Design of μ selective opioid dipeptide antagonists

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Abstract We have recently designed potent δ selective opioid antagonist dipeptides on the basis of a simple conformational analysis. Following a similar procedure we found a μ selective dipeptide antagonist, 2,6-dimethyl-Tyr-D-Phe-NH_2. Although its selectivity is not as high as those of the quoted δ selective dipeptides it has good in vitro activity and looks very promising for further development since the 2,6-dimethyl-Tyr-D-Phe message, like the δ selective 2,6-dimethyl-Tyr-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid counterpart, seems able to impart antagonism to longer peptides.

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1. Introduction

Opioid antagonists are generally obtained by substitution of a proton of the basic nitrogen of the tyramine moiety, common to all opioids, with an appropriate substituent, generally an allyl or a cyclopropylmethyl group. The first exception to this rule was furnished by the discovery [1] of two δ selective peptide antagonists related to dermorphin but containing L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) in the second position: Tyr-Tic-Phe-NH_2 (dubbed TIP) and Tyr-Tic-Phe-Phe-NH_2 (dubbed TIPP).

On the basis of a simple conformational analysis [2] we have shown that the antagonism displayed by all [Tyr-Tic^2] peptides can be attributed to the relative arrangement of the two aromatic rings of Tyr and Tic, i.e. to a specific two-residue message domain represented by the sequence Tyr-Tic. The spatial relationship of these rings is very similar to the characteristic 90° arrangement assumed by the corresponding rings of several δ selective naltrindole derivatives described by Portoghese and coworkers [3]. Our interpretation led to the synthesis of the first opioid dipeptide, Tyr-Tic-NH_2 [2], and to a series of simple and very potent δ selective antagonists containing the related 2,6-dimethyl-Tyr (Tyr(Me)$_2$) instead of Tyr [4]. In fact, Tyr-Tic-NH_2 itself behaves as a δ selective antagonist [2] and even more significantly introduction of Tic in the second position of the sequence converts enkephalin, a non-selective agonist, and dermorphin (a μ selective agonist) into δ selective antagonists [5]. The view of a specific 'antagonist message' was subsequently substantiated by the discovery of ultraselective δ antagonists containing the Tyr(Me)$_2$-Tic message [4,6] and by the design of a rigid antagonist lacking the basic charge of tyramine [7].

The conformations of [Tyr-Tic] and/or [Tyr(Me)$_2$-Tic] peptides are greatly influenced by the conformational preferences of Tic. Since the amide nitrogen is part of a six-membered ring similar to that of pipecolic acid, the populations of conformers containing a *cis* peptide bond are comparable to those of conformers containing *trans* peptide bonds. In addition, the Tic side chain conformation is confined to the single value of χ^1 imposed by cyclization, a circumstance drastically different from the conformational freedom typical of aromatic amino acid residues. Thus, the conformations accessible to Tyr-Tic peptides, notably the C1b+ *cis* conformer we previously identified [2] as a likely δ selective bioactive conformation, are forbidden or severely disfavored for opioid peptides containing an aromatic amino acid residue in the second position.

We decided to explore the conformational preferences (and possible biological activity) of peptides containing a aromatic amino acid residue (Xaa) in the second position, i.e. Phe, Tyr, Trp or His, since it is likely that other relative arrangements accessible to Tyr-Xaa, although not ideal for δ selectivity, may confer to the peptides good opioid activity and a different selectivity. The starting choice of this systematic search was Phe for the second residue (Xaa), because Tic was originally derived from this amino acid residue. Instead of Tyr we decided to use Tyr(Me)$_2$ as the first residue for all peptides since it ensures a far better binding without departing too much from the constitution of the parent residue (Tyr).

Based on a detailed conformational analysis of the model dipeptides Tyr(Me)$_2$-Phe-$NHCH_3$ and Tyr(Me)$_2$-D-Phe-$NHCH_3$ that cover the entire range of chiral isomers corresponding to Tyr(Me)$_2$-Phe, here we present the synthesis and biological properties of new μ selective opioid peptide antagonists characterized by a message domain of only two residues and by the absence of derivatization on the basic nitrogen.

