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Tyr¹-ψ[(Z)CF=CH]-Gly² Fluorinated Peptidomimetic Improves Distribution and Metabolism Properties of Leu-Enkephalin

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Abstract

Opioid peptides are key regulators in cellular and intercellular physiological responses, and could be therapeutically useful for modulating several pathological conditions. Unfortunately, the use of peptide-based agonists to target centrally located opioid receptors is limited by poor physicochemical (PC), distribution, metabolic, and pharmacokinetic (DMPK) properties that restrict penetration across the blood-brain-barrier *via* passive diffusion. To address these problems, the present manuscript exploits fluorinated peptidomimetics to simultaneously modify PC and DMPK properties, thus facilitating entry into the central nervous system. As an initial example, the present manuscript exploited the Tyr¹-ψ[(Z)CF=CH]-Gly² peptidomimetic to improve PC drug-like characteristics (computational), plasma and microsomal degradation, and systemic and CNS distribution of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). Thus, the fluoroalkene replacement transformed an instable *in vitro* tool compound into a stable and centrally-distributed *in vivo* probe. In contrast, the Tyr¹-ψ[CF₃CH₂-NH]-Gly² peptidomimetic decreased stability by accelerating proteolysis at the Gly³-Phe⁴ position.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS

R.A.A. conceived the research plan and wrote the initial draft of the manuscript. R.A.A. and K.K.S. interpreted data and revised the manuscript. L.G.R., P.C.T. and M.J.B. executed *in vitro* and *in vivo* experiments. M.P. and S.N.K. synthesized compounds 2–4.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXXXXXX. Microsomal stability without NADPH, and Caco-2, MDCK-MDR1, and PAMPA results.

Keywords

Opioid Peptides; Leu-Enkephalin; Peptidomimetics; Fluorination; Peptide Stability; Peptide CNS-Distribution

INTRODUCTION

Opioid peptides are key regulators in various cellular and intercellular physiological responses, and could be therapeutically useful for modulating pain, alcohol abuse, emotional and mood disorders, and other pathological conditions.^{1–3} Unfortunately, the use of peptide-based agents to target centrally located opioid receptors (ORs) is limited by poor physicochemical and pharmacokinetic (PK) properties,⁴ including low lipophilicity (logD), high topological polar surface area (TPSA), and extensive H-bonding with H₂O, which combined, lowers oral bioavailability and disfavors penetration of the blood-brain-barrier (BBB) *via* passive diffusion.^{5,6} Additionally, rapid proteolysis by endogenous peptidases provides a short half-life ($t_{1/2}$) that disfavors therapeutically relevant concentrations and/or tissue distribution.^{4,5,7,8} Thus for medicinal application of small peptides, the ability to rationally modify both the PK and physicochemical (PC) properties, and to facilitate entry into the central nervous system (CNS) remains an important goal. To address these challenges, fluorinated peptidomimetics (FPMs) might simultaneously modulate physicochemical [logD, TPSA, and HBD/HBA (Hydrogen-Bond Donors/Hydrogen-Bond Acceptors)] and metabolic properties of neuropeptides, and in turn, improve distribution.

As a prototypical example, Leu-enkephalin (**1**, Tyr-Gly-Gly-Phe-Leu), agonizes the δ -opioid receptor (DOR) with 1–5-fold binding affinity over the μ receptor and >1000-fold over the κ receptor.^{9,10} This pharmacological profile might be useful for treating chronic pain, inflammation, and cancer pain, while avoiding respiratory depression and addiction.¹¹ However, *in vivo* administration of Leu-enkephalin is limited by a poor PK profile, including rapid proteolysis of Tyr¹-Gly² by aminopeptidase N in human plasma,^{7,12} and of Gly³-Phe⁴ by angiotensin-converting enzyme at the BBB,⁸ both of which inhibit penetration into the CNS.⁵ To improve potency and selectivity, many cyclic and linear analogues of enkephalin have been developed that possess excellent affinities and selectivities for the DOR,^{9,13–15} and in some cases, the replacement of hydrolyzable amide bonds at various positions with peptidomimetics (e.g., *trans*-alkene,⁹ thioamide,¹⁶ ester,¹⁷ or *N*-methyl amide¹⁷) has modulated stability and PK properties, without major loss of agonist activity at the DOR. However, most of these peptidomimetic analogues either (a) still lack appropriate *in vivo* stability and CNS distribution to serve as the biological probes and/or therapeutic candidates, or (b) have not been assessed *in vivo*. To improve metabolic and distribution

properties of Leu-enkephalin, we report the use of Tyr¹- $\psi[(Z)CF=CH]$ -Gly² and Tyr¹- $\psi[CF_3CH_2-NH]$ -Gly² analogs (**2–4**), of which the former displays sufficient stability to enable systemic and CNS distribution from both intravenous (IV) and oral (PO) doses.

