Peripheral versus Central Antinociceptive Actions of 6-Amino Acid Substituted Derivatives of 14-O-Methyloxymorphone in Acute and Inflammatory Pain in the Rat

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Abbreviations: ED₅₀, antinociceptive dose necessary to produce a 50% response; HS-730, 2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]acetic acid bis(tetrafluoroborate); HS-731, 2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]acetic acid bis(tetrafluoroborate); HS-935, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]propanoic acid bis(tetrafluoroborate); HS-936, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]propanoic acid bis(tetrafluoroborate);
methylmorphinan-6β-yl)amino]propanoic acid bis(tetrafluoroborate); HS-937, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]-3-phenylpropanoic acid bis(tetrafluoroborate); HS-938, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]-3-phenylpropanoic acid bis(tetrafluoroborate); M6G, morphine-6-glucuronide.

**Recommended section:** Behavioral Pharmacology
Abstract

Opioid analgesics with restricted access to the central nervous system represent a new approach to the treatment of severe pain with an improved safety profile. The objective of this study was to investigate the peripheral and central components of the antinociceptive actions of the 6-amino acid conjugates (glycine, alanine, phenylalanine) of 14-O-methyloxymorphone. Their antinociceptive activities were compared to those of the centrally penetrating µ-opioid agonists morphine, fentanyl and 14-O-methyloxymorphone. In the tail flick test in rats, the 6-amino acid conjugates were 45- to 1170-fold more potent than morphine after intracerebroventricular (i.c.v.) administration, and 19- to 209-fold after subcutaneous (s.c.) administration. They showed potencies similar to fentanyl after s.c. administration, and were more potent after i.c.v. application. The time course of action was different between s.c. and i.c.v. administration, with significant long-lasting effects after i.c.v. administration. Systemic administration of the peripherally selective opioid antagonist naloxone methiodide antagonized the effects after s.c. but not after i.c.v administration in the tail flick test. Subcutaneous 6-amino acid derivatives also elicited antihyperalgesic effects in the formalin test in rats, which were reversed by systemically administered naloxone methiodide. While morphine exerts its analgesic effects by central and peripheral mechanisms, the investigated new opioids interact primarily with peripheral opioid receptors after s.c. administration. The present data indicate that the 6-amino acid conjugates of 14-O-methyloxymorphone have limited access to the central nervous system and can mediate antinociception at peripheral sites. Also, they might find clinical application when the central actions of opioids are unwanted.
Introduction

The potent antinociceptive actions of classical opioids such as morphine are traditionally considered to be mediated centrally through an action at the supraspinal or spinal level (for reviews see Fürst, 1999; Gutstein and Akil, 2001; Przewlocki and Przewlocka, 2001). Antinociceptive effects have also been demonstrated to result after local application of opioids in the periphery, for example in mouse writhing (Kolesnikov et al., 1996; Reichert et al., 2001), in rat models of inflammation (Stein et al., 1989; Perrot et al., 2001) or neuropathic pain (Obara et al., 2004). These effects have been attributed to opioid induced actions mediated by peripheral opioid receptors (Fields et al., 1980; Stein et al., 1995). Neuroanatomical, molecular and electrophysiological studies have shown that such receptors are expressed on peripheral terminals of sensory neurons where they can modulate both afferent and efferent neuronal functions resulting in antinociception (Schäfer et al., 1995; Stein et al., 1996).

Opioids interacting with peripheral opioid receptors without crossing the blood-brain barrier might be used as potent analgesics and should be devoid of centrally mediated side effects. Strategies to restrict the access of opioids to the central nervous system include quaternization and incorporation of highly polar hydrophilic substituents (Brown and Goldberg, 1985; Botros et al., 1989; Portoghese et al., 1995). Generally, it has been found that quaternary compounds have reduced access into the central nervous system, but they have considerably lower affinity and potency to opioid receptors (Herz and Teschemacher, 1971; Brown and Goldberg, 1985). In order to avoid this problem, opioids with hydrophilic groups attached at the C-6 position of the morphinan structure were synthesized (Botros et al., 1989, Portoghese et al., 1995; Schütz et al., 2003). Loperamide, asimadoline, morphine-6-glucoronide (M6G) and several peptidic κ-opioid agonists are examples of peripherally...
restricted opioids which have been investigated in experimental and clinical studies (for a review see Stein et al., 2003).

Since the numerous side effects (e.g. respiratory depression, sedation, constipation) and tolerance to morphine, fentanyl or oxycodone strongly limit their clinical use, there is a continuous search for analgesic drugs with an improved side effect profile (for reviews see Nicholson, 2003; Stein et al., 2003). Efforts to eliminate or minimize the undesired side effects of morphine led to the synthesis of a significant large number of compounds (for reviews see Schmidhammer, 1993; Fürst et al., 1995). A derivative of oxymorphone, 14-0-methyloxymorphone was developed by our group, and reported to be about 400- and 40-fold more potent than morphine and oxymorphone, respectively, in the hot plate test in mice (Schmidhammer et al., 1984; Fig. 1). Further chemical optimization led to 14-methoxymetopon (Schmidhammer et al., 1990; Fig. 1), a highly selective µ-opioid receptor agonist (Fürst et al., 1993; Spetea et al., 2003). This opioid was described as an extremely potent centrally acting analgesic, being 300- to 20,000-fold more potent than morphine, depending upon the analgesic paradigm used (Schmidhammer et al., 1990; Fürst et al., 1993; Zernig et al., 2000; King et al., 2003).

