Peripherally acting NMDA receptor/glycineB site receptor antagonists inhibit morphine tolerance∗

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Received 17 June 2004; received in revised form 29 October 2004; accepted 27 November 2004

Abstract

The present study focused on the role of peripheral ionotropic N-methyl-D-aspartate (NMDA) receptors in the development of tolerance to morphine-induced antinociception. An initial experiment revealed that NMDA channel blocker memantine, and NMDA receptor/glycineB site antagonist MRZ 2/576 inhibited maximal electroshock-induced convulsions (MES) in female NMR mice with respective potency of 5.93 and 20.8 mg/kg, while other NMDA receptor/glycineB site antagonists MRZ 2/596 and MDL 105,519 were ineffective, supporting lack of CNS activity of the latter two agents. This observation was also supported by blood–brain barrier experiments in vitro. In male Swiss mice, morphine (10 mg/kg) given for 6 days twice a day (b.i.d.) produced tolerance to its antinociceptive effects in the tail-flick test. The NMDA receptor/glycineB site antagonists, MRZ 2/576 at 0.03, 0.1, 0.3 mg/kg and MRZ 2/596 at 0.1, 0.3, 3 and 10 mg/kg attenuated the development of morphine tolerance. Similarly, in male C57/Bl mice, morphine (10 mg/kg) given for 6 days b.i.d. produced tolerance to its antinociceptive effects in the tail-flick test. Like in Swiss mice, in C57/Bl mice morphine tolerance was attenuated by both MRZ 2/576 and MRZ 2/596. Another NMDA receptor/glycineB site receptor antagonist, MDL 105,519 (that very weakly penetrates to the central nervous system) also inhibited morphine tolerance at the dose of 1 but not 0.1 mg/kg. Moreover, both naloxone hydrochloride (5 and 50 mg/kg) and centrally inactive naloxone methiodide (50 mg/kg) inhibited morphine tolerance suggesting the involvement of peripheral opioid receptors in this phenomenon. The present data suggest that blockade of NMDA receptor/glycineB sites in the periphery may attenuate tolerance to the antinociceptive effects of morphine.

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Keywords: Antinociception; Pain; Glutamate; NMDA receptor/glycineB site antagonist; Blood–brain barrier

1. Introduction

Over the last decade research has provided compelling evidence that glutamate receptors are crucially involved in phenomena related to opioid tolerance (see Mao, 1999; Price et al., 2000 for reviews). Antagonists of the ionotropic N-methyl-D-aspartate (NMDA) receptor complex, including memantine, the moderate affinity and highly voltage-dependent clinically used NMDA channel blocker (Parsons et al., 1999) inhibit the development of morphine tolerance (Trujillo and Akil,
NMDA receptor/glycineB site. These compounds reach morphine. As pharmacological tools we used recently antinociceptive effects of systemically administered nervous system (PNS) would inhibit tolerance to the antagonism of NMDA receptors in the peripheral aim of the present study was to investigate whether is more favorable from the therapeutic perspective, the systemic rather than local administration of compounds pounds may not be applicable to the inhibition of antagonists, (topical) application of uncompetitive NMDA receptor antagonists, (+)MK-801 or ketamine, inhibited tolerance to topically applied morphine (Kolesnikov et al., 1999). On the other hand, recent data reported by Kolesnikov and colleagues demonstrated that local (topical) application of uncompetitive NMDA receptor antagonists, (+)MK-801 or ketamine, inhibited tolerance to topically applied morphine (Kolesnikov et al., 1996; Kolesnikov and Pasternak, 1999b; Kolesnikov and Pasternak, 1999a), suggesting a peripheral component of antinociceptive morphine tolerance. Since systemic rather than local administration of compounds is more favorable from the therapeutic perspective, the aim of the present study was to investigate whether antagonism of NMDA receptors in the peripheral nervous system (PNS) would inhibit tolerance to the antinociceptive effects of systemically administered morphine. As pharmacological tools we used recently developed NMDA receptor antagonists acting at the NMDA receptor/glycineB site. These compounds reach relatively low brain levels after systemic application as compared to plasma values (MRZ 2/576: ~2% (Hesselink et al., 1999b), MDL 105,519: 0.01–0.08% (Opacakajuffry et al., 1998)) or seem to be lacking CNS activity as was the case with MRZ 2/596 (see Sections 3.1.3 and 4). In contrast, the free brain levels of memantine are over 50% of plasma concentration (Hesselink et al., 1999a).

2. Methods

2.1. In vitro

2.1.1. Receptor binding

Tissue preparation was performed according to Foster and Wong (1987) with some modifications. Male Sprague-Dawley rats (200–250 g, Janvier, Le Genest-Isle, France) were decapitated and their brains were removed rapidly. Tissue was then processed as described previously (Parsons et al., 1997). The amount of protein in the final membrane preparation was determined according to Hartree (1971) and adjusted to 250–500 μg/ml.

