Intermolecular Interactions between Peptidic and Nonpeptidic Agonists and the Third Extracellular Loop of the Cholecystokinin 1 Receptor

Craig Giragossian,[†] Elizabeth E. Sugg,[§] Jerzy R. Szewczyk,[§] and Dale F. Mierke[†,‡,*]

Department of Chemistry and Department of Molecular Pharmacology, Division of Biology & Medicine, Brown University, Providence, Rhode Island 02912, and Department of Medicinal Chemistry, GlaxoSmithKline, Five Moore Drive, PO Box 13398 RTP, North Carolina 27709

Received March 28, 2003

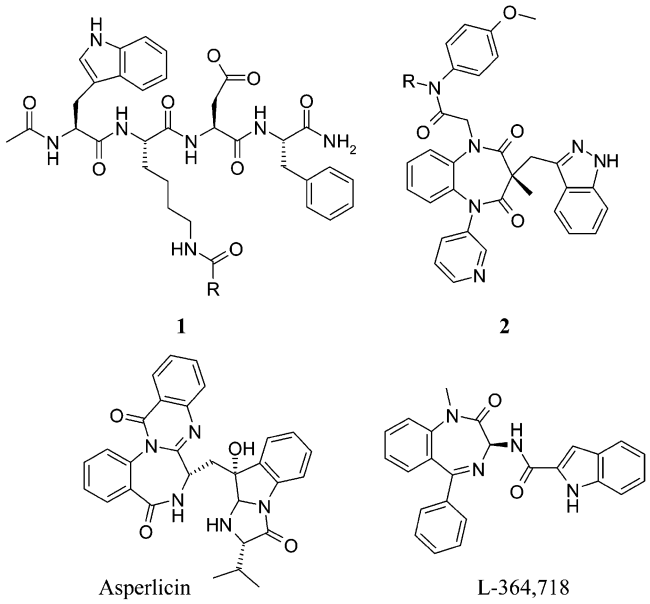
Intermolecular interactions were determined between a synthetic peptide corresponding to the third extracellular loop and several residues from the adjoining sixth and seventh transmembrane domains of the human cholecystokinin-1 receptor, CCK_1-R(329−357), and the synthetic agonists Ace-Trp-Lys[NH$^{\epsilon}$CONH-o-(MePh)]-Asp-MePhe-NH_2 (GI5269) and the C1 *N*-isopropyl-*N*−(4-methoxyphenyl)acetamide derivative of 3-(1*H*-Indazol-3ylmethyl)-3-methyl-5-pyridin-3-yl-1,5-benzodiazepine (GI0122), using high-resolution nuclear magnetic resonance spectroscopy and computer simulations. Addition of the ligands to CCK_1-R(329−357) in an aqueous solution of DPC micelles produced a number of intermolecular nuclear Overhauser enhancements (NOEs) to residues in TMs 6 and 7 of the receptor fragment. NOE-restrained molecular models of the GI5269 and GI0122/CCK_1-R complexes provide evidence for overlapping ligand-binding sites for peptidic and nonpeptidic agonists. The proposed binding modes of GI5269 and GI0122 are supported by the structure−activity relationship of analogues and mutagenesis data for the CCK_1-R selective antagonist L-364,718.

Introduction

The cholecystokinin-1 (CCK_1-R) and -2 (CCK_2-R) guanine nucleotide binding regulatory protein-coupled receptors (GPCRs) are pharmaceutical targets for metabolic diseases, such as obesity and central nervous system maladies, including anxiety and panic attacks.[1,2] The receptors share approximately 50% sequence identity and are activated by a series of C-terminal carboxyamidated peptides, derived from a 115-amino acid preprohormone (e.g., CCK-58, CCK-33, and CCK-8).[3] The sulfated C-terminal octapeptide of CCK is the minimal sequence necessary for full activation of the CCK_1-R, whereas for the CCK_2-R only the C-terminal tetrapeptide is required. The differential affinity of endogenous and exogenous ligands has been used to ensconce subtype-distinct ligand-binding sites.

The rational design of potent and selective ligands for the CCK_1-R has focused on peptidic derivatives of CCK and polycyclic derivatives of the natural product asperlicin,[4] a CCK_1-R antagonist identified through random screening (Scheme 1). Replacement of Met^{31} in the C-terminal tetrapeptide of CCK with Lys(NH$^{\epsilon}$CONHR)^{31} reversed subtype selectivity and led to the discovery of highly potent CCK_1-R agonists (**1**).[5−9] The vast majority of nonpeptidic CCK_1-R antagonists incorporate a benzodiazepine core with varied substituents in the 1, 3, and 5 positions (e.g., L-364,718).[10−14] Nominal changes in the *N*-phenylacetamide of **2** modulated the pharmacological effect, converting antagonists to agonists.[15−18] The molecular basis for altering subtype selectivity and potentiation in analogues of **1** and **2**, respectively, is of considerable interest, since knowledge of these salient ligand−receptor interactions will facilitate development of the next generation of ligands.

Scheme 1



As with other GPCRs, high-resolution structural studies of the CCK receptors have been hampered by the inability to form crystals suitable for X-ray diffraction. In lieu of determining the structure of the intact receptor, X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) have been used to characterize the structure of synthetic peptides corresponding to complete or partial domains of GPCRs.[19−22] Using this approach, intermolecular contacts between CCK-8 and receptor fragments corresponding to the N-terminal

* To whom correspondence should be addressed. Voice: (401) 863-2139, Fax: (401) 863−1595, e-mail: Dale_Mierke@Brown.edu.
† Department of Chemistry, Brown University.
‡ Department of Molecular Pharmacology, Brown University.
§ GlaxoSmithKline.

