Ligands for κ-Opioid and ORL1 Receptors Identified from a Conformationally Constrained Peptide Combinatorial Library*

Jérôme A. J. Becker‡, Andrew Wallace§§, Aaron Garzon§§, Paolo Ingallinella§, Elisabetta Bianchi§, Riccardo Cortese§, Frédéric Simonin‡‡, Brigitte L. Kieffer‡, and Antonello Pessi‡‡‡

From the %Ecole Supérieure de Biotechnologie de Strasbourg, 67400 Illkirch, France and 1IRBM P. Angeletti, 00040 Rome, Italy

We have screened a synthetic peptide combinatorial library composed of 2 × 10^7 β-turn-constrained peptides in binding assays on four structurally related receptors, the human opioid receptors μ, δ, and κ and the opioid receptor-like ORL1. Sixty-six individual peptides were synthesized from the primary screening and tested in the four receptor binding assays. Three peptides composed essentially of unnatural amino acids were found to show high affinity for human κ-opioid receptor. Investigation of their activity in agonist-promoted stimulation of [35S]guanosine 5'-3-O-(thio)triphosphate binding assay revealed that we have identified the first inverse agonist as well as peptidic antagonists for κ-receptors. To fine-tune the potency and selectivity of these κ-peptides we replaced their turn-forming template by other turn mimicetic molecules. This “turn-scan” process allowed the discovery of compounds with modified selectivity and activity profiles. One peptide displayed comparable affinity and partial agonist activity toward all four receptors. Interestingly, another peptide showed selectivity for the ORL1 receptor and displayed antagonist activity at ORL1 and agonist activity at opioid receptors. In conclusion, we have identified peptides that represent an entirely new class of ligands for opioid and ORL1 receptors and exhibit novel pharmacological activity. This study demonstrates that conformationally constrained peptide combinatorial libraries are a rich source of ligands that are more suitable for the design of nonpeptidal drugs.

Opines exert their pharmacological actions through three receptor types (1, 2), μ, δ, and κ. Their genes have been cloned (see Ref. 3), and the analysis of their amino acid sequence indicated that they belong to the G-protein-coupled receptor family and display a high degree of homology. The cloning of opioid receptors led to the discovery of an additional member for this receptor family referred to as opioid receptor like (ORL1; see Ref. 4). Although ORL1 shares high sequence similarities with opioid receptors, it does not bind opioid ligands with high affinity.

Opiate drugs, the prototype of which is morphine, are largely used in medicine for the treatment of pain, but their administration is associated with several side effects, including high abuse potential (see Ref. 5). Most nonanalgesic actions of opiates have been associated with the activation of μ-receptors (6), and the development of δ- and κ-compounds both as pharmacological tools and therapeutic agents is an extremely active research field. Unlike opioid receptors, there is only a small number of available ligands for ORL1 including the endogenous heptadecapeptide nociceptin/orphanin FQ (7, 8) hexapeptides, recently identified by Dooley et al. (9) using combinatorial chemistry techniques, and naloxone benzoylhydrazone (10), previously described as a μ- and κ-ligand (11). This recently discovered neurotransmitter system is likely to participate in a broad range of physiological and behavioral functions, with possible interactions with the opioid system (see Ref. 12). At present our comprehension of the in vivo functions of the ORL1 receptor is severely limited by the lack of ligands, agonists as well as antagonists, with high selectivity and bioavailability.

Combinatorial strategies are important new approaches to drug discovery, and synthetic peptide combinatorial libraries (SPCL) have repeatedly shown their usefulness as a source of new drug leads; in particular, when SPCL have been applied to the search for new ligands of the opioid receptors, potent hexapeptides (13–15) and tetrapeptides (15) were identified. In the latter work for example, a single library in positional scan-

* This work was funded by institutional grants from CNRS and by specific grants from Association pour la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡‡ To whom correspondence concerning the pharmacological evaluation should be addressed: École Supérieure de Biotechnologie, Parc d'innovation, Bld. Sébastien Brand, F-67400 Illkirch, France. Tel.: 33-388-655288; Fax: 33-388-655298; E-mail: simona@esbs.u-strasbg.fr.

‡‡ To whom correspondence concerning the combinatorial chemistry should be addressed: IRBM P. Angeletti, Via Pontina Km 30.600, 00040 Pomezia (Rome) Italy. Tel.: 39-06-91093445; Fax: 39-06-91093654; E-mail: pessi@irbm.it.

This paper is available on line at http://www.jbc.org
Peptides, however, generally display unfavorable pharmacological properties, like poor bioavailability, short duration of action, and lack of oral activity (17), prompting the effort to evolve them into peptidomimetics (18, 19). Moore (20) has divided the peptide-to-peptidomimetic transition into three logical steps: (a) identification of the amino acid side chains responsible for activity (“pharmacophoric groups”); (b) establishment of the spatial relationship between these groups (“pharmacophore model”); (c) selection of an organic template suitable for reproducing the geometry of the pharmacophore model. The most difficult and usually rate-limiting step is the second one, since only rarely can the biologically relevant peptide topology be deduced from direct observation of the receptor-ligand complex. Although, as noted above, SPCL are very effective to carry out step a, their use in steps b and c is still in its infancy (21, 22).

