Systemic Analgesic Activity and δ-Opioid Selectivity in [2,6-Dimethyl-Tyr^1,D-Pen^2,D-Pen^5]enkephalin

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The cyclic peptide [2,6-dimethyl-Tyr^1,D-Pen^2,D-Pen^5]enkephalin (2) was synthesized by solid-phase techniques and contains the optically pure unnatural amino acid 2,6-dimethyltyrosine (DMT) as a replacement for the Tyr^1 residue of [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 1). This structural modification resulted in a 10-fold increase in the potency of 2 at the δ opioid receptor and a 35-fold increase in potency at the μ receptor while substantial δ receptor selectivity was maintained. In addition, 2 was 86-fold more effective than 1 at inhibiting electrically stimulated contractions of the mouse vas deferens. In the hot plate test, 2 was 7-fold more potent than 1 after intracerebroventricular administration in the mouse. While 1 was inactive following systemic administration of doses as high as 30 mg/kg, subcutaneous administration of 2 significantly inhibited writhing with an ED_{50} of 2.6 mg/kg. These results demonstrate that the potency and systemic activity of DPDPE are significantly increased by replacement of Tyr^1 with DMT.

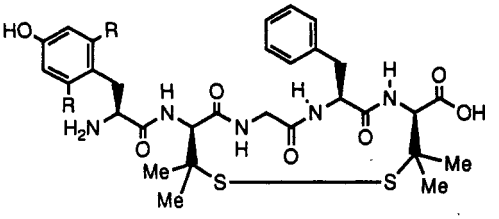
Introduction

The multiplicity of opioid receptor types in the central nervous system is now well established.[1,2] Though much work has been directed at defining the structural elements that determine receptor specificity and efficacy, these factors are still, at best, poorly understood. The rigid alkaloid opiates, typified by morphine, are generally believed to produce analgesia and multiple adverse side effects by interacting with the μ receptor.[3] It is now well-established that the δ opioid receptor mediates analgesia in the mouse.[4] Furthermore, evidence is emerging that activation of this receptor elicits less undesirable side effects, such as inhibition of gastrointestinal transit and physical dependence, than stimulation of μ opioid receptors.[4,5] For several years, the prototypic agonist for the δ opioid receptor has been the cyclic enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 1).[6] The recently discovered deltorphins, heptapeptides of frog-skin origin, are also highly selective and potent, in vitro, at this receptor.[7] The relatively large size of these peptides, however, suggests potential difficulty in crossing the blood–brain barrier to elicit analgesia after systemic administration, a desirable property for a useful analgesic. This has also hampered attempts to more fully define the functional role of δ receptors in the central nervous system.

In this paper we describe the design, synthesis, and biological properties of a δ-selective agonist which produces analgesia following systemic administration in the mouse. Our earlier efforts led to the discovery that replacement of the N-terminal tyrosine unit by 2,6-dimethyl-L-tyrosine (DMT) in several series of μ-selective linear tetrapeptide enkephalin analogues produced compounds with significantly enhanced potency and systemic bioavailability.[8] We have now applied this modification to DPDPE (1) to synthesize [2,6-dimethyltyrosyl^1]DPDPE (2).

Chemistry

Optically pure (*tert*-butyloxycarbonyl)-2,6-dimethyltyrosine (Boc-DMT) was prepared as previously described.[9] Peptides 1 and 2 were synthesized using solid-phase peptide synthesis methodology previously described for the preparation of 1.[6] Chloromethylated polystyrene resin (Merrifield resin), cross-linked with 1% divinylbenzene, was employed as the solid-phase support. All amino acids were Boc-protected, and the *p*-methylbenzyl protecting group was used to block the side-chain sulfur of penicillamine residues. The assembled peptides were cleaved from



1; R = H (DPDPE)
2; R = Me (DMT-DPDPE)

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Table I. In Vitro Characterization of DMT-DPDPE (2)

compd	receptor binding: K_i, nM μ^a	δ^b	$K_i(u)/K_i(\delta)$	mouse vas deferens: EC_{50}, nM
2	58.3	1.8	39.4	0.15 ± 0.02
1	2018	17.7	114.0	12.9 ± 3.8

[a] Based on displacement of [³H]DAMGO. [b] Based on displacement of [³H]DSLET.