Membranes were suspended and incubated in 50 mM Tris–HCl, pH 8.0 for 45 min at 4 °C with a fixed [3H]MDL-105,519 concentration of 2 nM. MDL-105,519 is a selective high affinity antagonist at the NMDA receptor/glycineB site and has recently been introduced as a commercially available radioligand (Amersham Biosciences, Freiburg, Germany) (Baron et al., 1996; Baron et al., 1997). Non-specific binding was defined by the addition of unlabeled glycine at 100 μM. Incubations were terminated using a Millipore filter system (Millipore, Schwabach Germany). The samples, all in duplicate, were rinsed three times with 2.5 ml ice-cold assay buffer over glass fibre filters (Schleicher and Schuell, Dassel, Germany) under a constant vacuum. Filtration was performed as rapidly as possible (max 2 s). Following separation and rinse, the filters were placed into scintillation liquid (5 ml; Ultima Gold) and radioactivity was determined with a liquid scintillation counter (both Packard BioScience, Dreieich, Germany).

2.1.2. Patch clamp

Patch clamp recordings were made from rat hippocampal neurons, after 12–15 days in vitro, with polished glass electrodes (3–5 mΩ) in the whole cell mode at room temperature (20–22 °C) with the aid of an EPC-7 amplifier (HEKA, Lambrecht, Germany) – detailed methods described in Parsons et al. (1999).

2.1.3. BBB permeability studies

An in vitro model of the BBB has been established as a first screen for BBB permeability.

2.1.3.1. Preparation and cultivation of BBCEC. Bovine brain capillary endothelial cells (BBCEC) were isolated from brains, purified, and cultured according to Meresse et al. (1989). Briefly, after mechanical homogenization microvessels were seeded onto dishes coated with an extracellular matrix secreted by bovine corneal endothelial cells (Gospodarowicz et al., 1976). Pure colonies of endothelial cells were seeded onto gelatin-coated dishes (Corning Costar, Bodenheim, Germany) in the presence of Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco Invitrogen GmbH, Karlsruhe, Germany) supplemented with 20% calf serum (Hyclone, Utah, USA), 2 mM glutamine, 50 μg/ml of gentamycin (Biochrom, Berlin, Germany), and bovine fibroblast growth factor (bFGF; Roche, Mannheim, Germany) and bovine fibroblast growth factor (bFGF; Roche, Mannheim, Germany; 1 ng/ml added every other day). BBCEC in passage 4–6 were used for co-cultivation/transport studies.

2.1.3.2. Preparation and cultivation of rat cortical astrocytes. Astrocytes were prepared mechanically from cortices of newborn rats as described by Booher and Sensenbrenner (1972). The cell suspension was seeded onto 12-well plates (Corning, Wiesbaden, Germany) and cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco Invitrogen GmbH, Karlsruhe, Germany) containing 10% FCS (HyClone, Utah,
USA), 2 mM glutamine and 50 μg/ml gentamycin (Biochrom, Berlin, Germany).

2.1.3.3. Co-cultivation of BBCEC with rat cortical astrocytes. For co-cultivation the BBCEC were seeded onto a collagen-coated filter (Transwell Col; Corning, Wiesbaden, Germany) and placed into 12-well plates (Corning, Wiesbaden, Germany) containing differentiated astrocytes in BBCEC culture medium.

2.1.3.4. Transport studies. The transport studies were performed between 8 and 12 days of co-cultivation in Krebs Ringer buffer. The amount of substance passing through the endothelial cell layers after 10, 20, and 30 min was quantified in samples taken from the abluminal (brain) side of the filter (n = 3).

As a control, collagen coated filters without cells were used.

Transport studies were performed with the following compounds: pyrido-phthalazine-diones – MRZ 2/501, its choline salt MRZ 2/596, MRZ 2/502 and its choline salt MRZ 2/576, all at a concentration of 10 μM, and compared to other NMDA receptor/glycine B site antagonist [3H]-MDL 105,519 (0.02 μM) (known to diffuse very slowly under physiological conditions into the brain) and NMDA receptor channel blocker memantine (10 μM) representing a drug which shows a good penetration through the BBB. For testing the BBB integrity, the permeability of 0.1 μM[^14C]-sucrose, a functional blood–brain barrier-impermeant marker, was determined every day before the actual experiments. The permeability calculations were based on the clearance principle published by Sillinger-Birnboim et al. (1987).

2.1.3.5. BBB integrity study. Co-cultures of BBCEC and astrocytes were incubated with morphine sulphate at concentrations of 3, 6 and 10 μg/ml for 1 h, 6 h, and 24 h (on the luminal side only) and the permeability of[^14C]-sucrose across the BBB in vitro was tested afterwards in the presence of morphine.

Drug concentrations were analyzed via HPLC in case of NMDA receptor/glycine B site antagonists (see Hesselink et al., 1999b), GC/MSD in case of memantine (Hesselink et al., 1999a), or liquid scintillation counting in case of radiolabelled substances having approximate detection limits of 100 nM, 10 nM and 2 nM, respectively.

2.2. In vivo

2.2.1. Maximal electroshock (MES), traction reflex and rotarod tests

2.2.1.1. Subjects. MES, traction reflex and rotarod tests were performed in NMR female mice (18–28 g, Janvier, Le Genest-Isle, France) housed 5 per cage. All animals were kept with water and food ad libitum under a 12-h light-dark cycle (light on: 06:00) and at a controlled temperature (20 ± 0.5 °C). Experiments were performed according to the animal rights commission allowance #F 77-51 (Hessen, Germany).

