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Irreversible labelling of the opioid receptors by a melphalan-substituted [Met^5]enkephalin-Arg-Phe derivative

Nana Sartania [a,1], Ildikó Szatmári [a], György Orosz [b], András Z. Rónai [b], Kálmán Medzihradszky [b], Anna Borsodi [a], Sándor Benyhe [a,*]

[a] *Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, P.O.B. 521 Szeged, H-6701, Hungary*
[b] *Group of Peptide Chemistry, Hungarian Academy of Sciences, P.O.B. 32, Budapest 112, H-1518, Hungary*

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Abstract

[Met^5]enkephalin-Arg-Phe (Tyr-Gly-Gly-Phe-Met-Arg-Phe) was modified with the methyl esther of melphalan (Mel; 4-bis(2-chloroethyl)amino-L-phenylalanine) and the resulting compounds were studied for their opioid binding properties in guinea pig and rat brain membranes. Three new peptides, with a substitution of a single amino acid, were synthesized (**Mel**-Gly-Gly-Phe-Met-Arg-Phe, Tyr-Gly-Gly-**Mel**-Met-Arg-Phe and Tyr-Gly-Gly-Phe-Met-Arg-**Mel**). In the rat brain, none of these ligands displayed any type specificity, whereas in guinea pig brain membranes the C-terminally modified peptide, Tyr-Gly-Gly-Phe-Met-Arg-Mel ([Mel^7]peptide), displayed a κ-binding profile and was a weak κ-opioid-receptor agonist in isolated guinea pig ileum. The effect of sodium ions on [Mel^7]peptide competition against [^{3}H]naloxone binding indicated a weak agonist nature of the compound. When guinea pig brain membranes were preincubated with 1–10 μM of [Mel^7]peptide, an apparently irreversible inhibition of [^{3}H]naloxone ligand binding was observed. These results suggest that the heptapeptide containing melphalan at the C-terminus can be used as a relatively high-affinity irreversible label for the κ-opioid receptor.

Keywords: Opioid peptide; κ-Opioid receptor; Ligand binding; Affinity labelling; Melphalan; Brain, guinea pig

1. Introduction

[Met^5]enkephalin-Arg-Phe (Tyr-Gly-Gly-Phe-Met-Arg-Phe) is a proenkephalin-derived naturally occurring heptapeptide generally found in the adrenal gland and brain. It has antinociceptive activity when administered directly into the cerebral ventricles of mice, supporting the opioid pharmacology of this heptapeptide (Inturrisi et al., 1980). [Met^5]enkephalin-Arg-Phe is found in high amounts in human, rat and bovine striatum in concentrations comparable or greater than those of [Leu^5]enkephalin (Rossier et al., 1980). The heptapeptide is also detected in human putamen and globus pallidus (Stern et al., 1979) as well as in the brain of some lower vertebrates (Kilpatrick et al., 1982). [Met^5]enkephalin-Arg-Phe has been found to be a selective ligand for κ_2-opioid receptors in opioid receptor binding assays performed with [^{3}H]etorphine or [^{3}H]ethylketocyclazocine and rodent spinal cord membranes (Attali et al., 1982; Gouarderes and Cros, 1984) as well as frog brain membrane preparations (Benyhe et al., 1990). [^{3}H][Met^5]enkephalin-Arg-Phe has been shown to label κ_2- and δ-opioid sites in rat (Benyhe et al., 1997a) and κ_2-sites in frog brain membrane preparations (Wollemann et al., 1994). The radiolabelled heptapeptide also interacts with σ_2-like non-opioid sites (Benyhe et al., 1997a).

Since the discovery of endogenous opioid peptides a number of synthetic analogues have been developed for structure-activity relationship studies, among them electrophilic affinity reagents, which are capable of interacting with the receptors in an irreversible manner. Such ligands, named 'affinity labels', should have high affinity and good receptor-type selectivity. In addition, they should possess a chemically reactive functional group targeting a nucleophilic center at or near the ligand binding site of the receptor protein. Besides the opioid peptides, halomethyl ketone enkephalins (Szücs et al., 1983; Newman and

* Corresponding author. Tel.: +36-62-432-232 Ext. 176; Fax: +36-62-433-432; E-mail: benyhe@nucleus.szbk.u-szeged.hu

[1] Present address: Research School of Biosciences, The University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, UK.

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Barnard, 1984; Benyhe et al., 1987) and dynorphins (Benyhe et al., 1997b), melphalan-containing enkephalins have been developed as potent affinity labels for opioid receptors.

Melphalan (**Mel**; 4-bis[2-chloroethyl]amino-L-phenylalanine), a nitrogen mustard derivative of phenylalanine, is a well-known alkylating agent and has antitumor activity (Samuels and Bitran, 1995). Melphalan-containing irreversible peptide ligands have been described for different receptors, e.g., melanotropin receptor (Süli-Vargha et al., 1990) and bombesin receptor (De Castiglione et al., 1991). Introduction of Mel into enkephalin sequences led to the development of powerful affinity reagents for opioid binding sites (Szücs et al., 1983, 1985; Lovett and Portoghese, 1986, 1987). Here, we report the synthesis of three different melphalan-containing analogues of the C-terminally elongated enkephalin [Met^5]enkephalin-Arg-Phe, where the N-terminal Tyr^1, Phe^4, or Phe^7 on the C-terminus was substituted with melphalan and the resulting compounds were studied for their opioid binding properties in rat and guinea pig brain membranes. Taking into consideration that the parent [Met^5]enkephalin-Arg-Phe is considered to be κ_2 selective (Attali et al., 1982; Gouarderes and Cros, 1984; Wollemann et al., 1993, 1994), similar features of the new heptapeptide derivatives were predicted.

2. Materials and methods

2.1. Chemicals

[^{3}H]Naloxone (57 Ci/mmol) was synthesized as described previously (Tóth et al., 1982). [^{3}H][D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin (45 Ci/mmol) and [^{3}H]U-69,593 (5α,7α,8β-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl)]benzene-acetamide) (44 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK), [^{3}H]ethylketocyclazocine (20 Ci/mmol) was from DuPont-NEN (Cambridge, MA, USA). [^{3}H]norbinaltorphimine (Márki et al., 1995), [^{3}H][Met^5]enkephalin-Arg-Phe (Wollemann et al., 1994) and [^{3}H]Ile^{5,6}deltorphin II (Nevin et al., 1994) were obtained from the Isotope Laboratory, BRC, Szeged, Hungary. The [Met^5]enkephalin-Arg-Phe derivatives were synthesized in our laboratory except the tyrosine and the arginine derivatives, which were from BACHEM (Switzerland) and Novabiochem (UK), respectively. HBTU (2-1*H*(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate) and benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate were from Richelieu Biotechnologies (Canada). Wang and 2-chlorotrityl chloride resin (200–400 mesh) were purchased from BACHEM (Switzerland). All other chemicals used in this study were of analytical grade and purchased from Sigma (St. Louis, USA) or Reanal/Egis Pharmaceuticals (Budapest, Hungary).

