Bioactivity of New μ and δ Opioid Peptides

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Abstract: Endogenous opioids have been studied extensively since their discovery, in the hope of findings a perfect analgesic, devoid of the secondary effects of alkaloid opioids. However, the design of selective opioid agonists and or antagonists has proved very difficult. First, structural studies of peptides in general are hampered by their intrinsic flexibility. Second, the relationship between constitution and the socalled "bioactive conformations" is far from obvious. Ideally, a direct structural study of the complex between a peptide and its receptor should answer both questions, but such a study is not possible, because opioids receptors are large membrane proteins, difficult to study by standard structural techniques. Thus, conformational studies of opioid peptides are still important for drug design and also for indirect receptor mapping. This review deals the pharmacological activity of :

a) a new μ and δ agonist: The single amino acid replacement of 2',6'-dimethyl-L-tyrosine in deltorphin B (H-Dmt-D-Ala-Phe-Glu-Val-Val-Gly-NH2) yielded high affinity for mu- and delta-binding sites. [Dmt1]Deltorphin B lacks activity at kappa-opioid binding sites. Bioactivity *in vitro* with guinea-pig ileum confirmed that [Dmt1]deltorphin B interacted with mu-opioid receptors by reducing electrically induced contractions in a naloxone-reversible manner and was 150-fold more potent than morphine and comparable to [D-Ala2,NMePhe4,Gly-ol5]enkephalin (DAGO). The inhibition of spontaneous contractions of rabbit jejunum provided evidence for delta-opioid receptor interaction. Analgesia (hot plate and tail flick tests) revealed that [Dmt1]deltorphin B was 180- to 200-fold more potent than morphine. Pretreatment with naloxone, naltrindole or H-Dmt-Tic-Ala-OH (a highly selective delta-opioid receptor antagonist) prevented [Dmt1]deltorphin B antinociception. Thus, [Dmt1]deltorphin B exhibited remarkably high dual affinity and bioactivity toward delta- and mu-opioid receptors.

b) two new δ opioid peptide receptor antagonists (Dmt-Tic-OH (DTOH) and Dmt-Tic-Ala-OH (DTAOH): Dmt-Tic-OH (DTOH) and Dmt-Tic-Ala-OH (DTAOH), effective antagonists *in vitro*, represent a new potent opioid dipeptides for the delta-opioid receptor (Ki delta of 0.022 nM and a selectivity, Ki mu/Ki delta, of 150,000 for DTOH; Ki delta of 0.285 nM and a selectivity Ki mu/Ki delta, of 20,4 for DTAOH). In the present study we considered the pharmacological activity of these two new delta opioid peptide receptor antagonists *in vivo*. Therefore, we have evaluated their possible antagonistic activity against the antinociception induced by the highly selective delta opioid receptor agonist, [D-Ala2]deltorphin II (DEL). Furthermore, these two delta opioid peptide receptor antagonists were injected centrally or peripherally in order to assess their ability to act also after systemic administration. Concurrent i.c.v. injection of DTOH or DTAOH (0.5-1.0-2.0 nM) with DEL (5 nmol) induced a significant reduction of DEL antinociception. By contrast, while DTOH (10-20-40 mg/kg) administered peripherally (i.p., s.c. or i.v.) was also able to reduce DEL antinociception, DTAOH failed. The present results indicate that DTOH is the first opioid dipeptide with delta antagonist activity after systemic administration and it could be important in the clinical and therapeutic applications.

c) a new μ selective opioid dipeptide antagonists: the potent delta selective opioid antagonist dipeptides were designed on the basis of a simple conformational analysis. Following a similar procedure we found a mu selective dipeptide antagonist, 2,6-dimethyl-Tyr-D-Phe-NH2. Although its selectivity is not as high as those of the quoted delta 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 delta selective 2,6-dimethyl-Tyr-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid counterpart, seems able to impart antagonism to longer peptides.

