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In vitro opioid activity profiles of 6-amino acid substituted derivatives of 14-*O*-methyloxymorphone

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Abstract

A series of 6-amino acid conjugates (glycine, alanine and phenylalanine) of the highly potent opioid analgesic 14-*O*-methyloxymorphone was developed in an effort to obtain agonists that would have potentially limited ability to cross the blood–brain barrier. Binding studies revealed that all derivatives displayed high affinities (0.77–2.58 nM) at the μ-opioid receptor in rat brain membranes. They were potent agonists in mouse vas deferens preparation (IC_{50} = 5.52–26.8 nM). While the α-amino acid epimers are favoured by μ-opioid receptors, the β-epimers proved to have increased interaction with δ-sites. Only the β-phenylalanine conjugate showed some preference for δ- over μ-opioid receptors and δ-opioid receptor agonist activity. The relatively high δ-opioid receptor affinity of this analogue was also predicted by molecular modelling studies. The newly developed ionizable derivatives could find clinical applications as potent analgesics without the adverse actions of centrally acting opioids.

Keywords: Opioid receptor; μ-Opioid receptor agonist; 14-*O*-methyloxymorphone; Binding affinity; Vas deferens, Mouse

1. Introduction

Opioid receptors exist as three main types, μ, δ and κ, and they have distinct anatomical distribution, pharmacological profile and ligand selectivity (Simon and Hiller, 1994). Traditionally, antinociception of endogenous and exogenous opioids has been associated with activation of opioid receptors in the central nervous system (CNS) (Reisine and Pasternak, 1996). In the past decades, experimental and clinical reports demonstrate that locally applied opioid agonists elicit pronounced analgesia by interacting with peripheral opioid receptors (Stein, 1995; Stein et al., 2001, 2003). Dose-dependency, stereospecificity and antagonist reversibility indicate an opioid receptor-specific mechanism of action.

Currently, treatment of severe pain mostly relies on the use of centrally acting opioid analgesics such as morphine, codeine, fentanyl and oxycodone. The clinical utility of these analgesics is, however, severely limited by a plethora of undesirable side effects which are mediated predominantly via the CNS (e.g. sedation, nausea, confusion, respiratory depression, tolerance, dependence) (MacPherson, 2002). An active search for opioid compounds exhibiting a favourable dissociation between analgesic activity and the development of dependence/tolerance and other side effects has been carried out during the past decades (Schiller, 1991; Schmidhammer, 1998). In 1984, a derivative of oxymorphone, 14-*O*-methyloxymorphone (Fig. 1) was developed by our group and described to be ca. 400 and 40 times more potent than morphine and oxymorphone, respectively, in the hot plate assay in mice (Schmidhammer et al., 1984). Further chemical optimization led to another

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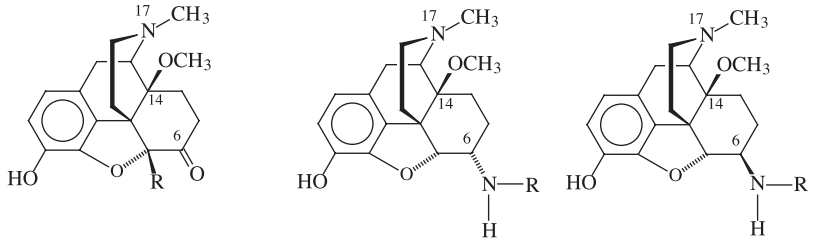
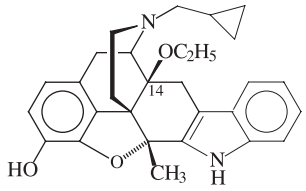


R = H: 14-*O*-Methyloxymorphone
R = CH_3: 14-Metoxymetopon

6-Amino acid substituted derivatives of 14-*O*-methyloxymorphone

α-series
1 R = CH_2COOH
3 R = (S)-$CH(CH_3)COOH$
5 R = (S)-$CH(CH_2Ph)COOH$

β-series
2 R = CH_2COOH
4 R = (S)-$CH(CH_3)COOH$
6 R = (S)-$CH(CH_2Ph)COOH$



HS 378

Fig. 1. Chemical structures of the studied compounds.

derivative of the 14-alkoxymorphinan series of opioids, 14-methoxymetopon (Fig. 1) (Schmidhammer et al., 1990), which was reported to be a highly selective and highly potent μ-opioid receptor agonist (Fürst et al., 1993; Spetea et al., 2003). 14-Methoxymetopon was described as an extremely potent centrally acting analgesic, which has the advantage of inducing minimal physical dependence, tolerance, respiratory depression, constipation and other side effects observed with the use of morphine or sufentanil (Fürst et al., 1993; Freye et al., 2000; Zernig et al., 2000; King et al., 2003).