2.2. Synthesis of H-Mel-GGFMRF (Mel-Gly-Gly-Phe-Met-Arg-Phe), H-YGG-Mel-MRF (Tyr-Gly-Gly-Mel-Met-Arg-Phe) and H-YGGFMR-Mel (Tyr-Gly-Gly-Phe-Met-Arg-Mel)

2.2.1. H-Mel-GGFMRF-OH × 2 HCl

The synthesis of the peptide was performed by standard manual fluorenylmethyloxycarbonyl solid-phase peptide synthesis procedures (the tyrosine hydroxyl group is protected in the form of *t*-butyl ether, whereas the arginine guanidine is protected by 2,2,5,7,8-pentamethylchroman-6-sulfonyl group) on Wang resin, using two equivalents of fluorenylmethyloxycarbonyl-amino acid and 1.9 equivalents of HBTU (2-1*H*(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate) in the presence of diisopropylethylamine. After the melphalan residue was coupled to the peptide chain in the form of Boc-Mel-OH, the resin was washed and dried. The peptide and the protecting groups were cleaved at 0°C with hydrogen fluoride (HF) containing 20% anisole. After evaporation of HF, the peptide was precipitated with diethyl ether. The peptide was dissolved in ice-cold 0.1 N HCl, separated from the resin particles and lyophilized. After purification by high-performance liquid chromatography and lyophilization, the peptide showed 95% homogeneity. [R_F(*n*-butanol–acetic acid–water = 4:1:1) = 0.34, R_F(Ethyl acetate–pyridine–acetic acid–water = 60:20:6:11) = 0.31, Merck Kieselgel 60 glass plates]. Amino acid analysis: Gly 2.25 (2), Met 1.02 (1), Phe 2.0 (2), Arg 1.03 (1). FAB-MS = 1000.5 (MH +).

2.2.2. H-YGG-Mel-RMF-OH × 2 HCl

H-Met-Arg(Tos)-Phe-OBzl × HCl was synthesized by solution-phase synthesis starting from phenylalanine benzyl ester hydrochloride and sequential coupling with Boc-Arg(Tos)-OH and Boc-Met-OH. The removal of the Boc group was accomplished with HCl (3.5 M) in ethyl acetate acid containing 20% anisole.

Boc-melphalan (450 mg, 1.1 mM) and the tripeptide hydrochloride (733 mg, 1 mmol) were coupled in 5 ml dimethyl formamide in the presence of benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate reagent (0.44 g, 1 mmol) and 388 mg (514 μl, 3 mmol) of diisopropylethylamine at 0°C for 30 min and further stirred at room temperature for another 30 min. The solution was evaporated, the residue was dissolved in ethyl acetate and washed sequentially with sodium bicarbonate, potassium hydrogen sulphate and brine and then the solution was evaporated to yield 1.17 g (> 100%) of thick oil. Without further purification, the oil was dissolved in 5 ml of ethyl acetate and the Boc group was removed by the addition of 25 ml of HCl/ethyl acetate (3.5 M) containing 20% anisole.

After trituration with diethyl ether, the precipitated oil solidified to yield 0.67 g of solid [R_F(*n*-butanol–acetic

acid–water = 4:1:1) = 0.88, R_F(Ethyl acetate–pyridine–acetic acid–water = 60:20:6:11) = 0.83]. Coupling of 166 mg (0.42 mmol) of Boc-Tyr-Gly-Gly-OH with 390 mg (0.38 mmol) of the tetrapeptide hydrochloride yielded 0.43 g (83%) of crude Boc-YGG-Mel-Met-Arg(Tos)-Phe-OBzl. [R_F(chloroform–acetic acid–methanol = 90:2:8) = 0.62, R_F(Ethyl acetate–pyridine–acetic acid–water = 240:20:6:11) = 0.66].

The protecting groups were removed by treating the peptide with HF in the presence of anisole at 0°C for 1 h. The peptide obtained after evaporation of HF and precipitation with diethyl ether was purified by high performance liquid chromatography to yield 118 mg of lyophilate which showed more than 95% homogeneity. Amino acid analysis: Gly 2.06 (2), Tyr 1.07 (1), Phe 1.0 (1), Arg 1.01 (1), Met 0.92 (1). FAB-MS: 1016.6 (MH +)

2.2.3. H-YGGFMR-Mel-OH × 2 HCl

Boc-Tyr(tBu)-Gly-Gly-Phe-Met-Arg(Pmc)-OH was synthesized on 2-chlorotrityl chloride resin (Barlos et al., 1989) by the general fluorenylmethyloxycarbonyl synthesis protocol described above. The protected peptide was cleaved from the resin in dichloromethane–methanol–acetic acid (8:1:1) mixture.

Boc-Tyr(tBu)-Gly-Gly-Phe-Met-Arg(Pmc)-OH (763 mg, 0.662 mmol) was coupled to melphalan benzyl ester hydrochloride (Hsieh and Marshall, 1981) (301 mg, 0.73 mmol) in 2 ml dimethyl formamide by condensing the components with benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate reagent and diisopropylethylamine at 0°C. After the reaction mixture was stirred for 30 min, the solvent was evaporated and the remaining oil was subjected to flash chromatography on silica gel using chloroform–methanol–acetic acid (9:0.8:0.2) to yield 522 mg of thick oil which solidified under vacuum. The protecting groups were cleaved in HF:anisole (8:2) mixture at 0°C for 1 h and the material obtained after evaporation of HF and precipitation of the peptide by ice-cold diethyl ether was purified by high-performance liquid chromatography to give 126 mg of peptide with 85% homogeneity. Amino acid analysis: Gly 2.13 (2), Tyr 0.96 (1), Phe 1.0 (1), Arg 0.98 (1), Met 0.96 (1). FAB-MS: 1016.6 (MH +).

The purity of the peptides was checked by analytical high-performance liquid chromatography (Vydac 4.6 × 250 mm C18 (5 μm) column, acetonitrile–water–0.1% trifluoroacetic acid solvent system (1% acetonitrile content increase/min) and by thin-layer chromatography on Merck Kieselgel 60 F_{254} plates in two different solvent systems. For the visualization of the peptides on the plates the standard ninhydrin, chlorine-*o*-tolidine and Epstein reactions (Epstein et al., 1955) were used.

