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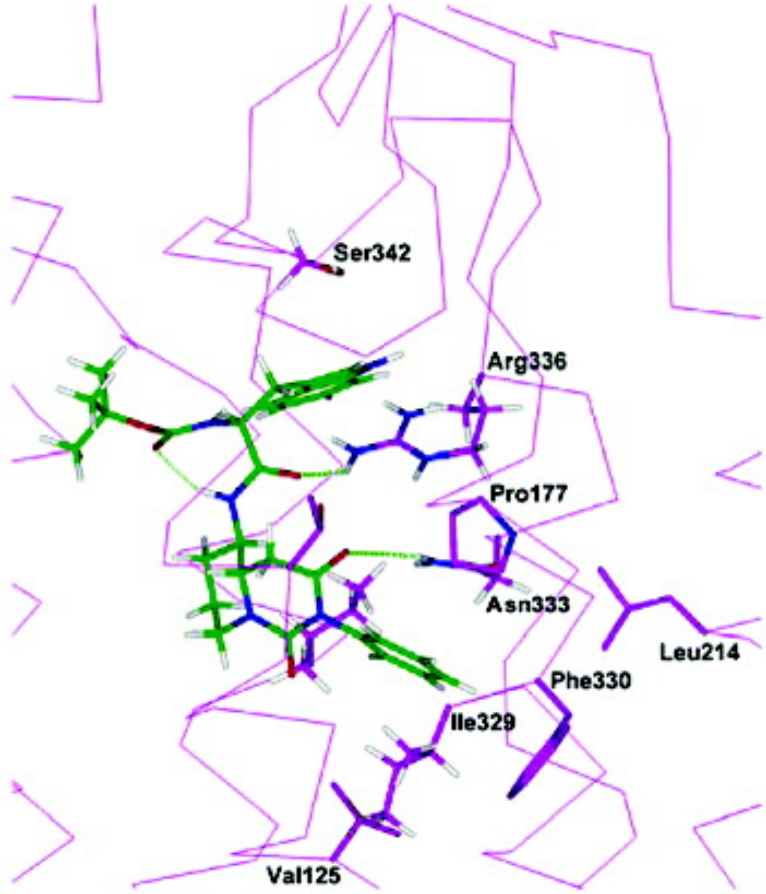

# Combination of Molecular Modeling, Site-Directed Mutagenesis, and SAR Studies To Delineate the Binding Site of Pyridopyrimidine Antagonists on the Human CCK1 Receptor

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A rational combination of site-directed mutagenesis studies, structure−activity relationships, and dynamic-based docking of pyridopyrimidine-derived CCK1R antagonists into a refined three-dimensional model of the CCK1R allowed us to identify the receptor residues and the ligand functional groups implicated in the molecular recognition process. Our results provided unambiguous evidence that the binding site of these antagonists is overlapping that of the C-terminal tetrapeptide of CCK. In particular, Asn333 and Arg336 residues of the CCK1R are essential for high-affinity binding of these ligands. Moreover, the 2-aryl group in the pyridopyrimidine derivatives shares the same binding pocket as the C-terminal Phe side chain of CCK. Our [pyridopyrimidine•CCK1R] complex model is consistent with previous suggestions concerning the molecular basis that governs functional activity and provides useful considerations about the high CCK1 versus CCK2 selectivity of our derivatives and could contribute to fine-tune the rational design of new molecules with optimized properties.

## Introduction

Cholecystokinin (CCK) is a neuropeptide composed of several molecular variants differing in length (CCK-58, -39, -33, -8) that are widely distributed in the central nervous system as well as in the intestine.[1−3] The actions of CCK are mediated by two receptors, the CCK1 receptor (abbreviated CCK1R) and the CCK2 receptor (abbreviated CCK2R), which are members of group I of the superfamily of G-protein-coupled receptors (GPCRs).[3,4] CCK1R activation by agonists initiates a cascade of signaling primarily resulting from coupling to the heterotrimeric protein Gq that activates phospholipase-C and subsequent signaling pathways.[4,7,8] CCK1R-mediated effects include control of gallbladder contraction, pancreatic growth and secretion, gastric emptying, gut motility, and satiety.[6−8] The wide spectrum of biological functions regulated by the CCK1R has made this receptor a candidate target for a therapeutic approach in a number of diseases related to nutrient assimilation.[9] This has prompted intensive research in the area that, during the past 2 decades, has generated a large variety of agonists and antagonists of the CCK1R, several of which are of therapeutic value and have reached advanced clinical evaluation.[9] Strategies aimed at discovering CCK1R antagonists have evolved from the initial CCK derived peptides toward peptidomimetics or non-peptide antagonists. Of note, the CCK1R is the first peptide-binding GPCR for which a non-peptide antagonist (asperlicin) was discovered by screening of fermentation products.[10] Subsequently, the asperlicin backbone was successfully used as a template to design high-affinity antagonists and agonists for the CCK1R.[11,12]

