Enkephalin Glycopeptide Analogues Produce Analgesia with Reduced Dependence Liability

Edward J. Bilsky,[†] Richard D. Egleton,[‡] Scott A. Mitchell,[§] Michael M. Palian,[§] Peg Davis,[‡] Jason D. Huber,[‡] Heather Jones,[†] Henry I. Yamamura,[‡] Jacqueline Janders,[‡] Thomas P. Davis,[‡] Frank Porreca,[‡] Victor J. Hruby,[§] and Robin Polt*[,§]

Department of Biological Sciences, University of Northern Colorado, Greeley, Colorado 80639, Department of Pharmacology, College of Medicine, University of Arizona, Tucson, Arizona 85724, and Department of Chemistry, University of Arizona, Tucson, Arizona 85721

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Endogenous peptides (e.g. enkephalins) control many aspects of brain function, cognition, and perception. The use of these neuroactive peptides in diverse studies has led to an increased understanding of brain function. Unfortunately, the use of brain-derived peptides as pharmaceutical agents to alter brain chemistry in vivo has lagged because peptides do not readily penetrate the blood−brain barrier. Attachment of simple sugars to enkephalins increases their penetration of the blood−brain barrier and allows the resulting glycopeptide analogues to function effectively as drugs. The δ-selective glycosylated Leu-enkephalin amide **2**, H_2N-Tyr-D-Thr-Gly-Phe-Leu-Ser(β-D-Glc)-$CONH_2$, produces analgesic effects similar to morphine, even when administered peripherally, yet possesses reduced dependence liability as indicated by naloxone-precipitated withdrawal studies. Similar glycopeptide-based pharmaceuticals hold forth the promise of pain relief with improved side-effect profiles over currently available opioid analgesics.

Introduction

Chronic pain represents a major health and economic problem throughout the world. Despite major advances in understanding the physiological and pathological basis of pain, an ideal analgesic does not yet exist. Opiates (e.g. morphine, Demerol) remain a mainstay for the treatment of moderate to severe pain. Use of these drugs, however, is associated with significant side effects such as respiratory depression, constipation, urinary retention, tolerance, physical dependence, and/or addiction. In addition, some pain states are resistant to the analgesic actions of currently available opioid analgesics.[1] Analgesics with improved preclinical therapeutic profiles have emerged from the study of structure−activity relationships (SAR) of peptide ligands with the opioid receptors, and peptide chemists have made significant improvements in the in vitro and in vivo pharmacology of drugs based on the enkephalin neurotransmitters.[2] The therapeutic potential of peptide ligands that can mimic the action of most endogenous peptide neurotransmitters and neuromodulators is notable, but disadvantages of peptide molecules include instability in serum and their inability to penetrate the blood−brain barrier (BBB) to gain access to the brain and spinal cord. Chemists have solved the first problem by modifying the amino acid sequence of the enkephalin molecule to produce peptides that are resistant to proteolytic enzymes (e.g. peptidases).[18,25,26]

Numerous endogenous opioid peptides have been isolated and structurally characterized, most of which have the same tetrapeptide Tyr-Gly-Gly-Phe N-terminal pharmacophore (e.g. Leu- and Met-enkephalin).[3−5] Using the enkephalin scaffold as a starting point, many analogues have been prepared with altered affinity for opioid receptor subtypes.[6,7] Considerable effort has been focused on mixed μ/δ-receptor ligands for several reasons. It is well-known that mixed μ/δ-agonists have increased antinociceptive potency and efficacy.[8] Furthermore, activation of the δ-receptor may attenuate μ-mediated side effects including respiratory depression and urinary retention.[9,10]

Beginning in the 1980s, several research groups began to explore the attachment of carbohydrate groups to opioid peptides in order to influence receptor binding (μ vs δ), improve serum stability, and enhance peptide delivery to the brain or spinal column.[11−13] From these studies it is clear that the carbohydrate can destroy receptor binding.[14−16] Recent work published by Polt et al. suggests that a glycosyl group, if properly placed, will preserve previously determined SAR of the native enkephalin peptides[17,18] and can simultaneously promote penetration of the BBB.[19,20] This notion has now been extended to deltorphin-based glycopeptides, which also show antinociception following systemic administration.[21−23]

The BBB is composed of capillaries with specialized endothelial cells, which have very few endocytotic vesicles and are coupled with tight junctions. These features allow the BBB to regulate the entry of solutes into the central nervous system and ultimately keep potential toxicants out of the brain. The BBB significantly impedes entry of most molecules, except those that are small and lipophilic or those for which transporters exist, which may include the enkephalins.[24] The BBB also acts as a metabolic barrier, containing several peptidases.[18,25,26] However, transport of substances across the BBB is often dependent upon lipophilicity.[27]

* To whom correspondence should be addressed. Tel: (520) 621-6322. Fax: (520) 621-8407. E-mail: polt@u.arizona.edu.
[†] University of Northern Colorado.
[‡] Department of Pharmacology, University of Arizona.
[§] Department of Chemistry, University of Arizona.

