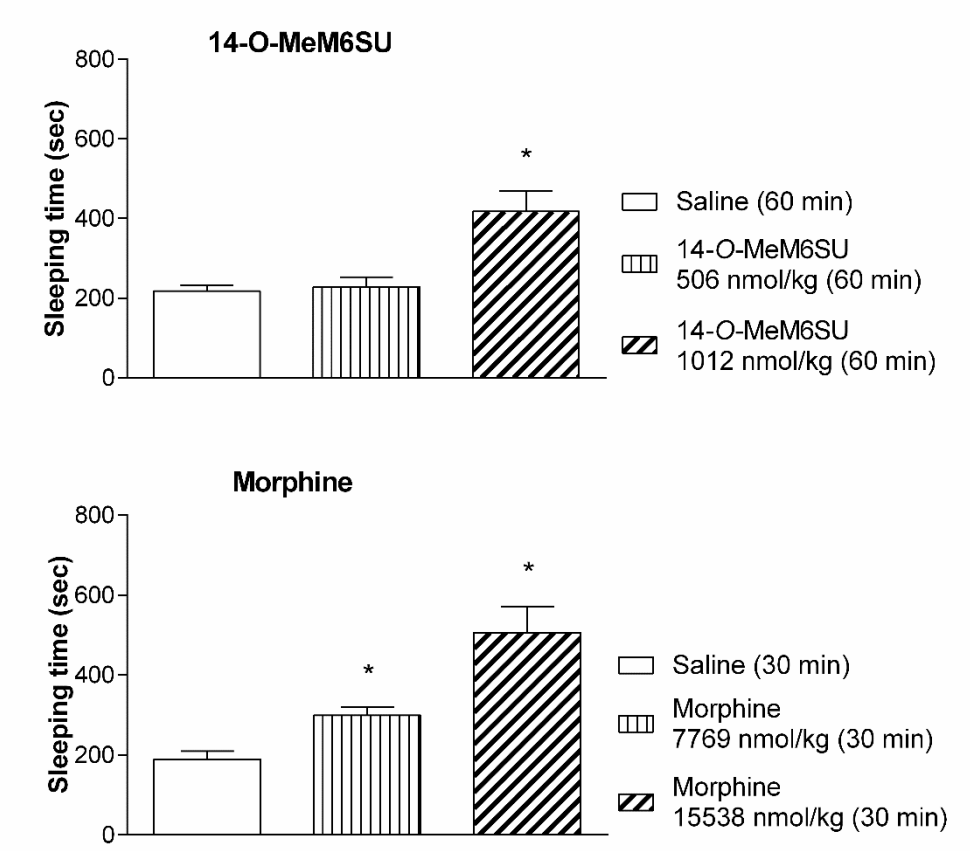

Figure 17. *Sleeping time of animals anaesthetized with inhaled isoflurane. Data were obtained 60 min after the injection of 14-O-MeM6SU and 30 min in the case of morphine injection (times of peak effect). Drugs were administered in a 2.5 ml/kg volume.*

Each value represents the mean ± SEM.

*: significant difference vs. saline treated control group ($p<0.05$)

+: significant difference vs. saline treated control group ($p<0.05$)

(one way ANOVA followed by Fisher's LSD post hoc test, n= 4-10)

4.4.4. Analgesic tolerance of 14-*O*-MeM6SU compared to morphine in mouse tail-flick test

The dose-effect relationships for s.c. administered 14-*O*-MeM6SU and morphine were determined in the mouse tail-flick test in the dose range 0.25–2 μmol/kg and 2.5–20 μmol/kg in 3 days saline treated mice, respectively. As shown in Fig. 18. and Table 4. s.c. administered 14-*O*-MeM6SU achieved peak analgesic effect at 60 min while morphine at 30 min. The calculated ED_{50} values reveal that 14-*O*-MeM6SU is a 17-fold more potent analgesic agent than morphine in mouse tail-flick test.

Table 4. *Antinociceptive potencies of 14-O-MeM6SU and morphine in mouse tail-flick test after 30 or 60 min of s.c. administration in saline, morphine or 14-O-MeM6SU treated mice.*

Compound	**Time (min)**	**ED_{50} (μmol/kg, s.c.)**				
		Saline treated	Morphine treated	Shift	14-*O*-MeM6SU treated	Shift
Morphine	30	8.01 (6.54-9.82)	27.34 (20.25–36.91)	3.41	46.95 (33.24–66.30)	5.86
14-*O*-MeM6SU	30	0.52 (0.37-0.74)	0.83 (0.67–1.04)	1.59*	1.46 (1.15–1.84)	2.78
	60	0.47 (0.35-0.64)	0.95 (0.74–1.24)	2.02	1.58 (1.29–1.93)	3.34

Shift: $ED_{50;\ treated}/ED_{50;\ control\ (saline)}$

*: not significant compared to saline (no overlap in confidence intervals)

3 days treatment of mice with 200 μmol/kg s.c. morphine resulted in a 3.41-fold increase of the morphine ED_{50} value after systemic administration. The calculated ED_{50} value for morphine in saline, morphine and 14-*O*-MeM6SU treated mice are shown in Fig. 18 and Table 4. 3 days treatment with morphine resulted in a 3.41− and a 2.02-fold decrease of the antinociceptive effect of morphine and 14-*O*-MeM6SU, respectively (Table 4.). Treatment for 3 days with 12 μmol/kg, 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.

The calculated ED_{50} values and the rightward shifts of the dose response curves are shown in Fig. 18. and Table 4.

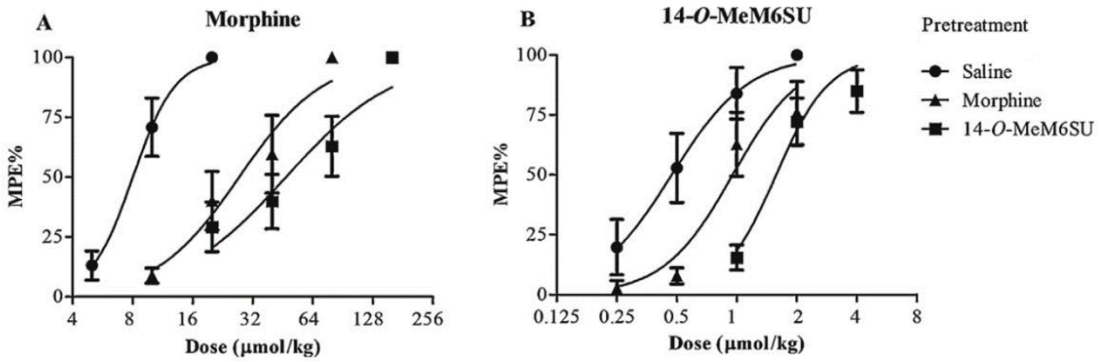

Figure 18. *Dose–response curves of morphine at 30 min (A) and 14-O-MeM6SU at 60 min (B) after treatment with saline, 200 µmol/kg morphine or 12 µmol/kg 14-O-MeM6SU twice daily for three days.* Each point represents the mean ± S.E.M. (n=5-12)

4.5. *In vitro* receptor binding assays

4.5.1. MOR immunoreactivity and binding sites in the spinal cord and DRG of diabetic and non-diabetic rats

Constant hyperglycemia resulted in apparent decrease in the number of MOR positive DRG neurons in rats developed allodynia (Fig. 19.). In parallel, there is apparent reduction in the MOR immunoreactivity within superficial layer of dorsal horn in spinal cord of diabetic rats (Fig. 19.).

Indeed, the radioligand binding assay demonstrated that the maximal of [^{3}H]DAMGO by membrane spanning MOR (B_{max}) was significantly decreased in the dorsal horn of diabetic rats (13.11±1.85 fmol/mg) compared to controls (23.55±4.36 fmol/mg) ($P < 0.001$; Fig. 20.). The dissociation constant (K_d) was 0.49 ± 0.18 for diabetic and 0.29 ± 0.17 for control rats. These data indicate no significant difference in the affinity of DAMGO to MOR between diabetic and control rats..

DOI:10.14753/SE.2019.2219

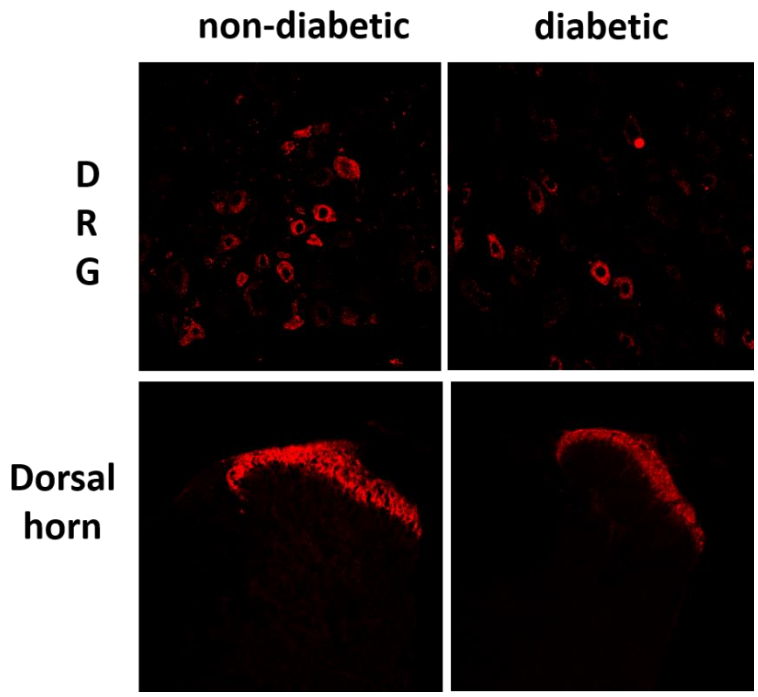

Figure 19. *Immunohistological assay shows reduction in MOR number in DRG and spinal tissues of STZ treated diabetic rats in comparison with non-diabetic animals*. (n= 5)

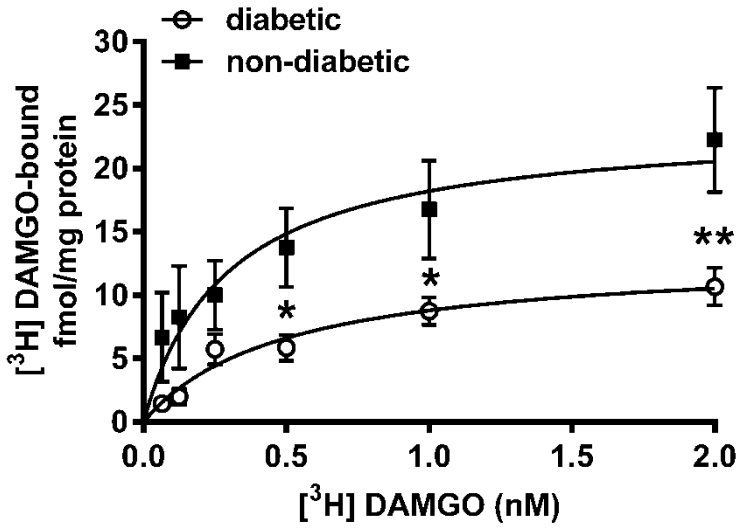

Figure 20. *[^{3}H]DAMGO binding in membrane tissues from dorsal spinal cord of diabetic and non-diabetic rats*. Data are shown as mean ± SEM.
: significant difference vs. non-diabetic control group (: $p<0.05$; **: $p< 0.01$)
(Two-way ANOVA followed by Fisher's LSD post hoc test, n= 3-5)

DOI:10.14753/SE.2019.2219

4.5.2. The G-protein coupling activity in presence of 14-*O*-MeM6SU, or morphine in spinal homogenates prepared from diabetic or control rats

MOR specific G protein coupling was measured by MOR agonist-stimulated [^{35}S]GTPγS binding assay. 14-*O*-MeM6SU produced similar G-protein coupling in spinal cord tissues prepared from STZ or vehicle treated rats after 9 or 12 weeks of treatment (Fig. 21.). On the other hand, morphine showed significantly reduced efficacy (E_{max}) of G-protein coupling in spinal cord tissues of diabetic rats. The calculated E_{max} for test compounds are presented in Table 5 and 6. The reduction in [^{35}S]GTPγS specific binding of morphine was also observed at certain concentration points of the concentration-response curves (Fig. 21.). In general, 14-*O*-MeM6SU showed significantly higher efficacy than morphine in the spinal cord samples (Table 5., 6.). Taken together, no difference exists in 14-*O*-MeM6SU-stimulated coupling but there is significant difference in morphine-stimulated coupling between diabetic and control rats.

