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Role of the sugar moiety on the opioid receptor binding and conformation of a series of enkephalin neoglycopeptides

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ABSTRACT

Glycosylation by simple sugars is a drug discovery alternative that has been explored with varying success for enhancing the potency and bioavailability of opioid peptides. Long ago we described two *O*-glycosides having either β-Glucose and β-Galactose of (D-Met^2, Pro^5)-enkephalinamide showing one of the highest antinociceptive activities known. Here, we report the resynthesis of these two analogs and the preparation of three novel neoglycopeptide derivatives (α-Mannose, β-Lactose and β-Cellobiose). Binding studies to cloned zebrafish opioid receptors showed very small differences of affinity between the parent compound and the five glycopeptides thus suggesting that the nature of the carbohydrate moiety plays a minor role in determining the binding mode. Indeed, NMR conformational studies, combined with molecular mechanics calculations, indicated that all glycopeptides present the same major conformation either in solution or membrane-like environment. The evidences provided here highlight the relevance for in vivo activity of the conjugating bond between the peptide and sugar moieties in opioid glycopeptides.

1. Introduction

Endogenous opioid neurotransmitters such as Met- and Leu-enkephalin represent one class of opioid receptor ligands that produce relatively non-selective agonist effects at both μ and δ receptors[1] but have inspired the design and development of many of both selective and non-selective μ/δ active peptides.[2] Although these peptides have played an important role in defining the pharmacological effects of combined μ/δ opioid receptor activation, natural and synthetic opioid peptides have historically been poor drug candidates, mainly because of their limited ability to cross the blood-brain barrier (BBB) that impairs their access to the corresponding central nervous system (CNS) sites of action after systemic administration.[3]

In spite of these hurdles, a number of structure-activity studies on opioid peptides suggest that glycosylation may facilitate biodistribution of these peptides across the BBB and enhance their potency after iv, ip or sc administration by producing centrally mediated behavioral effects.[4–6] A recent example is MMP2200, which is a glycosylated derivative of a Leu-enkephalin analog, that is approximately 10-fold more potent than the parent unglycosylated compound and twice as potent as morphine in producing antinociception in mice after systemic iv administration.[7–9]

Abbreviations: BBB, Blood brain barrier; CNS, Central nervous system; [3H]-DPN, [3H]-Diprenorphine; DMEM, Dulbecco's modified Eagle's medium; ENK, Enkephalin; HRMS, High resolution mass spectrometry; HPLC, High performance liquid chromatography; icv, intracerebroventricular administration; ip, intraperitoneal administration; it, intrathecal administration; iv, Intravenous administration; sc, Subcutaneous administration; Nx, Naloxone; NMR, Nuclear magnetic resonance; TSP, 2,2,3,3-Tetradeutero-3-trimethylsilylpropionic acid.

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However, when systemically administered in rhesus monkeys MMP2200 acted as peripheral μ/δ opioid receptor agonist with limited distribution to the central nervous system, suggesting the existence of species differences in the pharmacokinetics and BBB penetration of glycopeptides.[10]

These and other results on the opioid glycopeptide field are in good agreement with the more general realization that post-translational glycosylation of proteins and synthetic glycosylation of other peptides extend their half-life in serum,[11,12] enhance their potency,[13,14] and even influence their biodistribution,[15] including BBB penetration.[3,12–14,16–18]

Following this principle, a more than 20 years-old search for systemically active glycoopioids is now still under way and producing interesting compounds such as the above mentioned MMP2200. During this time, the initially difficult attempts to conjugate enkephalin analogs to sugar moieties have led to more amenable synthetic procedures to screen different glycosides of various complexities, most of them naturally occurring mono-, di- and trisaccharides. Also, different peptide to carbohydrate conjugation methods have been explored that range from native *O*- and *N*-glycosyl bond formation to chemical ligation at suitable functional groups on a rather limited number of enkephalin sequences. The analgesic potency of many of these compounds in mice is comparable to, or sometimes greater than, the potency of morphine after iv administration.[4] Regrettably, up to now, clear correlations between identity of the sugars, type and site of linkage to the peptide and enkephalin sequence with biological activity seem still far away.