The MES test was performed together with tests for myorelaxant action (traction reflex) and motor coordination (rotarod, Ugo Basile, Italy). For the traction reflex test, mice were placed with their forepaws on a horizontal rod and were required to place all 4 paws on the wire within 10 s. To test ataxia (motor coordination) mice were placed on rotarod (5 r.p.m.) and were required to remain on the rod for 1 min. Only mice not achieving the criteria in all three repetitions (performed with 1 min intervals) of each test were considered to exhibit myorelaxation or ataxia, respectively. In case of reaching the criterion on the 1st or 2nd trial, no further trials were performed. These tests were followed 1–2 min later by MES (100 Hz, 0.5 s shock duration, 50 mA shock intensity, 0.9 ms impulse duration, Ugo Basile) applied through corneal electrodes. The presence of tonic convulsions was scored (tonic extension of hind paws with minimum angle to the body of 90°). The aim was to obtain ED50 for all parameters scored (anticonvulsive activity and motor side effects) with use of the Litchfield Wilcoxon test for quantal dose responses. Tested agents were dissolved in distilled water and injected 15–30 min before MES.

An additional experiment was designed to exclude the possibility that after chronic treatment, MRZ 2/596 accumulates in the brain sufficiently to block central NMDA receptors. Mice were treated for 7 days twice daily with saline or MRZ 2/596 (10 mg/kg), 20 min after the last injection mice were subjected to MES and the occurrence of tonic seizures was scored. Additionally, MRZ 2/576 was injected at a dose of 20 mg/kg as a positive control since this agent penetrates sufficiently to the CNS.

2.2.2. Morphine tolerance

2.2.2.1. Subjects. Male Swiss mice (25–30 g, Institute of Pharmacology breeding facility, Krakow, Poland) were group-housed in standard laboratory cages and kept in a temperature-controlled colony room (21 ± 2 °C) with a 12-h light/dark cycle (light on: 07:00). Similarly housed and maintained male C57Bl mice (25–30 g) were obtained from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Commercial food and tap water were available ad libitum. Each experimental group consisted of at least 7 mice per treatment in morphine tolerance experiments and at least 5 mice per treatment in acute morphine antinociception experiment. All mice were used only once.

Morphine tolerance studies were carried out according to the National Institutes of Health Guide for Care
and Use of Laboratory Animals (revised 1996) and were approved by the Institute of Pharmacology PAN Animal Care and Use Bioethics Commission.

The tail-flick tests were performed as described previously (Popik et al., 2000). The assessments of antinociceptive ED\textsubscript{50} of morphine (test #1 and test #2) were carried out without pretreatment with glutamate and opioid antagonists.

2.2.2. Experimental design. The first experiment was carried out on Swiss mice to determine whether the development of tolerance to the antinociceptive effects of morphine could be inhibited by NMDA receptor/glycine\textsubscript{B} site antagonists not penetrating (MRZ 2/596) or weakly penetrating (MRZ 2/576) the BBB.

On day 1 the first measurement of morphine antinociceptive potency was performed (test #1), followed by 6 days of b.i.d. morphine injections (10 mg/kg, s.c., 9:00 and 17:30) (Elliott et al., 1994; Popik et al., 2000b). MRZ 2/596 (0.05, 0.1, 0.3, and 1 mg/kg) and MRZ 2/576 (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 mg/kg) were given s.c. 30 min prior to each morphine dose on days 2–7. On day 8 the second measurement of the antinociceptive potency of morphine was performed (test #2). The degree of morphine tolerance was assessed by comparing the antinociceptive potency of morphine (ED\textsubscript{50}) observed in tests #1 and #2.

The subsequent experiments were carried out several months later and due to technical reasons C57/Bl mice were used. In order to confirm results obtained in Swiss mice, the effect of MRZ 2/596 (0.3 mg/kg), MRZ 2/576 (1 mg/kg) on the development of morphine tolerance was tested in C57/Bl mice. To further confirm the usefulness of C57/Bl mice in the present experimental settings, we also used the NMDA channel blocker memantine (2.5 and 7.5 mg/kg) that has been shown previously to inhibit morphine tolerance in Swiss mice (Popik et al., 2000a). Since the effects obtained with Swiss mice were also observed in C57/Bl mice, further experiments were conducted using of C57/Bl mice. To investigate the possibility that the effects of MRZ 2/596 on the development of morphine tolerance were due to an inhibition of morphine’s antinociceptive action (naloxone-like effect), MRZ 2/596 was administered in a single injection of 1 mg/kg s.c. 30 min before single morphine 3 mg/kg s.c. administration. The antinociceptive effects of morphine were investigated 30, 60 and 120 min later. In another control experiment, we investigated if the inhibitory effects of MRZ 2/596 on the development of morphine tolerance could be attributed to an accumulation of this compound. To this end, groups of mice were treated for 7 days twice daily with saline or MRZ 2/596 (1 mg/kg). Twelve hours after the last injection, mice were injected with 3 mg/kg s.c. of morphine and tested in the tail-flick apparatus 30, 60 and 120 min.

