Receptorphin: A conserved peptide derived from the sequence of the opioid receptor, with opioid displacement activity and potent antiproliferative actions in tumor cells
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Abstract

Background: In addition to endogenous opioids, a number of peptide sequences, derived from endogenous (hemorphins, alphaS1-casomorphin), and exogenous proteins (casomorphins, exorphins) have been reported, possessing opioid activity. In the present work, we report the identification of a new peptide, receptorphin (Tyr-Ile-Phe-Asn-Leu), derived from the sequence of the second transmembrane loop of the opioid receptor. This sequence is unique for the opioid receptor, and conserved in all species and receptor-types.

Results and Discussion: Receptorphin competes for opioid binding, presenting a kappa-receptor interaction, while it binds equally to delta- and mu-opioid and somatostatin-binding sites, and inhibits the cell proliferation of a number of human cancer cell lines, in a dose-dependent and reversible manner, at the picomolar or the nanomolar range. Receptorphin shows a preferential action on prostate cancer cells.

Conclusion: Our work identifies, for the first time a peptide, in a receptor sequence, possessing ligand-agonistic activities. A hypothesis, based on receptorphin liberation after cell death, is presented, which could tentatively explain the time-lag observed during opioid antiproliferative action.

Background

Endogenous opioids derive from three main precursor proteins, namely proenkephalin A, proenkephalin B or prodynorphin and proopiomelanocortin (POMC), through alternative post-translational processing. These three precursor proteins give rise to at least seven endogenous opioid peptides [1]. All these peptides usually have a very brief half-life, with the exception of beta-endorphin, and therefore it is believed to act locally. Opioid binding sites, characterized pharmacologically, belong to three main categories, namely delta, mu and kappa. Different subtypes at each opioid site were equally reported (δ₁, δ₂, μ₁, μ₂, κ₁, κ₂, κ₃). A differential distribution of each opioid site was found in the central and the peripheral nervous system [2], while each endogenous opioid ligand binds with a different selectivity to each site [3,4].
The molecular characterization of opioid receptors was obtained in the '90s. The cDNA and the aminoacid sequence of the three main opioid receptors was reported, and found to follow the general scheme of the seven loops membrane receptors [5]. The homology among the reported opioid receptor sequences varies between 50 and 64%. It is interesting to note that transmembrane and intracellular domains, presents a greater homology (~69%) as compared to that of their extracellular part [5–7].

Opioids interfere with a number of physiological actions in the nervous system, including nociception, cognition, and release of hormones or neurotransmitters. In addition, a specific modulatory action of opioids was reported in the peripheral nervous system [8,9], and immune-related cells [10–12]. The biological actions of opioids necessitate the presence of opioid receptors, their ligand and the ligand-specific degradation system in the proximity of their site of action. This is true for the nervous and the immune system, while the hypothalamic-pituitary portal circulation provides opioids to the pituitary gland [13]. In addition, opioids act in other tissues, and recent reports indicate that these agents possess potent antiproliferative actions in tumor cells [14–22], inducing arrest of cell proliferation and apoptosis [23–25]. β-endorphin, an opioid peptide produced mainly by the pituitary and the immune system, with a sufficiently high biological half-life, and/or locally produced opioids were proposed as potential endogenous opioid mediators of this antiproliferative effect [15,17]. In addition to the endogenous classical opioid peptides, a number of other opioid agonists has been reported, derived from food (exorphins) [26,27], hemoglobin (hemorphins) [28–31] or milk-caseins (casomorphins) [16,32–39]. These peptides can also compete for opioid binding, and decrease cell proliferation in different human and animal cell systems, and could be additional sources of locally-acting opioids. Finally, a number of synthetic peptides, usually produced through combinatorial search of peptide libraries [40,41] have shown an increased affinity for one or multiple opioid binding sites.

In the present work, we report another peptide, derived from the conserved second transmembrane segment of the opioid receptor, with potent antiproliferative activity, in different human cancer cell lines. Its sequence is Tyr-Ile-Phe-Asn-Leu. We named it receptorphin, and we report that it further competes for opioid agonist binding.