We have contributed to these efforts by providing early examples of neoglycoenkephalins. In one case, we prepared a couple of neoglycopeptides by amide-linking a glucosyl-[19] and a galactosyl-amine residue[20] to the C-terminal of the μ-selective sequence H-Tyr-DMet-Gly-Phe-Pro-NH_2, that produces significant analgesic activity after systemic administration by acting upon both μ and δ receptors.[21] The Glc analog was about two orders of magnitude less potent by peripheral (ip) than by central (icv or it) administration in rats, but still about 2000 times more potent than morphine. By central administration (icv) the Gal analog was one order of magnitude more potent than the Glc analog and about 5000 times more potent than morphine. These data were obtained after experiments using the tail immersion analgesic test and were in fair agreement with the ones obtained on the paw pressure test.[19,20] In a second case, we synthesized a couple of positional analogs of the previous glycopeptides by *O*-glycosyl bond formation of Glc and Gal with the hydroxyl function of Hyp that substitutes Pro.[5] The most striking results were that the Gal analog was about 57000 times more potent than morphine, 1700 times more potent than the Glc analog and 10000 times more potent than the parent unglycosylated peptide, as assessed by the tail immersion analgesic test after icv administration in rats.[22] More recently, we have found that a α-mannoside of morphine is 100 fold more potent and twice long lasting as compared to morphine when ip injected to rats and assessed by the tail-flick and paw pressure analgesic tests.[23]

At the time of these early experiments with the glycopeptides, the molecular and structural characterization of opioid receptors have not been achieved yet, and cloning as well as the modern expression techniques were not available either. Thus, to shed light on the affinity and selectivity of these class of glycopeptides to bind to individual opioid receptor types and to study the role of the nature of the sugar moiety on their binding features, we have resynthesized the Glc and Gal *O*-linked Hyp[5] derivatives (**3** and **4**) above discussed and expanded these family with new compounds. Thus, owing to the improved pharmacological profile observed with our mannoside of morphine we have chosen to produce the enkephalin mannoside derivative **2**. Also, following other glycoopioid results that indicate that disaccharides seem to yield more dual δ and μ active compounds,[6] we have also prepared the corresponding *O*-linked glycopeptide analogs of lactose (β-Lac) **5** and cellobiose (β-Cel) **6**. The pharmacological properties of these new and the two old glycosylated derivatives (**3** and **4**) were assessed by radioligand binding assays on isolated μ and δ opioid receptors from zebrafish owing that this organism presents high receptor opioid homology respect to the human counterparts. A pilot study of the antinociceptive properties of the Man glycopeptide (**2**) was conducted on the tail-flick test, after ip administration in mice, to assess the potential permeability of this compound across the BBB. Finally, in an attempt to correlate their binding properties to their conformation in solution, NMR conformational studies combined with molecular mechanics calculations were also conducted.

2. Results and discussion

2.1. Chemistry

The five neoglycopeptides **2**–**6** depicted in Fig. 1 and their parent compound **1** were prepared by stepwise manual solid-phase peptide synthesis following standard Fmoc protocols from previously synthesized suitable glycosyl amino acid building blocks (Fig. 1).[24–27] The final products were purified (>98% by HPLC) and characterized by UPLC-TOF/MS. The analytical data from already reported products **3** and **4** which were previously prepared by solution phase methods was consistent with the new solid-phase synthesized materials.