2. Materials and methods

Tyr(Me)$_2$ (2′,6′-dimethyl-L-tyrosine) was a generous gift of J.H. Dygos (Searle and Co.). The racemic mixture of Tyr(Me)$_2$ was prepared as described in [8]. All peptides were prepared by solid phase peptide synthesis with a Milligen 9050 synthesizer using a (4-(2′,4′-dimethoxyphenyl)-Fmoc-aminomethyl-phenoxy resin (Rink amide

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Abbreviations: CNX, 6*N*-cinnamoyl-β-naltrexamine; DAGO, Tyr-D-Ala-Gly-NMePhe-Gly-ol; DPDPE, cyclo[-D-Pen2,D-Pen5-]enkephalin; EM, energy minimization; GPI, guinea pig ileum; MeNTI, *n*-methyl naltrindole; ICI 199441, 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-phenyl-2-(1-pyrrolidinyl)ethyl] acetamide; MM, molecular mechanics; MVD, mouse vas deferens; RJ, rabbit jejunum; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP, Tyr-Tic-Phe-NH_2; TIPP, Tyr-Tic-Phe-Phe-NH_2; Tyr(Me)$_2$, 2,6-dimethyl-Tyr

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MBHA resin) (0.55 mmol/g; 0.2 g in all syntheses) (Novabiochem AG). The resin was mixed with glass beads (1:15 w/w) (Sigma). Peptides were assembled using Fmoc protected amino acids (4-fold excess) (Novabiochem AG), 1,3-diisopropylcarbodiimide (DIPCDI, 4-fold excess) and 1-hydroxybenzotriazole (HOBt, 4-fold excess) as coupling agents (1 h for each coupling). Tyr(Me)$_2$ and L/D-Tyr(Me)$_2$ were protected as Boc and the side chain were left unprotected. Crude deprotected peptides (88% CF_3COOH, 5% H_20 and 7% triethylsilane) were purified by preparative HPLC. The synthetic peptides were homogeneous as assessed by analytical HPLC and TLC; amino acid analysis and NMR properties were consistent with the peptide sequence and purity was >98%. D-Tyr(Me)$_2$ containing peptides were prepared starting from the racemic mixture of Tyr(Me)$_2$ and diastereoisomeric peptides were separated by preparative HPLC and identified by comparison with an authentic sample of L-Tyr(Me)$_2$ containing peptides.

2.1. Biological tests

2.1.1. Radioreceptor assays. Rat brain membrane synaptosomes (P_2 fraction) were prepared as described by Salvadori et al. [9]. The δ and μ receptor binding sites were specifically labeled using [^{3}H]DPDPE (34.3 Ci/mmol) and [^{3}H]DAGO (60 Ci/mmol) respectively [10]. Competitive displacement assays were incubated for 2 h at room temperature (22–23°C); rapid filtration onto glass fiber filters and washing of the membranes occurred within 5 s. All assays were conducted in duplicate with 7–12 dosage points and using three synaptosomal preparations; repetitions (*n* values) are given in Table 1 together with K_i/nM determined according to Cheng and Prusoff [11].

2.1.2. Bioassays. Transmurally stimulated guinea pig ileum (GPI). Guinea pigs were killed with CO_2 and bled. Sections of GPI were prepared as described previously [12]. Segments of ileum 2–3 cm long were placed in a 10 ml organ bath containing Tyrode's solution with 5% CO_2 in 95% oxygen maintained at 37°C. The ileum preparation was placed between platinum electrodes and connected to a 85/2/50 MARB stimulator. A force displacement transducer and unirecord model polygraph were used for measurement of isotonic contraction. A resting tension of 0.5 g was applied and after a 30 min equilibration period, the preparation was stimulated with a 0.5 ms pulse delivered transmurally at a frequency of 10 s at supramaximal voltage (25 V). Under these conditions, the preparation allows a contraction mean of 60 mm ± 0.57. The inhibition of ileal contractions by drugs was expressed as percentage of basal value (mean ± S.E.M.). Each analog was tested for its ability to inhibit electrically evoked contractions (i.e. tested for agonist activity) and to antagonize the inhibitory effects of μ agonists (DAGO, morphine, dermorphin). pA_2 values were calculated according to the procedure reported by Tallarida and Murray [13].