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, 1993) to the development of opioid analgesics that would have limited access to the central nervous system. A family of 6-amino acid substituted (glycine, alanine, phenylalanine) derivatives of 14-0-methyloxymorphone (Fig. 2) was developed in order to obtain opioid agonists which are more hydrophilic than the parent compound, and have peripheral selectivity (Schütz et al., 2003; Spetea et al., 2004). Binding studies revealed that these 6-amino acid conjugates displayed very high affinities to the µ-opioid receptor, and were effective in inhibiting the electrically-evoked contractions of the mouse vas deferens.
through the µ-opioid receptor (Spetea et al., 2004). Based on the calculation of blood-brain distribution coefficients, these derivatives were predicted to have restricted access to the central nervous system after systemic administration (Schütz et al., 2003).

In the present study, we aimed to investigate the peripheral and central components of the antinociceptive action of the 6-amino acid conjugates of 14-O-methyloxymorphone after subcutaneous and intracerebroventricular administration in the tail flick test in rats. In addition, we have examined the dose-response correlations and the site of action of the 6-amino acid derivatives after subcutaneous administration in the formalin test in rats.

Methods
Materials

The 6-amino acid substituted derivatives of 14-O-methyloxymorphone (HS-730, 2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]acetic acid bis(tetrafluoroborate); HS-731, 2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]acetic acid bis(tetrafluoroborate); HS-935, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]propanoic acid bis(tetrafluoroborate); HS-936, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]propanoic acid bis(tetrafluoroborate); HS-937, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]-3-phenylpropanoic acid bis(tetrafluoroborate) and HS-938, (2S)-2-[(4,5α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]-3-phenylpropanoic acid bis(tetrafluoroborate)) were prepared as recently described (Schütz et al., 2003; Fig. 2). 14-O-Methyloxymorphone was synthesized as previously described (Schmidhammer et al., 1984; Fig. 1). Morphine hydrochloride, fentanyl and naloxone hydrochloride were obtained from Sigma Chemicals (St. Louis, MO, USA) or from Polfa, Kutno, Poland (morphine). Naloxone methiodide (N-methylnaloxonium
iodide; quaternary naloxone) was kindly provided by Dr. S. Hosztafi (Alkaloida-ICN, Hungary) or obtained from Sigma Chemicals (St. Louis, MO, USA). Formaldehyde was obtained from Odczynniki SA (Lublin, Poland).

All drugs were dissolved in physiological saline and administered to 5-10 rats/group. Rats received subcutaneous (s.c.) injections under the skin of the neck, in a volume of 5 ml/kg body weight in the tail flick test, or in a volume of 4 ml/kg in the formalin test. Intracerebroventricular (i.c.v.) injections were given directly into the cerebral ventricles in a volume of 5 µl. Control animals received the same volume of s.c. or i.c.v. saline.

Animals

Male Wistar rats (120-150 g) used in the tail flick test were purchased from Charles River (Budapest, Hungary). Male Wistar rats (250-270 g) used in the formalin test were obtained from the Institute of Pharmacology, Polish Academy of Sciences (Krakow, Poland). Animals were housed in macrolon cages (six animals per cage) with food and water ad libitum, in a temperature controlled room (22 ± 2°C), maintained on a 12 h light/dark cycle (the light on at 8:00 AM). Experiments were carried out in accordance to the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals. The experimental protocols were approved by the Semmelweis University (Budapest, Hungary), and the Institute of Experimental Animal Care Committee (Institute of Pharmacology, Krakow, Poland).

Tail Flick Test

The tail flick test in the rat was performed according to the described procedure (Fürst et al., 1993). A beam light was focused on the tip of the tail and the latency required for the rat to remove its tail was determined before (baseline) and after drug administration, using an arbitrary cut off time of twice the control reaction time and expressed as percentages. The
antinociceptive activity was assessed 30, 60, 120 and 180 min after s.c. drug administration, and 10, 20, 30, 60, 120, 180, 240, 300, 360, 420 and 480 min after i.c.v. administration.

In order to evaluate the site of action (peripheral or central) of the opioid agonists, the effect of naloxone, the tertiary opioid antagonist which readily crosses the blood-brain barrier, and that of the peripherally selective opioid antagonist naloxone methiodide on the antinociceptive action in the tail flick test was assessed after s.c. and i.c.v. administration. In experiments when the antagonist effect was assessed, i.c.v. naloxone or naloxone methiodide were given 10 min prior to the s.c. administration of the opioid agonist. Antagonists were co-administered with the respective agonist, when they were given by the same route of administration, s.c. or i.c.v. Measurements were performed 30 min after s.c. and 20 min after i.c.v. administration of the opioid agonists.