We have recently proposed the concept of selection-driven design of peptidomimetics (23–25), a process whereby a first generation peptide pharmacophore is rapidly derived from screening of a panel of libraries with predetermined ligand geometry. Our first example on the application of this strategy was the development of a conformationally homogeneous library of α-helical peptides and the concurrent selection of a peptide mimicking the lipopolysaccharide antigen of the human pathogen Shigella flexneri (26). Here we report the results of the screening of a β-turn SPCL on human μ-, δ- and κ-opioid receptors (hMOR, hDOR, hKOR) and ORL1 receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Naloxone, [α-Ala2,N-methyl-Phe4,Gly-ol3]enkephalin (DAMGO), GDP, and GTP-S were purchased from Sigma. BW575U86 was kindly provided by Dr. K. J. Chang (Burroughs Wellcome Co., Research Triangle Park, NC). CI-977 was a gift from John Hughes (Parke-Davis Neuroscience Research Center, Cambridge, UK). [3H]Diprenorphine (37 Ci/mmol; 1 Cu) and [leucyl-3H]nociceptin (172 Ci/mmol) were obtained from Amersham Pharmacia Biotech, and [3H]SCH23390 (1156 Ci/mmol) was from BN Life Science Products. The hMOR cDNA was a gift from Lei Yu (Department of Medical and Molecular Genetics, Indianapolis, IN). The carrier plasmid used in the electroporation procedure (pBluescript) was from Stratagene (La Jolla, USA).

**Peptide Synthesis**—All the Fmoc-protected amino acids were obtained from Novabiochem, Bachem (Bubendorf, Germany), or Nenysystem (Strasbourg, Germany). The SPCL and the individual peptides were synthesized as described previously (23, 26, 27) using PyBOP®/HOBt/DIPEA (1:1:2) activation, 5-fold excess, and a coupling time of 20 min to judge by the standard ninhydrin and TNBS color tests. The undefined or “mixed” (X) positions were incorporated by coupling a mixture of activated amino acids, with the relative ratios suitably adjusted to yield close to equimolar incorporation. 8-Amino-5,6,7,8-tetrahydro-2-naphthoic acid (ATA) was prepared using H2O, 0.1% trifluoroacetic acid and acetonitrile, 0.1% trifluoroacetic acid as eluents. Analytical HPLC was performed on a Ultrasphere C-18, 250 × 4.6-mm, 80-A, 5-mm column (Beckman). Purified (≥95%) peptides were characterized by mass spectrometry and amino acid analysis.

**CHO cells** were grown in Dulbecco’s modified Eagle’s-F-12 medium (Eurobio). CHO stably transfected with pCDNA3Neo (Invitrogen, Nu Leek, Netherlands) or hORL1 and hKOR were gifts from Lawrence Tull (Torrey Pines Institute for Molecular Biology, San Diego, CA) and C. Mollereau (Institut de Pharmacologie et de Biologie Structurale, Toulouse, France), respectively.

**Cell Transfections**—Cells were electroporated essentially as described (34). Briefly, 2 × 105 COS-1 cells were seeded the night before transfection at a density of 105 cells/140-mm dish. Cells were washed two times with phosphate-buffered saline and detached by applying trypsin/EDTA (Eurobio). Cells were collected by centrifugation for 10 min at 400 × g and resuspended at a density of 106 cells/ml in EP 1× buffer (50 mM K2HPO4, 20 mM CH3COOK, 20 mM KOH, pH 7.4). hMOR, hDOR, or hKOR plasmidic DNA, prepared using Nucleobond columns (Macherey Nagel, Düren, Germany) and consisting of variable amounts of receptor-encoding plasmid and a carrier plasmid (pBluescript) up to a final 20-μg DNA quantity was diluted into EP 1× buffer to a total volume of 300 μl. The DNA mix was then supplemented with 13 μl of 1 M MgSO4 and incubated with 200 μl of cell suspension for 20 min at room temperature. The cell/DNA mixture was then transferred to a 0.4-cm cuvette and electroporated using a Gene Pulser apparatus (Bio-Rad) at a capacitance setting of 2000 microfarads and voltage setting of 240 volts. Cells were then immediately transferred into 50 ml of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and seeded into 2 140-mm dishes. After 72 h of growth, the cells were harvested, and membranes were then prepared as described previously (34).

**Cell Membrane Preparations**—Transfected cells (4 140-mm dishes at a 50 to 100% confluency) were washed with 2× phosphate-buffered saline, scraped off the plates in phosphate-buffered saline, pelleted by centrifugation at 400 × g for 10 min at 4 °C, frozen at −80 °C for 30 min at least, and thawed in 30 ml of cold 50 mM Tris-HCl, pH 7, when membranes were prepared for ligand binding experiments, and 30 ml of cold 50 mM Tris-HCl, pH 7, 2.5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (added extemporaneously) was added for [35S]GTPγS binding experiments. All the following steps were performed at 4 °C. The cell lysate was then DNase-homogenized and spun at 400 × g for 10 min. The pellet was resuspended in 15 ml of buffer, Dounce-homogenized, and spun again at 400 × g for 10 min. Both supernatants were pooled and centrifuged at 100,000 × g for 30 min. The pellet was then resuspended in 4 ml of 50 mM Tris-HCl, pH 7.0, and the protein concentration was measured using the Bradford assay. Membranes were then aliquoted at 1-mg protein/ml concentration and stored at −80 °C.

