Identification of a series of CCK-2 receptor nonpeptide agonists: Sensitivity to stereochemistry and a receptor point mutation

Alan S. Kopin*, Edward W. McBride*, Ci Chen*, Roger M. Freidinger*, Duan Chen§, Chun-Mei Zhao§, and Martin Beinborn*

* Molecular Pharmacology Research Center, Department of Medicine, Tufts-New England Medical Center, 750 Washington Street, Box 7703, Boston, MA 02111; †Medicinal Chemistry Department, Merck Research Laboratories, West Point, PA 19486; and §Institutes of Intra-abdominal Diseases and Laboratory Medicine, Norwegian University of Science and Technology, 7006 Trondheim, Norway

Communicated by Ralph F. Hirschmann, University of Pennsylvania, Philadelphia, PA, March 3, 2003 (received for review November 7, 2002)

The search for small-molecule drugs that act at peptide hormone receptors has resulted in the identification of a wide variety of antagonists. In contrast, the discovery of nonpeptide agonists has been far more elusive. We have used a constitutively active mutant of the cholecytokinin 2 receptor (CCK-2R) as a sensitive screen to detect ligand activity. Functional assessment of structural analogs of the prototype CCK-2R antagonist, L-365,260 [3R-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′(3-methylphenyl)urea], resulted in the identification of a series of agonists. Each of the active molecules is an S enantiomer, whereas the corresponding R stereoisomers have little or no activity. Further in vitro and in vivo assessment at the wild-type receptor indicated that efficacy of the two most active ligands approached that of the endogenous hormone. The function of selected R and S enantiomers was differentially sensitive to a point mutation, N353L, within the putative CCK-2R ligand pocket. The results of this study highlight the potential of constitutively active receptors as drug screening tools and the interdependence of ligand stereochemistry and receptor conformation in defining drug efficacy.

The cholecystokinin (CCK) 2 receptor (CCK-2R, previously known as the “CCK-B/gastrin” receptor) is a G protein-coupled seven transmembrane domain protein that has subnanomolar affinity for two endogenous peptide ligands, CCK octapeptide (CCK-8) and gastrin (1, 2). Receptor stimulation with either CCK-8 or gastrin triggers activation of phospholipase C, which, in turn, leads to the production of inositol phosphates (IPs).

The CCK-2R is expressed in the stomach and central nervous system, where it is the predominant CCK receptor subtype (3, 4). The CCK-2R has been postulated to modulate a wide spectrum of physiologic functions including gastric acid secretion, differentiation of the gastric mucosa, and perception of pain. The physiology linked to the CCK-2R has generated longstanding interest in this receptor as an important target for drug discovery. These efforts have resulted in the identification of multiple structurally diverse synthetic antagonists (5). Among these small molecules, the 1,4-benzodiazepine derivative L-365,260 [3R,N- (2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′(3-methylphenyl)urea] is considered the prototype subtype-selective antagonist (6, 7). This compound has been widely used to define CCK-2R-regulated functions in vivo.

Given the physiologic significance of CCK-2R-mediated functions and the availability of a wide range of subtype-selective synthetic molecules, this receptor has become an important model system for studying structure–function relationships of peptide hormone G protein-coupled receptors (GPCRs). Success in identifying nonpeptide antagonists has stimulated efforts to address the next challenge: the identification of small molecule agonists, which mimic, rather than block, peptide hormone function. To achieve this objective, there has been considerable interest in developing functional assays to expedite the process of agonist drug discovery (2, 8–10).

A.K. and M.B. have used the CCK-2R as a model system to demonstrate that constitutively active peptide receptors provide a sensitive tool for detecting agonist activity (i.e., efficacy) in small molecules (2). GPCR mutants with an elevated basal level of signaling have a lower threshold for ligand-induced signaling (vs. the corresponding wild-type isoform), a property that can be applied to facilitate the discovery of agonist lead compounds (11). Using this approach we have shown that the putative antagonist, L-365,260, can trigger second-messenger signaling in cells expressing a constitutively active CCK-2R. The present study was undertaken to explore the hypothesis that L-365,260 may provide a template from which nonpeptide derivatives could be developed that have strong agonist activity not only at the constitutively active mutant, but also at the wild-type CCK-2R.

Here we report the identification of such CCK-2R agonists and demonstrate that the ability to trigger receptor activation is highly dependent on compound chirality. Extending from the in vitro cell-based assays, our results also illustrate biological activity of the most efficacious nonpeptide molecules in vivo. In addition, the current work highlights how the efficacies of individual ligands may be differentially altered by even a single amino acid change in the CCK-2R, an observation that has important implications for the rapidly evolving field of pharmacogenomics (12).