10.1021/jm030144z CCC: $25.00
Published on Web 07/08/2003

Table 1. Structurally Informative $^3J_{NH\alpha}$ Coupling Constants, Amide Proton Temperature Coefficients and NOEs from Spectra Collected at 298, 303, and 308 K. the Thickness of the Bars Is Proportional to the NOE Cross-Peak Intensity (weak, medium, strong)

	30	31	32	33
	Ace	Trp	Lyu	Asp	Nmf
$^3J_{NH\alpha}$(Hz)		6	8	10	
-ppb/K		9.4	3.5	1.8	
d_{NN}(i,i+1)					
$d_{\alpha N}$(i,i+1)					
$d_{\alpha N}$(i,i+2)					
$d_{\alpha N}$(i,i+3)					
$d_{\alpha\alpha}$(i,i+1)					
$d_{\alpha\beta}$(i,i+1)					
$d_{\beta\alpha}$(i,i+1)					
$d_{\beta N}$(i,i+1)					
$d_{N\beta}$(i,i+1)					

(NT) domain of the human CCK$_1$-R(1−47)[23] and the third extracellular loop (EC3) domains of the human CCK$_1$-R(329−357)[24] and CCK$_2$-R(352−379)[25] have been determined by NMR. Molecular models of the ligand−receptor complexes, incorporating the experimentally determined structures of the ligands and receptor domains, produced binding orientations in agreement with mutagenesis and photoaffinity labeling studies.[24−26]

In this study, the conformational features of the synthetic agonists Ace-Trp-Lys[NH$^{\epsilon}$CONH-o-(MePh)]-Asp-MePhe-NH$_2$ (GI5269) and the C1 *N*-isopropyl-*N*-(4-methoxyphenyl)acetamide derivative of 3-(1*H*-indazol-3ylmethyl)-3-methyl-5-pyridin-3-yl-1,5-benzodiazepine (GI0122) were determined by NMR in a biphasic solvent system composed of dodecylphosphocholine (DPC) micelles. Addition of the ligands to a synthetic peptide corresponding to the third extracellular loop and several residues from the adjoining sixth and seventh transmembrane domains of the human CCK$_1$-R, CCK$_1$-R(329−357), produced a number of intermolecular nuclear Overhauser enhancements (NOEs). The intermolecular NOEs were used to manually dock the ligands to a molecular model of the CCK$_1$-R. The binding orientations and intermolecular interactions in the ligand−receptor complexes are rationalized in terms of the available structure activity relationships (SAR) for analogues of GI5269 and GI0122 and mutagenesis data for the CCK$_1$-R selective antagonist L-364,718.

Results

Structure of GI5269. The NMR spectra of GI5269 displayed two sets of proton resonances corresponding to the cis and trans isomers of the *N*-methylated amide bond (Nmf[33]). The NOE intensities observed for the cis configuration were insufficient for structural calculations (1:10, cis:trans), so the structural analysis of GI5269 was restricted to the all-trans configuration. The $^3J_{HN\alpha}$ coupling constants, amide ^{1}H temperature coefficients, and many of the structurally informative NOEs are provided in Table 1. The $^3J_{HN\alpha}$ and NOEs were converted to dihedral angle and distance restraints,

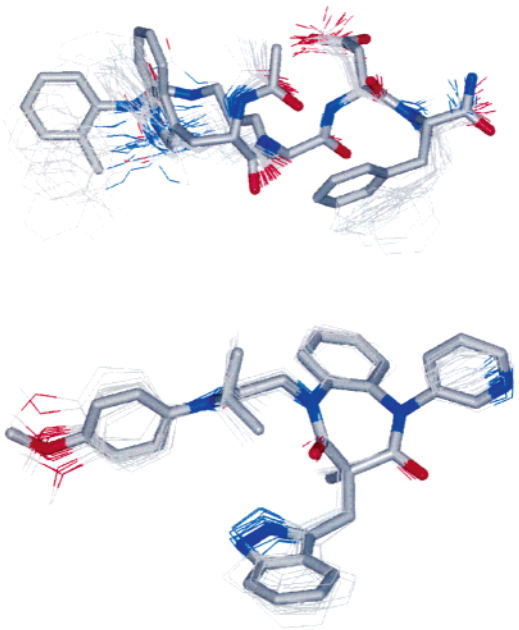


Figure 1. Superposition of the structures of GI5269 (top, RMSD 0.8 Å) and GI0122 (bottom, 1.4 Å) extracted from the last 50 ps of 200 ps restrained MD simulations in a water−decane simulation cell. The final structure in the trajectory is depicted in sticks.

Table 2. Mean Backbone Dihedral Angles of GI5269 during the Last 50 ps of a 200 ps Restrained MD Simulation, in a Water−Decane Simulation Cell

	phi	psi
Trp[30]	−64 ± 7	−38 ± 12
Lyu[31]	−104 ± 12	19 ± 10
Asp[32]	−114 ± 8	106 ± 8
Nmf[33]	−109 ± 10	91 ± 28

respectively. The temperature coefficient of Asp[32], being less than 2.0 ppb/K, is consistent with an amide proton in a solvent-shielded environment.[27,28] In accordance with the NOEs and coupling constants, which suggest a turn conformation, a hydrogen bond restraint was added between the acetyl oxygen and the amide proton of Asp[32]. While the amide proton temperature coefficient of Asp[32], by itself, is insufficient to determine the existence and location of an intramolecular hydrogen bond, the values obtained are in excellent agreement with those obtained for Asp[32] in sulfated CCK-9 (−1.8 ppb/K) and sulfated CCK-15 (−1.4 ppb/K), where hydrogen bonds to this residue and the carbonyl of the *i*-3 residue have been identified in high-resolution structural studies in the same solvent system.[29] The structures and mean backbone dihedral angles of GI5269 extracted from the last 50 ps of the 200 ps MD trajectory are provided in Figure 1 and Table 2. Throughout the entire simulation, the backbone dihedral angles for Trp[30] and the lysine phenyl urea (Lyu[31]) were consistent with a type I β-turn ($\phi_{i+1}/\psi_{i+1} = -60/-30$, $\phi_{i+2}/\psi_{i+2} = -90/0$).

Structure of GI0122. The NOESY spectra of GI0122 produced very few structurally informative NOEs; however, the semirigid 1,5-benzodiazepine core and the aromatic N1, N5, and C3 substituents limit the available degrees of conformational freedom. The initial configuration of the 1,5-benzodiazepine core was based on the X-ray structures of benzodiazepine analogues

Table 3. Intermolecular NOEs Observed upon Addition of GI5269 and GI0122 to CCK_1-R(329-357)

receptor	ligand		
	GI5269		
I329	Hβ	Trp[30]	HN
P351	Hα	Lyu[31]	urea
D339	HN	Nmf[33a]	Hα
	GI0122		
R336	Hγ	2*H*-Pyridinyl	
S353	Hβ	6*H*-Benzodiazepine	
L356	HN	6*H*-Benzodiazepine	
I355	Hδ	8*H*,9*H*−Benzodiazepine	

[a] Cis isomer.

reported in the literature.[11,12,30] NOEs were observed between the 2*H*,4*H*-pyridinyl and 4*H*-indazolylmethyl protons, the *N*-isopropyl-*N*-phenylacetamide and 7*H*-indazolylmethyl protons, and the C3-methyl and 1*H*-indazolylmethyl protons. The NOEs are consistent with a conformation in which the N1-, C3-, and N5-benzodiazepine substituents occupy equatorial positions on the same face of the benzodiazepine core. The C3-methyl group occupies an opposing axial position. The structures of GI0122 extracted from the last 50 ps of the 200 ps MD trajectory are provided in Figure 1. Considerable conformational flexibility is observed in the *N*-isopropyl-*N*-phenylacetamide and pyridinyl moieties.