Formalin Test

The formalin test was used as a model of tonic inflammatory pain. Pain-related behavior induced by local administration of formalin is characterized by the occurrence of two characteristic phases of increased pain sensitivity in rats. The first phase is related to a direct stimulation of nociceptors, primarily owing to the increased secretion of substance P, bradykinin and stimulatory amino acids. In the second phase, there is an increase in the levels of histamine, prostaglandins, 5-hydroxytryptamine and bradykinin, which lead to the development of a localized inflammatory response and progressive functional changes in the dorsal horn and the central nervous system.

Fifteen min after s.c. administration of the opioid agonists, rats were lightly anesthetized by inhalation of halothane (2-3% v/v/oxygen mixture, 5 l/min) for 2-3 min in a plexiglass chamber. Rats were then s.c. injected with 100 µl of 12% formalin into the dorsal part of the right hind paw as described (Dorazil-Dudzik et al., 2004). After formalin injection,
rats were placed in a single wire cage for observation. Pain-related behavior was quantified by counting the incidence of spontaneous flinches, shakes and jerks of the formalin-injected paw. Pain reactions were continuously counted for 90 min for each animal and then totaled over two characteristic periods: 5-15 min (first phase) and 35-40, 40-45, 45-50, 75-80, 80-85, 85-90 min (second phase) after formalin administration. Naloxone methiodide (2131 nmol/kg, s.c.) was injected simultaneously with the highest dose of each opioid agonist, 15 min before formalin injection.

Data Analysis

The differences between the results obtained both by the tail flick test or the formalin test were statistically determined by Analysis of Variance (ANOVA). For the significant differences between the groups we used Student's t-test with Bonferroni correction as post hoc test. The ED$_{50}$ values and 95% confidence limits were given in nmol. A p value <0.05 was considered statistically significant.

Results

Antinociceptive Effects in the Tail Flick Test in the Rat

Subcutaneous Administration. In the tail flick test in the rat, morphine, fentanyl, 14-\textit{O}-methyloxymorphone and five of the six 6-amino acid substituted derivatives of 14-\textit{O}-methyloxymorphone, elicited dose- and time-dependent antinociceptive effects after s.c. administration. The values of ED$_{50}$ are listed in Table 1. The peak ED$_{50}$ values of the amino acid conjugates were ranging between 29-315 nmol/kg. One compound, HS-938, failed to produce 50% antinociception between 30-120 min after s.c. administration of 3600 nmol/kg (Table 1). The calculated ED$_{50}$ values of morphine, fentanyl and 14-\textit{O}-methyloxymorphone at the peak of action (30 min) were 6053, 38.6 and 14.9 nmol/kg, respectively.
As shown in Table 1, morphine, fentanyl and 14-\textit{O}-methyloxymorphone produced their maximum antinociceptive effect 30 min after s.c. administration, while the new opioid agonists reached their peak of action at 60 min. The antinociceptive action of morphine, fentanyl, 14-\textit{O}-methyloxymorphone, HS-731 and -935 lasted about 120 min, whereas the effect of HS-730, -936, and -937 lasted for 3 h after a single s.c. administration. A 2-fold higher dose of morphine was required to produce the same effect at 2 h after s.c. administration as compared to the ED\textsubscript{50} dose at the peak of action. In the case of HS-731, -935, and -937, only a 1.0-1.5 fold ED\textsubscript{50} dose, as determined at 60 min, was required to produce antinociception at 120 min after s.c. administration. Notably, dose-dependent antinociceptive effects were still observed at 3 h after s.c. HS-730, -936, and -937. The shortest duration of action was elicited by fentanyl, and a 10-fold higher dose was required to induce 50\% analgesia at 120 min after s.c. administration than the dose determined at the peak effect (Table 1).

Antinociceptive potencies in the tail flick test of \textit{\alpha}- versus \textit{\beta}-epimers (Table 1; Fig. 2) did not differ significantly. The ratios are between 1.3-2.0, except for HS-938, the 6\textit{\beta}-phenylalanine derivative, which proved to be ineffective up to 3600 nmol/kg, while its \textit{\alpha}-counterpart HS-937 showed a dose-dependent effect after s.c. administration (ED\textsubscript{50} = 315 nmol/kg, at 60 min; Table 1).

\textbf{Intracerebroventricular Administration.} All tested opioid agonists produced dose- and time-related antinociceptive effects in the tail flick test in rats after i.c.v. administration (Table 2). The time-response curves of higher i.c.v. doses corresponding to the calculated ED\textsubscript{95} of the test opioids are shown in Fig. 3. The onset of the antinociceptive response elicited by fentanyl, morphine and 14-\textit{O}-methyloxymorphone was rapid, with a peak of action at 10 and 20 min, respectively (Fig. 3A). This effect rapidly declined and returned to the pre-injection value about 1 to 2 h after i.c.v. drug injection. In contrast, the 6-amino acid conjugates of 14-
O-methyloxymorphone exhibited a significantly increased duration of the antinociceptive effect (Fig. 3B). Their antinociceptive action showed a somewhat slower onset of action, which peaked at 30-120 min and lasted about 4-7 h. While HS-938 failed to induce antinociception by s.c. administration up to 3600 nmol/kg, it produced a time- and dose-dependent response when applied i.c.v. (Table 2; Fig. 3B).