When membranes were prepared for [35S]GTPγS binding experiments, the pellet was resuspended in 25 ml of 50 mM Tris-HCl, pH 7, Dounce-homogenized, and spun again at 100,000 × g for 30 min. The pellet was then resuspended in 4 ml of 50 mM Tris-HCl, pH 7, 0.32 mM sucrose, and the protein concentration was measured as described above.

**Receptor Binding Assay**—Binding experiments were done as described previously (35). For saturation experiments, various concentrations (from 5 × 10−11 to 6.4 × 10−9 M) of [3H]Diprenorphine (hMOR, hDOR, hKOR) or [leucyl-3H]nociceptin (hORL1) were used. For competition experiments, membrane proteins were diluted in 50 mM Tris-HCl, pH 7.4, and incubated with [3H]Diprenorphine (0.2 nM for hMOR and hDOR and 0.4 nM for hKOR) or 0.1 nM [leucyl-3H]nociceptin (for hORL1), and variable concentrations of competitor peptide (7.8 × 10−11 to 5 × 10−8 M) in a total volume of 0.2 ml for 1 h at 25 °C. Nonspecific bind-
A

FIG. 2. **Screening of the β-turn mimetic SPCL.** A, hMOR and hDOR membranes were labeled using a nonselective opioid antagonist [3H]diprenorphine (0.2 nM for hMOR and 0.4 nM for hDOR). B, hKOR and hORL1 membranes were labeled using 0.4 nM [3H]diprenorphine and 0.1 nM [leucyl-3H]nociceptin, respectively. Assays were carried out using target receptor either transiently expressed in COS-1 cells (hMOR, hDOR, hKOR) or stably expressed in CHO cells (hORL1). Each panel represents the screening of one of the four SPCL on one receptor. Each bar within a panel represents percent inhibition of binding by a peptide mixture (each individual peptide was at a concentration of 1.6 nM for hMOR, hDOR, hKOR and 0.16 nM for hORL1) defined in the O position with one of the 68 amino acids indicated below. Arrows indicated the selected amino acids for individual peptide synthesis. 1, L-Val; 2, L-Ile; 3, L-Trp; 4, L-Gln; 5, L-Asn; 6, L-Arg; 7, L-His; 8, L-Tyr; 9, L-Pro; 10, L-Phe; 11, L-Met; 12, L-Glu; 13, L-Asp; 14, L-Lys; 15, L-Thr; 16, L-Ser; 17, L-Leu; 18, L-Ala; 19, L-Gly; 20, L-a-aminobutyric; 21, L-aminisobutyric; 22, L-alanine; 23, L-g-aminobutyric; 24, L-6-aminohexanoic; 25, L-b-cyclohexyl-L-alanine; 26, L-3,4-dehydro-L-proline; 27, L-g-carboxyglutamic; 28, L-homo-L-phenylalanine; 29, L-hydroxy-L-proline; 30, L-norleucine; 31, L-norvaline; 32, L-ornithine; 33, L-p-chloro-L-phenylalanine; 34, L-p-nitro-L-phenylalanine; 35, L-phenylglycine; 36, sarcosine; 37, D-Val; 38, D-Ile; 39, D-Trp; 40, D-Gln; 41, D-Asn; 42, D-Arg; 43, D-His; 44, D-Tyr; 45, D-Pro; 46, D-Phe; 47, D-Met; 48, D-Glu; 49, D-Asp; 50, D-Lys; 51, D-Thr; 52, D-Ser; 53, D-Leu; 54, D-Ala; 55, D-cyclohexyl-D-alanine; 56, D-norleucine; 57, D-norvaline; 58, D-chloro-D-phenylalanine; 59, (3S,4S)-4-amino-3-hydroxy-5-cyclohexylpentanoic; 60, (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic; 61, 2,3-diaminopro-pionic; 62, D-phenylglycine; 63, (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic; 66, 1,2,3,4-tetrahydroisoquinoline-3-L-carboxylic; 67, 1,2,3,4-tetrahydroisoquinoline-3-L-carboxylic; 68, 2,3-diamino-β-l-propionic.
binding was determined in the presence of 1 μM naloxone (hMOR, hDOR, hKOR) or 1 μM nociceptin/orphanin FQ (hORL1). $K_i$ and $K_d$ values were determined using the EBDA/Ligand program (G. A. McPherson, Biosoft, Cambridge, UK). $K_d$ values were in good agreement with those described in the literature (7, 35–37).

**[^35S]GTP$gamma$S Binding Assay**—For the opioid receptors, 5 μg of hMOR, hKOR, and hDOR membrane proteins were incubated 1 h at 30 °C in 50 mM Hepes, pH 7.6, 5 mM MgCl$_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% bovine serum albumin, GDP (3 μM for hKOR, and 30 μM for hMOR and hDOR), 0.2 nM[^35S]GTP$gamma$S, and ligands ($1 \times 10^{-11}$ to $1 \times 10^{-5}$ M for the opioid ligand, and $2.8 \times 10^{-10}$ to $5 \times 10^{-5}$ M for the competitor peptides) in a final volume of 0.2 ml (34). For hORL1, 5 μg of membrane proteins were incubated 1 h at 37 °C in 50 mM Tris, pH 7.4, 5 mM MgCl$_2$, 1 mM EGTA, 100 mM NaCl, 0.1% bovine serum albumin, 40 μM GDP, 0.2 nM[^35S]GTP$gamma$S, and ligands ($1 \times 10^{-11}$ to $1 \times 10^{-5}$ M for nociceptin/orphanin FQ, and $2.8 \times 10^{-10}$ to $5 \times 10^{-5}$ M for the competitor peptides) in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of 10 μM GTP$gamma$S. Incubation mixtures were rapidly washed using a cell harvester (Brandell, Gaithersburg, MD) with cold 50 mM Tris-HCl, pH 7, 5 mM MgCl$_2$, 50 mM

