Improved Brain Uptake and Pharmacological Activity Profile of Morphine-6-Glucuronide Using a Peptide Vector-Mediated Strategy

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ABSTRACT

Morphine-6-glucuronide (M6G), an active metabolite of morphine, has been shown to have significantly attenuated brain penetration relative to that of morphine. Recently, we have demonstrated that conjugation of various drugs to peptide vectors significantly enhances their brain uptake. In this study, we have conjugated morphine-6-glucuronide to a peptide vector SynB3 to enhance its brain uptake and its analgesic potency after systemic administration. We show by in situ brain perfusion that vectorization of M6G (Syn1001) markedly enhances the brain uptake of M6G. This enhancement results in a significant improvement in the pharmacological activity of M6G in several models of nociception. Syn1001 was about 4 times more potent than free M6G (ED50 of 1.87 versus 8.74 μmol/kg). Syn1001 showed also a prolonged duration of action compared with free M6G (300 and 120 min, respectively). Furthermore, the conjugation of M6G results in a lowered respiratory depression, as measured in a rat model. Taken together, these data strongly support the utility of peptide-mediated strategies for improving the efficacy of drugs such as M6G for the treatment of pain.

The main metabolism pathway of morphine includes liver glucuronidation to morphine-6-glucuronide (M6G) and morphine-3-glucuronide. M6G is thought to contribute to the pharmacological effects of the parent drug (Abbott and Palmour, 1988; Paul et al., 1989; Frances et al., 1992), and various clinical trials have used M6G as the therapeutic drug in preference to morphine (Hanna et al., 1990; Thompson et al., 1995; Grace and Fee, 1996; Lötsh et al., 1997; Motamed et al., 2000; Penson et al., 2000). Antinociception studies in experimental animals have demonstrated that, although M6G and morphine are almost equally potent after systemic administration, the analgesic potency of M6G is more than 100-fold higher than morphine after intracerebroventricular injection, a route of administration that bypasses the blood-brain barrier (BBB) in vivo (Abbott and Palmour, 1988; Paul et al., 1989; Frances et al., 1992). These pharmacological data suggest that the brain penetration of M6G is significantly attenuated relative to that of morphine, probably due to the presence of the glucuronide moiety of M6G, conferring a higher hydrophilic character. Recently, a weak capacity and bidirectional transport by GLUT-1 and by a digoxin-sensitive transporter, which could be oatp2, was reported to be involved in the transport of M6G through the mouse BBB (Bourasset et al., 2003). However, several studies have shown that morphine has a better BBB permeability than M6G after i.v. injection (Bickel et al., 1996; Wu et al., 1997). Thus, enhancing the brain uptake of M6G would be expected to result in an improvement in its analgesic activity.

Brain delivery is still one of the major challenges for the pharmaceutical industry since many therapeutic drugs are unable to penetrate the BBB, a complex endothelial interface in vertebrates that separates the blood compartment from the extracellular fluid compartment of the brain parenchyma. The capillaries in the brain parenchyma possess a high electrical resistance due to tight junctions between the

ABBREVIATIONS: M6G, morphine-6-glucuronide; BBB, blood-brain barrier; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; DMF, dimethylformamide; DIEA, N,N-dimethylpropylylamine; U-69593, (+)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; % MPE, percent maximum possible effect; ANOVA, analysis of variance.
endothelial cells, and they also lack pores. Thus, the brain capillary endothelium behaves like a continuous lipid bilayer and diffusion through this BBB layer is largely dependent on the lipid solubility of the drug. Various strategies have been developed to enhance the brain uptake of therapeutic drugs, but most of these methods have been of limited use (Temsaman et al., 2001). Recently, we have shown that small peptide vectors can be used to enhance brain uptake of various drugs without opening the tight junctions (Rousselle et al., 2001, 2002). The potential of this approach as an effective brain delivery system has been demonstrated for various drugs (Rousselle et al., 2001, 2002; Blanc et al., 2004).