Relative Antinociceptive Potency after s.c. and i.c.v. Administration in the Tail Flick Test

The peak antinociceptive potencies of the 6-amino acid derivatives of 14-O-methyloxymorphone relative to morphine and fentanyl (Table 3). The differences in potency of the novel opioid agonists compared to morphine and fentanyl were much more remarkable after i.c.v. than after s.c. administration. While they were slightly less potent or equipotent when compared to fentanyl, they were about 19- to 209-fold more potent than morphine when administered s.c. (Table 3). However, the 14-O-methyloxymorphone derivatives proved to be 45- to 1170-fold more potent than morphine when given i.c.v. Furthermore, HS-938 which failed to produce antinociception by s.c. administration, was 45-fold more potent than morphine after i.c.v. injection (Tables 2 and 3). In contrast to s.c. administration, whereby the difference observed between the new opioids and fentanyl was negligible, the 6-amino acid derivatives of 14-O-methyloxymorphone were about 2- to 55-fold more potent analgesics than fentanyl after i.c.v. administration.

Antinociceptive s.c./i.c.v. Potency Ratios in the Tail Flick Test

As shown in Table 4, the difference between the dose required to elicit 50% antinociceptive effect after peripheral systemic (s.c.) versus central (i.c.v.) administration (ED$_{50}$ nmol/kg, s.c./ED$_{50}$ nmol/rat, i.c.v.) was 23 in the case of fentanyl, 172 for morphine,
and 87 for 14-O-methyloxymorphone, the parent molecule. The s.c./i.c.v. potency ratios of the 6-amino acid conjugates were found to be much higher compared to morphine, fentanyl and 14-O-methyloxymorphone, 569, 651, 967, 1887, >4600 and 5000 for HS-935, -936, -731, -730, 938, and -937, respectively. These ratios were observed to be remarkably high in the case of HS-938 and -937, the phenylalanine substituted β- and α-epimers, respectively (s.c./i.c.v. ED$_{50}$ ratios >4600 and 5000, respectively) (Table 4).

**Antagonist Effect on the Antinociceptive Action in the Tail Flick Test**

Among the new opioid agonists, HS-731 was selected for a more detailed investigation of the site of action, and compared to the action of morphine (Fig. 4). While systemically (s.c.) administered naloxone methiodide did not alter the effect of s.c. morphine (7769 nmol/kg), except in a very high dose (21308 nmol/kg), it antagonized the s.c. antinociceptive effect of HS-731 (183 nmol/kg) (Fig. 4, A and B). A similar degree of antagonism (34-42%), as determined for HS-731, was observed in rats after s.c. co-administration of equipotent antinociceptive doses of HS-730, -935, -936, and -937, with 320 nmol/kg naloxone methiodide (data not shown). Subcutaneous administration of 2131 and 21308 nmol/kg naloxone methiodide failed to antagonize the antinociceptive effects of i.c.v HS-731 (0.18 nmol/rat) and i.c.v. morphine (78 nmol/rat) (Fig. 4, C and D). Both naloxone methiodide (21.3 nmol/rat) and naloxone (2.75 nmol/rat) injected i.c.v. blocked the antinociception produced by HS-731 (0.18 nmol/rat) and morphine (78 nmol/rat) after i.c.v administration (Fig. 4, C and D). However, while i.c.v. injected naloxone (27.5 nmol/rat) completely antagonized the antinociceptive effect of s.c. morphine (7769 nmol/kg), it was ineffective to influence the action of s.c. HS-731 (183 nmol/kg) (Fig. 4, A and B). The same experiments were performed with HS-730, -935, -936,-937, and -938 and similar results were obtained (data not shown).
Antinociceptive Effects in the Formalin Test in the Rat after Subcutaneous Administration

Subcutaneous administration of morphine and 6-amino acid conjugates of 14-\(O\)-methylxymorphone, HS-730, -731, -937, and -938, produce a dose-dependent antinociceptive action in both phases of the formalin test in rats (Fig. 5A left panel; Fig. 6). The ED\(_{50}\) values of the 6-amino acid derivatives in the first and second phase of the formalin response were ranging between 72-171 nmol/kg and 110-292 nmol/kg, respectively (Table 5). The antinociceptive potencies of the new opioid agonists in the formalin test were 10- to 22- and 5- to 13-fold higher in the first and second phase, respectively, compared to morphine (Fig. 6). Their inhibition of pain behavior appears to be somewhat higher in the first phase than in the second phase, while morphine produced comparable inhibition of formalin-induced behavior in both phases. While s.c. administered 6\(\beta\)-phenylalanine epimer HS 938 was ineffective in eliciting antinociception in the tail flick test up to 3600 nmol/kg (Table 1), it produced antihyperalgesic effects in the formalin-injected paw after s.c. administration (ED\(_{50}\) first phase 79 nmol/kg and second phase 109 nmol/kg; Table 5).