4.5.3. The G-protein coupling activity in presence of 14-*O*-MeM6SU, or morphine in brain homogenates prepared from diabetic or control rats

MOR G-protein coupling in the presence of 14-*O*-MeM6SU or morphine was also determined in brain membrane homogenates from STZ or vehicle treated rats. Neither compounds showed significant differences in maximal efficacy (E_{max}) and ligand potency (EC_{50}) 9 or 12 weeks after STZ treatment (Table 5. and 6., Fig. 22.). Additionally, in the control brain samples, 14-*O*-MeM6SU showed significantly higher maximum efficacy compared to morphine. In the STZ treated brain samples this significance disappeared, though the tendency remained (Table 5., and 6., Fig. 22.).

DOI:10.14753/SE.2019.2219

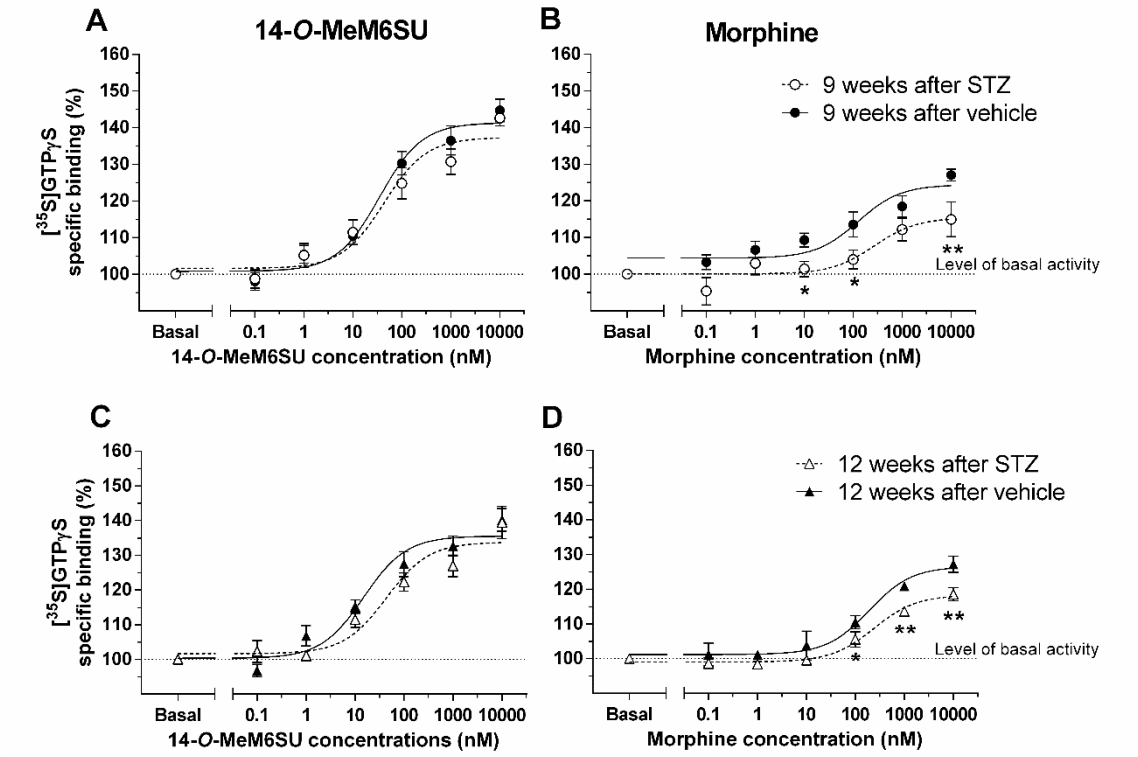

Figure 21. *Agonist activity of 14-O-MeMSU (A, C) compared to morphine (B, D) in vehicle and STZ treated rat whole* ***spinal cord*** *membrane homogenates in [^{35}S]GTPγS binding assay. Figure represent the specific binding of [^{35}S]GTPγS in the presence of increasing concentrations (0.1 nM-10 μM) of the indicated ligands. Points represent means ± S.E.M. for at least three experiments performed in triplicate. "Basal" on the x-axis indicates the basal activity of the monitored G-protein, which is measured in the absence of the compounds and also represents the total specific binding of [^{35}S]GTPγS. The level of basal activity was defined as 100% (indicated by dotted line).*

*: significant reduction of specific [^{35}S]GTPγS binding in STZ treated samples compared to control within the given concentration point with both compounds (Two-way ANOVA, uncorrected Fisher's LSD; *: $p<0.05$; **: $p<0.01$).

The calculated E_{max} and EC_{50} ± S.E.M. values are presented in Table 5. and 6.

DOI:10.14753/SE.2019.2219

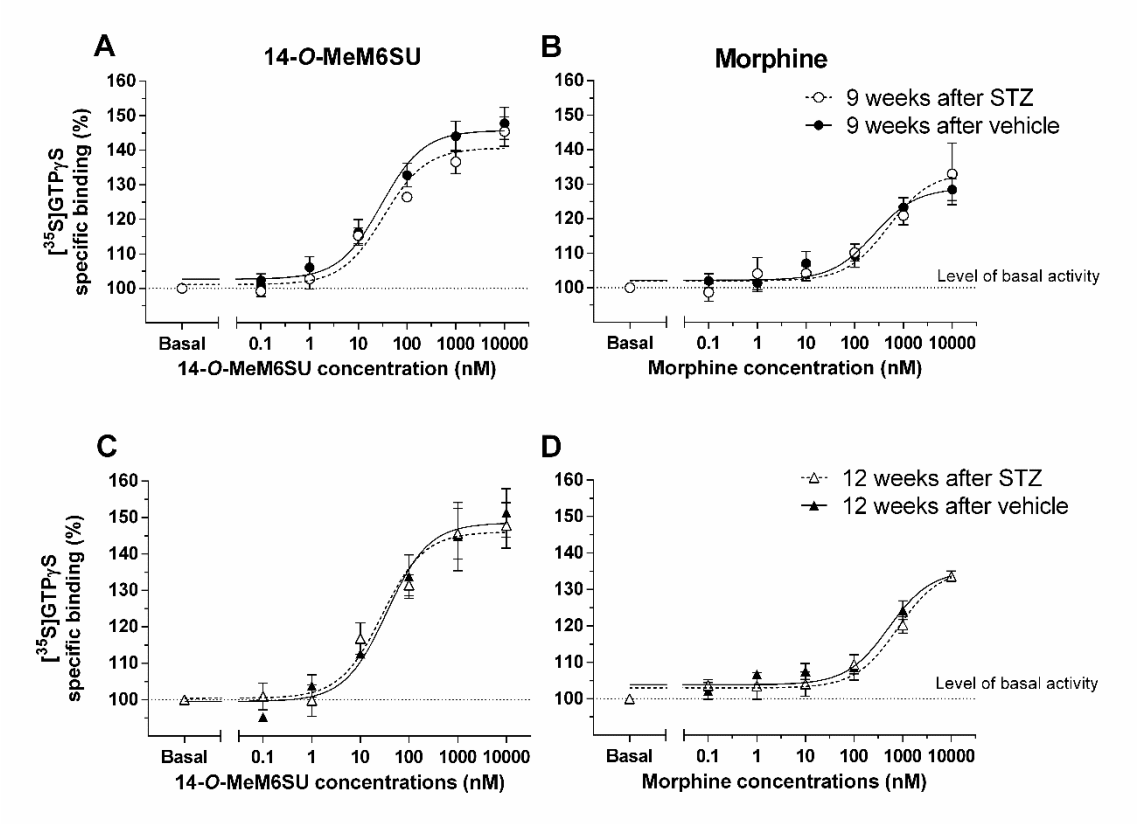

Figure 22. *Agonist activity of 14-O-MeMSU (A, C) compared to morphine (B, D) in vehicle and STZ treated rat whole* ***brain*** *membrane homogenates in [^{35}S]GTPγS binding assay 9 and 12 weeks after STZ treatment. Figure represents the specific binding of [^{35}S]GTPγS in the presence of increasing concentrations (0.1 nM-10 μM) of the indicated ligands. Points represent means ± S.E.M. for at least three experiments performed in triplicate. "Basal" on the x-axis indicates the basal activity of the monitored G-protein, which is measured in the absence of the compounds and also represents the total specific binding of [^{35}S]GTPγS. The level of basal activity was defined as 100% (indicated by dotted line).*

The calculated E_{max} and EC_{50} ± S.E.M. values are presented in Table 5. and 6.

DOI:10.14753/SE.2019.2219

Table 5. *Maximum G-protein efficacy (E_{max} ± S.E.M.) and potency (EC_{50} ± S.E.M.) of 14-O-MeM6SU, compared to morphine in vehicle and STZ treated rat brain and spinal cord performed in [^{35}S]GTPγS binding assay. Samples were taken **9 weeks after treatment**. Values were calculated according to Figure 21 and 22.*

	Morphine		**14-*O*-MeM6SU**	
Brain	**Control**	**Diabetes**	**Control**	**Diabetes**
E_{max} ± S.E.M. (%)	128.8 ± 2.65 (n=7)	133.5 ± 4.85 (n=6)	145.7 ± 2.4$^{###}$ (n=6)	140.7 ± 2.1 (n=6)
EC_{50} ± S.E.M. (nM)	N.D.[1]	N.D.[1]	29.51 ± 11.44 (n=6)	33.19 ± 19.09 (n=6)
Spinal cord				
E_{max} ± S.E.M. (%)	124.4 ± 2.09 (n=7)	115.4 ± 2.97* (n=7)	141.4 ± 2$^{###}$ (n=6)	137.3 ± 2.47^{+++} (n=5)
EC_{50} ± S.E.M. (nM)	N.D.[1]	N.D.[1]	35.08 ± 11.75 (n=6)	43.35 ± 19.94 (n=5)

*: significant difference in STZ treated samples compared to control. (*: $p<0.05$)

$^{\#}$: significant difference between morphine and 14-*O*-MeM6SU within control brain or spinal cord samples. ($^{###}$: $p<0.001$)

$^{+}$: significant difference between morphine and 14-*O*-MeM6SU within diabetic brain or spinal cord samples. ($^{+++}$: $p<0.001$)

[1]: not determined, since the EC_{50} values could not be interpreted

Unpaired t test, two-tailed P value.