Complexes with CCK_1-R(329−357). Addition of GI5269 and GI0122 to CCK_1-R(329−357) produced a number of specific intermolecular NOEs (Table 3) and concentration-dependent chemical shift perturbations (Figure 2). For both ligands, intermolecular NOEs were located in the putative TM6 and TM7 domains of the CCK_1-R(329−357). Chemical shift perturbations were evenly distributed throughout the receptor domain and suggest tight coupling in the ligand−receptor fragment complex. NOE-restrained MD simulations of the ligand−receptor fragment complexes revealed a number of stabilizing intermolecular interactions.

Consistent with cis−trans isomerization of the *N*-methylated amide bond in GI5269, intermolecular NOEs were observed between both isomers and CCK_1-R(329−357). Simultaneous application of the intermolecular distance restraints resulted in conversion of the penultimate amide bond from the trans to the cis configuration. Since GI5269 and non-N-methylated Phe[33] derivatives have similar binding affinities for the CCK_1-R, the peptide was modeled in the trans configuration with a concomitant reduction in the penalty function for the cis intermolecular distance restraint. In the GI5269/CCK_1-R(329−357) complex, the β-turn of the ligand was located between TMs 6 and 7, with the C-terminus in close spatial proximity to TM6 and the side chain of Lyu[31] spanning from TM6 to TM7 along a groove formed by the third extracellular loop helix and the extended strand leading to TM7. van der Waals interactions were observed between Nmf[33] and N333, R336, and A337; and Lyu[31] and N333, R336, E344, R345, and S348−F354. The side chain of Trp[30] was located in a hydrophobic pocket at the confluence of TMs 6 and 7, forming interactions with I329, F330, N333, I355 and L356. Coulombic interactions were present between the side chains of Asp[32] and R336, and hydrogen bonds were observed between the Lyu[31] urea and the carboxylate of E344.

The intermolecular NOEs observed for GI0122 were mutually consistent with two distinct binding orientations, differing by the relative placement of the *N*-isopropyl-*N*-phenylacetamide moiety. Simulated annealing calculations of the GI0122/CCK_1-R(329−357) complex starting with the acetamide in close spatial proximity to TM6 or TM7 converged to the same configuration in which the acetamide was near TM7. The benzodiazepine core was located in a hydrophobic pocket at the confluence of the TM domains, forming van der Waals interactions with N333, I352, S353, I355, and L356. Additional intermolecular interactions were observed between the pyridinyl substituent and N333, R336, and A337; and the C3-methyl substituent and N333, I355, and L356. π-Cation interactions were present between the guanidine of R336 and the aromatic rings of the pyridinyl and benzodiazepine moieties.

Molecular Models of the Ligand/Receptor Complexes. The experimentally determined structures of GI5269, GI0122, and CCK_1-R(329−357) were directly incorporated into molecular models of the full ligand−CCK_1-R complex. The NOE-derived intermolecular contacts were used to manually dock GI5269 and GI0122 to the putative ligand-binding pocket. The topological orientation of the ligands and the major binding interactions (≤3.6 Å) are depicted in Figures 3−5. The vast majority of the intermolecular interactions, in both complexes, are located in the upper third of the trans-

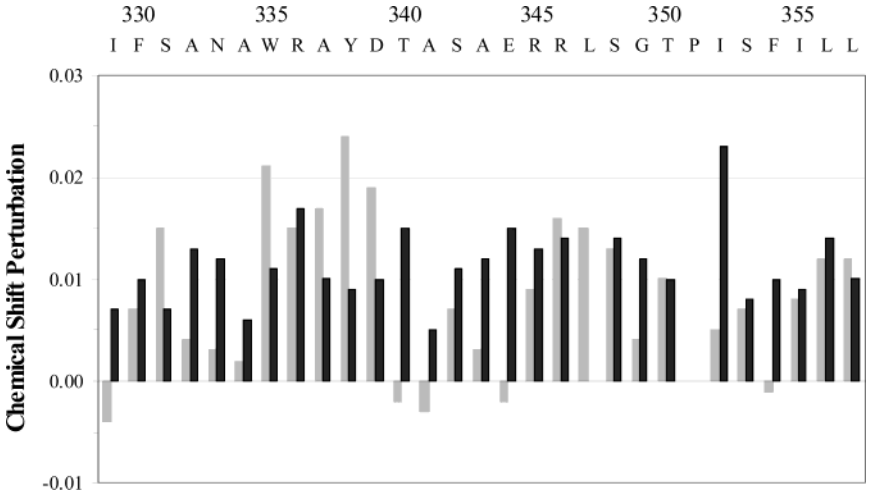


Figure 2. Amide proton chemical shift perturbations upon addition of GI5269 (gray) and GI0122 (black) to CCK_1-R(329−357).

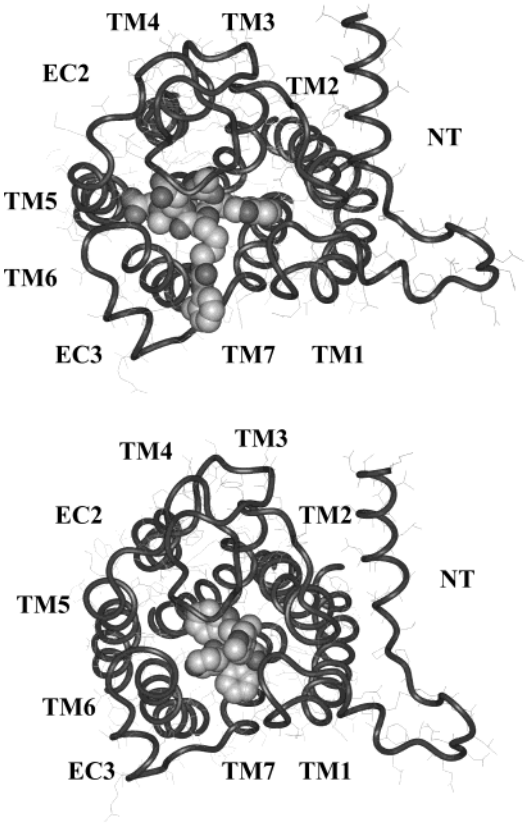


Figure 3. Extracellular face of the GI5269 (top) and GI0122 (bottom) complexes with CCK$_1$-R following 200 ps of restrained MD simulation in a water−decane−water solvent box. The backbone of the receptor and the heavy atoms of the ligands are displayed in ribbon and CPK, respectively.

membrane domains proximal to the extracellular face of the receptor.