**FIG. 2—continued**

B

| Peptide mixture (O$_1$ position) | Peptide mixture (O$_2$ position) | Peptide mixture (O$_3$ position) | Peptide mixture (O$_4$ position) |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| hKOR                            | hKOR                            | hKOR                            | hKOR                            |
| hORL1                           | hORL1                           | hORL1                           | hORL1                           |

[^35S]GTP$gamma$S Binding Assay—For the opioid receptors, 5 μg of hMOR, hKOR, and hDOR membrane proteins were incubated 1 h at 30 °C in 50 mM Hepes, pH 7.6, 5 mM MgCl$_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% bovine serum albumin, GDP (3 μM for hKOR, and 30 μM for hMOR and hDOR), 0.2 nM[^35S]GTP$gamma$S, and ligands ($1 \times 10^{-11}$ to $1 \times 10^{-5}$ M for the opioid ligand, and $2.8 \times 10^{-10}$ to $5 \times 10^{-5}$ M for the competitor peptides) in a final volume of 0.2 ml (34). For hORL1, 5 μg of membrane proteins were incubated 1 h at 37 °C in 50 mM Tris, pH 7.4, 5 mM MgCl$_2$, 1 mM EGTA, 100 mM NaCl, 0.1% bovine serum albumin, 40 μM GDP, 0.2 nM[^35S]GTP$gamma$S, and ligands ($1 \times 10^{-11}$ to $1 \times 10^{-5}$ M for nociceptin/orphanin FQ, and $2.8 \times 10^{-10}$ to $5 \times 10^{-5}$ M for the competitor peptides) in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of 10 μM GTP$gamma$S. Incubation mixtures were rapidly washed using a cell harvester (Brandell, Gaithersburg, MD) with cold 50 mM Tris-HCl, pH 7, 5 mM MgCl$_2$, 50 mM
RESULTS

Identification of New Opioid Ligands by Screening of a Reverse-turn SPCL—To find new ligands for the opioid and ORL1 receptors, we have screened a reverse-turn peptide SPCL composed of 2 × 10^7 N-terminal-acetylated and C-terminal-amidated peptides in a so-called positional scanning format (26). Each peptide of this library is constrained in a β-turn conformation by a rigid turn-forming mimetic block (ATA) in its center (see Fig. 1). The library is composed of four sublibraries: Ac-OX-ATA-XX-NH₂, Ac-XX-ATA-XX-NH₂, Ac-XX-ATA-XX-NO₂, and Ac-XX-ATA-XX-NO₂ (Ac, acetyl). Each sublibrary is composed of 68 peptide mixtures, in which the position labeled (O₁) is defined by the amino acids indicated in the legend of Fig. 2. We used an expanded combinatorial set, including many noncoded amino acids, and most of the residues were present both with L and D chirality.

The β-turn mimetic library was used in conjunction with a deconvolution selection process to identify individual peptides capable of inhibiting the binding of radioligands to membrane homogenates of COS-1 or CHO cells expressing recombinant human μ-, δ-, and κ-opioid receptors (hMOR, hDOR, and hKOR) and the human opioid-like receptor (hORL1, see "Experimental Procedures"). The sublibraries were screened at a fixed concentration of 500 μM (1.6 nm for each individual peptide). For hKOR, all the mixtures inhibited >90% of radioligand binding in this initial screening; the library was therefore screened again at a 10-fold lower concentration (50 μM).

Results of the screening of the four sublibraries with the four receptors are shown in Fig. 2. A lot of peptide mixtures in each sublibrary were found to be active (>75% inhibition) on either one or several receptors, particularly hKOR and hMOR. We therefore selected the most active and/or selective consensus sequences to synthesize individual peptides. For the first sublibrary (position O₁), L-Arg, L-Trp, and L-Cha were the most active residues on the four receptors and were then selected (excepted for hDOR for which we selected only L-Cha and D-Trp). More selective residues were also selected for hMOR (homol-phenylalanine and L-Arg) and for hKOR (L-Fno). The second sublibrary (position O₂) was the most active one on the four receptors, especially on hMOR and hKOR (all the peptide mixtures showed >75% inhibition of the binding). For these two receptors we chose the most active and selective unnatural residues L-Fcl (hMOR) and D-Trp (hKOR). The two most active residues were selected for hORL1 (6-aminohexanoic acid and L-Cha), and three were selected for hDOR (L-Glu, 1,2,3,4-tetrahydroisoquinoline-3-D-carboxylic acid, and 2,3-diamino-β-L-proline acid). In the third sublibrary (position O₃), Arg was by far the preferred residue for hORL1 (L- and D-Arg), hDOR (L-Arg), and to a lesser extent for hKOR (L-Arg). We therefore selected this residue for hORL1 and hDOR but not hKOR, for which D-Ile was preferred because of its better selectivity. For hMOR we chose the most active (L-Cha) and the most selective (L-Trp) residues. For the fourth sublibrary (position O₄) a strong activity of Arg residues at the four receptors was also observed.