The next experiment was carried out to find doses of opioid antagonists to affect acute morphine antinociception. Thus, naloxone hydrochloride (1, 5, 10 and 50 mg/kg) and naloxone methiodide (5, 10 and 50 mg/kg) were administered s.c. 15 min before 10 mg/kg of s.c. morphine (the dose used in chronic experiments) and the tail-flick test was conducted 30 min after morphine injection.

Further, it was determined if (a) another NMDA receptor/glycine\textsubscript{B} site antagonist, MDL 105,519, structurally dissimilar from both MRZ 2/576 and MRZ 2/596 and also weakly penetrating the CNS and (b) opioid receptor antagonists, naloxone hydrochloride and its quaternary derivative, naloxone methiodide affect the development of morphine antinociceptive tolerance. MDL 105,519 (0.1 and 1 mg/kg) was given s.c. 30 min prior to each morphine dose. Naloxone hydrochloride (1, 5 and 50 mg/kg) and naloxone methiodide (1, 5 and 50 mg/kg) were given s.c. 15 min prior to each morphine dose during its chronic administration. Doses of morphine and memantine were used based on previous observations (Popik and Skolnick, 1996; Popik et al., 2000b).

2.2.2.3. Data presentation and statistics. Latencies (in s) of the tail-flick responses were converted to %Maximum Possible Effect values [%MPE (Paronis and Holtzman, 1991)], according to the formula: 100[(post-injection latency – baseline latency)/(cut-off latency – baseline latency)]. %MPE values were used to construct morphine cumulative dose–response curves by non-linear regression; these curves were used to calculate antinociceptive ED\textsubscript{50} values using GraphPad Prism ver. 3.00 (GraphPad Software, CA, USA) software. The ED\textsubscript{50} values obtained for tests #1 and #2 were compared among groups, as were the fold shifts (determined by dividing individual test #2 ED\textsubscript{50} values by the test #1 ED\textsubscript{50} values) with one-way ANOVAs and post hoc Newman-Keul’s and LSD tests. Data are presented as mean ± S.E.M.

2.2.3. Drugs

MRZ 2/501 (8-chloro-1,4-dioxo-1,2,3,4-tetrahydropyridazino (4,5-b) quinoline), MRZ 2/596 (8-chloro-1,4-dioxo-1,2,3,4-tetrahydropyridazino (4,5-b) quinoline chloride salt), MRZ 2/502 (8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino (4,5-b) quinoline-5-oxide), and MRZ 2/576 (8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino (4,5-b) quinoline-5-oxide chloride salt) and memantine (1-amino-3,5-dimethyladamantane) were from Merz Pharmaceuticals GmbH, Frankfurt/M, Germany. Morphine sulphate was obtained from Sigma, Taufkirchen, Germany.

[H]-morphine was purchased from NEN-Research Products, Köln, Germany, [H]-MDL 105,519 ((E)-3-(2-phenyl-2-carboxyethyl)-4,6-dichloro-1H-indole-2-
carboxylic acid) and \(^{14}\text{C}\)-sucrose purchased from Amersham Life Science, Freiburg, Germany.

Morphine HCl (morphine tolerance studies) was obtained from Polfa, Kraków, Poland and naloxone hydrochloride, naloxone methiodide, and MDL 105,519 ((E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid) from Sigma–Aldrich, USA. All other compounds were obtained from Tocris, U.K. For morphine tolerance experiments morphine, MDL 105,519, memantine, naloxone hydrochloride and naloxone methiodide were dissolved in physiological saline (placebo). MRZ 2/596 and MRZ 2/576 were dissolved in sterile water. All injections were administered in a volume of 10 ml/kg. All NMDA receptor/glycine\(B\) site antagonist solutions were prepared fresh the day before the experiment started, aliquoted and stored at 4 °C but administered at room temperature. The dose of morphine is expressed as the base, the doses of all other compounds as their respective salts.

3. Results

3.1. In vitro

3.1.1. Receptor binding

In Scatchard analysis \(^{3}\text{H}\)MDL 105,519 had a \(K_d\) of 6.03 ± 0.14 nM and a \(B_{\text{max}}\) of 4.03 pmol/mg protein. MRZ 2/576 and MRZ 2/596 displaced \(^{3}\text{H}\)MDL-105,519 binding to rat cortical membranes with \(K_b\)s of 126 and 160 nM, respectively (Table 1, \(IC_{50}\) corrected according to the Cheng-Prussoff relationship for 2 nM \(^{3}\text{H}\)MDL 105,519). MDL-105,519 displaced its own binding with a \(K_i\) of 11.6 nM. Non-specific binding determined with glycine 100 µM was only 15–20% and standard compounds displaced binding to non-specific levels with potencies similar to the literature and Hill coefficients close to unity.

3.1.2. Patch clamp

Steady-state inward current responses of cultured hippocampal neurons to NMDA (200 µM with glycine 1 µM) were antagonized by MDL-105-519 with \(IC_{50}\) of 106 ± 13 nM (Table 1) whereas MRZ 2/576 and MRZ 2/596 with \(IC_{50s}\) of 540 ± 30 and 700 ± 35 nM, respectively, were 5–7-fold less potent (Table 1). When corrected for the affinity of glycine under these conditions (399 nM) all three antagonists had very similar \(K_b\) values to their \(K_b\)s assessed in the \(^{3}\text{H}\)MDL-105,519 binding experiments – \(K_b\):

MRZ 2/576 = 154 nM, MRZ 2/596 = 199 nM, MDL 105,519 = 30.3 nM.