The N- and C-termini of GI5269 were in close spatial proximity to TMs 2 and 5, respectively. The side-chain of Nmf[33] resided in a well-defined hydrophobic pocket defined by G122, M173, H210, L213, L214, L217, N333, and A337 at the confluence of TMs 3−6 and the EC2. The C-terminal carboxyamide formed hydrogen bonds with H210. Coulombic interactions were observed between Asp[32] and R336, with additional stabilizing contributions provided by Y119, T192, Q206, and H210. The side chain of Lyu[31] formed van der Waals interactions with TMs 6 (A332, N333, R336), 7 (I352, L355), and the EC3 (E344, S348−P351). The side chain of Trp[30] was positioned nearly in the center of the TM bundle, forming interactions with TMs 1 (Q40, Y48), 3 (T118, M121, G122), 6 (N333), and 7 (I355, L356, S359, Y360).

The benzodiazepine core of GI0122 was located near TMs 1, 6, and 7, producing intermolecular interactions with W39, Q40, R336, I352, I355, and L356. The isopropyl anilinoacetamide was in proximity to residues in TMs 1 (W39, Q44, Y48), 2 (C94, F97, N98, N102), and the EC2 (A193, M195). The indazolylmethyl formed interactions with residues in TMs 3 (T118, Y119, M121, G122) and 6 (F330, N333, S359). The pyridinyl and C-3 methyl substituents were in proximity to residues in TMs 6 (N333, R336, A337) and 7 (I355, L356, S359), respectively.

Discussion

The intermolecular NOEs in the GI5269/CCK$_1$-R(329−357) complex are consistent with those in the CCK-8/CCK$_1$-R(329−357) complex and provide further support for the proposed peptide-ligand binding mode, in which the C-terminus of CCK is located in close spatial proximity to TM 6 of the CCK$_1$-R.[23,24,31,32] NOE-restrained molecular models of the GI5269 and CCK-

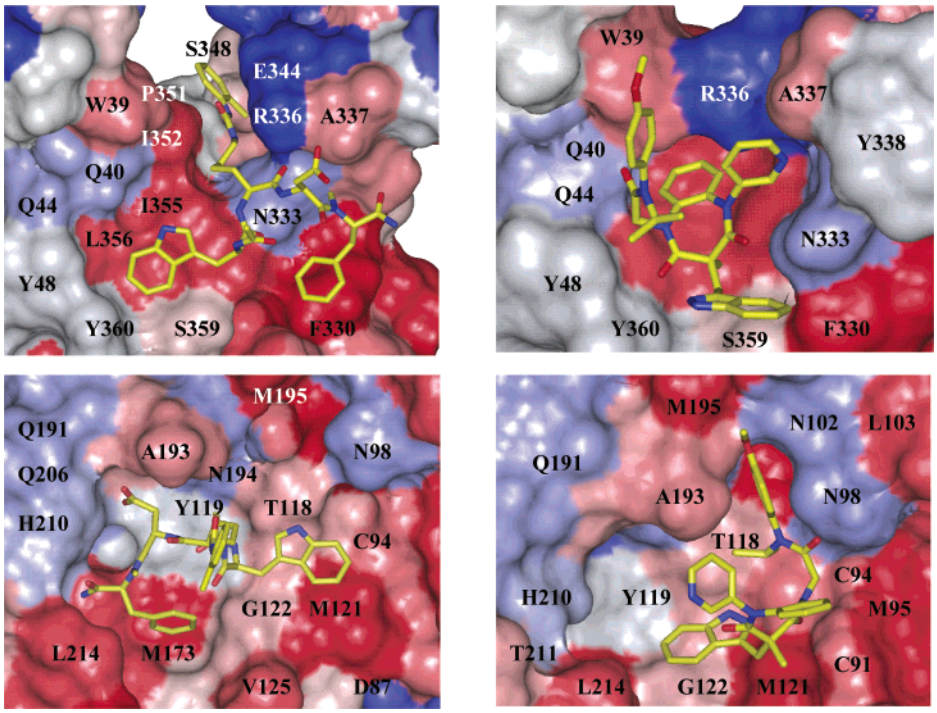


Figure 4. Surface-representation of the extracellular portions of TM domains in the molecular models of the GI5269 (left) and GI0122 (right) complexes with CCK$_1$-R following 200 ps of restrained MD simulation in a water−decane−water solvent box. Amino acids are color coded according to hydrophobicity (blue = polar, red = nonpolar), and the ligands are depicted as yellow sticks.

