Anthranilic Acid Based CCK_1 Receptor Antagonists and CCK-8 Have a Common Step in Their "Receptor Desmodynamic Processes"

Stefania De Luca,[†] Michele Saviano,[†] Lucia Lassiani,[‡] Konstantina Yannakopoulou,[§] Penny Stefanidou,[§] Luigi Aloj,[†] Giancarlo Morelli,*[,†] and Antonio Varnavas*[,‡]

Interuniversity Research Center on Bioactive Peptides (CIRPeB), University of Naples "Federico II", and Institute of Biostructures and Bioimaging of CNR, Via Mezzocannone, 16 I-80134 Naples, Italy, Department of Pharmaceutical Sciences, University of Trieste, P.le Europa 1, 34127 Trieste, Italy, and Institute of Physical Chemistry, NCSR "Demokritos" Ag. Paraskevi 15310 Athens, Greece

Received October 18, 2005

The interaction between the 1−47 N-terminus of the CCK_1-R and the anthranilic acid based antagonists has been investigated by fluorescence spectroscopy. These antagonists interact with W39 of the N-terminal domain of the CCK_1-R like that of the endogenous ligand CCK-8. This specific interaction was not found in other nonpeptide ligands of the CCK_1-R. Conformational studies, using NMR and energy minimization procedures, have allowed formulation of a new hypothesis on the CCK_1-R binding mode of the anthranilic antagonists.

Introduction

Cholecystokinin (CCK) is an endogenous gut−brain peptide hormone used as a messenger from the endocrine and nervous system.[1] The hormonal effects of CCK in the gastrointestinal tract are associated to gallbladder contraction and pancreatic secretion and are mediated by a receptor subtype known as CCK_A or CCK_1 (abbreviated CCK_1-R). In the central nervous system CCK acts as neurotransmitter /neuromodulator and seems to be involved in different neuro-dysfunctions through activation of another receptor subtype known as CCK_B or CCK_2 (abbreviated CCK_2-R). Both CCK receptors, which belong to the G-protein coupled receptors (GPCRs) superfamily, bind the C-terminal octapeptide fragment of CCK (CCK-8) with nanomolar affinity.

During the past few years, increasing efforts have been directed toward developing selective peptide or nonpeptide CCK mimetics with agonist or antagonist activity. The application of different design strategies had led to several classes of CCK receptor antagonists.[2,3] Structurally, these active compounds present large dissimilarities that could be considered responsible for the different pharmacokinetic (ADME: absorption, distribution, metabolism, excretion) and pharmacodynamic (affinity, selectivity, and receptor recognition approach) properties and for more or less complexity in their synthesis.

Recently, we described the discovery of an innovative class of CCK_1 receptor ligands, characterized by the presence of anthranilic acid, used as a molecular scaffold, and of two pharmacophores selected from the C-terminal tetrapeptide of CCK. The lead compound VL-0395 (Figure 1), endowed with sub-micromolar affinity ($IC_{50} = 0.197 \pm 0.107$ μM) toward CCK_1-R, was characterized by the presence of Phe and 2-indole moiety at the C- and N-termini of anthranilic acid, respectively.[4] The antagonist nature of VL-0395 was confirmed by an in vivo functional test. Its potency was comparable with that exhibited by the reference CCK_1 selective antagonist loxiglumide (Figure 1) under the same experimental condition.[4]

At the same time, since high-resolution structural studies by X-ray crystallography were not possible, several other methods (including site-directed mutagenesis, photoaffinity labeling, and NMR (nuclear magnetic resonance) spectroscopy) have been employed in order to investigate the CCK-8/CCK_1-R recognition approach.[5,6]

These studies have revealed specific residues in both CCK-8 and CCK_1-R which are involved in the peptide−protein recognition interactions. In particular, site directed mutagenesis experiments have revealed that the CCK_1-R binding site is composed of amino acids both from extracellular loops and transmembrane helices (TM) (TM III, V, VI, and VII).[7,8] Similar studies employing site directed mutagenesis, molecular modeling, and SAR or a combination of them showed that small nonpeptide CCK_1-R ligands and CCK-8 shared common binding sites.[9,10]

However, the entire and precise molecular determinants of receptor recognition approach remain not fully characterized since these interactions cannot be directly observed, even by NMR spectroscopy, due to the difficulty of working with full-length CCK_1-R.

Thus, to circumvent this problem, NMR spectroscopy has been employed to reconstruct the CCK-8/CCK_1-R recognition interface using a series of synthetic peptides derived from the extracellular portions of CCK_1 receptors. Using this technique, intermolecular contacts between CCK-8 and CCK_1-R fragments corresponding to the N-terminal domain of the human CCK_1-R(1−47) and the third extracellular loop domain have been investigated, and new details on the CCK-8/CCK_1-R binding sites were obtained.[11,12] The results of this study show that amino acid residues belonging to the extracellular N-terminal domain of CCK_1-R are involved in the interaction with CCK-8, and therefore the CCK_1-R(1−47) fragment, constituted by the entire extracellular N-terminal domain and a few residues of the first transmembrane helix (TM1), could be considered a valid receptor system model to investigate the binding of new receptor ligands.

In this regard we have recently developed a CCK-8 cyclic analogue which has been designed on the basis of the NMR structure of the biomolecular complex between the N-terminal fragment of CCK_1-R and its natural CCK-8 ligand. The interaction between the CCK_1-R(1−47) extracellular fragment

* Corresponding authors. For G. M.: phone, +39 081 2536650; fax, +39 081 2534574; e-mail, morelli@chemistry.unina.it. For A. V.: phone, +39 040 558 7861; fax, +39 040 52572; e-mail, varnavas@units.it.

[†] University of Naples "Federico II" and Institute of Biostructures and Bioimaging of CNR.