Table II. In Vivo Antinociceptive Activity (ED_{50}) of DMT-DPDPE (2) in the Mouse

compd	PBQ writhing:[a] sc (mg/kg)	hot plate[a] sc (mg/kg)	hot plate[a] icv (μg)
2	2.6 (0.9–5.7)	33[b]	0.2 (0.02–0.4)
1	inactive[c]	NT[d]	1.4 (0.8–2.4)
morphine	0.2 (0.1–0.4)	3.2 (2.8–3.7)	0.6 (0.5–0.9)

[a] ED_{50} value and 95% confidence limits. [b] Minimum effective dose as ED_{50} values could not be determined. [c] Inactive at 30 mg/kg sc, the highest dose tested. [d] Not tested in view of the lack of effect of 1 in the less stringent writhing test.

the resin and simultaneously deprotected by HF treatment. The resulting linear free sulfhydryl peptides were cyclized by oxidation with $K_3Fe(CN)_6$ to provide the desired cyclic pentapeptides which were purified by reverse-phase HPLC.[10]

Results and Discussion

Table I summarizes the potencies of 1 and 2 at displacing the binding of ³H[D-Ala²,MePhe⁴,Gly-ol⁶]enkephalin ([³H]DAMGO) and ³H[D-Ser²,Leu⁵,Thr⁶]enkephalin ([³H]DSLET) to the μ and δ opioid receptors, respectively, in homogenates of rat brain and the potency of these compounds at inhibiting the contraction of the electrically stimulated isolated mouse vas deferens. Replacement of the Tyr of 1 by DMT results in a 10-fold increase in affinity at the δ receptor and a 35-fold increase in affinity at the μ receptor as compared to 1. In addition, 2 was on the order of 86-fold more potent than 1 at inhibiting electrically stimulated contractions of the mouse vas deferens. Although 2 retains substantial selectivity for the δ receptor, the degree of selectivity (39-fold preference for the δ site) is significantly less than that of DPDPE (1).

Table II summarizes the analgesic activities of 1 and 2. The analgesic activities of morphine are also provided for comparative purposes. When administered intracerebroventricularly in the hot plate test, 2 was 7-fold more potent than 1 and 3-fold more potent than morphine in prolonging response latency in the mouse. This finding is consistent with the 10- and 35-fold greater potency of 2 at the μ and δ opioid binding sites, respectively, and the 86-fold greater potency of 2 in the mouse vas deferens. Notably, 2 was also effective in the hot plate assay following subcutaneous administration. In this test, a dose of 33 mg/kg sc significantly increased response latency to nearly 25 s, thus approximating an ED_{50} dose. In the writhing test, 2 produced a dose-dependent inhibition of writhing with an ED_{50} of 2.6 mg/kg sc, indicating that it is approximately one-tenth as potent as morphine in this test. By comparison, doses as high as 30 mg/kg sc of 1 were without effect in the writhing test. These data demonstrate that incorporation of DMT results in significantly enhanced potency and endows this cyclic enkephalin analogue with systemic activity.