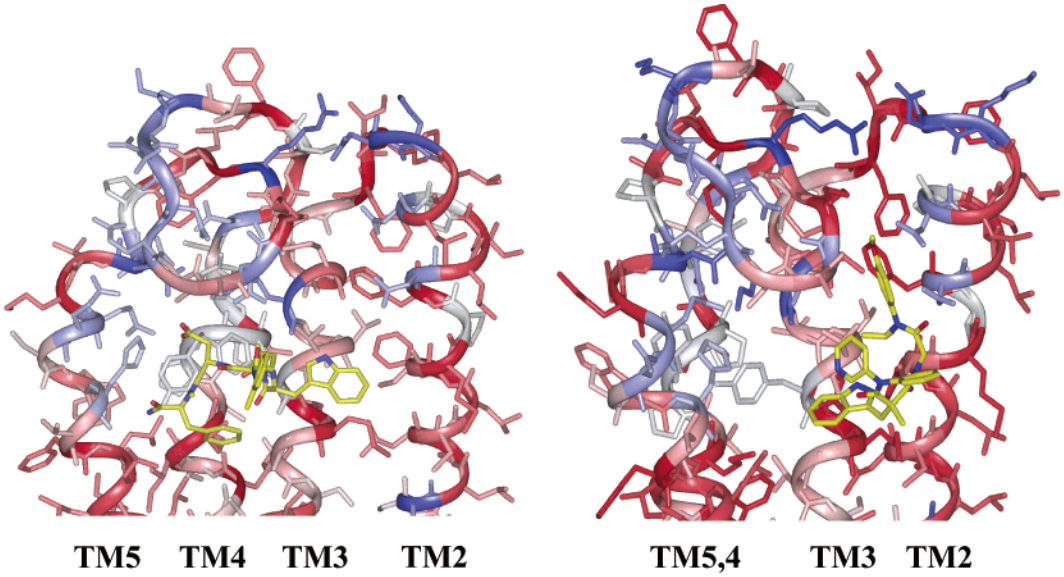


Figure 5. Ribbon-representation of the extracellular portions of TMs 2, 3, 4, and 5 in the molecular models of the GI5269 (left) and GI0122 (right) complexes with CCK_1-R following 200 ps of restrained MD simulation in a water−decane−water solvent box.

8/CCK_1-R complexes, produced nearly the same set of intermolecular contacts for the C-terminal tetrapeptide, though slight deviations were observed in the disposition of the backbone atoms in the ligand-binding pocket. Concurrent with the replacement of Met[31], additional intermolecular interactions were present between Lyu[31] and the EC3 of the CCK_1-R. These interactions may account for the 400-fold enhanced binding affinity of GI5269 as compared to CCK-4.

The proposed binding mode of GI5269 is supported by a number of features in the SAR of **1**.[5−8] These studies suggest a preference for hydrophobic substituents in Lys(NH$^{\epsilon}$CONHR), varying in size from cyclohexane to naphthalene.[7] The length of the linker between the Cα and urea moiety appears to be critical, with four carbons optimal.[7] The presence of a relatively small group in the ortho position of the phenyl urea analogues (CH_3, CF_3, Cl, and Br) also resulted in preferential binding.[5,7] In the molecular model of the GI5269/CCK_1-R complex, the side chain of Lyu[31] is located in a binding groove between the EC3 and TMs 6 and 7 that is terminated by a cluster of hydrophobic residues. The size of this hydrophobic pocket is slightly flexible and is large enough to accommodate both planar and nonplanar substituents. Interestingly, the distance between the hydrophobic pocket and the Cα of Lyu[31] is optimal for four carbons in an extended conformation. Conformational studies of GI5269 indicate a slight out of plane bend in the *o*-methylphenyl urea, that further enhances the interaction of this moiety with the binding groove; however, the hydrophobic pocket cannot accommodate ortho-substituents much greater than methyl. The neutral influence of larger ortho-substituents most likely arises from a 180 degree rotation of the phenyl ring, placing the ortho-substituent on the outer edge of the binding pocket, where it no longer forms contacts with the EC3 of the receptor.

NOE-restrained molecular models of the GI5269 and GI0122/CCK_1-R(329−357) complexes support overlapping binding sites for peptidic and nonpeptidic agonists. Superposition of the backbone atoms in the molecular models of the ligand−receptor complexes revealed common structural features. The 3-pyridinyl and indazolyl moieties interact with many of the same residues of the receptor as the side chains of Asp[32] and Trp[30], respectively, whereas the anilinoacetamide mimics the backbone disposition of the N-terminal residues (Asp[26],Tyr[27]) of CCK-8. The N-substituent of the anilinoacetamide functions as a "trigger", converting analogues of GI0122 from antagonist to agonist.[18] The size of the substituent appears to be critical with *N*-methyl and *N*-ethyl analogues functioning as antagonist and agonist, respectively. In the molecular model of the GI0122/CCK_1-R complex, the *N*-isopropyl substituent is involved in intermolecular interactions with residues in TMs 2 (C94, F97, N98), 3 (T118), and the EC2 (A193). The methine hydrogen of the *N*-isopropyl-*N*-phenylacetamide does not form intermolecular interactions with the receptor. On the basis of these results, intermolecular interactions in TMs 2 and 3 appear to be necessary for inducing agonism in the CCK_1-R. The facile modulation in pharmacology also implies a common binding mode for benzodiazepine-based antagonists and agonists.

Chimera and site-directed mutagenesis studies of GPCRs have established intermolecular contacts between synthetic ligands and the TM7 domain of the A_1 adenosine,[33] neurokinin-1,[34] and α_2 adrenergic receptors,[35] as well as the CCK_1-R and CCK_2-R.[36−39] The proposed binding mode of GI0122 is supported by mutagenesis data for the CCK_1-R selective antagonist L-364,718. Replacement of TM7 in the rat CCK_2-R with the corresponding residues in the CCK_1-R resulted in a 10-fold increase in the binding affinity of L-364,718, without altering the binding affinity of the CCK_2-R selective antagonist L-365,260.[38] Mutagenesis of H381L/H376L in the rat/human CCK_2-R with the corresponding residues in CCK_1-R(L372/L356), resulted in a 13 and 60-fold increase, respectively, in the binding affinity of L-364,718.[36,37] A double mutant V349I/Y350F, swapping residues in TM 6 of the human CCK_2-R with residues in the CCK_1-R(I329,F330), also increased the binding affinity of L-364,718.[37] Each of these residues has been identified in the ligand-binding pocket of the NOE-restrained molecular model of the GI0122/CCK_1-R

complex and is involved in intermolecular interactions with GI0122.

In contrast to sulfated CCK, N-terminal truncation (Δ43)[40] and mutagenesis of R197 in the EC2[41] of the CCK$_1$-R did not significantly alter the binding affinity of L-364,718. However, N333A and R336M mutants decreased the affinity of both CCK and L-364,718.[31] Multiple binding interactions to N333 and R336 have been identified in the molecular model of the GI0122 complex providing additional support for overlapping peptidic and nonpeptidic ligand-binding sites in the upper third of the TM domains proximal to the extracellular face of the receptor.

Conclusion

Direct intermolecular contacts in the synthetic agonist−CCK$_1$-R(329−357) molecular complexes have been discerned in TMs 6 and 7 using NMR. NOE-restrained molecular models of the ligand−receptor complexes support overlapping binding sites for peptidic and nonpeptidic agonists. The binding orientation and intermolecular interactions deduced in the molecular models are consistent with the available SAR for analogues of GI5269 and GI0122 and mutagenesis data for L-364,718. The high correlation in binding contacts established by mutagenesis and NMR for L-364,718 and GI0122, respectively, suggests a common binding mode for benzodiazepine-based agonist and antagonists. Conversion from antagonism to agonism is dependent upon additional intermolecular interactions in the TM domains.

