Melphalan potently substitutes the N-terminal Tyr of D-Ala^2-Leu^5-enkephalin methyl ester

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In search of an affinity label of the opioid receptor, the nitrogen mustard melphalan, Mel, was built into the peptide chain of D-Ala^2-Leu^5-enkephalin (DALE) methyl ester in different positions. We report now that in contrast to the previous observations that an intact Tyr in position 1 is essential for opioid activity [(1980) Annu. Rev. Pharmacol. Toxicol. 20, 81–110], substitution of Tyr by Mel did not result in a loss of the binding affinity. Mel^1, Leu^5-enkephalin-OMe competed for the binding sites of [^{3}H]naloxone as potently as DALE did; IC_{50} values for both compounds were 50 nM. Mel substitution has led to one order potency decrease in binding to the δ-sites. 0.5–1 μM of the compound irreversibly inactivates 50% of the binding sites of [^{3}H]naloxone, and 5–10 μM of that of [^{3}H]DALE. These results shed new light on the structural requirements established for opioid peptides. In addition, the new derivative can be used as an affinity label of the opioid receptor.

Opioid receptor *New enkephalin analog* *Affinity labeling*

1. INTRODUCTION

Since the discovery of the enkephalins as naturally occurring ligands of opioid receptors [2], hundreds of analogues have been synthesized. These studies have led to the conclusion that an intact tyrosyl residue at the N-terminus is essential for significant opioid activity [1]. Thus, its substitution by tryptophan, phenylalanine, or dopa-dihydroxyphenylalanine results in a loss of binding affinities of 2 or 3 orders of magnitude [3]. More precisely, the aromatic hydroxyl group of tyrosine seems essential, and its removal [3], or replacement by a *p*-amino, -nitro, -chloro, or -iodo group [1] leads to large affinity losses. To date, the only exception was a cyclic enkephalin analogue, Phe-c(-N$^\epsilon$-D-Lys-Gly-Phe-Leu-), which showed twice the potency of Leu-enkephalin in the GPI assay [4]. The same substitution in the linear analogue resulted in a drastic reduction in potency [4]. An intact Tyr seems to be important for casomorphins, the other peptide family with morphine-like properties des-Tyr-β-casomorphin shows no affinity to the μ-receptor [5].

In search of a potent affinity label for the opiate receptor, the nitrogen mustard melphalan (4-bis/-3-chloro-ethyl/-amino-L-phenylalanine), Mel, was coupled to the C-terminal end of D-Ala^2-Leu^5-enkephalin [6]. The compound, DALA-Mel-OMe, was shown to bind with high affinity and irreversibly to rat brain opioid receptors [6]. We report now that substitution of the N-terminal Tyr for melphalan results in a highly active enkephalin analogue. Moreover, as an affinity label it might provide a valuable tool for characterizing the interaction of opioid ligands with their respective receptor size.

2. MATERIALS AND METHODS

2.1. *Synthesis of Mel-D-Ala-Gly-Phe-Leu-OMe (Mel^1, Leu^5-enkephalin-OMe)*

Boc-protected melphalan was coupled to H-D-Ala-Gly-Phe-Leu-OMe with the aid of i-butyl-

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chlorocarbonate in the presence of *N*-methylmorpholine. Boc was removed from the pentapeptide by HCl in formic acid. The purity of the peptide was checked by amino acid analysis and by TLC. The ionic and covalent chloro content of the compound was also determined. Details of the synthesis will be described elsewhere.

2.2. *Rat brain membrane preparation*

Rat brain membranes were prepared according to [7]. Briefly, brains (without cerebella) were homogenized in 30 vols of 50 mM Tris-HCl (pH 7.4) buffer by a Teflon potter. After filtration through gauze, the homogenate was pelleted at $40\,000 \times g$ for 20 min. The pellet, suspended in the original volume of buffer, was incubated at 37°C for 30 min, then recentrifuged. The final pellet was suspended in 5 vols (for affinity labeling) or 80 vols (for displacement studies) of 50 mM Tris-HCl (pH 7.4).

2.3. *Affinity labeling*

The reaction mixture contained 200 μl of the ligand at the appropriate concentration, 100 μl ethanol, 50 mM Tris-HCl (pH 7.4) and 500 μl of 5 vols rat brain membranes in a final volume of 2 ml. After 120 min incubation at 30°C, samples were diluted with 28 ml of 4°C Tris-HCl buffer and centrifuged at $25\,000 \times g$ for 10 min. The pellets were suspended in 30 ml buffer, incubated for 10 min at room temperature and centrifuged again. This washing step was repeated 4 times. The final pellets were homogenized in 8 ml of 50 mM Tris-HCl (pH 7.4) buffer and used immediately for binding assay.