Results

Opioid receptor binding selectivity of receptorphin

Figure 1 (upper panel) shows the competition of receptorphin for opioid ligand binding on different sites in the rat brain. As shown, the peptide displaces radiolabelled DPDPE and DAGO (selective ligands for the delta and mu opioid binding site) in a dose-dependent manner, with IC_{50} s of 8.25 × 10^{-8} and 1.81 × 10^{-7} M respectively. In addition, [3H]U69-593 was equally displaced by receptorphin from kappa opioid sites. This peptide competes equally with kappa opioid sites, as [3H]diprenorphine binding, in the presence of micromolar concentrations of DADLE (which masks kappa, delta and mu sites, on which the radioligand could equally bind [4]) does not modify the competition curves of receptorphin. The analysis of the binding curves is presented in Table 1.

Previous results have shown that opioid binding sites could also been identified in a number of breast [17,19] and prostate cell lines [38]. Binding of opioid agonists on these sites induces a decrease of cell proliferation. Figure 1 (lower panel) shows the competition of receptorphin for opioid binding on T47D cells. The interaction of the peptide with the different subtypes of the kappa opioid site was obtained by the use of a combination of radiolabelled ligands (ethylketocyclazocine and diprenorphine) and effectors (DADLE), as described under Material and Methods. Receptorphin competes for binding to kappa opioid sites, with a very high affinity, presenting in this cell line too a main interaction with kappa, and kappa subtypes, as microsomal concentrations of DADLE do not modify substantially opioid binding. The interaction of receptorphin with delta and mu sites in T47D cells was inexistant, due to the minimal number of these sites in T47D cells. In contrast, receptorphin displaces [3H]DP-
DPE and [3H]DAGO, in MCF7 cells, bearing delta and mu binding sites. A summary of the interaction of receptorphin with opioid sites in other cell lines is presented in Table 1.

Cell proliferation
The above results indicate that receptorphin competes for opioid binding on human breast and prostate cancer cells, bearing opioid receptors. A main effect of opioid ligands in these cancer cell lines is the decrease of cell proliferation [16,17,19,38]. We have therefore examined the antiproliferative action of receptorphin, in the same cell lines. Figure 2 and Table 2 present the effect of receptorphin on the proliferation of breast (MCF7 and T47D) and prostate cancer derived cell lines (PC3 and DU145). As shown, this peptide, at concentrations ranging from $10^{-12}$ to $10^{-6}$ M inhibits cell proliferation of breast (MCF7 and T47D) and prostate (DU145, PC3) cell lines by 41, 49, 59 and 27% respectively. IC$_{50}$ was 0.15, 0.07, 0.44 and 6.98 nM respectively. The general opioid antagonist diprenorphine ($10^{-6}$ M) shifted the effect of receptorphin by one log in breast cancer cell lines, while its action was much more pronounced in DU145 and PC3 cancer cell lines, indicating that opioid antagonists reverse, at least partially the antiproliferative effect of the peptide. In contrast, the selective kappa opioid antagonist Nor-Binaltorphine (NorBNI) ($10^{-6}$ M) produced only a partial inhibition of receptorphin binding. This result indicates that receptorphin might interact equally with other sites, different from classical opioid receptors.

Table 1: Inhibitory concentrations 50% (IC$_{50}$) ± SE of receptorphin competition on different opioid sites, assayed by displacement binding of a variety of opioid ligands.

| Opioid Source | DPDPE or | DAGO | EKC | Diprenorphin | EKC +DADLE | Diprenorphin +DADLE |
|---------------|-----------|------|-----|-------------|------------|-------------------|
| MCF7          | 3.25 ± 0.42 × 10$^{-9}$ | 1.43 ± 0.20 × 10$^{-10}$ | 6.65 ± 1.01 × 10$^{-11}$ | 5.78 ± 0.64 × 10$^{-13}$ | 1.59 ± 0.14 × 10$^{-10}$ | >10$^{-12}$ |
| T47D          | ND        | ND   | 6.52 ± 0.58 × 10$^{-11}$ | 5.14 ± 0.74 × 10$^{-10}$ | >10$^{-12}$ | >10$^{-12}$ |
| DU145         | ND        | ND   | 4.30 ± 0.97 × 10$^{-11}$ | >10$^{-6}$ | >10$^{-6}$ | ND |
| PC3           | 8.25 ± 0.81 × 10$^{-8}$ | 1.81 ± 0.23 × 10$^{-7}$ | 1.85 ± 0.11 × 10$^{-10}$ | >10$^{-10}$ | >10$^{-12}$ | 5.41 ± 0.57 × 10$^{-11}$ |
| Rat Brain     | 5.77 ± 0.87 × 10$^{-10}$ | 2.65 ± 0.13 × 10$^{-9}$ | ND | ND |