Experimental Section

NMR Methods. A peptide corresponding to the putative EC3 domain of the human CCK$_1$-R, CCK$_1$-R(329−357), was synthesized using Fmoc chemistry (Protein Chemistry Facility at Tufts University, Boston, MA). The N- and C-termini of the peptide were acetylated and amidated, respectively. NMR quantities of the receptor fragment were purified using reverse-phase high-performance liquid chromatography. The receptor fragment (1 mM) was dissolved in 50 mM phosphate buffer (90% H_2O/10% D_2O) with 150 mM DPC-d_{38} (98.6%, Cambridge Isotopes). The pH of the solution was 5.2, excluding corrections for the deuterium isotope effect. The ligands were titrated into the receptor fragment, until the concentrations of the ligand and the receptor fragment were equimolar. NOESY and TOCSY spectra (298−308 K, 50−300 ms, f_2 2048, f_1 640, 8−64 scans) were collected on Bruker Avance 400 and 600 MHz spectrometers in the phase sensitive mode using the method from States.[42] $^3J_{HN\alpha}$ coupling constants were extracted from the absorptive and dispersive cross-peak multiplets observed in phase sensitive DQF COSY spectra (f_2 4096, f_1 512).[43] The spectra were processed using nmrPipe[44] and integrated using Sparky.[45]

Structure Calculations. NOESY cross-peaks were converted to distances using the isolated two-spin approximation and the geminal cross-peak volumes of the Asp^{32} β protons (1.75 Å). $^3J_{N\alpha}$ coupling constants were converted to dihedral angles using the Karplus relation.[46] Amide proton temperature coefficients less than 2.0-ppb/K were considered solvent shielded hydrogen bond donors. The NMR-derived distances and dihedral angles were included as restraints during MD simulations in a water−decane membrane mimetic solvent box with periodic boundary conditions (Gromacs v3.1).[47] Initial simulations were conducted using annealing (800 to 300 K over 50 ps) followed by MD simulations at 300 K for 200 ps. The integration time-step was 1 fs, the neighbor list was updated every 10 fs, and Lennard−Jones and Coulomb potentials were truncated with a polynomial switch function between 1.0 and 1.2 nm. Initial velocities were assigned randomly to each atom from a Maxwellian distribution and scaled to reflect the initial temperature of the system. The temperature was coupled to an external bath using a time constant of 20 fs.[48] Force constants of 2000 kJ mol^{-1} nm^{-2} and 50 kJ mol^{-1} rad^{-1} were applied to the distance and dihedral angle restraints, respectively.

Models of the Ligand−CCK$_1$-R(329−357) Complexes. The experimentally determined structure of CCK$_1$-R(329−357) was template forced to the corresponding TM 6 and 7 residues in the crystal structure of bovine rhodopsin.[24] Following several cycles of energy minimization and template forcing, NOE-restrained MD simulations were performed with annealing (1200 to 300 K, 50 ps). The ligands were docked to CCK$_1$-R(329−357) using the intermolecular NOEs as restraints. An additional electrostatic restraint was added between the carboxylate and guanidine side-chains of Asp^{32} and R336. The backbone atoms in the putative TM domains were restrained to initial positions using a force constant of 200 kJ mol^{-1} nm^{-2}. The average backbone dihedral angles of GI5269 and the intramolecular NOEs of GI0122 were used to restrain the ligands during the docking experiments. Upon satisfying the intra- and intermolecular restraints, MD simulations were performed at 300 K for 500 ps.

Models of the Ligand−CCK$_1$-R Complexes. As described previously,[24] the backbone atoms of CCK$_1$ were template forced to the crystal structure of bovine rhodopsin.[49] The experimentally determined structures of the amino-terminal (1−47) and the third extracellular loop (329−357) domains were incorporated into the molecular model, and the entire receptor was subjected to several cycles of energy minimization and template forcing. Extensive NOE-restrained MD simulations of the CCK-8/CCK$_1$ complex, including several cycles of simulated annealing, were used to generate a model of the ligand binding pocket and to remove inter-helical steric conflicts. After achieving a stable structure for the CCK-8/CCK$_1$-R complex (500 ps, 300 K), CCK-8 was replaced with GI5269 and GI0122. The ligands were manually docked using the intermolecular NOEs and binding orientations deduced from NOE-restrained-MD simulations of the ligand−receptor fragment complex. NOE-restrained MD simulations of the ligand−receptor complex were conducted for 200 ps at 300 K. The structure coordinates for the molecular model of the CCK$_1$-R have been deposited in the Brookhaven Protein Data Bank (filename 1PB2).

References

(1) Noble, F.; Roques, B. P. CCK−B receptor: chemistry, molecular biology, biochemistry and pharmacology. *Prog. Neurobiol.* **1999**, *58*, 349−379.
(2) Wank, S. A. G protein-coupled receptors in gastrointestinal physiology. I. CCK receptors: an exemplary family. *Am. J. Physiol.* **1998**, *274*, G607−613.
(3) Rehfeld, J. F.; van Solinge, W. W. The tumor biology of gastrin and cholecystokinin. *Adv. Cancer Res.* **1994**, *63*, 295−347.
(4) Chang, R. S.; Lotti, V. J.; Monaghan, R. L.; Birnbaum, J.; Stapley, E. O.; Goetz, M. A.; Albers-Schonberg, G.; Patchett, A. A.; Liesch, J. M.; Hensens, O. D.; et al. A potent nonpeptide cholecystokinin antagonist selective for peripheral tissues isolated from Aspergillus alliaceus. *Science* **1985**, *230*, 177−179.
(5) Shiosaki, K.; Lin, C. W.; Kopecka, H.; Bianchi, B.; Miller, T.; Stashko, M.; Witte, D. Minor structural differences in Boc-CCK-4 derivatives dictate affinity and selectivity for CCK-A and CCK−B receptors. *J. Med. Chem.* **1997**, *40*, 1169−1172.
(6) Sugg, E. E.; Kimery, M. J.; Ding, J. M.; Kenakin, D. C.; Miller, L. J.; Queen, K. L.; Rimele, T. J. CCK-A receptor selective antagonists derived from the CCK-A receptor selective tetrapeptide agonist Boc-Trp-Lys(Tac)-Asp-MePhe-NH2 (A-71623). *J. Med. Chem.* **1995**, *38*, 207−211.
(7) Shiosaki, K.; Lin, C. W.; Kopecka, H.; Tufano, M. D.; Bianchi, B. R.; Miller, T. R.; Witte, D. G.; Nadzan, A. M. Boc-CCK-4 derivatives containing side-chain ureas as potent and selective CCK-a receptor agonists. *J. Med. Chem.* **1991**, *34*, 2837−2842.
(8) Lin, C. W.; Shiosaki, K.; Miller, T. R.; Witte, D. G.; Bianchi, B. R.; Wolfram, C. A.; Kopecka, H.; Craig, R.; Wagenaar, F.; Nadzan, A. M. Characterization of two novel cholecystokinin tetrapeptide (30−33) analogues, A-71623 and A-70874, that exhibit high potency and selectivity for cholecystokinin-A receptors. *Mol Pharmacol* **1991**, *39*, 346−351.