To establish the site of action of the 6-amino acid derivatives and morphine, the peripherally selective opioid antagonist naloxone methiodide was s.c. co-administered with the opioid agonists, and the response in the formalin-injected paw was assessed. As shown in Fig. 5B (right panel), the antihyperalgesic effects of s.c. 366 nmol/kg of HS-730 or HS-731 were significantly antagonized by s.c. 2131 nmol/kg naloxone methiodide. Similar inhibitory effects of naloxone methiodide were observed after co-injection with the other tested amino acid derivatives (data not shown). The antinociceptive activity of s.c. 5278 nmol/kg morphine in the formalin test was also significantly reversed by s.c. naloxone methiodide (Fig. 5B, right panel).
Discussion

The major finding of the present study is that the 6-amino acid conjugates (glycine, alanine, phenylalanine) of 14-\(O\)-methyloxymorphone produce potent antinociceptive effects in acute nociceptive and inflammatory pain in rats. These effects were demonstrated to be mediated preferentially via activation of opioid receptors located in the periphery after systemic (s.c.) administration.

The 6-amino acid derivatives elicited antinociception in a dose-dependent fashion after s.c. administration. The peripherally selective opioid antagonist naloxone methiodide reversed the antinociceptive effects of these opioid agonists. Typically, the pain ceased very rapidly upon s.c. and i.c.v. administration of morphine, fentanyl and 14-\(O\)-methyloxymorphone, but in the case of the new opioids, there was some delay in the onset of the effect after s.c. administration. Extremely long-lasting effects were measured in the tail flick test after i.c.v. administration. In the tail flick test, potencies of the 6-amino acid derivatives did not significantly differ between the \(\alpha\)- and \(\beta\)-epimers, in agreement with in vitro biological data whereby also no major changes in opioid binding and agonist activity were detected (Spetea et al., 2004). In the case of the phenylalanine conjugates, the \(\beta\)-epimer HS-938 failed to elicit antinociception up to 3600 nmol/kg after s.c. administration. However, its \(\alpha\)-counterpart HS-937 produced dose- and time-dependent antinociceptive action. In contrast to the inactivity of the \(\beta\)-epimer HS-938 determined after s.c. administration, it proved to be a potent antinociceptive agent after i.c.v. administration. The 6\(\beta\)-phenylalanine derivative was reported to have some preference for \(\delta\)- over \(\mu\)-opioid receptors in binding and \(\delta\)-opioid agonism in mouse vas deferens bioassays (Spetea et al., 2004). The increased activity at \(\delta\)-opioid receptors might explain the virtual ineffectiveness detected in the tail flick test after systemic administration. However, a potent antihyperalgesic effect of s.c. HS-938 was also found in the
formalin pain model. These observation corroborate with earlier reports on the lack of antinociceptive effects of other δ-opioid agonists after s.c. application in the tail flick test, which are, however, more active in visceral pain models such as the writhing test (Quock et al., 1999). Antinociceptive effects of δ-opioid agonists are enhanced in hyperalgesia and allodynia after spinal and supraspinal administration (Desmeules et al., 1993; Mika et al., 2001).

The potent antinociceptive effect of this class of opioids might be attributed to the substitution pattern in position C-6 of the morphinan skeleton. It was reported that numerous opioids bearing various substituents in position C-6, e.g. azido (Knoll et al., 1975) and 6-O-glucuronide in morphine (Abbott and Palmour, 1988; Frances et al., 1992), or amino in oxymorphamine (Botros et al., 1989), induce strong analgesic actions. As recently established in structure-activity relationship studies, the substitution at position C-6 with ionizable groups such as amino acid residues dose not have any detrimental effect on opioid activity at the receptor level (Spetea et al., 2004) and on antinociceptive potency as described here. The parent molecule, 14-O-methyloxymorphone proved to be a more potent antinociceptive agent after s.c. administration, than the new 6-amino acid substituted derivatives, while it was observed to produce similar potency after i.c.v administration.

Characteristic differences occurred in the time course of the antinociceptive action of the new opioids depending on the route of application, s.c. or i.c.v., in the tail flick test. The rapid onset with a maximum effect early, which waned very shortly after drug administration, are typical characteristics of the time course of action of opioid agonists (e.g. morphine, fentanyl, etorphine) that mediate analgesia via central mechanisms, and were described under both normal and inflammatory conditions (Millan et al., 1987; Aceto et al., 1997). In contrast, the new opioids administered s.c. acted longer with a some delay in onset.

Intracerebroventricular administration of the 6-amino acid conjugates produced a different
time course of action in the tail flick test than the centrally acting opioids, with extremely prolonged effects. Our observations are consistent with previous findings on the effect of i.c.v. morphine and its active metabolite M6G in different pain tests (Abbott and Palmour, 1988; Frances et al., 1992). Significantly long-lasting analgesia was reported for i.c.v. M6G, a hydrophilic molecule, which poorly penetrates the blood-brain barrier (Abbott and Palmour, 1988; Frances et al., 1992; Tegeder et al., 2003). One possible explanation of the long-lasting action might be a slow elimination of opioids containing hydrophilic groups from the brain based upon their reduced lipid solubility. It was observed that the differences in the duration of action of various opioids, after i.c.v. administration, are largely determined by their hydrophilic/lipophilic character and the time that the compound remains in the ventricular system (Herz and Teschemacher, 1971).