2.4. *Binding assay*

To assess the binding affinity of the compounds, 800 μl of rat brain membrane preparation (400–800 μg protein) was co-incubated with different concentrations of the ligands and 0.5 nM [7,8,9,20-^{3}H]naloxone (spec. act. 83.7 Ci/mmol), [8] or 1 nM [3,5-^{3}H-Tyr]^{1}D-Ala^2-Leu^5-enkephalin (spec. act. 32.3 Ci/mmol) (Benyhe et al. submitted) in 50 mM Tris-HCl (pH 7.4) in a final volume of 1 ml. The reaction was continued for 1 h at 4°C for [^{3}H]naloxone and for 45 min at 23°C for [^{3}H]-DALE binding and was stopped by filtration through Whatman GF/B or GF/C filters, respectively. Non-specific binding was measured with 10 μM unlabeled naloxone or 100 μM DALE and subtracted from total binding. Only specific binding is reported.

In the binding assay after affinity labeling, 800 μl of pretreated membrane preparation (200–350 μg protein) was incubated with 0.5 nM [^{3}H]naloxone or 1 nM [^{3}H]DALE and continued as above. The protein content was determined as in [9].

3. RESULTS

Surprisingly, substitution of Tyr for melphalan in position 1 resulted in a compound with high affinity to rat brain opioid receptors (fig.1). The new

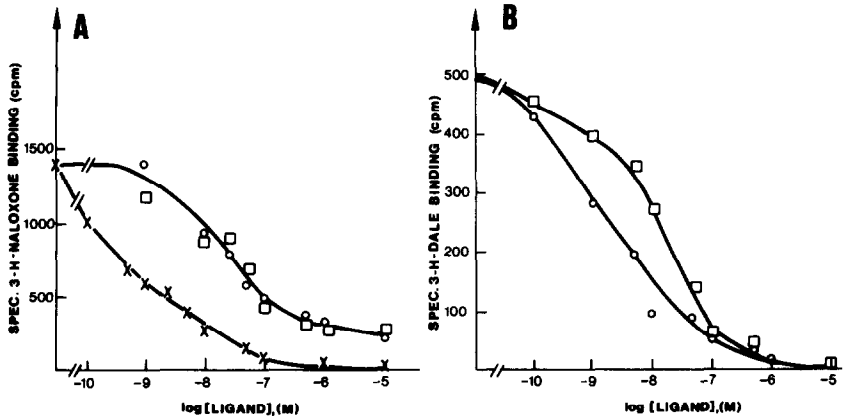

Fig.1. Competition of Mel-D-Ala-Gly-Phe-Leu-OMe for rat brain opioid receptors (A) 800 μl of rat brain membranes were incubated with 0.5 nM [^{3}H]naloxone and competing ligands for 1 h at 4°C and specific binding was determined. (B) Competition curve with 1 nM [^{3}H]DALE after 45 min incubation at 23°C. (×) Naloxone, (○) DALE, (□) Mel^1,Leu^5-enk.-OMe. One representative experiment of 3 others, each measured in duplicate.

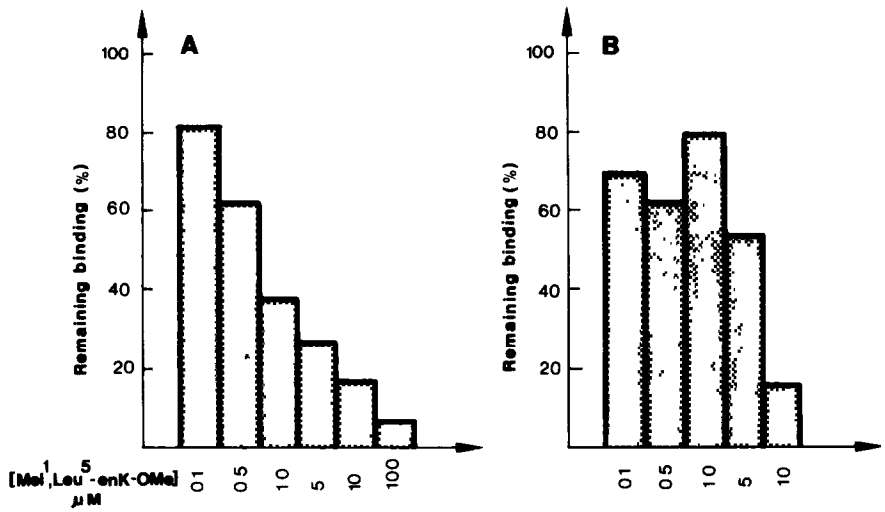

Fig.2. Concentration dependence of irreversible interaction of opioid receptors by Mel^1,Leu^5-enk.-OMe. Rat brain membranes were incubated with various concentrations of Mel^1,Leu^5-enk.-OMe for 120 min at 30°C for (A) and for 90 min for (B) in a final volume of 2 ml in 50 mM Tris-HCl (pH 7.4) buffer. After 4 washes, the remaining binding was measured with (A) 0.5 nM [^{3}H] naloxone after an additional 1 h incubation at 4°C, and (B) 1 nM [^{3}H]DALE after 2 h incubation at 4°C. Remaining specific binding was normalized by protein concentration and expressed as percentage of control binding (preincubation in buffer and 4 washes).