[‡] University of Trieste.

[§] Institute of Physical Chemistry.

10.1021/jm051050n CCC: $33.50
Published on Web 03/24/2006

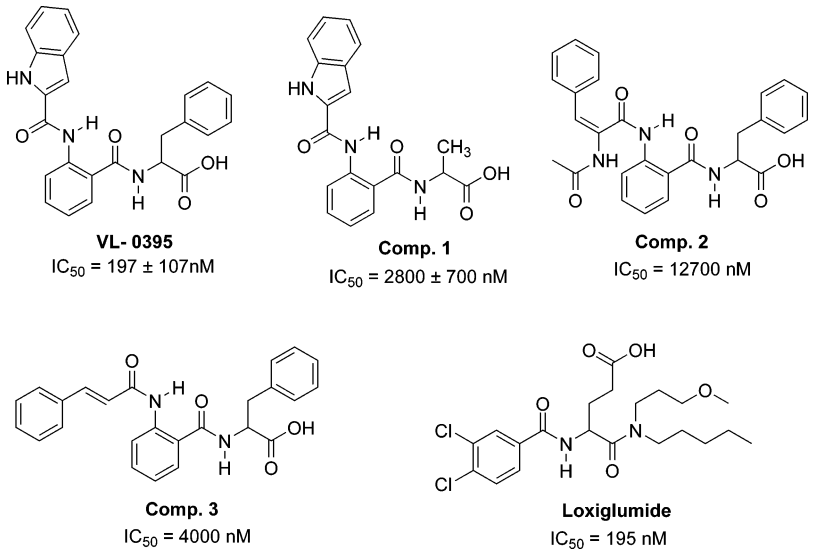

Figure 1. Chemical structures of the investigated compounds and their binding parameters (IC_{50} values) to CCK_1-R.

and either the endogenous CCK-8 peptide or the newly developed CCK-8 cyclic analogue has been studied by fluorescence spectroscopy, a suitable technique to investigate the protein fragment/ligand binding in the receptor fragment approach.[13]

On this basis and in order to investigate the possibility that the CCK_1-R N-terminal interaction observed in the CCK-8/CCK_1-R recognition pathway may be a common step even for selective and competitive CCK_1-R antagonists, we decided to perform an analogous fluorescence study using the peptide CCK_1-R(1−47) as a receptor system model. In the present work we tested the recently described anthranilic acid based antagonists, for which has been hypothesized a bioactive organization similar to that of CCK C-terminal sequence, taking as a reference compound the well-known and structurally different CCK_1-R antagonist loxiglumide. In addition, to provide a preliminary model of the interaction between the anthranilic acid based antagonists and CCK_1-R, conformational studies by NMR techniques and energy minimization procedures have been performed. The chemical structures of the investigated derivatives and their binding parameters (IC_{50} values) are shown in Figure 1.

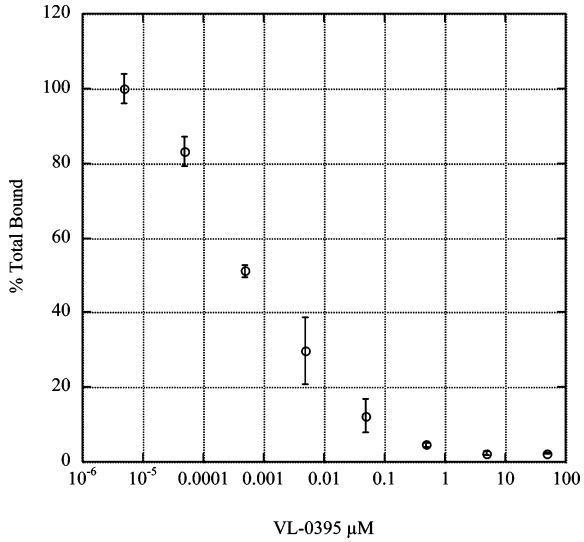

Figure 2. Displacement of ^{111}In-DTPAGlu-Gly-[Tyr^{27}(SO_3)]-CCK8 on A431 cells transfected with a plasmid containing the cDNA for the human CCK_1 receptor with VL-0395 at concentrations ranging from 10^{-10} to 10^{-3} M.

Results

Biological Assay. Since the reported IC_{50} value of the lead compound VL-0395 was obtained employing rat pancreatic acini preparations, additional competition binding experiments were carried out in order to estimate its binding specificity toward human CCK_1-R. Fixed tracer amounts of the ^{111}In-labeled peptide DTPAGlu-Gly-[Tyr^{27}(SO_3)]-CCK8 (DTPAGlu = *N*,*N*-bis[carboxyethyl)amino]ethyl]-L-glutamic acid)[14] were incubated with the receptor expressing cells in the presence of VL-0395 at concentrations ranging from 10^{-10} to 10^{-3} M. Measurements of the bound radioactivity amount are shown in Figure 2.

These data show a typical pattern of competitive interaction with reduction in binding of the radiolabeled tracer at low concentration of the unlabeled VL-0395. Nonlinear regression analysis using a model for homologous competition binding allowed us to derive the 50% inhibitory concentration (IC_{50}) for the anthranilic acid derivative; this value is in the range of 0.1−1 nM.

