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

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a) Running Title
Improved brain uptake of M6G

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c)
Number of text pages: 32
Number of tables: 0
Number of figures: 6
Number of references: 40
Number of words:

  Abstract: 178
  Introduction: 474
  Discussion: 1327

d) Abbreviations
DIEA: Diisopropylethylamine; M6G: morphine-6-glucuronide; MPE: maximum possible effect; PyBop: benzotriazol-1-yl-oxypyrrolidinephosphonium hexafluorophosphate; SPDP: N-succinimidyl 3-(2-pyridyldithio)propionate.
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 in order to enhance its brain uptake and its analgesic potency after systemic administration. We show by in situ brain perfusion that vectorisation 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 μmol/kg versus 8.74 μmol/kg). Syn1001 showed also a prolonged duration of action compared to free M6G (300 min 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.
INTRODUCTION

The main metabolism pathway of morphine includes liver glucuronidation to morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G). 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; Lötsch 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 bi-directional 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 intravenous 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 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 (Temsamani 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; 2003; Blanc et al., 2004).

In order 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.
METHODS

Animals

Adult OF1 mice (30-40g, 6-8 weeks old) and OFA rats (200-220g) were obtained from Iffa-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 N° 87-848).

Preparation and Characterization of Peptide Conjugates

Synthesis of Syn1001 (M6G-Cya-3MP-RRLSYSRRRF)

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 a 9-fluorenylmethoxycarbonyl/tertiobutyl-protection scheme. After trifluoroacetic acid (TFA) cleavage/deprotection, the crude peptide was purified on preparative C18 reverse phase HPLC (Waters LC40). Purity of the lyophilized products was assessed by C18 reverse phase analytical HPLC, and the molecular weight was checked by Matrix-Assisted Laser Desorption-Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF, Elite-DE-RP Perseptive Biosystems). MALDI TOF spectra were recorded in linear mode, using the matrix 2’-(4-Hydroxyphenylazo) benzoïc acid (HABA) (Fluka, Buchs, Switzerland).

Addition of the CyA-3MP linker: CyA-3MP-SynB3 was obtained in a two-steps one-pot reaction: One molar equivalent of SynB3, 6TFA was dissolved in dry DMF
(dimethylformamide, peptide synthesis grade), and mixed with 1 equivalent of SPDP (N-
succinimidyl 3-(2-pyridyldithio)propionate) (Fluka; Buchs, Switzerland). Then 4-6
equivalents of DIEA (N,N-diisopropylethylamine) 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;
Buchs, Switzerland) 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. 1.2 molar equivalents of morphine-6-glucuronide
di-hydrate were resuspended in DMF using ultrasound. Four to six-equivalents of DIEA were
added to the M6G suspension, followed by 1.5 equivalent of benzotriazol-1-yl-
oxopyrrolidinophosphonium hexafluorophosphate (PyBop) (Novabiochem; Läufelfingen,
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 PyBOP
activated M6G. Purification, lyophilisation and assessment of the conjugate Syn1001 (M6G-
CyA-3MP-SynB3, 2002.33 Da) were performed as described above.

**Radiolabelled compounds:** Preparations were performed as described above, except that 17-
[14CH3]M6G (Biodynamics, U.K. custom synthesis, 28.7 Ci/mol) was kept limiting 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 unlabelled conjugate, the specific activity of the compound was 14.3 µCi/mg.

**Receptor binding assay**

Radio-receptor 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 *mu* receptor, membrane homogenates of rat cerebral cortex (200 µg of protein) were incubated for 60 min at 22°C with 1 nM [³H]DAMGO 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 [³H]DPDPE 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 DAMGO. For *kappa* 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 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. Nonspecific binding was determined in the presence of naloxone (1 µM for mu-receptor and 10 µM for kappa 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) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard, Rungis, France). 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 non-linear regression analysis of the competitive curves. These parameters were obtained by Hill equation curve fitting. The inhibition constants ($K_i$) were calculated from the Cheng Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + L/K_D} \quad (1)$$

where $L=$ concentration of radioligand in the assay, and $K_D =$ affinity of the radioligand for the receptor). $K_i$ values were determined using GraphPad Prism, (San Diego, CA). The data were fitted by 1-site binding model.