3.1.3. BBB permeability studies

As expected, the impermeant marker sucrose showed a low permeability (pe) coefficient of 2.27 × 10\(^{-5}\) cm/s (Fig. 1) in the established BBB model. The lipophylic NMDA channel blocker memantine had good penetration through the in vitro BBB (pe-coefficient 50.32 × 10\(^{-5}\) cm/s) (Fig. 1). In contrast, the hydrophylic NMDA receptor/glycine\(B\) site antagonist MDL 105,519 diffused slowly through the endothelial monolayer (pe-coefficient 2.00 × 10\(^{-5}\) cm/s) (Fig. 1).

MRZ 2/576 and its free base MRZ 2/502 showed pe-coefficients of 24.33 × 10\(^{-5}\) and 25.07 × 10\(^{-5}\) cm/s, respectively. The quinoline, MRZ 2/596 and its free base MRZ 2/501 demonstrated pe-coefficients of 3.60 × 10\(^{-5}\) and 3.33 × 10\(^{-5}\) cm/s, respectively (Fig. 1). In the case of MRZ 2/596, the accuracy of the calculated pe-coefficient was limited by the sensitivity of the HPLC-method used. The concentrations of most analytical samples taken

![Fig. 1. In vitro BBB penetration of NMDA receptor/glycine\(B\) site antagonists in comparison to standards – sucrose poor penetration, memantine good penetration.](image_url)
from the BBCEC monolayers were under the detection limit (100 nM). Therefore the concentration of the detection limit had to be used for pe-coefficient calculation which leads to a slightly higher pe-value as the actual one.

3.1.4. BBB integrity study

BBCEC treated with morphine on the luminal side (3 μg/ml for 1 h, 3 μg/ml for 6 h, 6 μg/ml for 6 h and 10 μg/ml for 24 h) showed no significant increase in [14C]-sucrose permeability in comparison to untreated endothelial cells (data not shown).

3.2. In vivo

3.2.1. Maximal electroshock (MES), traction reflex and rotarod tests

Memantine and MRZ 2/576 inhibited MES with ED50s of 5.93 and 20.8 mg/kg, respectively, while both MRZ 2/596 and MDL 105,519 were ineffective (Table 2).

Memantine disturbed the traction reflex with an ED50 = 12.5 mg/kg and ataxia was observed with an ED50 = 14.2 mg/kg. MRZ 2/576 produced myorelaxation with an ED50 = 21.1 mg/kg and ataxia with an ED50 = 22.3 mg/kg. MRZ 2/596 and MDL 105, 519 administered even at very high doses did not show any activity in these tests (Table 2).

All mice treated semi-chronically with MRZ 2/596 (10 mg/kg) for 7 days showed tonic convulsions in the absence of drug as in control animals (8/8 mice). In contrast, in mice treated acutely with MRZ 2/576 (20 mg/kg) occurrence of tonic seizures was much lower (1/8 mice).

3.2.2. Morphine tolerance

The initial experiment carried out on Swiss mice demonstrated no differences among groups in antinociceptive morphine ED50 values in test #1. The following ED50 values (mg/kg) were observed in test #1 for placebo + placebo: 5.40 ± 0.68; for placebo + morphine: 5.17 ± 0.62; for MRZ 2/596 (at 0.05, 0.1, 0.3, 1 mg/kg + morphine): 5.83 ± 1.97, 4.76 ± 0.70, 5.03 ± 1.36, 4.87 ± 0.95, respectively; and for MRZ 2/576 (at 0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/kg) + morphine: 3.43 ± 0.62, 5.10 ± 0.75, 3.95 ± 0.49, 4.99 ± 0.67, 2.88 ± 0.65, 4.53 ± 0.61, 2.99 ± 0.50, respectively (ANOVA F(12, 158) = 0.92). Six-day b.i.d. treatment with 10 mg/kg of morphine produced a 3.47-fold increase in the morphine antinociceptive ED50 values as determined in test #2. MRZ 2/596 and MRZ 2/576 inhibited morphine antinociceptive tolerance at doses starting at 0.1 and 0.03 mg/kg, respectively. This was revealed by smaller differences between tests #1 and #2 (ED50 test #2/ED50 test #1 fold changes, presented in Fig. 2) in respective groups as compared to control group that received placebo + morphine. The raw data of the representative groups are also shown in Fig. 3.

For reasons explained in the methods, all the subsequent experiments were carried out in C57/Bl mice. In the acute morphine antinociception experiment, naloxone hydrochloride at doses of 5, 10 and 50 mg/kg and naloxone methiodide at a dose of 50 mg/kg prevented acute antinociception induced by morphine (10 mg/kg) (Fig. 4).