Strategies to restrict the access of opioids to the CNS include the incorporation of highly polar hydrophilic substituents. First efforts to minimize the CNS effects of opioids while retaining their actions in peripheral tissue have led to the synthesis of quaternary derivatives (Iorio and Frigni, 1984; Brown and Goldberg, 1986). These studies to develop peripherally selective opioids have primarily focused on the quaternization of known agonists or antagonists. Generally, it has been found that quaternary compounds have considerably lower affinity to opioid receptors and substantially reduced access into the CNS relative to their tertiary amine precursors (Brown and Goldberg, 1986; Shaw et al., 1989). In order to avoid this problem of lower affinity, opioids with hydrophilic groups attached to the C-6 position of the morphinan skeleton have been synthesized from β-oxymorphamine (Botros et al., 1989), β-naltrexamine (Botros et al., 1989) and β-funaltrexamine (Larson et al., 1993). It was reported that such compounds, particularly opioids with zwitterionic moieties, show a greatly reduced access to the CNS, without substantially decreased opioid receptor activity (Botros et al., 1989; Portoghese et al., 1995). Similar approaches have been employed in the benzeneacetamide (Shaw et al., 1989) and phenylpiperidine (Zimmerman et al., 1994) classes of opioids.

A series of 6-amino acid conjugates (glycine, alanine and phenylalanine) of the highly potent opioid analgesic 14-*O*-methyloxymorphone (Fig. 1) was developed in an effort to obtain opioid agonists with high potency that would have negligible access to the CNS (Schütz et al., 2003). In the present study, their binding affinities to opioid receptors (μ, δ, κ) in rat brain and in vitro pharmacological profile (agonist potency) in mouse vas deferens preparation are described.

2. Materials and methods

2.1. Materials

The 6-amino acid substituted derivatives of 14-*O*-methyloxymorphone (Fig. 1) were prepared as recently described (Schütz et al., 2003). [D-Ala^2,Me-Phe^4,Gly-ol^5] enkephalin (DAMGO), [D-Ala^2,Leu^5]enkephalin (DADLE), naloxone, naltrexone and naltrindole were obtained from Sigma (St. Louis, MO, USA). [^{3}H][$Ile^{5,6}$]deltorphin II (30 Ci/mmol) was prepared by Dr. G. Tóth (Biological Research Center, Szeged, Hungary) according to the described procedure (Nevin et al., 1994). [^{3}H]DAMGO (50 Ci/mmol) and [^{3}H]5α,7α,8β-(−)-*N*-methyl-*N*[7-(1-pyrrolidinyl)-1-oxaspiro(4-5)dec-8-yl]benzeneacetamide ([^{3}H]U69,593; 47 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). All other chemicals were of

analytical grade and obtained from standard commercial sources.

2.2. Receptor binding assay

A crude membrane fraction was prepared from Sprague–Dawley rat brains according to the method previously described (Spetea et al., 2001). All binding experiments were performed in 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 1 ml containing 300–500 μg protein. Rat brain membranes were incubated either with [^{3}H]DAMGO (1 nM, 45 min, 35 °C), [^{3}H][Ile^{5,6}]deltorphin II (0.5 nM, 45 min, 35 °C) or [^{3}H]U69,593 (1 nM, 30 min, 30 °C) and 10 concentrations of the test ligand. Reactions were terminated by rapid filtration through Whatman glass fibre filter type GF/B for [^{3}H]U69,593 or type GF/C ([^{3}H]DAMGO and [^{3}H][Ile^{5,6}]deltorphin II), using a Brandel Cell Harvester followed by washing with 3 × 5 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4). The bound radioactivity was measured in Ultima Gold scintillation cocktail, using a Beckman LS1701 liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM unlabelled naloxone. Protein concentration was measured by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.3. Mouse vas deferens bioassay

Male CFLP mice weighing 25–30 g were used for the vas deferens bioassay, performed as earlier described (Hughes et al., 1975; Ronai et al., 1977). The abdominal/pelvic cavity was opened and the testis was expelled from the scrotum. The whole length of vas was dissected and placed at room temperature in a modified Mg^{2+}-free Krebs-bicarbonate buffer, consisting of 113 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4 and 11 mM glucose, and aerated with 95% oxygen + 5% carbon dioxide. The whole vas was cut in the middle and the two sides were mounted separately. The double-jacketed organ bath had a capacity of 5 ml and had a built-in coil to pre-warm the Krebs buffer entering the bath. A ring-shaped wire electrode was fixed to the top of the bath. The buffer was kept in a double-jacketed, thermostated reservoir, where it was aerated with carbon dioxide at 31 °C. The bathing fluid in the working space was changed by overflow (a flush with 25–30 ml). The upper thread of the mounted organ was attached to a non-auxotonic force transducer fed into a preamplifier. The following system settings were used: initial tension: 0.1 g, re-setting after 2 min; stimulation: field, rectangular, 1 ms pulse width, constant voltage, 9 V/cm (supramaximal); pairs of stimuli with 100 ms (10 Hz), pulse distance by 10 s; equilibration: 25–30 min under stimulation, wash by 5 min. The inhibitory actions of the opioid compounds on the electrically evoked contractions were measured and IC_{50} values were estimated for the agonist activity and K_e values for the antagonist activity.