To assess this strategy as a brain delivery method for M6G, we have conjugated M6G to a 10-amino acid peptide SynB3 via a disulfide linker and measured its brain uptake and pharmacological effect in mice. We also measured its effect on respiratory depression in rats.

Materials and Methods

Animals

Adult OF1 mice (30–40 g, 6–8 weeks old) and OFA rats (200–220 g) were obtained from Ifa Credo (L’Arbresle, France). Eight-week-old male Swiss mice were obtained from Janvier (Le Genest-Saint-Isle, France). Animals were maintained under standard conditions of temperature and lighting and had free access to food and water. The research adhered to the ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (law no. 87-848).

Preparation and Characterization of Peptide Conjugates: Synthesis of Syn1001 (M6G-Cya-3MP-RRLSYSSRRRF)

Synthesis of the Peptide SynB3. The peptide SynB3 (H-RRLSYSRRRF-NH2; molecular mass, 1395 Da) was assembled on a carboxamide resin by conventional automated solid phase chemistry using 9-fluorenylmethoxycarbonyl/tertiobutyl-protection scheme. After trifluoroacetic acid (TFA) cleavage/deprotection, the crude peptide was purified on preparative C18 reverse-phase HPLC (Waters LC40; Waters, Milford, MA). Purity of the lyophilized products was assessed by C18 reverse-phase analytical HPLC, and the molecular mass was checked by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF; Elite-DE-RP; Applied Biosystems, Foster City, CA). MALDI-TOF spectra were recorded in linear mode, using the matrix 2-(4-hydroxyphenylazo)benzoic acid (Fluka, Buchs, Switzerland).

Addition of the Cya-3MP Linker. Cya-3MP-SynB3 was obtained in a two-step, one-pot reaction. One molar equivalent of SynB3, 6TFA, was dissolved in dry dimethylformamide (DMF; peptide synthesis grade), and mixed with 1 Eq of N-succinimidyl 3-(2-pyridylidithio)propionato (Fluka). Then, 4 to 6 Eq of N,N-diisopro- pylyetamine (DIEA) were added to start the reaction. The resulting product, S-pyridyl-3-mercaptopropionyl-SynB3, was monitored by HPLC and MALDI-TOF and was not isolated. Five equivalents of cysteamine hydrochloride (Fluka) dissolved in H2O/DMF 50% were then added, with enough DIEA to maintain alkaline conditions in the reaction mixture. The resulting Cya-3MP-SynB3 (1821 Da) was purified on preparative C18 reverse-phase HPLC and lyophilized.

Coupling of the Active Principle Morphine-6-Glucuronide. One molar equivalent of Cya-3MP-SynB3, 6TFA was dissolved in DMF. Equivalents (1.2 molar) of morphine-6-glucuronide dihydrate were resuspended in DMF using ultrasound. Four to six Eq of DIEA were added to the M6G suspension, followed by 1.5 Eq of benzotriazole-1-yl-oxyproplydiphenosmionium hexafluorophosphate (Novabiochem, Laufelfingen, Switzerland) dissolved in DMF. After 5 min, the peptide Cya-3MP-SynB3 dissolved in DMF was added to the reaction mixture and left a further 20 min for coupling of the benzotriazole-1-yl-oxypropyrolidinephosphonium hexafluorophosphate-activated M6G. Purification, lyophilization, and assessment of the conjugate Syn1001 (M6G-Cya-3MP-SynB3, 2002.33 Da) were performed as described above.

Radiolabeled Compounds. Preparations were performed as described above, except that 17-[14CH3]M6G (custom synthesis, 28.7 Ci/mmol; Biodynamics, Milton Keynes, UK) was kept limited by raising the stoichiometry of peptide, to 1.5 Eq in the coupling reactions. The resulting products were analyzed as described above, and the radiochemical purity was assessed by an HPLC fitted with a liquid scintillation counting detector (Flow One Packard, Rungis, France). After isotopic dilution with the unlabeled conjugate, the specific activity of the compound was 14.3 μCi/μg.