Six-day administration of morphine induced a 2.67 ± 0.42 fold change in morphine potency in test

<table>
<thead>
<tr>
<th>Agent</th>
<th>MES ED50 (mg/kg)</th>
<th>Traction ED50 (mg/kg)</th>
<th>Ataxia ED50 (mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>Memantine</td>
<td>5.93 (3.7–9.5)</td>
<td>12.5 (8.0–19.4)</td>
<td>14.2 (10.2–19.9)</td>
</tr>
<tr>
<td>MRZ 2/576</td>
<td>20.8 (15.2–28.4)</td>
<td>21.1 (17.2–25.9)</td>
<td>22.3 (19.9–25.6)</td>
</tr>
<tr>
<td>MRZ 2/596</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>MDL 105,519</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
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Values are ED50s in mg/kg (95% confidence limits are shown in parentheses).
#2 (as compared to test #1) that was significantly higher than the placebo treated group (fold change 1.04 \( \pm \) 0.13). MRZ 2/596 at 0.3 mg/kg and MRZ 2/596 at 1 mg/kg given prior to each morphine dose inhibited the development of morphine antinociceptive tolerance (fold changes 1.52 \( \pm \) 0.16 and 1.44 \( \pm \) 0.17, respectively) (ANOVA \( F(3, 54) = 6.45, p < 0.001 \)). This confirms that the phenomenon previously observed in albino Swiss mice can also be demonstrated in C57/Bl mice. Thus, further experiments were continued on C57/Bl mice using the same morphine tolerance paradigm.

In the next set of experiments, there were no differences among C57/Bl mice in the antinociceptive morphine ED\( \text{50} \) values in test #1. Test #1 ED\( \text{50} \) values (mg/kg) were obtained: for placebo + placebo, 1.92 \( \pm \) 0.16; placebo + morphine 1.51 \( \pm \) 0.13; memantine (2.5 and 7.5 mg/kg) + morphine 1.40 \( \pm \) 0.22, 1.59 \( \pm \) 0.29, respectively; MDL 105,519 (0.1, 1 mg/kg) + morphine 1.53 \( \pm \) 0.22, 1.86 \( \pm \) 0.31, respectively; naloxone hydrochloride (1, 5, 50 mg/kg) + morphine 1.66 \( \pm \) 0.23,
Treatment with 10 mg/kg b.i.d. of morphine produced a 4.55-fold increase in the morphine antinociceptive ED$_{50}$ values as determined in test #2. Morphine tolerance was attenuated by memantine at a dose of 7.5 but not at 2.5 mg/kg, and MDL 105,519 at the dose of 1 but not at 0.1 mg/kg (Fig. 5A).

Naloxone hydrochloride prevented the development of morphine antinociceptive tolerance at doses of 5 and 50 but not 1 mg/kg. Naloxone methiodide prevented morphine tolerance at doses of 50 but not 5 and 1 mg/kg (Fig. 5B).

Additionally, the possible acute effect of MRZ 2/596 on morphine-induced antinociceptive activity was investigated. Area Under the Curve calculated on %MPE on morphine-induced antinociceptive activity was increased by 4.55-fold, which was significantly different toward “Placebo” group that received saline and morphine during the development of morphine tolerance (*p < 0.05, **p < 0.01).

It is unlikely that repeated administration of MRZ 2/596 for 7 days has an effect on the pharmacokinetics of morphine as there was no change in the AUC for morphine antinociception (values for saline and 1 mg/kg of MRZ 2/596 were, respectively, 4100 ± 401 and 2930 ± 440, t(18) = 1.97, NS).

4. Discussion

NMDA receptor binding and patch clamp studies confirmed that MRZ 2/576 and MRZ 2/596 bind with moderate (nanomolar) affinity to NMDA receptor/glycine$_B$ site whereas MDL 105,519 was some 5–10-fold more potent. Because of the use of glycine (1 μM) in the patch clamp studies, IC$_{50}$ values obtained in these experiments were 4–9-fold higher than the $K_i$ values obtained in binding experiments. However, correction of these IC$_{50}$ values to $K_i$s taking into account a $K_d$ for glycine in these patch clamp experiments of 399 nM revealed very similar potency of all three antagonists in both assays. The effective concentrations in vivo will clearly depend on the endogenous levels of glycine and could differ in the CNS and the periphery. Certainly, considerable evidence suggests that the glycine$_B$ site is not saturated in the CNS in vivo (Danysz and Parsons, 1998).

The antagonism observed with MRZ 2/576 and MRZ 2/596 was typical for moderate affinity NMDA receptor/glycine$_B$ antagonists, i.e. they induced glycine-sensitive desensitization whereas MDL-105,519 was almost equally effective against peak and plateau – an effect probably related to its higher affinity – see Parsons et al. (1997).

Neurochemical studies using radiolabelled MDL 105,519 demonstrate that its brain penetration (plasma vs. brain levels) is low, and the uptake of radioactivity into the brain is in a range of 0.01–0.08% of the injected dose (Opackajuffry et al., 1998). The MES experiment in the present study is in line with this data and also indicates that MRZ 2/596 has negligible CNS effects. Thus, both MRZ 2/596 and MDL 105,519 seem to be devoid of CNS activity, at least at the doses used. This assumption could also be confirmed by the present BBB permeability study where both compounds pass the BBB very slowly as compared with memantine. The effect of morphine on BBB integrity was also investigated as Oishi et al. (1989) reported that morphine treatment affects BBB permeability. The present results indicate that morphine treatment did not affect BBB integrity in vitro since there was no difference in sucrose permeability after incubation with morphine.