To obtain non-peptide CCKR ligands, we used a different strategy, which was based on the incorporation of conformationally restricted structures into the sequence of the CCK2R-selective agonist CCK-4 ($Trp^{30}$-$Met^{31}$-$Asp^{32}$-$Phe^{33}$-$NH_2$), as spacers between the key amino acids $Trp^{30}$ and $Phe^{33}$. Thus, the replacement of the $Met^{31}$-$Asp^{32}$ fragment by the 5-amino-1,3-dioxoperhydropyrido[1,2-*c*]pyrimidine skeleton led to a series of potent and highly selective CCK1R antagonists.[13] The prototype of this series, compound **1** (IQM-95,333, Figure 1), is one of the most selective CCK1R antagonists described to date, and its pharmacological activity was studied both in vitro and in vivo. This compound was able to antagonize the hypophagia and the hypolocomotion induced by CCK-8S in rats and, more interestingly, showed anxiolytic-like properties in different animal models,[14] supporting the suggestion of some authors that the anxiogenic effects of CCK-8S are, at least in part, mediated by the CCK1R.[15,16] Subsequent modifications of compound **1** led to analogues with even improved properties.[17−19] However, to date, these molecules had not been evaluated on the human CCK1R, a test that is crucial owing to interspecies difference polymorphisms in G-protein-coupled receptors that can alter drug affinity and/or activity, as previously illustrated for the CCK2R.[20] Furthermore, in the drug development process of our synthetic ligands, it is

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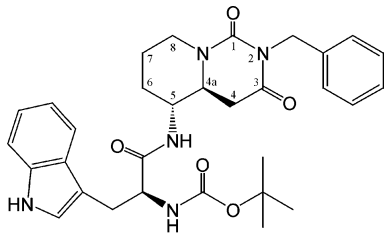


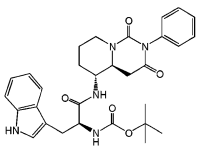
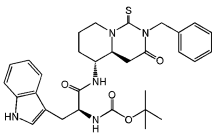
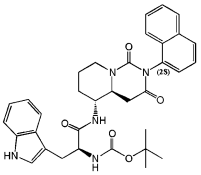
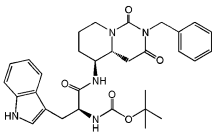
**1** (IQM-95,333)
$IC_{50}$ (rat CCK1R) =1.59 nM
$IC_{50}$ (rat CCK2R) > 10000 nM

**Figure 1.** IQM-95,333, the prototype of the pyridopyrimidine-derived CCK1R antagonists.

crucial to identify their binding site on the human CCK1R target. Besides this specific interest, works aimed at identification of the binding site of any antagonist and agonist of a given GPCR represent a prerequisite for a better understanding of how ligands activate/inactivate this GPCR and of how, eventually, they differentially direct its coupling to distinct signaling pathways. Regarding the CCK1R, during the past few years, some of us have mapped its binding site for CCK using site-directed mutagenesis and molecular modeling. The CCK1R binding site is composed of amino acids both from extracellular loops and transmembrane helices (TM), particularly TM III, V, VI, VII. Major anchoring points that account for 100−1000-fold in the binding affinity constant of CCK to CCK1R are constituted by Arg197, Arg336, and Asn333, which pair with the tyrosine sulfate, the penultimate Asp residue, and the C-terminal amide group of CCK, respectively.[21−23]