### Table I

Amino acids chosen for synthesis of individual peptides for hMOR, hDOR, hKOR, and hORL1

|            | hMOR         | hDOR         | hKOR         | hORL1        |
|------------|--------------|--------------|--------------|--------------|
| Ox-ATA-XX  | L-Cha, L-Hph, D-Trp, L-Cha, d-Trp, L-Cha, d-Trp, d-Trp, L-Fcl, L-Arg, L-Fno | | | |
| XX-ATA-OX  | L-Cha, L-Hph, L-Fno | | | |
| XX-ATA-XX  | L-Cha, L-Hph | | | |
| XX-ATA-XX  | L-Cha | | | |

### Table II

Binding affinities for hKOR, hMOR, hDOR, and hORL1 of the three peptides selected from the library and the peptide III-derived peptides further selected from the turn-scans process and binding affinities of selective agonists and antagonists of the opioid receptors

Experiments were conducted on hKOR, hMOR, hDOR transiently transfected into COS-1 cells and hORL1 stably expressed into CHO cells. Values are mean ± S.E. from three or more separate experiments performed in duplicate. ε, 6-aminohexanoic acid.
We therefore selected d-Arg for hKOR, hORL1, and hDOR and l-Arg for hORL1. The strongly active residues d-Fcl (hDOR and hORL1) and d-Lys (hKOR) were also selected. In addition we chose the most selective residue l-Trp for hMOR. The selected residues for the four receptors are summarized in Table I.

We synthesized 66 peptides corresponding to all possible combinations of the active residues selected from the screening (Table I). In a first step, two concentrations (5 and 500 nm) of each peptide were tested in competition experiments with hKOR, hMOR, hDOR, and hORL1 cell membranes (not shown). The most active peptides (IC50 ≤ 500 nm) were then selected and purified by HPLC, and three concentrations (5, 50, and 500 nm) were tested again with the four receptors (not shown). From these experiments three compounds, l-Arg-6-aminohexanoic acid-ATA-d-Arg-d-Fcl (peptide I), l-Fno-d-Trp-ATA-d-Ile-d-Arg (peptide II), and l-Arg-d-Cha-ATA-d-Arg-d-Fcl (peptide III), showed an IC50 ≤ 50 nm for hKOR. We then determined Ki values of these compounds for the four receptors (see Table II).

As expected, they displayed good affinities for hKOR with Ki values of 40, 87, and 114 nm, respectively. Peptide II and peptide I showed very weak affinity for hDOR (IC50 ≥ 50000 nm) and weak affinity for hORL1 (Ki of 8100 and 5500 nm, respectively), whereas peptide III displayed higher affinities for these receptors (1100 and 517 nm, respectively). Peptide II showed a weak affinity for hMOR (3000 nm) as well and was therefore the most selective hKOR peptide compared with peptide I and peptide III, which had somewhat higher affinities for hMOR (504 and 1300 nm).

Optimization of the Lead Compounds—To improve the affinity and selectivity of the three compounds for hKOR, we adopted a process called turn-scan that consists in replacing the β-turn mimetic of the tetrapeptides by other turn mimetic molecules to induce slight modifications in their conformation. The structure of the turn-forming templates (TFT) are represented in Fig. 3.

To identify the most active compounds from the turn-scan of peptides I, II, and III, we again tested three concentrations (5, 50, and 500 nm) of each peptide in competition experiments with the four receptors (not shown). We purified the most active peptides by HPLC and determined their Ki values. This procedure did not lead to any significant improvement neither of the affinity nor of the selectivity of the original peptide II and peptide I (not shown). In contrast, BZA, 4BZD, and Haic derivatives of peptide III displayed higher affinities for hKOR (67, 60, and 58 nm, respectively; see Table II). In addition, the III-4BZD and III-BZA peptides had better selectivity for hKOR (Kc (hKOR)/Kc (hMOR)/Kc (hDOR)/Kc (hORL1) ratio of 1:45:112:13 and 1:15:36:8, respectively) compared with peptide III (Kc (hKOR)/Kc (hMOR)/Kc (hDOR)/Kc (hORL1) ratio of 1:11:10:4.5). The III-Haic peptide showed an almost complete loss of selectivity (Kc (hKOR)/Kc (hMOR)/Kc (hDOR)/Kc (hORL1) ratio of 1:1.43:5.9:0.9). Interestingly the III-BTD derivative, which also lost hKOR selectivity, displayed a good affinity and some, although modest, selectivity for hORL1 (Kc (hORL1)/Kc (hKOR)/Kc (hMOR)/Kc (hDOR)/Kc (hORL1) ratio of 1:5:22:6).

[^35]S[GTP]γS Binding Assay—We further characterized these peptides in a functional assay consisting in agonist-promoted stimulation of the[^35]S[GTP]γS binding to hMOR, hDOR, hKOR, or hORL1 cell membranes. This assay has been shown to be a sensitive and reliable method to study the agonist or antagonist activity of opioid ligands with recombinant receptors expressed in mammalian cells (34, 38–41).

