Peptide and non-peptide μ-opioid agonists: peripheral and central analgesia

Doctoral theses

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Budapest
2016

INTRODUCTION

Opioids are still the most widely used analgesics, both in acute and chronic pain. However, unfortunately, apart from their strong pain-relieving effect, we have to take into account their considerable side effects as well, such as constipation, respiratory depression, nausea, vomiting, sedation, development of addiction and tolerance, which limit their daily use to a great extent. In many respects, relieving acute pain can be looked upon as solved, unlike chronic pains for which no really effective and safe medication has been found so far, though intensive research carried out worldwide. In pain research, it was a great breakthrough when, at the end of the 1980s, besides central opioid receptors, researchers also identified opioid receptors in peripheral sensory neurons.

Thus, novel opioid agonists came into the centre of interest that produced antinociceptive effect in the periphery but not CNS, avoiding the central side effects.

The aim of our work was the analysis of the site of action of compounds poorly penetrating through the blood-brain barrier such as opioid peptides and newly synthesized *non-peptide* opioid compounds after systemic administration, and also that of their possible peripheral antinociceptive effect.

In our experiments, we studied the peripheral effect of μ-opioid receptor selective agonist peptide DAMGO ([D-Ala^{2,} N-MePhe^{4}, Gly-ol^{5}] enkephalin) in a new rat writhing test induced by acetic acid modified by us. The applied visceral pain test is considered to be the model of persistent pain. The results obtained stimulated us to develop a non-peptide compound with similar chemical properties. Following this, we conducted the detailed study of the spectrum of opioid effect of the new compound, 14-*O*-Methylmorphine-6-*O*-sulphate (14-*O*-MeM6SU) in *in vitro* (receptor-binding studies, isolated organs) and *in vivo* (tail-flick) tests.

OBJECTIVES

I. Study of pharmacological effect of DAMGO and morphine in our new test system (rat writhing test):

1) Measuring antinociceptive effect of opioid prototype peptide, DAMGO compared to non-peptide opioid, morphine after intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injections.
2) Demonstrating the peripheral versus central opioid effect of i.p. or i.c.v. injected agonists by use of i.p. or i.c.v. injected naloxone methiodide (NAL-M), a peripherally acting opioid antagonist after systemic administration.

II. Study of pharmacological effect of 14-*O*-MeM6SU compared to reference compounds

1) In vitro
a) Biochemical assays (receptor-binding and G-protein activation):
Affinity, selectivity and potency
b) In *in vitro biologyical assays* (isolated mouse *vas deferens* and rat *vas deferens*)*:* selectivity, potency and efficacy
2) In vivo test (rat tail-flick test):
a) measuring antinociceptive effect of 14-*O*-MeM6SU compared to morphine-6-sulphate or morphine after systemic (subcutaneous, s.c.) and central (i.c.v.) administration.
b) Determining the ratio of i.c.v./ s.c. for the individual agonists.

METHODS

I. *In vivo* experiments

1. Acetic acid writhing (visceral pain) test on rats

Experiments were carried out on male Wistar rats weighing 100-140g. The experiments were performed in accordance with the regulations of the Ethical Committee of Semmelweis University, which are based on the Helsinki Treaty (EC Directive 86/609/EEC, number of permit: 22.1/605/001/2010).

Compounds used in this series of experiments: morphine hydrochloride, nalaxone hydrochloride (Alkaloid-ICN), DAMGO, NAL-M (Sigma-Aldrich). The compounds were dissolved in saline solution of 0.9 %.
Saline, morphine and DAMGO were injected i.p. (5 ml/kg body weight) or i.c.v. (10 µl/rat).
At zero minute, animals were injected with 2 % of acidic acid i.p. in an amount of 3 ml/kg each. Following the injection of i.p. acetic acid, the observation time of 120 minutes were divided into 12 periods, each lasting 10 minutes. The number of writhings was stabilised by the 50^{th} minute and this stability persisted until the 90^{th} minute. Saline and test compounds were injected in the 55^{th} minute i.p. or i.c.v., then antinociceptive effect was determined by the number of writhings between the 60^{th}-80^{th} minutes. I.p./i.c.v. rates were calculated based on ED_{50} values obtained from dose effect curves [ED_{50} (nmol/kg) i.p./ED_{50} (nmol/rat) i.c.v.].