DOI:10.14753/SE.2019.2219

Table 6. *Maximum G-protein efficacy (E_{max} ± S.E.M.) and potency (EC_{50} ± S.E.M.) of 14-O-MeM6SU, compared to morphine in vehicle and STZ treated rat brain and spinal cord performed in [^{35}S]GTPγS binding assay. Samples were taken **12 weeks after treatment**. Values were calculated according to Figure 21 and 22.*

	Morphine		**14-*O*-MeM6SU**	
Brain	**Control**	**Diabetes**	**Control**	**Diabetes**
E_{max} ± S.E.M. (%)	135.2 ± 2.65 (n=4)	135.6 ± 3.08 (n=4)	148.5 ± 3.59$^{\#}$ (n=4)	146.1 ± 3.22 (n=5)
EC_{50} ± S.E.M. (nM)	520 ± 222.26 (n=4)	785.24 ± 321.72 (n=4)	34.99 ± 17.74 (n=4)	27.86 ± 13.87 (n=5)
Spinal cord				
E_{max} ± S.E.M. (%)	126.5 ± 2.12 (n=4)	118.3 ± 1.41** (n=6)	135.5 ± 1.88$^{\#}$ (n=6)	133.8 ± 2.4^{+++} (n=5)
EC_{50} ± S.E.M. (nM)	N.D.[1]	N.D.[1]	14.96 ± 5.66 (n=6)	43.45 ± 21.17 (n=5)

*: indicates the significant difference in STZ treated samples compared to control. (**: P<0.01)

$^{\#}$: indicates the significant difference between morphine and 14-*O*-MeM6SU within control brain or spinal cord samples ($^{\#}$: P < 0.05)

$^{+}$: indicates the significant difference between morphine and 14-*O*-MeM6SU within diabetic brain or spinal cord samples. ($^{+++}$: P < 0.001)

[1]: not determined, since the EC_{50} values could not be interpreted

Unpaired t test, two-tailed P value.

DOI:10.14753/SE.2019.2219

5. Discussion

5.1. Inflammatory pain alleviation with high efficacy opioid of limited CNS penetration

The present work could clearly demonstrate for the first time that 14-*O*-MeM6SU, a novel compound of high efficacy and limited CNS penetration, produced strong antinociception in different models of inflammatory pain. Also, in certain doses produced antinociception that stemmed from the activation of peripheral opioid receptors. We can proclaim this, since the antagonist effect of NAL-M on the antinociception of test compounds clearly reveals that. Three different inflammatory pain models support the outcome of the mentioned character: mouse acetic acid induced writhing test, rat formalin test and CFA-evoked hyperalgesia.

In **mouse writhing test** the antinociceptive effect of 14-*O*-MeM6SU was investigated in comparison with M6SU. M6SU similarly to 14-*O*-MeM6SU is a zwitterionic compound with limited CNS penetration, although its efficacy is lower than the novel compound's [22, 70]. The acetic acid-evoked writhing assay is one of the most well-established and widely used experimental models of visceral pain to assess the pain relieving actions of either NSAIDs or opioids [71, 72]. Of note, the effects of 14-*O*-MeM6SU and M6SU have never been analyzed before in this model. After systemic (s.c.) or central (i.c.v.) administration 14-*O*-MeM6SU showed more potent antinociceptive action than M6SU in accordance with data previously published by our group [70]. 14-*O*-MeM6SU proved to be 23 times more potent than M6SU after systemic administration and only 5 times higher than M6SU after central dosing. However, the s.c./i.c.v. ratio was higher for M6SU than for 14-*O*-MeM6SU (Table 2.). Regarding the antinociceptive effect, the results are in agreement with data reported previously by our group in thermal pain model [70]. In previous studies lower s.c./i.c.v. ratio for morphine (4215) and larger for M6G (58400) that is similar to that of 14-*O*-MeM6SU was shown [44, 98]. The systemic/central ratio of the novel compound is high in comparison with other opioids like morphine or fentanyl [43, 70]. Under the present experimental conditions, 14-*O*-MeM6SU has shown limited CNS penetration, similarly to M6SU (high s.c./i.c.v. ratio indicates limited CNS penetration). Brown and his coworkers reported on the weak antinociceptive action of M6SU and related it to its limited CNS penetration [99]. Indeed, 14-*O*-MeM6SU is more

advantageous than M6SU since it has higher efficacy and affinity reflecting its stronger antinociceptive action as previously described [70] and showed in the present thesis in different animal models of pain diseases.

Applying systemic opioid antagonists of limited CNS penetration is a widely used method to investigate the peripheral antinociceptive component of opioids [100–102]. 14-*O*-MeM6SU (136 nmol/kg) or M6SU (3043 nmol/kg) showed peripheral antinociceptive effects after s.c. administration, since the co-administered quaternary opioid antagonist, NAL-M significantly reversed the effects of the test compounds (Fig. 3.). NAL-M in the applied dose does not penetrate the blood brain barrier after s.c. administration [101, 103].

In the **rat formalin test** the effects of 14-*O*-MeM6SU were analyzed in comparison with morphine. This model mimics the conditions of not just acute inflammatory pain but also somatic pain caused by the irritating effect of the locally applied formalin solution. The pain reactions in this model are classified into two phases, namely phase I and II. In the first phase the pain reactions are mostly mediated by the direct irritating effect of noxious agent, while in phase II inflammatory mediators (e.g. histamine, bradykinin) are released [73, 80]. Indeed, NSAIDs show antinociceptive action in the second phase, whereas opioids are able to alleviate the pain in both phases [80].

14-*O*-MeM6SU or morphine in the present study produced similar and dose dependent antinociceptive properties in both phases following systemic (s.c.) or local (i.pl.) administration. Co-administered NAL-M completely abolished the systemic (s.c.) antinociceptive effect of a certain dose of 14-*O*-MeM6SU (506 nmol/kg) (Fig 6.), indicating the contribution of the peripheral opioid system. On the other hand, the effect of morphine (15538 nmol/kg) was partially affected by NAL-M co-administration indicating both peripheral and central components in the antinociceptive action of morphine. We could conclude that, 14-*O*-MeM6SU but not morphine showed peripheral antinociceptive action at certain doses. A similar antinociceptive tendency was shown previously utilizing the same method - though the dose of morphine was smaller (5278 nmol/kg) [43]. The effect of morphine is also in accordance with previous work reported by Riba et al., where morphine showed similar, dual-site antinociceptive effect (both central and peripheral) in mouse tail-flick test (acute thermal antinociception) [100]. These data indicate the importance of CNS-actions in the antinociceptive effect of morphine, supporting previous studies [46, 96]. On the other side, NAL-M failed to affect

the antinociception of 14-*O*-MeM6SU when tested in higher doses. On the basis of this, 14-*O*-MeM6SU but not morphine seems to have peripheral antinociception at certain systemic doses.

Furthermore, in certain locally administered antinociceptive doses 14-*O*-MeM6SU but not morphine failed to produce antinociceptive action, when was injected into the contralateral paw (Fig. 7.). This might indicate that this dose is too small to achieve antinociceptive effect on the ipsilateral (formalin treated) paw after contralateral administration. As this dose has antinociceptive action when administered to the ipsilateral paw (Fig. 5.), then we can conclude that the site of hitting the pain is in the periphery for 14-*O*-MeM6SU in the dose of 50.6 nmol/rat.

In order to further model the clinical conditions of inflammatory pain we've set out to apply **CFA model** in addition to the above mentioned tests. In this pain model **(**CFA-induced inflammatory pain) the effects of 14-*O*-MeM6SU were compared to that of M6SU. In this study 14-*O*-MeM6SU and M6SU produced dose dependent antinociceptive action after systemic administration (Fig. 8.). The peripheral component of measured antinociception was analyzed in the presence of systemically co-administered NAL-M and also after local injections of the quaternary antagonist. The co-administered NAL-M blocks the antinociceptive action of certain doses of test compounds, indicating that they produce peripheral antinociception in a certain dose range. To localize the peripheral site of antinociceptive action of test compounds, i.pl. NAL-M was applied. The locally injected NAL-M also abolished the analgesic effects of s.c. 14-*O*-MeM6SU or M6SU (Fig. 10.). These results suggest that, the site where the test compounds produce their antinociception is at the inflamed paws.

Our data are in agreement with previous studies using this experimental model of pain and the same route of administration with other opioid compounds [104]. However, in the present work, test compounds could also elicit central antinociception at higher doses. The differences in the antinociceptive effects of 14-*O*-MeM6SU and M6SU between inflamed and non-inflamed paws gradually declined but at a lower dose range a clear peripheral action was demonstrated in the inflamed paws (Fig. 10.). Also, similarly to the formalin test the antinociceptive effect of higher systemic doses of the test compounds was not reversed by NAL-M (Fig 11.). These results show that careful dose titration of the MOR agonists, 14-*O*-MeM6SU and M6SU during their systemic administration can

reveal a distinct dose range in which antinociceptive effects are exerted exclusively by the activation of peripheral MOR at the inflammation site. At these doses PPT on the contralateral side were not significantly elevated, while significant elevation in the inflamed paws was observed (Fig. 9.). It is well established, that during inflammation the number of opioid receptors is elevated [13, 17]. This might offer an explanation why 14-*O*-MeM6SU and M6SU produced antinociception in inflamed paws compared to non-inflamed paws in Randall-Selitto test. The increase in the number of accessible opioid receptors results in enhanced peripheral opioid antinociceptive efficacy in inflammatory pain as it was already reported by others [105–107].

Similarly to formalin test – the model of acute somatic- and inflammatory pain – 14-*O*-MeM6SU showed significant peripheral antinociceptive action, even after systemic administration. These results further support the hypothesis that inflammatory pain can be alleviated satisfactorily through the activation of peripheral opioid receptors [44, 96, 108]. Therefore 14-*O*-MeM6SU - and similar compounds from the aspect of physicochemical properties - might offer analgesia of high clinical value, even after systemic administration especially in the cases of severe acute inflammatory conditions. In contrast to locally injected opioids systemic administration might offer a possibility to avoid the risk of infections and physical damages [17].