2.4. Molecular modelling

The entire molecular modelling study was performed using the Sybyl 6.7 software package (St. Louis, USA) running on a Silicon Graphics O2 R5000 workstation. The molecules were assembled de novo from the Tripos standard fragment library. Hydrogen atoms were placed at standard bond distances and angles. Flexible side chains were set to a fully extended conformation. The potential energy of each structure was refined by a molecular mechanics procedure (MAXIMIN2 energy minimization procedure using the Sybyl 6.7 Tripos standard force field based on Clark et al., 1989 until the root mean energy gradient was less than 0.005 kcal/mol Å). Partial atomic charges were calculated using the method of Gasteiger and Marsili (1980) and coulomb terms were included in the potential energy minimization process. Conformational analysis was performed using the systematic search procedure with a torsion angle increment value of 6° for the single bonds in the flexible side chain. In order to visualize the conformational space occupied by the aromatic group of the phenylalanine moiety, sweep plots were generated showing energetically possible positions of the phenyl centroid and superimposed with the fully relaxed structure model of compound HS 378 (Fig. 1) (Schmidhammer et al., 1998). For clarification purposes, a filter of 30° was set for plotting the centroid positions of investigated compounds **5** and **6**.

2.5. Data analysis

Inhibition constants (K_i) were calculated from displacement binding curves using the nonlinear least-square curve fitting by GraphPad Prism (version 3.0, San Diego, CA. USA) program. In mouse vas deferens bioassay, the IC_{50} concentrations of the agonists were estimated from each dose–response curve by logarithmic regression. The antagonist potencies (K_e) were calculated with the single dose method (Kosterlitz and Watt, 1968). All experiments were repeated at least three times and values are expressed as the mean ± S.E.M.

3. Results

3.1. Opioid receptor binding affinities and selectivities

The binding affinities of compounds **1–6** (Fig. 1) to opioid receptors were determined using competitive binding assays in rat brain membranes employing [^{3}H]DAMGO (μ), [^{3}H][Ile^{5,6}]deltorphin II (δ) and [^{3}H]U69,593 (κ) as radioligands. Morphine, 14-*O*-methyloxymorphone (Schmidhammer et al., 1984) and 14-methoxymetopon (Schmidammer et al., 1990) were also tested for direct comparison. The binding results expressed as K_i values are shown in Table 1.

Table 1
Opioid binding affinities and selectivities of 6-amino acid substituted derivatives of 14-*O*-methyloxymorphone in rat brain membranes

Compound	K_i (nM)[a]			Selectivity ratio	
	[^{3}H]DAMGO (μ)	[^{3}H][Ile^{5,6}]Deltorphin II (δ)	[^{3}H]U69,593(κ)	δ/μ	κ/μ
Morphine[b]	6.55 ± 0.74	217 ± 19	113 ± 9	33	17
14-*O*-methyloxymorphone	0.10 ± 0.01	4.80 ± 0.22	10.2 ± 2.0	48	102
14-Methoxymetopon[b]	0.15 ± 0.01	13.3 ± 0.2	25.2 ± 4.9	89	168
HS 378[c]	340 ± 27	0.78 ± 0.06	134 ± 30	0.002	0.39
1	0.89 ± 0.09	15.4 ± 1.4	43.2 ± 7.0	17	49
2	0.83 ± 0.02	7.86 ± 0.64	44.8 ± 0.1	9.5	54
3	0.77 ± 0.09	26.9 ± 0.8	142 ± 43	35	184
4	1.90 ± 0.08	7.71 ± 0.94	63.7 ± 7.8	4.1	34
5	0.95 ± 0.07	3.67 ± 0.32	28.5 ± 4.2	3.9	30
6	2.58 ± 0.09	1.03 ± 0.13	151 ± 17	0.40	59

[a] Values represent the mean ± S.E.M. of three to four independent experiments.
[b] Data taken from Spetea et al. (2003).
[c] Data taken from Spetea et al. (2001).

The derivatives **1**–**6** all displayed high potency to inhibit [^{3}H]DAMGO binding to the μ-opioid receptor with K_i values in the nanomolar range (K_i values from 0.77 to 2.58 nM). The K_i values at the δ-opioid binding sites were higher except for compound **6**, and the affinities were the lowest towards the κ-opioid receptor (Table 1). All six compounds showed a significant increase in affinity for the μ-opioid receptor as compared to that of morphine and somewhat lower μ-opioid receptor affinity than that of their parent compounds, 14-*O*-methyloxymorphone and 14-methoxymetopon. It was observed that the α-amino acid epimers **1**, **3** and **5** displayed very similar affinities for the μ-opioid receptor (0.89, 0.77 and 0.95 nM, respectively), whereas in the case of β-amino acid epimers **2**, **4** and **6** differences were noted (0.83, 1.90 and 2.58 nM, respectively). Moreover, the β-epimers showed higher affinity to the δ-opioid receptor compared to the corresponding α-epimers (Table 1). Notably, compound **6** exhibited similar binding affinity to both μ- and δ-opioid receptors with slight preference for the latter one. Moreover, this derivative showed comparable affinity to the δ-binding site (K_i = 1.03 nM) as that of the highly selective δ-opioid receptor antagonist, HS 378 (K_i = 0.78 nM) (Spetea et al., 2001) (Table 1).

Selectivity ratios were calculated for the new opioid compounds and their values are shown in Table 1. All of the 6-amino acid derivatives showed lower δ/μ selectivity ratios than morphine, 14-*O*-methyloxymorphone and 14-methoxymetopon. However, they were found to have higher κ/μ selectivity ratios compared to morphine and somewhat lower ratios than 14-*O*-methyloxymorphone and 14-methoxymetopon. Again, compound **6** showed preference for the δ-opioid receptor with a δ/μ selectivity ratio of 0.40. Of the 6-amino acid derivatives, the α-alanine analogue **3** was the most selective for the μ-opioid receptor with δ/μ and κ/μ selectivity ratios of 35 and 184, respectively, which are equivalent to or higher than those of the parent compounds (Table 1).