2.3. Membrane preparations

Wistar laboratory rats of both sexes and non-albino R9 guinea pigs were used throughout this study. Brain membrane preparations were prepared as described previously (Benyhe et al., 1997a). Briefly, the rats or guinea pigs were decapitated following CO_2 narcosis, and the brains without cerebella were rapidly removed, washed with chilled physiological saline and homogenized in 30 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4). After 20 min centrifugation at 40 000 × *g*, the pellet was resuspended in fresh buffer and incubated at 37°C for 30 min, then recentrifuged and the final pellet was suspended in five volumes of fresh buffer containing 0.32 M sucrose. Aliquots (5 ml) were frozen in liquid nitrogen and stored at −70°C. Before use, the membranes were thawed and washed by centrifugation to remove the sucrose. Protein content was estimated according to the method of Bradford (1976), using bovine serum albumin as a standard.

2.4. Ligand binding assay

The membrane suspension (200–400 μg protein) was incubated with the radioligand in a final volume of 1 ml in polypropylene assay tubes. Incubations were carried out in the presence of a mixture of peptidase inhibitors (1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsufonylfluoride, 40 KiU/ml trasylol, 20 μg/ml bacitracin, 1 μM bestatin, 1 μM phosphoramidone, 4 g/ml soybean trypsin inhibitor) and terminated by rapid filtration through Whatman GF glass fiber filters, using a Brandel M24R Cell Harvester. Filters were quickly washed with 3 × 7 ml ice-cold Tris–HCl (50 mM, pH 7.4) buffer. The radioactivity was measured in a toluene-based scintillation cocktail, using a Wallac 1409 spectrophotometer with 55% counting efficiency. Potencies of competing ligands were determined by co-incubation with 10^{-11}–10^{-4} M freshly prepared solutions of unlabelled drugs with 0.5–1 nM tritiated type-specific ligands. Non-specific binding was defined as the radioactivity bound in the presence of 10 μM unlabelled naloxone. All assays were performed in duplicate and repeated 3–5 times.

2.5. Affinity labelling

Incubations were carried out in 40-ml polycarbonate centrifuge tubes (Sorvall) for 30 or 45 min at 24°C. The incubation mixture contained 1 ml of the suspension of brain membranes (3–4 mg protein), 200 μl of the peptide at an appropriate concentration in 50 mM Tris/HCl buffer supplemented with inhibitors (buffer D), in a final volume of 2 ml. After incubation in a shaking water-bath, the samples were diluted with 28 ml buffer, incubated for 10 min at room temperature and centrifuged at 25 000 × *g* for 15 min in a Sorvall RC5C centrifuge with rotor model SS34. Pellets were suspended, by vortexing, in a small amount of 50 mM Tris/HCl buffer supplemented with the inhibitors, diluted with the buffer to a volume of 30 ml, incubated for 15 min at room temperature in order to

Table 1
Affinity of [Met^5]enkephalin-Arg-Phe and its derivatives for [^{3}H]naloxone binding sites in rat brain membranes and their opioid agonist activity in guinea pig ileum assay

Compound	$-Na^+$ IC_{50} (nM)	$+Na^+$ IC_{50} (nM)	Na^+-index	ED_{50} (nM) on guinea pig ileum
Tyr-Gly-Gly-Phe-Met-Arg-Phe [Met^5]enkephalin-Arg-Phe	32 ± 7	116 ± 23	3.6	679 ± 107
Mel-Gly-Gly-Phe-Met-Arg-Phe [Mel^1]peptide	1120 ± 298	2890 ± 480	2.6	N.D.
Tyr-Gly-Gly-**Mel**-Met-Arg-Phe [Mel^4]peptide	1370 ± 338	6310 ± 379	4.6	N.D.
Tyr-Gly-Gly-Phe-Met-Arg-**Mel** [Mel^7]peptide	142 ± 37	1330 ± 176	9.4	1117 ± 239

Membranes were incubated in the presence of 1 nM radioligand for 60 min at 0°C. Sodium ions were added as NaCl in 100 mM final concentration. Competition experiments were analyzed by the program GraFit using the 'four parameter logistic' fitting option. Values (±S.E.M.) are the means of at least three experiments.
N.D.: not determined.

dissociate loosely bound ligand, and then centrifuged as above. This washing step was repeated four times. The final pellets were homogenized in 8 ml buffer by vortexing the centrifuge tubes followed by passing the suspension successively through 21 G needles by means of plastic syringes. The preparation was used immediately for receptor binding assays.

2.6. Data analysis

Experimental data from competition experiments were analyzed to determine binding parameters for unlabelled compounds (IC_{50}) and were evaluated by the GraFit computer program, a non-linear least-squares curve fitting program (Leatherbarrow, 1992).

2.7. Physiological tests on isolated organs

Longitudinal muscle strips taken from the ilea of male guinea pigs weighing 400–600 g were prepared according to Paton and Vizi (1969) and used as described previously (Rónai et al., 1977). A range of enzyme inhibitors, like bestatin (3×10^{-5} M) and Leu-Leu-OH (2×10^{-3} M), was added as recommended by McKnight et al. (1983). Opioid agonist effects were characterized in terms of 50% inhibitory concentration (IC_{50}), calculated from the logarithmic regression of dose–response curves. Antagonist properties were quantified by calculating the K_e (equilibrium dissociation constant) values according to Arunlakshana and Schild (1959), using the 'single dose' method (Kosterlitz and Watt, 1969). The equilibration period with the antagonists was 20 min. For a set of data, the geometric mean and 95% confidence intervals were calculated (Fleming et al., 1972).

3. Results

Three derivatives of [Met^5]enkephalin-Arg-Phe containing melphalan (Mel) at different positions were synthesized and their potencies were compared in receptor binding studies as well as in pharmacological experiments with isolated guinea pig ileum assay. Binding of the Mel-substituted heptapeptides was first examined in rat brain membranes by using the general opioid antagonist radioligand, [^{3}H]naloxone (Table 1). They exhibited moderate to weak affinity compared with that of the parent compound, [Met^5]enkephalin-Arg-Phe. The affinity of the peptides in displacing [^{3}H]naloxone was substantially inhibited in the presence of 100 mM NaCl, which suggests that these compounds have opioid agonist properties.