That the presence of hydrophilic groups is indeed important in limiting penetration into the central nervous system was demonstrated by comparing the activity ratios of the peripheral (s.c.) versus central (i.c.v.) potencies in the tail flick test of the 6-amino acid conjugates with those of centrally penetrating µ-opioid agonists morphine, fentanyl and 14-O-methyloxymorphone. There was a marked enhancement of potency of the new agonists after i.c.v. in comparison to s.c. administration. Very good correlations between hydrophilicity and the intravenous (i.v.)/i.c.v. activity quotients were earlier described for various opioids (Herz and Teschemacher, 1971; Botros et al., 1989; Frances et al., 1992; Portoghese et al., 1995).

To further explore the peripheral and central components of antinociceptive actions of the 6-amino acid conjugates of 14-O-methyloxymorphone after s.c. and i.c.v. administration, the antagonist effects of naloxone methiodide, which has restricted access to the central nervous system, were investigated in the tail flick test. Antinociceptive effects of peripherally administered HS-731 was dose-dependently antagonized by s.c. naloxone methiodide. However, an approximately 10-fold higher dose of antagonist was required to antagonize the
effects of morphine to the same extent as the s.c. HS-731. A low dose of naloxone methiodide (320 nmol/kg, s.c.) also significantly attenuated the s.c. action of the other 6-amino acid conjugates, in contrast to s.c. morphine, indicating that these ionizable opioids have a peripheral site of action. The lack of antagonism by low doses of naloxone methiodide on the antinociception elicited by s.c. morphine, and the attenuation in nociception with significantly higher antagonist dose in the tail flick test corroborate with previous findings in hyperalgesia and inflammation (Kayser et al., 1991; Perrot et al., 2001; Reichert et al., 2001; Shannon and Lutz, 2002). Subcutaneous naloxone methiodide was ineffective in antagonizing the effects of the i.c.v. administered agonists. In addition, even a 10-fold higher dose of naloxone methiodide failed to block the antinociceptive effects of i.c.v. HS-731.

On the other hand, antinociceptive effects of HS-731 after i.c.v. administration were antagonized by i.c.v. naloxone, demonstrating an opioid mechanism of action. While i.c.v. injected naloxone inhibited almost completely the effects of systemically administered morphine, it failed to antagonize s.c. HS-731. Moreover, i.c.v. co-administration of the agonists with naloxone methiodide also resulted in significant antagonism. Naloxone was about 10-fold more potent than naloxone methiodide in antagonizing HS-731 and morphine. This is in agreement with observations that much higher doses of quaternary opioid antagonists compared to their tertiary congeners are required for equivalent antagonism (Brown and Goldberg, 1985; Shannon and Lutz, 2002). Taken together, these data are consistent with the interpretation that systemically administered morphine exerts its analgesic effects by interacting with both central and peripheral opioid receptors (Perrot et al., 2001; Shannon and Lutz, 2002), whereas the 6-amino acid derivatives of 14-O-methyloxymorphone interact primarily with peripheral opioid receptors after s.c. administration.

Several studies demonstrated that opioids can produce analgesia through peripheral mechanisms following inflammation of peripheral tissues (Stein et al., 1995; 2003). The new
opioid agonists produced potent antihyperalgesic effect in the formalin test in rats after s.c.
administration, being more potent than morphine. These effects were inhibited by s.c.
naloxone methiodide indicating that such opioids produce antihyperalgesia by activating
peripheral opioid receptors. The present findings are in agreement with other studies where
antihyperalgesic effects of a peripherally acting µ-opioid agonist, loperamide, were detected
in the formalin test after s.c. administration (Shannon and Lutz, 2002).

The 6-amino acid conjugates of 14-\(O\)-methyloxymorphone possess groups that should
be highly ionizable at physiological pH. The presence of these ionized groups increases
hydrophilicity and thus reduces the penetration into the central nervous system, by having
greater selectivity than the non-ionized molecules towards peripheral tissues upon peripheral
administration. The peripheral nature of antinociception produced by the new opioids is
supported by the calculated blood-brain distribution coefficients (Schütz et al., 2003), which
indicate that they have a limited ability to enter the central nervous system after systemic
administration. In most of the published experimental and clinical reports, peripheral
analgesic effects were achieved after local injection of opioids (Stein et al., 2003). Systemic
rather than local administration of opioids with exclusive or predominant peripheral action
would be more convenient in many cases.

In conclusion, the pharmacological profile described for the 6-amino acid conjugates
of 14-\(O\)-methyloxymorphone indicate that such opioids have limited access to the central
nervous system and can mediate antinociception at peripheral sites. Opioid agonists in this
series can find clinical application where the central actions of opioids are unwanted.

Acknowledgments

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References


Footnotes

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Legends for figures

Fig. 1. Chemical structures of 14-O-methyloxymorphone and 14-methoxymetopon.

Fig. 2. Chemical structures of the 6-amino acid substituted derivatives of 14-O-methyloxymorphone.