**In situ mouse brain perfusion study**

**Surgical procedure:** The uptake of $[^{14}\text{C}]$M6G and $[^{14}\text{C}]$Syn1001 (vectorised M6G) to the luminal side of six-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. x 0.70 mm o.d., Biotrol Diagnostic, Chennevrières-les-Louvres, France) filled with heparin (25 U/mL) and mounted on a 26G needle. The syringe containing the perfusion fluid was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus, Holliston, MA) and connected to the catheter. Brains of anesthetized mice were perfused for 60 sec at a flow rate of 2.5 mL/min. At the end of the perfusion time, the mouse was decapitated and the brain removed. Brain and perfusion samples were then digested for 2 hours in 1 ml of Solvable (Packard, Rungis, France) at 50°C and mixed with 9 ml of Ultima Gold XR scintillation cocktail (Packard, Rungis, France). Total $[^{14}\text{C}]$ and $[^{3}\text{H}]$ were determined simultaneously in a Packard Tri-Carb Model 1900 TR Liquid Scintillation
The perfusate consisted of a Krebs-bicarbonate buffer, in mM: 128 NaCl, 24 NaHCO₃, 4.2 KCl, 2.4 NaH₂PO₄, 1.5 CaCl₂, 0.22 MgSO₄ and 9 D-glucose added before infusion. The solution was gassed with 95% O₂ and 5% CO₂ 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 [³H]sucrose, the latter being a vascular marker with poor BBB penetration.

Drug uptake was expressed as a single time point unidirectional transfer constant (Kin). Briefly, calculations were accomplished as described previously (Smith, 1996), from the following relationship:

\[
\text{Kin} = \frac{(Q_{\text{tot}} - V_v . C_{\text{pf}})}{(T . C_{\text{pf}})}, \quad (2)
\]

where \( Q_{\text{tot}} \) is the measured quantity of \([^{14}\text{C}]\) free or vectorised drug in brain (\([^{14}\text{C}]\)tracer per gram of right brain hemisphere) at the end of the experiment, \( V_v \) is the cerebral vascular volume (microliters per gram), \( C_{\text{pf}} \) is the perfusion fluid concentration of \([^{14}\text{C}]\) free of vectorised drug (disintegrations per minute per microliter), and \( T \) is the perfusion time in seconds. \( V_v \) was evaluated by the sucrose space and calculated by the ratio between radioactivity of \([^{3}\text{H}]\)sucrose (expressed in dpm of sucrose 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). Naïve 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 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...
base-line tail-flick latencies ranged between 2 to 3 sec. Mice not responding after 10 sec were removed from the apparatus and assigned a latency of 10 sec in order to minimize tissue damage to the animal’s tail. Base-line latencies were determined just before drug administration and again at the indicated times. The compounds were administered by subcutaneous route in saline solution (volume of injection: 5 ml/kg). In a first series of experiments, 4 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, 2 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 pre-administration of naloxone (1 mg/kg; sc) and 3-methoxynaltrexone (0.2 mg/kg; sc) 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; sc) and beta-funaltrexamine (10 mg/kg; sc) respectively 3 hours and 23 hours before administration of Syn1001 at the dose of 4.2 µmol/kg (10 mice per group) (Pick et al, 1991; Paul 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 base-line latencies, the antinociceptive data (latencies) were converted to percentage maximum possible effect (% MPE) using the following formula (Brady and Holtzman, 1982):
The ED50 was calculated with non-linear regression equation using Sigma Plot® v.2.0.

Syn1001 was compared to M6G using an unpaired t-test and the significance was set at 0.05.

The antagonism of Syn1001 by opiate antagonists was analysed using the analysis of variance ANOVA followed by the Dunnett’s test and significance set at 0.01.

