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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF POSITION ONE AND THREE TRANSPOSED ANALOGS OF THE OPIOID PEPTIDE YKFA

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Abstract: Tyr-c[D-Lys-Phe-Ala], YKFA, is a potent opioid peptide analog with subnanomolar IC_{50}s toward mu and delta receptors. Transposing Phe and Tyr, a modification found to promote mu antagonist activity in opioid/somatostatin hybrids, gave surprisingly high mu agonist activities for several related analogs, considering the lack of a 1-position hydroxyl function.
Keywords: Agonists; analgesics; peptides and polypeptides; receptors

Since the discovery of the enkephalins,[1] endogenous peptides that bind to and activate each of the three types of opioid receptors (μ, δ, and κ) have been isolated and pharmacologically characterized.[2,3] In the years since the first discovery of endogenous opioid peptides, there has been considerable interest in the development of alternative analgesics based upon these peptide leads, which would lack the side effects of the opioids that are in clinical use today. In the search for alternative analgesics based upon peptide lead structures it is necessary to make structural modifications in order to address some of the problems associated with the use of peptides as drug candidates, but which are still compatible with the desired biological activity. For instance, peptides may exhibit a lack of resistance to proteolytic degradation, limited bioavailability, and lack of receptor subtype selectivity.

One of the most common strategies that has been employed to improve upon the biological activities and pharmacokinetic properties of peptides has been the use of conformational constraints.[4] Conformational constraints (especially cyclizations) have been applied extensively in the field of opioid peptides to produce compounds that have longer biological half-lives, increased affinity, and increased selectivity for opioid receptors.[4,5] In 1980, Schiller and DiMaio reported the first cyclic enkephalin analog,[6] Tyr-c[D-A_2bu-Gly-Phe-Leu]. This compound was 17-fold more potent than leucine enkephalin in the μ selective guinea pig ileum (GPI) assay and 7-fold less potent in the δ selective mouse vas deferens (MVD) assay. The resulting μ opioid selectivity was shown to be a consequence of the conformational constraint imposed by the side chain to C-terminus cyclization. Systematic variation of the ring size in this cyclic peptide resulted in increased selectivity for the μ opioid receptor, with the D-Lys^2 analog being approximately 5-fold more μ selective than the parent D-A_2bu containing compound.[7] Mosberg et al.[8] reported the development of a potent δ selective enkephalin analog through the use of a disulfide bridge in Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE). DPDPE is 3000-fold more potent at the δ opioid receptor than at the μ receptor as a result of the conformational constraint induced by the

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disulfide bridge and the *geminal* dimethyl groups of the penicillamine residues. Thus, cyclization has provided a powerful means for the development of both μ[6,7] and δ[8] opioid selective peptides derived from the same parent, leucine enkephalin.

First reported in 1991,[9] Tyr-c[D-Lys-Phe-Ala], or YKFA (Figure 1), is a super potent agonist at both μ and δ opioid receptors. This molecule has been conformationally constrained by virtue of cyclization between the side chain ε-amine of D-lysine in position two and the C-terminus, as well as the removal of the flexible glycine spacer present in peptides such as DPDPE. Even though the small 13-membered ring system of YKFA is constrained by cyclization, this compound is capable of facile interconversion between the two conformations required for bioactivity at the μ and δ opioid receptors[10] as evidenced by the fact that YKFA exhibits subnanomolar potencies in both bioassays. Due to its subnanomolar potency at both receptors, however, YKFA is non-selective.

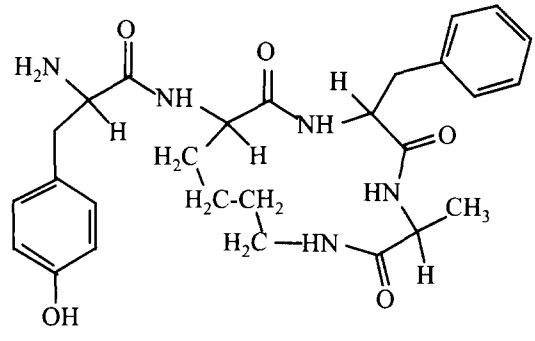


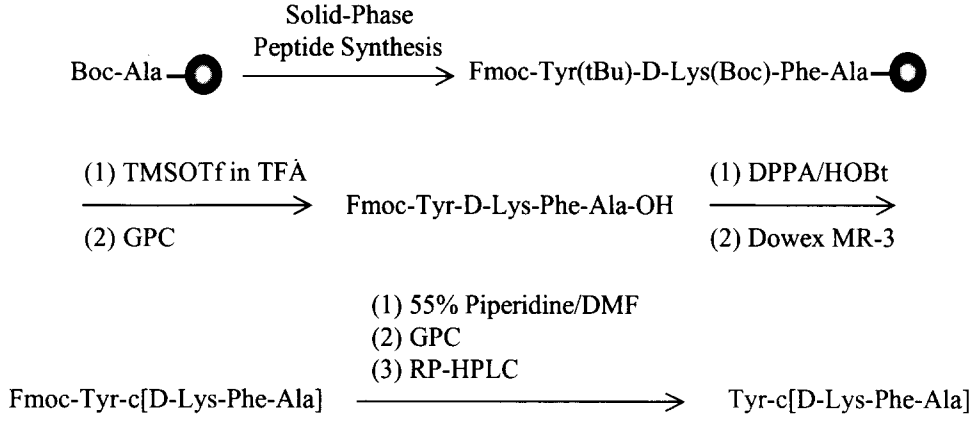
Figure 1. Structure of Tyr-c[D-Lys-Phe-Ala] (YKFA)