5.2. Neuropathic pain alleviation with high efficacy opioid of limited CNS penetration

Another huge clinical challenge facing physicians is the treatment of neuropathic pain, particularly diabetic neuropathy [69]. In our work, we also investigated the antinociceptive effect of 14-*O*-MeM6SU in comparison with morphine in the model of diabetic polyneuropathic pain: the STZ induced diabetes in rats [78]. Biochemical and histochemical assessments of the consequences of disease on MOR number at the spinal and supraspinal levels were also performed. Indeed, our idea to treat painful diabetic neuropathy was based on the efficacy of opioids. Our group have previously reported on the high efficacy of the novel compound 14-*O*-MeM6SU and low efficacy of morphine in different *in vitro* assays [70]. Opioid analgesic effectiveness in the management of neuropathic pain so far is a matter of controversy in both clinical practice and opioid research. Nevertheless, opioids and a related compound, tramadol are considered by some

guidelines as second line agents in the management of painful diabetic neuropathy [109]. The first task in the present study was to follow the changes in blood glucose level, water and food intake, body weight and development of allodynia for a 12 weeks period following STZ-treatment. As a consequence of β-cell destruction by STZ, diabetic animals developed hyperglycemia, gained significantly less weight than the non-diabetic ones, consumed significantly more water and food. These results are in agreement with our previous work [68]. The significantly developed allodynia appeared 3 weeks following STZ treatment and peaked at the 9^{th}-12^{th} weeks in the present study (Fig. 12.). These symptoms are indicative for development of DNP and are in accordance with previous studies [78]. However, only a few studies can be found in the literature regarding the analgesic effect of opioids at advanced diabetes (9-12 weeks after STZ treatment). The developed allodynia was effectively alleviated by high systemic doses of both 14-*O*-MeM6SU and morphine. This analgesic action is consistent with other studies reported on the effects of different opioids on DNP [110, 111]. However, a study reported on the ineffectiveness of morphine in doses up to 10 mg/kg (approx. 31 μmol/kg) seven weeks following STZ treatment [112].

The results of *in vivo* and *in vitro* studies in the present work give new information about diabetic neuropathy in two aspects. The first aspect is based on the calculated ED_{30} values which indicate that morphine was 7 times less effective in diabetic animals than non-diabetic ones, whereas 14-*O*-MeM6SU showed no difference in the analgesic action in diabetic or non-diabetic ones (Fig 13., $ED_{30diabetic}/ED_{30non\text{-}diabetic}$). This indicates a significant reduction in the antinociceptive effect of morphine, which is in accordance with previous studies [64, 113], however, the novel compound remained highly effective under neuropathic conditions. This is also strengthened by the observation that 14-*O*-MeM6SU (253 nmol/kg) but not morphine produced significant antiallodynic action only in DNP and no impact on PPT of weight match rats (Fig. 14.). In addition, under the present experimental circumstances, systemic NAL-M failed to affect the antiallodynic effect of systemic14-*O*-MeM6SU or morphine (Fig. 15.). Consequently, if we accept that NAL-M does not penetrate the blood brain barrier in the applied doses [43, 101], then, MOR in CNS might mediate the measured antinociceptive effect of higher systemic doses of test compounds that abolished allodynia in diabetic animals.

The second aspect is based on our ***in vitro*** **results** which depict that at the level of spinal cord, 14-*O*-MeM6SU but not morphine caused remarkable agonist effect in G-protein coupling in spinal tissues prepared from rats with DNP (Fig. 21. and Table 5., 6.). In our previous work in rats with advanced diabetic neuropathy and mechanical hyperalgesia (Randall-Selitto test), we demonstrated a decrease in fentanyl-mediated spinal antinociception in mechanical hyperalgesia associated with reduction of sensory neuron MOR number and G-protein coupling [68]. In addition, many studies reported on the lowered opioid analgesic efficacy in animal neuropathic models [113, 114]. In the present work we also detected a decrease in MOR density both in the DRG and the dorsal horn of the spinal cord of rats with DNP (Fig. 19.). 14-*O*-MeM6SU has higher intrinsic efficacy than morphine or fentanyl meaning that even if there is a decrease in MOR reserve it might activate MORs and produce measurable analgesia [96]. Our data from G-protein activation experiments reveal that 14-*O*-MeM6SU in comparison with morphine in spinal cord homogenates produced significantly higher efficacy, which was assessed from the maximal activation of functional opioid receptors from control and diabetic rats. In the spinal cord, morphine displayed very weak G-protein activation compared to that of 14-*O*-MeM6SU. On the other hand, neither morphine nor 14-*O*-MeM6SU showed any difference in efficacy at the supraspinal level of diabetic rats compared to control. Interestingly, 14-*O*-MeM6SU, but not morphine showed similar efficacy at the spinal level in control and diabetic rats. This latter tendency seen in case of 14-*O*-MeM6SU might be an advantage, since spinal cord is a crucial point in pain transmission [81].

Taken together, large reduction in antinociception of morphine but not of 14-*O*-MeM6SU in diabetic rats compared to control rats was observed. Large alterations on the antiallodynic effect of morphine but not 14-*O*-MeM6SU were shown in diabetic neuropathic rats. Diabetes results in reduced MOR G-protein coupling by morphine but not 14-*O*-MeM6SU at the level of spinal cord, key traffic point in the pain transmission. These data further support that the spinal cord is essential target in the treatment of DNP. In this pain traffic point MORs are found in the presynaptic central terminals of primary afferent neurons, which are a target for spinally administered opioids and other drugs prescribed for NP, like gabapentinoids [115]. These analgesic agents block the voltage gated calcium channels, and consequently the release of transmitters that further process the pain toward the brain. Since opioid receptors are localized in the presynaptic

membrane of primary afferent fibers, their activation will results in the inhibition of transmitter release and consequently peripheral signal propagation toward the brain. Voltage gated calcium channels (VGCCs) have been reported to be over expressed in the dorsal horn of diabetic animals [116]. In addition, in spinal cord slice preparations MOR activation on central terminals of Aδ- and C-fibers by opioid agonists results in the blockage of VGCCs, which in turn inhibits transmitter release and consequently nociceptive traffic toward the brain [117]. Therefore, we can hypothesize that 14-*O*-MeM6SU might block the pain effectively at this point.

5.3. The side effect profile of the novel compound, 14-*O*-MeM6SU

Opioid agonists beside their pain alleviation, cause several central and peripheral undesired effects. Therefore, it is important to pay attention to and investigate these effects. Opioid induced constipation is a very common side effect in opioid-treated patients, which can be a limiting factor in the chronic therapy of pain disorders [76]. 14-*O*-MeM6SU, M6SU and morphine inhibited the **gastrointestinal transit** in a dose dependent manner. Based on our study there is no significant difference in this inhibitory action between the novel and reference compounds in analgesic doses. However, 14-*O*-MeM6SU and M6SU also induced significant peripheral antinociception at the same dose range, clearly indicating that they are superior to an other peripherally acting opioid, loperamide, which failed to produce antinociception in doses producing constipation in mice [118]. It is worth noting that in the present work we could show that opioid analgesic action is mediated at sites within the CNS as well as the periphery. A possible solution to overcome the constipation causing effect of opioids is the co-administration of the non-selective opioid antagonist naloxone [119, 120]. Due to the low bioavailability of naloxone, caused by quick first-pass elimination, the antagonistic effect is implemented at the intestinal level [121], meaning this effect would not interfere with the observed peripheral analgesia of the novel compound.

An other clinically significant side effect caused by opioids is **respiratory depression** [46]. Although with proper dose titration opioids rarely show clinically relevant respiratory depressive effects, it is known that opioids penetrating into the CNS can cause respiratory depression, especially at higher doses and in “opioid naïve” patients. Therefore, we aimed to assess the tested compounds for their respiratory effects in opioid-

naïve rats. 14-*O*-MeM6SU and M6SU were tested at doses producing NAL-M reversible antinociceptive effect under inflammatory pain conditions. Test compounds showed no significant alterations in respiratory parameters compared to the control group, indicating that the drugs did not cause respiratory depression in the tested dose range, under the given circumstances. This dose of 14-*O*-MeM6SU (253 nmol/kg) also showed antinociception in the model of diabetic neuropathy. Based on the fact that these doses of 14-*O*-MeM6SU and M6SU elevated thiobutabarbital induced sleeping time (see below and section 4.4.3.) we cannot exclude CNS penetration of test compounds. Still, proper titration of systemic doses of high efficacy opioid compounds with limited access to the brain might offer peripheral analgesia of clinical importance without unwanted CNS effects, like respiratory depression.

CNS depressing drugs have been reported to have longer action by co-administration of opioids [122, 123]. Therefore, we utilized this approach to assess the penetration of systemically administered 14-*O*-MeM6SU or M6SU compared to morphine in one hand and also assess the **sedative effects** of test compounds on the other hand. For this aim, we studied the central actions of anesthetics (i.v. thiobutabarbital and inhaled isoflurane) in the presence of test opioids. In this test, NAL-M reversible antinociceptive doses of 14-*O*-MeM6SU or M6SU failed to potentiate the sleeping time induced by thiobutabarbital (Fig. 16.). However, at higher doses both compounds lengthened the sleeping time. We also tested the impact of certain analgesic doses of morphine or 14-*O*-MeM6SU on the sleeping time evoked by inhaled isoflurane. 14-*O*-MeM6SU in contrast to morphine in some analgesic doses failed to prolong the sleeping time of isoflurane (Fig. 17.). Morphine in the dose of 7769 nmol/kg (the smallest effective dose in both phases) prolonged isoflurane induced sleeping time, whereas 14-*O*-MeM6SU did not alter the sleeping time in the dose of 506 nmol/kg under the present circumstances. In the case of thiobutabarbital, 14-*O*-MeM6SU in dose of 253 nmol/kg or higher did prolong thiobutabarbital-induced sleeping time. Indeed, presently our explanation for this issue is based on pharmacokinetic properties since the two anesthetic agents have different routes of administration and different sleeping induction properties [70, 73, 124]. However, these differences need to be elaborated in the future but the substantial result regarding the analgesia of 14-*O*-MeM6SU in the present work remains clear because the systemic dose of 506 nmol/kg was antagonized by the peripherally acting opioid antagonist, NAL-

M in rat formalin test. Thus, 506 nmol/kg and lower doses showed peripheral analgesia, regardless their impact on sleeping time evoked by anesthetics.