**Hot plate:** In the hot-plate assay, naïve 6-8 weeks old OF1 mice (n=15) were placed on a 54°C surface (Harvard Apparatus, Holliston, MA) and the time to lick one of the paws or escape jump was recorded as the response latency. Pre-dosing latency was determined before administration of the compounds and was 4.6 ± 1.6 sec. The compounds (Syn1001 and M6G: 2.2 µmol/kg and morphine 2.6 µmol/kg) were administered intravenously (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 sec to prevent tissue damage. To correct for individual differences in baseline latencies, the antinociceptive data (latencies) were converted to percentage maximum possible effect (% MPE) as described above.

\[
\text{% MPE} = \frac{(\text{Postdrug latency}) - (\text{Predrug latency})}{(\text{Maximum latency}) - (\text{Predrug latency})} \times 100
\]

(3)

Syn1001 was compared to M6G using an unpaired t-test and the significance was set at 0.05.
Formalin test: Naïve eight-week-old male Swiss mice (n=10 per group) were injected subcutaneously (5 ml/kg) with either the vehicle (saline solution) or test compounds (2.18, 5.46 and 10.9 µmol/kg) 45 min before receiving a 10 µl intraplantar injection of 2% formalin solution (Sigma, France) 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 one hour after the formalin injection.

Results are expressed as the mean ± the standard error of the mean (s.e.m). A global analysis of the data was performed using one factor or repeated measures analysis of variance (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 naïve OFA rats (200-220 g) as described by Ling et al. (1989). Animals were anaesthetized with valium/ketamine (8/50 mg/kg; ip). A vinyl cannulae was inserted in the femoral artery 24 hours before drug administration to obtain arterial blood samples and tunneled subcutaneously to the back of the neck where it was exteriorized and kept patent with heparinized saline (50 U/ml). The compounds were given by subcutaneous 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 and at different time after compound administration, arterial blood (0.2 ml) was harvested for analysis. Blood pO₂, pCO₂ and pH were measured using a Blood Gas Analyzer.
RESULTS

Receptor binding assay

The affinity of M6G and vectorised 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 nM and 23 nM, respectively). Surprisingly, vectorisation 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 (Ki >10 µM).

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 sec) (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 has not been altered. BBB permeabilities of M6G and Syn1001 were then assessed after 60 sec perfusion. The brain uptake of free M6G was very low after 60 sec of perfusion (Kin = 0.024 +/- 0.02 µl/g/sec). In contrast, conjugation of M6G to the SynB3 vector significantly enhanced its brain uptake, giving a Kin of 1.27 +/- 0.5 µl/g/sec.
Antinociceptive activity

First, we measured the effect of Syn1001, administered subcutaneously, in mice using the tail flick assay at different doses ranging from 1 to 4.24 µmol/kg. Figures 1 A and B show that the effect of Syn1001 is dose-dependent and lasts for about 300 min. The calculated ED50 from this experiment was 3.6 mg/kg (1.87 µmol/kg) (Figure 1B). During the course of our studies, the ED50 varied from 1.87 to 3.2 µmol/kg. A similar experiment with free M6G (Figure 1C) showed that the ED50 of this compound is 4 mg/kg (8.74 µmol/kg). We then compared the effect of free M6G and Syn1001 by subcutaneous route at an equimolar dosing of 3.2 µmole/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 subcutaneous route in the tail-flick assay. Interestingly, time-course studies with Syn1001 revealed a longer duration of action compared to M6G. The effect of Syn1001 lasted about 300 min while 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 intravenous route in mice. Syn1001 displayed a significant analgesic effect compared to morphine or M6G (Figure 3).

Antagonism of Syn1001 antinociception by opiate antagonists.