RESULTS AND DISCUSSION

Selection of Tyr¹–Gly²

To stabilize and improve distribution of Leu-enkephalin (**1**), we modified Tyr¹–Gly², which undergoes rapid proteolysis by aminopeptidase N (AP-N), thus providing an insufficient half-life to sustain therapeutically useful systemic and/or CNS concentrations (Figure 1A).^{5–8} Additionally, this site tolerates substitution,^{9,18–20} which suggests that other modifications of this amide linkage might be tolerated, such as fluorinated peptidomimetics (FPMs). Specifically, FPMs, including fluoroalkenes and β,β,β -trifluoroethylamines, should improve physicochemical and biophysical properties of target molecules, and should not be substrates for the endogenous peptidases. Fluoroalkenes possess similar dipole moments, electrostatic potential,²¹ steric, resonance, and inductive contributions to amides.^{22–24} However, the sigma-electron withdrawing effect of the fluorine atom decrease the basicity of neighboring functional groups, which makes these groups less prone to H-bond with H₂O and possibly the catalytic domains of metabolizing enzymes.^{19,25} As a result, this substitution should increase lipophilicity, and facilitate membrane permeability by passive diffusion.^{22,26} Trifluoroethylamines^{27,28} also match the polar nature of the amide, and can alter a probe by imparting greater lipophilicity, and thermal and proteolytic stability to a peptide.²⁹ For this substructure, the strong inductive effect of the CF₃ group renders neighboring amines sufficiently non-basic to remain neutral under physiological conditions, and thus, not alter the ionization state of the peptide. In addition, the strong electron-withdrawing CF₃ group acidifies the N–H group, thus increasing the H-bond donating capability of the trifluoroethylamine.³⁰ Finally, the conversion of the sp²-hybridized amide to the sp³-hybridized trifluoroethylamine can alter the conformational preference of the peptide. All combined, when judiciously applied to the appropriate location of a peptide, these FPMs should improve metabolism and distribution properties. In our previous work, Tyr¹- $\psi[(Z)CF=CH]$ -Gly² substitution provided a 5 nM DOR full agonist (**2**) with similar selectivity to the endogenous peptide though 60 fold less potent than the parent peptide (**1**), though both (*R*)- and (*S*)-isomers of Tyr¹- $\psi[CF_3CH_2-NH]$ -Gly² analogs (**3–4**) did not activate the DOR.³¹ Despite the decreased activities, the stability, metabolism, and distribution properties of all analogs were not previously assessed.

Physicochemical Perturbations Imparted by Tyr¹–Gly² FPMs

Substitution of Tyr¹–Gly² with FPMs moves computationally predicted physicochemical properties toward CNS drug-like space (Table 1).³² Specifically, replacement of the amide linkage with FPMs increased clogD 1.1–1.6 units, and decreased topological polar surface area (TPSA) by 17–29 Å². Replacement with Tyr¹- $\psi[(Z)CF=CH]$ -Gly² eliminated a hydrogen-bond donor and acceptor group, while substitution with Tyr¹- $\psi[CF_3CH_2-NH]$ -Gly² only reduced the hydrogen-bond acceptor count. Finally, substitution with FPMs increased overall molecular weight (MW), though considering the emerging concept of fluorine-adjusted molecular weight,³³ the FPM analogs might behave as smaller molecules

than the endogenous peptide (**1**). Further, β,β,β -trifluoroethylamine substitution at the Tyr¹-Gly² site increases the pK_a of the N-terminal amine one unit, while fluoroalkene substitution does not modulate the pK_a relative to **1**. Combined, these PC descriptors suggest that the FPM enkephalin analogs fit within orally available drug-space for therapeutic peptides,^{34,35} though outside of CNS drug-like space for small molecules that enter the CNS by passive diffusion.^{36–38}

FPM Analogs Demonstrate Excellent Media Stability and Solubility

All FPMs demonstrate excellent stability towards various solvents and assay media. All FPM analogs were stable over 48 h in PBS, simulated gastric fluid (pH = ~2), Captisol (200 mg/mL), 1:3 isopropanol:H₂O, 1:1 acetonitrile:H₂O. Thus, the investigational compounds should be stable in the acid environment of the stomach, as well as various solvents that might be useful for formulation or assay development. Further, all analogs displayed excellent solubility (>160 μ M) in PBS, SGF, Captisol, IPA:H₂O, MeCN:H₂O, which minimizes challenges for assay-development.