Based on our results, we could show that inflammatory pain in contrast to neuropathic pain can be alleviated by targeting peripheral opioid receptors following acute drug administration. Also, it is well known that chronic and repeated opioid administration results in **analgesic tolerance** development [125]. Tolerance is indicated by a significant loss in the analgesic effect of administered opioid drugs, causing the need for dose elevation during treatment. Opioid tolerance became a major clinical problem generating continuous effort to find major analgesics with less tolerance developing potency [125–127]. To investigate this issue, the tolerance profile of 14-*O*-MeM6SU and morphine was assessed applying the mouse tail-flick test after 3 days twice daily administration of test compounds. Mouse tail-flick test is a widely used and accepted method to study the antinociceptive properties of opioid compounds [80]. The antinociception of 14-*O*-MeM6SU was assessed previously with rat tail-flick test. Based on previous and present results the ED_{50} values of 14-*O*-MeM6SU and morphine are relatively close in rat- and mouse tail-flick assay: 6.8 μmol/kg vs. 8 μmol/kg for morphine and 0.18 μmol/kg vs. 0.47 μmol/kg for 14-*O*-MeM6SU, in rat- and mouse tail-flick tests, respectively [70]. For inducing tolerance such high doses were chosen, that surely penetrate into the CNS in mice and also rats in order to be able to interpret the results. In mouse tail-flick assay 14-*O*-MeM6SU was 17 times more potent in analgesic action than morphine (Table 4.). Treatment with morphine resulted in a decreased magnitude of morphine analgesia by 3.41-fold indicating the development of analgesic tolerance. These results are in agreement with other studies demonstrating the development of tolerance upon chronic morphine treatment [89]. 14-*O*-MeM6SU showed promising analgesia either in morphine or 14-*O*-MeM6SU pretreated mice compared to morphine. The analgesic tolerance developed for morphine is higher than that developed for 14-*O*-MeM6SU, indicating an another advantage of clinical significance for 14-*O*-MeM6SU. A possible explanation is based on the high efficacy of the novel compound. Considering that 14-*O*-MeM6SU has higher intrinsic efficacy than morphine [70] and in the tolerant mice the opioid receptor reserve is decreased [127], 14-*O*-MeM6SU under these circumstances still activates sufficient number of opioid receptors. Based on these results in neuropathic conditions (e.g. diabetic neuropathy), when repetitive administration of opioids might be indicated,

14-*O*-MeM6SU and similar compounds can be effective even after chronic administration. It is a future plan to further investigate the tolerance profile of the novel compound in different animal models of different pain diseases (e.g. STZ induced diabetic neuropathy in rats).

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.

DOI:10.14753/SE.2019.2219

6. 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 role of peripheral opioid receptors** in antinociception differs in inflammatory and neuropathic conditions.

In animal **inflammatory pain models**, in distinct dose ranges systemically applied 14-*O*-MeM6SU and M6SU but not morphine **elicit significant peripheral antinociceptive effect** that was proved as follows:

- In the mouse visceral pain model (mouse writhing test) indicated by high systemic/central (s.c./i.c.v.) ratio and antagonist action of the peripherally acting opioid antagonist NAL-M.
- In rat formalin test indicated by the effects of locally applied 14-*O*-MeM6SU but not morphine, and also the antagonist effect of NAL-M on systemic 14-*O*-MeM6SU.
- In rat CFA model (model of subchronic inflammatory pain), indicated by the significant peripheral antinociceptive effect in inflamed rat paws compared to non-inflamed paws. The antinociception was localized to inflamed paws as proven by systemically or locally administered NAL-M.

The 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. To further analyze the CNS effects of test compounds in diabetic neuropathic conditions **G protein activity assay** was performed in spinal and supraspinal tissues. 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. Also,

reduction of MOR number and binding in the DRG and dorsal horn of diabetic animals was shown.

Developing novel opioids with high efficacy in the management of advanced painful diabetes is an unmet medical need. The reduction of spinal opioid receptors concomitant with reduced analgesic effect of morphine in the treatment of diabetic neuropathy 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.

DOI:10.14753/SE.2019.2219

7. Summary

Growing data support the peripheral opioid antinociceptive effect - particularly in inflammatory pain models -, which is a possible way to avoid central opioid side effects (e.g. respiratory depression, dependence). Here, we examined the antinociceptive effects of the recently synthesized 14-*O*-methylmorphine-6-*O*-sulfate (14-*O*-MeM6SU) compared to morphine-6-*O*-sulfate (M6SU) after subcutaneous (s.c.) and intracerebroventricular (i.c.v.) administration in a mouse model of visceral pain evoked by acetic acid. In addition, we examined the effects of 14-*O*-MeM6SU in comparison with morphine in another acute inflammatory pain model (rat formalin test) after systemic and local administration. Rat model of subchronic inflammatory pain, induced by the injection of Complete Freund's Adjuvant was also applied. Our results indicate that 14-*O*-MeM6SU and M6SU are able to alleviate pain in inflammatory conditions via activating peripheral opioid receptors.

The analgesic action of opioids in diabetic neuropathic pain (DNP) is impaired due to the reduction of μ-opioid receptor (MOR) reserve. Therefore, high efficacy opioids having spare receptors may be promising analgesics. Herein, we examined the degree of the antinociception impairment and antiallodynic action of a novel high efficacy opioid agonist, 14-*O*-MeM6SU compared to morphine in rats with streptozocin-evoked DNP following s.c. administration. Significant reduction in the antinociceptive effect of morphine, but not 14-*O*-MeM6SU was shown. Co-administered naloxone methiodide (NAL-M), a peripherally acting opioid receptor antagonist failed to affect the antiallodynic effect of 14-*O*-MeM6SU or morphine, indicating the contribution of central opioid receptors. Significant reduction in spinal MOR binding sites and loss in MOR immunoreactivity of nerve terminals in the spinal cord and dorsal root ganglia in diabetic rats were observed. Significant reduction in G-protein activation for morphine, but not for 14-*O*-MeM6SU at spinal level was also observed. Taken together, the reduction of spinal opioid receptors concomitant with reduced analgesic effect of morphine in the treatment of diabetic neuropathy may be circumvent by using high efficacy opioids, such as 14-*O*-MeM6SU, which provide superior analgesic effect over morphine. In terms of side effects, 14-*O*-MeM6SU shows a promising tolerance profile, and is devoid of respiratory depressive and sedative effects in a lower antinociceptive dose range.

DOI:10.14753/SE.2019.2219

8. Összefoglalás

Egyre több irodalmi adat támasztja alá a periférián található opioid receptorokon keresztül megvalósuló fájdalomcsillapító hatást, amely a központi idegrendszeri (KIR) mellékhatások tekintetében lehet igen előnyös (pl.: légzésdepresszió, dependencia). E célból vizsgáltuk a nemrégiben szintetizált 14-*O*-metilmorfin-6-*O*-szulfát (14-*O*-MeM6SU) és a morfin-6-*O*-szulfát (M6SU) antinociceptív hatását akut viszcerális fájdalom modellen (egér ecetsav indukálta writhing teszt) szisztémás, valamint centrális adagolás után. Emellett vizsgáltuk a 14-*O*-MeM6SU-ot a morfinnal összevetésben akut gyulladásos patkány modellen is (formalin teszt) szisztémás és lokális adagolás mellett. Patkány szubkrónikus gyulladásos modellen (CFA modell) szintén végeztünk kísérleteket. Eredményeink alapján a 14-*O*-MeM6SU és a M6SU képes a gyulladásos fájdalom csillapítására perifériás támadásponton keresztül.

Diabeteses neuropátia során a különböző opioidok hatása csökken a μ-opioid receptorok (MOR) számának következményeként. Ezért olyan nagy hatékonyságú opioidok, melyek képesek spare receptorokat aktiválni ígéretes analgetikumok lehetnek. Vizsgáltuk a nagy hatékonyságú 14-*O*-MeM6SU és a morfin hatékonyságának csökkenését streptozocin indukálta diabeteses neuropátia modellen, patkányokon, szisztémás adagolás mellett. A morfinnal ellentétben az új analóg nem mutatott hatáscsökkenést diabeteses állatokon. Együtt adott naloxon-metiljodid (periférián ható opioid antagonista) nem befolyásolta a hatást, mely a KIR szerepére utal. A diabetes állatok gerincvelő mintáin MOR kötőhelyek csökkenését mutattuk ki, valamint az immunhisztokémiai vizsgálatok MOR csökkenést igazoltak gerincvelői, valamint hátsó gyökér ganglion (DRG) mintákon. Ezzel összefüggésben csökkent G-protein aktivációt mutattunk ki morfin esetén diabetes állatok gerincvelő mintáin, azonban a 14-*O*-MeM6SU nem mutatott ilyen hatáscsökkenést. Következésképpen: a morfin diabeteses neuropátia során, a MOR csökkenés következtében kialakuló hatásvesztése kiküszöbölhető lehet olyan nagy hatékonyságú molekulák alkalmazásával, mint a 14-*O*-MeM6SU.

Az új vegyület ígéretes mellékhatásprofillal is rendelkezik. A 14-*O*-MeM6SU a morfinnál kedvezőbb toleranciaprofilt mutatott. Alacsony antinociceptív dózistartományban a légzést nem befolyásolta, valamint szedatív hatást nem okozott.

9. Bibliography

1. Debono DJ, Hoeksema LJ, Hobbs RD (2013) Caring for patients with chronic pain: pearls and pitfalls. J Am Osteopath Assoc 113:620–7 . doi: 10.7556/jaoa.2013.023
2. Chou R, Fanciullo GJ, Fine PG, Adler JA, Ballantyne JC, Davies P, Donovan MI, Fishbain DA, Foley KM, Fudin J, Gilson AM, Kelter A, Mauskop A, O'Connor PG, Passik SD, Pasternak GW, Portenoy RK, Rich BA, Roberts RG, Todd KH, Miaskowski C (2009) Clinical Guidelines for the Use of Chronic Opioid Therapy in Chronic Noncancer Pain. J Pain 10:113–130.e22 . doi: http://dx.doi.org/10.1016/j.jpain.2008.10.008
3. Schmitz R (1985) Friedrich Wilhelm Sertürner and the Discovery of Morphine. Pharm Hist 27:61–74 . doi: 10.2307/41109546
4. Busse GD., Triggle DJ (2006) Morphine (Drugs: The Straight Facts)
5. Meldrum M (2003) Opioids and pain relief : a historical perspective
6. Ossipov MH, Lai J, King T, Vanderah TW, Malan TP, Hruby VJ, Porreca F (2004) Antinociceptive and nociceptive actions of opioids. J. Neurobiol. 61:126–148
7. Gaveriaux-Ruff C (2014) Opiate-Induced Analgesia: Contributions From Mu, Delta and Kappa Opioid Receptors Mouse Mutants. Curr Pharm Des 19:7373–7381 . doi: 10.2174/13816128194214010516372 7
8. Manchikanti L, Kaye AM, Kaye AD (2016) Current State of Opioid Therapy and Abuse. Curr. Pain Headache Rep. 20:34
9. Joranson DE, Ryan KM, Gilson AM, Dahl JL (2000) Trends in Medical Use and Abuse of Opioid Analgesics. JAMA 283:1710 . doi: 10.1001/jama.283.13.1710
10. Wardhan R, Chelly J (2017) Recent advances in acute pain management: understanding the mechanisms of acute pain, the prescription of opioids, and the role of multimodal pain therapy. F1000Research 6:2065 . doi: 10.12688/f1000research.12286.1
11. Nagasaka H, Awad H, Yaksh TL (1996) Peripheral and spinal actions of opioids in the blockade of the autonomic response evoked by compression of the inflamed knee joint. Anesthesiology 85:808–816 . doi: 10.1097/00000542-199610000-00016
12. Stein C, Schäfer M, Hassan AHS (1995) Peripheral opioid receptors. Ann Med