*[^3H]U60-593 was used as a selective kappa opioid ligand. **Displacement curves were best fitted with a two-site model. See text for details. IC$_{50}$ (in Moles/L) were calculated by sigmoidal fitting of displacement experiments. ND = not detected. Mean ± SE of three different experiments performed in duplicate.

Discussion
In recent years, a number of endogenous and food-derived proteins have been identified as potential sources of opioid or opiomimetic peptides. They include gluten, hemoglobin and caseins [26,27,32–37,39]. By limited proteolysis of such proteins, a number of peptides have been identified, which presents features of opiomimetic action: binding to opioid receptors, competition for opioid ligands, opiomimetic effects, and reversion of their action by the addition of opioid antagonists. These opioid peptide sources could resolve the problem of opioid availability in organs, in which classical opioid precursor were not identified. Indeed, the blood flow disposition of
endogenous opioid peptides is limited, due to the very short half-life of these peptides, with the exception of beta-endorphin.

The results of the present investigation identify another peptide, receptorphin, derived from the opioid receptor itself. Receptorphin sequence is comprised of the conserved structure of the second transmembrane segment of the opioid receptor (see Table 3 and Figure 5). Receptorphin exhibits some characteristics of an opioid ligand:

1. It competes for opioid ligand binding on opioid sites, exhibiting principally selectivity for kappa opioid receptors (especially kappa, and kappa3). Its affinity, in human breast and prostate cancer cell lines, is higher than that of the prototype ligands [14,17,19]. In contrast, in rat brain membranes, the affinity of receptorphin is lower than that of DAGO or DPDPPE. It is not actually known whether this discrepancy is due to the difference of normal and neoplastic tissue, in which a possible alternative splicing of the opioid receptor could occur, to the conditions of binding (hypotonic medium in rat brain as compared to isotonic medium in whole cell binding) or to species differences. A similar discrepancy was also observed in the case of αS1-casomorphin and other casomorphin peptides [16,37,38]. In addition, receptorphin interacts with somatostatin binding, a result explaining the non-complete reversion of its effect by opioid antagonists. A similar interaction was equally observed with a number of other food-derived peptides, in the same system [16,38].

2. Receptorphin decreases cell proliferation in different breast and prostate cell lines, in a dose-dependent and reversible manner, as reported for other opioid alkaloids and casomorphin ligands [16,17,38]. Comparing the IC50 of receptorphin with that obtained in the same cell lines by a number of other opioid peptides [16,37,38] it is derived that this new opioid agonist is almost as potent as alphaS1-casomorphin in the prostate, while it is much less potent (at least by a factor of 20) in the breast. Comparable maximal inhibition of growth was obtained by receptorphin and other casomorphin peptides, both in the breast and the prostate [16,38], representing possibly, the maximum opioid-related effect on these cell lines. The physiological relevance of this prostate selectivity of receptorphin is not known.

The above results are, of course, not enough to characterize fully a possible new opioid peptide. Further work, implying more classical opioid effects in cells (GTP binding, cAMP inhibition) and organs or animals might be necessary for the complete identification of receptorphin as an opioid agonist. Nevertheless, the fact that receptorphin binds with a high affinity to opioid sites in different organs (brain and cancer cell lines) in which opioid receptors were identified, and its antiproliferative action in different cancer cell lines is inhibited by the general opioid antagonist diprenorphine apply for an opioid activity of this peptide.

