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NEW POTENT ENKEPHALIN ANALOGS CONTAINING TRIFLUOROMETHYL-AMINO ACID RESIDUES

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Abstract: *A series of new methionine-enkephalin analogs containing trifluoronorvaline (TFNV) or trifluoronorleucine (TFNL) in place of Gly^2 or Gly^3 are synthesized. The new analogs bearing (D)-TFNV in place of Gly^2 exhibit ca. 100,000 times enhancement in in vivo analgesic activity in comparison with methionine-enkephalin. The in vitro binding assays for mu, delta and kappa receptors reveal that this enhancement is not based on the much stronger binding to these receptors, but mainly due to the extremely efficient inhibition of degradation by aminopeptidase(s).*

Extensive studies have been performed on structurally modified enkephalins in the hope of developing non-toxic, non-addictive, and effective analgesics, replacing morphine, and a variety of analogs have been developed, including those with potencies much greater than the parent enkephalin as well as morphine.[1] Neurobiological studies have revealed the presence of the major opiate receptors *mu*, *delta* and *kappa* which mediate the analgesic effects of opiates and opioid peptides including enkephalins,[2] and receptor specific ligands are of active current interest in medicinal chemistry as well as neurobiology.

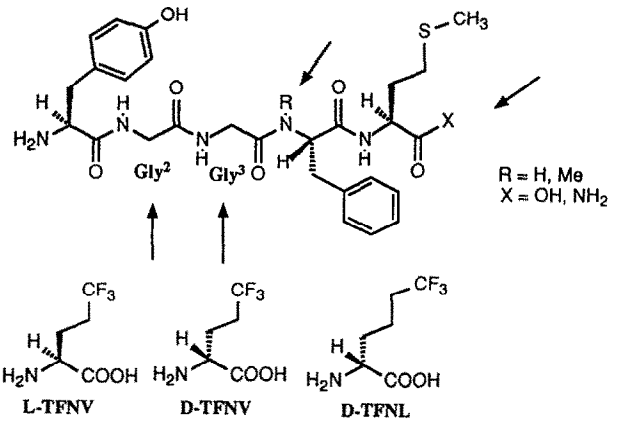
The major nemesis of these opioid peptides is a series of degrading enzymes, which cleave the peptide into inactive fragments.[3] These enzymes include (i) aminopeptidase M and membrane-bound aminopeptidase(s), cleaving enkephalins at the Tyr^1-Gly^2 bond, which is responsible for 80% of the degradation pathway,[3b,4] (ii) enkephalinase, cleaving the Gly^3-Phe^4 bond,[3a] and (iii) dipeptidylaminopeptidase, cleaving the Gly^2-Gly^3 bond.[3b] Besides these degrading enzymes in the brain, carboxypeptidases degrade enkephalins from their carboxyl termini, especially when the peptides are administrated orally, intravenously or subcutaneously.[3b] Accordingly, the development of inhibitors for these degrading enzymes is another important approach to the enhancement of analgesic activity by increasing the duration of the effect. In addition to these, other equally important pharmacological requirements such as improved distribution and transport properties should be taken into account in order to obtain more potent analogs.

In the course of our study on the synthetic and medicinal chemistry of trifluoromethyl-containing amino acids, enzyme inhibitors and peptide hormones by exploring unique effects of trifluoromethyl group,[5] we designed and synthesized a series of new enkephalin analogs bearing 5,5,5-trifluoronorvaline (TFNV) and 6,6,6-trifluoronorleucine (TFNL), and their *in vivo* analgesic activity as well as *in vitro* receptor binding ability examined. In spite of extensive studies on enkephalin analogs, little attention has been paid to the fluoro-analogs of enkephalins.[6] Since it has been shown that trifluoromethyl group is one of the most lipophilic

substituents, it was originally expected that the incorporation of trifluoromethyl-amino acid residue would greatly enhance the hydrophobic binding of such enkephalin analogs to receptors as well as transportation ability.[7] In fact, a couple of analogs were found to exhibit extremely strong analgesic activity *in vivo*. We communicate here the results of our SAR study on the new fluoro-enkephalin analogs and discuss the origin of the observed remarkable enhancement in potency (100,000 times with regard to parent methionine-enkephalin).