Materials and Methods

Ligands. Synthetic sulfated CCK-8 and human gastrin-17 were purchased from Bachem. Nonpeptide small molecules were a generous gift from Merck. The absolute stereochemistry of these compounds was inferred by correlation of their optical rotations with the rotations of the enantiomers of the amidinobenzodiazepine L-740,093 (13). All enantiomers described were >99% enantiomer excess (ee), as determined by chiral HPLC. Stock solutions of these compounds (5.0 mM) were prepared by dissolving the ligands in DMSO.

Radioligand Binding Experiments. The cDNA expression constructs encoding the wild-type human CCK-2R and the L325E (third intracellular loop) and N353L (sixth transmembrane domain) mutants have been described (2, 14). COS-7 cells (10⁶) were transfected with 5 μg of either wild-type or mutant human CCK-2R cDNA subcloned into the expression vector pcDNA1.1 (Invitrogen). The following day, transfected cells were split into 24-well plates (4–50 × 10⁴ cells per well) and binding experiments were performed by using 20 nM ¹²⁵I-labeled CCK-8 (¹²⁵I-CCK-8; Perkin–Elmer) as the radioligand. Affinities for the 1,4-benzodiazepine-based nonpeptide compounds were determined by competition binding experiments with increasing concentrations of

Abbreviations: CCK, cholecystokinin; CCK-2R, CCK-2 receptor; CCK-B, CCK octapeptide; GPCR, G protein-coupled receptor; HDC, histidine decarboxylase; IP, inositol phosphate.

*To whom correspondence should be addressed. E-mail: akopin@tufts-nemc.org.
the unlabeled ligands. IC₅₀ values were calculated by computerized nonlinear curve fitting (PRISM 3.0, GraphPad, San Diego).

**Luciferase Activity.** By using LipofectAMINE (Invitrogen), HEK293 cells were cotransfected with receptor cDNA and a multimerized serum response element (SRE) cloned upstream from the coding sequence for firefly luciferase, SRE-Luc. Cells were transfected and stimulated in serum-free DMEM (GIBCO/BRL) at 37°C. Twenty-four hours after transfection, the cells were incubated for an additional 18 h with (i) CCK-8 (3 × 10⁻⁶ M), (ii) one of the nonpeptide compounds (1 × 10⁻⁵ M), or (iii) no ligand (to assess “basal” activity). After stimulation, the cells were lysed and assayed for light emission by using the LucLite luciferase assay kit (Packard).

**Measurement of IP Production.** One day after transfection (see Radioligand Binding Experiments), COS-7 cells were seeded into 12-well plates (2 × 10⁵ cells per well). After an additional 6 h, the cells were labeled overnight with 3 μCi/ml (1 Ci = 37 GBq) myo-[³²P]inositol in serum-free DMEM. Cells were then stimulated for 1 h at 37°C with either CCK-8 (3 × 10⁻⁷ M) or the specified nonpeptide compound (1 × 10⁻⁵ M) in PBS (GIBCO/BRL) containing 10 mM LiCl. After stimulation, the cells were lysed and extracted with methanol/chloroform. The upper phase was analyzed for IPs by strong anion exchange chromatography as described (2).

**Measurement of Gastric pH and Oxyntic Mucosal Histidine Decarboxylase (HDC) Activity.** Male C57BL/6J mice [~3 months old, 18.8 ± 0.1 grams body weight (mean ± SEM, n = 47)] were purchased from Møllegaard’s Breeding Center (Skensved, Denmark). Groups of six mice were housed in wire-mesh-bottom cages at 20°C and 40–45% humidity. The animals were maintained with a 12-h light/12-h dark cycle and were provided free access to commercial standard mouse/rat food pellets (B & K Universal, Hull, U.K.) and water. After a 20-h fast (free access to water was provided), a pyloric ligation was performed under anesthesia with methoxyflurane (Metofane, Mallinckrodt, Mundelein, IL; ref. 15). Through an abdominal midline incision, the junction between the pylorus and the duodenum was ligated with silk. Immediately after surgery, nonpeptide compounds (8 μmol/kg body weight) or gastrin (1 μmol/kg body weight) were administered by s.c. injection into the neck.