2.2. Biological activity

As pointed out, CNS bioavailability is on the main focus on current glycoopioid studies, however, owing the mounting evidence of a functional interaction between μ and δ opioid receptors and a possible regulatory role for δ agonists, interests in opiod research

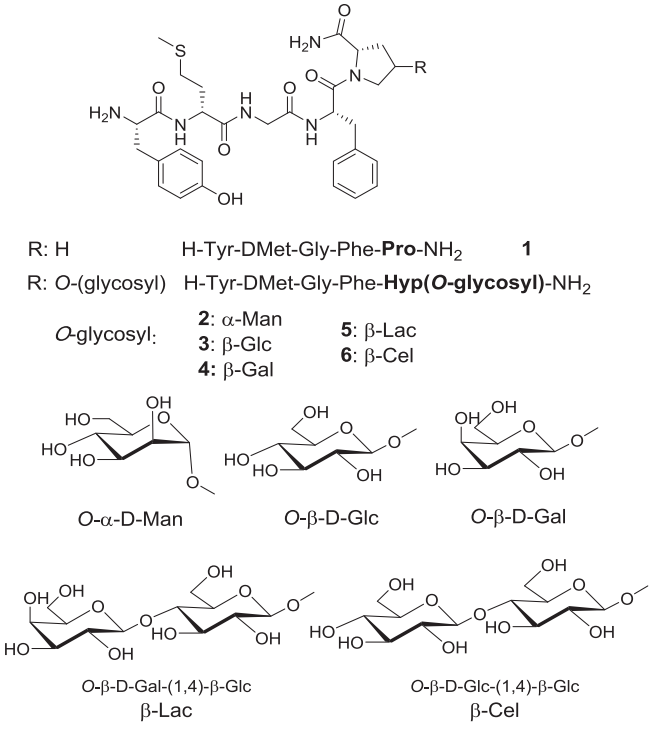

Fig. 1. Structures of the peptide (**1**) and glycopeptides (**2**–**6**) prepared in this work.

Table 1
Binding affinities, expressed as Ki-values (mean ± SEM), found for a series of glycopeptides (**2**–**6**) and their parent compound (**1**) when tested on the μ and δ opioid receptors from zebrafish. Data taken from our previous work: *(Ref. 28), #(Ref. 29) and †(Ref. 30). n.d. not determined.

No.	Peptide and glycopeptides and references	Sequence	μ receptor K_I (nM)	δ1a receptor K_I (nM)	δ1b receptor K_I (nM)
1	(DMet^2,Pro^5)-enkephalinamide parent compound	H-Tyr-DMet-Gly-Phe-Pro-NH_2	269.0 ± 40.9	565.8 ± 38.0	256.4 ± 39.4
2	α-D-Man	H-Tyr-DMet-Gly-Phe-Hyp(Man)-NH_2	239.4 ± 37.0	608.6 ± 58.4	536.2 ± 5.3
3	β-D-Glc	H-Tyr-DMet-Gly-Phe-Hyp(Glc)-NH_2	312.6 ± 67.6	389.5 ± 118.8	158.9 ± 18.9
4	β-D-Gal	H-Tyr-DMet-Gly-Phe-Hyp(Gal)-NH_2	383.3 ± 57.1	468.2 ± 105.0	518.5 ± 39.9
5	β-Lac	H-Tyr-DMet-Gly-Phe-Hyp(Lac)-NH_2	202.5 ± 3.8	634.6 ± 109.7	559.4 ± 1.1
6	β-Cel	H-Tyr-DMet-Gly-Phe-Hyp(Cel)-NH_2	223.1 ± 24.8	843.1 ± 116.5	528.3 ± 16.2
	Morphine		187*	223#	1427†
	Met-ENK	H-Tyr-Gly-Gly-Phe-Met-OH	684*	n.d.	45†
	Leu-ENK	H-Tyr-Gly-Gly-Phe-Leu-OH	1317*	73	175†

have also shifted to the δ opioid receptor. Thus, more contemporary efforts have also focused to produce compounds possessing dual actions at δ and μ receptors that may show a broader spectrum of analgesic efficacy with less side effects as compared with μ-selective agonists.[31] Accordingly, we have examined opioid receptor selectivity in our series of compounds.