Naloxone, a non-selective mu antagonist, administered subcutaneously immediately before Syn1001 reversed significantly its analgesic effect (Figure 4A). Interestingly, the analgesic activity of Syn1001 was also reversed by the antagonist 3-methoxynaltrexone (3-MTNX) (Figure 4A). Since Syn1001 displays a higher kappa affinity in vitro compared to free M6G, the contribution of kappa activity to the antinociception was assessed using the kappa
selective antagonist nor-binaltorphimine (Nor-BNI). The \textit{mu} antagonist, beta-funaltrexamine (\(\beta\)-FNA), was used as a control. As expected, antinociception produced by Syn1001 was inhibited by the \textit{mu} antagonist but no inhibition was observed in the presence of the \textit{kappa} antagonist (Figure 4B). This suggests that the analgesic effect observed with Syn1001 is mediated by the \textit{mu} 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 subcutaneously administered with Syn1001, morphine, or M6G. Morphine and M6G were both administered at 1, 2.5 and 5 mg/kg while Syn1001 was administered at 4, 10 and 20 mg/kg in order to have an equivalent molar concentration of M6G (2.18, 5.46 and 10.9 \(\mu mol/kg\), 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 to M6G or morphine-treated animals. This decrease in licking time was dose-dependent. At 2.18 \(\mu mol/kg\), the total paw licking time for Syn1001 was about 50\% in the chronic phase and at 10.9 \(\mu mol/kg\), it was reduced almost to 0\%. Figure 5 shows a comparison of the three compounds at equimolar dosing of 10.9 \(\mu mol/kg\). In the acute phase, although animals treated with Syn1001 displayed less difference in paw-licking time compared to 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 subcutaneous route at an equimolar dosing of 43 µmol/kg for M6G and Syn1001 (20 mg/kg M6G and 80 mg/kg 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 mmHg before administration to 62 mmHg at one hour post-administration, for both M6G and morphine (Figure 6A). This increase in pCO2 was accompanied by a decrease in pO2 levels (Figure 6B). Interestingly, no significant increase in pCO2 levels was observed with vectorised M6G (Figure 6A). Measurement of blood pH showed a decrease in pH levels after M6G and morphine administration while no significant effect was obtained with Syn1001 administration (Figure 6C). These preliminary data point out to a significant decrease in respiratory depression after vectorisation of M6G.
DISCUSSION

Our results confirm our previous reports that vectorisation 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 in order 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). As the affinity of both substances for the mu 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 to 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 vectorised M6G crosses the BBB is not yet clear but could involve adsorptive-mediated endocytosis, a mechanism previously proposed for doxorubicin vectorised 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.

Vectorisation 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 noxious 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 vectorisation 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 vectorisation 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 to M6G and morphine. The almost total abolition of a chronic phase response with Syn1001 supports the conclusion that vectorisation 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 mu receptor in vitro. Surprisingly, it was noted that in the presence of the SynB3 vector, M6G exhibited a high affinity for the kappa 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 kappa antagonist Nor-BNI, it was
clear that the \textit{mu} 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, which 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 towards the action of Syn1001 being mediated by the same receptors as M6G and heroin.

The fact that vectorised M6G binds to the \textit{mu} receptor \textit{in vitro} indicates that free M6G does not need to be cleaved from the vector in order 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 \textit{in vivo}. Further studies are needed to assess the mechanism and rate of cleavage of vectorised M6G within the brain.

As demonstrated in this study and by others, M6G has been shown to have a slightly longer antinociceptive effect compared to morphine (150 min 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 vectorised M6G in the extracellular fluid, thereby increasing its availability to bind to the \textit{mu} 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 30-60 min post-administration. 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 mu and kappa receptors. Several lines of evidence support this hypothesis. First, the respiratory depression effect has been shown to be associated with mu receptors, and that the predictability of the degree of respiratory depression of an opioid appears to decrease with its selectivity for mu opioid receptors (Stott and Pleuvry, 1991). Secondly, activation of mu and kappa opioid receptors leads to functionally opposite effects. Verborgh et al., (1997) have shown that combination of mu and kappa receptor agonists can be additive with respect to antinociception with additionally less risk for respiratory side effects. It will be interesting to see whether the kappa binding seen with Syn1001 is agonist or antagonist and to measure the effect of respiratory depression in the presence of kappa antagonists.