Receptor Binding Assay

Radioreceptor assays were carried out in which competition between labeled opioid ligands and the cold test compound was measured using an opioid receptor-containing membrane preparation as described previously (Cotton et al., 1985; Kinouchi and Pasternak, 1991; Yoburn et al., 1991). The concentration of the test compounds ranged from 10−12 to 10−5 M. For opiate μ-receptor, membrane homogenates of rat cerebral cortex (300 μg of protein) were incubated for 60 min at 22°C with 1 nM [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl, pH 7.7. For delta receptor, membrane homogenates of guinea pig cerebral cortex (300 μg of protein) were incubated for 120 min at 22°C with 1.5 nM [d-Pen2,d-Pen5]-enkephalin in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, and 30 nM [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin. For κ-receptor, membrane homogenates of guinea pig cerebellum (250 μg of protein) were incubated for 80 min at 22°C with 0.7 nM [3H]U-69593 (or in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 1 mM EDTA. Non-specific binding was determined in the presence of naloxone (1 μM for μ-receptor and 10 μM for κ-receptor) and naltrexone (10 μM) for the delta receptor.

Following incubation, the samples were filtered rapidly under vacuum through glass-fiber filters (GF/B; Packard, Rungis, France) presoaked with 0.3% polyethyleneimine and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters were dried then counted for radioactivity in a scintillation counter (Topcount; Packard) using a scintillation cocktail (Microscint O; Packard). IC50 values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by nonlinear regression analysis of the competitive curves. These parameters were obtained by Hill equation curve fitting. The inhibition constants (Ki) were calculated from the Cheng Prusoff equation (Ki = IC50/1 + L/Kd), where L is the concentration of radioligand in the assay and Kd is the affinity of the radioligand for the receptor. Ki values were determined using GraphPad Prism (GraphPad Software Inc., San Diego, CA). The data were fitted by one-site binding model.

In Situ Mouse Brain Perfusion Study: Surgical Procedure

The uptake of [14C]M6G and [14C]Syn1001 (vectorized M6G) to the luminal side of 6-week-old OF1 mouse brain capillaries was measured using the in situ brain perfusion method previously adapted in our laboratory for the study of drug uptake in the mouse brain (Rousselle et al., 2001). Briefly, mice were anesthetized with ketamine/xylazine (140/8 mg/kg i.p.), and the right common carotid was exposed and ligated at the heart side. The common carotid artery was catheterized rostrally with polyethylene tubing (0.30-mm i.d. × 0.70-mm o.d.; Biotrol Diagnostic, Chemin enrverrres-les-Louvres, France) filled with heparin (25 U/ml) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid was placed in an

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infusion pump (Harvard pump PHD 2000; Harvard Apparatus Inc., Holliston, MA) and connected to the catheter. Brains of anesthetized mice were perfused for 60 s at a flow rate of 2.5 ml/min. At the end of the perfusion time, the mouse was decapitated, and the brain was removed. Brain and perfusion samples were then digested for 2 h in 1 ml of Solvable (Packard) at 50°C and mixed with 9 ml of Ultima Gold XR scintillation cocktail (Packard). Total $^{14}$C and $^3$H were determined simultaneously in a Packard Tri-Carb model 1900 TR Liquid Scintillation.

The perfusate consisted of a Krebs-bicarbonate buffer: 128 mM NaCl, 24 mM NaHCO$_3$, 4.2 mM KCl, 2.4 mM Na$_2$HPO$_4$, 1.5 mM CaCl$_2$, 0.22 mM MgSO$_4$, and 9 mM D-glucose added before infusion. The solution was gassed with 95% O$_2$ and 5% CO$_2$ for pH control (7.4) and warmed at 37°C in a water bath. Tracers were added to perfusate at concentrations of 0.3 μCi/ml for free M6G, 0.1 μCi/ml for Syn1001, and 0.3 μCi/ml for $[^3]$H-sucrose, the latter being a vascular marker with very small BBB penetration.