After 4 h, the mice were again anesthetized with Metofane. Gastric juice from each mouse was collected by gastrogavage for assessment of pH. The mice were then killed by cervical dislocation, and the stomachs were removed, opened, and washed in ice-cold saline. The oxyntic mucosa was scraped from the gastric wall of each mouse, weighed, and homogenized in cold (4°C) 0.1 M sodium phosphate buffer (pH 7.0). The final concentration was adjusted to 100 mg (wet weight)/ml. Eighty-microliter aliquots of each homogenate were incubated for 1 h with 0.02 mCi/ml L-[¹⁴C]histidine (specific activity, 50 μCi/mmol; Perkin-Elmer Life Sciences) in the presence of 5 × 10⁻⁴ M l-histidine and 10⁻⁴ M pyridoxal-5-phosphate at 37°C under nitrogen. The total reaction volume was 160 μl. The ¹⁴CO₂ formed during the reaction was trapped in protosol solution and measured by liquid scintillation counting (16, 17). The enzyme activity was expressed as pmol of CO₂ per mg of mucosa per h. The experiment was approved by the University Hospital of Trondheim Animal Research Committee.

**Results**

A serum response element (SRE)-luciferase reporter gene assay was used to screen structural analogs of L-365,260 for agonist activity (Fig. 1). Evaluation of drug function revealed that ligand efficacy at a constitutively active CCK-2R (L325E) was amplified relative to the corresponding values in cells expressing the wild-type receptor. Six compounds that showed activity greater than that of L-365,260 (1S–6S) were selected for further study. Variability among these compounds was limited to the C5 substituent of the core benzodiazepine structure (X in Table 1). In addition, to investigate the role of compound chirality, corresponding R stereoisomers (1R–6R) were assayed in parallel.

The binding affinity of each compound was assessed at the human wild-type receptor. All R enantiomers had higher affinity for the CCK-2R than the corresponding S stereoisomers. The R compounds (including L-365,260) had IC₅₀ values ranging from 0.25 to 3.3 nM, whereas the respective values for the S enantiomers ranged from 6.2 to 48 nM (Table 1). The binding of each compound was also determined at a mutant CCK-2R, N353L, that is known to have reduced affinity (21-fold) for L-365,260 (1S–6S). As observed with the prototype CCK-2R antagonist, the affinity of each R compound is highly sensitive to the N353L mutation. The observed decreases in affinity were between 33- and 500-fold (Table 1). In contrast, several of the S enantiomers (3S–6S) show conserved affinity at
the N353L mutant, suggesting that the overall tertiary structure of the modified receptor remained intact. The two other S compounds, 1S and 2S, had a 61- and 9-fold reduction, respectively, in affinity at the mutant CCK-2R.

The efficacy of each L-365,260 derivative was further explored by measuring ligand-induced production of IP in COS-7 cells expressing the recombinant human CCK-2R (Fig. 2A). The activity of each ligand was compared with that of the endogenous peptide hormone, CCK-8. The S enantiomers had activities ranging from 14% to 77% of the CCK-8-induced maximum, whereas the efficacies of the R enantiomers ranged from 5% to 9%, which is comparable to the trace activity observed with the wild-type CCK-2R.

In addition to demonstrating agonist efficacy in vitro, complementary experiments were designed to assess in vivo activity of the most active compounds (1S and 2S). Because these experiments were to be carried out in mice, it was important to first document that the agonist activity of 1S and 2S, as initially observed at the recombinant human CCK-2R, was conserved at the mouse recep-

The structure of the L-365,260 template molecule and respective CS substituents ("X") are illustrated. A wavy line marks the C3 chiral center of the molecule; R and S denote the respective enantiomers. IC50 values of each enantiomer were assessed by radioligand (125I-CCK-8) competition experiments using COS-7 cells transiently expressing either the wild-type CCK-2R or the N353L mutant. Data represent mean ± SEM from at least three independent experiments.

*Previously reported (14).

Table 1. Comparison of 1,4-benzodiazepine-based ligand structures and corresponding affinities at the wild-type human CCK-2 receptor vs. the N353L mutant