Table 2
Inhibitory potency of [Met^5]enkephalin-Arg-Phe and its derivatives in equilibrium competition experiments performed with type-specific opioid radioligands in rat brain membranes

Compound	IC_{50} (nM)		
	μ	κ	δ
Tyr-Gly-Gly-Phe-Met-Arg-Phe ([Met^5]enkephalin-Arg-Phe)	186 ± 23	9.9 ± 1.3	298 ± 49
Mel-Gly-Gly-Phe-Met-Arg-Phe ([Mel^1]peptide)	7523 ± 768	> 10 000	1077 ± 83
Tyr-Gly-Gly-**Mel**-Met-Arg-Phe ([Mel^4]peptide)	> 10 000	> 10 000	5910 ± 928
Tyr-Gly-Gly-Phe-Met-Arg-**Mel** ([Mel^7]peptide)	2052 ± 487	268 ± 71	361 ± 98

Binding to μ-opioid receptors was measured with [^{3}H][D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin (45 min, 35°C).
κ-Opioid receptors were labelled by [^{3}H]ethylketocyclazocine (45 min, 24°C in the presence of 100 nM [D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin and 100 nM [D-Ala^2,D-Leu^5]enkephalin) in order to block crossbinding to mu and delta sites).
δ-Opioid receptor activity was determined with [^{3}H]Ile^{5,6}deltorphin II (45 min, 35°C).
IC_{50} values were calculated by mathematical analysis of data from heterologous competition experiments.
Data are means ± S.E.M. of three determinations.

Table 3
Affinity of peptides in competing with type-specific opioid radioligands in guinea pig brain membranes

Compound	IC_{50}(nM)		
	μ	κ	δ
Tyr-Gly-Gly-Phe-Met-Arg-Phe ([Met^5]enkephalin-Arg-Phe)	275 ± 76	9.1 ± 0.6 6.2 ± 0.5[a]	335 ± 28
Mel-Gly-Gly-Phe-Met-Arg-Phe ([Mel^1]peptide)	3796 ± 939	> 10 000	1160 ± 213
Tyr-Gly-Gly-**Mel**-Met-Arg-Phe ([Mel^4]peptide)	9220 ± 666	> 10 000	7599 ± 513
Tyr-Gly-Gly-Phe-Met-Arg-**Mel** ([Mel^7]peptide)	1350 ± 98	25.4 ± 2.5	670 ± 84

μ-Receptor activity was measured with [^{3}H][D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin.
κ-Opioid receptors were labelled by [^{3}H]norbinaltorphimine (45 min, 24°C).
δ-Opioid receptor affinity was determined with [^{3}H]Ile^{5,6}deltorphin II.
[a]κ_2-Affinity was measured with [^{3}H][Met^5]enkephalin-Arg-Phe (40 min, 0°C).
Values are the means ± S.E.M. of at least three experiments performed in duplicate.

The agonist effects of the peptides were further studied on isolated longitudinal muscle strips of guinea pig ileum (Table 1). Kosterlitz et al. have demonstrated the existence of two types of opioid receptors—μ and κ—in the guinea pig ileum (Lord et al., 1977; Kosterlitz et al., 1980). The K_e of the slightly μ-opioid receptor preferring antagonist naltrexone at the μ-opioid receptor type is approximately 0.3 nM. A K_e higher than 2 nM for naltrexone against an agonist in guinea pig ileum is a clear indication of a very significant κ-opioid receptor contribution to the action of the agonist. The IC_{50} of [Mel^7]peptide in guinea pig ileum was 1.120 nM (710–1750, $n = 4$); the K_e of naltrexone against it was 12.2 nM (7.4–20.0, $n = 3$). The corresponding values for [Met^5]enkephalin-Arg^6-Phe^7 in the same preparation were 680 nM (530–870, $n = 3$) and 0.9 nM (0.8–1.1, $n = 3$).

The receptor-type selectivity of the compounds was investigated by the use of appropriate radioligands in rat brain membranes. The Mel-containing peptides exhibited only low potencies in inhibiting μ-, δ- and κ-binding sites labelled by [^{3}H][D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin, [^{3}H]Ile^{5,6}-deltorphin II and [^{3}H]ethylketocyclazocine, respectively (Table 2). Yet, the moderate affinity of [Mel^7]peptide observed in [^{3}H]ethylketocyclazocine binding assays suggested a κ-like binding preference for the peptide. Therefore the binding properties of the compounds were subsequently examined in membrane fractions of guinea pig brain.

In guinea pig brain membranes, only the C-terminally modified [Mel^7]peptide showed high affinity, with IC_{50} values in the nanomolar range, when κ-selective radioligands were used (Table 3). [Mel^1]peptide and [Mel^4]peptide had no notable interaction with the opioid binding sites, whereas [Mel^7]peptide displayed quite good affinity for κ-sites. On the other hand, μ- and δ-binding sites were less affected by these peptides, as indicated by the low potency of the compounds for competing reversibly with [^{3}H][D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin and [^{3}H]Ile^{5,6}-deltorphin II binding (Table 3).

The affinity of [Mel^7]peptide was compared with that of the parent [Met^5]enkephalin-Arg-Phe as well as with that of several opioid ligands by measuring the inhibition of the

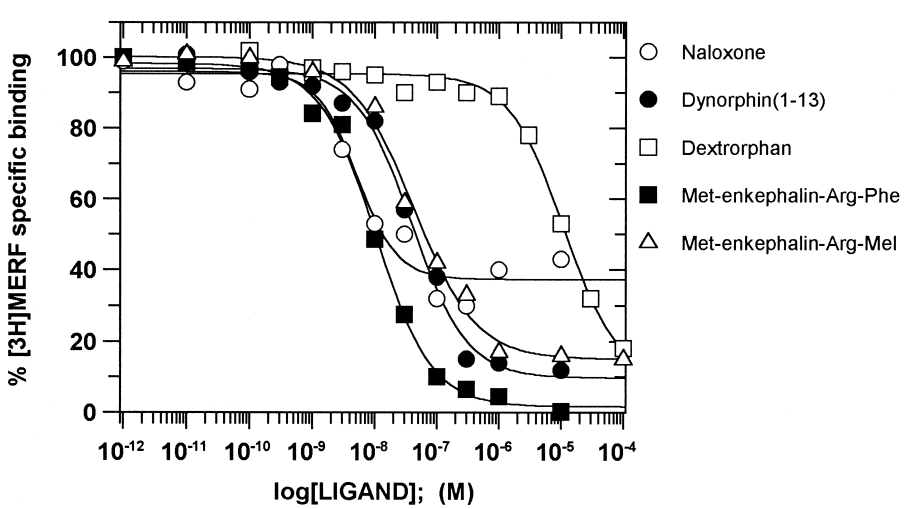

Fig. 1. Inhibition by various ligands of [^{3}H][Met^5]enkephalin-Arg-Phe specific binding to guinea pig brain membrane preparations. Membrane samples were incubated in the presence of 1 nM radioligand and the stated concentrations of unlabelled compounds for 40 min at 0°C. Each point represents the mean of duplicate values in a single experiment that was repeated several times.