Drug uptake was expressed as a single time point unidirectional transfer constant ($K_v$). Briefly, calculations were accomplished as described previously (Smith, 1996), from the following relationship: $K_v = (Q_t - V) / Cpf (T - Cpf)$, where $Q_t$ is the measured quantity of $^{14}$C free of vectorized drug in brain ($^{14}$C tracer per gram of right brain hemisphere) at the end of the experiment, $V$ is the cerebral vascular volume (microliters per gram), Cpf is the perfusion fluid concentration of $^{14}$C free of vectorized drug (disintegrations per minute per microliter), and $T$ is the perfusion time in seconds. $V$ was evaluated by the scruce volume and calculated by the ratio between radioactivity of $[^3]$H-sucrose (expressed in disintegrations per minute per gram of brain) and the perfusate sucrose concentration.

**Measurement of the Antinociceptive Effect**

**Tail Flick.** Responsiveness to radiant heat was determined using a modification of the procedure of Ling and Pasternak (1983). Naive mice (OF1; 6–8 weeks old) were restrained in a paper handkerchief with the hand. A constant heat intensity (hot lamp) was applied to the ventral of the mouse tail, and when the animal flicked its tail in response to the noxious thermal stimulus, both the heat source and the ventral of the mice tail, and when the animal flicked its tail in response to the noxious thermal stimulus, both the heat source and the timer stopped automatically. The stimulus intensity was adjusted so that the baseline tail flick latencies ranged between 2 and 3 s. Mice not responding after 10 s were removed from the apparatus and assigned a latency of 10 s to minimize tissue damage to the animal’s tail. Baseline latencies were determined just before drug administration and again at the indicated times. The compounds were administered by s.c. route in saline solution (volume of injection, 5 ml/kg). In a first series of experiments, four groups ($n = 8$) of mice received Syn1001 at the doses of 1.06, 2.1, 3.2, and 4.24 μmol/kg, and the antinociceptive activity was measured at 30, 60, 180, and 300 min after compound administration. In a second series of experiments, two groups ($n = 10$) of mice received M6G or Syn1001 at the dose of 3.2 μmol/kg, and antinociceptive activity was measured 30, 60, 120, and 240 min after compound administration.

Antagonism of Syn1001 antinociception by opiate antagonists was determined in a first experiment by preadministration of naltrexone (1 mg/kg, s.c.) and 3-methoxynaltrexone (0.2 mg/kg, s.c.) 15 min before administration of Syn1001 at the dose of 2.8 μmol/kg (10 mice per group) and in a second experiment by administration of nor-binaltorphimine (6 mg/kg, s.c.) and β-funaltrexamine (10 mg/kg, s.c.), respectively, 3 and 23 h before administration of Syn1001 at the dose of 4.2 μmol/kg (10 mice per group) (Paul et al., 1991; Pick et al., 1991).

At each time point, two measurements (in a different place of the tail) were performed, and the mean was calculated.

To correct for individual differences in baseline latencies, the antinociceptive data (latencies) were converted to percent maximum possible effect (% MPE) using the following formula (Brady and Holtzman, 1982):

$$\% \text{MPE} = \left( \frac{\text{postdrug latency} - \text{predrug latency}}{\text{maximum latency} - \text{predrug latency}} \right) \times 100$$

The ED$_{50}$ was calculated with nonlinear regression equation using Sigma Plot v.2.0. Syn1001 was compared with M6G using an unpaired Student’s t test, and the significance was set at 0.05. The antagonist of Syn1001 by opiate antagonists was analyzed using the analysis of variance (ANOVA) followed by the Dunnnett’s test and significance set at 0.01.

**Hot Plate.** In the hot-plate assay, naive 6- to 8-week-old OF1 mice ($n = 15$) were placed on a 54°C surface (Harvard Apparatus Inc.), and the time to lick one of the paws or escape jump was recorded as the response latency. Predosing latency was determined before administration of the compounds and was 4.6 ± 1.6 s. The compounds (Syn1001 and M6G, 2.2 and 2.6 μmol/kg/mouse) were administered i.v. (into the tail vain, volume of injection: 2 ml/kg). The hot plate latency was determined at various times (5, 10, 15, 30, 45, 90, 120, and 180 min) after compound administration. A maximal cut-off time of the heat was 30 s to prevent tissue damage. To correct for individual differences in baseline latencies, the antinociceptive data (latencies) were converted to % MPE as described above. Syn1001 was compared with M6G using an unpaired Student’s t test, and the significance was set at 0.05.