2. Rat tail-flick test (RTF)

We used rats of body-weights of 100-140g. Substances dissolved in physiological saline were injected to the rats s.c. or i.c.v. At the beginning of the experiment, the basic latency values of tail flicks were determined. The effect of substances were measured at the 20^{th}, 30^{th}, 60^{th} and 120^{th} minutes following s.c. administration. Following i.c.v. dosing, measurements were carried out at the 10^{th}, 20^{th}, 30^{th}, 60^{th} and 120^{th} minutes. We took the 2-fold increase of latency as maximal effect. The values obtained were expressed in percentage.

II. *In vitro* experiments

1. Receptor-binding experiments

Receptor-binding experiments were performed at the Institute of Biochemistry at the Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary.
Brain homogenates were taken from male Wistar rats or R9 guinea pigs weighing 250-300g.
14-*O*-methylmorphine-6-*O*-sulphate (14-*O*-MeM6SU) and morphine-6-*O*-sulphate (M6SU) were synthesized at the department of Pharmaceutical Chemistry, Semmelweis University, Budapest by Dr Sándor Hosztafi and Dr András Váradi under the guidance of Dr Béla Noszál.

Compounds used were as follows: naloxone hydrochloride (NAL), naltrexone hydrochloride (NTX), morphine hydrochloride (Alkaloid-INC), ethyl ketocyclazocine – EKC (Sterling Winthrop), [d-Ala^2, d-Leu^5] enkephalin – DADLE (Sigma-Aldrich), naltrindole – NTI (Sigma-Aldrich), 5-α,7-α,8-α-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)-dec-8yl]-benzeneacetamide -U-69,593 (Upjohn Co), [D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin (DAMGO), Ile^{5,6}deltorphin II (DIDII), [^{3}H]DAMGO-t (41 Ci/mmol), [^{3}H]DIDII (49 Ci/mmol) (Isotope Laboratory of the Biological Centre, Szeged), [3H] U-69,593 (44 Ci/mmol) (Amersham). Each compound was dissolved in physiological saline.

a) Competitional binding experiments. Competitional binding experiments were carried out on the homogenised brain membrane preparations of rats and guinea pigs. From these, by centrifuging, we obtained saccharose-free pellets, then the obtained pellets were suspended in 50nM Tris-HCl buffers (pH 7.4). Membrane homogenates, in correspondence with radioactive ligands, were incubated – [^{3}H] DAMGO and [^{3}H] DIDII radioligands at 35 °C for 45 minutes, whereas [^{3}H] U-69.593 radioligand at 30 °C for 30 minutes. The final volume of the incubation mixture with the unlabeled 14-*O*-MeM6SU and M6SU 10^{-11} – 10^{-5} M) ligands and the 1 nM [^{3}H] DAMGO, [^{3}H] DIDII or the 3 nM [^{3}H] U-69.593 were 1 ml altogether in each case. The complete bond of radioactive ligands was established without the presence of the unlabeled ligands, while the level of non-specific bond, depending on the opioid receptor studied, was determined in the presence of 10 μM of unlabeled DAMGO, DIDII or U-69.593. Following incubation, radioligands bound or not bound to the receptor were separated by fast vacuum screening, then the radioactivity of the dried filter discs was detected.