In this study we describe the synthesis and biological activities of four new analogs of YKFA in which the usual Tyr[1]-Phe[3] combination found in opioid peptides such as dermorphin and deltorphin has been transposed. The rationale for this modification is based upon the observation that the D-Phe[1]-Tyr[3] combination found in the somatostatin analogs of Pelton et al.[11] results in compounds which display high affinity for μ opioid receptors, and a large degree of selectivity for μ opioid receptors over δ opioid receptors and somatostatin receptors. Furthermore, these compounds, typified by D-Phe-c[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2 (CTOP) are antagonists at the μ opioid receptor.[12] Therefore, we have synthesized and assayed these new compounds in an effort to modulate the selectivity and/or intrinsic activity of YKFA.

Peptide Synthesis and Purification

The compounds in this study were synthesized through a combination of solid phase synthesis of a protected linear peptide and solution-phase cyclization (Scheme 1). Protected linear peptides were synthesized

by stepwise elongation on Merrifield resin, using both Boc and Fmoc strategies, with BOP/HOBt as the condensing agent. Cleavage of the Fmoc protected linear peptides from the resin using anhydrous HF at room temperature led unexpectedly to the removal of the Fmoc protecting group. Due to the premature loss of the N-α protecting group, an alternative cleavage method employing trimethylsilyl trifluoromethanesulfonate (TMSOTf) in TFA[13] was attempted, providing good yields of the desired N-α-Fmoc protected linear peptides in excellent purities.



Scheme 1. Synthesis of YKFA and Analogs.

Linear peptides were cyclized in solution using diphenylphosphoryl azide[14] under conditions of high dilution and low temperature in order to favor the intramolecular cyclization reaction over intermolecular dimerization. The progress of the cyclization reactions was monitored using analytical reverse-phase, high-performance liquid chromatography (RP-HPLC) and found to be complete within two to three days. After treatment with Dowex® MR-3 (an equimolar mixture of strong cation and strong anion exchange resin), the N-terminal Fmoc protecting group was cleaved with piperidine in DMF and the final compounds were purified using a combination of gel permeation chromatography (GPC) and semi-preparative RP-HPLC. The purity of the final compounds was assessed using analytical RP-HPLC with monitoring at both 220 and 254 nm and thin layer chromatography (TLC) in three different solvent systems. The structural integrity was confirmed by amino acid analysis (AAA) and electrospray mass spectrometry (ES-MS) in the positive mode, which showed that the experimental molecular weight was in agreement with the calculated molecular weight for each peptide. In addition, each compound exhibited an NMR spectrum that was in all cases consistent with the proposed structure.

Results and Discussion

The opioid activities of the compounds that have been synthesized in this study were determined by their abilities to inhibit the electrically induced contractions of smooth muscle preparations.[15] Specifically, activity at

the μ opioid receptor was measured using the guinea pig ileum (GPI) and the mouse vas deferens (MVD) was used to measure δ opioid activity. The ratio of the IC_{50}s in the two assays, $IC_{50}(\delta)/IC_{50}(\mu)$, is taken as a measure of the selectivity of the compounds. The results of the in vitro bioassays are listed in Table 1 and clearly show these compounds to be agonists at both the μ and δ opioid receptors, though their potencies vary over a wide range. As shown previously[9] YKFA is a super potent agonist at both receptors, displaying subnanomolar IC_{50}s in both the GPI and the MVD. As a result of its high degree of potency in both assays, YKFA shows no clear preference for either receptor, as reflected in its low selectivity ratio ($IC_{50}(\delta)/IC_{50}(\mu) = 4.9$).

Table 1. Bioassay Results of YKFA and Phe[1] Substituted Analogs.