**Formalin Test.** Naive 8-week-old male Swiss mice ($n = 10$ per group) were injected s.c. (5 ml/kg) with either the vehicle (saline solution) or test compounds (2.1, 5.46, and 10.9 μmol/kg) 45 min before receiving a 10-μl intraplantar injection of 2% formalin solution (Sigma-Aldrich) into the right hind paw. The amount of time that the mice licked the injected paw was monitored. Both the acute and chronic phases were examined. The incidence of licking was measured during the first 7 min (acute phase) and in 2-min periods at 5-min intervals for 60 min (chronic phase). The observations were carried out for a period of 1 h after the formalin injection.

Results are expressed as the mean ± S.E.M. A global analysis of the data was performed using one-factor or repeated-measures ANOVA. A Dunnett’s test was used when the ANOVA indicated a significant difference. The level of significance was set at $p < 0.05$.

**Respiratory Depression.** The respiratory depression was measured in naive OFA rats (200–220 g) as described by Ling et al. (1989). Animals were anesthetized with Valium/ketamine (8/50 mg/kg i.p.). A vinyl cannulae was inserted in the femoral artery 24 h before drug administration to obtain arterial blood samples and tunneled s.c. to the back of the neck where it was exteriorized and kept patent with heparinized saline (50 U/ml). The compounds were given by s.c. route (5 ml/kg) (43 μmol/kg for Syn1001 and M6G and 65 μmol/kg for morphine). During the respiratory depression studies, all animals remained unrestrained, were housed individually, and were not handled. Prior to and at different times after compound administration, arterial blood (0.2 ml) was harvested for analysis. Blood pO$_2$, pCO$_2$, and pH were measured using a Blood Gas Analyzer.

**Results**

**Receptor Binding Assay.** The affinity of M6G and vectorized M6G (Syn1001) to the main opioid receptors was investigated in radioligand competition binding assays. The data show that Syn1001 binds to $\mu$-receptors with a higher affinity than free M6G (0.1 versus 3.8 nM). The $\delta$ receptor binding was similar for both Syn1001 and M6G (19 and 23 nM, respectively). Surprisingly, vectorization of M6G exhibited a high affinity for the $\kappa$-receptor not shown by free M6G (1.1 nM for Syn1001 and 1860 nM for M6G). This increase in affinity to the $\kappa$-receptor was not related to the free peptide since we did not observe any $\kappa$-affinity for free SynB3 ($K_i > 10 \mu M$).
BBB Permeability. We measured the brain uptake of free M6G and Syn1001 using the in situ brain perfusion technique in mice. To assess the integrity of the BBB, [3H]sucrose was used as a marker of brain vascular volume since it does not measurably penetrate the BBB during brief periods of perfusion (e.g., 60–120 s) (Rousselle et al., 2001). When M6G or Syn1001 were perfused, the distribution volume of [3H]sucrose into the right cerebral hemisphere was less than 20 μl/g, indicating that the permeability of the BBB had not been altered. BBB permeabilities of M6G and Syn1001 were then assessed after 60-s perfusion. The brain uptake of free M6G was very low after 60 s of perfusion (K_in = 0.024 ± 0.02 μl/g/s). In contrast, conjugation of M6G to the SynB3 vector significantly enhanced its brain uptake, giving a K_in of 1.27 ± 0.5 μl/g/s.