Fig. 4 shows the results obtained with CHO-hKOR membranes. In this experiment CI-977, a potent alkaloid agonist of KOR, stimulated the[^35]S[GTP]γS binding with an EC50 value of 3.5 ± 0.5 nM and a maximal activity corresponding to 194 ± 10% that of the basal level of[^35]S[GTP]γS binding. From the seven tested peptides, peptide II, peptide III-Haic, and to a less extent peptide III-BTD stimulated the[^35]S[GTP]γS binding at low concentrations, with EC50 values of 138 ± 4, 110 ± 11, and 434 ± 2 nm, respectively. The maximal activity of each peptide was found to be 146 ± 4%, 137 ± 2%, and 110 ± 1% (respectively) that of the basal level of[^35]S[GTP]γS binding. These values were significantly less than that found for CI-977 (194%), indicating that these peptides were partial agonists for hKOR (see Table III). In contrast, peptide I was found to decrease the basal level of[^35]S[GTP]γS binding down to 82 ± 3% that of control, with an EC50 value of 220 ± 8 nm. This result strongly suggests that we have identified an inverse agonist for KOR. To our knowledge, no κ-ligand with inverse agonist activity has been described previously.

Peptide III-4BZD, peptide III-BZA, and peptide III neither increased nor decreased the[^35]S[GTP]γS binding at low concentrations. It is of note that the higher concentration (50 μM) of peptide III-4BZD stimulated this binding up to approximately 130% that of basal level. To further confirm the antagonist activity of the three latter peptides, we performed concentration-effect curves of CI-977 in presence of 100 Kc of each competitor peptide (see Fig. 5). Peptide III-BZA (5 μM) and peptide III (10 μM) and peptide III-4BZD (6 μM) shifted the concentration-effect curve of CI-977 to the right by about 8-, 40-, and 160-fold, respectively. This result therefore confirms that peptide III and its BZA or 4BZD derivatives have potent antagonist activity. These peptides represent the first κ-receptor antagonists with a peptidomimetic structure.

The activity of five peptides with reasonable affinity for ORL1 (Ki values < 1 μM, see Table II) was also assessed in the[^35]S[GTP]γS binding assay using CHO-hORL1 membranes (see Fig. 6). Under our conditions (see “Experimental Procedures”) nociceptin/orphin FQ stimulated the[^35]S[GTP]γS binding (Fig. 6) with an EC50 value of 12 ± 1 nm and a maximal activity corresponding to 231 ± 5% that of the basal level of[^35]S[GTP]γS binding. These values are in good agreement with those described in the literature (39, 42). As shown in Fig. 6, three from the five tested peptides (peptide III and its -BZA and -Haic derivatives) slightly but significantly stimulated the
[35S]GTPγS binding to CHO-hORL1 membranes, with EC50 values >1 μM and maximal activity of about 125% that of the basal level of [35S]GTPγS binding. In contrast, peptides III-BZA and III-BTD did not stimulate this binding, suggesting that they have antagonist activity. We therefore performed concentration-effect curve of nociceptin/orphanin FQ in the presence of 100 K of peptide III-BTD, which displayed the best affinity and selectivity for hORL1 (see Table II). The results presented in Fig. 7 show that this peptide (2.5 μM) shifted the concentration-effect curve of nociceptin/orphanin FQ to the right by about 65-fold. This indicates that the peptide III-BTD is a potent antagonist of hORL1 in this test.

The two peptides III-BTD and III-Haic also displayed sub-micromolar affinities for hMOR and hDOR (see Table II). We therefore tested their activity at those receptors in the [35S]GTPγS binding assay. As shown in Table III these two peptides stimulated the [35S]GTPγS binding to COS-hMOR membranes (EC50 values of 611 and 116, respectively) and to COS-hDOR membranes (EC50 values of 79 and 30 nM, respectively) at low concentrations. Maximal activation values obtained with peptide III-BTD (158%) and peptide III-Haic (178%) on COS-hMOR membranes were lower than those obtained with DAMGO (270%), a classical peptidic MOR agonist, indicating partial agonist activity of these two peptides at hMOR. Further maximal activation values obtained with these peptides (126% and 117, respectively) on COS-hDOR membranes were close to that obtained with BW373U86 (129%), a potent alkaloid DOR agonist, suggesting that these peptides were full hDOR agonists.

In conclusion the peptide III-Haic exhibits agonist activity and no selectivity toward all four receptors. More interestingly, the peptide III-BTD (structure shown in Fig. 8), which is the only peptide from this study showing ORL1 selectivity, displays antagonist activity toward ORL1 and agonist activity at all three opioid receptors.

**DISCUSSION**

Although linear peptides constitute an attractive starting point for the development of peptidomimetics, their use as drug leads is severely limited by their flexibility in solution, which makes it difficult if not impossible to discriminate among an ensemble of almost iso-energetic conformations the one biologically most relevant (43). Against this background, major progress would come from moving the analysis of constrained sequences earlier in the process, i.e. during the selection phase. To this aim, we have proposed a strategy, “selection-driven design of peptidomimetics,” which is based on the use of a series of conformationally constrained libraries, each one corresponding to a predetermined structure shared by all the peptide sequences (23–25). A positive hit from any such library would immediately yield not only the identity of the side chain pharmacophores but their three-dimensional arrangement as well, i.e. the information that is necessary for the design of the corresponding “scaffolded” peptidomimetic (20). We further argued that this information could be either directly converted into a first generation peptidomimetic or probed and refined by the synthesis of secondary libraries spanning a narrower shape space. Overall, this iterative process would be analogous to traditional drug design but applied to populations of molecules instead of individuals (44). The first example of a conformationally homogeneous peptide combinatorial library was based on the CysHIS2 zinc finger fold and displayed α-helical geometry (23); we could select a carbohydrate-mimicking ligand and show that it had the expected pharmacophoric structure (45).