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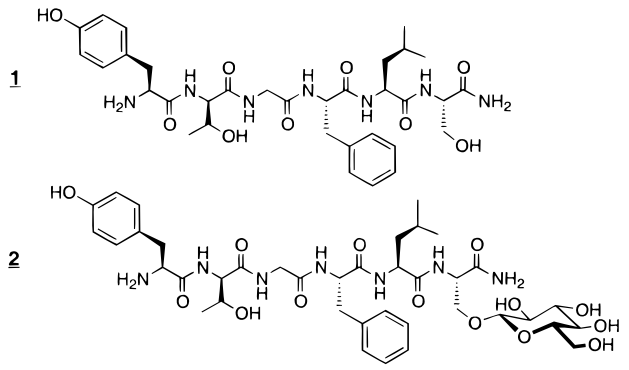

Figure 1. Chemical structures of peptide **1** and the corresponding glycopeptide analogue **2**.

Enzymatically labile transport vectors have been used to temporarily increase the lipophilicity of peptides, but the resulting lipophilic prodrugs (chimeric peptides) lacked the aqueous solubility necessary for effective serum transport.[28] Thus, simply increasing the lipophilicity of a peptide has proven to be self-limiting, since the drug must also be water-soluble. Paradoxically, the incorporation of a hydrophilic glucoside moiety into enkephalin analogues (e.g. **2**) has been found to increase BBB penetration and increase serum lifetime without significantly perturbing the pharmacology of the parent peptide pharmacophore **1**. (Figure 1).

Opioid receptor selectivities of **1** and **2** were initially assessed using radioligand binding methods and smooth muscle bioassays as previously described.[16,29,30] Both compounds bind with low-nanomolar affinity to δ- and μ-opioid receptors with an approximate 2-fold preference for δ-receptors (Table 1). The functional smooth muscle bioassays indicated that both compounds were potent agonists at δ (MVD) and μ (GPI) opioid receptors with a moderate (~20-fold) preference for δ-receptors (Table 1).

BBB permeability of the two peptides was assessed by in situ perfusion studies. A significantly higher amount of peptide **2** entered the brain than peptide **1** over a 20-min perfusion. Both **1** and **2** had significantly higher association with the brain than sucrose, the vascular space marker (Figure 2). The radioactivity was found to be greater than 80% associated with intact peptide by RP-HPLC (data not shown). Capillary depletion analysis revealed no significant difference in trapping in the capillaries of the two peptides. Stability studies in both brain and plasma showed that **2** was significantly more stable than **1** in both media, with metabolic half-lives for **2** of 164 and >500 min compared to 89 and 331 min for **1** (plasma and brain, respectively).

Peptide **1** and glycopeptide **2** both showed potent, full agonist effects in the 55 °C tail-flick test[31] following intracerebroventricular (icv) injection (Table 1). Glycosylation of the parent peptide increased the icv potency more than 3-fold. When administered intravenously (iv), both **1** and **2** produced dose-related antinociception, with glycopeptide **2** showing approximately 4.1-fold increase in potency compared to the unglycosylated peptide **1**. The calculated iv A_{50} value for glycopeptide **2** was only 1.8 times greater than the A_{50} value for morphine (Table 1). The antinociceptive effects of iv injections of all three drugs (systemic administration) were sensitive to antagonism by an icv injection (central administration) of naloxone (3 nmol), thus indicating a supraspinal site of action (data not shown). Both **1** and **2** produced full agonist effects in the 55 °C tail-flick test following intraperitoneal (ip) or subcutaneous (sc) administration. In each case, the glycosylated peptide **2** was significantly more potent than the unglycosylated peptide **1**. In fact, **2** was 1.8 times more potent than morphine following sc administration. This may be due to saturation of the BBB transport mechanism during iv administration, while sc administration allows the glycopeptide to slowly infuse into the bloodstream, keeping serum concentrations lower so that more glycopeptide can ultimately reach the brain. Also, the iv bolus may lead to a rapid redistribution of the drug or inactivation,[32] lowering blood levels quickly after the infusion.