The sequence of receptorphin (Tyr-Ile-Phe-Asn-Leu) is conserved in all reported opioid receptors, from a variety of species (Table 3). In contrast, this sequence is restricted to the opioid receptor, and is not detected in other seven-loop membrane receptors [43]. It is interesting that, at the same position (second transmembrane segment) of the different types of the somatostatin receptor (SSTR-1 to 5) which present the greater homology with opioid receptors [5], in different species, a peptide with the sequence Tyr-Ile(or-Leu)-Leu-Asn-Leu exists, presenting an homology in structure with receptorphin [44–48]. Furthermore, two or three amino acids of receptorphin (Phe-Asn-Leu) interfere with the agonist or antagonist binding, at least indirectly, participating in the formation of the ligand envelope [43]. This might be the reason of the conservation of the receptorphin sequence in all opioid receptor sequences (Table 3). Comparing receptorphin (Tyr-Ile-Phe-Asn-Leu) with other opioid peptides, it is observed that it shares Tyr at position 1 and Leu in position 5. In contrast, the presence of Ile and Asn at positions 2 and 4 and the non-classical Phe at position...
3 are unique in receptorphin. This non-classical amino-acid composition may confer the described binding characteristics to this new peptide (Table 1), which bears the classical pharmacophoric groups of classical opioid agonists (Tyr, Phe, Leu).

**Conclusions**

In the present paper, we report the identification of a ligand-specific peptide, in the sequence of its cognitive receptor structure. Receptorphin (Tyr-Ile-Phe-Asn-Leu) has a unique sequence, conserved in all opioid receptor types and species. The peptide competes with a high affinity for opioid binding, showing a preferential interaction with kappa (κ₁ and κ₃), while it competes with delta, mu, and somatostatin sites with a lower affinity. It can therefore be considered as a non-selective opioid ligand. Although this finding is interesting per se, the current knowledge permits only speculations about a possible biological role of the peptide, if any. Receptorphin is flagged by Ile at position -1 and always by Ala at position 6 (see Figure 5), making it a putative target of peptidases, while its location, in the hydrophobic membrane environment, protects it from a possible hydrolytic-enzyme action. A possible hypothesis could be that receptorphin might act as an opioid peptide after cell death. In this case, after intra- and extracellular domains of opioid receptor destruction by different liberated proteolytic enzymes, and rupture of the membrane structure, the leading Ile⁻¹ of receptorphin could be cleaved by exopeptidases, while the following Ala⁶ might be removed by basic endopeptidases, leading to the liberation of the active peptide. If such a mechanism occurs, opioid action could be potentiated after an opioid- and/or other inducers-related cell death. Indeed, it is tentative to assume that after an opioid mediated cellular death [23–25], receptorphin liberation might trigger a positive feedback

### Table 2: Inhibitory concentrations 50% (IC₅₀) and maximal inhibition of cell growth of different breast and prostate cancer cell lines by receptorphin.

| Receptorphin | Maximum Inhibition | Receptorphin+ Diprenorphin | Maximum Inhibition |
|--------------|--------------------|---------------------------|--------------------|
| MCF7         | 1.51 ± 0.18 × 10⁻¹₀ | 0.41                      | 1.69 ± 0.10 × 10⁻⁹ | 0.49               |
| T47D         | 7.01 ± 0.36 × 10⁻¹₁ | 0.44                      | 3.61 ± 0.13 × 10⁻⁹ | 0.48               |
| LNCaP        | 8.70 ± 0.87 × 10⁻⁴   | 0.58                      | 2.88 ± 0.15 × 10⁻⁹ | 0.61               |
| DU145        | 4.36 ± 0.19 × 10⁻¹₀  | 0.59                      | 3.44 ± 2.32 × 10⁻⁷ | 0.53               |
| PC3          | 6.98 ± 0.11 × 10⁻⁹   | 0.27                      | 7.00 ± 0.14 × 10⁻⁷ | 0.31               |

Curves were obtained by sigmoidal fitting of data presented in Figure 2. Concentrations are expressed in Moles/L. Mean ± SE of three different experiments performed in triplicate.