The binding affinity of the different glycosylated derivatives for the μ and δ receptors has been assessed by competition binding assays using [^{3}H]-DPN. The obtained Ki-values are summarized in Table 1; and the binding affinities of morphine, Met-ENK, and Leu-ENK have also been included for comparison (See also Supplementary information Figs. S3 and S4). All glycopeptides tested showed Ki-values on the nanomolar range, and were able to displace up to 100% of the specific binding, thus confirming their opioid nature. Statistical analysis has been performed to determine whether the differences in the binding affinities for these glycoenkephalins are statistically significant (See Supplementary information Table S5). Disaccharides, especially the Lac derivative (**5**), display higher affinities for the μ opioid receptor than the unglycosylated parent compound (**1**). In contrast, glycosylation with monosaccharides does not greatly improve the binding of these analogs to the μ opioid receptor; just the Man derivative (**2**) yields a slight lower Ki-value. In the case of the δ receptors, glycosylation does not improve the binding affinity of the parent compound, except for the Glc analog (**3**). Therefore, it seems that disaccharides shift the binding affinity of the unglycosylated peptide towards the μ opioid receptor, while the presence of a single Glc shifts the binding profile towards the δ receptor.

The observed affinities of these glycopeptides are not higher than those found for longer enkephalin-containing peptides, such as MEGY[28] or β-endorphin, for the μ opioid receptor. This led us to think that the improvement in binding affinity is not due to the disaccharide per se, but to the fact that bulkier ligands yield better affinities for the μ opioid receptor.[1] In any event, this similarity among the binding data is puzzling owing that when some of these products where directly administered into the CNS large differences in their antinociceptive properties where observed. Thus, in our early experiments the Gal analog was more potent than morphine (57000 times), the Glc analog (1700 times) and the parent unglycosylated peptide (10000 times).[22] In an attempt to rationalize these discrepancies and to better understand the mechanisms of action of these products we have carried out a series of new in vivo and new conformational studies in solution.

To explore the possibility that the glycosyl moieties may be facilitating the BBB passage of these glycopeptides a pilot study of their activity after peripheral administration was conducted. The Man derivative (**2**) was chosen because, in our hands, an analogous derivative of morphine was found to be more potent, long lasting and with less side effects than morphine after ip peripheral administration.[23] A set of experiments with just two doses of the compound (5 and 8 mg/kg) and a reference dose of morphine (5 mg/kg) were performed to minimize the use of test animals. The compounds were ip administered in mice and the antinociceptive effects monitored by the tail-flick test for two hours. As seen in Fig. 2 and corresponding Table 2, morphine showed a 50% of maximum activity while the mannoside (**2**) a modest 62% at the higher assayed dose.

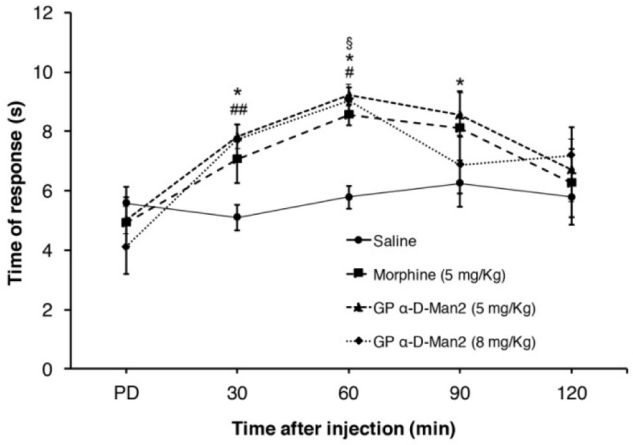

Fig. 2. Antinociceptive actions of morphine and glycopeptide α-D-Man 2 following ip administration in the tail-flick test. Pre-drug (PD) measurement was taken before the saline or drug administration, followed up by the four subsequent post-injection measurements every 30 min (30, 60, 90 and 120 min). Each point represents the mean ± SEM of 4–6 animals. At 30 min post-injection, GP α-D-Man **2** (5 mg/kg) (*) and GP α-D-Man **2** (8 mg/kg) (##) latency was significantly higher than saline. After 60 min, morphine (§), GP α-D-Man **2** (5 mg/kg) (*) and GP α-D-Man **2** (8 mg/kg) (#) also showed a statistical difference versus saline. At 90 min time point, only GP α-D-Man **2** (5 mg/kg) dose (*) showed a significant analgesic effect compared to saline treatment. To assess the statistical differences (p-value ≤ 0.05) between saline and the corresponding treatment, a non-paired, one tailed *Student's t-test* was performed (∗, #, § < 0.05; ## < 0.01).