Synthesis of new fluoro-enkephalin analogs. As shown in Scheme 1, a series of new enkephalin analogs were synthesized by replacing either Gly^2 or Gly^3 by D-TFNV, L-TFNV or D-TFNL. An enkephalin amide analog bearing (N-Me)Phe^4 in place of Phe^4 was also synthesized. Enantiomerically pure D- and L-TFNV and D-TFNL were prepared through enzymic resolution of the corresponding racemic *N*-Ac-TFNV and *N*-Ac-TFNL using acylase I, which were obtained from 4,4,4-trifluorobutanal via amidocarbonylation and Erlenmyer's azlactone method, respectively, by following the procedures reported previously from this laboratory.[5a] Those new fluoro-enkephalin analogs were obtained through solid-phase peptide synthesis using *N*-Fmoc-amino acid pentafluorophenyl esters on a Du Pont's RaMP™ system.

Scheme 1



Analgesic activity assay in vivo. The *in vivo* analgesic activity of new fluoro-enkephalin analogs was evaluated by the standard writhing test using ddY male mice (5 weeks old; average weight 26 g). Thus, the mice were given 0.7% acetic acid by an intraperitoneal injection, followed by intracerebroventricular (i.c.v.) administration of fluoro-enkephalin analog, and writhing reaction was observed to evaluate the analgesic activity of the peptide in comparison with the mice in the control group. Results are summarized in Table 1. As Table 1 shows, the substitution of Gly^2 by D-TFNV exhibited a remarkable increase in potency, i.e., 100,000 times stronger than methionine-enkephalin, and even one order of magnitude stronger than morphine (Entry 8). The configuration of TFNV at this position is very important, thus [L-$TFNV^2$, Met^5-NH_2]enkephalin shows only ca. 30 times increase in potency (Entry 7). The substitution of Gly^3 by TFNV improves potency by the factor of 30-60, in which the D-TFNV analog is twice as better as the L-TFNV analog (Entries 10,11). The modification of carboxyl terminus to amide shows 5-6 times improvement in potency (Entries 5,6,7,10). The substitution of Gly^2 by D-TFNL, a homolog of D-TFNV, exhibits 10,000 times increase in potency, but it is one order of magnitude lower than the corresponding D-TFNV analog (Entry 12).

Table 1. *In vivo* analgesic activity of fluoro-enkephalin analogs (i.c.v.)

Entry	Enkephalins	$ED_{50}(10^{-9}$ mol/mouse)
1	Methionine-Enkephalin	700
2	(Morphine.HCl)	0.07
3	Tyr-(D)Ala-Gly-Phe-Met-NH_2	0.05
4	Sedapain™ (Morphine analog)	0.05
5	Tyr-(L)TFNV-Gly-Phe-Met	120
6	Tyr-Gly-(L)TFNV-Phe-Met	140
7	Tyr-(L)TFNV-Gly-Phe-Met-NH_2	25
8	Tyr-(D)TFNV-Gly-Phe-Met-NH_2	0.007
9	Tyr-(D)Nval-Gly-Phe-Met-NH_2	0.04
10	Tyr-Gly-(L)TFNV-Phe-Met-NH_2	22
11	Tyr-Gly-(D)TFNV-Phe-Met-NH_2	12
12	Tyr-(D)TFNL-Gly-Phe-Met-NH_2	0.07
13	Tyr-(D)TFNV-Gly-(N-Me)Phe-Met-NH_2	0.002