Rabbit jejunum (RJ) preparation. This is a new in vitro model to study δ opioid activity that we prefer to the customary MVD assay since the jejunum contains mainly δ receptors [14–16] whereas MVD also contains μ and κ receptors [17,18]. The preparation of rabbit jejunum was according to Valeri et al. [14]. The animals were killed with CO_2 and bled. The abdomen was opened with a midline incision and three or four segments of jejunum (3 cm long) were removed from the same animal and placed in 10 ml tissue baths containing Tyrode's solution. The tissues were connected to an isotonic transducer by 1 g loading and allowed to equilibrate for 45 min; during this period regular spontaneous activity was recorded. Under these conditions, the preparation showed a contraction mean of 60 mm ± 0.57, and the inhibition of rabbit contractions by the standard δ agonist [D-Ala$_2$] deltorphin II was as reported by Guerrini et al. [15].

Molecular mechanics (MM). MM calculations were based on the all atoms parametrization of the AMBER force field [19,20] as implemented in the SYBYL package. EM calculations were performed with a distance dependent dielectric constant (ε=r) and no distance cut-off for non-bonded interactions.

3. Results and discussion

As already done in the design of δ selective antagonists containing the Tyr-Tic message [2], in order to limit the synthetic efforts we performed a conformational analysis of peptides containing the initial Tyr(Me)$_2$-Phe sequence and compared the resulting energy minima with appropriate rigid compounds. The choice for a δ selective mold is easy since all naltrindole analogs described by Portoghese et al. [3,21] are suitable candidates. In the present work we used MeNTI as already done for the [Tyr-Tic] [2,5] and [Tyr(Me)$_2$-Tic] peptides [4,6].

The choice of an appropriate μ selective mold is more difficult since most μ selective opioids derived from alkaloids have but one aromatic ring (i.e. that of tyramine). In our case, to have a meaningful comparison with the Tyr(Me)$_2$-Phe sequence, we wanted to have a rigid mold with two aromatic rings. A reasonable compromise is to use some of the many synthetic κ opioids that have fairly low κ/μ selectivity. In particular we chose 6*N*-cinnamoyl-β-naltrexamine (CNX) for which the ratio of $(K_i)_{\kappa 1}$ to $(K_i)_\mu$ is 0.2/0.07, i.e. slightly in favor of μ [22], and ICI 199441 [23], which is more κ selective.

We selected the model dipeptides Tyr(Me)$_2$-Phe-NHCH$_3$ and Tyr(Me)$_2$-D-Phe-NHCH$_3$ as representative of all possible chiral isomers of the Tyr(Me)$_2$-Phe message, i.e. Tyr(Me)$_2$-Phe, D-Tyr(Me)$_2$-D-Phe, D-Tyr(Me)$_2$-Phe and Tyr(Me)$_2$-D-Phe. This is rigorously true if we deal with dipeptides only, whereas all four possibilities should be taken into account in the case of longer peptides. However, it is safe to assume that the conformational preferences of the two initial residues are predominant for receptor selectivity, particularly if the C-terminal sequences contain no aromatic residue. Accordingly we performed a detailed energy calculation only for the quoted dipeptides Tyr(Me)$_2$-Phe-NHCH$_3$ and Tyr(Me)$_2$-D-Phe-NHCH$_3$, whose preferences also correspond to D-Tyr(Me)$_2$-D-Phe-NHCH$_3$ and D-Tyr(Me)$_2$-Phe-NHCH$_3$ respectively.

Table 1
Binding and functional bioactivity[a] of [Tyr(Me)$_2$-Phe] peptides

Peptide	K_i/nM δ	K_i/nM μ	GPI[b] pA_2
Tyr(Me)$_2$-Phe-NH$_2$	386.5 ± 52 (3)	209.6 ± 48 (3)	i
D-Tyr(Me)$_2$-Phe-NH$_2$	1977 ± 412 (5)	866.4 ± 141 (4)	i
Tyr(Me)$_2$-D-Phe-NH$_2$	15.5 ± 1.1 (4)	3.56 ± 0.4 (3)	7.2; 7.0[c]; 6.5[d]
D-Tyr(Me)$_2$-D-Phe-NH$_2$	2543 ± 334 (4)	501 ± 92 (4)	4.9
Tyr(Me)$_2$-Phe-G-V-V-NH$_2$	118.3 ± 22 (4)	75.3 ± 8.8 (4)	5.7
D-Tyr(Me)$_2$-Phe-G-V-V-NH$_2$	246.6 ± 55 (5)	113.8 ± 18 (3)	5
Tyr(Me)$_2$-D-Phe-G-V-V-NH$_2$	2.32 ± 0.5 (5)	0.53 ± 0.08 (3)	7.3; 7.2[c]; 6.7[d]
D-Tyr(Me)$_2$-D-Phe-G-V-V-NH$_2$	22.3 ± 5.6 (5)	2.1 ± 0.4 (3)	5.2