Although this approach looks conceptually appealing, one major issue must be addressed. Restricting the conformational space available to the peptide sequence inevitably lowers the possibilities of finding suitable ligands: to what extent? We have chosen the opioid receptors as a test case because of their therapeutic importance and because linear peptide libraries have allowed successful selection of ligands for these receptors (13–15, 46). In addition it has been reported that enkephalins and enkephalin analogs have a β-turn conformational preference (47–49). The library used in this study is based on this ubiquitous conformational motif. We searched a compromise

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Stimulation of [35S]GTPγS binding to hKOR by CI-977 and 7 synthetic peptides selected from the turn-scan process. CHO-hKOR membranes (5 μg of protein) were incubated 1 h at 30 °C with [35S]GTPγS (0.2 nM) and GDP (3 μM), with increasing concentrations of ligands: CI-977 (●), peptide II (■), peptide III-Haic (○), peptide III-BTD (+), peptide III-BZA (∇), peptide III (△), and peptide I (▲). Data are expressed as percentage of basal [35S]GTPγS binding and represent mean ± S.E. from at least two separate experiments.

| TABLE III
| --- |
| Activity of two peptides with low receptor selectivity was tested on membrane preparations expressing each receptor. COS-hMOR, COS-hDOR, CHO-hKOR, and CHO-hORL1 membranes (5 μg of protein) were incubated 1 hr at 30 °C with [35S]GTPγS (0.2 nM) and GDP (see “Experimental Procedures”) with increasing concentrations of ligands: DAMGO, BW373U86, CI-977, nociceptin/orphanin FQ, peptide III-BTD and peptide III-Haic. Maximal activation is expressed as percentage of basal [35S]GTPγS binding, and values represent the mean ± S.E. from at least two separated experiments performed in triplicate. ND, not determined. |
| hMOR | hDOR | hKOR | hORL1 |
| --- | --- | --- | --- |
| **EC50** | **Maximal activation** | **EC50** | **Maximal activation** | **EC50** | **Maximal activation** | **EC50** | **Maximal activation** |
| DAMGO | 12.3 ± 0.1 | 270 ± 10 | n.d. | n.d. | 2.4 ± 0.7 | 129 ± 8 | 3.5 ± 0.5 | 194 ± 10 | 12 ± 1 | 231 ± 5 |
| BW373U86 | 2.0 ± 0.1 | 117 ± 6 | 11 ± 1 | 126 ± 2 | n.d. | n.d. | 110 ± 1 | 126 ± 10 | 100 |
| CI-977 | 116 ± 30 | 178 ± 11 | 110 ± 11 | 114 ± 4 | >1000 | >1000 |
| Nociceptin/orphanin FQ | 611 ± 116 | 158 ± 6 | 79 ± 11 | 126 ± 2 | 434 ± 2 | 110 ± 1 | 100 |
| III-BTD | 611 ± 116 | 158 ± 6 | 79 ± 11 | 126 ± 2 | 434 ± 2 | 110 ± 1 | 100 |
| III-Haic | 116 ± 30 | 178 ± 11 | 110 ± 11 | 114 ± 4 | >1000 | >1000 |
between too much and too little constraint and decided to insert a rigid TFT in the middle of a tetrapeptide. The resulting ligands thus have the size of a hexapeptide, having in the middle a dipeptide mimetic occupying the corner positions of the reverse turn. The TFT used is ATA, which was designed as a β-turn inducer (28) and used in the synthesis of a cyclic peptide template for protein engineering (50). Further analysis on the conformational preferences of ATA has been performed by Floegel and Mutter (51) and Gillespie et al. (52). We report here the screening of this library for binding at the three recombinant human opioid receptors (μ, δ, or κ) and ORL1 receptor. The data show that our library contains peptidomimetic compounds with high affinities for the receptors and therefore demonstrate that our strategy was successful. Here we have identified an entirely novel class of ligands for members of the opioid receptor gene family.

The four sublibraries showed a different binding profile for each receptor, but several peptide mixtures were active on more than one receptor. Particularly, Arg residues from the first, third, and fourth sublibraries as well as several hydrophobic residues were active on the four receptors. This confirms that the receptors under study, which display high protein sequence homology (see 3), also share common spatial structural characteristics. Previous studies using chimeric (53) and point-mutated (54) receptors have demonstrated closest structural similarity between KOR and ORL1 receptors. In the course of our peptide screening process, peptides I and III originated from the primary screening on hORL1, and the pure peptides finally displayed best affinity for hKOR. Also we observed that many active mixtures identified from the screening on hORL1 were also active in the hKOR screening. Therefore, our results further support the notion of similar recognition mechanisms at hKOR and hORL1 receptors.

The structures of active ATA peptides do not exhibit any obvious commonalities with the canonical Tyr-Gly-Gly-Phe N-terminal portion of opioid peptides, which has been proposed to interact with the opioid receptor binding site (55). Interestingly however, the peptides described here contain L- and D-Arg amino acid residues and, thus, are highly basic peptides. To this respect, they share the strong basic properties of the C-terminal portion of dynorphins, the endogenous κ-prefering peptides. In dynorphins these residues have been proposed to interact with the positively charged second extracellular loop of KOR (56). The κ-selectivity of the ATA tetrapeptides may therefore arise from specific interactions with this extracellular domain of the receptor protein. Furthermore, the peptides identified in this study also contain hydrophobic residues, with aliphatic (L-cyclohexylalanine) and aromatic (tryptophan, p-chlorophenylalanine) side chains. Those amino acid residues could interact with a number of hydrophobic amino acids located within the transmembrane helical bundle of the receptor, as previously suggested for alkaloid or peptidic opioid compounds (57).