INTRODUCTION

The opioid system consists of three main receptor types (μ, δ, κ) and their corresponding ligands (enkephalins, endorphins, dynorphins) which govern diverse physiological functions in peripheral tissues and central nervous system (CNS). [1-4] Recently a number of natural opioid peptides have been discovered endowed with significant receptor speificity: in particular endomorphins that have been found highly selective mu-receptor ligands [5] and two major classes of opioid peptides secreted from the skin of *Phyllomedusa* amphibians [6]. These heptapeptides,[6,7] called dermorphins (dermal morphine-like substances) DER, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 [8] and deltorphins [9] (delta-specific opioids), DEL C, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 are potent and selective μ and δ agonists, respectively.

μ, δ and κ receptors are inordinately sensitive to morphine and a veriety of alkaloid-derived agonists and antagonists [10] They are traditionally applied extensively for the

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treatment of chronic [11,12] and acute [13] pain, as well as in the current therapy regimes to combat alcohol dependency [14] during immunosuppression in organ transplants [15] and in mediating the effects of narcotic addiction [16]. Alcaloid analgesic are very powerful and difficult to substitute with other drugs, especially for acute pain, but are plagued by several undesirable side effects: respiratory depression, gastrointestinal depression, and the development of tolerance and addiction.

All attempts to find the perfect analgesic, that is, an alkaloid retaining analgesic power comparable to that of morphine, but devoid of its secondary effects, failed. Notwithstanding the discovery that all opioids act in the framework of a complex neurotrasmitter system that comprises at least three opioid receptors, that in principle might correspond to different actions, rekindled the hope of finding a powerful analgesic devoid of side effects. In effect many evidences indicate that analgesia mediated by agonist activation of delta opioid receptors [17] produces less marked side effects than analgesia mediated by mu-opioid receptors [18]. Consistently, since the discovery of natural selective opioid substances starting from these analogues, many efforts were aimed to the synthesis of compounds retaining opioid activity but improved farmacodinamic and farmacocinetic properties.

The basic step in the finding of the perfect analgesic is the knowledge of the bioactive conformation as a starting point for the synthesis of a rigid and powerful analog devoid of side effects. The bioactive conformation is consistent with the farmacoforic points that are identified through structure activity relationship studies (SAR). SAR studies include the synthesis of many peptide and/or non peptide analogues displaying selectivity for one or another of the receptors and their pharmacologically defined subtypes [19] such as delta1- and delta2-opioid receptor subtypes [20].

Due to their high selectivity, DER and DEL were the basis for an extensive structure activity relationship study aimed to understand the essential requisites of μ and δ activity. More than 200 analogues of DER and DEL C were synthesized, and the role of each amino acid in binding to μ and δ receptors and bioactivity was defined [21-31]. New synthetic products were prepared such as oxymorphindole and related derivatives [32], and (+)-4-[(alfa R)-alfa-((2S,5R)-4-allyl-2.5-dimethyl-l-piperazinyl)-3-methoxy-benzyl]-N, diethylbenzamide [33]. These products are under investigation for their clinical relevance and provide potential non-peptide ligands for studies on delta-opioid-receptor.

As briefly reported the synthesis of an ideal analgesic asks for an improvement of both farmacodinamic and in farmacocinetic peculiarities. Sometimes if farmacodinamic properties are improved, the farmacocinetic characters can be still far from those of a perfect analgesic molecule. The quantities of opioids normally transported into the CNS can be low, and inadequate blood-brain barrier (BBB) permeation can be responsible for not optimized or for the different analgesic potencies among different opioid analogues. Consistently one major avenue of investigation involves the development of opioid analogues that penetrate the blood-brain-barrier (BBB).

DER and DEL as well as their synthetic analogues are generally resistant to enzymatic degradation, [34-35] and showed potent antinociceptive effects following intracerebroventricular administration, but only moderate or negligible central actions after systemic administration in mice and rats [36-38].

Alteration of the physiochemical properties of opioids, such as potentiating lipophilicity [39-42] or glycosylation in order to take advantage of the glucose transporter [43] represented efforts to enhance passage through the BBB. Following the latter strategy, Polt *et al.* [42] designed and prepared glycopeptide enkephalin analogues able to cross the BBB and to produce prolonged analgesia in mice.[42]These considerations have prompted to prepare and test DER and DEL C analogues in which D-glucopyranosyl units are -O-glycosidically linked to Thr^4 or Thr^7 side chains.