Tyr¹- ψ [(Z)CF=CH]-Gly² Substitution Improves Stability

As expected, fluoroalkene analog **2** also demonstrated excellent stability in mouse, rat and human plasma (Figure 2). In mouse plasma, both Leu-enkephalin (**1**) and **2** showed excellent stability (>90% of peptide remaining at 4 h). However, in this experiment, the intrinsic stability of the control peptide (**1**) suggested that this model is not appropriate for demonstrating the improved stability of **2**, which supports previous reports describing low expression of peptidases in mouse plasma.³⁹ However, due to interspecies differences in peptidase expression, Leu-enkephalin hydrolyzes more quickly in rat and human plasma samples.⁷ Thus, both human and rat plasma clearly demonstrated the improved stability imparted by Tyr¹- ψ [(Z)CF=CH]-Gly² substitution. In Sprague-Dawley rat plasma, 76% of **2** remained at 4 h, while $t_{1/2}$ for **1** was less than 5 min. In human plasma (female donor), 68% of **2** remained at 4 h, while $t_{1/2}$ for **1** was 12 min. These rat plasma half-lives compared favorably to Leu-enkephalin ($t_{1/2}$ = 4.6 min), as well as Phe⁴-OLeu⁵ ($t_{1/2}$ = 3.3 min)¹⁷ and Phe⁴-(NMe)Leu⁵ ($t_{1/2}$ = 10.7 min)¹⁷ analogs.

Similarly, the Tyr¹- ψ [(Z)CF=CH]-Gly² substitution stabilized Leu-enkephalin (**1**) to mouse, rat and human NADPH-charged microsomes (Figure 3). Using all three microsomal samples, **2** demonstrated excellent stability with <10% of the material metabolized at 2 h. In contrast, **1** was 38–50% metabolized by all microsomal samples at 2 h. In the absence of NADPH, **2** demonstrated no microsomal degradation, while **1** slowly degraded (see Supporting Information for more details).

Tyr¹- ψ [CF₃CH₂-NH]-Gly² Substitution Decreases Stability

Considering that Tyr¹-Gly² is the primary site of proteolysis,^{5,6,8,40} and that the trifluoroethylamine substructure is not subject to proteolytic metabolism, we expected both (*R*)- and (*S*)- analogs of Tyr¹- ψ [CF₃CH₂-NH]-Gly²-Leu-enkephalin (**3–4**) to increase stability towards proteolysis. However, **3** and **4** unexpectedly worsened stability in both plasma and microsomes (Figure 4). The main pathway of this degradation involved proteolysis of the Gly³-Phe⁴ linkage, as indicated by LC-MS analysis of the degradation

mixture. Thus, despite stabilizing Tyr¹-Gly², this substitution accelerated metabolism at the known secondary site of metabolism,⁵ possibly by forcing a conformational change in the peptide that facilitates recognition by an alternate protease. As a result of the poor stability, both **3** and **4** were not promoted for further *in vivo* studies.