Antinociceptive Activity. First, we measured the effect of Syn1001, administered s.c., in mice using the tail flick assay at different doses ranging from 1 to 4.24 μmol/kg. Figure 1, A and B, shows that the effect of Syn1001 is dose-dependent and lasts for about 300 min. The calculated ED_{50} from this experiment was 3.6 mg/kg (1.87 μmol/kg) (Fig. 1B). During the course of our studies, the ED_{50} varied from 1.87 to 3.2 μmol/kg. A similar experiment with free M6G (Fig. 1C) showed that the ED_{50} of this compound is 4 mg/kg (8.74 μmol/kg). We then compared the effect of free M6G and Syn1001 by s.c. route at an equimolar dosing of 3.2 μmol/kg (1.5 mg/kg M6G and 6 mg/kg Syn1001). Figure 2 shows that Syn1001 is more potent, on a molar basis, than free M6G by the s.c. route in the tail flick assay. Interestingly, time course studies with Syn1001 revealed a longer duration of action compared with M6G. The effect of Syn1001 lasted about 300 min, whereas the effect of M6G was for 120 min. We also measured the antinociceptive effect of Syn1001 in the hot plate model in comparison with free M6G and morphine at equimolar dosing (2.2 μmol/kg for M6G and 2.6 μmol/kg for morphine) by the i.v. route in mice. Syn1001 displayed a significant analgesic effect compared with morphine or M6G (Fig. 3).

Antagonism of Syn1001 Antinociception by Opiate Antagonists. Naloxone, a nonselective μ-antagonist, administered s.c. immediately before Syn1001 reversed significantly its analgesic effect (Fig. 4A). Interestingly, the analgesic activity of Syn1001 was also reversed by the antagonist 3-methoxynaltrexone (Fig. 4A). Since Syn1001 displays a higher κ-affinity in vitro compared with free M6G, the contribution of κ-activity to the antinociception was assessed using the κ-selective antagonist nor-binaltorphimine. The μ-antagonist, β-funaltrexamine, was used as a control. As expected, antinociception produced by Syn1001 was inhibited by the μ-antagonist, but no inhibition was observed in the presence of the κ-antagonist (Fig. 4B). This suggests that the analgesic effect observed with Syn1001 is mediated by the μ-opioid receptor.

Formalin Assay. To see whether the enhanced analgesic activity of Syn1001 over either M6G or morphine was also observed in a different nociceptive pain model, we assessed their relative activities in the formalin mouse pain model. Mice were s.c. administered with Syn1001, morphine, or M6G. Morphine and M6G were both administered at 1, 2.5, and 5 mg/kg, whereas Syn1001 was administered at 4, 10, and 20 mg/kg to have an equivalent molar concentration of M6G (2.18, 5.46, and 10.9 μmol/kg, respectively). At all doses studied, administration of Syn1001 decreased the licking time both in the acute (1–7-min) and in the chronic (7–60-min) phases, compared with M6G- or morphine-treated animals. This decrease in licking time was dose-dependent. At 2.18 μmol/kg, the total paw licking time for Syn1001 was about 50% in the chronic phase, and at 10.9 μmol/kg, it was reduced almost to 0%. Figure 5 shows a comparison of the three compounds at equimolar dosing of 10.9 μmol/kg. In the acute phase, although animals treated with Syn1001 displayed less difference in paw licking time compared with other groups, this difference was not statistically significant. However, a significant difference was observed in the chronic phase. The total paw licking time in this phase was about 25, 30, and 0% for morphine, M6G, and Syn1001, respectively.
Respiratory Depression. We compared the effects of M6G and Syn1001 on respiratory depression in a rat model. Rats were administered with the compounds by the s.c. route at an equimolar dosing of 3.2 μmol/kg (n = 10 per group) for M6G and Syn1001. Morphine was also administered as a control at a dose of 25 mg/kg (65 μmol/kg). The side effects observed in animals were usually scabbing at the injection site, subdued behavior, and Straub tail. Respiratory depression in the rat injected with free M6G and morphine is typically reflected in an initial increase in pCO2. The levels of pCO2 increased from 38 mm Hg before administration to 62 mm Hg at 1 h postadministration for both.
M6G and morphine (Fig. 6A). This increase in pCO₂ was accompanied by a decrease in pO₂ levels (Fig. 6B). Interestingly, no significant increase in pCO₂ levels was observed with vectorized M6G (Fig. 6A). Measurement of blood pH showed a decrease in pH levels after M6G and morphine administration, whereas no significant effect was obtained with Syn1001 administration (Fig. 6C). These preliminary data point out to a significant decrease in respiratory depression after vectorization of M6G.