In our effort to get further insight into the mechanism by which the CCK1R is differentially activated or blocked by ligands, we were interested in delineating the binding site of the pyrido[1,2-*c*]pyrimidine-based antagonists. We had also interest in studying if there were any interspecies differences between rat and human CCK receptors related to the binding of these derivatives. For this purpose, we selected pyridopyrimidine derivatives with high affinity and selectivity for rat CCK1R bearing different substituents at the N-2 position, in particular compounds **2**−**4** (Table 1).[17,18] We also chose derivative **5** that possess low rat CCK1R and CCK2R affinity,[13] for comparative purposes. For derivatives **2**−**5** functional assays on human CCKRs were also performed to study if they follow a similar functional behavior as on the rat CCK receptors. As a first approximation to study the binding site of these antagonists on the human CCK1R, we assumed that it could overlap, at least partially, the binding site of the endogenous ligand, CCK. Accordingly, our experimental approach included, as a first step, recognition tests of several pyridopyrimidine-based antagonists by CCK1Rs mutated at positions that are important for recognition of CCK. Then, on the basis of results from these recognition tests, the pyridopyrimidine-based ligands were docked into a validated model of CCK1R.[24] Finally, the position of the pyridopyrimidine-derived ligands was refined on the foundation of additional site-directed mutagenesis studies, as well as on previous structure−activity relationship (SAR) results of this series.

**Table 1.** Pharmacological Properties of Pyridopyrimidine Ligands: Inhibition of CCK Binding to Cos-7 Cells Expressing either the Human CCK1R or CCK2R and Inhibition of CCK-Induced Production of Inositol Phosphates in Cos-7 Cells Expressing the Human CCK1R

| Entry | Compound | Inhibition of CCK binding ($IC_{50}$, nM)[a] | | Inhibition of InsP production ($IC_{50}$, nM)[a] |
|---|---|---|---|---|
| | | $CCK_1R$ | $CCK_2R$ | CCK1R |
| **2** |  | 5.2 ± 2.9 | >10000 | 35.8 ± 1.9 |
| **3** |  | 3.8 ± 2.8 | >10000 | 7.3 ± 1.2 |
| **4** |  | 2.1 ± 0.5 | >10000 | 8.8 ± 0.7 |
| **5** |  | 6850 ± 3570 | 6025 ±1500 | 20860 ±11600 |

[a] $IC_{50}$ values correspond to concentration of compounds inhibiting 50% of binding or inositol phosphate response. Results are the mean ± SEM of three to six individual experiments.

## Results

**1. Pharmacological Properties of Pyridopyrimidine-Based Antagonists toward Human CCK Receptors.** To determine the binding properties of pyridopyrimidine-based antagonists toward human CCK receptors, Cos-7 cells were transiently transfected with plasmids containing cDNA encoding either human CCK1R or CCK2R cloned from human pancreas and gallbladder, respectively.[25,26] As illustrated in Figure 2a,b, the four derivatives **2**−**5** dose-dependently and competitively inhibited the binding of [$^{125}$I]BH-(Thr,-Nle)-CCK9 to Cos-7 cells expressing the CCK1R. As shown in Table 1, concentrations inhibiting 50% of binding ($IC_{50}$) were 5.2 ± 2.9, 3.8 ± 2.8, 2.1 ± 0.5, and 6850 ± 3570 nM, respectively. Of note, analysis of competition binding plots yielded a Hill coefficient of 1.0, which demonstrates binding of the compounds with an homogeneous affinity to the CCK1R, whereas binding of CCK occurs with two binding affinity values ($K_{d1}$ = 0.132 ± 0.012 nM, $K_{d2}$=11.0 ± 9.5 nM, Figure 2a). As previously observed in rats, compound **5**, having a 4a*R*,5*S* configuration on the bicyclic skeleton of the molecule core, exhibited a nearly 1000-fold lower affinity than the other compounds of the series having 4a*S*,5*R*-configuration. Since CCK1R and CCK2R recognize CCK-8 with the same high affinity and present a reasonable degree of structural homology, we checked