Fluorescence Spectroscopy. By analogy to the previously studied interactions between CCK_1-R(1−47) and peptidic ligands, i.e., the natural substrate CCK-8 and the analogue Cyclo^{29,34}[Dpr.^{29}Lys^{34}]-CCK-8, the interaction between the protein fragment CCK_1-R(1−47) and the anthranilic acid derivatives was studied by monitoring selectively the fluorescence variation of the Trp residue. Thus, fluorescence was excited at 295 nm where it can be assumed that the emission spectra originate only from the Trp of the CCK_1-R(1−47) fragment, since in this emission region the fluorescence spectrum of the each anthranilic ligand is negligible.[13]

Binding experiments were performed according to the limiting reagent method. Namely, increasing amounts of anthranilic ligand were added to the peptide CCK_1-R(1−47) in the presence of micellar concentrations of sodium dodecyl sulfate (8 mM) that create a membrane-like environment. In fact, at the ionic strength of the titration experiments (0.01 M), it has been found that micellar aggregation of SDS, occurring at concentration close to 5 mM, overlaps with secondary and tertiary structural rearrangement of CCK_1-R(1−47). It has been then inferred that the detergent binds to the peptide CCK_1-R(1−47) in micellar form, thus mimicking the membrane-like environment.

Figure 3 (left) shows typical saturation experiments performed for the anthranilic acid derivatives under the conditions described

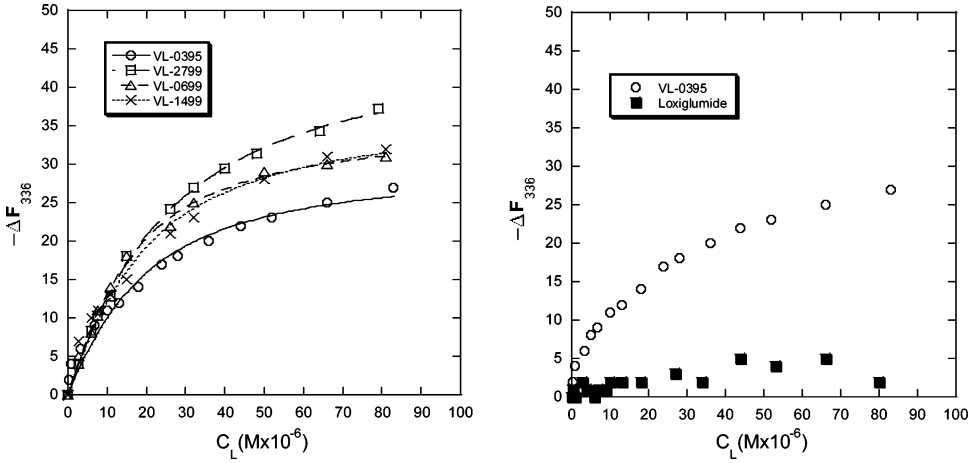

Figure 3. (Left) Binding of the anthranilic ligands to CCK_1-R(1−47). Compounds 1, 2, and 3 are indicated as VL-2799, VL-0699, and VL-1499, respectively. The receptor concentration was 10.6 μM in 10 mM phosphate buffer, pH = 7.1, as judged by absorbance at 280 nm. A certain volume of SDS was added from a 400 mM mother solution dissolved in the same buffer to a final concentration of about 8 mM SDS. Increasing amounts of the different anthranilic ligands were added from concentrated buffer solutions containing 10% DMSO and 8 mM SDS. The ordinate in all cases represents the residual fluorescence signal at 336 nm after subtraction of the individual contributions of CCK_1-R(1−47) and anthranilic ligand. The hyperbolic trends for each ligand reflect binding, with a dissociation constant in the range of 8−23 μM. (Right) Titration of CCK_1-R(1−47) with VL-0395 and loxiglumide. Fluorescence modification, upon titration of CCK_1-R(1−47), was monitored under the same experimental conditions described for Figure 3, left. The fluorescence signal does not change for loxiglumide, suggesting that binding does not take place.

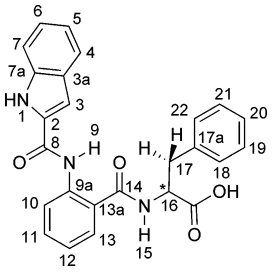

Figure 4. Numbering scheme of VL-0395.

above. The titration of CCK_1-R(1−47) with each ligand is characterized by a fluorescence signal quenched on binding, and therefore a negative fluorescence modification, after subtraction of the separate contributions of CCK_1-R(1−47) and anthranilic ligand from the total fluorescence signal, was observed. Binding curves, obtained using the receptor fragment at 10^{-5} M concentration, were independent of the emission wavelength chosen to perform the calculations. The apparent dissociation constant for each anthranilic ligand, K_d, was estimated to be in the range of 8−23 μM.

Using the same fluorescence method we investigate the binding of the known selective nonpeptide antagonist loxiglumide to CCK_1-R(1−47). The structure of this molecule is unrelated to the structures of the anthranilic-type antagonists, although it has shown the same IC_{50} as the lead compound VL-0395 (VL-0395: $IC_{50} = 197 \pm 107$ nM;[4] loxiglumide: $IC_{50} = 195$ nM[15]).

Therefore, a loxiglumide fluorescence titration was performed under the same conditions described above for the anthranilic acid derivatives. The results of a typical experiment are shown in Figure 3 (right). After subtraction of the individual signals of the two noninteracting species, no hyperbolic modification of the residual fluorescence has been detected. This indicates that binding between loxiglumide and CCK_1-R(1−47) is absent.

Conformational Analysis. NMR spectroscopy in 90% H_2O−10% D_2O and in DMSO-d_6 was used to probe the conformational behavior of the sodium salt of VL-0395. The atom numbering is shown in Figure 4. The proton chemical shifts and the assignments of ^{1}H NMR spectra are listed in Table 1, and the NOE interactions observed are summarized in Table 2.