27:219–221 . doi: 10.3109/07853899509031962

13. Stein C, Lang LJ (2009) Peripheral mechanisms of opioid analgesia. Curr Opin Pharmacol 9:3–8 . doi: 10.1016/j.coph.2008.12.009
14. Hua S, Cabot PJ (2010) Mechanisms of peripheral immune-cell-mediated analgesia in inflammation: Clinical and therapeutic implications. Trends Pharmacol Sci 31:427–433 . doi: 10.1016/j.tips.2010.05.008
15. Koulousakis A, Kuchta J, Bayarassou A, Sturm V (2007) Intrathecal opioids for intractable pain syndromes. Acta Neurochir Suppl 97:43–8
16. Kalso E, Smith L, McQuay HJ, Andrew Moore R (2002) No pain, no gain: Clinical excellence and scientific rigour - Lessons learned from IA morphine. Pain 98:269–275 . doi: 10.1016/S0304-3959(02)00019-2
17. Stein C, Schäfer M, Machelska H (2003) Attacking pain at its source: New perspectives on opioids. Nat. Med. 9:1003–1008
18. Likar R, Koppert W, Blatnig H, Chiari F, Sittl R, Stein C, Schäfer M (2001) Efficacy of peripheral morphine analgesia in inflamed, non-inflamed and perineural tissue of dental surgery patients. J Pain Symptom Manage 21:330–337 . doi: 10.1016/S0885-3924(01)00251-2
19. Peyman GA, Rahimy MH, Fernandes ML (1994) Effects of morphine on corneal sensitivity and epithelial wound healing: implications for topical ophthalmic analgesia. Br J Ophthalmol 78:138–41 . doi: 10.1136/bjo.78.2.138
20. Smith TW, Buchan P, Parsons DN, Wilkinson S (1982) Peripheral antinociceptive effects of N-methyl morphine. Life Sci 31:1205–1208 . doi: 10.1016/0024-3205(82)90343-5
21. Spetea M, Schmidhammer H (2012) Recent advances in the development of 14-alkoxy substituted morphinans as potent and safer opioid analgesics. Curr Med Chem 19:2442–57 . doi: CMC-EPUB-20120328-004 [pii]
22. Mori M aki, Oguri K, Yoshimura H, Shimomura K, Kamata O, Ueki S (1972) Chemical synthesis and analgesic effect of morphine ethereal sulfates. Life Sci 11:525–533 . doi: 10.1016/0024-3205(72)90003-3
23. Wenk HN, Nannenga MN, Honda CN (2003) Effect of morphine sulphate eye drops on hyperalgesia in the rat cornea. Pain 105:455–465 . doi: 10.1016/S0304-3959(03)00260-4

24. Stiles J, Honda CN, Krohne SG, Kazacos EA (2003) Effect of topical administration of 1% morphine sulfate solution on signs of pain and corneal wound healing in dogs. Am J Vet Res 64:813–818 . doi: 10.2460/ajvr.2003.64.813
25. Bourne S, Machado AG, Nagel SJ (2014) Basic anatomy and physiology of pain pathways. Neurosurg. Clin. N. Am. 25:629–638
26. Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384:360–364 . doi: 10.1038/384360a0
27. Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE (1976) The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther 197:517–532
28. Lord JAH, Waterfield AA, Hughes J, Kosterlitz HW (1977) Endogenous opioid peptides: Multiple agonists and receptors. Nature 267:495–499 . doi: 10.1038/267495a0
29. Wang JB, Johnson PS, Persico AM, Hawkins AL, Griffin CA, Uhl GR (1994) Human μ opiate receptor. cDNA and genomic clones, pharmacologic characterization and chromosomal assignment. FEBS Lett 338:217–222 . doi: 10.1016/0014-5793(94)80368-4
30. Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, Bell GI (1993) Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. Proc Natl Acad Sci U S A 90:6736–40 . doi: 10.1073/pnas.90.14.6736
31. Evans C, Keith D, Morrison H, Magendzo K, Edwards R (1992) Cloning of a delta opioid receptor by functional expression. Science (80-) 258:1952–1955 . doi: 10.1126/science.1335167
32. Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG (1992) The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci U S A 89:12048–52
33. Chen Y, Chen Y, Mestek A, Mestek A, Liu J, Liu J, Hurley JA, Hurley JA, Yu L, Yu L (1993) Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol Pharmacol 44:8–12
34. Min BH, Augustin LB, Felsheim RF, Fuchs JA, Loh HH (1994) Genomic structure

and analysis of promoter sequence of a mouse mu opioid receptor gene. Pharmacology 91:9081–9085

35. Womack MD, McCleskey EW (1995) Interaction of opioids and membrane potential to modulate Ca2+ channels in rat dorsal root ganglion neurons. J Neurophysiol 73:1793–1798
36. Schneider SP, Eckert III WA, Light AR (1998) Opioid-activated postsynaptic, inward rectifying potassium currents in whole cell recordings in substantia gelatinosa neurons. J Neurophysiol 80:2954–2962
37. Miotto K, Kaufman D, Anton B, Keith Jr DE, Evans CJ, Keith DE (1996) Human opioid receptors: chromosomal mapping and mRNA localization. Mol Approaches to Drug Abus Res Vol III Recent Adv Emerg Strateg 161:72
38. Tzschentke TM (2000) The medial prefrontal cortex as a part of the brain reward system Review Article. Amin Acid 19:211–219 . doi: 10.1007/s007260070051
39. Xie Y-F, Huo F-Q, Tang J-S (2009) Cerebral cortex modulation of pain. Acta Pharmacol Sin 30:31–41 . doi: 10.1038/aps.2008.14
40. Berry PH, Chapman CR, Covington EC, Dahl JL, Katz JA, Miaskowski C, McLean MJ (2001) Pain : Current Understanding of Assessment, Management and Treatment. Pharm. Counc. Jt. Comm. Accredit. Healthc. Organ. VA, USA
41. Mansour A, Fox CA, Akil H, Watson SJ (1995) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci 18:22–29 . doi: 10.1016/0166-2236(95)93946-U
42. Coggeshall RE, Zhou S, Carlton SM (1997) Opioid receptors on peripheral sensory axons. Brain Res 764:126–132 . doi: 10.1016/S0006-8993(97)00446-0
43. Fürst S, Riba P, Friedmann T, Timar J, Al-Khrasani M, Obara I, Makuch W, Spetea M, Schutz 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. J Pharmacol Exp Ther 312:609–618 . doi: 10.1124/jpet.104.075176
44. Al-Khrasani M, Spetea M, Friedmann T, Riba P, Király K, Schmidhammer H, Furst S, Kiraly K, Schmidhammer H, Furst S (2007) DAMGO and 6B-glycine substituted 14-O-methyloxymorphone but not morphine show peripheral, preemptive antinociception after systemic administration in a mouse visceral pain

model and high intrinsic efficacy in the isolated rat vas deferens. Brain Res Bull 74:369–375 . doi: 10.1016/j.brainresbull.2007.07.008

45. Tegeder I, Meier S, Burian M, Schmidt H, Geisslinger G, Lötsch J (2003) Peripheral opioid analgesia in experimental human pain models. Brain 126:1092–1102 . doi: 10.1093/brain/awg115
46. Ghelardini C, Di Cesare Mannelli L, Bianchi E (2015) The pharmacological basis of opioids. Clin. Cases Miner. Bone Metab. 12:219–221
47. Balogh M (2015) Egy új opioid, a 14-O-metilmorfin-6-szulfát tolerancia és kereszttolerancia vizsgálata egéren. Semmelweis University
48. Lanas A (2009) Nonsteroidal Antiinflammatory Drugs and Cyclooxygenase Inhibition in the Gastrointestinal Tract: A Trip From Peptic Ulcer to Colon Cancer. Am J Med Sci 338:96–106 . doi: 10.1097/MAJ.0b013e3181ad8cd3
49. Schäfer M, Imai Y, Uhl GR, Stein C (1995) Inflammation enhances peripheral mu-opioid receptor-mediated analgesia, but not mu-opioid receptor transcription in dorsal root ganglia. Eur J Pharmacol 279:165–169
50. Pol O, Puig MM (2004) Expression of opioid receptors during peripheral inflammation. Curr Top Med Chem 4:51–61 . doi: 10.2174/1568026043451519
51. Hurley RW, Hammond DL (2000) The analgesic effects of supraspinal mu and delta opioid receptor agonists are potentiated during persistent inflammation. J Neurosci 20:1249–1259
52. Gupta A, Bodin L, Holmström B, Berggren L (2001) A systematic review of the peripheral analgesic effects of intraarticular morphine. Anesth Analg 93:761–770 . doi: 10.1097/00000539-200109000-00042
53. Kalso E, Tramèr MR, Carroll D, McQuay HJ, Moore RA (1997) Pain relief from intra-articular morphine after knee surgery: A qualitative systemic review. Pain 71:127–134 . doi: 10.1016/S0304-3959(97)03344-7
54. Skarke C, Darimont J, Schmidt H, Geisslinger G, Lötsch J (2003) Analgesic effects of morphine and morphine-6-glucuronide in a transcutaneous electrical pain model in healthy volunteers. Clin Pharmacol Ther 73:107–121 . doi: 10.1067/mcp.2003.5
55. Gaskin DJ, Richard P (2012) The economic costs of pain in the United States. J Pain 13:715–724 . doi: 10.1016/j.jpain.2012.03.009
56. Bouhassira D, Lantéri-Minet M, Attal N, Laurent B, Touboul C (2008) Prevalence

of chronic pain with neuropathic characteristics in the general population. Pain 136:380–387 . doi: 10.1016/j.pain.2007.08.013

57. Häuser W, Schug S, Furlan AD (2017) The opioid epidemic and national guidelines for opioid therapy for chronic noncancer pain. PAIN Reports 2:e599 . doi: 10.1097/PR9.0000000000000599
58. Vallejo R, Barkin RL, Wang VC (2011) Pharmacology of opioids in the treatment of chronic pain syndromes. Pain Physician 14:E343–E360
59. Furlan AD, Sandoval JA, Mailis-Gagnon A, Tunks E (2006) Opioids for chronic noncancer pain: a meta-analysis of effectiveness and side effects. CMAJ 174:1589–94 . doi: 10.1503/cmaj.051528
60. Wiffen PJ, Carr DB, Aldington D, Cole P, Derry S, Moore RA (1996) Morphine for neuropathic pain in adults. In: Derry S (ed) Cochrane Database of Systematic Reviews. John Wiley & Sons, Ltd, Chichester, UK
61. Derry S, Stannard C, Cole P, Wiffen PJ, Knaggs R, Aldington D, Moore RA (2016) Fentanyl for neuropathic pain in adults. Cochrane Database Syst. Rev. 2016
62. Dworkin RH, O'Connor AB, Audette J, Baron R, Gourlay GK, Haanpää ML, Kent JL, Krane EJ, LeBel AA, Levy RM, Mackey SC, Mayer J, Miaskowski C, Raja SN, Rice ASC, Schmader KE, Stacey B, Stanos S (2010) Recommendations for the pharmacological management of neuropathic pain: An overview and literature update. Mayo Clin. Proc. 85:S3–S14
63. Eriksen J, Sjøgren P, Bruera E, Ekholm O, Rasmussen NK (2006) Critical issues on opioids in chronic non-cancer pain:. An epidemiological study. Pain 125:172–179 . doi: 10.1016/j.pain.2006.06.009
64. Courteix C, Bourget P, Caussade F, Bardin M, Coudore F, Fialip J, Eschalier A (1998) Is the reduced efficacy of morphine in diabetic rats caused by alterations of opiate receptors or of morphine pharmacokinetics? J Pharmacol Exp Ther 285:63–70
65. Hama A, Sagen J (2007) Altered antinociceptive efficacy of tramadol over time in rats with painful peripheral neuropathy. Eur J Pharmacol 559:32–37 . doi: 10.1016/j.ejphar.2006.11.047
66. Zurek JR, Nadeson R, Goodchild CS (2001) Spinal and supraspinal components of opioid antinociception in streptozotocin induced diabetic neuropathy in rats.