3.2. Agonist potencies

The opioid agonist activities of the 6-amino acid derivatives of 14-*O*-methyloxymorphone were assessed in mouse vas deferens bioassay. The prototypical agonists DAMGO (μ) and DADLE (δ) were used as standard ligands. The in vitro pharmacological data for the derivatives **1**–**6** and parent compounds are shown in Table 2. In agreement with previous findings, 14-*O*-methyloxymorphone and 14-methoxymetopon displayed high agonist potency in mouse vas deferens (Schmidhammer et al., 1984, 1990; Fürst et al., 1993) with IC_{50} values of 7.76 and 12.7 nM, respectively. All compounds **1**–**6** were effective in inhibiting the electrically stimulated twitch in mouse vas deferens tissue in a concentration-dependent manner, indicating that they acted

Table 2
Agonist potencies of 6-amino acid substituted derivatives of 14-*O*-methyloxymorphone in mouse vas deferens preparation

Compound	IC_{50} (nM)[a]	K_e (nM)[a]	
		Naltrexone[b]	Naltrindole
DAMGO	75.6 ± 6.9	0.33 ± 0.017	9.52 ± 0.63[c]
DADLE	0.90 ± 0.08	8.00 ± 0.49[d]	0.091 ± 0.013[e]
Normorphine	248 ± 11	0.79 ± 0.04	
14-*O*-methyloxymorphone	7.76 ± 0.88	0.33 ± 0.04	
14-Methoxymetopon	12.7 ± 3.9	0.35 ± 0.05	
1	26.8 ± 3.6	0.70 ± 0.18	
2	7.00 ± 0.18	1.20 ± 0.12	
3	12.2 ± 3.2	0.53 ± 0.13	
4	19.6 ± 1.3	1.71 ± 0.14	
5	5.52 ± 1.00	1.56 ± 0.07	
6	6.07 ± 0.60	4.44 ± 1.18[f]	0.46 ± 0.11[g]

[a] Values represent the mean ± S.E.M. of three to six independent experiments.
[b] Antagonism (K_e) with naltrexone (3 nM).
[c] Antagonism (K_e) with naltrindole (50 nM).
[d] Antagonism (K_e) with naltrexone (50 nM).
[e] Antagonism (K_e) with naltrindole (1 nM).
[f] Antagonism (K_e) with naltrexone (10 nM).
[g] Antagonism (K_e) with naltrindole (4 nM).

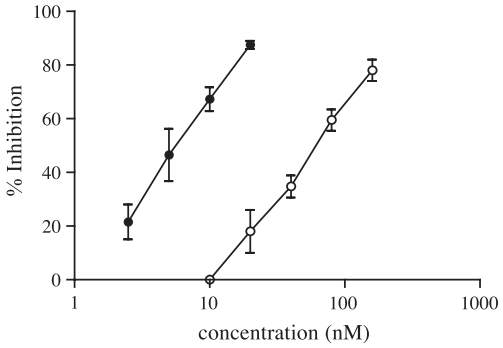


Fig. 2. Concentration–response curves of compound **6** in the absence (●) and in the presence of 4 nM naltrindole (○) in the mouse vas deferens preparation.

as potent agonists with IC_{50} values from 5.52 to 26.8 nM. Moreover, the agonist potencies of all new derivatives were substantially greater (9- to 45-fold) than the potency of normorphine.

The agonist activity of the 6-amino acid conjugates was reversed by the μ-opioid receptor preferring antagonist naltrexone (3 nM, except for compound **6** when a concentration of 10 nM was used) with K_e values ranging between 0.53 and 4.4 nM (Table 2). The antagonist potency of naltrexone was similar to that against μ-opioid receptor agonists DAMGO and normorphine for compounds **1** and **3**, while against compounds **2**, **4** and **5**, the K_e values were slightly higher. Since the K_e value of naltrexone was more than 13-times greater against compound **6** than against DAMGO (4.44 vs. 0.33 nM), the possibility of a δ-opioid receptor activity was assessed. Antagonizing the analogue **6** in mouse vas deferens with the selective δ-opioid receptor antagonist naltrindole (4 nM), a K_e value of 0.46 nM was determined by the single dose method (0.42 nM form the mean curve), suggesting a δ-opioid receptor agonist effect. The concentration–response curve of compound **6** with naltrindole is shown in Fig. 2, and it can be noted that naltrindole afforded a parallel shift of the agonist response curve. This finding is also supported by the binding results where the β-phenylalanine conjugate **6** showed slight preference for the δ- over μ-opioid receptor in rat brain membranes (Table 1) and was predicted by molecular modelling studies (see below).