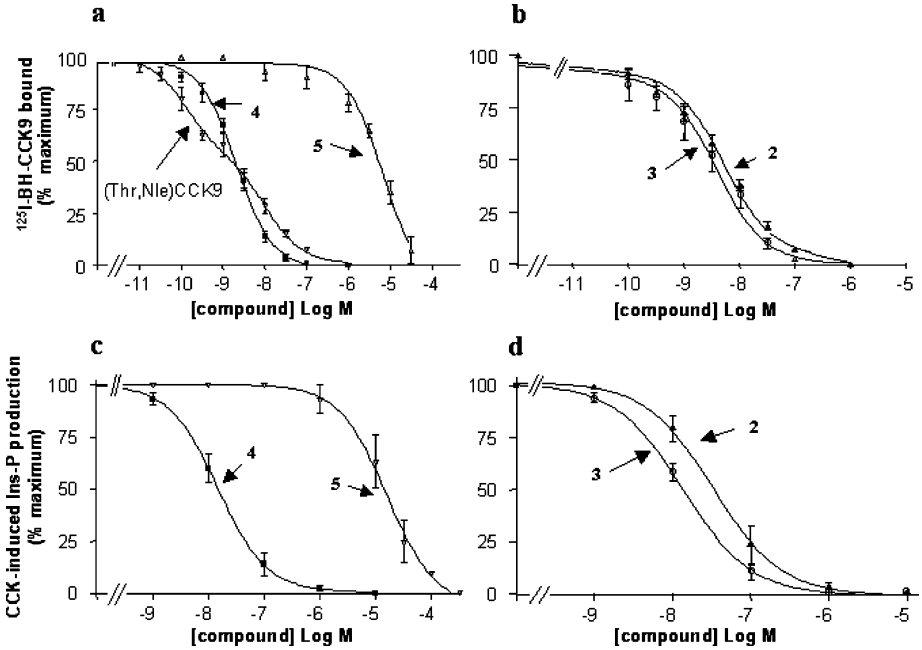



**Figure 2.** Ability of the pyridopyrimidine ligands **2**−**5** to displace CCK binding to the CCK1R and to inhibit CCK-induced production of inositol phosphates. (a, b) COS-7 cells expressing the human wild-type CCK1R were incubated with [$^{125}$I]BH-(Thr,-Nle)-CCK-9 alone or in the presence of increasing concentrations of competiting compound as described in the experimental procedures. Specific binding in each assay is expressed as a percent of specific binding in absence of competitor. Data are the mean of three to six individual experiments. (c, d) Cos-7 cells transiently transfected with the wild-type CCK1R were stimulated by a concentration of CCK fixed at the $EC_{50}$, i.e., 2 nM. Inhibition of inositol phosphate production in the presence of increasing concentration of pyridopyrimidine derivatives was then studied. Results are expressed as the percentage of CCK-induced inositol phosphates production with each compound.

if the pyridopyrimidine derivatives could bind to the human CCK2R. Compounds **2**−**4** tested at concentrations up to 1 $\mu$M, failed to displace binding of [$^{125}$I]BH-(Thr,Nle)-CCK9 to Cos-7 cells expressing the human CCK2R. Compound **5** bound to the human CCK2R with the same low affinity as to the CCK1R (Table 1). Therefore, the pyridopyrimidine-based antagonists, except compound **5**, are at least 1000-fold selective for the human CCK1R over the human CCK2R. Then, we determined whether these ligands for the CCK1R were devoid of agonist activity by evaluating the production of inositol phosphates. Cos-7 cells expressing the CCK1R did not respond to stimulations with the pyridopyrimidine-based ligands in experiments where they responded to CCK. In contrast, Cos-7 cells expressing the human CCK2R responded to stimulations with 10 $\mu$M concentrations of the tested compounds. Increases in inositol phosphates with compounds **2**−**4** reached 2.7, 9.0, and 30% of maximum responses achieved with the full agonist, CCK (not illustrated). These sets of experiments demonstrate that the pyridopyrimidine-based ligands bind with high affinity to human CCK1R, but they are unable to trigger coupling of this receptor to phospholipase-C, a key effector enzyme of CCK1R signaling. In contrast, albeit they have low affinity for the human CCK2R, compounds **2**-**4** showed weak agonist properties at this receptor, given that these pyridopyrimidine derivatives were able to couple the CCK2R to phospholipase-C, as does the endogenous agonist CCK.