Pain 90:57–63 . doi: 10.1016/S0304-3959(00)00386-9

67. Zhang X, Bao L, Shi T-J, Ju G, Elde R, Hökfelt T (1998) Down-regulation of μ-opioid receptors in rat and monkey dorsal root ganglion neurons and spinal cord after peripheral axotomy. Neuroscience 82:223–240 . doi: 10.1016/S0306-4522(97)00240-6
68. Shaqura M, Khalefa BI, Shakibaei M, Winkler J, Al-Khrasani M, Fürst S, Mousa SA, Schäfer M (2013) Reduced number, G protein coupling, and antinociceptive efficacy of spinal mu-opioid receptors in diabetic rats are reversed by nerve growth factor. J Pain 14:720–730 . doi: 10.1016/j.jpain.2013.01.776
69. Zeng L, Alongkronrusmee D, van Rijn RM (2017) An integrated perspective on diabetic, alcoholic, and drug-induced neuropathy, etiology, and treatment in the US. J Pain Res 10:219–228 . doi: 10.2147/JPR.S125987
70. 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 . doi: 10.2174/092986712803306376
71. Koster R, Anderson M, De Beer E (1959) Acetic acid for analgesic screening. Fed Proc 18:412–430 . doi: 10.1071/ZO9820427
72. Porreca F, Mosberg HI, Omnaas JR, Burks TF, Cowan A (1987) Supraspinal and spinal potency of selective opioid agonists in the mouse writhing test. J Pharmacol Exp Ther 240:890–4
73. Tjølsen A, Berge O-G, Hunskaar S, Rosland JH, Hole K (1992) The formalin test: an evaluation of the method. Pain 51:5–17 . doi: 10.1016/0304-3959(92)90003-T
74. Zhou LI, Zhang QIN, Stein C, Scha M, Schäfer M (1998) Contribution of opioid receptors on primary afferent versus sympathetic neurons to peripheral opioid analgesia. J Pharmacol Exp Ther 286:1000–1006
75. Holmdahl R, Lorentzen JC, Lu S, Olofsson P, Wester L, Holmberg J, Pettersson U (2001) Arthritis induced in rats with non-immunogenic adjuvants as models for rheumatoid arthritis. Immunol Rev 184:184–202 . doi: 10.1034/j.1600-065x.2001.1840117.x
76. Charnogursky G, Lee H, Lopez N (2014) Diabetic neuropathy. Handb. Clin. Neurol. 120:773–785

77. Horowitz M, O'Donovan D, Jones KL, Feinle C, Rayner CK, Samsom M (2002) Gastric emptying in diabetes: Clinical significance and treatment. Diabet Med 19:177–194 . doi: 10.1046/j.1464-5491.2002.00658.x
78. Courteix C, Eschalier A, Lavarenne J (1993) Streptozocin-induced diabetic rats: behavioural evidence for a model of chronic pain. Pain 53:81–88 . doi: 10.1016/0304-3959(93)90059-X
79. Tulunay FC, Takemori AE (1974) The increased efficacy of narcotic antagonists induced by various narcotic analgesics. J Pharmacol Exp Ther 190:395–400
80. Le Bars D, Gozariu M, Cadden SW (2001) Animal models of nociception. Pharmacol Rev 53:597–652 . doi: 10.1111/j.1476-5381.2011.01386.x
81. Pasternak GW (1993) Pharmacological mechanisms of opioid analgesics. Clin Neuropharmacol 16:1–18 . doi: 10.1097/00002826-199302000-00001
82. Coderre TJ, Yashpal K (1994) Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model. Eur J Neurosci 6:1328–1334 . doi: 10.1111/j.1460-9568.1994.tb00323.x
83. Mousa SA, Cheppudira BP, Shaqura M, Fischer O, Hofmann J, Hellweg R, Schäfer M (2007) Nerve growth factor governs the enhanced ability of opioids to suppress inflammatory pain. Brain 130:502–513 . doi: 10.1093/brain/awl330
84. Fülöp K, Zádori Z, Rónai AZ, Gyires K (2005) Characterisation of α2-adrenoceptor subtypes involved in gastric emptying, gastric motility and gastric mucosal defence. Eur J Pharmacol 528:150–157 . doi: 10.1016/j.ejphar.2005.10.025
85. Asano T, Aida S, Suemasu S, Mizushima T (2016) Anethole restores delayed gastric emptying and impaired gastric accommodation in rodents. Biochem Biophys Res Commun 472:125–130 . doi: 10.1016/j.bbrc.2016.02.078
86. de Novellis V, Vita D, Gatta L, Luongo L, Bellini G, De Chiaro M, Marabese I, Siniscalco D, Boccella S, Piscitelli F, Di Marzo V, Palazzo E, Rossi F, Maione S (2011) The blockade of the transient receptor potential vanilloid type 1 and fatty acid amide hydrolase decreases symptoms and central sequelae in the medial prefrontal cortex of neuropathic rats. Mol Pain 7:7 . doi: 10.1186/1744-8069-7-7
87. Király K, Kozsurek M, Lukácsi E, Barta B, Alpár A, Balázsa T, Fekete C, Szabon

J, Helyes Z, Bölcskei K, Tékus V, Tóth ZE, Pap K, Gerber G, Puskár Z (2018) Glial cell type-specific changes in spinal dipeptidyl peptidase 4 expression and effects of its inhibitors in inflammatory and neuropatic pain. Sci Rep 8:3490 . doi: 10.1038/s41598-018-21799-8

88. Scheibner J, Trendelenburg A-U, Hein L, Starke K, Blandizzi C (2002) α_2 -Adrenoceptors in the enteric nervous system: a study in α_{2A} -adrenoceptor-deficient mice. Br J Pharmacol 135:697–704 . doi: 10.1038/sj.bjp.0704512
89. Szentirmay AK, Kiraly KP, Lenkey N, Lacko E, Al-Khrasani M, Friedmann T, Timar J, Gyarmati S, Toth G, Furst S, Riba P (2013) Spinal interaction between the highly selective mu agonist DAMGO and several delta opioid receptor ligands in naive and morphine-tolerant mice. Brain Res Bull 90:66–71 . doi: 10.1016/j.brainresbull.2012.09.006
90. 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
91. Zádor F, Kocsis D, Borsodi A, Benyhe S (2014) Micromolar concentrations of rimonabant directly inhibits delta opioid receptor specific ligand binding and agonist-induced G-protein activity. Neurochem Int 67:14–22 . doi: 10.1016/j.neuint.2013.12.005
92. Shaqura M, Li X, Al-Khrasani M, Shakibaei M, Tafelski S, Fürst S, Beyer A, Kawata M, Schäfer M, Mousa SA (2016) Membrane-bound glucocorticoid receptors on distinct nociceptive neurons as potential targets for pain control through rapid non-genomic effects. Neuropharmacology 111:1–13 . doi: 10.1016/j.neuropharm.2016.08.019
93. Mousa SA, Shaqura M, Al-Madol M, Tafelski S, Khalefa BI, Shakibaei M, Schäfer M (2017) Accessibility of axonal G protein coupled mu-opioid receptors requires conceptual changes of axonal membrane targeting for pain modulation. J Control Release 268:352–363 . doi: 10.1016/j.jconrel.2017.10.016
94. Mousa SA, Shaqura M, Khalefa BI, Zöllner C, Schaad L, Schneider J, Shippenberg TS, Richter JF, Hellweg R, Shakibaei M, Schäfer M (2013) Rab7 silencing prevents μ-opioid receptor lysosomal targeting and rescues opioid responsiveness to strengthen diabetic neuropathic pain therapy. Diabetes 62:1308–1319 . doi:

10.2337/db12-0590

95. Endres-Becker J, Heppenstall PA, Mousa SA, Labuz D, Oksche A, Schäfer M, Stein C, Zöllner C (2007) Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. Mol Pharmacol 71:12–18 . doi: 10.1124/mol.106.026740
96. Khalefa BI, Shaqura M, Al-Khrasani M, Furst S, Mousa SA, Schafer M (2012) Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. Eur J Pain 16:690–705 . doi: 10.1002/j.1532-2149.2011.00070.x
97. Strange PG (2010) Use of the GTPγS ([35S]GTPγS and Eu-GTPγS) binding assay for analysis of ligand potency and efficacy at G protein-coupled receptors. Br. J. Pharmacol. 161:1238–1249
98. Frances B, Gout R, Monsarrat B, Cros J, Zajac J-M (1992) Further Evidence That Morphine-6b-Glucuronide is a More Potent Opioid Agonist Than Morphine. J Pharmacol Exp Ther 262:25–31
99. Brown CE, Roerig SC, Burger VT, Cody RB, Fujimoto JM (1985) Analgesic potencies of morphine 3- and 6-sulfates after intracerebroventricular administration in mice: Relationship to structural characteristics defined by mass spectrometry and nuclear magnetic resonance. J Pharm Sci 74:821–824 . doi: 10.1002/jps.2600740804
100. Riba P, Ben Y, Nguyen T, Furst S, Peter BSP, Schiller W, Lee N (2002) [Dmt^1]DALDA is Highly Selective and Potent at μ Opioid Receptors, but is not Cross-Tolerant with Systemic Morphine. Curr Med Chem 9:31–39 . doi: 10.2174/0929867023371445
101. Lewanowitsch T, Irvine RJ (2002) Naloxone methiodide reverses opioid-induced respiratory depression and analgesia without withdrawal. Eur J Pharmacol 445:61–67 . doi: 10.1016/S0014-2999(02)01715-6
102. Bianchi G, Fiocchi R, Tavani A, Manara L (1982) Quaternary narcotic antagonists' relative ability to prevent antinociception and gastrointestinal transit inhibition in morphine-treated rats as an index of peripheral selectivity. Life Sci 30:1875–1883 . doi: 10.1016/0024-3205(82)90467-2
103. Khalefa BI, Mousa SA, 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 . doi: 10.1016/j.ejphar.2013.04.043