3.3. Molecular modelling-comparison of epimeric compounds 5 and 6 with the δ-opioid receptor antagonist HS 378

In addition to the biological evaluation of the 6-amino substituted derivatives of 14-*O*-methyloxymorphone, a molecular modelling study has been performed. As expected, the phenylalanine side chains of both compounds **5** and **6** are able to adopt a large number of energetically comparable low energy conformations. Remarkably, the conformational space occupied by the phenyl ring of compound **5** is larger than the region occupied by the same group in compound **6**. A graphic representation of superposition of HS 378 with phenyl centroid positions of compounds **5** and **6** is shown

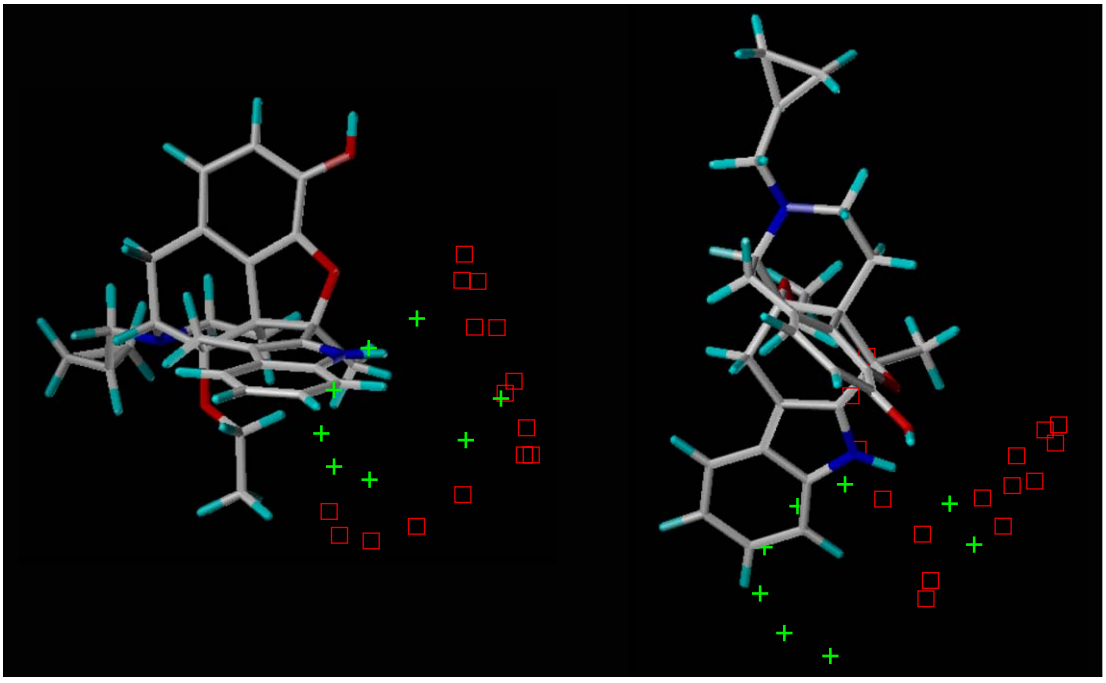


Fig. 3. Alignment of compounds **5** and **6** to HS 378. The structure of HS 378 is shown together with phenyl centroid positions (i.e. the computed center of the phenyl ring in the amino acid side chains) of **5** (red boxes) and **6** (green crosses). Boxes/crosses represent the space which can be occupied by the phenyl centroid in energetically reasonable conformers. It can be observed that the green crosses (**6**) are situated in close proximity to the aromatic moiety of HS 378, while the red boxes (**5**) are not, supporting the results of the biological studies.

in Fig. 3. As seen, the phenyl moiety of the β-epimer **6** can clearly adopt a position in close vicinity to the aromatic indole ring of the δ-opioid receptor antagonist HS 378 (Schmidhammer et al., 1998; Spetea et al., 2001). This observation is in good agreement with the experimental results (Tables 1 and 2) indicating a more than 3-fold higher affinity for the δ-opioid receptor of the β-phenylalanine analogue **6** in comparison to the corresponding α-analogue **5**.

4. Discussion

In the present study, our research efforts in the morphinan series have shifted from the development of highly potent and selective μ-opioid receptor agonists with a central site of action (Fürst et al., 1993; Schmidhammer et al., 1984, 1990) to the development of opioid analgesics that would have limited access to the CNS. Our approach involved the attachment of an amino acid residue to the C-6 position of the morphinan skeleton to afford derivatives of the highly potent analgesic 14-*O*-methyloxymorphone (Schmidhammer et al., 1984) that are more polar than their parent compound. Structure–activity relationship studies were pursued for a series of 6-amino acid substituted (gylcine, alanine and phenylalanine) derivatives of 14-*O*-methyloxymorphone that are expected to exhibit lower penetration through the blood–brain barrier. The strategy involved investigations of the influence of the substituent in position 6, and α vs. β orientation of the amino acid residue at this position on the opioid activity.

The 6-amino acid conjugates **1**–**6** (Fig. 1) were examined with regard to opioid binding properties in rat brain membranes and in vitro bioactivities to inhibit the electrically induced contractions of mouse vas deferens. As expected and in agreement with previous determinations in receptor binding and functional assays, 14-*O*-methyloxymorphone (Schmidhammer et al., 1984) and 14-methoxymetopon (Schmidhammer et al., 1990; Fürst et al., 1993; Spetea et al., 2003) showed very high affinity and selectivity for the μ-opioid receptor, and agonist potency. Moreover, these two opioid agonists displayed significantly greater selectivity for the μ-opioid receptor than morphine (Table 1). Binding studies revealed that all 6-amino acid derivatives described in this study displayed high affinities for the μ-opioid receptor which were about 2.5- to 8-fold greater than that of morphine. Having established the binding properties of the newly developed derivatives of 14-*O*-methyloxymorphone, the agonist profile was assessed in mouse vas deferens. They were found to possess high μ-opioid receptor agonist potency comparable to that of 14-*O*-methyloxymorphone and 14-methoxymetopon and several times greater than that of normorphine.