EFFECTS OF DMT^1 IN DELTORPHIN B

Current evidence indicates that analgesia mediated by agonist activation of δ-opioid receptors [44] produces less marked side-effects than analgesia mediated by μ-opioid receptors [18].

Deltorphin which represent the potent family of opioid peptides originally isolated from frog skin [45] are the most selective δ-opioid receptor peptides. Thus they have served as parent peptide in the synthesis of numerous δ active peptide analogues.

Structural investigations had a basic role in driving the synthesis of new peptide and/or not peptide analogues. Essential indications on kinds and positions of new chemical moieties were given. Consistently it was found that the insertion of the 2',6'-dimethyl L- tyrosine (DMT) in the selective δ-opioid di and tri- peptides receptor antagonists [43, 46] confers a conformation to the peptide similar to that of its bioactive conformer [47]. On this basis deltorphin B was modified replacing Tyr with DMT and its activity was determined in pharmacological bioassay and radioligand binding assays for μ and δ opioid receptor.

BIOASSAYS

Transmurally stimulated guinea pig ileum (GPI). Guinea pig were killed with CO_2 and bled. Sections of GPI were preparated as follows. 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 85/5/20 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 30 min equilibration period, the preparation was simulated with a 0,5 ms pulse delivered transmurally at a frequency of 10 s at supramaximal voltage (25 V). Under these condictons, 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 (means +/- 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.

Rabbit jejunum (RJ) preparation. This is a new *in vitro* model to study δ opioid actiyity that we prefer to the customary MVD assay since the jejunum contains mainly δ receptors whereas MVD also contains μ and κ receptors. 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 tessue 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 condictions, the preparation showed a contraction mean of 60 mm+/- 0.57, and the inhibition of rabbit contractions by the standard δ agonist (o- Ala_2) deltorphin II.

ANTINOCICEPTION

To determine the nociceptive threshold of the mice, the hot plate test and the tail flick test were used.

Drugs used are dissoved in sterile distilled water from icv administration immediately prior to injection at 5 μl/kg or 5μl mouse, respectively. The icv injection was performed and the site of administration of the peptide was verified in all animals by the injection of 1% methylene blue and the examination of the dye distribution in the cerebral ventricles at the termination of the experiment. To evaluate the hot plate and tail flick test responses detailed below, a control latency (T_0) was obtained from the mean of two latencies determined prior to drug injection; test latencies (T_1) were determined at various times after injection for each animal. The percentage of analgesia was calculated as $(T_1 - T_0)/ (T_2 - T_0) \times 100$ where the cut off times (T_2) for the hot plate and tail flick test were 60 and 15s, respectively. The median antinociceptive dose (ED50) and 95% confidence limits were calculated according to the method of Litchfield and Wilcox.

BIOACTIVITY

The high μ-opioid receptor affinity of **1** was confirmed in the guinea- pig ileum bioassay *in vitro*. These experiments indicated that peptide 1 reduced the electrically induced contractions of guinea pig-ileum while peptides **2** and **3** failed to cause significant inhibition up to μM concentrations (Table **1**). Since naloxone at a concentration of 0.05 nM reversed the inhibition effect of **1**, the results are entirely consistent with an interaction of μ- opioid receptors. Furthermore, in a competitive study performed with morphine (a preferential μ- opiate recetor alkaloid ligand) and DAGO (a highly μ-selective opioid peptide), peptide **1** appears to be 150- fold more active than morphine (Table **1**) and as active as DAGO. The strong inhibition of the spontaneous contractions of rabbit jujunum by peptide **1**, also indicated an interaction with δ-opioid receptors since this effect was totally blocked by naltrindole, an established δ- opioid receptor antagonist, at an equimolecular concentration. Moreover, in comparison to peptides **2** and **3**, compound **1** appeared to be moderately more active as an inhibitor than either of the deltorphins, which were comparable to each other as to activity (Table **1**).