**Discussion**

Our results confirm our previous reports that vectorization of drugs with SynB vectors results in an enhancement of brain uptake and pharmacological activity (Rousselle et al., 2001, 2002, 2003; Blanc et al., 2004). In the present study, our rationale was to attach M6G to the SynB3 peptide vector to enhance its brain uptake. M6G was chosen because it has been reported to be more potent than morphine after central administration (Abbott and Palmour, 1988; Paul et al., 1989; Frances et al., 1992). Because the affinity of both substances for the µ-receptor has been reported to be similar, a possible explanation for this observation could involve differences in the permeability of the blood-brain barrier to M6G. In fact, several reports have indicated a significant lower BBB permeability to M6G by systemic administration, in comparison with morphine (Bickel et al., 1996; Wu et al., 1997). Therefore, a peptide vector that would enhance the BBB permeability of M6G would most likely enhance its pharmacological activity.

Our study shows that SynB3 enhances significantly the brain uptake of M6G as measured by the in situ brain perfusion in mice. The mechanism whereby vectorized M6G crosses the BBB is not yet clear but could involve adsorptive-mediated endocytosis, a mechanism previously proposed for doxorubicin vectorized with SynB3 (Rousselle et al., 2001). The SynB3 vector used in this study is positively charged (five arginines), and this net positive charge is likely to play a major role in electrostatic interactions between the peptide vector and the negative surface charges of the endothelial cells composing the BBB.

Vectorization of M6G with the SynB3 vector resulted in a significant enhancement in the analgesic effect of M6G. We show by the tail flick assay in mice that the effect of Syn1001 is dose-dependent. We then compared the antinociceptive activity of M6G, morphine and Syn1001 in various tests using the tail flick and hot plate tests. This comparison was useful because the rank order of potency of opioids may vary with the nature and/or the intensity of the nociceptive stimulus (Porreca et al., 1987; Millan, 1990; South and Smith, 1998). In fact, South and Smith (1998) have shown that systemic administration of M6G resulted in high levels of antinociception using the tail flick, whereas no significant antinociception was detected using the hot plate test. In our study, Syn1001 was more potent than free M6G or morphine in the different tests used. The ratio of the antinociceptive ED₅₀ of M6G over Syn1001 was approximately 4 on a molar basis. This indicates that vectorization leads not only to improvement of brain uptake but also to an enhancement in the antinociceptive activity of M6G. This enhancement was due to the vectorization of M6G since free peptide (SynB3) had no antinociceptive effect and no affinity for the opioid receptors. The enhanced analgesic activity of Syn1001 over M6G can also be obtained in another animal nociceptive pain model: the formalin pain model. At all doses studied, administration of Syn1001 decreased the licking time both in the acute and in the chronic phases. A significant difference was observed in the chronic phase compared with M6G and morphine. The almost total abolition of a chronic phase response with Syn1001 supports the conclusion that vectorization of M6G leads to a significant enhancement of its antinociceptive effect.

The mechanism of action of Syn1001 was further explored by receptor binding studies both in vitro and in vivo. Using a radioligand-binding assay, Syn1001 was demonstrated to bind with higher affinity than M6G to the µ-receptor in vitro. Surprisingly, it was noted that in the presence of the SynB3 vector, M6G exhibited a high affinity for the κ-receptor that it is not shown by free M6G or SynB3 vector. In vivo, the analgesic effect of Syn1001 was reversed easily by naloxone and β-funaltrexamine, confirming the opioid nature of the antinociception. Since no inhibition was obtained with the κ-antagonist nor-binaltorphimine, it was clear that the µ-receptor has been reported to be similar, a possible explanation for this observation could involve differences in the permeability of the blood-brain barrier to M6G.