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wild type IC50, nM</th>
<th>N353L IC50, nM</th>
<th>Fold Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-365,260 (R)</td>
<td>3.3*</td>
<td>69*</td>
<td>21*</td>
</tr>
<tr>
<td>1S</td>
<td>7.6 ± 1.2</td>
<td>460 ± 35</td>
<td>61</td>
</tr>
<tr>
<td>1R</td>
<td>0.85 ± 0.25</td>
<td>420 ± 34</td>
<td>500</td>
</tr>
<tr>
<td>2S</td>
<td>48 ± 3.6</td>
<td>450 ± 61</td>
<td>9.3</td>
</tr>
<tr>
<td>2R</td>
<td>0.99 ± 0.25</td>
<td>120 ± 18</td>
<td>120</td>
</tr>
<tr>
<td>3S</td>
<td>17 ± 3.0</td>
<td>14 ± 2.9</td>
<td>0.81</td>
</tr>
<tr>
<td>3R</td>
<td>1.0 ± 0.22</td>
<td>143 ± 19</td>
<td>140</td>
</tr>
<tr>
<td>4S</td>
<td>42 ± 10</td>
<td>44 ± 12</td>
<td>1.1</td>
</tr>
<tr>
<td>4R</td>
<td>3.0 ± 0.70</td>
<td>98 ± 16</td>
<td>33</td>
</tr>
<tr>
<td>5S</td>
<td>6.2 ± 0.63</td>
<td>8.8 ± 0.64</td>
<td>1.4</td>
</tr>
<tr>
<td>5R</td>
<td>0.25 ± 0.04</td>
<td>34 ± 9.5</td>
<td>140</td>
</tr>
<tr>
<td>6S</td>
<td>21 ± 2.0</td>
<td>50 ± 9.5</td>
<td>2.4</td>
</tr>
<tr>
<td>6R</td>
<td>0.60 ± 0.09</td>
<td>83 ± 8.3</td>
<td>140</td>
</tr>
</tbody>
</table>
tor homolog. It is well documented that interspecies differences in GPCRs may alter ligand activity (12). Ligand-induced IP production was assessed in transiently transfected COS-7 cells expressing the mouse CCK-2R (18). The efficacies of 1S and 2S were 88 ± 6.2% and 92 ± 7.5% (mean ± SEM of three independent experiments), respectively, of the CCK-8-induced maximum. These values are comparable to, or even higher than, those observed at the recombinant human CCK-2R (Fig. 2A). Further experiments at the recombinant mouse CCK-2R revealed that both 1S and 2S stimulated IP production with subnanomolar potencies (mean EC50 values of 0.2 and 0.4 nM, respectively, of two independent experiments), suggesting that in vivo application of these drugs would be likely to result in receptor activation.

Both acid secretion and activation of HDC are well established physiologic consequences of CCK-2R stimulation in the gastric mucosa. Each of these parameters has been used as a readily detectable correlate of CCK-2R-mediated signaling in vivo (17, 19, 20). As an index of the in vivo function of 1S and 2S in mice, the gastric luminal pH, as well as the activity of mucosal HDC, was measured 4 h after s.c. administration of each drug. Observed changes in pH and HDC after administration of either 1S or 2S were comparable to the effects induced by the full peptide agonist, gastrin (Fig. 3).
Discussion
A significant challenge in molecular pharmacology has been to identify small-molecule ligands that mimic the action of peptide hormones. We have used a constitutively active CCK-2R as a sensitive screen to detect intrinsic activity of L-365,260. Functional assessment of structurally related analogs led to the identification of a series of nonpeptide agonists (Fig. 1). Characterization of these small molecules at the wild-type receptor revealed efficacy both in vitro and in vivo (Figs. 2 and 3).

A common theme among CCK-2R compounds 1–6 (Table 1) is the critical role of stereochemistry in defining ligand function. In cells expressing either the constitutively active or the wild-type receptor, each of the S enantiomers stimulated significant ligand-induced IP production, whereas the corresponding R stereoisomers triggered little, if any, signaling (Fig. 2). Parallel findings were observed with the luciferase reporter gene assay (Fig. 1). Within the CCK receptor literature, other examples of contrasting functional activities between enantiomers have been reported. The CCK-1 receptor ligand PD-149,164 is an agonist, whereas the enantiomer of this compound (PD-151,932) is an antagonist (21). In addition, S and R enantiomers of the CCK-2R ligand, L-740,093, were demonstrated to have weak agonist and inverse agonist function, respectively (2).

The identification of enantiomers with opposite functional activities at a given GPCR is not unique to CCK receptor ligands. At the α2A-adrenergic receptor, medetomidine enantiomers have been demonstrated to possess agonist and inverse agonist activity, respectively (22). In addition, α1A-muscarinic receptor ligands, stereoisomers of compound BN225 have been reported to have agonist vs. antagonist function (23). Our results, taken together with the literature, suggest that among the enantiomers of known antagonists and related compounds are molecules with detectable intrinsic activity. The use of constitutively active receptors provides a sensitive screen for detecting such potential lead compounds. These ligands, in turn, provide promising templates for the development of related small molecules, which may show full agonist activity at the wild-type receptor.