The observed differences in receptor binding affinities, selectivities and agonist potencies of the investigated compounds are related to specific structural features as described below. In agreement with earlier reports from our group of the positive influence of 14-alkoxy substitution in *N*-methyl-6-ketomorphinans in opioid receptor activity (Schmidhammer, 1993, 1998), all 6-amino acid conjugates **1**–**6**, which possess a methoxy group in the 14-position (Fig. 1), were found to interact with high affinity to opioid receptors (K_i values in low nanomolar range). Compounds **1**–**5** displayed preference for the μ-opioid receptor (see the δ/μ selectivity ratios in Table 1 and the low K_e values of naltrexone in Table 2). Only the β-phenylalanine substituted epimer **6** exhibited some preference for δ- over μ-opioid receptors (2.5-fold difference in the binding assay and the relatively high K_e value of naltrexone in mouse vas deferens bioassay). The δ-opioid receptor agonist activity of compound **6** was indicated by the difference in K_e values of naltrexone and naltrindole in mouse vas deferens preparation. Since both K_e values of naltrexone and naltrindole were similar to affinities at μ- and δ-opioid receptors, it can be suggested that compound **6** acted on both μ- and δ-opioid receptors in mouse vas deferens. The involvement of κ-opioid receptors can be ruled out since the affinity to these receptors as determined in binding assay was 60- and 150-fold lower than affinities to μ- or δ-opioid receptors, respectively.

Following the pattern of structure–activity relationships, the amino acid substitution in position 6 does not seem to have a detrimental effect on affinity to the μ-opioid receptor. This observation suggests that the 6-keto group is not a requirement for the high affinity to the μ-opioid receptor. However, changes in binding affinities are rather associated with the α/β substitution of the amino acid at position 6 of the morphinan skeleton. While the α-epimers are favoured by the μ-opioid receptor, the β-epimers proved to have increased interaction with the δ-opioid binding site. Comparison of glycine (**1** vs. **2**), alanine (**3** vs. **4**), and phenylalanine (**5** vs. **6**) conjugates revealed that the change from the α- to the β-isomer produces a decrease in the μ-opioid receptor affinity up to 3-fold (except for the pair **1** vs. **2**), and an increase up to 4-fold in the δ-opioid receptor affinity. In addition, while the affinities for μ- and δ-opioid receptors remained somewhat similar in comparison to the parent compounds, affinities at the κ-opioid receptor were significantly decreased, independent of α- or β-substitution. Among the investigated compounds, the α-alanine conjugate **3** was the most μ-opioid receptor selective analogue showing a similar or improved selectivity profile compared to 14-*O*-methyloxymorphone and 14-methoxymetopon. Notably, within the pairs of α- or β-amino acid analogues, there was no large difference in the potency in mouse vas deferens. Only in the case of glycine conjugates, the β-epimer **2** was about 4-fold more potent then the corresponding α-epimer **1** (Table 2).

The combination of the α/β substitution and the nature of the amino acid substituent at position 6 fully account for the binding differences between the different conjugates. Among the studied derivatives, compound **6** displayed increased affinity for the δ-opioid receptor and a reduction

in the activity for the μ-opioid receptor compared to the other analogues. Molecular dynamic simulations supported the experimental findings when the conformational space occupied by the phenyl moiety of analogue **6** was restricted to the region of the aromatic indole ring of the highly selective δ-opioid receptor antagonist HS 378 (Schmidhammer et al., 1998; Spetea et al., 2001) (Fig. 3). This might permit binding of the phenyl group of compound **6** to a δ-address site in the locus of the indolic benzene moiety. Similarly, it was previously observed that the Phe^4 phenyl residue of leucine-enkephalin can simulate the benzene moiety of naltrindole or naltriben, two δ-opioid receptor antagonists, in a conformational rigid setting (Takemori and Portoghese, 1992).

Pharmacological properties of the investigated compounds correlated very well with their structure. If the nature of the substituent at positions 6 and 14 is important for opioid receptor affinities (Schmidhammer, 1993, 1998), the substituent at the morphinan nitrogen plays a major role concerning opioid activity of the compound (Casy and Parfitt, 1986; Takemori and Portoghese, 1992; Schmidhammer, 1993). Large groups such as cyclopropylmethyl or allyl on the nitrogen have been commonly associated with an antagonist character in this series of opioids, whereas small substitutents e.g. methyl confer an agonist character. Like morphine, all of the 6-amino acid derivatives possess a methyl group at the *N*-17 position (Fig. 1).