b) Functional [^{35}S] GTPγS binding experiments. In order to obtain ~ 10 μg of protein sample, we diluted brain membrane preparations of rats and guinea pigs by a 50 mM Tris HCl buffer (pH 7.4). Following this, samples were incubated in a Tris-EGTA buffer (pH 7.4) at 30 °C for 60 minutes in a final volume of 1 ml. The buffer also contained 20 MBq/0.05 cm^3 of [^{35}S] GTPγS radioactive nucleotide analogue (0.05 nM) and 14-*O*-MeM6SU, M6SU, DAMGO, DIDII and U-69.593 unlabeled ligands in increasing concentrations (10^{-10} – 10^{-5} M). The value of the complete bond was determined without the presence of unlabeled ligands, whereas the level of non-specific bond was determined in the presence of 10 μM of unlabeled GTPγS. The value of the specific bond of [^{35}S] GTPγS was given by the

difference between the two (T-NS). The specific binding value of the [^{35}S]GTPγS determined without the presence of the unlabelled ligands indicates the basic activity of the receptor G-protein, which was taken as 100 %. The separation of bound and unbound [^{35}S] GTPγS and the detection of samples were carried out similarly to those of competition binding assays.

2. Isolated organs

a) Mouse vas deferens (MVD). Vasa deferentia were taken from NMRI male mice weighing 35-45g. Then the preparations were suspended between two electrods in 31 °C Mg^{2+}-free Krebs solution composed of (mM/ml): NaCl = 118; NaHCO3 = 25; KCl = 4.7; KH_2PO_4 = 1.2; glucose = 11; $CaCl_2$ = 2.5). The isolated organs were exposed to rest tension of 0.1 g. In these experiments, we studied the effects of test compounds on mouse vas deferens muslce contractions evoked by electric field stimulation. The parameters of electric stimulation were as follows: paired rectangular waves (of 1 ms, signal strength of 9 V/cm) were used in a pulse distance of 100 ms, repeated every 10 seconds. We followed the amplitude of muscle contractions on computer.

b) Rat vas deferens (RVD). In RVD tests we used rats weighing 170-250 g. The protocol, except for a few modifications, was the same as in case of MVD. In this case, Krebs solution was used (NaCl = 118; $NaHCO_3$ = 25; KCl = 4.7; KH_2PO_4 = 1.2; glucose = 11; $CaCl_2$ = 2.5; $MgSO_4$ = 1.2 mM/L). The initial organ tension was 0.5 g and the parameters of electrical stimulation were as follow: rectangular waves, (of a width of 1 ms and of an intensity of 9 V/cm) at frequency of 0.1 Hz.

III. Statistical analysis

The results obtained in rat writhing test were expressed as mean ± S.E.M. (standard error of mean), while antinociceptive effect was expressed in percentage. The antinociceptive (ED_{50}) values were calculated from the linear dose effect curves. Confidence interval of 95 % was determined based on the way described in the literature (Litchfield-Wilcoxon's method). The difference between animal groups treated with saline, agonist and agonist + NAL-M was determined by analysis of variance (one way ANOVA), followed by Tukey's post hoc test. Results were considered statistically significant if p was < 0.05.

In tail-flick test, the antinociceptive (ED_{50}) values and 95 % confidence intervals were calculated as described by Litchfield-Wilcoxon's method.
Competition binding (assay) experiments were evaluated with the help of GraphPad Prism 5.0. We determined the compound concentration which brought down the 50 % (IC_{50}) of the appropriate radioactive ligands. At functional [35S]GTPγS binding experiments Log EC_{50} and E_{max} values and significance levels were also calculated with GraphPad Prism 5.0.
In MVD and RVD experiments, 50 % effective concentration (EC_{50}) and maximal effect (E_{max}) were calculated with nonlinear regression. In case of MVD, the dissociational constant (K_e) of NAL was calculated with the single-dose method. Calculation of K_e value = [concentration of antagonist]/dose ratio-1.

RESULTS

I. Study of pharmacological effects of DAMGO and morphine

Antinociceptive effect of morphine and DAMGO on visceral pain model. We studied the antinociceptive effects of morphine and DAMGO in a rat writhing test induced by i.p. injected acetic acid. Compounds were injected in the 55^{th} minute following acetic acid; their effect was measured in the period between the 60^{th} and 80^{th} minutes. Following i.p. injections, DAMGO showed a dose-dependent antinociceptive effect, similar to that of morphine. The value of ED_{50} of morphine and DAMGO was 238.57 and 289.52 nmol/kg, respectively. These data show that the antinociceptive effect of the two compounds is equipotent following i.p. dosing. After i.c.v. injections, too, both compounds demonstrated dose-dependent antinociceptive effect. Based on the calculated ED_{50} values, both morphine (2.02 nmol/animal) and DAMGO (0.006 nmol/animal) proved to be more potent than in case of i.p. injection. It can also be stated that, following i.c.v. injectins, DAMGO is substantially more potent analgesic than morphine. In correspondence with the above facts, that the i.p./i.c.v. ratio of ED_{50} values of DAMGO is also higher than that of morphine.