We further characterized the pyridopyrimidine-derived ligands by measuring their ability to inhibit CCK-stimulated production of inositol phosphates in Cos-7 cells expressing the CCK1R. As illustrated in Figure 2, the three compounds **2**−**4** dose-dependently inhibited CCK-induced production of inositol phosphates. Concentrations inhibiting 50% of inositol phosphate formation ($IC_{50} \pm$ SEM) were in agreement with dissociation constants of the compounds measured in binding assays: $35.8 \pm 1.9$, $7.3 \pm 1.2$, and $8.8 \pm 0.7$ nM, respectively. Therefore, according to these results, molecules **2**−**4** behave as potent and selective antagonists on the human CCK1R.

**2. Identification of Amino Acids of the Human CCK1R Involved in the Recognition of Pyridopyrimidine-Based Antagonists by Site-Directed Mutagenesis.** As mentioned above, in a first attempt to identify the binding site for pyridopyrimidine-based antagonists, we hypothesized that this binding site could overlap, at least partially, the binding pocket of the endogenous peptide CCK, as has been demonstrated for other nonpeptide antagonists.[23,27,28] To this end, we tested the effect of mutations of the CCK1R, which we had previously confirmed to compose the binding site of CCK, on the potency of pyridopyrimidine-based antagonist to inhibit CCK-stimulated formation of inositol phosphates. In a first instance, we decided to evaluate the effect of the N333A mutation, since it has been verified that the binding of different CCK1R agonists and antagonists is affected by this single point mutation.[22,23] The results showed that the pyridopyrimidine derivatives failed to inhibit CCK-induced production of inositol phosphates in Cos-7 cells expressing the (N333A)-CCK1R (Table 2), suggesting that Asn333 is also a key residue for the binding of our derivatives to the CCK1R.

To verify whether other previously identified amino acids of the CCK1R binding site were effectively in-

**Table 2.** Effects of CCK1R Mutations on the Potency of Pyridopyrimidine Ligands To Inhibit CCK-Stimulated Production of Inositol Phosphates

| | compound **2** | | compound **3** | | compound **4** | |
|---|---|---|---|---|---|---|
| CCK1R | $IC_{50}$ (nM)[a] | $F_{mut}$[b] | $IC_{50}$ (nM)[a] | $F_{mut}$[b] | $IC_{50}$ (nM)[a] | $F_{mut}$[b] |
| F107A | 23.3 ± 4.7 | 0.7 | 31.5 ± 2.2 | 4.6 | 26.8 ± 3.4 | 3.0 |
| T117A | 71.6 ± 17.4 | 1.9 | 28.4 ± 0.8 | 1.6 | 24.9 ± 5.8 | 2.0 |
| M195L | 35.2 ± 1.6 | 0.4 | 5.8 ± 0.8 | 0.3 | 5.2 ± 1.2 | 0.2 |
| R197M | 23.0 ± 1.7 | 0.7 | 11.8 ± 2.3 | 0.4 | 8.9 ± 0.7 | 0.9 |
| I329A | 1106.8 ± 112.5 | 30.8 | 128.8 ± 19.0 | 6.6 | 9.3 ± 0.6 | 1.0 |
| I329F | 514.3 ± 92.0 | 14.3 | 183.2 ± 18.2 | 24.9 | 801.2 ± 99.7 | 90.8 |
| N333A | no inhibition | | no inhibition | | no inhibition | |
| R336M | 4855.7 ± 471.3 | 125.4 | 2059.4 ± 575.5 | 138.2 | 582.4 ± 38.0 | 40.1 |
| S342A | 33.2 ± 5.5 | 0.8 | 27.5 ± 3.2 | 0.8 | 7.9 ± 0.9 | 0.7 |
| S348A | 36.6 ± 2.5 | 1.2 | 5.7 ± 0.3 | 0.2 | 3.6 ± 0.9 | 0.5 |
| I352A | 2288 ± 235.3 | 65.5 | 831.2 ± 22.8 | 61.6 | 227.4 ± 18.7 | 21.2 |