ANTINOCICEPTION

The results obtained with the hot plate test indicated that 10 min after i.c.v. administation, peptide 1 induced a significant and dose- related analgesia in the mouse (Table **2**), which lasted for the entire experimental period (45 min). In a companion study with morphine, peptide **1** was 180-fold more potent thant this narcotic opiate alkaloid, thus confirming the observations on the interaction with μ-opioid receptors with the *in vitro* bioassays (Table **1**).

These observations were furthen confirmed with the tail flick test, in wich peptide **1** had an analgesic activity comparable to that demonstrated in the hot plate test. Whereas peptide **2** was about **3** times more active than peptide **1**, all three deltorphin analogues **(1-3)** were about 300 to over 800- fold more analgesically potent than morphine (Table **2**). A concurrent study then considered the effects of naltrindole (δ-opioid receptor antagonist), naloxone (μ-opioid receptor antagonist), and H-Dmt-Tic-Ala-OH (DTA), a new highly selective δ-opioid receptor antagonist on the analgesic effect induced by peptides **1** and **3** (Table **3**). While DTA and naltrindole (0,05 nM, i.c.v.) produced no measurable antinociception, both antagonized the antinociceptive action during co-administration of peptides **1** or **3** in the tail flick test (Table **3**). Identical treatment with reported δ receptor antagonists failed to affect antinociception in the hot plate test (Table **3**). On the other hand, pretreatment with noloxone (0.05 nM i.c.v.), which does not produce antinociception, antagonized the activity of peptide **1** in both the hot plate and tail flick tests (Table **3**), but it antagonized peptide 3 in the tail flick test and not in the hot plate test (Table **3**). Statistical

Table 1. ***In Vitro*** **Characterization of [Dmt1]Deltorphin B and Related Peptides**

Compound	Ki Nm		Kiμ /Kiδ	$ED_{50,Nm}$	
	μ	δ		Guinea- pig ileum	Rabbit jejunum
(1) [Dmt^1]Deltorphin B	1,01 ± 0,06 (3)	0,13 ± 0,027 (3)	7,7	300 (216-353)	360 (297-395)
(2) Deltorphin B	638 ± 55 (5)	0,12±0,03 (6)	5317	/	850 (775-915)
(3) Deltorphin C	272 ±50 (11)	0,24 ± 0,06 (6)	1135	/	730(665-794)
Morphine	/	/		43μM (38-52)	/
DAGO	1,51±0,18 (8)	376,3 ± 81,8 (4)	0,004	22,0 (18,3-24,9)	/

Table 2. ***In Vitro*** **Antinociception Activity of [Dmt] Deltorphin B and Related Peptides**

Compound	Hot Plate Test		Talk flick test	
	i.c.v. ED_{50}nM	Rel. Prot.	i.c.v. ED_{50}nM	Rel. Pot.
(1)[Dmt^1]Deltorphin B	0,016 (0,011-0,030	180	0,011 (0,0087-0,023)	300
(2)Deltorphin B	0,021 (0,016-0,042)	140	0,0038 (0,0016-0,008)	840
(3)Deltorphin C	0,035 (0,020-0,051)	80	0,017 (0,01-0,021)	190
Morphine	2,88 (1,75-3,9)	1	3,18 (2,9-3,7)	1

analysis comparing data of Tables **2** and **3** showed significant ($P<0.01$) differences between peptides tested alone and the same in the presence of each antagonists except for deltorphin C in the hot plate test versus deltorphin C plus each antagonist in the hot plate test.

The peptides subtly modified in the side chain at N-terminus residue yielded an opioid with high dual affinities to both δ- and μ-opioid receptor types. At present, it appears that the replacement of the Tyr residue by the innatural Dmt analogue in the δ- selective opioid agonist, deltorphin B, produced a peptide with high affinity for both μ- and δ-opioid receptors, thereby yielding a δ-opioid receptor ligand which, to all intents and purposes, lacks δ-opioid receptor selectivity. While the high affinity for the μ-opioid receptor was confirmed in the vitro pharmacological assay (guinea-pig ileum), *in vitro* antinociception apparently failed to correlate with high μ-opioid receptor activity; however, the activity profile of peptide)1 (DMT1 deltorfin B was more closely related to that of the δ- selective opioid peptides 2 (deltorfin B) and 3 (deltorfin C) In summary, [Dmt^1] deltorphin B possesses remarkable agonist activity and exerts effects on both μ- and δ-opioid receptors *in vitro* ant *in vivo*, and also that the newly discovered opioid tripeptide antagonist, DTA, exerts antagonist activity primarily toward the δ-opioid receptor, in keeping with *in vitro* data.