Fig. 3. Time course of the antinociceptive effects produced by i.c.v. administration of morphine (78 nmol/rat), fentanyl (6 nmol/rat) and 14-O-methyloxymorphone (0.25 nmol/rat) (A), and HS-730 (0.18 nmol/rat), HS-731 (0.18 nmol/rat), HS-935 (0.34 nmol/rat), HS-936 (0.34 nmol/rat), HS-937 (0.29 nmol/rat) and HS-938 (1.44 nmol/rat) (B) in the tail flick test in the rat. Each point represents the mean ± S.E.M. n = 5-8.

Fig. 4. Antagonism of the antinociceptive effects of HS-731 and morphine after s.c. and i.c.v. administration by naloxone methiodide (QNX) and naloxone (NX) in the tail flick test in the rat. (A) The antinociceptive effects of s.c. HS-731 (183 nmol/kg) were determined alone, after s.c. co-administration with QNX, or after i.c.v. administration of NX. (B) The antinociceptive effects of s.c. morphine (7769 nmol/kg) were determined alone, after s.c. co-administration with QNX, or after i.c.v. administration of NX. (C) The antinociceptive effects of HS-731 (0.18 nmol/rat) and (D) morphine (78 nmol/rat) injected i.c.v. were determined alone, after s.c. administration of QNX, after i.c.v. co-administration with QNX or after i.c.v. co-administration with NX. Measurements were performed 30 min after s.c. and 20 min after i.c.v. administration of the opioid agonists. When antagonists were administered i.c.v., they were injected 10 min prior to the s.c. administration of the agonists. Antagonists were co-
administered with the respective agonist, when they were given by the same route of
administration, s.c. or i.c.v. All data are presented as means ± S.E.M. n = 5-10.

*Denotes significance between agonist + antagonist group versus agonist group. *p<0.05,
**p<0.01 (ANOVA with Bonferroni test).

Fig. 5. Effect of the 6-amino acid conjugates of 14-\(O\)-methyloxymorphone in the formalin test
in the rat. Opioid agonists were administered 15 min before the formalin injection and number
of paw flinching were counted during a 5 min observation period over the first (5-15 min) and
second phase (35-90 min) of formalin-induced behavior. The time-effect curves of s.c.
morphine, HS-730 and HS-731 in the formalin test in the rat. (A, left panel). The antagonist
effect of s.c. administered naloxone methiodide (QNX, 2131 nmol/kg) on the antinociceptive
effects induced by morphine, HS-730 and HS-731 in the first and second phase of the
formalin test in rats. (B, right panel). All data are presented as means ± S.E.M. n = 8-10.

*Denotes significance versus saline treated group. *p<0.05, **p<0.01, ***p<0.001 (ANOVA
with Bonferroni test).

+Denotes significance between group treated with agonist alone versus group treated with
agonists and antagonist +p<0.05, ++p<0.01, +++p<0.001 (ANOVA with Bonferroni test).

Fig. 6. Dose-response curves of morphine and 6-amino acid derivatives of 14-\(O\)-
methyloxymorphone after s.c. administration in the first (5-10 min) and second phase (40-45
min) of the formalin test. Rats were given the respective opioid agonist s.c. 15 min before
intraplantar injection of formalin. Data are presented as percentage inhibition of pain behavior
after formalin injection. Each point represents as means ± S.E.M. n = 8-10.
TABLE 1
Antinociceptive potencies of 6-amino acid conjugates of 14-O-methyloxymorphone against radiant heat induced nociception in the tail flick test in the rat after s.c. administration as a function of time

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (nmol/kg, s.c.)</th>
<th>Time after s.c. administration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Morphine</td>
<td></td>
<td>6053$^a$</td>
</tr>
<tr>
<td>Fentanyl</td>
<td></td>
<td>38.6$^a$</td>
</tr>
<tr>
<td>14-O-Methyloxymorphone</td>
<td></td>
<td>14.9$^a$</td>
</tr>
<tr>
<td>HS-730</td>
<td></td>
<td>137</td>
</tr>
<tr>
<td>HS-731</td>
<td></td>
<td>50.8</td>
</tr>
<tr>
<td>HS-935</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>HS-936</td>
<td></td>
<td>&gt;850</td>
</tr>
<tr>
<td>HS-937</td>
<td></td>
<td>641</td>
</tr>
<tr>
<td>HS-938</td>
<td></td>
<td>&gt;3600</td>
</tr>
</tbody>
</table>

Figures in parentheses are 95% confidence limits. n = 5-8.

$^a$ Peak of effect.

- No dose-response relationship.
TABLE 2

Antinociceptive potencies of 6-amino acid conjugates of 14-O-methyloxymorphone against radiant heat induced nociception in the tail flick test in the rat after i.c.v. administration as a function of time