The effect of i.p.-injected, peripherally acting opioid antagonist NAL-M on i.p. administered morphine or DAMGO. As expected, i.p. injected NAL-M (2130.83 nmol/kg) by itself had no effect on the number of writhes, however, it significantly decreased the antinociceptive effect of the i.p. injected morphine or DAMGO.

The effect of i.c.v. injected, opioid antagonist NAL-M on i.p. injected morphine or DAMGO. NAL-M was injected i.c.v., whereas, simultaneously, morphine or DAMGO was injected i.p. I.c.v. injected NAL-M (21.31 nmol/animal) significantly reduced the effect of the i.p. injected morphine, at the same time, it had no effect on the antinociceptive effect of the i.p. injected DAMGO.

II. Study of the pharmacological effects of 14-*O*-MeM6SU

Receptor-binding experiments. We studied binding affinity and selectivity of 14-*O*-MeM6SU for MOR and DOR on rat brain preparations, while for KOR we studied them on guinea pig brain membrane preparations. We used [^{3}H] DAMGO (μ), [^{3}H] DIDII (δ) and [^{3}H] U-69.593 (K) as radioligands. 14-*O*-MeM6SU inhibited the specific binding of [^{3}H] DAMGO, [^{3}H] DIDII and [^{3}H] U-69.593. Based on competition binding curves, we obtained the following K_i values: 1.12; 10.23; and 295.12 nM, respectively. We state that, compared to the data of M6SU, morphine and homologous opioid ligands, 14-*O*-MeM6SU had the higher MOR affinity, but its affinity to DOR 9 times lower compared to MOR.

[^{35}S]GTPγS binding stimulated by 14-O-MeM6SU. In rat brain membrane preparations, the maximal activation (E_{max}) of receptor-mediated G-protein induced by 14-*O*-MeM6SU was substantially higher than that of morphine or M6SU ($p < 0.0001$) and it was similar to that of DAMGO, which is an extremely effective synthetic MOR agonist peptide. The E_{max} values of morphine and M6SU, compared to the effect of DAMGO, correspond to the partial agonist property. Their potency order was as follows: morphine > 14-*O*-MeM6SU > M6SU > DAMGO > DIDII. The opioid antagonist NAL (10 μM), added to rat brain membrane homogenates, as expected, shifted the binding curve of [^{35}S]GTPγS stimulated by 14-*O*-MeM6SU to the right, confirming the opioid specificity of the effect. 14-*O*-MeM6SU effectively stimulated G-protein activation mediated by KOR, with greater efficacy than U-69.593, KOR selective ligand.

The activity of opioid agonists on MVD and RVD tests. In MVD, the EC_{50} (nM) values of 14-*O*-MeM6SU, M6SU, morphine and DAMGO were 4.38, 102.81, 346.63 and 238.47, respectively. Similarly to DAMGO, 14-*O*-MeM6SU strongly inhibits muscle contractions induced electrically on MVD in a concentration-dependent way. On the contrary, morphine and M6SU were unable to exert maximal effect. The obtained E_{max} ± S.E.M.

values in percentage were as follows: 99.10 ±0.90 for 14-*O*-MeM6SU; 96.99 ± 1.88 for DAMGO; 36.87 ± 3.36 for M6SU and 42.51 ± 6.43 for morphine. The values of NAL K_e were fallen between 0.66 - 1.92 range for the four compounds.
In RVD, the electrically induced muscle contractions were inhibited by both 14-*O*-MeM6SU and DAMGO in a concentration-dependent manner, whereas morphine and M6SU failed to produce inhibition. 14-*O*-MeM6SU was approximately 5 times more effective than DAMGO. E_{max} (%) was 85.16 for 14-*O*-MeM6SU and 80.58 for DAMGO. 14-*O*-MeM6SU and DAMGO showed high efficacy and potency, whereas morphine and M6SU had no effect in this test.