In conclusion, our results show that vectorisation 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
ACKNOWLEDGEMENTS

We thank our colleagues at Synt:em and at the INSERM U26 for their helpful discussions.

We are also thankful to Pr. Gavril Pasternak for helpful advice.
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FOOTNOTES

a) Part of this work was carried out at Synt:em, Institut de Génétique Moléculaire of Montpellier.

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LEGENDS FOR FIGURES

Figure 1: A: Antinociceptive activity of subcutaneous Syn1001 in the tail flick in mice (n=8 per group) at different doses (1.06; 2.1; 3.2 and 4.24 µmol/kg). The antinociceptive effect was measured at 30, 60, 180 and 300 min post-administration and is expressed as a percentage of the maximum possible effect (% MPE) versus time.

B and C: Dose-dependent antinociceptive activity of subcutaneous Syn1001 and M6G in the tail flick in mice. The effect was measured at 60 min. Values are means ± sem.

Figure 2: Antinociceptive activity of subcutaneous M6G and Syn1001 in the tail flick in mice at a dose of 3.2 µmol/kg (n=10 per group). The antinociceptive effect was measured at 30, 60, 120 and 240 min post-administration and was expressed as a percentage of the maximum possible effect (% MPE) versus time. Values are means ± sem. The effect seen with Syn1001 was significantly different from the response observed with M6G (* P< 0.05; **P<0.01).

Figure 3: Antinociceptive activity of intravenous Syn1001, M6G and morphine in the hot plate in mice (n=15 per group) at a dose of 2.6 µmol/kg for morphine and 2.2 µmol/kg for M6G and Syn1001. The antinociceptive effect was measured at 5, 10, 15, 30, 45, 90, 120 and 180 min post-administration and was expressed as a percentage of the maximum possible effect (% MPE) versus time. Values are means ± sem. The effect seen with Syn1001 was significantly different from the response observed with M6G (*P<0.05).

Figure 4: Antagonism of Syn1001 antinociception. Mice (10 per group) were injected subcutaneously with Syn1001 (2.8 µmol/kg in panel A and 4.2 µmol/kg in panel B). The different antagonists were administered to mice subcutaneously before administration of
Syn1001 as described in Methods. The antinociceptive effect was measured using the tail flick assay and was expressed as a percentage of the maximum possible effect (% MPE) versus time. Values are means ± sem. *P<0.01.

Figure 5: Formalin test. Comparison of paw-licking time in acute and chronic phase after subcutaneous administration of either the vehicle or the test compounds at 10.9 µmol/kg to mice (n=15 per group). Values are means ± sem. *P<0.05, ****P<0.001.

Figure 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 sc administration of the compounds to rats at a dose of 43 µmol/kg for M6G and Syn1001 and 65 µmol/kg for morphine.
Figure 1A

- 1.06 µmol/kg
- 2.1 µmol/kg
- 3.2 µmol/kg
- 4.24 µmol/kg
Figure 2

The figure shows a graph plotting the percentage of maximum possible effect (% MPE) over time (min). Two lines are depicted: one for M6G and another for Syn1001. The graph includes error bars and markers indicating statistical significance with asterisks (* and **) at specific time points.
Figure 3

The graph shows the % MPE over time for Syn1001, M6G, and morphine. Syn1001 shows a significant decrease in % MPE over time, with markers indicating statistical significance. M6G shows a moderate decrease, while morphine shows a minimal decrease compared to Syn1001 and M6G.
Figure 4

A

% MPE

Syn1001

Syn1001 + Naloxone

Syn1001 + 3-MNTX

B

% MPE

Syn1001

Syn1001 + β-FNA

Syn1001 + Nor-BNI

*
Figure 5

Acute Phase (0-7 min)

Chronic Phase (7-60 min)

Paw-licking Time (s)

vehicle  morphine  M6G  Syn1001

vehicle  morphine  M6G  Syn1001
Figure 6A

- Syn1001
- M6G
- morphine

Time (min)

pCO2 (mmHg)
Figure 6C

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