At present, it is not clear whether the analgesic activity of 2 observed after subcutaneous administration is mediated by peripheral or central opioid receptors. Hardy et al.[14] recently reported that the inhibition of writhing produced by a series of polar pentapeptide enkephalin analogues in the mouse was antagonized by the quaternary opioid antagonist *N*-methylnalorphine, suggesting that the analgesic activity of these compounds was mediated by peripheral opioid receptors. Thus, an involvement of peripheral opioid receptors in the analgesic action of 2 in the writhing test cannot be excluded at this time. However, the ability of subcutaneously administered 2 to significantly prolong response latencies in the hot plate test, an action typically associated with activation of central opioid receptors, suggests that 2 crosses the blood–brain barrier to some extent. A comparison of the equieffective subcutaneous dose (33 mg/kg or 990 μg total dose/mouse) to the icv ED_{50} dose (0.2 μg total dose/mouse) in the hot plate test indicates that the central bioavailability of 2 after sc administration may approach 0.02%. Recently, Roques and colleagues reported that the central bioavailability of BUBU [Tyr-D-Ser(*O-tert*-butyl)-Gly-Phe-Leu-Thr(*O-tert*-butyl)-OH], another more δ-selective opioid agonist with systemic analgesic activity, was 0.065% after iv administration.[15] More importantly, despite the high selectivity of BUBU for the δ opioid receptor in vitro, its in vivo analgesic activity appears to be mediated principally by the μ subtype of the opioid receptor.[16] Although the analgesic activity of 2 can be antagonized by naloxone (unpublished observations), indicating that the analgesic activity is opioid mediated, the exact subtype of the opioid receptor responsible for this effect remains to be determined.

In summary, DMT-DPDPE (2) is a reasonably potent opioid ligand that exhibits significant selectivity for the δ type of the opioid receptor and produces analgesia following sc as well as icv administration. Incorporation of

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the unnatural amino acid 2,6-dimethyltyrosine diminishes, but does not abolish, the selectivity of the resulting peptide for the δ receptor.

Experimental Section

Proton (^{1}H) NMR spectra were recorded at 400 and 300 MHz on Varian VXR-400 and General Electric Model QE-300 instruments using DOAc as solvent. Infrared spectra of KBr disks were measured on Perkin-Elmer Model 283B and Model 681 instruments. Specific optical rotations were measured in MeOH at 365 nM in a cell 10 cm in length on a Perkin-Elmer Model 241 digital polarimeter. Mass spectra were taken on a Finnigan-MAT Model 8430 system with high resolution, FAB, and chemical ionization capability. Elemental analyses were determined by the Searle Laboratories Microanalytical Department under the direction of Mr. E. Zielinski. Preparative high-pressure liquid chromatography (HPLC) was performed on a Rainin Model-HPX system. Analytical HPLC was carried out on a Waters Model-45 instrument equipped with a Kratos Sprectroflow Model 773 detector and a Hewlett-Packard Model 3390A integrator.

DPDPE (1) was synthesized as described previously[6] and purified on the system described above using a 20 × 500 mm YMC ODS-AQ 120-S50 column and eluting with a solution gradient of 2% to 30% acetonitrile in water with 0.2% acetic acid. The purity of the product was assessed by analytical HPLC using the system described above and an analytical YMC ODS-AQ 303 S-5 120A column. The 1 used in the assays discussed in this paper was determined to be >98% pure by HPLC, NMR, and microanalytical analyses.