On conformational point of view the activity of the analogues including DMT in deltorphin B can be explained on the basis of a conformation of the N-terminal portion of deltorphin B where the rotation of the Tyr residue is sensibly reduced.

ROLE OF THE TIC MOIETY

The synthesis of an antagonist with high δ- selectivity and without the substitution of the basic nitrogen of the tyramine moiety was performed for the first time inserting a Tic (1,2,3,4-tetrahydroisoquinoline-3- carobxylic acid) moiety in the second position of a dermorphin related peptide Tyr-Tic-Phe-NH2 (dubbed TIP) and Tyr-Tic-Phe-Phe NH2 (dubbed TIPP).

Conformational studies of the Tyr-Tic analogs showed 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 [48]. This interpretation led to the synthesis of the first opioid dipeptide, Tyr-Tic-Phe-NH_2 [43], and to a series of simple and very potent δ selective antagonists. In fact Tyr-Tic-Phe-NH_2 itself behaves as a δ selective antagonists [43] and even more significantly introduction of Tic in the second position of the sequence converts enkephalin, a non-selective agonist, and dermorphin (a μ-selec-

Table 3. Co-Administration of, or Pretratment with, H- Dmt- Tic- Ala- OH (DTA), Naltrindole (NTI) or Naloxone (NAL) on the $ED_{50}$for Antinociception Produced by i.c.v. Dmt Deltorphin B and Deltorphin C in the Hot Plate Test and Tail Flick Test

Peptide	Hot plate test, ED_{50} Nm		
	plus DTA	plus NTI	plus NAL
(1)[Dmt^1]Deltorphin B	0.097 (0.065 - 0.11)	0,067 (0.042 - 0,081)	1.76 (1.23 - 1,97)
(2)Deltorphin C	0.053 (0.031 - 0.083)	0.042 (0.016 - 0.072)	0.055 (0.01 - 0,073)

Tail Flick test, ED_{50}, nM		
plus DTA	plus NTI	plus NAL
2.7 (2.9 - 3.1)	1.9 (1.3 - 2.1)	1.58 (1.1 - 1.84)
2.3 (1.9 - 2.9)	1.8 (1.1 - 2.3)	0.77 (0.65 - 0.83)

tive agonist) into δ selective antagonists [49]. The view of a specific "antagonist message" was subsequently substantiated by the discovery of ultraselective δ antagonists containing the Tyr-$(Me)_2$-Tic message [46,50] and by the design of a rigid antagonist lacking the basic charge of tyramine [51].

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 population 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 ciclization, a circumstance drastically different form the conformational freedom typical of aromatic aminoacid residues. Thus, the conformation accessible to Tyr-Tic peptides, notably the C1b+ cis conformer we previously identified [43] as a likely δ-selective bioactive conformation, are forbidden or severely disfavoured for opioid peptides containing an aromatic amino acid residue in the second position.

EFFECTS OF TIC AND DMT INSERTION IN MESSAGE DOMAIN

The rationale in designing peptides with the substitution of methyl groups at the 2' and 6' position of Tyr (Dmt) Dmt-Tic-OH (DTOH) and Dmt-Tic-Ala-OH (DTAOH), was to minimize the message domain of opioid peptides while maintaining activity. Thus a class of highly potent and selective opioid di- and tripeptide δantagonist [52] were synthesized whose physiochemical properties might be amenable to passage through the BBB.