The observed remarkable increase in potency for [D-TFNV^2, Met^5-NH_2]enkephalin is not necessarily totally exceptional since a known analog, [D-Ala^2, Met^5-NH_2]enkephalin, shows 10,000 increase in potency in the same *in vivo* assay (Entry 3). In order to assess a "fluorine effect" on potency, [D-Nval^2, Met^5-NH_2]enkephalin (D-Nval = D-norvaline) was synthesized and assayed. As Entry 9 shows, this analog exhibits almost equivalent potency as the D-Ala^2 analog which is one order of magnitude *weaker* than the D-TFNV^2 analog. *Accordingly, it is clear that there is such a "fluorine effect", which can improve the potency by a half order of magnitude even after major enhancement factor, i.e., D-amino acid residue at Gly^2 position, is introduced.* The most potent analog in this series is [D-TFNV^2, (N-Me)Phe^4, Met^5-NH_2]enkephalin (Entry 13), which is ca. 4 times more potent than [D-TFNV^2, Met^5-NH_2]enkephalin.

Receptor binding assay in vitro. In order to investigate the origin of the remarkable enhancement in potency by the introduction of D-TFNV at the Gly^2 position, the *in vitro* receptor binding assays for [D-TFNV^2, Met^5-NH_2]enkephalin were carried out against *mu*, *delta*, and *kappa* receptors using tritium labeled standard ligands.[2] Results are summarized in Table 2. As Table 2 shows, [D-TFNV^2, Met^5-NH_2]enkephalin exhibits the IC_{50} of 10^{-10}M level against *mu*-receptor, which is only a half order of magnitude enhancement in the binding ability compared with methionine-enkephalin. For *delta*-receptor, [D-TFNV^2, Met^5-NH_2]enkephalin shows almost the same level binding ability as methionine-enkephalin. Interestingly, [D-TFNV^2, Met^5-NH_2]enkephalin binds to *kappa*-receptor with the IC_{50} of 10^{-7}M level, whereas methionine-enkephalin does not show any appreciable binding.

The results clearly indicate that the observed remarkable enhancement in *in vivo* potency of [D-TFNV^2, Met^5-NH_2]enkephalin is not based on much stronger binding to receptor sites, but mainly due to the extremely efficient inhibition of degradation by aminopeptidase(s). Possible enhancement of the rates of absorption and transport, arising from the lipophilicity of trifluoromethyl group should also be taken into account as the secondary effect. The fact that [D-TFNL^2, Met^5-NH_2]enkephalin shows one order of magni

Table 2. *In vitro* receptor binding assay for fluoro-enkephalin analogs

Enkephalin	Receptor	Tissue	Ligand[a]	IC_{50} (nM)
[D-TFNV^2, Met^5-NH_2]enkephalin	mu	cerebrum[b]	[^{3}H]-PL-017	0.5
Methionine-enkephalin	mu	cerebrum[b]	[^{3}H]-PL-017	2
[D-TFNV^2, Met^5-NH_2]enkephalin	delta	cerebrum[b]	[^{3}H]-DPDPE	2
Methionine-enkephalin	delta	cerebrum[b]	[^{3}H]-DPDPE	1
[D-TFNV^2, Met^5-NH_2]enkephalin	kappa	cerebellum[c]	[^{3}H]-U-69593	400
Methionine-enkephalin	kappa	cerebellum[c]	[^{3}H]-U-69593	>10,000

[a] [^{3}H]-PL-017 = [^{3}H]Tyr-Pro-(N-Me)Phe-(D)Pro-NH_2; [^{3}H]-DPDPE = [^{3}H][(D)Pen^2, (D)Pen^5]enkephalin; [^{3}H]-U-69593 = [^{3}H](5α,7α,8β)-(-)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide. [b] rat. [c] guinea pig.

tude weaker activity than [D-TFNV^2, Met^5-NH_2]enkephalin may indicate a limit of tolerance either at the receptor or at the binding site of aminopeptidase(s) provided that this peptide acts as an enzyme inhibitor.

It should be noted that [D-TFNV^2, Met^5-NH_2]enkephalin exhibits ED_{50} of 0.1 μM/mouse by intravenous administration and that of 0.12 μM/mouse by subcutaneous administration, which indicates that [D-TFNV^2, Met^5-NH_2]enkephalin can cross the blood-brain barrier.

Further studies on the design and synthesis of newer fluoro-enkephalin analogs as well as their SAR studies are actively underway.

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