Tyr¹-ψ[(Z)CF=CH]-Gly² Improves Distribution

The excellent *in vitro* stability of Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-enkephalin (**2**) also improved *in vivo* distribution and PK properties. Using a mouse model [BALB/c], plasma concentrations of a 50 mg/kg IV dose were detected for 2 h, while the Leu-enkephalin (**1**), was completely consumed within 5 min (Figure 5A). Additionally, **2** was orally bioavailable, with a 400 mg/kg PO dose providing maximum plasma concentrations of 28 ng/mL. Using the PO dose, plasma concentrations were detected up to 30 min. Despite the short half-life of 22 min, this compound provides a distinct strategy for designing orally bioavailable analogs of Leu-enkephalin. Critically, both IV and PO doses of Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-enkephalin (**2**) crossed the BBB (Figure 5B). By IV, a 50 mg/kg dose of **2** provided measurable levels (60–140 ng/mL tissue), which eliminated to undetectable levels within 20 min. This dose of 50 mg/Kg resides within the range of previous IV doses (30–60 mg/kg) of DPDPE, [*p*-Cl-Phe⁴]DPDPE, and [*p*-¹²⁵I-Phe⁴]DPDPE that demonstrated *in vivo* antinociceptive activity (hotplate) within 5 min in Male CD1 mice.⁴¹ Additionally, the PO dose (400 mg/kg) provided detectible concentrations of **2** (5–8 ng/mL tissue) at 5 min, though the compound was no longer detected by 10 min. In both cases, the detectible concentrations of Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-enkephalin show promise compared to the endogenous concentration of Leu-enkephalin (**1**) in the non-stressed mouse brain (4–40 ng/gm tissue, depending on region of the brain).⁴²

For **2**, maximum brain uptake was slightly lower than analogs of DPDPE and biphalin that have previously been explored *in vivo* (Table 2).^{41,43,44} Specifically, calculated brain uptake values for **2** of 0.008%/g tissue (IV) are within an order of magnitude of several related enkephalin analogs, including [³H] DPDPE^{41,43} (IV: 0.08%, Tail Vein Injection: 0.059–0.067%), [*p*-¹²⁵I-Phe⁴]DPDPE⁴¹ (Tail Vein Injection: 0.021–0.027%), and [¹²⁵I-Tyr¹]biphalin⁴⁴ (Tail Vein Injection: 0.0045–0.053%). Though, maximum brain concentrations of **2** peaked more quickly (5 min) than [³H] DPDPE⁴³ (60 min) and [¹²⁵I-Tyr¹]biphalin⁴⁴ (20 min), respectively. However, due to interspecies differences (Balb/c mice vs. CD1 mice vs. Sprague–Dawley rats), and differences in assays (LCMS vs. radioactivity) and injection strategies (IV vs. tail vein injection), direct comparisons of the data are not possible.

Additionally, a safety/dose-escalation study suggested engagement of centrally-located DOR. Specifically, convulsions are a side-effect associated with DOR agonists, which might derive from β-arrestin2 signaling,⁴⁵ and/or from rapid engagement of centrally localized DOR.^{45–47} At a 50 mg/kg IV dose, no side effects were observed, although higher doses induced brief seizures from which the animals recovered fully. These observations are consistent with DOR activation, though from this preliminary experiment, an appropriate safety window cannot yet be determined.

Potential Mechanisms of Membrane Transport

To demonstrate the mechanisms by which compound **2** entered systemic distribution and penetrated the BBB, standard PAMPA, Caco-2, and MDCK transwell assays were conducted. However, in these assays, minimal penetration in either the A→B or B→A directions discounted processes involving passive diffusion (see Supporting Information for more details).^{48,49} Further, considering that other enkephalin analogs are not substrates for P-gp,⁴⁹ we tentatively believe that **2** should also not be subject to efflux.

Combined, (a) the poor penetration in transwell assays, (b) the excellent plasma and microsomal stability, (c) the rapid *in vivo* plasma uptake and CNS penetration (PO dose), and (d) short *in vivo* plasma and CNS half-life in (IV and PO doses) might best be explained by an active bidirectional transport mechanism. At present, we suspect OATP or LAT might mediate transport of **2** across the BBB. OATP facilitates entry of DPDPE and some structurally modified Leu-enkephalin analogs into the CNS,^{50,51} while, LAT, which normally transports large amino acids (e.g. phenylalanine, tyrosine, and leucine), can also transport enkephalin analogs (e.g. biphallin and DPDPE).^{52,53} This bidirectional transport mechanism might provide opportunities for lower PO and IV doses while retaining similar CNS concentrations, particularly a high concentrations of **2** that might saturate the transporter. However, future work is required to determine the mechanisms of uptake for both oral availability and CNS distribution, and to generate new analogs with improved distribution properties, which would provide concrete justification for exploring lower doses.