The design of DTOH peptide was performed on the basis of an 1H-NMR solution conformation of DTOH that supported the idea of DTOH similar to naltrindole (a nonapeptide delta antagonist) and suggested a viable binding conformation at the delta opioid receptor [53]. DTOH and DTAOH could be much more rigid inside the receptor with the methyl groups of Dmt favoring a better fit of the tyramine moiety in the T subsite of the opioid receptor [46 ,53]. The good fit of Dmt for the subsite might force the carboxyl group into close contact with the anion site of the μ receptor, explaining the high δ selectivity of these compounds [52]. Furthermore lipophilic aminoacids and water solubility imply a potential to transit the brain capillary endothelium.

Previous experiments performed *in vitro* with receptor binding assay [46, 54] mouse vas deferens (MVD) and guinea-pig ileum (GPI) [52] showed a marked delta opioid receptor preference.

Although receptor binding assay (brain membranes) revealed that DTOH and DTAOH exhibited high affinity and extraordinary selectivity for the δ opioid receptor and pharmacological experiments *in vitro* (MVD and GPI) further indicated their δ antagonist activity [52, 54] DTOH and DTAOH, showed a different behaviour against DEL antinociception, depending on the route of the administration used.

Both DTOH and DTAOH if injected centrally were able to antagonize DEL antinociception. On the contrary, after systemic administration only DTOH was able to antagonize DEL antinociception whereas DTAOH failed (Table **4**).

The different behaviour after systemic administration of di- (DTOH) and tripeptide (DTAOH) treatment could reflect different pharmacokinetic of these two molecules containing a tyrosine surrogate, Dmt and a constrained phenylalanine, Tic.

DTOH is an active δ-opioid dipeptide antagonist upon peripheral administration due to a combination of its unique physiochemical properties (lipophilic, water-solubility, stability).

ROLE OF PHE^2 IN μ-SELECTIVITY

Many experimental evidences suggest that in opioid peptides the second position is strategic to orient the selectivity of the biological response. As previously reported the insertion of Tic residue in 2 position of the message sequence plays a basic role in driving δ selectivity.

The conformational preferences of analogs containing an aromatic residue (Xaa) in second position have been examined, i.e. Phe, Tyr, Trp or His, since it is likely that other relative arrangements confer to the peptides good opioid activity and a different selectivity.

The starting choice of the systematic search was Phe for the second residue (Xaa), because Tic was originally derived from this aminoacid residue.

Instead of Tyr, Tyr-$(Me)_2$ was used as the first residue for all peptides, since it ensures a far better binding without de-

Table 4. Antagonistic Effect of DTOH and DTAOH After Central (A) or (B) Systemic Administration

Compound	A	B
	Tail Flick Test	Talk flick test
	i.c.v. ED_{50}nM	i.c.v. ED_{50}nM
Deltorphin II	5	5
DTOH+DEL II	20	25
DTAOH+DEL II	22	5

parting too much from the constitution of the parent residue (Tyr).

As already done in the design of the δ selective antagonists containing the Tyr-Tic message [55], in order to limit the synthetic efforts, a conformational analysis was performed by means of energy calculation of peptides containing the initial Tyr-$(Me)_2$-Phe sequence and the resulting energy minima were compared with appropiate rigid compounds. To have a meaningful comparison with the Tyr-$(Me)_2$-Phe sequence, a rigid mold with two aromatic rings was chosen. Many synthetic k opioids that have fairly low k/μ selectivity were used: in particular 6N-cinnamoyl-β-naltrexamine (CNX) for which the ratio of (Ki)μ is 0.2/0.07, i.e. slightly in favor of μ [56] , and ICI 199441 [57], which is more k selective.

A complete conformational search was done using intervals of 10° for all relevant internal rotation angles, i.e. χ^1_1, ψ_1 φ_2, ψ_2, χ^1_2. Other internal coordinates were minimized at each step. ψ_1 and Φ_2. were chosen as most representative to describe the conformational preferences of the dipeptides, since they are the central rotation angles. The energy map of Tyr-$(Me)_2$-Phe-$NHCH_3$ as a function of ψ_1 and φ_2 shows 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). 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 the 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 (II) have a fairly good overlay with the molecular model of ICI 199441. The fact that the fit is no 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 [51].