Table 1. Chemical Shifts and Assignments of the ^{1}H NMR Spectra of VL-0395 at 298 K

no.	δ^1_H (H_2O)[a]	δ^1_H (DMSO)	no.	δ^1_H (H_2O)[a]	δ^1_H (DMSO)
1 (NH)	10.81 (br s)	11.91 (s)	12	7.047 (t)	∼7.25[b]
3	6.726 (s)	6.93 (s)	13	7.366	7.75 (d)
4	7.552 (d)	7.70 (d)	15 (NH)	8.25 (d)	9.08 (d)
5	6.987 (t)	7.15	16	4.680[c]	4.75 (m)
6	7.152 (t)	∼7.25[b]	17a	3.055 (d)	3.34 (dd)
7	7.350	7.46(d)	17b	2.582 (dd)	3.18 (dd)
9 (NH)	10.08 (br s)	12.21 (s)	18, 22	6.884 (t)	7.33 (d)
10	7.654 (d)	8.62 (d)	19, 21	6.667 (t)	7.25 (m)[b]
11	7.309	7.55			

[a] 90% H_2O−10% D_2O, pH 5. [b] Mutual overlapping. [c] Under the water peak.

It was found that the protons NH1 and NH9 exchange rapidly with H_2O (signals disappear in D_2O) and do not show NOE with any of the other protons (except for NH9 with H3 at 283 K) and therefore can be regarded as "free" in the sense that they interact primarily with the solvent.

On the contrary, NH15 displays NOE interactions with several protons of the molecule and therefore can be regarded as hydrogen bonded intramolecularly. The most enlightening interactions listed in Table 2 are those denoting proximity of NH15 with H13 and 17a,b, of H13 with H17a,b, and of NH9 with H3.

These interactions indicate that the molecule actually forms a twist on each side of the anthranilic core. Similar interactions are observed in DMSO-d_6, in which, however, the order of appearance of the NH protons is different in that NH9 is more deshielded than NH1 (Table 2).

Since there are no structural data on the VL-0395/CCK_1-R complex, the NMR structure of the complexes between CCK-8 and the CCK_1-R fragment 1−47 [CCK-8/ CCK_1-R(1−47)] proposed by Mierke et al.[6] was used as a starting point to clarify the CCK_1-R affinity toward VL-0395. In particular, attention was focused on CCK-8 conformation as derived from NMR data in DMSO for the CCK-8/ CCK_1-R(1−47) model.

The model of VL-0395 was obtained using the NOE distances derived from NMR data as restraints in a restrained energy minimization procedure.

In particular, the analysis of the spatial arrangement of the main pharmacophoric groups, i.e., the indole moiety and phenyl

Table 2. NOE Interactions Observed in H_2O[a] and in DMSO-d_6[a]

H_2O[a]			DMSO-d_6[a]		
strong	medium	weak	strong	medium	weak
NH15/H13	NH15/H17b	H17a/H18,22	NH15/H13	NH15/H17b	NH15/H17a[b]
H17b/H18,22	H18, 22/17b	NH15/H17a[d]	NH9/H3	H17b/H18,22	NH9/H10
H10/H11[c]	H13/17a,b	NH9/H3[d]	NH15/H18,22		
	H4/H3	NH15/H18,22 (at 283K)			

[a] Classified as strong (2.5−3.0 Å), medium (3.0−4.0 Å), weak interactions (4−5 Å). [b] Weak to medium. [c] Similarly observed for H13/H12, H5/H4, H19,21/H18,22, H6/H5. [d] Medium at 283 K.

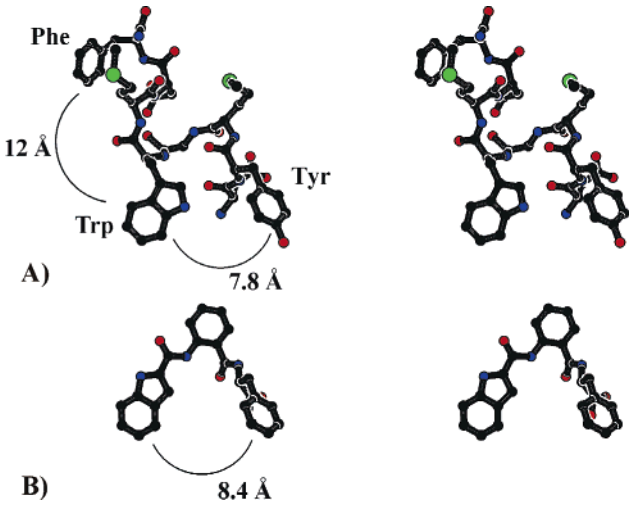

Figure 5. Stereo drawings of (A) CCK-8 and (B) VL-0395 as obtained from NMR data. The centroid distances between pharmacophoric groups are reported.

ring of Phe, shows that the mean distance between the centroid of the indole group and the phenyl ring is 8.46 Å (Figure 5B). A similar analysis carried out on the CCK-8 NMR structure shows that the distances between the centroid of the Tyr^{27} phenyl ring and of the Trp^{30} indole ring, and between centroid of the Trp^{30} indole ring and of the Phe^{33} phenyl ring, are 7.8 Å and 12.0 Å (Figure 5A), respectively.

Discussion

Currently, the receptor−ligand recognition is generally treated as a process rather than a fixed "lock and key" interaction or as the static structure derived from X-ray crystallography, both being far from that observed under physiological conditions. In fact, one important feature that is currently accepted is the dynamic nature of the receptors. Therefore, considering that the ligand−receptor "recognition approach" is a multistep and dynamic process involving interactions through noncovalent bonds, we prefer to use the new and more appropriate terminology of the "receptor desmodynamic process" (from Greek *δεσμός*: relation; bond). In particular, the desmodynamic process should describe the sequence of all the interactions between the ligand and the receptor, including those energetically instable (transient), that jolt the ligand into its own final and energetically more stable complex with the receptor. In this context, we can reasonably hypothesise that different ligands capable of binding the same receptor should be characterized by desmodynamic pathways differing by the number and the type of interactions, by the global energy minimum of the ligand−receptor complex, and by the final receptor conformation which may be able or not to engage the signal transduction machinery. Clearly, the differences of the desmodynamic pathways depend on the structural features of the ligands including the number and the nature of functional groups, the shape, and the type of the interfaces created in the formation of the ligand−receptor complex.