Table 4
Potency of Tyr-Gly-Gly-Phe-Met-Arg-Mel ([Mel^7]peptide) in equilibrium competition experiments performed with κ-selective opioid radioligands

Labelled drug used	IC_{50} (nM)
[^{3}H]ethylketocyclazocine[a] (0.5 nM)	1.78 ± 0.54
[^{3}H]norbinaltorphimine (0.1 nM)	25.4 ± 2.5
[^{3}H][Met^5]enkephalin-Arg-Phe (1 nM)	46.8 ± 13.6
[^{3}H]U-69,593 (0.5 nM)	62.7 ± 10.2

[a][^{3}H]ethylketocyclazocine binding assays were carried out in the presence of 100 nM [D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin and 100 nM [D-Ala^2,D-Leu^5]enkephalin.

Guinea pig brain membranes were incubated with the indicated concentrations of [^{3}H]ethylketocyclazocine (40 min), [^{3}H]nor-binaltorphimine (60 min), [^{3}H][Met^5]enkephalin-Arg-Phe (45 min) each at 24°C and [^{3}H]U69.593 for 30 min at 30°C in the presence of increasing concentrations of [Mel^7]peptide.

IC_{50} values were determined by GraFit analysis. Each value represents the mean ± S.E.M. of three to six independent determinations carried out under identical conditions.

binding of [^{3}H][Met^5]enkephalin-Arg-Phe, a radioligand designed for the characterization of κ_2-binding sites (Wollemann et al., 1994; Benyhe et al., 1997a). Homologous and heterologous competition curves for [^{3}H][Met^5]enkephalin-Arg-Phe binding in guinea pig brain are illustrated in Fig. 1. The rank order potency of ligands tested here was [Met^5]enkephalin-Arg-Phe > naloxone > dynorphin$_{(1-13)}$ > [Mel^7]peptide > U-50,488 > dextrorphan.

When various radioligands with κ-selectivity were used, [Mel^7]peptide was the most efficient in displacing [^{3}H]ethylketocyclazocine, followed by [^{3}H]norbinaltorphimine, [^{3}H][Met^5]enkephalin-Arg-Phe and [^{3}H]U-69,593 with minor differences in the IC_{50} values (Table 4).

In order to characterize the irreversible portion of the binding of Mel-containing peptides, the wash resistance of prelabelling was examined (Szücs et al., 1983). In such experiments, small volumes of brain membranes were preincubated in the presence of the affinity reagents and the reversibly bound ligands were removed by extensive washing of the membranes with buffer. Washed membranes were then subjected to radioligand binding to estimate their remaining opioid binding activity. The binding of [Mel^7]peptide to guinea pig brain membranes was found to be apparently irreversible. As shown in Fig. 2, [Mel^7]peptide caused a dose-dependent, wash-resistant inhibition of specific [^{3}H]naloxone binding. The apparent IC_{50} value for the irreversible blockade by [Mel^7]peptide was approximately 5–10 μM, depending upon the preincubation time. Maximal inhibition was observed at 10 μM ligand concentration (Fig. 2).

To determine the efficiency of the washing procedure, membranes were preincubated with reversible opioid ligands such as naloxone or norbinaltorphimine and the remaining binding activity was measured after extensive washing. Control values were obtained, proving that the washing procedure was sufficient to remove reversibly

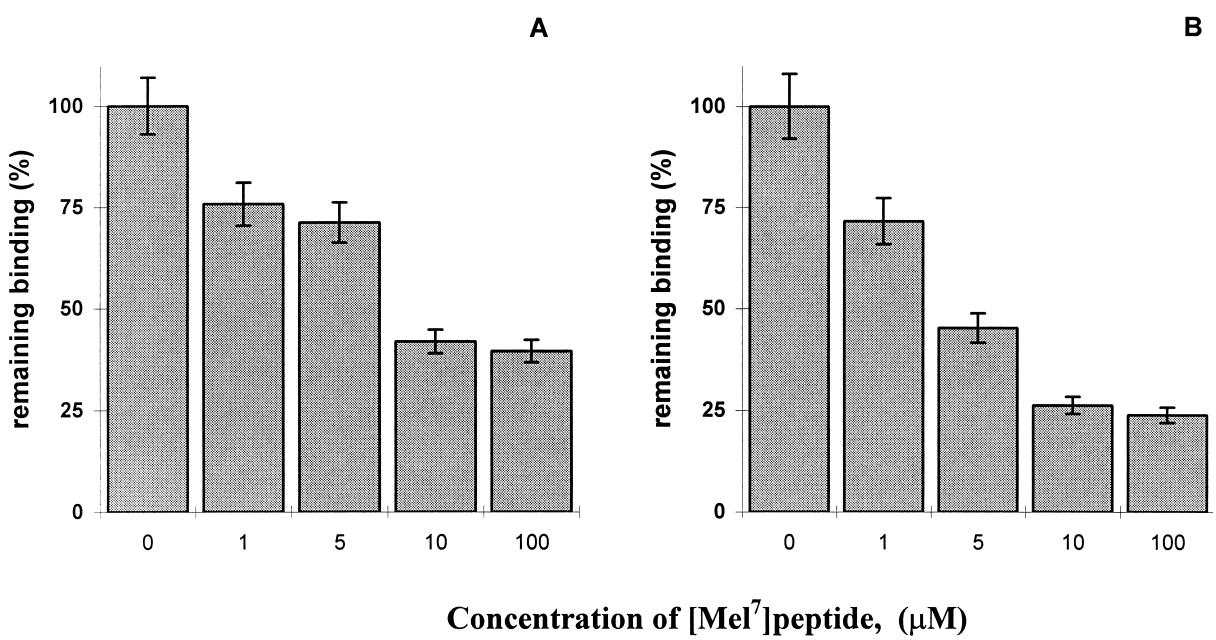

Fig. 2. Concentration dependence of the irreversible interaction of opioid receptors with [Mel^7]peptide ligand. Guinea pig brain membranes were incubated with various concentrations of [Mel^7]peptide at 24°C for 30 min (A) and for 45 min (B) in a final volume of 4 ml in 50 mM Tris–HCl (pH 7.4) buffer containing 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsufonylfluoride, 20 μg/ml bacitracin, 1 μM bestatin, 1 μM phosphoramidone, 4 μg/ml soybean trypsin inhibitor, 40 KIU/ml trasylol as protease inhibitors. After four washes the remaining binding was measured with 1 nM [^{3}H]naloxone after an additional 1-h incubation at 4°C. Remaining binding was normalized to the protein concentration and expressed as a percentage of control binding. Control values are the specific binding of [^{3}H]naloxone to membranes preincubated with buffer and treated in the same way. Results are the averages (± 'range') of two independent experiments.