Peptide	IC_{50} ± SEM (nM)		Selectivity
	GPI (μ)	MVD (δ)	$IC_{50}(\delta)/IC_{50}(\mu)$
1. Tyr-c[D-Lys-Phe-Ala]	0.11 ± 0.013	0.54 ± 0.031	4.9
2. D-Phe-c[D-Lys-Tyr-Ala]	1827 ± 58	9327 ± 2829	5.1
3. Phe-c[D-Lys-Tyr-Ala]	30.4 ± 8.6	367 ± 27	12.1
4. D-Phe-c[D-Lys-Tyr-Trp]	627 ± 124	8129 ± 1438	13.0
5. Phe-c[D-Lys-Tyr-Trp]	17.7 ± 1.9	228 ± 62	12.9

The four compounds (**2-5**) containing the Phe^1-Tyr^3 combination retained opioid agonist activity, the magnitude of which was dependent upon the chirality of the phenylalanine in position one. While the somatostatin based μ-antagonists of Hruby et al. contain a D-Phe in position one[11] and lose all activity when the D-Phe is replaced with L-Phe,[16] compounds **2-5** here show the opposite structure/activity relationship. Thus, the potency of D-Phe-c[D-Lys-Tyr-Ala] (**2**) is increased 60-fold in the GPI assay, and 25-fold in the MVD, upon substitution of the D-Phe with L-Phe (compound **3**). This larger increase in activity at the μ receptor results in a selectivity ratio for **3**, which is more than twice that of the D-Phe containing compound **2**. Similarly, the replacement of the D-Phe in position one of D-Phe-c[D-Lys-Tyr-Trp] (**4**) with L-Phe (**5**) results in a 35-fold increase in μ receptor activity, and an identical increase in δ receptor activity. Though the similar increase in activity at both receptor types leads to a compound with the same selectivity ratio, the change in chirality of the phenylalanine in position one provides a moderately potent μ-receptor agonist ($IC_{50}(\mu)$ = 18 nM). The substitution at the C-terminus of tryptophan (**5**) for alanine (**3**), designed in order to make the primary sequence more closely resemble that of the μ-antagonists of Hruby et al., increased the opioid activity to a similar extent at both receptors. Thus, the tryptophan containing compound **5** is almost twice as potent in the GPI as the alanine containing analog **3**. Due to a similar increase in activity in the MVD, these two analogs display an almost

identical selectivity ratio, each of them being approximately 2.5-fold more selective for the μ opioid receptor than the parent YKFA.

The chirality effect of the phenylalanine in position one (**2** vs **3**, **4** vs **5**) mirrors that of traditional opioid peptides, in that there is a clear preference for the L-amino acid in this position. Furthermore, the somatostatin-based peptides are antagonists at the μ receptor while YKFA and analogs are clearly agonists. Taken together, the data indicate that the Phe[1] substituted analogs of YKFA are probably interacting with opioid receptors via traditional modes of binding and signal transduction, and not through a novel mechanism such as the one proposed for the somatostatin based μ antagonists.[16]

The loss of activity for the phenylalanine containing compounds compared with the parent YKFA is not surprising due to the well-known propensity of linear opioid peptides to exhibit dramatic losses in activity upon substitution of phenylalanine for tyrosine. For example, it was quickly discovered that [Phe[1]]-Met-enkephalin was 500-fold less potent in the GPI and 3300-fold less potent in the MVD than Met-enkephalin.[17] Therefore, the drop in potency for the Phe[1] substituted analogs of YKFA in this study is in keeping with the normal structure-activity relationships of opioid peptides in which the N-terminal tyrosine residue has been replaced with phenylalanine.[5]

It is interesting to note that compounds **3** and **5** are among a small group of opioid peptides that retain significant opioid activity after the loss of the N-terminal phenolic hydroxyl group. Though the hydroxyl group of the tyrosine in position one has long been thought to be indispensable for opioid activity,[5] there is precedence for retention of significant opioid activity in Phe[1] substituted analogs. In particular, early work by Schiller and DiMaio resulted in two cyclic disulfide compounds which retained moderate potency at the μ receptor (IC_{50}(GPI) = 13 nM for Phe-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]-NH_2 and 49 nM for Phe-c[D-Cys-Gly-Phe-D-Cys]-NH_2) even after the loss of the side chain hydroxyl in position one.[18] Mosberg et al.[19] recently reported the binding affinities for the Phe[1] analog of the μ selective opioid peptide JOM-6 (Tyr-c[D-Cys-Phe-D-Pen]-NH_2) which is cyclized via an ethylene dithioether bridge. The new compound, Phe-c[D-Cys-Phe-D-Pen]-NH_2 (JH-54), was shown to retain high affinity for the μ receptor in a binding assay (K_i = 1.36 nM vs 0.29 nM for JOM-6). Additionally, JH-54 was still capable of activation of the μ receptor, as evidenced by its IC_{50} (9.1 nM) in the GPI bioassay. The significant retention of μ activity was ascribed to a mode of binding in which the peptide shifted within the μ receptor binding pocket in such a way as to minimize the contribution of the hydrogen bond between the phenolic oxygen of Tyr[1] and its presumed hydrogen bond donor (His[297] from the previously developed model of the μ opioid receptor[20]). This implies that the overall contribution of this interaction to opioid ligand binding and signal transduction may not be as important as has previously been believed. The structural similarities between JH-54 and compounds **3** and **5** raises the possibility that they are binding to the μ opioid receptor in a similar manner, and that a shift in the mode of binding similar to that described by Mosberg et al. may be operative in the case of μ receptor binding and activation for these compounds.

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