The potent actions of glycopeptide **2**, along with the ability to synthesize gram quantities of the compound, allowed us to assess the physical dependence liability of the compound using an acute model.[33a] Administration of naloxone (10 mg/kg, ip) to mice pretreated 4 h prior with a 20 × A_{50} dose of sc morphine (100 mg/kg) produced a withdrawal syndrome characterized by vertical jumping (Figure 3). Using a similar model, naloxone injection precipitated much less jumping in mice pretreated (−4 h) with a 20 × A_{50} dose of sc **2** (122 mg/kg). The morphine dose produced near maximal antinociception for 4 h, while **2** produced near maximal analgesia for 2 h. To address these pharmacokinetic differences, a third group of mice received two injections of **2** (122 mg/kg, sc, 4 and 2 h prior to naloxone precipitation) which maintained near maximal antinociception for the entire 4-h pretreatment time. This group of mice also jumped significantly less than the morphine control mice, indicating less physical dependence with **2**.

In summary, our results show that glycosylation of an enkephalin-derived peptide increases penetration of the peptide across the rat BBB in situ and increases systemic bioavailability of the compound in vivo. A significant feature of the present work is the use of reliable methods[36,37] for the synthesis of gram quantities of glycopeptide **2**, which permits further study in vivo. This has allowed us to observe the increased potency of the opioid glycopeptide following iv or sc administration and assessment of the physical dependence liability of glycopeptide **2** following systemic administration. By administering the compound systemically, the compound presumably accesses opioid receptors in both the brain and spinal cord, an important consideration in the development of physical dependence in mice.[33] While preliminary, the results indicate that glycopeptide **2** produced less physical dependence in mice than equivalent doses of morphine using an acute model, and additional studies are needed to assess the development of physical dependence using chronic models. Other typical opioid side effects such as tolerance, respiratory depression, and urinary depression will also be assessed.[34] Glycopeptide **2** will be an important tool for evaluating what therapeutic advantages neuropeptide-based pharmaceuticals may possess, versus narcotics currently in use such as morphine.[26,35]

Experimental Section

Synthesis and Purification of Glycopeptide 2. The required FMOC-protected glycosyl amino acids were synthe-

Table 1. Summary of in Vitro and in Vivo Assays for Opioid Receptor Activity[a]

assay	morphine	peptide **1**	glycopeptide **2**
GPI IC_{50}, nM	54.7	25	33.8
MVD IC_{50}, nM	258	2.7	1.6
μ binding IC_{50}, nM	ND	4.0	8.2
δ binding IC_{50}, nM	ND	2.4	3.4
icv A_{50}, nmol (95% CL)	2.7 (1.8−4.2)	0.07 (0.05−0.09)	0.02 (0.01−0.04)
iv A_{50}, mmol/kg (95% CL)	6.3 (4.9−7.9)	46.4 (35.4−60.7)	11.4 (8.5−15.2)
ip A_{50}, mmol/kg (95% CL)	14.6 (11.8−18.0)	137.2 (124.0−151.8)	34.3 (21.2−55.5)
sc A_{50}, mmol/kg (95% CL)	13.2 (10.2−17.0)	20.3 (15.9−26.0)	7.2 (4.9−10.0)

[a] A_{50} is the amount of drug required to produce 50% antinociception (% antinociception = 100 × [test latency − control latency]/[15 − control latency]); ND, not determined.