Taking into account the above considerations, we have good reason to think that, in the case of the CCK$_1$-R, every type of ligand including the endogenous peptide CCK-8 has their own desmodynamic pathway since they differ in chemical structure, shape, size, and number of functional groups.

Nevertheless, the results of the investigation reported here revealed that the anthranilic type CCK$_1$-R antagonists and the endogenous ligand CCK-8 have a common step in their desmodynamic processes. Indeed, these results show that, contrary to loxiglumide, the compound VL-0395 and all the other anthranilic acid derivatives studied, are capable of interacting with Trp^{39} (W39) of the N-terminal extracellular domain of human CCK$_1$-R as previously observed for the endogenous ligand CCK-8.[6]

A possible explanation could involve a recognition motif of CCK-8 that is, in structural terms, very similar to that of the VL-0395 ligand. In this perspective, we undertake a conformational analysis study comparing the spatial arrangement of the main pharmacophoric groups of the VL-0395 with similar recognition motifs of CCK-8.

On the basis of the conformational analysis results, a new receptor binding hypothesis, different to that previously reported for the anthranilic derivatives can be provided. In fact, the CCK$_1$-R affinity of VL-0395 was ascribed to the conformational ability of the anthranilic acid to keep the main pharmacophoric groups (indole moiety and phenyl ring of Phe) in the correct spatial orientation mimicking therefore the regnylogical bioactive organization of the C-terminal tetrapeptidic sequence of CCK. On this basis, the proposed receptor binding model involves at least two hydrophobic pockets in order to accommodate the main pharmacophoric components shared by VL-0395 in the final step of its desmodynamic process.[4]

Here, since the distance in VL-0395 between the two hydrophobic groups (indole and Phe aromatic ring) is much more similar to that found in CCK-8 between the Tyr^{27} phenyl ring and Trp^{30} indole ring, rather than that found between the CCK-8 Trp^{30} indole ring and Phe^{33} phenyl ring, seems reasonable to consider the pharmacophoric system of VL-0395 as a much more faithful mimic of the Tyr^{27}-Trp^{30} regnylogic type assembly of CCK-8.

Thus, using these findings and the NMR structure of CCK-8/ CCK$_1$-R(1−47) complex, it can be proposed that Phe side chain of VL-0395 interacts with the W39 residue of the CCK$_1$-R extracellular N-terminal domain, while the 2-indole ring may be responsible of the interaction with the CCK$_1$-R(329−357) third extracellular loop, as found in the NMR structure of CCK-8/CCK$_1$-R(329−357) complex.[11]

This new hypothesis is supported by accumulated data including SAR study on the anthranilic derivatives. Indeed, this model also accounts for the SAR observation that every modification of the indole ring of VL-0395 has a concomitant high negative effect on binding affinity. Moreover, since the 2-indole group is the unique moiety able to establish very specific interactions with the receptor we have hypothesized

that it may be viewed as a "needle" type pharmacophoric group in this new class of CCK_1 receptor antagonists.[16]

Here, despite the structural diversity between the indole and the other N-terminal groups of the anthranilic derivatives, the K_i values obtained by fluorescence spectroscopy are comparable. This opposite behavior of the same compounds on these two different CCK_1-R models (i.e full-length CCK_1-R and the CCK_1-R(1−47) fragment) suggest that the interaction with W39 is not due to the N-terminal moieties of the anthranilic acid derivatives but probably to the second pharmacophoric group of the molecules (amino acid side chain).

On the other hand, compound **1** (Figure 1) bearing a different amino acid side chain (methyl group instead of the benzyl moiety of Phe) showed a K_i value almost comparable to that of VL-0395 employing the CCK_1-R(1−47) model. This fact could be mainly due to the type of interaction with W39 by these two different side chains. Clearly, although the benzyl group could interact in various modes with W39 including its π-system, in this case it probably adopts the classic edge-to-face (T-shaped) interaction, similar to the CH−π monotropic type interaction of the methyl group, rather than the parallel-displaced or face-to-face (stacking) arrangement.[17]

This hypothesis is supported by previous SAR study regarding the importance of the Phe phenyl ring of VL-0395 showing that the π-system and therefore the electrostatic potential of the aromatic ring is irrelevant for the receptor recognition.[18] In this regard, a recent study of Wilcox and co-workers highlights how no electronic effect was involved in edge-to-face interactions, suggesting in contrast an important role of London dispersion forces.[19]

Further examination of the K_i values obtained for VL-0395 and for the other anthranilic derivatives, according to the "three ligand concept"[20] and therefore on thermodynamic basis, suggests that these compounds have at least two "touch points" with the CCK_1-R(1−47) fragment. In fact, on a thermodynamic basis, a ligand−receptor affinity on the order of 10^{-9} M, corresponds, through the Gibbs−Helmholtz equation, to binding energy of about 12.3 kcal/mol at 298 K. This energy, according to the "three ligand concept" could be easily achieved by three ligand or receptor-binding groups, assuming that formation of a single ionic bond releases 5 kcal/mol while hydrophobic interactions release about 1 kcal per methylene group. Thus, the micromolar range binding costants (K_i) obtained here by the fluorescence study correspond to a range of binding energy of about 6.4−6.9 kcal/mol, suggesting that besides to the edge-to-face type interaction between the amino acid side chain and the W39 residue of the CCK_1-R(1−47) should be an additional contact with the receptor. This interaction has been detected by the conformational analysis study and involves the free carboxy group of the anthranilic derivatives and the Q40 side chain of the CCK_1-R(1−47). Additional data supporting the importance of the free carboxy group and its contribution to receptor affinity was derived by the SAR study of VL-0395. Indeed, any modification of the free carboxy group was associated with a dramatic drop of the receptor affinity (unpublished results). Hence, we think that the micromolar range binding costants (K_i) observed here correspond to the sum of the binding energies of the individual interactions established by the amino acid side chain and the free carboxy group.