To explore how the respective function of paired enantiomers is altered by a mutation within the putative ligand pocket of the CCK-2R, we compared the activity of each compound at the wild-type receptor with the activity at the N353L receptor. We have previously shown that small-molecule ligands recognize a putative binding pocket within the CCK-2R that is comprised of the side chains of transmembrane domain amino acids (24). Mutation of individual residues within this pocket, including N353, may differentially alter the affinity and/or efficacy of small-molecule ligands (14). Extending this theme, we now demonstrate that steric configuration of a given ligand may markedly influence the magnitude and even the direction of affinity/efficacy changes resulting from a point mutation. Paired enantiomers may show divergent alterations in functional activity (e.g., 1S vs. 1R) and/or affinity (e.g., 3S vs. 3R) in response to a single amino acid substitution within the target receptor (e.g., N353L). These results highlight the importance of stereochemistry as a critical determinant of ligand–receptor interaction both at the wild-type receptor and mutant receptors or, by analogy, at naturally occurring polymorphic GPCRs.

There is an increasing number of studies reporting that slight modifications in ligand structure may translate into loss or gain of agonist function (25–30). At the wild-type CCK-2R, the addition of two methyl groups to the S enantiomer of compound 3 markedly enhances ligand function (compare 2S and 3S in Table 1 and Fig. 2). The addition of these methyl groups likely acts by increasing hydrophobic bulk, although it cannot be excluded that this structural change indirectly influences ionic interactions between the receptor and other domains of the ligand. It is of note that there is a growing list of nonpeptide ligands where functional activity can be markedly enhanced with the addition of small aliphatic side chains. In a CCK-1 receptor ligand derived from a 1,5-benzodiazepine template, the addition of a single methyl group converts an antagonist (11b) to a relatively strong partial agonist (11c) that has ∼66% of the activity of the endogenous hormone, CCK-8 (26). Similarly, at the angiotensin AT1 receptor, a biphenyl-based nonpeptide antagonist can be converted into an agonist by the addition of a single methyl group (27).

The optimal degree of hydrophobic bulk for a given agonist molecule appears to depend both on the configuration of the ligand and that of the receptor binding pocket. This is illustrated by comparing the effects of selected ligand modifications on efficacy at the wild-type CCK-2R to those at the N353L mutant. Comparison of compounds 3S and 2S reveals that activity at the wild-type CCK-2R increases with hydrophobic bulk (e.g., with the addition of two methyl groups on 3S, as discussed above). In contrast, the same modification has no effect on compound efficacy at the N353L mutant. Another example illustrating the interplay between stereochemistry and the configuration of the ligand pocket in determining small-molecule function is illustrated by comparing compounds 2R and 3R. At the N353L receptor, removal of two methyl groups from 2R increases activity, whereas the same change in ligand structure has little consequence at the wild-type protein.

The principle that optimal agonist efficacy may in part be a function of hydrophobic bulk is further supported in the literature on opioid receptors. Replacement of an allyl group (CH2=CH—CH2) with a methyl side chain converts naltorphine, a potent antagonist, to morphine, the prototype nonpeptide agonist (31). As with the CCK receptor ligands presented in this study, activity is highly dependent on the size and composition of the selected aliphatic group.

In summary, we have demonstrated that a constitutively active CCK-2R mutant can be used as a highly sensitive tool for identifying small molecules with intrinsic activity, thus providing a structural template for the discovery of full agonists. These compounds could be further assessed and optimized (e.g., for bioavailability and receptor/subtype selectivity) with the goal of developing therapeutically useful drugs. The proposed strategy for identifying nonpeptide agonists should be applicable to other GPCRs and, therefore, has the potential to expedite drug discovery efforts for a wide range of targets. Furthermore, the differential responsiveness of the wild-type CCK-2R vs. the N353L mutant to compound enantiomers highlights the interplay between receptor conformations and ligand chirality in defining efficacy. This example, although not based on a naturally occurring CCK-2R variant, may presage how polymorphic variation(s) in a target receptor can markedly alter drug response.

We thank Yong Ren and Amanda Doran for helpful discussions and Sylvie Bourrain, Sarah Hobbs, and Kevin Moore (Merck Sharp & Dohme Research Laboratories, Terlings Park, U.K.) for chemical synthesis. This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (Grants DK46767 and DK56674 and GRASP Digestive Disease Center Grant P30 DK34928). A.S.K. and M.B. are Tufts–New England Medical Center MCRI investigators.