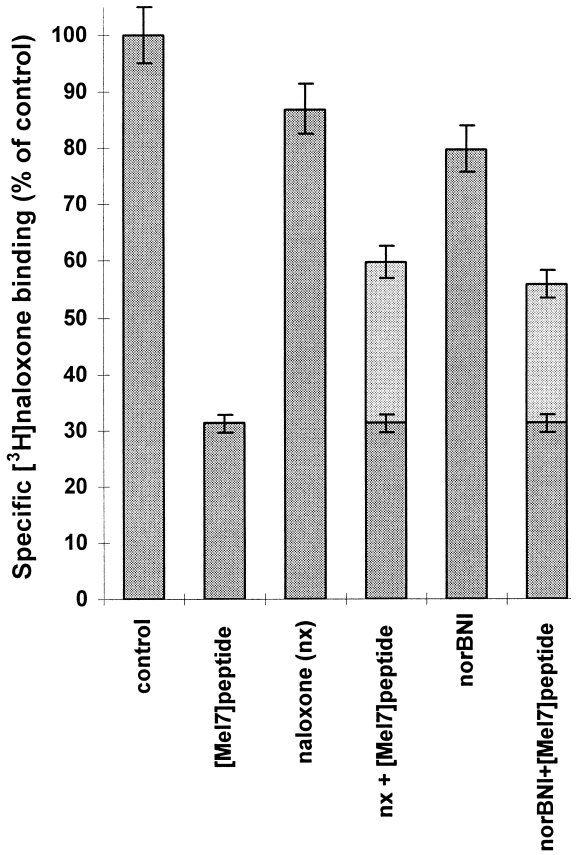

Fig. 3. Protection of [^{3}H]naloxone binding sites against inactivation by [Mel^7]peptide. Guinea pig brain membranes were treated with either 10 μM [Mel^7]peptide or 10 μM reversible opioids (naloxone, norbinaltorphimine) for 45 min at 24°C. In protection studies (5th and 6th column), 10 μM naloxone or norbinaltorphimine (norBNI) was added 15 min prior to incubation with 10 μM [Mel^7]peptide. The reactions were terminated by centrifugation of the suspensions followed by four washing (dilution/centrifugation/resuspension) steps. The remaining binding was measured with 1 nM [^{3}H]naloxone in duplicate. Specific binding was normalized to the protein content of the samples. Control samples (1st column) were run under identical conditions, substituting buffer for the ligand. Bars represent the mean values (±S.E.M.) of three duplicate experiments.

bound ligands (Fig. 3). Naloxone and norbinaltorphimine were also tested for protecting the binding sites against inactivation by [Mel^7]peptide. As can be seen in Fig. 3, pretreatment of the membranes with 10 μM naloxone or norbinaltorphimine was able to decrease the irreversible blockade caused by the alkylating agent in equimolar concentrations.

4. Discussion

New derivatives of the endogenous opioid heptapeptide [Met^5]enkephalin-Arg^6-Phe^7, with single amino acid substitutions by melphalan in three different positions, were synthesized and tested for their in vitro binding activities. In rat brain membranes Tyr-Gly-Gly-**Mel**-Met-Arg-Phe ([Mel^4]peptide) showed only low potency in competing with the opioid antagonist ligand [^{3}H]naloxone. **Mel**-Gly-Gly-Phe-Met-Arg-Phe ([Mel^1]peptide) was also found to be ineffective in the binding assays, despite the fact that melphalan replaced Tyr^1 in the structure of [Leu^5]enkephalin (Szücs et al., 1985). One compound, however, Tyr-Gly-Gly-Phe-Met-Arg-**Mel** ([Mel^7]peptide), displayed comparable affinity in opioid binding assays, exhibiting a preference for κ binding sites. This peptide retained the opioid activity and type specificity of its parent compound; moreover its binding to the receptor was shown to be partially irreversible.

Opioid peptides including [Met^5]enkephalin-Arg-Phe are subjected to rapid inactivation by membrane-bound and intracellular peptide hydrolysing enzymes (Dupont et al., 1977; Benuck et al., 1981; Hiranuma et al., 1997). Binding and prelabelling studies with the heptapeptide derivatives were therefore undertaken in the presence of various peptidase inhibitors. Inhibitors were selected on the basis of previous studies with elongated enkephalins and dynorphins (Hiranuma et al., 1997; Gillan et al., 1985; Benyhe et al., 1997b). Thiorphan and captopril, however, were omitted from the incubation mixtures since they can chemically react with the alkylating group of melphalan.

In [^{3}H]naloxone binding assays [Mel^7]peptide showed moderate affinity in comparison with [Met^5]enkephalin-Arg-Phe in rat brain membrane fractions. Its potency was even weaker when binding affinities were measured with type-selective opioid radioligands (Table 1). [Mel^7]peptide retained its high affinity for κ-binding sites in guinea pig brain membranes (Table 3). This tissue has been shown to be rich in κ-opioid receptors (Kosterlitz et al., 1981; Zukin et al., 1988), whereas in rat brain κ-sites represent the smallest population of opioid sites. The key structure for the κ selectivity of endogenous opioid peptides is an arginine residue in position 6, which is shared by [Met^5]enkephalin-Arg-Phe, dynorphins, α-neo-endorphin and [Met^5]enkephalin-Arg-Gly-Leu, but it is not present in β-endorphin, pentapeptide enkephalins or endomorphins. Recently this positively charged region of opioid peptides was postulated as a core sequence essential for κ-selectivity (Mansour et al., 1995). Natural opioid peptides containing Arg^6 are known to exhibit fairly high κ preference in a variety of systems, but endorphins, enkephalin pentapeptides and endomorphins act mainly through δ- and μ-opioid receptors. Since Arg^6 is maintained in the structure of all three synthetic Mel-derivatives, including [Mel^7]peptide, κ-selectivity of the compounds was supposed. Indeed, [Mel^7]peptide was capable of interacting with κ-sites labelled by different type-selective primary ligands in guinea pig brain membranes (Table 4). The apparent affinities of the compound, as represented by IC_{50} values (ligand concentration required for 50% inhibition of specific radioligand binding), were similar to one another regardless of the radioligand used for the labelling of κ-opioid recep-

tors. Affinities at κ-sites were substantially higher than those observed at μ- or δ-sites labelled by [^{3}H][D-Ala^2-*N*-Me-Phe^4,Gly^5-ol]enkephalin or [^{3}H]Ile^{5,6}deltorphin II, respectively. Comparison of affinities obtained in [^{3}H]ethylketocyclazocine (κ_2) and [^{3}H]U-69593 (κ_1) assays showed that the ligand possesses some selectivity for κ_2-sites. Mel^7 substitution, however, caused a loss by about an order of magnitude in the affinity (46.8 nM) compared with that of the parent compound (6.2 nM) in [^{3}H][Met^5]enkephalin-Arg-Phe binding assays (Fig. 1).