[a]All compounds were inactive on the RJ at concentrations $>10^{-4}$ M.
μ antagonism measured in relation to: [b]DAGO, [c]morphine or [d]dermorphin;
i=inactive at concentrations $>10^{-4}$ M.

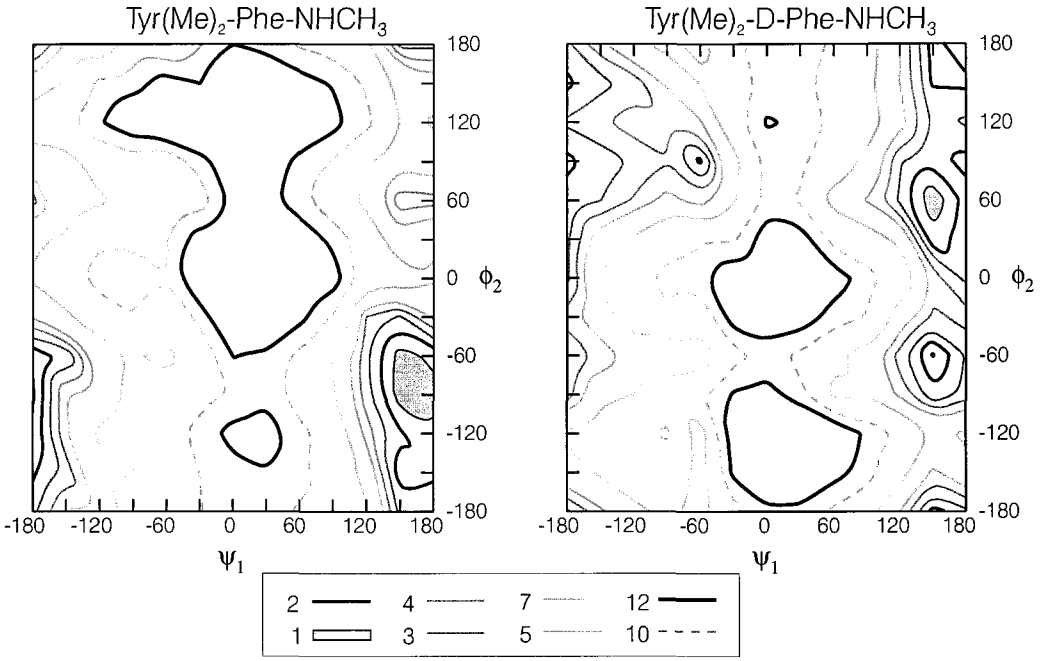

Fig. 1. Energy maps of $Tyr(Me)_2$-Phe-$NHCH_3$ (left) and $Tyr(Me)_2$-D-Phe-$NHCH_3$ (right). The energy (kcal/mol) is plotted as a function of ψ_1 and ϕ_2. The energy levels are indicated by the legend at the bottom of the figure.

A complete conformational search for a dipeptide presents no technical problems; we performed complete searches for our dipeptides using intervals of 10° for all relevant internal rotation angles, i.e. χ^1_1, ψ_1, ϕ_2, ψ_2 and χ^1_2. Other internal coordinates were minimized at each step. However, in the representation of the results it is convenient to describe the conformers as a function of only two internal coordinates, i.e. ψ_1 and ϕ_2. These two internal coordinates were chosen as most representative to describe the conformational preferences of the dipeptides, since they are the central rotation angles. Fig. 1 shows that the energy map of $Tyr(Me)_2$-Phe-$NHCH_3$ as a function of ψ_1 and ϕ_2 is characterized by one broad minimum centered approximately at 160/−80 (a). The energy map of $Tyr(Me)_2$-D-Phe-$NHCH_3$ on the other hand shows three distinct minima of comparable energy centered at 150/60 (i), 150/−60 (ii) and −60/90 (iii).