104. Stein C, Millan MJ, Herz A (1988) Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: Alterations in behavior and nociceptive thresholds. Pharmacol Biochem Behav 31:445–451 . doi: 10.1016/0091-3057(88)90372-3
105. Rittner HL, Amasheh S, Moshourab R, Hackel D, Yamdeu RS, Mousa SA, Fromm M, Stein C, Brack A (2012) Modulation of tight junction proteins in the perineurium to facilitate peripheral opioid analgesia. Anesthesiology 116:1323–1334 . doi: 10.1097/ALN.0b013e318256eeeb
106. Rittner HL (2005) Leukocytes in the regulation of pain and analgesia. J Leukoc Biol 78:1215–1222 . doi: 10.1189/jlb.0405223
107. Antonijevic I, Mousa SA, Schäfer M, Stein C (1995) Perineurial defect and peripheral opioid analgesia in inflammation. J Neurosci 15:165–72 . doi: 10.1523/JNEUROSCI.15-01-00165.1995
108. Iwaszkiewicz KS, Schneider JJ, Hua S (2013) Targeting peripheral opioid receptors to promote analgesic and anti-inflammatory actions. Front Pharmacol 4:132 . doi: 10.3389/fphar.2013.00132
109. Moulin D, Boulanger A, Clark AJ, Clarke H, Dao T, Finley GA, Furlan A, Gilron I, Gordon A, Morley-Forster PK, Sessle BJ, Squire P, Stinson J, Taenzer P, Velly A, Ware MA, Weinberg EL, Williamson OD (2014) Pharmacological management of chronic neuropathic pain: revised consensus statement from the Canadian Pain Society. Pain Res Manag 19:328–35 . doi: 10.1016/j.pain.2009.05.014
110. Rutten K, Tzschentke TM, Koch T, Schiene K, Christoph T (2014) Pharmacogenomic study of the role of the nociceptin/orphanin FQ receptor and opioid receptors in diabetic hyperalgesia. Eur J Pharmacol 741:264–271 . doi: 10.1016/j.ejphar.2014.08.011
111. Shaqura M, Khalefa BI, Shakibaei M, Zöllner C, Al-Khrasani M, Fürst S, Schäfer M, Mousa SA, Zollner C, Al-Khrasani M, Furst S, Schafer M, Mousa SA (2014) New insights into mechanisms of opioid inhibitory effects on capsaicin-induced

TRPV1 activity during painful diabetic neuropathy. Neuropharmacology 85:142–150 . doi: 10.1016/j.neuropharm.2014.05.026

112. Yamamoto H, Shimoshige Y, Yamaji T, Murai N, Aoki T, Matsuoka N (2009) Pharmacological characterization of standard analgesics on mechanical allodynia in streptozotocin-induced diabetic rats. Neuropharmacology 57:403–408 . doi: 10.1016/j.neuropharm.2009.06.037
113. Cegielska-Perun K, Bujalska-Zadrożny M, Gąsińska E, Makulska-Nowak HE (2014) Enhancement of antinociceptive effect of morphine by antidepressants in diabetic neuropathic pain model. Pharmacol Rep 66:228–34 . doi: 10.1016/j.pharep.2013.09.003
114. Yadlapalli JSK, Ford BM, Ketkar A, Wan A, Penthala NR, Eoff RL, Prather PL, Dobretsov M, Crooks PA (2016) Antinociceptive effects of the 6-O-sulfate ester of morphine in normal and diabetic rats: Comparative role of mu- and delta-opioid receptors. Pharmacol Res 113:335–347 . doi: 10.1016/j.phrs.2016.09.012
115. Perret D, Luo ZD (2009) Targeting Voltage-Gated Calcium Channels for Neuropathic Pain Management. Neurotherapeutics 6:679–692 . doi: 10.1016/j.nurt.2009.07.006
116. Luo ZD (2002) Injury Type-Specific Calcium Channel alpha 2delta -1 Subunit Up-Regulation in Rat Neuropathic Pain Models Correlates with Antiallodynic Effects of Gabapentin. J Pharmacol Exp Ther 303:1199–1205 . doi: 10.1124/jpet.102.041574
117. Schroeder JE, Fischbach PS, Zheng D, McCleskey EW (1991) Activation of μ opioid receptors inhibits transient high- and low-threshold Ca2+currents, but spares a sustained current. Neuron 6:13–20 . doi: 10.1016/0896-6273(91)90117-I
118. Sánchez-Fernández C, Nieto FR, González-Cano R, Artacho-Cordón A, Romero L, Montilla-García Á, Zamanillo D, Baeyens JM, Entrena JM, Cobos EJ (2013) Potentiation of morphine-induced mechanical antinociception by σ1receptor inhibition: Role of peripheral σ1receptors. Neuropharmacology 70:348–358 . doi: 10.1016/j.neuropharm.2013.03.002
119. Merchan C, Altshuler D, Papadopoulos J (2017) Methylnaltrexone Versus Naloxone for Opioid-Induced Constipation in the Medical Intensive Care Unit. Ann Pharmacother 51:203–208 . doi: 10.1177/1060028016677310

120. Nee J, Zakari M, Sugarman MA, Whelan J, Hirsch W, Sultan S, Ballou S, Iturrino J, Lembo A (2018) Efficacy of Treatments for Opioid-induced Constipation: A Systematic Review and Meta-Analysis. Clin Gastroenterol Hepatol. doi: 10.1016/j.cgh.2018.01.021
121. Meissner W, Schmidt U, Hartmann M, Kath R, Reinhart K (2000) Oral naloxone reverses opioid-associated constipation. Pain 84:105–109 . doi: 10.1016/S0304-3959(99)00185-2
122. Craft RM, Leitl MD (2006) Potentiation of morphine antinociception by pentobarbital in female vs. male rats. Pain 121:115–125 . doi: 10.1016/J.PAIN.2005.12.009
123. McGuire JL, Awouters F, Niemegeers CJ (1978) Interaction of loperamide and diphenoxylate with ethanol and methohexital. Arch Int Pharmacodyn Ther 236:51–59
124. Lacko E, Riba P, Giricz Z, Varadi A, Cornic L, Balogh M, Kiraly K, Csek K, Mousa SA, Hosztafi S, Schafer M, Zadori ZS, 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 . doi: 10.1124/jpet.116.233551
125. Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, Koch T, Evans CJ, Christie MJ (2013) Regulation of -Opioid Receptors: Desensitization, Phosphorylation, Internalization, and Tolerance. Pharmacol Rev 65:223–254 . doi: 10.1124/pr.112.005942
126. Riba P, Ben Y, Smith AP, Furst S, Lee NM (2002) Morphine tolerance in spinal cord is due to interaction between mu- and delta-receptors. J Pharmacol Exp Ther 300:265–72 . doi: 10.1124/JPET.300.1.265
127. Martini L, Whistler JL (2007) The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. Curr Opin Neurobiol 17:556–564 . doi: 10.1016/J.CONB.2007.10.004

10. List of own publications

10.1. 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)

10.2. 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 [^{3}H]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)

DOI:10.14753/SE.2019.2219

11. Acknowledgements

First, I would like to thank my supervisor **Mahmoud Al-Khrasani** for his tremendous help, patients and leadership.

I also have to thank **Kornél Király, Zoltán Zádori**, and **Pál Riba** for their continuous support.

I thank **Júlia Timár** for her careful work in the finalization of the present thesis.

I thank **Prof. Péter Ferdinandy,** head of Department for his support.

I am grateful for the support of **Prof. Zsuzsanna Fürst** and **Prof. Klára Gyires.**

For their scientific and experimental work I thank: **Erzsébet Lackó** (Randall-Selitto test), **Prof. Sándor Benyhe, Ferenc Zádor** and their co-workers (Hungarian Academy of Sciences; *in vitro* binding experiments), **Prof. Michael Schäfer** and his co-workers (Charité University, Berlin; immunohistochemistry), **Sándor Hosztafi** and his co-workers (synthesis of novel compounds).

I thank our students for their effective work: **Bence Varga, Dávid Karádi, Erzsébet Varga, Róbert Stollmayer, Amir Mohammadzadeh.**

I would like to thank all fellow co-workers of the **Department of Pharmacology and Pharmacotherapy**.

I also thank **Zsuzsanna Kalmár** and all other workers of the Doctoral Office for their help.

Finally, I am grateful for the never-ending support of my family.

I thank the support of Semmelweis University Doctoral School (EFOP-3.6.3.-VEKOP-16-2017-00009) and Richter Gedeon Plc. (Centenáriumi Alapítvány).

DOI:10.14753/SE.2019.2219

12. Köszönetnyílvánítás

Elsőként köszönettel tartozom témavezetőmnek, **Al-Khrasani Mahmoudnak**. Köszönöm TDK-s korom óta tartó türelmes, folyamatos útmutatását, támogatását, segítségét.

Köszönöm **Király Kornélnak**, **Zádori Zoltánnak** és **Riba Pálnak**, hogy szinte társ-témavezetőként mindig számíthattam segítségükre, tanácsaikra.

Köszönöm **Timár Júliának**, hogy gondos munkájával segítette a dolgozat véglegesítését.

Köszönöm **Prof. Ferdinandy Péter** intézetvezetőnek, hogy munkámat figyelemmel kísérte és támogatta.

Köszönöm **Prof. Fürst Zsuzsanna** és **Prof. Gyires Klára** támogatását.

Köszönöm a kísérletes munkát **Dr. Lackó Erzsébetnek** (Randall-Selitto teszt); **Prof. Benyhe Sándornak, Zádor Ferencnek** és munkatársaiknak (MTA, Szeged; G-protein aktivitási vizsgálatok); **Prof. Michael Schäfernek** és munkatársainak (Charité Egyetem, Berlin; immunhisztokémiai és receptorkötési vizsgálatok). Köszönöm **Dr. Hosztafi Sándornak** és munkatársainak az új vegyület szintézisét.

Köszönöm TDK hallgatóink, **Varga Bence, Karádi Dávid, Varga Erzsébet, Stollmayer Róbert** és **Amir Mohammadzadeh** sok segítségét.

Köszönettel tartozom a **Farmakológiai és Farmakoterápiás Intézet** szinte összes munkatársának, különös tekintettel a Transzlációs Neurofarmakológiai Kutatócsoport munkatársaira. Köszönöm Pol-Maruzs Veronika kísérletek során nyújtott segítségét, Ph.D hallgatótársaimnak a jó hangulatú közös munkát.

Köszönöm **Kalmár Zsuzsanna** és a Doktori Iskola Hivatala többi munkatársának segítségét.

Végül, de nem utolsó sorban hálával tartozom családom soha el nem múló támogatásáért. Köszönöm barátaim lelkesítő ösztönzését.

Köszönöm a Semmelweis Egyetem Doktori Iskola (EFOP-3.6.3.-VEKOP-16-2017-00009) és a Richter Gedeon Centenáriumi Alapítvány anyagi támogatását.