[2,6-Dimethyl-Tyr^1,D-Pen^2,D-Pen^5]enkephalin (2). The title compound was prepared by solid-phase peptide synthesis methods using procedures similar to those employed in the previously reported synthesis of DPDPE.[6] Chloromethylated (1.34 mequiv Cl/g) polystyrene resin cross-linked with 1% divinylbenzene (Lab Systems, San Mateo, CA) was used as the solid support. N^α-Boc-(*S*-*p*-MeBzl)-D-penicillamine was attached to the solid-phase resin support via an ester linkage using a modification of the procedure of Gisin.[17] N^α-Boc-(*S*-*p*-MeBzl)-D-penicillamine (7.96 g, 22.5 mmol) was dissolved in 160 mL of dry, N_2-purged dimethylformamide (DMF). To this solution was added 20 g of resin and 5.15 g (26.5 mmol) $CsHCO_3$, and the suspension was stirred at 50 °C under anhydrous conditions for 72 h. Progress of the reaction was followed by disappearance of N^α-Boc-(*S*-*p*-MeBzl)-D-penicillamine assessed by analytical HPLC which indicated >99% completion at 72 h. The product, N^α-Boc-(*S*-*p*-MeBzl)-D-penicillamine-Merrifield resin, was filtered, washed with 3 × 75 mL of DMF, 3 × 75 mL of DMF/H_2O (9:1), 3 × 75 mL, DMF, and 3 × 75 mL of ethanol, and dried under vacuum. A 1.06-g sample of N^α-Boc-(*S*-*p*-MeBzl)-D-penicillamine-resin was placed in the reaction vessel of a Vega Biotechnologies 250C automated solid-phase peptide synthesizer, and the fully protected, resin-bound pentapeptide, N^α-Boc-2,6-Me_2Tyr-D-Pen(*S*-*p*-MeBzl)-Gly-Phe-D-Pen(*S*-*p*-MeBzl)-resin was prepared by stepwise addition of the appropriate N^α-Boc-protected amino acids. Dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were used in the coupling reactions, and trifluoroacetic acid/dichloromethane (1:1, v/v) was employed for removal of the N^α-Boc group following each coupling. Coupling reactions were monitored by the ninhydrin test.[18] A 1.3-g sample of the resin-bound peptide, N^α-Boc-2,6-Me_2Tyr-D-Pen(*S*-*p*-MeBzl)-Gly-Phe-D-Pen(*S*-*p*-MeBzl)-resin, was treated with 0.65 g of *p*-thiocresol, 0.65 g of cresol, and 20 mL of anhydrous HF at 0 °C for 45 min to effect cleavage from the resin and removal of the N-terminal Boc as well as deprotection of the D-penicillamine sulfurs. Following evaporation of the HF, the resin was extracted with 100 mL Et_2O (and the filtrate discarded) followed by extraction with 20 mL of a mixture of DMF and 80% acetic acid (90/10). This latter extract was diluted with 200 mL of a solution of 0.1% trifluoroacetic acid (TFA) in H_2O and was purified on a Vydac 218TP reversed-phase HPLC column (2.2 cm × 25 cm) using a linear gradient of 10–50% solvent B (solvent B = 0.1% TFA in CH_3CN; solvent A = 0.1% TFA in H_2O). The linear, disulfhydryl-containing pentapeptide, eluting in ca. 65 mL at 33% solvent B, was collected and diluted with 200 mL of H_2O, and the pH of the solution was adjusted to 8.5 with NH_4OH. To the solution was added 60 mL of 0.01 M $K_3Fe(CN)_6$ in water, and the reaction was allowed to proceed with stirring for 2 h. An additional 30 mL of 0.01 M $K_3Fe(CN)_6$ solution was added, and the reaction was allowed to continue for an additional 1 h. Analytical HPLC showed that the oxidation reaction to the cyclic disulfide-containing pentapeptide was essentially complete. The mixture was acidified to pH 4 by addition of acetic acid, stirred for 20 min with 10 mL (settled volume) of anion-exchange resin (AG 3x4A, Cl^- form), and filtered. The resin was washed with 20 mL of a mixture of DMF and 80% acetic acid (90:10). Following lyophilization, the resulting crude product was purified by HPLC on a Vydac 218TP reversed-phase HPLC column (2.2 cm × 25 cm) using a linear gradient of 10–50% solvent B. This procedure yielded 54 mg of the title compound: $[\alpha]^{25}_D = -45.1°$ (*c* = 0.12, MeOH); ^{1}H NMR (400 MHz, DOAc) δ 0.76 (s, 3 H), 1.37 (s, 3 H), 1.40 (s, 3 H), 1.42 (s, 3 H), 2.27 (s, 6 H), 3.00 (dd, 1 H, *J* = 14, 10 Hz), 3.16 (dd, 1 H, *J* = 14, 5.5 Hz), 3.27 (dd, 1 H, *J* = 14, 4.5 Hz), 3.29 (dd, 1 H, *J* = 14, 11 Hz), 3.58 (br d, 1 H, *J* = 15 Hz), 4.46 (br d, 1 H, *J* = 15 Hz), 4.52 (dd, 1 H, *J* = 11, 5.5 Hz), 4.53 (s, 1 H), 4.71 (s, 1 H), 4.74 (dd, 1 H, *J* = 10, 4.5 Hz), 6.51 (s, 2 H), 7.19–7.30 (m, 5 H); IR (KBr) 3400, 1660, 1610, 1520 cm^{-1}; MS (chemical ionization, NH_3) *m*/*z* 674 (MH^+). Anal. Calcd for $C_{32}H_{43}N_5O_7S_2 \cdot 1.5HOAc \cdot 3.0H_2O$: C, 51.39; N, 8.56; S, 7.84; H; calcd, 6.78; found, 6.16.