In principle, the present data indicate that structurally similar substances with different penetration to the CNS can be discriminated in the BBB in vitro model. Of the glycine$_B$ antagonists tested, only those with a
NO-substitution could pass the blood–brain barrier (MRZ 2/502 and MRZ 2/576) in contrast to the quinolines (MRZ 2/501 and MRZ 2/596) and MDL 105,519 confirming results of functional studies such as MES.

However, it should be stressed that this model can only be regarded as a “first line screen” for CNS penetration since it does not reflect all aspects of governing CNS bioavailability in vivo such as e.g. probenecid sensitive transport out of the brain in the choroid plexus.

The present study revealed that NMDA receptor antagonists acting at the NMDA receptor/glycineB site with modest (MRZ 2/576) or likely no CNS activity such as MRZ 2/596 and MDL 105,519 inhibited tolerance to the antinociceptive effects of morphine. The effects of the structurally similar moderate affinity agents MRZ 2/576 and MRZ 2/596 were compared to the structurally different high affinity NMDA receptor/glycineB site antagonist MDL 105,519. All three NMDA receptor/glycineB site antagonists inhibited morphine tolerance suggesting that the inhibition of morphine tolerance by both MRZ 2/576 and MRZ 2/596 is related to their common pharmacodynamic action, i.e. blockade of NMDA receptors at the glycineB site, and not via actions at an unrelated site due to chemical similarity.

The inhibition of morphine tolerance by glycineB antagonists has been well documented. The full glycineB antagonist, ACEA-1328 (20 mg/kg) completely blocks morphine-induced antinociception in the tail-flick test in CD-1 mice (Lutfy et al., 1999). Similarly, another antagonists GV 196771A prevented development of tolerance to morphine antinociception in the formalin test in mice (Quartaroli et al., 2001). Even the low intrinsic activity partial agonist, (R, +)-HA966, was able to inhibit morphine tolerance in neuropathic rats (Christensen et al., 2000).

It is certain that the doses of MRZ 2/576 that effectively inhibited morphine tolerance (starting at 0.03 mg/kg) do not reach sufficient concentrations in the brain to block CNS NMDA receptors. Brain microdialysis experiments revealed that only 2% of systemically applied MRZ 2/576 is found in the extracellular fluid in the CNS (Hesselink et al., 1999b).

Despite good BBB permeability properties, as described also in our study in the in vitro BBB model, MRZ 2/576 is quickly removed from the brain by probenecid-sensitive transporters (Hesselink et al., 1999b). Moreover, a number of behavioral studies showed that CNS-related effects of MRZ 2/576 occur at doses much higher than the minimal effective dose in the present report. MRZ 2/576 shows neuroprotective properties at 5 mg/kg (Wenk et al., 1998), anticonvulsive activity at 2.8 mg/kg after intravenous administration (Parsons et al., 1997). In the present study, anticonvulsive activity was observed at doses of 0.03 mg/kg given s.c. at the dose of 0.03 mg/kg is present in the brain at a concentration sufficient to block NMDA receptors to the degree producing inhibition of morphine tolerance. With regard to MRZ 2/596 and MDL 105,519, these compounds show no CNS-related anticonvulsive activity against maximal electroshock at 50 mg/kg in adult mice (present study).

The present study revealed that NMDA receptor antagonists acting at the NMDA receptor/glycineB site with modest (MRZ 2/576) or likely no CNS activity such as MRZ 2/596 and MDL 105,519 inhibited tolerance to the antinociceptive effects of morphine.
It was also previously reported that systemic administration of morphine for 4 days (10 mg/kg) produces downregulation of mu receptor mRNA in dorsal root ganglia accompanied by tolerance to the analgesic effects in the hot plate, and the authors suggested that the peripheral nervous system may be important site of opioid tolerance development (Meuser et al., 2003). This form of tolerance is also not surprising when considering that peripheral mu receptors seem to contribute to analgesia produced by systemically administered morphine (Lewanowitsch and Irvine, 2002).

In contrast to studies demonstrating behavioral activity of MRZ 2/576 at doses ≥ 5 mg/kg, Olivar and Laird (1999) reported that this compound inhibited the effects of visceral noxious stimuli (as measured by an increase in blood pressure) with an ED₅₀ of 0.2 mg/kg. These findings support the notion that MRZ 2/576 has peripheral actions, at least in the case of processing of nociceptive signaling.

Another surprising aspect of the present findings is that despite the short half life (of about 15 min) and also the short duration of anticonvulsive activity (Parsons et al., 1997; Hesselink et al., 1999b), MRZ 2/576 was active in the present study when given 30 min before morphine. It is possible that administration of MRZ 2/576 leaves “finger prints”, an effect that outlasts the presence of the compound in the body. The “finger print” concept is supported by the data of Belozertseva et al. (2000a), demonstrating that MRZ 2/576 at a dose of 1 mg/kg inhibited spontaneous morphine withdrawal in mice 45–60 min after injection when less than 25% of the injected dose is still present in the body (Parsons et al., 1997; Hesselink et al., 1999b). The “finger print” effect of MRZ 2/576 was also described by Chizh and colleagues in rats in the chronic constriction nerve injury model. In this study MRZ 2/576 (1–10 mg/kg, i.p.) produced an antiallodynic effect more than 24 h after administration of the drug despite the fact that the inhibition of responses to iontophoretically applied NMDA in the spinal cord only lasted about 10–15 min (Chizh et al., 2001). All these and the present findings suggest that even a short lasting blockade of the NMDA receptor/glycineB site may lead to long lasting consequences.