Based on this conformational analysis the simple dipeptides Tyr-$(Me)_2$-D-Phe-$NHCH_3$ and Tyr-$(Me)_2$-Phe-$NHCH_3$ 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 were synthesized. Table **5** 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 in 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.

On the other hand it is very interesting to observe that, similar to what we have recently reported for δ selective antagonists [55], a simple dipeptide sequence can behave as a kind of "antagonist message". In fact, although all compounds are inactive as a 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 dipeptides, 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 IC50 of 730nM in the case of δ antagonist naltridole (unpublished results). This behaviour is particularly surprising for the Tyr-$(Me)_2$-D-Phe di and pentapeptide that bind to δ receptors with Ki values of 15.5 and 2.32 nM respectively (Table **5**).

The μ opioid antagonist activity of the dipeptide is remarkable also considering that the only know 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÷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 [58, 59]. However, the recent discovery of a

Table 5. Binding and Functional Bioactivity of [Tyr(Me)$_2$-Phe] Peptides

Peptide	Ki/Nm δ	Ki/nM μ	GPI[b]
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)	65.3)3(4.0±	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-NH2	22.3 ± 5,6 (5)	2.1 ± 0.4 (3)	5,2

very selective μ agonist tetrapeptide [60] 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.

CONCLUSIONS

The results of the present rewiev have demonstrated that:

a) The replacement of the Tyr residue by the innatural Dmt analogue in the δ- selective opioid agonist, deltorphin B, produced a peptide with high affinity for both μ- and δ-opioid receptors, thereby yielding a δ-opioid receptor ligand which, to sall intents and purposes, lacks δ-opioid receptor selectivity. While thehigh affinity for the μ-opioid receptor was confirmed in the vitro pharmacological assay (guinea-pig ileum), *in vitro* antinociception apparently failed to correlate with high μ-opioid receptor activity; however, the activity profile of peptide **1** was more closely related to that of the δ- selective opioid peptides **2** and **3**. Similarly, the analgesic activity of the δ- opioid-selective ankephalin analogue [D-Ser $(O\text{-tert-butyl})^2$, Leu^5] enkephalyn- $Thr(O\text{-tert-butyl})^6$ (BUBU) and [Dmt^1] DPDPE is mediated through different receptor types: the μ-opioid receptor in the case of BUBU and an unknown δ-opioid receptor subtype for the latter peptide. In summary, our results indicate that [Dmt^1] deltorphin B possesses remarkable agonist activity and exerts effects on both μ- and δ-opioid receptors *in vitro* ant *in vivo*, and also that the newly discovered opioid tripeptide antagonist, DTA, exerts antagonist activity primarily toward the δ- opioid receptor, in keeping with *in vitro* data.

b) Both DTOH and DTAOH if injected centrally were able to antagonize DEL antinociception. On the contrary, after systemic administration only DTOH was able to antagonize DEL antinociception whereas DTAOH failed.

The different behaviour after systemic administrationof di- (DTOH) and tripeptide (DTAOH) treatmentcould reflect different pharmacokinetic of these two molecules containing a tyrosine surrogate, Dmt and a constrained phenylalanine, Tic. A detailed conformational analysis of DTOH in solution shows a highly selective non peptide δ antagonist reported by Portoghese that is active upon peripheral administration (11). Also, DTOH and DTAOH could be much more rigid inside the receptor with the methyl groups of Dmt favouring a better fit of the tyramine moiety in the T subsite of the opioid receptor. The good fit of Dmt for the subsite might force the carboxyl group into close contact with the anion site of the μ receptor, explaining the high δ selectivity of these compounds. Our results provide evidence that DTOH is an active δ-opioid dipeptide antagonist upon peripheral administration due to a combination of its unique physiochemical properties (lipophilic, water-solubility, stability). This peptide could be clinically and therapeutically useful because, while opioid peptides generally antagonize an antinociceptive action, following systemic injection.

c) The μ opioid antagonist activity of the dipeptide is remarkable also considering that the only know 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÷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. However, the recent discovery of a very selective μ agonist tetrapeptide hints that the searchfor 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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