Preincubation of guinea pig brain membranes in the presence of micromolar concentrations of [Mel^7]peptide followed by extensive washing produced a dose-dependent inhibition of [^{3}H]naloxone binding sites. This set of binding sites is considered to be alkylated by the preincubating ligand under appropriate conditions, which makes binding sites inaccessible for the subsequent occupation by radioligands. The *N,N*,-bis-chloroethyl group in melphalan can attack nucleophilic centers such as sulphydryls or imidazoles located close to the ligand binding pocket, but the detailed mechanism and the exact site of its action needs further investigation including radiolabelling of the reagent. Examination of the concentration and time dependence of the irreversible effect of [Mel^7]peptide revealed that relatively high concentrations of the alkylating reagent are necessary for irreversible blockage (Fig. 3). Similarly to the chloromethyl ketone-containing opioid peptides, irreversible binding was observed by preincubating the membranes with the peptide at a concentration of at least 5–10 μM (Benyhe et al., 1986, 1987, 1997b). To determine the opioid receptor specificity of [Mel^7]peptide labelling, membranes were pretreated with naloxone or with the selective κ-opioid receptor antagonist ligand norbinaltorphimine before the addition of [Mel^7]peptide. The reversible opioid added first should occupy the receptors, protecting them from covalent inactivation by [Mel^7]peptide. In our hands, only partial protection by these opioids was achieved under the reaction conditions applied (Fig. 3). This is probably due to the irreversible nature of [Mel^7]peptide binding, which makes protection by a reversibly bound ligand inherently inefficient.

Halomethyl ketone and melphalan-containing opioid peptides are valuable tools in opioid receptor research. In this respect, [Mel^7]peptide represent a novel synthetic compound among [Met^5]enkephalin-Arg-Phe-related peptides. These peptides are of particular interest because they can interact with a subpopulation of κ-opioid sites termed κ_2-sites (Wollemann et al., 1993) and also recognize non-opioid sites (Benyhe et al., 1997a). [Mel^7]peptide is the first affinity reagent with nitrogen mustard alkylating function developed for κ_2-binding sites. Its structure was designed on the basis of the endogenous opioid [Met^5]enkephalin-Arg-Phe, which is suggested to be the natural agonist ligand for κ_2-opioid receptors. In order to increase the enzymatic stability and selectivity of these peptides, introduction of D-amino acids into the heptapeptide sequence is in progress in our laboratory (manuscript in preparation).

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References

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. 14, 48–58.

Attali, B., Gouarderes, C., Mazarguil, H., Audigier, Y., Cros, J., 1982. Evidence for multiple kappa binding sites by use of opioid peptides in the guinea pig lumbo-sacral spinal cord. Neuropeptides 3, 53–64.

Barlos, K., Gatos, D., Papathotiu, G., Schafer, W., Wenqing, Y., 1989. Veresterung von partiell Geschutzten peptid-fragmenten mit harzen. Einsatz von 2-chlorotritylchlorid zur synthese von Leu^5-gastrin. Tetrahedron Lett. 30, 3947–3950.

Benuck, M., Berg, M.J., Marks, N., 1981. Met-enkephalin-arg^6-phe^7: conversion to met-enkephalin by brain and kidney dipeptidyl carboxypeptidase. Biochem. Biophys. Res. Commun. 99, 630–636.

Benyhe, S., Hepp, J., Szucs, M., Simon, J., Borsodi, A., Medzihradszky, K., Wollemann, M., 1986. Irreversible labelling of rat brain opioid receptors by enkephalin chloromethyl ketones. Neuropeptides 9, 173–184.

Benyhe, S., Hepp, J., Simon, J., Borsodi, A., Medzihradszky, K., Wollemann, M., 1987. Tyr-D-Ala-Gly-(Me)Phe-chloromethyl ketone: a *mu* specific affinity label for the opioid receptor. Neuropeptides 9, 225–235.

Benyhe, S., Varga, É., Hepp, J., Magyar, A., Borsodi, A., Wollemann, M., 1990. Characterization of *kappa*$_1$ and *kappa*$_2$ opioid binding sites in frog (*Rana esculenta*) brain membrane preparation. Neurochem. Res. 15, 899–904.

Benyhe, S., Farkas, J., Tóth, G., Wolleman, M., 1997a. Met^5-Enkephalin-Arg^6-Phe^7, an endogenous neuropeptide, binds to multiple opioid and nonopioid sites in the rat brain. J. Neurosci. Res. 48, 249–258.

Benyhe, S., Ketevan, A., Simon, J., Hepp, J., Medzihradszky, K., Borsodi, A., 1997b. Affinity labelling of frog brain opioid receptors by dynorphin(1–10) chloromethyl ketone. Neuropeptides 31, 52–59.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

De Castiglione, R., Gozzini, L., Galantino, M., Corradi, F., Arlandini, E., Molinari, I., Ciomei, M., 1991. Bombesin receptor antagonists: 3. Irreversible alkylating analogues: melphalan derivatives. Farmaco 46, 743–757.

Dupont, A., Cusan, L., Garon, M., Alvarado-Urbina, G., Labrie, F., 1977. Extremely rapid degradation of [^{3}H]methionine-enkephalin by various rat tissues in vivo and in vitro. Life Sci. 21, 907–914.

Epstein, J., Rosenthal, R.W., Ess, R.J., 1955. Use of g (4-nitrobenzyl) pyridine as analytical reagent for ethylenimines and alkylating agents. Anal. Chem. 27, 1435–1439.

Fleming, W.W., Westfall, D.P., De la Lande, I.S., Jellett, L.B., 1972. Lognormal distribution of equieffective doses of norepinerphine and acetylcholine in several tissues. J. Pharmacol. Exp. Ther. 181, 339–345.

Gillan, M.G., Robson, L.E., McKnight, A.T., Kosterlitz, H.W., 1985. Kappa-binding and degradation of [^{3}H]dynorphin A (1–8) and

[^{3}H]dynorphin A (1–9) in suspensions of guinea pig brain membranes. J. Neurochem. 45, 034–1042.

Gouarderes, C., Cros, J., 1984. Opioid binding sites in different levels of rat spinal cord. Neuropeptides 5, 113–116.