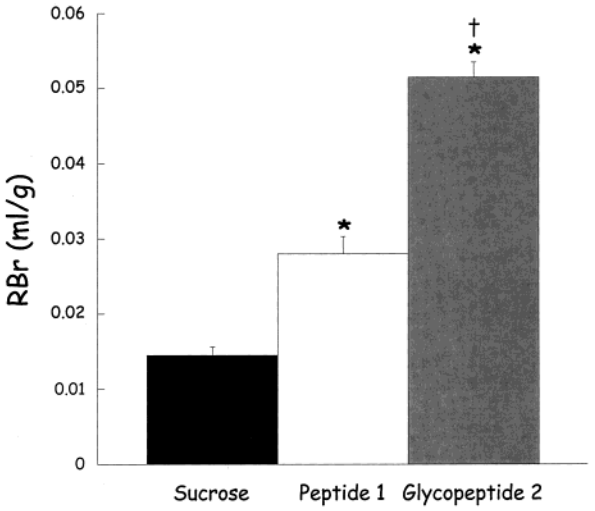

Figure 2. Uptake of the vascular space marker [^{14}C]sucrose, **1**, and **2** into rat brain after a 20-min in situ perfusion as R_{Br} = ratio of the radioactivity per unit mass of brain (dpm/g) to the radioactivity per unit volume of perfusate (dpm/mL). Each point represents the mean ± SEM of 4 rats. Both peptides show a significantly higher accumulation in the brain than sucrose (*p < 0.01 ANOVA). There is a significantly higher accumulation of **2** than **1** (†p < 0.01 ANOVA, followed by Newman−Keuls analysis).

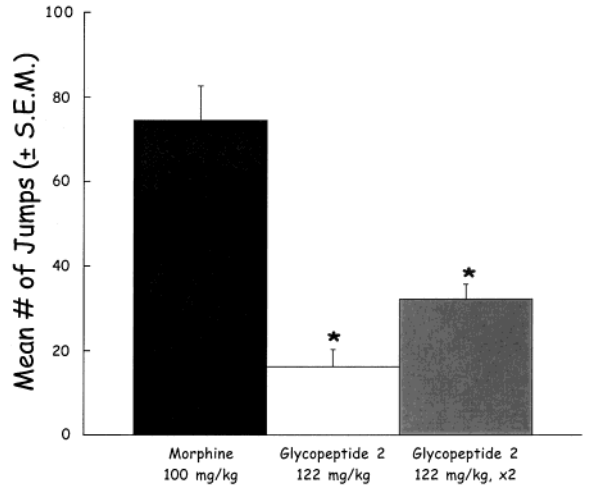

Figure 3. Assessment of the physical dependence liability of morphine and glycopeptide **2** in an acute model of physical dependence. The ANOVA yielded an $F(2,66) = 13.4$, $p < 0.001$, with both glycopeptide **2** treatment groups having significantly less jumps than the morphine control group ($p < 0.05$, Newman−Keuls analysis).

sized using published methods.[36−38] FMOC-Rink resin (2.50 g, 0.55 mmol/g substitution) was swollen in DMF for 30 min. FMOC deprotection and washes were followed by coupling of Ser^6 with FMOC-serine[O-β-D-Glc(OAc)$_4$] (1.13 g, 1.72 mmol), BOP (0.76 g, 1.72 mmol), HOBt (0.27 g, 1.72 mmol), and ^{i}PrNEt$_2$ (0.60 mL, 3.44 mmol) in 25 mL of 1:1 DMF:NMP for 2 h. FMOC deprotection and washes were followed by coupling of Leu^5 with FMOC-leucine (1.94 g, 5.50 mmol), BOP (2.43 g, 5.50 mmol), HOBt (0.86 g, 5.50 mmol), and ^{i}PrNEt$_2$ (1.0 mL, 11.0 mmol) in 25 mL of 1:1 DMF:NMP for 40 min. FMOC deprotection and washes were followed by coupling of Phe^4 with FMOC-phenylalanine (2.13 g, 5.50 mmol), BOP (2.43 g, 5.50 mmol), HOBt (0.86 g, 5.50 mmol), and ^{i}PrNEt$_2$ (1.0 mL, 11.0 mmol) in 25 mL of 1:1 DMF:NMP for 2 h. FMOC deprotection and washes were followed by coupling of Gly^3 with FMOC-glycine (1.64 g, 5.50 mmol), BOP (2.43 g, 5.50 mmol), HOBt (0.86 g, 5.50 mmol), and ^{i}PrNEt$_2$ (1.0 mL, 11.0 mmol) in 25 mL of 1:1 DMF:NMP for 1 h. FMOC deprotection and washes were followed by coupling of D-Thr^2 with FMOC-(O-*t*-butyl)-D-threonine (2.19 g, 5.50 mmol), BOP (2.43 g, 5.50 mmol), HOBt (0.86 g, 5.50 mmol), and ^{i}PrNEt$_2$ (1.0 mL, 11.0 mmol) in 25 mL of 1:1 DMF:NMP for 1.5 h. FMOC deprotection and washes were followed by coupling of Tyr^1 with FMOC-(O-*t*-butyl)-tyrosine (2.53 g, 5.50 mmol), BOP (2.43 g, 5.50 mmol), HOBt (0.86 g, 5.50 mmol), and ^{i}PrNEt$_2$ (1.0 mL, 11.0 mmol) in 25 mL of 1:1 DMF:NMP for 1.5 h. N$^\alpha$-FMOC deprotection with piperidine was followed by acetate removal while on the resin via treatment with $H_2N-NH_2 \cdot H_2O$ in MeOH. Washing and vacuum-drying gave 3.326 g of protected peptide resin, for a synthetic yield of 87%. Standard cleavage and precipitation with Et_2O gave crude **2**, which yielded analytically pure **2** in 30−35% after chromatography on a preparative Vydac C_{18} column with 10−50% CH_3CN (2%/min gradient)/0.1% $CF_3COOH-H_2O$: $[\alpha]^D = +21.82°$ ($c = 0.165$, H_2O); FAB HRMS (M + H) for $C_{39}H_{57}O_{14}N_7$ calcd 848.4042, found 848.4053. In this way, 1-g batches of **2** were prepared and could be purified (>99% by HPLC) in 100-mg quantities with a 22-mm diameter HPLC column in a routine fashion.