(9) Shiosaki, K.; Lin, C. W.; Kopecka, H.; Craig, R.; Wagenaar, F. L.; Bianchi, B.; Miller, T.; Witte, D.; Nadzan, A. M. Development of CCK-tetrapeptide analogues as potent and selective CCK-A receptor agonists. *J. Med. Chem.* **1990**, *33*, 2950−2952.
(10) Bock, M. G.; DiPardo, R. M.; Evans, B. E.; Rittle, K. E.; Veber, D. F.; Freidinger, R. M.; Chang, R. S.; Lotti, V. J. Cholecystokinin antagonists. Synthesis and biological evaluation of 4-substituted 4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepines. *J. Med. Chem.* **1988**, *31*, 176−181.
(11) Evans, B. E.; Rittle, K. E.; Bock, M. G.; DiPardo, R. M.; Freidinger, R. M.; Whitter, W. L.; Lundell, G. F.; Veber, D. F.; Anderson, P. S.; Chang, R. S.; et al. Methods for drug discovery: development of potent, selective, orally effective cholecystokinin antagonists. *J. Med. Chem.* **1988**, *31*, 2235−2246.
(12) Evans, B. E.; Rittle, K. E.; Bock, M. G.; DiPardo, R. M.; Freidinger, R. M.; Whitter, W. L.; Gould, N. P.; Lundell, G. F.; Homnick, C. F.; Veber, D. F.; et al. Design of nonpeptidal ligands for a peptide receptor: cholecystokinin antagonists. *J. Med. Chem.* **1987**, *30*, 1229−1239.
(13) Bock, M. G.; DiPardo, R. M.; Rittle, K. E.; Evans, B. E.; Freidinger, R. M.; Veber, D. F.; Chang, R. S.; Chen, T. B.; Keegan, M. E.; Lotti, V. J. Cholecystokinin antagonists. Synthesis of asperlicin analogues with improved potency and water solubility. *J. Med. Chem.* **1986**, *29*, 1941−1945.
(14) Evans, B. E.; Bock, M. G.; Rittle, K. E.; DiPardo, R. M.; Whitter, W. L.; Veber, D. F.; Anderson, P. S.; Freidinger, R. M. Design of potent, orally effective, nonpeptidal antagonists of the peptide hormone cholecystokinin. *Proc Natl Acad Sci U.S.A.* **1986**, *83*, 4918−4922.
(15) Sherrill, R. G.; Berman, J. M.; Birkemo, L.; Croom, D. K.; Dezube, M.; Ervin, G. N.; Grizzle, M. K.; James, M. K.; Johnson, M. F.; Queen, K. L.; Rimele, T. J.; Vanmiddlesworth, F.; Sugg, E. E. 1,4-Benzodiazepine peripheral cholecystokinin (CCK-A) receptor agonists. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1145−1148.
(16) Willson, T. M.; Henke, B. R.; Momtahen, T. M.; Myers, P. L.; Sugg, E. E.; Unwalla, R. J.; Croom, D. K.; Dougherty, R. W.; Grizzle, M. K.; Johnson, M. F.; Queen, K. L.; Rimele, T. J.; Yingling, J. D.; James, M. K. 3-[2-(*N*-phenylacetamide)]-1,5-benzodiazepines: orally active, binding selective CCK-A agonists. *J. Med. Chem.* **1996**, *39*, 3030−3034.
(17) Henke, B. R.; Willson, T. M.; Sugg, E. E.; Croom, D. K.; Dougherty, R. W., Jr.; Queen, K. L.; Birkemo, L. S.; Ervin, G. N.; Grizzle, M. K.; Johnson, M. F.; James, M. K. 3-(1*H*-indazol-3-ylmethyl)-1,5-benzodiazepines: CCK-A agonists that demonstrate oral activity as satiety agents. *J. Med. Chem.* **1996**, *39*, 2655−2658.
(18) Aquino, C. J.; Armour, D. R.; Berman, J. M.; Birkemo, L. S.; Carr, R. A.; Croom, D. K.; Dezube, M.; Dougherty, R. W., Jr.; Ervin, G. N.; Grizzle, M. K.; Head, J. E.; Hirst, G. C.; James, M. K.; Johnson, M. F.; Miller, L. J.; Queen, K. L.; Rimele, T. J.; Smith, D. N.; Sugg, E. E. Discovery of 1,5-benzodiazepines with peripheral cholecystokinin (CCK-A) receptor agonist activity. 1. Optimization of the agonist "trigger". *J Med Chem* **1996**, *39*, 562−569.
(19) West, A. P., Jr.; Llamas, L. L.; Snow, P. M.; Benzer, S.; Bjorkman, P. J. Crystal structure of the ectodomain of Methuselah, a Drosophila G protein-coupled receptor associated with extended lifespan. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3744−3749.
(20) Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **2000**, *407*, 971−977.
(21) Yeagle, P. L.; Albert, A. D. Use of nuclear magnetic resonance to study the three-dimensional structure of rhodopsin. *Methods Enzymol.* **2002**, *343*, 223−231.
(22) Mierke, D. F.; Giragossian, C. Peptide hormone binding to G-protein-coupled receptors: structural characterization via NMR techniques. *Med. Res. Rev.* **2001**, *21*, 450−471.
(23) Pellegrini, M.; Mierke, D. F. The molecular complex of cholecystokinin-8 and N-terminus of the cholecystokinin A receptor by NMR spectroscopy. *Biochemistry* **1999**, *38*, 14775−14783.
(24) Giragossian, C.; Mierke, D. F. Intermolecular interactions between cholecystokinin-8 and the third extracellular loop of the cholecystokinin A receptor. *Biochemistry* **2001**, *40*, 3804−3809.
(25) Giragossian, C.; Mierke, D. F. Intermolecular interactions between cholecystokinin-8 and the third extracellular loop of the cholecystokinin-2 receptor. *Biochemistry* **2002**, *41*, 4560−4566.
(26) Giragossian, C.; Pellegrini, M.; Mierke, D. F. NMR studies of CCK-8/CCK1 complex support membrane-associated pathway for ligand−receptor interaction. *Can. J. Physiol. Pharmacol.* **2002**, *80*, 383−387.