Table 2
Percentage of analgesia in response to different compounds administration calculated following the formula below included.

Compound	ANALGESIA (%) Time after injection (min)			
	30	60	90	120
Saline	−7	3	10	−8
Morphine (5 mg/kg)	30	51	45	30
GP α-D-Man **2** (5 mg/kg)	40	60	50	40
GP α-D-Man **2** (8 mg/kg)	45	62	35	45

$$\%\,Analgesia = \frac{(Post - drug\ score - (control\ score))}{(cut - off\ value) - (control\ score)} \times 100$$

This narrow potency difference was not expected from a compound that has a similar binding affinity than others such as Glc (**3**) and Gal (**4**) which have potencies of various orders of magnitude above morphine when the BBB is bypassed by central administration. These findings seem suggestive of a poor BBB permeability of these *O*-linked glycopeptides and are in clear opposition to our own previous results observed with *N*-linked positional analogs that show substantial BBB permeability.[19,20] These results are reasonable proof of the critical role of the nature of the linking bond between the sugar and the peptide moieties.

2.3. Conformational studies in solution

The apparent contradiction seen in some of these glycopeptides that in spite of having similar binding affinities as morphine display an unusual antinociceptive activity when centrally administered, prompted us to examine the possibility that certain structural features of some of these glycopeptides may be in the cause of this phenomena. Thus, NMR-based conformational studies in solution or membrane model media have been conducted. A particular aim was to evaluate the effects of the sugar moieties on the peptide backbone conformation and side chain orientations in solution.

Careful analysis of NMR data, including 1H chemical shifts (Table S9 and Figs. S5-S9), coupling constants (Table S10) and NOEs, did not show any significant differences between enkephalinamide **1** and its hydroxyproline derivatives **2**–**6**. In fact, no significant differences were apparent in the NOE fingerprints in the absence (glycopeptide **3**, Fig. S8) and in the presence of SDS membrane-like environment (glycopeptide **3**, Fig. S9). This evidence strongly suggests that the conformation of these peptides in the membrane-like environment corresponds to the major conformation existing in water solution, which was deduced by standard NOE analysis. In particular, the global 3D model was generated using the key NOE data for all the glycopeptides. In particular, the following NOEs were employed to build the 3D model shown in Fig. 3: NH D-Met^2 - HδTyr^1; NH Gly^3 – NH Phe^4; NH Gly^3 – Hβ2 D-Met^2; NH Gly^3 Hβ3 D-Met^2; NH Phe^4 – Hα D-Met^2; H1 – Hβ Hyp^5 and H1 –Hγ Hyp^5. Thus, no long-range NOEs were observed, strongly suggesting that these peptides do not adopt a well defined secondary or tertiary structure in solution or in the presence of SDS micelles. The measured coupling constants also displayed medium size values, also in agreement with the above mentioned lack of a defined structure. From these experimental data, model 3D structures for the peptides were generated by employing a standard conformational search protocol with Macromodel[32] as integrated in the Maestro package.[33] The global minimum structure found for **3** is shown in Fig. 3.

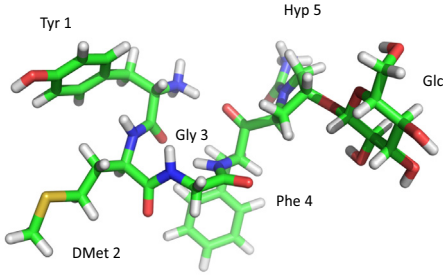

Fig. 3. Representative model structure for glycopeptide **3**, the major conformation obtained from NMR-derived data after molecular mechanics calculations.