In Situ BBB Studies. Peptides **1** and **2** were monoiodinated on the N-terminal tyrosine using a standard chloramine-T procedure[39] and purified by RP-HPLC. Both peptides had specific activities of 2175 Ci/mmol.

Female Sprague−Dawley rats (250−300 g) were anesthetized with 1 mL/kg of a cocktail of acepromazine (0.6 mg/mL), ketamine (3.1 mg/mL), and xylasine (78.3 mg/mL) and then heparinized (10 000 U/kg). The carotid arteries were exposed cannulated and the animal was perfused with oxygenated mammalian Ringers (117.0 mM NaCl, 4.7 mM KCl, 0.8 mM $MgSO_4 \cdot 3H_2O$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2 \cdot 6H_2O$, 10 mM D-glucose, 3.9% dextran (MW 70 000) and 1% bovine serum albumin) at 70−90 mmHg.[40] The jugular veins were sectioned, and iodinated peptide or [^{14}C]sucrose (10 μCi, 492 Ci/mmol) was infused into the perfusion medium. At 20 min the rat was decapitated, and brain and perfusate samples were taken for radioactive counting. The γ-samples (iodinated) were counted on a Beckman 5500 (Beckman Instruments, Fullerton, CA). The β-samples (^{14}C) were incubated overnight with TS-2 tissue solubilizer (RPI; Budget-Solve, Mount Prospect, IL), neutralized with acetic acid, and then mixed with liquid scintillation fluid (RPI; Budget-Solve, Mount Prospect, IL) prior to counting on a LS 5000 TD counter (Beckman Instruments, Fullerton, CA). The uptake into the brain was calculated as the ratio (R_{Br}) of the radioactivity per unit mass of brain (dpm/g) to the radioactivity per unit volume of perfusate (dpm/mL). R_{Br} values for sucrose and the two

peptides were compared by ANOVA and Newman−Keuls analysis using the Pharmacological Calculation Software.[41]

Brain extractions were performed using a modified method of Davis and Cullin-Berglund.[42] The protein concentration was determined to be 6.8 mg/mL using the Lowry method.[43] Blood was collected from the rat via the abdominal aorta and centrifuged at 4000*g* for 12 min. The plasma was separated and stored at −80 °C. Aliquots (180 μL) of resuspended 15% rat brain homogenate or plasma were placed into 1.5-mL centrifuge tubes and, together with a buffer control, warmed to 37 °C in a rolling water bath incubator. At time 0, **1** and **2** were added to each tube to achieve a final concentration of 100 μM and incubated for 0, 60, 120, 240, and 360 min. At the end of the set incubation period, enzyme activity was terminated by the addition of 200 μL of CH_3CN and 200 μL of 0.5% HOAc and the tubes were placed on ice. Each tube was then centrifuged at 3000*g* for 12 min, and 300 μL of the supernatant was transferred to a clean 1.5-mL centrifuge tube. An equal volume of distilled water was added to reduce the final concentration of acetonitrile to <25%, and the samples were then taken for HPLC analysis.

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Supporting Information Available: HPLC traces of purified glycopeptide **2**, complete descriptions of the radioligand binding studies, mouse vas deferens (MVD) and guinea pig ileum (GPI) bioassays, and in vivo antinociception studies, including dose−response curves for icv, iv, ip, and sc administration. This material is available free of charge via the Internet at http://pubs.acs.org.

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