The κ-selective peptides I and III display novel pharmacological properties. Peptide III and its two derivatives III-4BZD and III-BZA are hKOR antagonists, as shown by [35S]GTPγS binding experiments. Norbinaltorphimine, the prototypal κ-antagonist, is a nonpeptide compound that was synthesized on the basis of alkaloid (morphinic) structure (58). Despite the availability of many κ agonist compounds, both of peptidic or nonpeptidic type (59), few κ agonists have been developed, and peptide III may represent a lead compound for a novel class of κ antagonists. Peptide I (structure shown in Fig. 8) decreases basal [35S]GTPγS binding in hKOR membrane preparations and therefore acts as an inverse agonist for this receptor. At present, a single compound has been described with inverse agonist activity at an opioid receptor. This peptide, referred as to ICI174864 (60), inhibits basal GTPase activity in membranes of cells expressing the endogenous (61) or recombinant (62, 63) δ-receptor. To our knowledge, no inverse agonist has been described for μ- and κ-receptors. Thus peptide I, which has comparable affinity and selectivity for the κ-receptor as does ICI174864 for the δ-receptor, represents a unique pharmacological tool for the study of ligand-independent activity at κ-receptors.

To fine-tune the potency and selectivity of the selected li-
gands for hKOR, we systematically replaced the original TFT with other TFTs in a process that we call turn-scan. We used the TFTs shown in Fig. 3, which are all commercially available with the exception of AMTA, which was prepared as described by Ernest et al. (28). Their features as dipeptide mimetics are discussed in Gillespie et al. (52). From the turn-scan we obtained a series of peptide III-derived compounds with novel pharmacological characteristics. More specifically, modification of the turn structure markedly altered the selectivity profile of the original peptide III-ATA. Introduction of the Haic moiety generated a peptide that bound and activated the four receptors similarly, suggesting that opioid and ORL1 receptors share common structural motifs that are involved both in ligand recognition and receptor signaling processes. This is the first example of a “universal” ligand for the four members of this receptor family. On the other hand introduction of the BTD-turn revealed the existence of distinct activation mechanisms that oppose the ORL1 receptor to opioid receptors. The peptide III-BTD acts as an agonist for $\mu$-, $\delta$-, and $\kappa$-opioid receptors but clearly blocks ORL1 activation. This result is particularly interesting since numerous studies suggest “anti-opioid” actions of nociceptin/orphanin FQ, particularly in pain control (see Ref. 12). Therefore, one can assume that ligands with dual selectivity (ORL1/opioid receptors) and dual activity (antagonist for ORL1/agonist for opioid receptors) could produce strong anti-nociceptive effects. This hypothesis will be tested in future studies. Moreover, few antagonists have been described for ORL1 (10), and further optimization of the peptide III-BTD should permit the development of a highly specific antagonist ligands devoid of opioid activity. Such compounds will be extremely useful for the study of in vivo functions of the ORL1-nociceptin/orphanin FQ neurotransmitter system.

In conclusion, these results validate our contention that selection-driven design of peptidomimetics is a viable strategy to drug discovery. After showing selection of a carbohydrate mimetic from a $\alpha$-helical library, we have shown here selection from a reverse-turn library of ligands for an important class of G-protein-coupled receptors. These initial findings are being extended through the synthesis of new reverse-turn libraries based on different TFT, including the ones here used in the turn-scan procedure.

The peptides identified in this study generally exhibit lower affinities than prototypic selective opioid ligands (see Table II) and than the $\kappa$ and ORL1 peptides identified by Dooley et al. (9, 46) from natural peptide combinatorial libraries. However they represent a novel class of $\kappa$-opioid and ORL1 ligands with a well-defined secondary structure and provide good templates for the development of a nonpeptide drugs with innovative and useful biological activities.

Acknowledgment—We thank F. Naimo and F. Bonelli for mass spectrometry and S. Pesci for NMR. We gratefully acknowledge B. Illien, J-L. Galzi, and K. Befort for their helpful discussions. We especially thank P. Chambon for supporting our work.

FIG. 7. Stimulation of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding by nociceptin/orphanin FQ on hORL1 in presence of a putative antagonist peptide. CHO-hORL1 membranes (5 $\mu$g of proteins) were incubated 1 h at 37 °C with $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ (0.2 nM) and GDP (40 $\mu$M), with increasing concentrations of nociceptin/orphanin FQ (O) and 2.5 $\mu$M peptide III-BTD (●). Peptide III-BTD shifted the concentration effect curve of nociceptin/orphanin FQ to the right by 65-fold. Data are expressed as percentage nociceptin/orphanin FQ-induced maximal $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding and represent mean ± S.E. from at least two separate experiments.

FIG. 8. Structures of compounds with original pharmacological properties obtained from this study. Peptide I is a $\kappa$-selective compound with inverse agonist activity. Peptide III-BTD binds with high affinity to the four receptors, shows weak ORL1 selectivity, and acts as an antagonist at ORL1 and agonist at opioid receptors.

Peptide I

Peptide III-BTD

Peptide II

Conformationally Constrained Peptides for Opioid Receptors