### Table 3: Position of receptorphin in different reported opioid receptor sequences.

| Receptor | Source      | Length (a.a.) | Receptorphin position | Reference |
|----------|-------------|---------------|-----------------------|-----------|
| Delta    | Zebrafish   | 373           | 90–94                 | [51]      |
| Delta    | Human       | 372           | 87–91                 | [52]      |
| Mu       | Human       | 400           | 98–102                | [53][54][55] |
| Mu variant | Human     | 392           | 107–112               | [55]      |
| Mu       | Mouse       | 367           | 86–90                 | [56]      |
| Mu       | Rat         | 398           | 69–100                | [57][58][59][60][61][62][63][64] |
| Mu       | Bovine      | 401           | 109–113               | [65]      |
| Kappa    | Rat         | 380           | 97–101                | [66]      |
| Kappa    | Guinea Pig  | 380           | 97–101                | [67]      |
| Kappa    | Mouse       | 380           | 97–101                | [56]      |
| Kappa    | Human       | 380           | 97–101                | [68][69] |
| Orphan   | Human       | 370           | 89–93                 | [70][71] |
| Orphan   | Rat         | 357           | 86–90                 | [72]      |
| Orphan   | Mouse       | 367           | 87–92                 | [56][73][74][75] |
| Orphan   | Rat         | 367           | 86–90                 | [76][77][78][79][80][81] |
loop, propagating the opioid effects to a number of adjacent opioid receptor-positive cells. This mechanism could possibly explain the time-lag found in opioid-related cell proliferation and apoptosis, reported in a number of malignant cell lines [15–17,37,38]. Nevertheless, this putative receptorphin implication in cell proliferation remains highly speculative, until its presence could be detected in cell cultures. In addition, it might be of interest to detect similar structures in the sequence of other members of the seven-loop superfamily, in order to investigate whether this finding is unique to the opioid receptor itself, or could be extended to its other members.

Methods

Peptide synthesis

Receptorphin (Tyr-Ile-Phe-Asn-Leu) was synthesized by conventional peptide chemistry methods. t-butoxycarbonyl (Boc) groups were used for protection of the α-amino-groups, while a t-butyl group was used for the protection of the phenolic hydroxy-group. The dichloro-carbodi-imiole/1-hydroxybenzo-triazo method was used for the coupling of the protected amino acids. Removal of the t-butyl and Boc groups was achieved by trifluoroacetic acid. Peptide purification was made by semipreparative HPLC on a reverse phase C-18 Nucleosil column, on a 30 min linear gradient of 15–85% methanol in 0.1% aqueous trifluoroacetic acid.

Cell lines and culture conditions

Two breast cancer (MCF7 and T47D), and two prostate cell lines (PC3 and DU145), bearing opioid binding sites [16,17,19,38], were used, in order to assay the opioid activity of receptorphin. DU145 and MCF7 cell lines were purchased from DSMZ (Braunschweig, Germany), while T47D, and PC3 cells were from the European Collection of Cell Cultures (Salisbury, UK). T47D and DU145 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). PC3 cells were cultured in DMEM medium with 10% FBS, while, for MCF7 cell culture, DMEM/F12 medium was used, supplemented with 5 µg/ml insulin (Sigma, St Louis, MI) and 10% FBS. All cell lines were maintained in a humidified atmosphere of 5% CO2 in air. All culture media were from Gibco BRL (Life Technologies, Paisley, UK). Medium, supplemented or not with receptorphin, was changed every day. Without addition of any drug, the proliferation time of all cell lines was two days. Receptorphin was dissolved in phosphate buffered saline shortly before use.

Cell proliferation

Cells were plated in 24-well plates, at an initial density of 2 × 104 cells, with 1.0 ml medium per well. All drugs were added to cultures one day after seeding (designated as day 0), in order to ensure uniform attachment of cells at the onset of the experiments. Cell growth was measured by the tetrazolium salt assay [49]. Cells were incubated for 4 h at 37°C with the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), and metabolically active cells reduced the dye to purple formazan. Dark blue crystals were dissolved with propanol, and the absorbance was measured at 570 nm and compared against a standard curve of known numbers of cells. All experiments were performed, at minimum, three times in triplicate.

Binding conditions

Cancer cell lines

Ligand binding assays on whole cells (10^6 cells/well) were performed as described in Hatzoglou et al [14,16,17], in a total volume of 0.5 ml phosphate buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4), containing radioactive opioids or somatostatin, without (total binding), with 10^-5 M of the same unlabelled agent (non specific binding), or with varying concentrations of receptorphin, ranging from 10^-12 to 10^-6 M. Cells were incubated for 2 h at room temperature (18–22°C). At the end of the incubation period, cells were washed twice with cold buffer, in order to eliminate the unbound radioactivity. Cells were removed from plates with 0.4 ml 2N NaOH, mixed with 4 ml scintillation cocktail (Sigma-Fluor, Sigma, St Louis, MI) and counted in a scintillation counter (Tricarb, Series 4000, Packard), with a 60% effi-
efficiency for Tritium, for opioids and in a Tricarb (Packard) gamma counter with a 95% efficiency for $^{125}$I (somatostatin binding). Binding was repeated three times (in duplicate).