compound, Mel^1-Leu^5-enkephalin-OMe has the same affinity as the parent compound, DALE, for the binding sites of [^{3}H]naloxone ($IC_{50} = 5 \times 10^{-8}$ M), and shows a somewhat lower affinity ($IC_{50} = 1 \times 10^{-8}$ M) than DALE ($IC_{50} = 1.5 \times 10^{-9}$ M) for the binding sites of [^{3}H]DALE.

The irreversible binding of Mel^1,Leu^5-enkephalin-OMe was tested with [^{3}H]naloxone (which, under the circumstances described, labels mainly μ-sites) and with [^{3}H]DALE (which labels mainly δ-sites) to membranes preincubated with the compound. As shown in fig.2, Mel^1,Leu^5-enkephalin-OMe caused a dose-dependent irreversible blockade of both binding sites. Mel^1,Leu^5-enkephalin-OMe blocked 50% of the [^{3}H]naloxone sites at 0.5–1 μM and caused 93% inhibition at 100 μM. On the other hand, it was less active in irreversibly inactivating the δ-sites, having an IC_{50} of 5–10 μM. To determine the efficiency of the washing procedure, membranes were preincubated with the reversible opioid ligands, naloxone and DALE. A complete restoration of the binding was observed after 4 washes (not shown). Melphalan itself, at 50 μM, did not cause any inhibition [6].

To determine the specificity of labeling, membranes were pretreated with naloxone or DALE

Table 1

Protection of naloxone binding sites

Treatment	Specific [^{3}H]naloxone binding	
	dpm · mg protein^{-1}	% of control
Control	3111	100
Mel^1,Leu^5-enk.-OMe (50 μM)	318	10
Mel^1,Leu^5-enk.-OMe (50 μM) + naloxone (100 μM)	964	32
Mel^1,Leu^5-enk.-OMe (50 μM) + DALE (100 μM)	894	29

Naloxone and DALE were added 10 min before Mel^1,Leu^5-enk.-OMe to rat brain membranes. After 120 min of further incubation at 30°C, the membranes were washed by 4 centrifugation/resuspension steps and the remaining specific binding was measured with 0.5 nM [^{3}H]naloxone in quadruplicate. Control samples were run under identical conditions, substituting buffer for the ligand. Results are the average of two parallel incubations

before the addition of Mel[1],Leu[5]-enkephalin-OMe. The opioid added first should occupy the receptors, thus protecting them from inactivation by Mel[1],Leu[5]-enkephalin-OMe. As shown in table, only partial protection was achieved under the reaction conditions applied. 50 μM Mel[1],Leu[5]-enkephalin-OMe caused 90% inhibition, which decreased to 70% when naloxone or DALE was given first.

4. DISCUSSION

A new analogue of D-Ala[2]-Leu[5]-enkephalin was synthesized and tested for its in vitro binding activity. The compound, Mel[1],Leu[5]-enkephalin-OMe competed with high affinity for both [^{3}H]naloxone and [^{3}H]DALE binding sites. Thus, the generally accepted rule, that an intact Tyr residue is necessary for opioid activity, has to be reconsidered. The fundamental role of Tyr was explained such [10] that the benzene ring of morphine which bears a hydroxyl group is in precisely the same orientation as the benzene ring of tyrosine, suggesting that this group binds to the opioid receptor in both cases. Conformational reasons might cause the extraordinary property of melphalan, which allow retainment of opioid activity upon replacing Tyr. The chloroethylamino group in the *para* position of phenylalanine might induce a conformational change of the peptide which then fits to the receptor. It is not a suprise, on the other hand, that melphalan substitution resulted in a compound which is able to bind irreversibly to the receptor, melphalan being a well-known alkylating agent. We have found that other analogues of D-Ala[2]-Leu[5]-enkephalin containing melphalan in different positions of the peptide chain are also able to bind irreversibly to the opioid receptor (submitted). Experiments are in progress to elucidate the mechanism of Mel[1],Leu[5]-enkephalin-OMe binding.

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