In the present study, both naloxone hydrochloride and methiodide prevented the development of morphine tolerance with minimal effective doses of 5 and 50 mg/kg, respectively. These doses also inhibited acute morphine antinociception although the former agent was more effective in this regard (Fig. 4). The inhibitory effect of naloxone methiodide on morphine antinociceptive tolerance may additionally suggest that this phenomenon involves peripheral sites considering that its purity was at least 99% (Sigma–Aldrich communication). The difference in potencies between naloxone hydrochloride and naloxone methiodide can be attributed to the difference in the affinity at mu-opioid receptors, for which naloxone methiodide has much lower affinity than naloxone hydrochloride ca. 50-fold in mu-opioid receptor binding assay and ca. 26 in functional assay (Valentino et al., 1983).

One could also consider the possibility that the NMDA receptor/glycineB site antagonists used in the present study mediate their effects of morphine tolerance in a similar manner to opioid antagonists, but they should then have an inhibitory effect on morphine-induced antinociception per se (“naloxone-like” effect). However, in previous reports, NMDA receptor/glycineB site antagonists, either did not affect, or even potentiated morphine antinociceptive effects in the mice tail-flick test (Lutfy et al., 1999; Belozertseva et al., 2000b), for review see Kozela and Popik (2002), thus making this possibility unlikely. Nevertheless, we investigated this possibility for MRZ 2/596 and this compound did not change morphine’s antinociceptive activity.

Moreover, one cannot exclude the possibility that glycine site NMDA receptor antagonists not penetrating to the brain after acute administration, could show tissue accumulation after chronic administration, leading to concentrations that have central activity. This was investigated in two types of experiments. Treatment with MRZ 2/596 for 7 days did not affect morphine antinociception in the absence of acute MRZ 2/596 and similarly such treatment even at a much higher dose did not influence MES convulsions suggesting insufficient NMDA receptor blockade in the CNS. Thus, the possibility of brain accumulation leading to brain levels sufficient to block NMDA receptors can be rather excluded.

In conclusion, it appears that both the NMDA receptor/glycineB site and opioid receptors in the PNS play a role in the development of antinociceptive tolerance to morphine applied systemically. This further supports previous findings about peripheral (topical) components of morphine tolerance by Kolesnikov and Pasternak group (Kolesnikov et al., 1996; Kolesnikov and Pasternak, 1999a,b).

It can be further hypothesized that the use of NMDA receptor antagonists to inhibit morphine antinociceptive tolerance could be restricted to centrally inactive NMDA receptor antagonists e.g. acting at the NMDA receptor/glycineB sites. Such an approach would allow the avoidance of centrally-mediated side effects known for many NMDA receptor antagonists which penetrate to the brain well.

Acknowledgements

The study was supported in part by Institute of Pharmacology Statutory Activity.
References


Baron, B.M., Siegel, B.W., Harrison, B.L., Gross, R.S., Hawes, C., Towers, P., 1996. \[^{1}H\]MDL 105,519, a high-affinity radioligand for the N-methyl-D-aspartate receptor-associated glycine recognition site. Journal of Pharmacology and Experimental Therapeutics 279, 62–68.


Bienkowski, P., Danysz, W., Kostowski, W., 1998. Study on the role of glycine, strychnine-insensitive receptors (glycine (b) sites) in the discriminant stimulus effects of ethanol in the rat. Alcohol 15, 87–91.


Bienkowski, P., Danysz, W., Kostowski, W., 1998. Study on the role of glycine, strychnine-insensitive receptors (glycine (b) sites) in the discriminant stimulus effects of ethanol in the rat. Alcohol 15, 87–91.


Bienkowski, P., Danysz, W., Kostowski, W., 1998. Study on the role of glycine, strychnine-insensitive receptors (glycine (b) sites) in the discriminant stimulus effects of ethanol in the rat. Alcohol 15, 87–91.


Bienkowski, P., Danysz, W., Kostowski, W., 1998. Study on the role of glycine, strychnine-insensitive receptors (glycine (b) sites) in the discriminant stimulus effects of ethanol in the rat. Alcohol 15, 87–91.
Parsons, C.G., Danysz, W., Quack, G., 1999. Memantine is a clinically well tolerated N-methyl-D-aspartate (NMDA) receptor antagonist – a review of preclinical data. Neuropharmacology 38, 735–767.
Patierno, S., Zellalem, W., Ho, A., Parsons, C.G., Tonini, M., Sternini, C. N-methyl-D-aspartate (NMDA) receptors mediate endogenous opioid release in enteric neurons of guinea pig ileum induced by abdominal surgery, submitted for publication.