Study of antinociceptive effect on a tail-flick test in rats. Following s.c. administration, 14-*O*-MeM6SU, M6SU and morphine produced dose-dependent antinociceptive effect. Based on the s.c. ED_{50} values 14-*O*-MeM6SU was 51 and 34-fold more potent than M6SU and morphine, respectively. After i.c.v. injections, 14-*O*-MeM6SU proved to be 23-times more potent than M6SU and 2456-times than morphine. NAL (1 mg/kg, s.c.) abolished the antinociceptive effect of test compounds (14-*O*-MeM6SU, M6SU and morphine). At maximal effect, s.c./i.c.v. dose ratio was 12242 for 14-*O*-MeM6SU, 26137 for M6SU and 161 in the case of morphine.

CONCLUSIONS

- In our test system, on the writhing test, DAMGO and morphine administered ystemically (i.p.) or centrally (i.c.v.) inhibited pain dose-dependently. When applied systemically, the antinocieptive effect of DAMGO and morphine was comparable. On the contrary, following central administration, DAMGO proved to be more potent analgesic. The quaterner opioid antagonist NAL-M, having limited penetration through the blood-brain barrier, inhibited the antinociceptive effect of systemically administered morphine when injected i.p. or i.c.v., indicating that morphine has peripheral and central analgesic effects. Furthermore, NAL-M injected i.c.v. inhibited the antinociceptive effect of morphine but not of DAMGO administered systemically, revealing the peripheral analgesic effect of DAMGO. In our experiments, we used visceral pain test, modified by us, as model of persistent pain. It is to be underlined that the applied method is unique in that in its practice it

is identical with the clinical conditions, as the application of compounds takes place when *the pain has already developed*. We were the first to demonstrate that MOR agonists inhibit persistent visceral pain already developed in rats.

- We established that a morphine derivative, 14-*O*-MeM6SU, newly synthesised in cooperation, showed low (δ/μ) and high (κ/μ) selectivity in receptor-binding tests.
- We ascertained that 14-*O*-MeM6SU showed opioid properties on *in vitro* MVD and RVD tests. In MVD, 14-*O*-MeM6SU was more potent than the reference compounds. The obtained naloxone K_e value refers to MOR-mediated effect of 14-*O*-MeM6SU, although its selectivity, compared to DOR-mediated effect, was very low (10-fold). In RVD, based on E_{max} values, 14-*O*-MeM6SU and DAMGO are full agonists, whereas M6SU and morphine are partial agonists. This observation was supported by our G-protein activation experiments as well.
- In *in vivo* thermal RTF tests, after systemic (s.c.) and central (i.c.v.) administration, 14-*O*-MeM6SU showed dose-dependent antinociceptive effect. We established that in this test, compared to morphine or M6SU, 14-*O*-MeM6SU proved to be the most potent analgesic. Furthermore, the calculated i.c.v./s.c. ratio for 14-*O*-MeM6SU and M6SU was significantly higher than in case of morphine; this fact may refer to limited penetration into the central nervous system of the former ones.
- Based on the results obtained, it seems to be promising that 14-*O*-MeM6SU and also its developed derivatives really possess reduced central side effects, which we wish to analyse in detail in the next stage of our work, thus, in future, there will be a chance to produce safe and effective analgesics suitable for clinical practice as well.

LIST OF OUR OWN PUBLICATIONS
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[1]These authors equally contributed to the work

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5. Alberti, A., Beni, S., Lacko, E., Riba, P., Al-Khrasani, M., Kery, A., 2012. Characterization of phenolic compounds and antinociceptive activity of Sempervivum tectorum L. leaf juice. Journal of pharmaceutical and biomedical analysis 70, 143-150

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