A number of different opioid ligands were used in order to identify specific opioid binding sites. $[^3]$H] $\text{[D-Pen}^2\text{, D-Pen}^5\text{]enkephalin (DPDPE)}$ was used as a selective delta ligand, and $[^3]$H] $\text{[N-me-Phe}^4\text{, Gly}^5\text{ol]enkephalin (DAGO)}$ as a selective mu ligand. $[^3]$H]ethylketocyclazocine (EKC) and $[^3]$H]diprenorphine were used as general opioid ligands, sharing nevertheless, at nanomolar concentrations, a different receptor recognition spectrum: ethylketocyclazocine recognizes delta, mu, and kappa$_2$ opioid sites, permitting the differentiation of the effect of the tracer with the kappa$_1$ and the kappa$_3$ opioid site respectively [3,4]. Control experiments, in our laboratory have proven that by the use of these combinations we obtain similar results for the detection of kappa1 and kappa3 opioid sites, as with the use of the selective ligands U50488 and Mepet-enkephalin-Arg$^6$-Phe$^7$.

### Rat brain membranes

Rat brain membrane preparation and opioid binding was performed as described previously by Loukas et al. [39]. Briefly, binding was performed in Tris-HCl buffer (10 mM, pH 7.4), in a final volume of 1.0 ml. The protein concentration was 300 $\mu$g/assay. Binding was initiated by the addition of 2 nM of the selective ligand ($[^3]$H]DPDPE for $\delta$ or $[^3]$H]DAGO for $\mu$ sites, $[^3]$H]U69-593 for kappa$_1$ site and $[^3]$H]diprenorphine as a general opioid ligand, as discussed above). Non specific binding was estimated in the presence of 10$^{-5}$ M naloxone or diprenorphine. Peptide concentrations varied from 10$^{-12}$ to 10$^{-6}$ M.

After the incubation, bound radioactivity was separated by filtration, under reduced pressure, through GF/D filters, previously soaked in Tris-HCl buffer, and rinsed twice with ice-cold buffer.

The results of binding assays were analyzed by the Origin (MicroCal, Northampton, MA.) V 5 package, using equations described by Munson and Rodbard [50].

### Radiochemicals and chemicals

$[^3]$H]ethylketocyclazocine (S.A. 18 Ci/mmol), $[^3]$H] $\text{[D-Pen}^2\text{, D-Pen}^5\text{]enkephalin (DPDPE)}$ (S.A. 37 Ci/mmol) and $[^3]$H]U69-593 (S.A. 39.7 Ci/mmol) were bought from New England Nuclear Co (Zaventum, Belgium). $[^3]$H]diprenorphine (S.A. 29 Ci/mmol), $[^3]$H] $\text{[N-me-Phe}^4\text{, Gly}^5\text{ol]enkephalin (DAGO)}$, S.A. 47.7 Ci/mmol) and $[^125]$Tyrl$^{11}$somatostatin14 (S.A. 2000 Ci/mmol) were from Amersham (Buckinghamshire, UK). Ethylketocyclazocine was a gift from Sterling-Winthrop. Diprenorphine was from Reckit and Coleman Co. All other chemicals

### Figure 4

Interaction of receptorphin with somatostatin binding sites. A: Effect of receptorphin on the proliferation of LNCaP cells, not-presenting opioid binding sites [38]. Conditions of cell growth were similar with those presented in the legend of Figure 2, with the exception that cells were assayed at day 6, as the doubling time of LNCaP cells is longer (3.02d). Mean ± SEM of three experiments in triplicate. B. Competition of receptorphin for somatostatin binding. Displacement of radiolabelled $[^12]$Tyr$^{11}$Somatostatin14 by varying concentrations of receptorphin. See text for details of binding.

### Figure 5

Alignment of the sequences of the second transmembrane loop of different opioid receptors. Sequences are derived from references in Table 3. The sequence of receptorphin is indicated in bold letters.
were either from Merck (Darmstadt, Germany) or from Sigma (St Louis MO).

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