Reduction of drug lipophilicity usually was shown to result in decreased penetration into the CNS. Attempts to confer peripheral selectivity by chemical modification have been reported (Botros et al., 1989; Shaw et al., 1989; Zimmerman et al., 1994; Portoghese et al., 1995). A well-established means of limiting the access to the CNS of drugs is to prepare their quaternary derivatives. The quaternary analogues of opioids such as morphine, nalorphine and naltrexone have been demonstrated to have restricted access to the CNS, but they have considerably lower affinity and potency at the opioid receptor than their parent compounds (Iorio and Frigni, 1984; Brown and Goldberg, 1986). A more successful approach is represented by the development of opioids with hydrophilic groups attached to the C-6 position of the morphinan skeleton. Such compounds were reported to show limited ability to cross the blood–brain barrier without significant changes in the opioid receptor affinity (Botros et al., 1989; Shaw et al., 1989; Zimmerman et al., 1994; Portoghese et al., 1995). According to our results, the 6-amino acid substituted derivatives of 14-*O*-methyloxymorphone retained the high affinity for the μ-opioid receptor of the non-ionizable parent compounds, in agreement with earlier finding in the series of β-oxymorphamine (Botros et al., 1989) and β-naltrexamine (Larson et al., 1993) that contain ionizable moieties. Since the agonist potencies of these opioid compounds **1**–**6** are in the range of 14-*O*-methyloxymorphone and 14-methoxymetopon, it appears that C-6 substituent with ionizable groups do not have a significant adverse effect on agonist activity at the receptor level. This was also reported in the case of oxymorphamine derivatives containing ionizable moieties coupled to the 6β-amino group (Botros et al., 1989).

Since ionized groups are expected to lower lipid solubility and should reduce access into the CNS, the newly developed compounds are expected to exhibit greater selectivity than non-ionized molecules towards peripheral tissues. Very recently, an index of CNS penetration was estimated and reported by our group for compounds **1**–**6** and morphine (Schütz et al., 2003). Based on the calculated blood–brain distribution coefficients, derivatives **1**–**6** were predicted to have a very limited ability to enter the CNS after systemic administration, being ca. 98% distributed in peripheral tissue and ca. 2% in brain tissue, while morphine is distributed ca. 75% in the periphery and ca. 25% in the brain (Schütz et al., 2003). This low CNS penetration appears to be a consequence of the increased hydrophilicity of these compounds.

In conclusion, our present data on 6-amino acid substituted (glycine, alanine and phenylalanine) derivatives of the highly potent analgesic 14-*O*-methyloxymorphone indicate that this class of compounds exhibit high binding affinities to the μ-opioid receptor and potent agonist activity. The newly developed ionizable derivatives could find clinical applications as potent analgesics without the adverse actions of centrally acting opioids.

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References

Botros, S., Lipkowski, A.W., Larson, D.L., Stark, A.P., Takemori, A.E., Portoghese, P.S., 1989. Opioid agonist and antagonist activities of peripherally selective derivatives of naltrexamine and oxymorphamine. J. Med. Chem. 32, 2068–2071.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brown, D.R., Goldberg, L.I., 1986. The use of quaternary narcotic antagonists in opiate research. Neuropharmacology 24, 181–191.

Casy, A.F., Parfitt, R.T., 1986. Opioid Analgesics: Chemistry and Receptors. Plenum, New York, NY.

Clark, M., Cramer, R.D., van Opdenbosch, N., 1989. Validation of the general purpose TRIPOS 5.2 force field. J. Comput. Chem. 10, 982–1012.

Freye, E., Schmidhammer, H., Latasch, L., 2000. 14-Methoxymetopon, a potent opioid, induces no respiratory depression, less sedation, and less bradycardia than sufentanil in the dog. Anesth. Analg. 90, 1359–1364.

Fürst, S., Búzás, B., Friedmann, T., Schmidhammer, H., Borsodi, A., 1993.

Highly potent novel opioid receptor agonist in the 14-alkoxymetopon series. Eur. J. Pharmacol. 236, 209–215.

Gasteiger, J., Marsili, M., 1980. Iterative partial equalization of orbital electronegativity—a rapid access to atomic charges. Tetrahedron 36, 3219–3228.

Hughes, J., Kosterlitz, H.W., Leslie, F.N., 1975. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. Br. J. Pharmacol. 53, 371–381.

Iorio, M.A., Frigni, V., 1984. Narcotic agonist/antagonist properties of quaternary diastereoisomers derived from oxymorphone and naloxone. Eur. J. Med. Chem. 19, 301–303.

King, M.A., Su, W., Nielan, C., Chang, A.H., Schütz, J., Schmidhammer, H., Pasternak, G.W., 2003. 14-Methoxymetopon, a very potent μ-opioid analgesic with an unusual pharmacological profile. Eur. J. Pharmacol. 459, 203–209.

Kosterlitz, H.W., Watt, A.J., 1968. Kinetic parameters of narcotic agonists and antagonists, with particular reference to *N*-allylnoroxymorphone (naloxone). Br. J. Pharmacol. 33, 266–276.

Larson, D.L., Hua, M., Takemori, A.K., Portoghese, P.S., 1993. Possible contribution of a glutathione conjugate to the long-duration action of β-funaltrexamine. J. Med. Chem. 36, 3669–3673.

MacPherson, R.D., 2002. New directions in pain management. Drugs Today 38, 135–145.

Nevin, S.T., Kabasakal, L., Ötvös, F., Tóth, G., Borsodi, A., 1994. Binding characteristics of the novel highly selective δ-agonist, [^{3}H]IIe^{5,6}deltorphin II. Neuropeptides 26, 261–265.