The rest of the analogs displayed similar features. In addition, and independently of its chemical nature, in all cases, the sugar moiety was always observed to move freely and not contacting the peptide backbone nor the amino acid side chains of these glycopeptides. Thus, the presentations of the sugar or the peptide moieties of these glycopeptides to possible targets are likely to be unrelated.

3. Conclusions

The small differences of binding affinity along this series of glycopeptides suggest that the nature of the carbohydrate moiety plays a minor role in determining the binding mode, as the affinities found for the glycopeptides are of the same order of magnitude than the parent compound. This has been corroborated by the fact that no major conformation changes on the peptide backbone structure were observed by NMR along the series, thus suggesting that the saccharide part of the molecule does not modulate this enkephalin receptor recognition. Also, these minor differences in binding affinities cannot explain the substantial potency changes found for the in vivo antinociceptive activities that we reported for the Glc (**3**) and Gal (**4**) analogs as well as for the ones of the Man (**2**) analog here disclosed. These results suggest that these *O*-linked glycopeptides do not easily diffuse through the BBB in contrast to the *N*-linked series previously reported by us, thus highlighting the relevance for activity of the bonding nature between the peptide and sugar moieties in opioid glycopeptides.

4. Materials and methods

4.1. Chemicals

Naloxone (Nx) was purchased from Sigma-Aldrich and [^{3}H]-diprenorphine ([^{3}H]-DPN) 50 Ci/ mmol from Perkin-Elmer. All other reagents used were from analytical grade.

4.2. Cell culture

Stably transfected HEK293 cells expressing the isolated μ receptor (dre-oprm1, UniProt entry name Q98UH1_DANRE[28]), the δ1a receptor (dre-oprd1a, UniProt entry name O57585_DANRE[29]) or the δ1b receptor (dre-oprd1b, UniProt entry name B3DH72_DANRE[30]) from zebrafish were used for this study. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 250 μg/mL geneticin (G-418) (all from Gibco-BRL, Life Technologies), at 37 °C in humidified atmosphere containing 5% (v/v) CO_2 in a Forma incubator. Cells were grown to 80% confluence, harvested with 2 mM EDTA in PBS and collected by centrifugation at 500 g. The cell pellet was frozen at −80 °C until use.

4.3. Membrane preparation

Cell pellets were resuspended and homogenized with a Potter-Elvehjem tissue grinder in assay buffer (Tris HCl 50 mM pH 7.4 with protease inhibitors: 0.1 mg/mL bacitracin, 3.3 μM captopril and protease inhibitor cocktail, all from Sigma-Aldrich), and homogenates were centrifuged at 500*g* for 10 min at 4 °C. The nuclear pellet was homogenized again, centrifuged and discarded. The two supernatants were combined, homogenized again with the tissue grinder and the membrane pellet was collected upon centrifugation at 18000*g* for 1 h at 4 °C. The crude membrane fraction was

resuspended in ice-cold assay buffer with protease inhibitors and protein concentration was determined by Bradford (BioRad).

4.4. Competition binding assays and data analysis

Radioligand binding was performed as previously described[28] 7–30 μg protein were incubated with different concentrations of unlabelled ligand ranging from 0.3 nM to 10 μM, and using [^{3}H]-DPN as radioligand. The working concentration for [^{3}H]-DPN was similar to the affinity constant obtained for the different receptors: 1 nM for the μ opioid receptor[28] and 3.4 nM for both δ receptors.[34] Reactions were incubated for 1 h in the case of the μ and δ1a receptors and for 4 h for δ1b receptor at 25 °C in 250 μL assay buffer. 10 μM Nx was used to determine nonspecific binding. After incubation, the reaction was stopped by adding 4 mL of ice-cold 50 mM Tris HCl buffer pH 7.4, the mixture was rapidly filtrated using a Brandel Cell Harvester and washed two times onto GF/B glass-fiber filters that were pre-soaked with 0.2% (v/v) polyethylenimine for at least 1 h. The filters were placed in scintillation vials and incubated overnight at room temperature in EcoScint A scintillation liquid (National Diagnostics). Radioactivity was counted using a Beckman Coulter 6500 scintillation counter. Experiments were performed in duplicate and repeated three times.