In Vitro Characterization. Binding at μ and δ receptors was measured in a twice-washed P2 membrane fraction obtained from whole brain (minus cerebellum) using a 50 mM Tris-HCl buffer (pH 7.4 and 37 °C). Assay tubes contained 0.8 mL of membrane homogenate (0.5 mg of protein), 0.1 mL of ^{3}H-ligand (1.0 nM DSLET for δ or 2.0 nM DAMGO for μ), and 0.1 mL of the test compound in replicates of three. After incubation for 60 min at 37 °C, the reactions were terminated by rapid filtration on Whatman GF/B glass fiber filters and a subsequent 10-mL wash of ice-cold buffer. Filters were prepared for liquid scintillation counting. Specific binding was calculated as the difference in radioactivity bound in the absence and presence of 10 μM levorphanol. IC_{50} values, the concentration of test compound that inhibited ^{3}H-ligand binding by 50%, were obtained by regression analysis of a log–logit transformation of binding data. The IC_{50} values were converted to K_i's according to the formula $K_i = IC_{50}/(1 + L/K_D)$ where *L* and K_D are the concentration and dissociation constant of the ^{3}H-ligand.[19] The K_D's for [^{3}H]DSLET and [^{3}H]DAMGO were 1.6 nM and 1.2 nM, respectively. Values are the mean ± SE of between three and six determinations.

The method of the electrically stimulated mouse vas deferens assay is a modification of that described by Smith et al.[11] Male albino ICR mice (25 g to 30 g) were sacrificed by decapitation, and their vasa deferentia were removed, cleaned of connective tissue, and suspended in 25-mL organ baths containing a Krebs physiological solution bubbled with 5% CO_2 and 95% O_2 and maintained at 37 °C. The physiological media consisted of the following mM concentrations: NaCl, 188; KCl, 4.75; $CaCl_2$, 2.54; $MgSO_4$, 0.58; KH_2PO_4, 1.19; $NaHCO_3$, 25; glucose, 11; pargyline hydrochloride, 0.3; EDTA, 0.03. The segments were suspended between two platinum electrodes and attached to isometric transducers. The segments were allowed to equilibrate for 30 min with bath changes at 0 and 15 min. Contractions were induced by a stimulus consisting of twin electrical pulses of 2-ms duration at supramaximal voltage and a frequency of 0.1 Hz. Agonists were cumulatively added to the baths 15 min after initiation of contractions. EC_{50}'s, the dose of agonist necessary to inhibit contractions by 50%, were calculated by probit analysis.[20] All values

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are the mean and standard error of at least three determinations.

In Vivo Characterization. The writhing test was performed using male albino mice (Charles River Laboratories, CD-1/HAM/1LR) weighing between 20 and 30 g. Twenty-five minutes after sc administration of the test compound (0.1 mL/10 g body weight), 0.025% (w/v) phenylbenzoquinone was injected intraperitoneally (0.1 mL/g body weight). Five minutes later each mouse was placed in a large glass beaker, and the number of writhes that occurred in the subsequent 10 min was counted. A test compound was considered to have produced antinociception in a mouse if the number of writhes elicited by phenylbenzoquinone was equal to or less than $^1/_2$ the median number of writhes recorded for the saline-treated group that day. Each dose of test compound was administered to 10 mice, and the results were expressed as the number of mice (out of a possible 10) in which the test compound produced antinociception. The ED_{50} value, defined as the dose that inhibited writhing in 50% of the mice, and 95% confidence limits, were calculated using a maximum likelihood function.