Portoghese, P.S., Farouz-Grant, F., Sultana, M., Takemori, A.E., 1995. 7′-Substituted amino acid conjugates of naltrindole. Hydrophilic groups as determinants of selective antagonism of δ_1-opioid receptor-mediated antinociception in mice. J. Med. Chem. 38, 402–407.

Reisine, T., Pasternak, G., 1996. Opioid analgesics and antagonists. In: Hardman, J.G., Goodman Gilman, A., Limbird, L.E. (Eds.), The Pharmacological Basis of Therapeutics. McGraw-Hill, New York, NY, pp. 521–555.

Ronai, A.Z., Berzetei, I., Bajusz, S., 1977. Differentiation between opioid peptides by naltrexone. Eur. J. Pharmacol. 45, 393–394.

Schiller, P.W., 1991. Development of receptor-specific opioid peptide analogues. In: Ellis, G.P., West, G.B. (Eds.), Progress in Medicinal Chemistry, vol. 28. Elsevier, Amsterdam, pp. 301–339.

Schmidhammer, H., 1993. 14-Alkoxymorphinans—a series of highly potent opioid agonists, antagonists and partial agonists. Curr. Top. Med. Chem. 1, 261–276.

Schmidhammer, H., 1998. Opioid receptor antagonists. In: Ellis, G.P., Luscombe, D.K., Oxford, A.W. (Eds.), Progress in Medicinal Chemistry, vol. 35. Elsevier, Amsterdam, pp. 83–132.

Schmidhammer, H., Aeppli, L., Atwell, L., Fritsch, F., Jacobson, A.E., Nebuchla, M., Sperk, G., 1984. Synthesis and biological evaluation of 14-alkoxymorphinans: 1. Highly potent opioid agonists in the series of (−)-14-methoxy-*N*-methylmorphinan-6-ones. J. Med. Chem. 27, 1575–1579.

Schmidhammer, H., Schratz, A., Mitterdorfer, J., 1990. Synthesis and biological evaluation of 14-alkoxymorphinans: 8. 14-Methoxymetopon, an extremely potent opioid agonist. Helv. Chim. Acta 73, 1784–1787.

Schmidhammer, H., Krassnig, R., Greiner, E., Schütz, J., White, A., Berzetei-Gurske, I.P., 1998. Synthesis and biological evaluation of 14-alkoxymorphinans: 15. Novel δ opioid receptor antagonists with high affinity and selectivity in the 14-alkoxy-substituted indolomorphinan series. Helv. Chim. Acta 81, 1064–1069.

Schütz, J., Brandt, W., Spetea, M., Wurst, K., Wunder, G., Schmidhammer, H., 2003. Synthesis of 6-amino acid substituted derivatives of the highly potent analgesic 14-*O*-methyloxymorphone. Helv. Chim. Acta 86, 2142–2148.

Shaw, J.S., Carroll, J.A., Alcock, P., Main, M.G., 1989. ICI 204448: A κ-opioid agonist with limited access to the CNS. Br. J. Pharmacol. 96, 986–992.

Simon, E.J., Hiller, J.M., 1994. Opioid peptides and opioid receptors. In: Siegel, G.J., Agranoff, B.W., Albers, R.W. (Eds.), Basic Neurochemistry: Molecular, Cellular and Medical Aspects. Raven Press, New York, NY, pp. 321–340.

Spetea, M., Erlandsson Harris, H., Berzetei-Gurske, I.P., Klareskog, L., Schmidhammer, H., 2001. Binding, pharmacological and immunological profiles of the δ-selective opioid receptor antagonist HS 378. Life Sci. 69, 1775–1782.

Spetea, M., Tóth, F., Schütz, J., Ötvös, F., Tóth, G., Benyhe, S., Borsodi, A., Schmidhammer, H., 2003. Binding characteristics of [^{3}H]14-methoxymetopon, a high affinity μ-opioid receptor agonist. Eur. J. Neurosci. 18, 290–295.

Stein, C., 1995. The control of pain in peripheral tissue by opioids. N. Engl. J. Med. 332, 1685–1690.

Stein, C., Machelska, H., Binder, W., Schäfer, M., 2001. Peripheral opioid analgesia. Curr. Opin. Pharmacol. 1, 62–65.

Stein, C., Schäfer, M., Machelska, H., 2003. Attaching pain at its source: new perspectives on opioids. Nat. Med. 9, 1003–1008.

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Takemori, A.E., Portoghese, P.S., 1992. Selective naltrexone-derived opioid receptor antagonists. Annu. Rev. Pharmacol. Toxicol. 32, 239–269.

Zernig, G., Saria, A., Krassnig, R., Schmidhammer, H., 2000. Signal transduction efficacy of the highly potent μ-opioid agonist 14-methoxymetopon. Life Sci. 66, 1871–1877.

Zimmerman, D.M., Gidda, J.S., Cantrell, A.B., Schoepp, D., Johnson, B.G., Leander, J.D., 1994. Discovery of a potent, peripherally selective trans-3,4-dimethyl-4-(3-hydroxyphenyl) piperidine opioid antagonist for the treatment of gastrointestinal motility disorders. J. Med. Chem. 37, 2262–2265.