Specific binding was defined as the difference between total binding and non-specific binding, as measured in presence of 10 μM Nx. Radioligand binding data were analyzed by computer-assisted non linear regression analysis using GraphPad PRISM software (GraphPAd Software Inc., San Diego, U.S.A.), and inhibition constants[1] were obtained for each ligand using Cheng and Prusoff's equation, which corrects for the concentration of radioligand used in each experiment as well as for the affinity of the radioligand for its binding site (KD).[35]

4.5. Antinociceptive tail-flick assay

The tail-flick test was performed in male C57Bl6/J mice weighing 22–28 g. They were housed in a room with controlled temperature on a 12 h light cycle and access to water and food *ad libitum*. Prior to the antinociceptive test, habituation was carried out for a week, always by the same operators, during the following periods of time: 11:00h–12:00h and 15:00h–17:30 h. Experiments were always performed during the afternoon timeframe. The Ugo Basile tail-flick testing apparatus was set up to emit a 129 mW/cm^2 infrared light intensity with a cut-off time of 12 s (approximately twice the control response latency).

Mice were divided into four different groups depending on the drug to be administrated. Thirty and fifteen minutes before the intraperitoneal injection, two pre-drug measurements which correspond to the basal response were taken. Following treatment, a single reading for each mouse was performed after 30, 60, 90 and 120 min. The observers were blind to the treatment. To assess the statistical differences (p-value ≤ 0.05) between every group, a non-paired, one tailed Student's *t*-test was performed.

All animals were housed and bred in an Animal Facility of the University of Salamanca. Proper measures were taken to reduce the pain or discomfort of experimental animals. All animal care and procedures were done in accordance with protocols approved by the Bioethics Committee of the University of Salamanca and following the European Community guidelines.

4.6. NMR conformational studies

Experiments were recorded in H_2O/D_2O 90:10 on a Bruker Avance 500 MHz at 278 K. NMR assignments were accomplished using standard 2D-TOCSY experiments at different mixing times (20 and 60 ms), assisted with 2D-ROESY experiments (200 ms). The concentration of the natural peptide enkephalin and its glycosylated analogs was set to 2 mM. The pH was adjusted in order to detect the amide exchangeable protons. Additional NMR experiments were performed in the presence of SDS micelles to mimic a membrane-like environment. In this case, the NMR samples were prepared with ca. 1 mM of peptide in 105 mM of SDS in a H_2O/D_2O (90:10) solution. These experiments were recorded on the same spectrometer and at 288 K. The NMR assignments were accomplished combining 2D-TOCSY (mixing times of 20, 60 ms) with 2D-NOESY experiments (mixing time of 300 ms). The resonance of 2,2,3,3-tetradeutero-3-trimethylsilylpropionic acid (TSP) was used as a chemical shift reference in the 1H NMR experiments (δTSP = 0 ppm).

4.7. Molecular mechanics calculations

Molecular mechanics were conducted using Macromodel 9.6, (MacroModel, version 9.6; Schrödinger, LLC: New York, 2008) as implemented in version 8.5.110 of the Maestro suite[32,33] using OPLS-2005[36] as the force field. The starting coordinates for conformational search calculations (OPLS-2005 as the force field) were those obtained after energy minimization. The continuum GB/SA solvent model[37] was employed, and the general PRCG (Polak-Ribière conjugate gradient) method for energy minimization was used. A conformational search protocol was then performed, using the Monte Carlo torsional sampling (MCMM) method, with the same force field and minimization conditions.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.02.052.

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