CONCLUSIONS

In conclusion, Tyr¹- $\psi[(Z)CF=CH]$ -Gly² substitution of Leu-enkephalin increased plasma and microsomal stability sufficiently to provide an orally-active CNS-distributed peptide probe. Considering the improved stability and distribution, and 5 nM potency of this probe, future studies will evaluate *in vitro* signaling bias, *in vivo* anti-nociceptive and anti-alcohol abuse properties, and mechanism of transport. Combined, this data will provide a basis for designing potent, stable, long acting and CNS-distributed DOR-selective biased probes with improved safety profiles. In contrast, Tyr¹- $\psi[CF_3CH_2-NH]$ -Gly² substitution decreased stability by accelerating metabolism at the Gly³-Phe⁴ site, which combined with poor potency, limits future investigations of this probe.

METHODS

Leu-Enkephalin Analogs

Leu-enkephalin acetate salt (**1**) was purchased from Sigma Aldrich. Tyr¹- $\psi[(Z)CF=CH]$ -Gly²-Leu-enkephalin (**2**), Tyr¹- $\psi[(R)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**3**), and Tyr¹- $\psi[(S)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**4**) were synthesized according to previously reported procedures.³¹ Confirmation of the identity and purity of **2–4** was accomplished by a combination of ¹H, ¹⁹F, and ¹³C NMR, HRMS, and UPLC.

Microsomal Stability

Microsomal stability of Leu-enkephalin (**1**), Tyr¹- $\psi[(Z)CF=CH]$ -Gly²-Leu-enkephalin (**2**), Tyr¹- $\psi[(R)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**3**), and Tyr¹- $\psi[(S)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**4**) were monitored in vitro.^{54–56} Solutions of the compounds (0.50 μ M) were incubated with mouse, rat or human microsomes (0.50 mg/mL, Gibco) and two cofactors UDPGA (250 μ M, Sigma Aldrich) and NADPH (1 mM, Sigma Aldrich) in PBS at 37 °C. The compounds and microsomes, in the absence of co-factors, were prepared as negative controls. Samples were taken at predetermined time points, quenched with acetonitrile, and the parent compound was analyzed by LC-MS/MS. Samples were prepared in triplicate. Positive controls were set up with compounds known to be susceptible to mouse, rat, and human microsomal metabolism. These included positive controls as diphenhydramine (Sigma Aldrich) for mouse and rat microsomes, and verapamil (Sigma Aldrich) for human microsomes.

Plasma Stability

Stability of Leu-enkephalin (**1**), Tyr¹- $\psi[(Z)CF=CH]$ -Gly²-Leu-enkephalin (**2**), Tyr¹- $\psi[(R)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**3**), and Tyr¹- $\psi[(S)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**4**) were monitored in mouse, rat, or human plasma (Innovative Research) at 37 °C. Compounds were spiked into plasma to obtain a concentration of 50 μ M. At predetermined time points, samples were taken and quenched with acetonitrile, and the compounds were analyzed by LC-MS/MS. Samples were prepared in duplicate.

LCMS Analysis Method

Analysis was performed on Shimadzu 20A Series HPLC with binary pump, column oven, autosampler, controller and AB Sciex 3200 Qtrap mass spectrometer. A Zorbax SB-C18 column (2.1 mm $\times$ 50 mm, 5 μ m particle size) was used for the assay. The injection volume was 5 μ L. Mobile phase A consisted of 5/95/0.1 acetonitrile/water/formic acid and mobile phase B consisted of 100% methanol. The column was kept at 40 °C with a column oven and the autosampler temperature was set to 15 °C.

For Tyr¹- $\psi[(Z)CF=CH]$ -Gly²-Leu-enkephalin (**2**) and Leu-enkephalin (**1**), the initial flow rate was 0.6 mL/min and initial mobile phase B concentration was 10%. After a 0.25 min hold, the gradient was ramped from 10% to 65% B over 4.25 min; the column was washed at 98% B and re-equilibrated to 10% B prior to the next injection. These two compounds were used as internal standards for each other. For Tyr¹- $\psi[(Z)CF=CH]$ -Gly²-Leu-enkephalin (**2**) the mass transition from 557.3 $\rightarrow$ 120.2 was monitored with a collision energy of 50 eV ($\sim$ R_t 3.03 min); for Leu-enkephalin (**1**) the mass transition 556.2 $\rightarrow$ 397.2 was monitored with a collision energy of 28 eV ($\sim$ R_t 2.76 min). Additional MS/MS parameters include curtain gas (25), CAD (MED), IS (2500), TEM (600), GS1 (50), GS2 (60).