To summarize, this proposed model, supported by fluorescence and classic SAR studies, offers an insight into the CCK_1-R desmodynamic process of VL-0395 and of the other anthranilic derivatives. In fact, although it is not possible to work with the full length receptor, use of the single receptor fragment CCK_1-R(1−47) model demonstrates that the anthranilic derivatives interact with the extracellular N-terminal domain of the receptor, which represents an important region of the CCK_1-R that binds the N-terminal sequence of CCK. This specific interaction was not found in other potent and selective nonpeptide ligands of the CCK_1-R. On the contrary, as recently reported, docking nonpeptide antagonists such as SR-27897 and devazepide into a new and more accurate model of human CCK_1-R showed that these compounds most likely occupy a region of CCK_1-R that interacts with the C-terminal tripeptide of CCK.[9]

Conclusions

In this report we have described binding studies performed by fluorescence spectroscopy using the receptor fragment CCK_1-R(1−47) as receptor model. We have investigated on the binding affinity of the lead compound VL-0395 as well as of other anthranilic acid derivatives which are characterized by different substituents on the C- and N-terminal site of the molecular scaffold. The changes in fluorescence spectra that have been observed upon titration of the receptor fragment CCK_1-R(1−47) with the investigated compounds revealed their interaction with W39 of CCK_1-R(1−47) fragment. A negative control has been obtained employing the equipotent glutamic acid derivative "loxiglumide" as reference compound.

These results together with conformational studies, performed by using NMR data and energy minimization procedures, have allowed the formulation of a new hypothesis on the CCK_1-R binding mode of the anthranilic antagonists. Nevertheless, although these findings undoubtedly contribute to a better understanding of the CCK_1-R recognition mechanism and will help in the design of new lead compounds with enhanced properties further structural studies, with different CCK_1-R fragments, are necessary in order to validate the formulated hypothesis on CCK_1-R/ VL-0395 desmodynamic pathway. Hopefully in the future, application of time-resolved biophysical techniques will permit the study of rapid dynamic changes in the GPCR structure enabling a better and easier characterization of the ligand−receptor desmodynamic processes.

Experimental Section

Materials. The 47 residue peptide CCK_1-R(1−47), having the following sequence: MDVVDSLLVNGSNITPPCELGLENETLF-CLDQPRPSKEWQPAVQILL, and the anthranilic compounds 2(*R,S*)-{2-[(1*H*-indole-2-carbonyl)amino]benzoylamino}-3-phenylpropionic acid (VL-0395), 2(*R,S*)-{2-[(1*H*-indole-2-carbonyl)amino]-benzoylamino}propionic acid (compound **1**), 3-phenyl-2(*R,S*)-[2-(cinnamoylamino)benzoylamino]propionic acid (compound **3**), and 2(*R,S*)-{2-[(2-acetylaminocinnamoyl)amino]benzoylamino}-3-phenylpropionic acid (compound **2**), were synthesized in our laboratory as previously reported.[4,13,16] Loxiglumide was kindly donated by Rotta Research Laboratorium (Monza, Italy), and the conjugate DTPAGlu-Gly-[$Tyr^{27}(SO_3)$]-CCK8 was purchased from INBIOS (Pozzuoli, Italy).

Biological Measurements. Competition binding experiments were performed on A431 cells that had been stably transfected with a plasmid containing the cDNA for the human CCK_1 receptor. DTPAGlu-Gly-[$Tyr^{27}(SO_3)$]-CCK8 was labeled with ^{111}In as previously described.[14] Fixed tracer amounts of the ^{111}In-labeled peptide DTPAGlu-Gly-[$Tyr^{27}(SO_3)$]-CCK8 were incubated with the receptor expressing cells in the presence of VL-0395 at concentrations ranging from 10^{-10} to 10^{-3} M. The amount of bound radioactivity was determined after 1 h at 4 °C. Nonlinear regression analysis using a model for homologous competition binding was performed by using the GraphPad Prism program (version 3.0a for Macintosh; GraphPad Software, san Diego, Ca) to calculate the IC_{50}.

Absorption Spectroscopy. Concentrations of all solutions were determined by absorbance measurements on a Jasco Model V-550

spectrophotometer. For the peptide CCK$_1$-R(1−47) a molar absorptivity (ϵ_{280}) of 5630 M^{-1} cm^{-1} was used, taking into account only the contribution from tryptophan, present in the primary structure, at 280 nm.[21] Before measurements, all solutions were centrifuged and filtered, and their limpidity was checked by absorbance at 325 nm, where absorption should be negligible.

The molar absorptivity determination for the anthranilic acid derivatives was performed by using the following procedure. After preparation of a stock solution for each compound in 10 mM phosphate buffer (pH = 7.1) with 10% DMSO, a set of solutions were obtained by dilution with the buffer. The absorbance of the resulting samples was measured at the corresponding absorption maxima, χ = 315 nm, χ = 311 nm, χ = 294 nm, and χ = 294, respectively for VL-0395, compound **1**, compound **2**, and compound **3**. The molar coefficients of absorbance were determined by linear regression using the Lambert−Beer low, and the correlation coefficients were in all cases less than 0.995: ϵ_{315}(VL-0395) = 0.2285×10^5; ϵ_{311}(compound **1**) = 0.2590×10^5; ϵ_{294}(compound **3**) = 0.2797×10^5; ϵ_{294}(compound **2**) = 0.1629×10^5. Before measurements, all solutions were centrifuged and filtered, and their limpidity was checked by absorbance at 400 nm, where absorption should be negligible.