(27) Bovey, F. A. *High-Resolution NMR of Macromolecules*; Academic Press: New York, 1972.
(28) Kessler, H. Peptide Conformation: Conformation and biological activity of cyclic peptides. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512−523.
(29) Giragossian, C.; Stone, S.; Papini, A. M.; Moroder, L.; Mierke, D. F. Conformational and molecular modeling studies of sulfated cholecystokinin-15. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1053−1059.
(30) Ursini, A.; Capelli, A. M.; Carr, R. A.; Cassara, P.; Corsi, M.; Curcuruto, O.; Curotto, G.; Dal Cin, M.; Davalli, S.; Donati, D.; Feriani, A.; Finch, H.; Finizia, G.; Gaviraghi, G.; Marien, M.; Pentassuglia, G.; Polinelli, S.; Ratti, E.; Reggiani, A.; Tarzia, G.; Tedesco, G.; Tranquillini, M. E.; Trist, D. G.; Van Amsterdam, F. T. Synthesis and SAR of new 5-phenyl-3-ureido-1,5-benzodiazepines as cholecystokinin-B receptor antagonists. *J. Med. Chem.* **2000**, *43*, 3596−3613.
(31) Gigoux, V.; Escrieut, C.; Fehrentz, J. A.; Poirot, S.; Maigret, B.; Moroder, L.; Gully, D.; Martinez, J.; Vaysse, N.; Fourmy, D. Arginine 336 and asparagine 333 of the human cholecystokinin-A receptor binding site interact with the penultimate aspartic acid and the C-terminal amide of cholecystokinin. *J. Biol. Chem.* **1999**, *274*, 20457−20464.
(32) Kennedy, K.; Gigoux, V.; Escrieut, C.; Maigret, B.; Martinez, J.; Moroder, L.; Frehel, D.; Gully, D.; Vaysse, N.; Fourmy, D. Identification of two amino acids of the human cholecystokinin-A receptor that interact with the N-terminal moiety of cholecystokinin. *J. Biol. Chem.* **1997**, *272*, 2920−2926.
(33) Townsend-Nicholson, A.; Schofield, P. R. A threonine residue in the seventh transmembrane domain of the human A1 adenosine receptor mediates specific agonist binding. *J. Biol. Chem.* **1994**, *269*, 2373−2376.
(34) Sachais, B. S.; Snider, R. M.; Lowe, J. A., 3rd; Krause, J. E. Molecular basis for the species selectivity of the substance P antagonist CP-96,345. *J. Biol. Chem.* **1993**, *268*, 2319−2323.
(35) Suryanarayana, S.; Daunt, D. A.; Von Zastrow, M.; Kobilka, B. K. A point mutation in the seventh hydrophobic domain of the alpha 2 adrenergic receptor increases its affinity for a family of beta receptor antagonists. *J. Biol. Chem.* **1991**, *266*, 15488−15492.
(36) Jagerschmidt, A.; Guillaume-Rousselet, N.; Vikland, M. L.; Goudreau, N.; Maigret, B.; Roques, B. P. His381 of the rat CCKB receptor is essential for CCKB versus CCKA receptor antagonist selectivity. *Eur. J. Pharmacol.* **1996**, *296*, 97−106.
(37) Kopin, A. S.; McBride, E. W.; Quinn, S. M.; Kolakowski, L. F., Jr.; Beinborn, M. The role of the cholecystokinin-B/gastrin receptor transmembrane domains in determining affinity for subtype-selective ligands. *J. Biol. Chem.* **1995**, *270*, 5019−5023.
(38) Mantamadiotis, T.; Baldwin, G. S. The seventh transmembrane domain of gastrin/CCK receptors contributes to non-peptide antagonist binding. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1382−1389.
(39) Beinborn, M.; Lee, Y. M.; McBride, E. W.; Quinn, S. M.; Kopin, A. S. A single amino acid of the cholecystokinin-B/gastrin receptor determines specificity for non-peptide antagonists. *Nature* **1993**, *362*, 348−350.
(40) Kennedy, K.; Escrieut, C.; Dufresne, M.; Clerc, P.; Vaysse, N.; Fourmy, D. Identification of a region of the N-terminal of the human CCKA receptor essential for the high affinity interaction with agonist CCK. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 845−852.
(41) Gigoux, V.; Maigret, B.; Escrieut, C.; Silvente-Poirot, S.; Bouisson, M.; Fehrentz, J. A.; Moroder, L.; Gully, D.; Martinez, J.; Vaysse, N.; Fourmy, A. D. Arginine 197 of the cholecystokinin-A receptor binding site interacts with the sulfate of the peptide agonist cholecystokinin. *Protein Sci.* **1999**, *8*, 2347−2354.
(42) States, D. J.; Haberkorn, R. A.; Ruben, D. J. A Two-Dimensional Nuclear Overhauser Experiment with Pure Absorption Phase in Four Quadrants. *J. Magn. Reson.* **1982**, *48*, 286−292.
(43) Kim, Y.; Prestegard, J. H. Measurement of Vicinal Couplings from Cross-Peaks in COSY Spectra. *J. Magn. Reson.* **1989**, *84*, 9−13.
(44) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **1995**, *6*, 277−293.
(45) Goddard, T. D.; Kneller, D. G. Sparky 3.0. University of California, San Francisco, 2001.
(46) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley: New York, 1986.
(47) Lindahl, E.; Hess, B.; van der Spoel, D. GROMACS 3.0: A package for molecular simulation and trajectory analysis. *J. Mol. Mod.* **2001**, *7*, 306−317.
(48) Berendsen, H. J. C.; Postma, J. P. M.; DiNola, A.; Haak, J. R. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* **1984**, *81*, 3684−3690.
(49) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **2000**, *289*, 739−745.

JM030144Z