For Tyr¹- $\psi[(R)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**3**) and Tyr¹- $\psi[(S)-CF_3CH_2-NH]$ -Gly²-Leu-enkephalin (**4**), the initial flow rate was 0.5 mL/min and initial mobile phase B concentration was 25%. After a 0.25 min hold, the gradient was ramped from 10% to 60% B over 4.25 min; the column was washed at 98% B and re-equilibrated to 25% B prior to the next injection. These two compounds were used as internal standards for each other. For

both Tyr¹- $\psi[(R)\text{-CF}_3\text{CH}_2\text{-NH}]\text{-Gly}^2\text{-Leu-enkephalin}$ (**3**), and Tyr¹- $\psi[(S)\text{-CF}_3\text{CH}_2\text{-NH}]\text{-Gly}^2\text{-Leu-enkephalin}$ (**4**) the mass transition from 610.3 $\rightarrow$ 120.1 was monitored with a collision energy of 62 eV. The retention time for Tyr¹- $\psi[(S)\text{-CF}_3\text{CH}_2\text{-NH}]\text{-Gly}^2\text{-Leu-enkephalin}$ (**4**) was ~3.29 min and for Tyr¹- $\psi[(R)\text{-CF}_3\text{CH}_2\text{-NH}]\text{-Gly}^2\text{-Leu-enkephalin}$ (**3**) was ~3.06 min). Additional MS/MS parameters were identical as for Tyr¹- $\psi[(Z)\text{CF=CH}]\text{-Gly}^2\text{-Leu-enkephalin}$ (**2**) and Leu-enkephalin (**1**).

Dose Tolerance and Pharmacokinetic Studies

For the dose escalation and tolerance studies, Balb/c mice received a single IV or oral gavage dose of the compound. For the oral gavage dosing, a low dose of isoflurane was administered as a chemical restraint. The dose started at 1 mg/kg and was gradually escalated until drug related side effects were observed. The dose used in the pharmacokinetic studies was selected at a level just below where drug related side effects were observed. The mice used in the dose escalation studies were continuously monitored for 4 h and then euthanized using inhaled isoflurane to induce deep anesthesia followed by cardiac puncture. No mice exhibited severe drug related side effects.

For the pharmacokinetic studies, Balb/c mice were dosed by IV or oral gavage using a well-tolerated dose identified by the procedure above. Each mouse was used for one time point and received a single drug dose. Blood was collected at a pre-selected time point by cardiac puncture following deep anesthesia with isoflurane inhalation and exsanguination. Each time point required 3 mice and 10 time points for a total of up to 30 mice per study. Plasma was collected from each of the blood samples and assayed for the compound using a validated LCMS/MS procedure.

After death, the brains of the euthanized mice were perfused with PBS to remove as much blood as possible prior to harvesting the brains. A perfusion needle was inserted in the left ventricle of the heart and the right atrium was clipped to allow for the remaining blood from the mouse to be flushed out. Perfusion was done for approximately 20 min or until the liver became very pale and no blood was visible in the tissue around the eyes. The brain was ivory in color and had no visible signs of residual blood. The brain was harvested, and tissue was extracted. These extraction samples were assayed using a validated LCMS/MS procedure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACE Angiotensin Converting Enzyme

AP-N	Aminopeptidase N
BBB	blood-brain-barrier
CNS	central nervous system
DMPK	distribution, metabolic, and pharmacokinetic
DOR	δ -opioid receptor
DPDPE	[D-Pen ² ,D-Pen ⁵]-enkephalin
FPMs	fluorinated peptidomimetics
HAC	Heavy Atom Count
HBD	Hydrogen-Bond Donors
HBA	Hydrogen-Bond Acceptors
IPA	isopropanol
IV	intravenous
LAT	large neutral amino acid transporter
LCMS	Liquid Chromatography Mass Spectroscopy
logD	lipophilicity at pH 7.4
logP	lipophilicity
NADPH	nicotinamide adenine dinucleotide phosphate
OATP	organic-anion-transporting polypeptide
ORs	opioid receptors
PAMPA	parallel artificial membrane permeability assay
PC	physicochemical
PK	pharmacokinetic
PO	oral
PBS	phosphate buffered saline
TPSA	topological polar surface area
UPLC	Ultra-Performance Liquid Chromatography

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Leu-Enkephalin (1) **Problematic Physicochemical Properties**