The hot plate test was conducted using male albino mice (Charles River Laboratories, CD-1/HAM/1LR) weighing between 20 and 30 g. Hot plate latency was defined as the time that elapsed between placement of the mouse on a 55 °C surface and a lick of the hindpaw or a jump. Animals that achieved the cut-off latency of 40 s were removed from the hot plate to prevent tissue damage and assigned this value. Hot plate latency was determined before and at fixed intervals after either sc or icv administration of the test compound. One measurement was made at each time point. Two-way analyses of variance were used to determine the significance of the drug effect on response latencies as compared to saline-treated animals. The ED_{50} value was defined as the dose that produced $^1/_2$ the maximum possible increase in latency (i.e. 25 s). Calculations of ED_{50} values were based on a least squares linear regression equation computed for the data obtained at 10 min, the time of peak effect.

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Registry No. 1, 88373-73-3; 2, 137666-07-0; Boc-D-Pen(*p*-MeBzl)-OH, 115962-34-0.

Novel Anticancer Prodrugs of Butyric Acid. 2[1]

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The antitumor activity of novel prodrugs butyric acid was examined. The in vitro effect of the compounds on induction of cytodifferentiation and on inhibition of proliferation and clonogenicity showed that (pivaloyloxy)methyl butyrate (**1a**) (labeled AN-9) was the most active agent. SAR's suggested that its activity stemmed from hydrolytically released butyric acid. In vivo, **1a** displayed antitumor activity in B16F0 melanoma primary cancer model, manifested by a significant increase in the life span of the treated animals. Murine lung tumor burden, induced by injection of the highly metastatic melanoma cells (B16F10.9), was decreased by **1a**. It also displayed a significant therapeutic activity against spontaneous metastases which were induced by 3LL Lewis lung carcinoma cells. Moreover, **1a** has the advantage of low toxicity, with an acute $LD_{50} = 1.36 \pm 0.1$ g/kg ($n = 5$). These results suggest that **1a** is a potential antineoplastic agent.

Many well-recognized adverse effects accompany current conventional cancer chemotherapy treatment using aggressive cytotoxic agents, as a result of being nonspecific toward neoplastic cells and affecting normal cells as well. Cancer research seeks alternative methods of directly or indirectly stimulating the host's immune system, with minimal systemic toxicity and maximum specificity, and efficiency in eliminating tumor cells. Since cancer can be considered a disorder of cell differentiation, another approach which satisfies the requirements for specificity and low toxicity is induction of neoplastic cell differentiation. With the arrest of maturation, immature cells continue to proliferate, resulting in the emergence of clinically manifested cancer cells. Experimental evidence has demonstrated that tumor cells can be induced to differentiate, indicating that the malignant process can be at least partially reversed.[2] Retinoic acid, phorbol esters, DMSO, and butyric acid (BA) are among the compounds which have been shown to induce differentiation.

Butyric acid is a nontoxic natural product found in food (e.g. in butter it constitutes up to 5%). In the digestive system, it is secreted as a byproduct of microbial fermentation, and in the human colon it can reach millimolar concentrations. In a variety of human tumor cells grown in vitro, BA displayed antitumor activity, reflected in growth arrest, decreased clonogenicity, and induction of morphological and biochemical changes.[3]

Clinical trials with high doses of BA produced no detectable toxicity. In a child with acute myeloid leukemia,[4] it induced partial and temporary remission. However, no clinical activity was detected in an adult with myelomonocytic leukemia[5] or in nine adults with acute leukemia.[6]

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