Hiranuma, T., Iwao, K., Kitamura, K., Matsumiya, T., Oka, T., 1997. Almost complete protection from [Met^5]-enkephalin-Arg^6-Gly^7-Leu^8 (Met-enk-RGL) hydrolysis in membrane preparations by the combination of amastatin, captopril and phosphoramidon. J. Pharmacol. Exp. Ther. 281, 769–774.

Hsieh, K.-H., Marshall, G.R., 1981. Alkylating angiotensin analogues: synthesis, analysis and biological activity of angiotensin II analogues containing the nitrogen mustard melphalan in position 8. J. Med. Chem. 24, 1304–1310.

Inturrisi, C.E., Umans, J.G., Wolff, D., Stein, A.S., Lewis, L.V., Stern, S., Udenfriend, S., 1980. Analgetic activity of the naturally occurring heptapeptide Met-enkephalin-Arg^6-Phe^7. Proc. Natl. Acad. Sci. USA 77, 5512–5514.

Kilpatrick, D.L., Howells, R.D., Lahm, H.-W., Udenfriend, S., 1982. Evidence for proenkephalin-like precursor in amphibian brain. Proc. Natl. Acad. Sci. U.S.A. 80, 5772–5775.

Kosterlitz, H.W., Watt, A.J., 1969. Kinetic parameters of narcotic agonists and antagonists with particular reference to *N*-allylnoroxymorphone (naloxone). Br. J. Pharmacol. 33, 266–276.

Kosterlitz, H.W., Lord, J.A.H., Paterson, S.J., Waterfield, A.A., 1980. Effects of changes in the structure of enkephalins and narcotic analgesic drugs on their interaction with mu- and delta-receptors. Br. J. Pharmacol. 68, 333–342.

Kosterlitz, H.W., Paterson, S.J., Robson, L.E., 1981. Characterization of the k subtype of the opiate receptor in the guinea pig brain. Br. J. Pharmacol. 73, 939–949.

Leatherbarrow, R.J., 1992. GraFit Version 3.0, Erithacus Software, Staines, UK, pp. 1–287.

Lord, J.A., Waterfield, A.A., Hughes, J., Kosterlitz, H.W., 1977. Endogenous opioid peptides: multiple agonists and receptors. Nature 267, 495–499.

Lovett, J.A., Portoghese, P.S., 1986. Melphalan-containing *N,N*-dialkylenkephalin analogs as potential irreversible antagonists of the delta opioid receptor. NIDA Res. Monograph 75, 185–188.

Lovett, J.A., Portoghese, P.S., 1987. Synthesis and evaluation of melphalan-containing *N,N*-dialkylenkephalin analogues as irreversible antagonists of the delta opioid receptor. J. Med. Chem. 30, 1668–1674.

Mansour, A., Hoversten, M.T., Taylor, L.P., Watson, S.J., Akil, H., 1995. The cloned mu-receptors, delta-receptors and kappa-receptors and their endogenous ligands—evidence for two opioid peptide recognition cores. Brain Res. 700, 89–98.

Márki, Á., Ötvös, F., Tóth, G., Hosztafi, S., Borsodi, A., 1995. Characterization of kappa opioid receptors with tritiated norbinaltorphimine. Analgesia 1, 557–560.

McKnight, A.T., Corbett, A.D., Kosterlitz, H.W., 1983. Increase in potencies of opioid peptides after peptidase inhibition. Eur. J. Pharmacol. 86, 393–402.

Nevin, S.T., Kabasakal, L., Ötvös, F., Tóth, G., Borsodi, A., 1994. Binding characteristics of the novel highly selective delta-agonist [^{3}H]Ile(5,6)deltorphin-II. Neuropeptides 26, 261–265.

Newman, E.L., Barnard, E.A., 1984. Identification of an opioid receptor subunit carrying the *mu*-binding site. Biochemistry 23, 5385–5389.

Paton, W.D., Vizi, E.S., 1969. The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea pig ileum longitudinal muscle strip. Br. J. Pharmacol. 35, 10–28.

Rónai, A.Z., Gráf, L., Székely, J.I., Dunai-Kovács, Z., Bajusz, S., 1977. Differential behaviour of LPH-(61–91)-peptide in different model systems: comparison of the opioid activities of LPH-(61–91) peptide and its fragments. FEBS Lett. 74, 182–184.

Rossier, J., Audigier, Y., Ling, N., Cros, J., Udenfriend, S., 1980. Met-enkephalin-Arg^6-Phe^7, present in high amounts in brain of rat, cattle and man, is an opioid agonist. Nature 288, 88–90.

Samuels, B.L., Bitran, J.D., 1995. High-dose intravenous melphalan: a review. J. Clin. Oncol. 13, 1786–1799.

Stern, A.S., Lewis, R.V., Kimura, S., Rossier, J., Gerber, L.D., Brink, L., Stein, S., Udenfriend, S., 1979. Isolation of the opioid heptapeptide Met-enkephalin [Arg^6, Phe^7] from bovine adrenal medullary granules and striatum. Proc. Natl. Acad. Sci. U.S.A. 76, 6680–6683.

Süli-Vargha, H., Botyánszky, J., Medzihradszky-Schweiger, H., Medzihradszky, K., 1990. Synthesis of alpha-MSH fragments containing phenylalanine mustard for receptor studies. Int. J. Pept. Prot. Res. 36, 308–315.

Szücs, M., Di Gléria, K., Medzihradszky, K., 1983. A new potential affinity label for the opiate receptor. Life Sci. 33, 435–438, Suppl. 1.

Szücs, M., Di Gléria, K., Medzihradszky, K., 1985. Melphalan potently substitutes the N-terminal Tyr of D-Ala^2-Leu^5-enkephalin methyl ester. FEBS Lett. 179, 87–90.

Tóth, G., Krámer, M., Sirokmán, F., Borsodi, A., Rónai, A.Z., 1982. Preparation of (7,8,19,20-^{3}H)naloxone of high specific activity. J. Label Comp. Radiopharm. 19, 1021–1030.

Wollemann, M., Benyhe, S., Simon, J., 1993. Minireview: the kappa-opioid receptor: evidence for the different subtypes. Life Sci. 52, 599–611.

Wollemann, M., Farkas, J., Tóth, G., Benyhe, S., 1994. Characterization of [^{3}H]Met-enkephalin-Arg^6-Phe^7 binding to opioid receptors in frog brain membrane preparations. J. Neurochem. 63, 1460–1465.

Zukin, R.S., Eghbali, M., Olive, D., Unterwald, E.M., Tempel, A., 1988. Characterization and visualization of rat and guinea pig brain *k* opioid receptors: evidence for k_1 and k_2 opioid receptors. Proc. Natl. Acad. Sci. U.S.A. 85, 4061–4065.