- logP/logD • HAC • HBD/HBA
- TPSA • Proteolytic Stability

FMPs Analogs **Improved Properties**

- logP/logD • HAC • HBD/HBA
- TPSA • Proteolytic Stability

Figure 1. Hypothesis: FMPs Should Stabilize and Increase Distribution of Leu-Enkephalin (HAC = Heavy Atom Count, HBD = Hydrogen-Bond Donors, HBA = Hydrogen-Bond Acceptors, TPSA = Topological Polar Surface Area, ACE = Angiotensin Converting Enzyme, AP-N = Aminopeptidase N)

Figure 2. Tyr¹-ψ[(Z)CF=CH]-Gly² Substitution Improves Plasma Stability of Leu-Enkephalin
(■) = Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-Enkephalin (●) = Leu-Enkephalin

Figure 3. Tyr¹-ψ[(Z)CF=CH]-Gly² Substitution Improves Microsomal Stability
(■) = Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-Enkephalin, (●) = Leu-Enkephalin, (▲) = Diphenhydramine (Mouse and Rat) or Verapamil (Human).

Figure 4. Tyr¹-ψ[CF₃CH₂-NH]-Gly² Substitution Worsens Plasma and Microsomal Stability
 (●) = Leu-Enkephalin (1), (●) = Tyr¹-ψ[(R)-CF₃CH₂-NH]-Gly²-Leu-enkephalin (3), (●) = Tyr¹-ψ[(S)-CF₃CH₂-NH]-Gly²-Leu-enkephalin (4), (●) = Diphenhydramine

Figure 5. Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-Enkephalin Is Orally Available and Distributes into the CNS

(♦) = IV 50 mg/kg Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-Enkephalin, (▲) = PO 400 mg/kg Tyr¹-ψ[(Z)CF=CH]-Gly²-Leu-Enkephalin

Table 1

Physicochemical Descriptors for FPM Analogs of Leu-Enkephalin.

Compound	Structure	clogD ^a	clogP ^a	MW [Da]	F-Adj MW	TPSA [$\text{\AA}^2$] ^a	HBD	HBA	pK _a ^a
1		-2.3	-2.3	555	555	204	6	7	7.7
2		-1.2	-1.2	556	537	175	5	6	7.7
3		-0.7	0.7	609	552	187	6	7	8.7
4		-0.7	0.7	609	552	187	6	7	8.7
–	CNS-MPO ^{36,38} (Sm. Molecules)	2–4	3–5	360–500	–	40–90	0–4	–	8–10

Compound	Structure	PO Peptides ³⁴ 80 th Percentile	clogD ^a	clogP ^a	MW [Da]	F-Adj MW	TPSA [$\text{\AA}^2$] ^a	HBD	HBA	pK _a ^a
-				-	<5.1	<1,217	-	<301	<9	<23	-

^aCalculated using MarvinSketch (<https://www.chemaxon.com/products/calculators-and-predictors>).

(CNS-MPO = Central Nervous System Multiparameter Optimization, PO Peptides = Calculated percentiles of main descriptors for oral peptide drugs, clogD = calculated lipophilicity in pH 7.4 buffer, clogP = calculated lipophilicity, MW = Molecular Weight, F-Adj MW = Fluorine Adjusted Molecular Weight, TPSA = Topological Polar Surface Area, HBD = Hydrogen-Bond Donors, HBA = Hydrogen-Bond Acceptors, pK_a = acid dissociation constant)

Table 2

Maximum Brain Concentrations of 2 Compared to Other Reported Leu-Enkephalin Analogs.

Entry	Peptide	Time Points (min)	Brain Concentrations (% uptake/g tissue)	Comments
1	2	5, 15	IV: 0.008% (5 min)	a. Balb/c mice b. Dose: 50 mg/kg IV
2	[³ H] DPDPE ⁴³	60, 120, 240	IV: 0.08% at 60 min (maximum)	a. Male CD1 mice b. Dose: 1.5–2.0 μCi IV
3	[³ H] DPDPE ⁴¹	5, 10, 20, 40	0.059–0.067%	a. Male CD1 mice b. Tail vein injection
4	[<i>p</i> - ¹²⁵ I Phe ⁴]DPDPE ⁴¹	5, 10, 20, 40	0.021–0.027%	a. Male CD1 mice b. Tail vein injection
5	[¹²⁵ I-Tyr ¹]biphalin ⁴⁴	5, 20, 40	(0.0045–0.053%)	a. Adult Sprague—Dawley rats b. Dose: 10 μCi Tail vein injection