**Fig. 5.** Formalin test. Comparison of paw licking time in acute and chronic phase after s.c. administration of either the vehicle or the test compounds at 10.9 μmol/kg to mice (n = 15 per group). Values are means ± S.E.M. *, P < 0.05; ****, P < 0.001.
opioid receptor was mediating the analgesic effect of Syn1001. Interestingly, the analgesic effect was also antagonized by 3-methoxynaltrexone. This antagonist has been described to antagonize the action of M6G and heroin at a dose that is inactive against morphine (Brown et al., 1997). These observations led to the hypothesis of the presence of a novel receptor, which is responsible for M6G and heroin analgesia (Brown et al., 1997). Since this antagonist also acts on the action of Syn1001, this points toward the action of Syn1001 being mediated by the same receptors as M6G and heroin.

The fact that vectorized M6G binds to the µ-receptor in vitro indicates that free M6G does not need to be cleaved from the vector to have a pharmacological effect. M6G was conjugated to the SynB3 vector via a linker containing a disulfide bond. The disulfide-based linker system has been shown to be stable in plasma for several hours though labile in brain (Letvin et al., 1986). It is not clear yet in which form Syn1001 binds to its opioid receptors in vivo. Further studies are needed to assess the mechanism and rate of cleavage of vectorized M6G within the brain.

As demonstrated in this study and by others, M6G has been shown to have a slightly longer antinociceptive effect compared with morphine (150 and 90 min, respectively) (Paul et al., 1989; Frances et al., 1990). This longer action is probably the result of a slower rate of elimination of M6G from the brain and its entrapment in the extracellular fluid (Frances et al., 1992; Van Crugten et al., 1997; Stain-Texier et al., 1999). The fact that Syn1001 induces a longer duration of action could be due to the enhancement of the bioavailability of the vectorized M6G in the extracellular fluid, thereby increasing its availability to bind to the µ-receptors. Further investigations are needed to measure the concentrations of Syn1001 and M6G within the brain.

Respiratory depression is one of the most disturbing side effects associated with opioid drugs. Case reports have implicated M6G in respiratory depression (Osborne et al., 1986) and have reported on the respiratory depressant properties of M6G after cerebroventricular administration to dogs (Pelligrino et al., 1989) and rats (Gong et al., 1991). In one study in humans, M6G was shown to produce fewer respiratory effects than morphine (Peat et al., 1991; Thompson et al., 1995). However, this study was not compatible with the other observations where respiratory depression was observed after intrathecal administration of M6G in human subjects (Grace and Fee, 1996). In the present study, rats that received a high dose of M6G exhibited a significant increase in the pCO₂. Respiratory depression occurred at 30 to 60 min postadministration. On the other hand, no significant effect on respiratory depression was seen after Syn1001 administration. The difference in the respiratory effect between M6G and Syn1001 is not clear yet, but a likely explanation may be the combined affinity of Syn1001 at the µ- and κ-receptors. Several lines of evidence support this hypothesis. First, the respiratory depression effect has been shown to be associated with µ-receptors and that the predictability of the degree of respiratory depression of an opioid seems to decrease with its selectivity for µ-opioid receptors (Stott and Pleuvry, 1991). Secondly, activation of µ- and κ-opioid receptors leads to functionally opposite effects. Verborgh et al. (1997) have shown that combination of µ- and κ-receptor agonists can be additive with respect to antinociception with additionally

![Fig. 6. Effect of Syn1001, M6G, and morphine on arterial blood gases (A, pCO₂; B, pO₂) and pH (C). Femoral arterial lines were placed, and arterial blood gases were taken 30, 60, and 90 min after s.c. administration of the compounds to rats at a dose of 43 μmol/kg for M6G and Syn1001 and 65 μmol/kg for morphine.](resources/fig6.png)
less risk for respiratory side effects. It will be interesting to see whether the κ-binding seen with Syn1001 is agonist or antagonist and to measure the effect of respiratory depression in the presence of κ-antagonists.

In conclusion, our results show that vectorization of M6G enhances its brain delivery. This enhancement in brain uptake results in a significant improvement in the analgesic activity of M6G and reduces respiratory depression. This study supports the usefulness of peptide-mediated strategies for improving the efficacy and safety of M6G for the treatment of pain.

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References


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