In spite of the similarity of most of the internal coordinates of the absolute minimum (a) of $Tyr(Me)_2$-Phe-$NHCH_3$ with those of the T1b+ conformer of Tyr-Tic-NH_2, none of these minima (a, i–iii) gave a satisfactory overlap with MeNTI. This finding may be viewed as an indirect confirmation that the bioactive conformation for δ selective dipeptides corresponds to the *cis* C1b+ conformer of Tyr-Tic-NH_2, which however is not energetically accessible to $Tyr(Me)_2$-Phe-$NHCH_3$. On the other hand, minima (iii) and to a lesser extent (ii) have a fairly good overlay with the molecular model of CNX whereas minima (i) and to a lesser extent (ii) have a fairly good overlay with the molecular model of ICI 199441. The fact that the fit is not perfect, particularly at the basic nitrogen and the second aromatic ring (distinct from that of tyramine), hints that high agonist activity may be prevented whereas antagonism is still likely [7].

Based on this conformational analysis we synthesized the simple dipeptides $Tyr(Me)_2$-D-Phe-NH_2 and $Tyr(Me)_2$-Phe-NH_2 and two analogs containing the same messages but a longer, hydrophobic address sequence, i.e. $Tyr(Me)_2$-D-Phe-Gly-Val-Val-NH_2 and $Tyr(Me)_2$-Phe-Gly-Val-Val-NH_2. Table 1 summarizes the binding data and in vitro activity of these peptides. For comparison, the binding data of the corresponding D-$Tyr(Me)_2$-Phe and D-$Tyr(Me)_2$-D-Phe peptides are also reported.

All compounds show some preference for μ vs. δ opioid receptors, and all pentapeptides have better opioid receptor binding than the corresponding dipeptides, i.e. C-terminal elongation with hydrophobic residues (Val-Val) improves binding at both μ and δ opioid receptors. These data are somewhat surprising if compared to the behavior of peptides containing the Tyr-D-Xaa-Phe (Xaa = Ala, Met, Leu) typical of amphibian opioid peptides, in which changing the chirality of the second residue leads to a drastic decrease of affinity to opioid receptors.

On the other hand, it is very interesting to observe that, similar to what we have recently reported for δ selective antagonists [2], a simple dipeptide sequence can behave as a kind of 'antagonist message'. In fact, although all compounds are inactive as agonists in the GPI assay at concentrations $> 10^{-4}$ M, consistent with binding data the two compounds with a L-$Tyr(Me)_2$-D-Phe chirality message have pA_2s ranging from 6.5 to 7.3, depending on the μ agonist used. Intrinsic activity does not change from the dipeptide, $Tyr(Me)_2$-D-Phe-NH_2 to the pentapeptide $Tyr(Me)_2$-D-Phe-Gly-Val-Val-NH_2 in the stimulated GPI tissue. On the other hand, all compounds are inactive in the tissue containing δ opioid receptors (RJ) at concentrations $> 10^{-4}$ M. These compounds can be considered neither δ agonists nor δ antagonists since the typical values for RJ are an IC_{50} of 730 nM in the case of the δ agonist [D-Ala_2] deltorphin II [15] and a pA_2 of 7.2 for the δ antagonist naltrindole (unpublished results). This behavior is particularly surprising for the $Tyr(Me)_2$-D-Phe di- and pentapeptide that bind to δ receptors with K_i values of 15.5 and 2.32 nM respectively.

The μ opioid antagonist activity of the dipeptide is remark-

able also considering that the only known μ antagonist of peptide structure, i.e. H-D-Phe-c(Cys-Tyr-D-Trp-Arg-Thr-Pen)-Thr-NH_2 ($pA_2 = 6.4 \div 7.9$), a cyclic octapeptide derived from somatostatin [24], contains the Tyr-D-Trp motif in its sequence. The fairly small μ selectivity is not too surprising if one takes into account the role of a further locus of interaction (different from the two aromatic residues) present in longer peptides [25,26]. However, the recent discovery of a very selective μ agonist tetrapeptide [27] hints that the search for short selective peptides is still realistic. Owing to its simple chemical constitution $Tyr(Me)_2$-D-Phe-NH_2 looks to be a very promising μ antagonist both as a lead structure and for practical applications.

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