Fluorescence Measurement. The interaction between CCK$_1$-R(1−47) and the anthranilic acid derivatives was monitored by modification of the Trp fluorescence. Emission spectra were recorded at room temperature using a Varian Model Cary Eclipse spectrofluorimeter with the excitation wavelength set at 295 nm to selectively excite the Trp of the CCK$_1$-R(1−47) fragment. Five nanometers excitation and emission bandwidths were used throughout experiments, with a recording speed of 120 nm/min and automatic selection of the time constant. Small aliquots of each concentrated anthranilic ligand dissolved in 10 mM phosphate buffer solution (pH = 7.1) containing 10% DMSO were added to a fixed volume of the peptide CCK$_1$-R(1−47) dissolved in the same buffer in the presence of 8 mM SDS to mimic membrane environment. Measurements were always performed after allowing receptor/ligand mixture to stand until an apparent equilibrium was reached, as judged by the constancy of the fluorescence which took place in less than 1 min. For each ligand addition, final spectra were obtained after blank correction, adjustment for dilution, and subtraction of the separate contributions of CCK$_1$-R(1−47) and the anthranilic ligands from the total fluorescence of the assayed mixture. This allowed us to discriminate the Trp signal modifications produced by CCK$_1$-R(1−47)/anthranilic ligand interaction. It is worth noting that the emission spectrum of each anthranilic ligand, obtained under the same experimental conditions described above, was negligible in the range of Trp emission. In all case, during the fluorescence titration, the Trp absorbance at 295 nm was less than 0.1, which ensured fluorescence linearity. The binding between the CCK$_1$-R(1−47) and the loxiglumide antagonist was investigated by a fluorescence study as it was done for the anthranilic derivatives.

Treatment of Binding Data. Treatment of binding data was performed as previously described,[13,22] adopting a quantitative description of the CCK$_1$-R(1−47)/Anthranilic ligands titration pattern in terms of complex formation according to P + L = PL. Here P, L, and PL denote CCK$_1$-R(1−47), anthranilic ligand, and the bimolecular complex, respectively. In a titration experiment, the concentration of anthranilic ligand, L_0, and the fraction of anthranilic ligand-bound CCK$_1$-R(1−47), α, increase in steps of δL_0 and $\delta\alpha$, respectively, while the total CCK$_1$-R(1−47) concentration, P_0, remains constant (except for small dilution effects). Then, the equilibrium can be written in terms of the apparent dissociation constant as $K_d = [P][L]/[PL] = (1 - \alpha)(L_0 - \alpha P_0)/\alpha$, where the free peptide equilibrium concentration, [L], equals $L_0 - \alpha P_0$. Fluorescence intensities at 336 nm were used to evaluate α as $\Delta F_i/\Delta F_{max}$, where ΔF_i and ΔF_{max} are the excess fluorescence observed after the *i*th ligand addition and on saturation of binding sites, respectively. Finally, K_d was evaluated by fitting ΔF_i vs L_0 data to the function

$$\Delta F_i/\Delta F_{max} = [L_0 - \Delta F_i/\Delta F_{max} P_0]/(K_d + [L_0 - \Delta F_i/\Delta F_{max} P_0]) \quad (1)$$

which was implemented in the program KaleidaGraph for Windows Version 3.09.

^{1}H NMR Experiments. The assignment of the ^{1}H NMR spectra was based on information derived from 1D and 2D spectra in DMSO-d_6 and D_2O (COSY, HETCOR, NOESY, 250 MHz) and 1D and 2D spectra (COSY, TOCSY, HSQC, NOESY, ROESY) in H_2O-D_2O (90%-10% at pH 5, at 298 K and 283 K, 500 MHz). The latter were all acquired with excitation sculpting to reduce the residual water signal in both 1D and 2D spectra. NMR spectra were recorded on Avance DRX 500 (Bruker) and AC 250 (Bruker) spectrometers.

Computational Procedures. Energy minimizations were carried out with the program DISCOVER, version 2.98, implemented in the INSIGHT II software package. Calculations were performed using CVFF[23−25] without cross- or Morse terms on a Silicon Graphics Octane workstation. The program INSIGHT II, version 2000 (Accelrys Co, San Diego, CA), was employed for model-building procedures and as a graphic interface. The minimization procedure consisted of a conjugate gradient minimization until the root means square (rms) gradient of the potential energy was <0.001 kcal mol^{-1}.

Acknowledgment. We thank Dr. Anna Esposito who was partially involved in the experimental work. This work was supported by grants from Italian MIUR − P.S. Oncologia. The corresponding author A.V. wishes to thank all of his teachers of "Ancient Greek" of the "Hellenic Ministry of Education" starting with Aphrodite Varnava.