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (nmol/rat, i.c.v.)</th>
<th>Time after i.c.v. administration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Morphine</td>
<td></td>
<td>66.2 (44.1-99.4)</td>
</tr>
<tr>
<td>Fentanyl</td>
<td></td>
<td>1.66$^a$ (1.01-2.75)</td>
</tr>
<tr>
<td>14-O-Methyloxymorphone</td>
<td>0.479 (0.280-1.79)</td>
<td>0.172$^a$ (0.109-0.270)</td>
</tr>
<tr>
<td>HS-730</td>
<td>0.205 (0.121-0.348)</td>
<td>0.101 (0.067-0.152)</td>
</tr>
<tr>
<td>HS-731</td>
<td>0.122 (0.068-0.218)</td>
<td>0.089 (0.052-0.157)</td>
</tr>
<tr>
<td>HS-935</td>
<td>0.299 (0.193-0.463)</td>
<td>0.157 (0.102-0.244)</td>
</tr>
<tr>
<td>HS-936</td>
<td>1.05 (0.567-1.94)</td>
<td>0.126 (0.077-0.208)</td>
</tr>
<tr>
<td>HS-937</td>
<td>0.234 (0.124-0.445)</td>
<td>0.136 (0.072-0.260)</td>
</tr>
<tr>
<td>HS-938</td>
<td>10.6 (7.08-15.9)</td>
<td>1.44 (0.905-2.30)</td>
</tr>
</tbody>
</table>

Figures in parentheses are 95% confidence limits. n = 5-8.

$^a$ Peak of effect.

- No dose-response relationship.
TABLE 3

The relative antinociceptive (peak) potencies of 6-amino acid conjugates of 14-O-methyloxymorphone derivatives compared to morphine and fentanyl in the tail flick test in the rat after s.c. and i.c.v. administration

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative potency&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MORPHINE = 1</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
</tr>
<tr>
<td>14-O-Methyloxymorphone</td>
<td>406</td>
</tr>
<tr>
<td></td>
<td>2.56</td>
</tr>
<tr>
<td>HS-730</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>HS-731</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>1.33</td>
</tr>
<tr>
<td>HS-935</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>HS-936</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>0.72</td>
</tr>
<tr>
<td>HS-937</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td>HS-938</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Relative potencies were calculated at the peak of action (ED<sub>50</sub>) in the tail flick test in rats after s.c. (nmol/kg) and i.c.v. (nmol/rat) administration.
TABLE 4

The s.c./i.c.v. potency ratios of 6-amino acid conjugates of 14-O-methylxymorphone derivatives compared to morphine and fentanyl in the tail flick test in the rat

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$</th>
<th>s.c./i.c.v.$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s.c. (nmol/kg)</td>
<td>i.c.v. (nmol/rat)</td>
</tr>
<tr>
<td>Morphine</td>
<td>6053</td>
<td>35.1</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>38.6</td>
<td>1.66</td>
</tr>
<tr>
<td>14-O-Methylxymorphone</td>
<td>14.9</td>
<td>0.172</td>
</tr>
<tr>
<td>HS-730</td>
<td>58.5</td>
<td>0.031</td>
</tr>
<tr>
<td>HS-731</td>
<td>29.0</td>
<td>0.030</td>
</tr>
<tr>
<td>HS-935</td>
<td>68.9</td>
<td>0.121</td>
</tr>
<tr>
<td>HS-936</td>
<td>53.4</td>
<td>0.082</td>
</tr>
<tr>
<td>HS-937</td>
<td>315</td>
<td>0.063</td>
</tr>
<tr>
<td>HS-938</td>
<td>&gt;3600</td>
<td>0.776</td>
</tr>
</tbody>
</table>

$^a$s.c./i.c.v. was calculated as the ratio of ED$_{50}$ (nmol/kg, s.c.)/ED$_{50}$ (nmol/rat, i.c.v.).
TABLE 5

Antinociceptive effect (ED$_{50}$) of morphine and 6-amino acid conjugates of 14-\textit{O}-methyloxymorphone after s.c. administration on formalin-induced pain behavior in the rat observed in the first (5-10 min) and second (40-45 min) phase

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (nmol/kg, s.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first phase</td>
</tr>
<tr>
<td>Morphine</td>
<td>1613</td>
</tr>
<tr>
<td>HS-730</td>
<td>72</td>
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<tr>
<td>HS-731</td>
<td>125</td>
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<tr>
<td>HS-937</td>
<td>171</td>
</tr>
<tr>
<td>HS-938</td>
<td>79</td>
</tr>
</tbody>
</table>
FIG. 1

![Chemical structure diagram]

R = H: 14-\textit{O}-Methyloxymorphone
R = CH\textsubscript{3}: 14-Methoxymetopon
FIG. 2

α-conjugates

β-conjugates

<table>
<thead>
<tr>
<th>R</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂COOH</td>
<td>HS-730 (α)</td>
</tr>
<tr>
<td></td>
<td>HS-731 (β)</td>
</tr>
<tr>
<td>(S)-CH(CH₃)COOH</td>
<td>HS-935 (α)</td>
</tr>
<tr>
<td></td>
<td>HS-936 (β)</td>
</tr>
<tr>
<td>(S)-CH(CH₂Ph)COOH</td>
<td>HS-937 (α)</td>
</tr>
<tr>
<td></td>
<td>HS-938 (β)</td>
</tr>
</tbody>
</table>
FIG. 3

A

Antinociceptive effect %

Time after i.c.v. administration (min)

- morphine
- fentanyl
- 14-O-Methyloxymorphone
- saline

B

Antinociceptive effect %

Time after i.c.v. administration (min)

- HS-730
- HS-731
- HS-935
- HS-936
- HS-937
- HS-938
- saline
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