References

(1) Wank, S. A. Cholecystokinin receptors. *Am. J. Physiol.* **1995**, *269*, 628−646.
(2) Herranz, R. Cholecystokinn antagonists: Pharmacological and teraputic potential. *Med. Res. Rev.* **2003**, *23*, 559−605.
(3) de Tullio, P.; Delarge, J.; Piroette, B. Recent advances in the chemistry of cholecystokinin receptor ligands (agonists and antagonists). *Curr. Med. Chem.* **1999**, *6*, 433−455.
(4) Varnavas, A.; Lassiani, L.; Valenta, V.; Berti F.; Mennuni, L.; Makovec, F. Anthranilic acid derivatives: a new class of non-peptide CCK$_1$ receptor antagonists. *Bioorg. Med. Chem.* **2003**, *11*, 741−751.
(5) Kennedy, K., Gigoux, V.; Escrieut, C.; Maigret, B.; Martinez, J.; Moroder, L..; Fréhel, 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.
(6) Pellegrini, M.; Mierke, D. F. Molecular complex of cholecystokinin-8 and N-terminus of the cholecystokinin A receptor by NMR spectroscopy. *Biochemistry* **1999**, *38*, 14775−14783.
(7) Gigoux, V.; Maigret, B.; Escrieut, C.; Silvente-Poirot, S.; Bouisson, M.; Fehrentz, J.-A.; Moroder, L.; Gully, D.; Martinez, J.; Vaysse, N.; Fourmy, 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.
(8) Gigoux, V.; Escrieut, C.; Fehrentz, J.-A.; Poirot, S.; Maigret, B.; Moroder, L.; Gully, D.; Martinez, J.; Vaysse, N. and 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*, 20547−20464.
(9) Archer-Lahlou, E.; Tikhonova, I.; Escrieut, C.; Dufresne, M.; Seva, C.; Pradayrol, L.; Moroder, L.; Maigret, B.; Fourmy, D. Modeled structure of a G-protein-coupled receptor: the cholecystokinin-1 receptor. *J. Med. Chem.* **2005**, *48*, 180−191.
(10) Martín-Martínez, M.; Marty, A.; Jourdan, M.; Escrieut, C.; Archer, E.; González-Muñiz R.; García-López, M. T.; Maigret, B.; Herranz, R. Fourmy, D. Combination of Molecular Modeling, Site-Directed Mutagenesis, and SAR Studies To Delineate the Binding Site of Pyridopyrimidine Antagonists on the Human CCK1 Receptor. *J. Med. Chem.* **2005**, *48*, 4842−4850.
(11) Giragossian, C.; Sugg, E. E.; Szewczyk, J. R.; Mierke, D. F. Intermolecular interactions between peptidic and nonpeptidic agonists and the third extracellular loop of the cholecystokinin 1 receptor. *J. Med. Chem.* **2003**, *46*, 3476−3482.

(12) 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.
(13) Ragone, R.; De Luca, S.; Tesauro, D.; Pedone, C.; Morelli, G. Fluorescence studies on the binding between 1 and 47 fragment of cholecystokinin receptor CCK(A)-R(1−47) and nonsulfated cholecystokinin octapeptide. CCK8. *Biopolymers* **2001**, *56*, 47−53.
(14) Aloj, L.; Caracò, C.; Panico, M. R.; Zannetti, A.; Del Vecchio, S.; Tesauro, D.; De Luca, S.; Arra, C.; Pedone, C.; Morelli G.; Salvatore M. In vitro and in vivo evaluation of 111In-DTPAGlu-G-CCK8 for cholecystokinin-B receptor imaging. *J. Nucl. Med.* **2004**, *45*, 485−494.
(15) Revel, L.; Makovec, F.; Castaner, J. Dexloxiglumide. CCK1 (CCKA) receptor antagonist, Treatment of irritable bowel syndrome. *Drugs Fut*. **1999**, *24*, 725−728.
(16) Varnavas, A.; Lassiani, L.; Valenta, V.; Berti, F.; Tontini, A.; Mennuni, L.; Makovec, F. Antranilic acid based CCK1 antagonists: the 2-indole moiety may represent a "needle" according to the recent homonymous concept. *Eur. J. Med. Chem*, **2004**, *39*, 85−97.
(17) Meyer, E. A.; Castellano R. K.; Diederich, F. Interactions with aromatic rings in chemical and biological recognition. *Angew. Chem., Int. Ed.* **2003**, *42*, 1210−1250.
(18) Varnavas, A.; Lassiani, L.; Valenta, V.; Mennuni, L.; Makovec, F.; Hadjipavlou-Litina, D. Anthranilic Acid Based CCK_1 Receptor Antagonists: Preliminary Investigation On Their Second "Touch point"**.** *Eur. J. Med. Chem.* **2005**, *40*, 563−581.
(19) Kim, E.; Paliwal, S.; Wilcox, C. S. Measurements of Molecular Electrostatic Field Effects in Edge-to-Face Aromatic Interactions and CH-π Interactions with Implications for Protein Folding and Molecular Recognition. *J. Am. Chem. Soc.* **1998**, *120*, 11192−11193.
(20) Farmer, P. S.; Ariëns, E. J. Speculations on the design of nonpeptidic peptidomimetics. *Trends Pharmacol. Sci*. **1982**, *5*, 362−365.
(21) Pace, C. N.; Vajados, F.; Fee, L. Grinsley, G.; Gray, T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **1995**, *4*, 2411−2423.
(22) De Luca, S.; Sanseverino, M.; Zocchi, I.; Pedone, C.; Morelli, G.; Ragone R. Receptor fragment approach to the binding between CCK8 peptide and cholecystokinin receptors: a fluorescence study on type B receptor fragment CCK(B)-R (352−379). *Biopolymers* **2005**, *77*, 205−211.
(23) Hagler, A. T.; Dauber, P.; Lifson, S. Consistent force field studies of intermolecular forces in hydrogen-bonded crystals. 3. The C═O···H−O hydrogen bond and the analysis of the energetics and packing of carboxylic acids. *J. Am. Chem. Soc.* **1979**, *101*, 5131−5141.
(24) Hagler, A. T.; Lifson, S.; Dauber, P. Consistent force field studies of intermolecular forces in hydrogen-bonded crystals. 2. A benchmark for the objective comparison of alternative force fields. *J. Am. Chem. Soc*. **1979**, *101*, 5122−5130.
(25) Hagler, A. T.; Stern, P. S.; Sharon, R.; Becker, J. M.; Naider, F. Computer simulation of the conformational properties of oligopeptides. Comparison of theoretical methods and analysis of experimental results. *J. Am. Chem. Soc*. **1979**, *101*, 6842−6852.

JM051050N