[a] $IC_{50}$ values correspond to the concentration of compounds inhibiting 50% of binding or inositol phosphate response. Results are the mean ± SEM of three to six individual experiments. [b] The mutation factors ($F_{mut}$) were calculated as $IC_{50}$(mutated receptor)/$IC_{50}$(wild-type-CCK1R). Results are the mean ± SEM from at least three experiments performed in duplicate.

volved in the binding pocket of pyridopyrimidines, a second round of mutagenesis studies was carried out. Results of these experiments, which are summarized in Table 2, revealed two categories of CCK1R mutations. A first series, including M195L, R197M, and S348A, did not affect the inhibitory action of pyridopyrimidine ligands. Such results support that amino acids Met195, Arg197, and Ser348 of the CCK1R do not contribute to recognition of the pyridopyrimidine-based ligands, while they strongly contribute to that of CCK.[21,24,29] A second set of mutations, namely F107A, I329A, I329F, R336M, and I352A, affected the inhibitory action of pyridopyrimidine-based compounds. Furthermore and interestingly, in this second set, drops in inhibitory potencies ($F_{mut}$) differed according to both the mutated amino acid and the structure of the ligand. This behavior was well illustrated for mutation of Ile329. Exchange of Ile329 for Ala did not affect potency of compound **4**, whereas it affected that of compounds **2** and **3** by 30.8- and 6.6-fold, respectively. Exchange of Ile329 for a Phe strongly diminished potency of compound **4** ($F_{mut}$ = 90.8) and moderately affected that of compounds **2** and **3** ($F_{mut}$ = 14.3 and 24.9, respectively). Two other mutations, R336M and I352A, also clearly discriminated among the pyridopyrimidine-based ligands. Mutation of Arg336 affected potency of compound **4** by 40-fold and decreased equally that of compounds **2** and **3** ($F_{mut}$ = 125 and 138). Mutation of Ile352 affected potency of compound **4** by 21-fold and equally decreased that of compounds **2** and **3** ($F_{mut}$ = 65.5- and 61.6-fold). Such structure−activity data, particularly the differential effects of certain mutations on the antagonistic activity of the pyridopyrimidine-based ligands strongly support an involvement of amino acids Ile329, Asn333, Arg336, and Ile352 in the binding site of these ligands. Therefore, among the eight amino acids of the CCK1R that highly contribute to the binding site of CCK, only four, Ile329, Asn333, Arg336, and Ile352, seem to be involved in the recognition of the pyridopyrimidine-derived compounds. It is interesting to note that all these four residues are located in transmembrane helices VI and VII of the CCK1R.

**3. Molecular Model of [Pyridopyrimidine·CCK1R] Complex.** For the selection of the initial docking orientation, we paid attention to the first series of mutagenesis data that identified the Asn333, at the top of helix VI, as a crucial residue for the binding of compounds **2**−**4** to human CCK1R (Table 2). Additionally, we also took into account the hypothesis of an overlapping binding site with that of the CCK. This led to the first modeled structure of the [pyridopyrimidine·CCK1R] complex, model I (Figure 3), in which the pyridopyrimidine antagonists were buried within the transmembrane region of the CCK1R and formed three intermolecular H-bonds with the receptor. In particular, the side chains of Asn333, Thr117, and Ser348 of the CCK1R participated in H-bonds with the carbonyl group at position 1 of the bicyclic ring and those of the Trp and Boc moieties, respectively. This [pyridopyrimidine·CCK1R] model was also in agreement with previous SAR studies. For example, the carbonyl group of the Trp residue, whose H-bonding acceptor character was shown to be important for high affinity,[19] was hydrogen bonded to the hydroxyl group of the Thr117 side chain. Moreover, model I positioned the key substituent at position 2 into a hydrophobic pocket, constituted by residues Val125 (III), Leu214 (V), Ile329 (VI), Phe330 (VI), and Ile352 (VI), large enough to accommodate a naphthyl group.

In model I, Thr117 and Ser348 are involved in interactions with the pyridopyrimidine ligands. However, mutations of these two residues did not significantly affect the antagonist potency of the pyridopyrimidine-based compounds, thus refuting the initial [pyridopyrimidine·CCK1R] structural model I (Table 2). Moreover, in model I, Arg336, which in pharmacological experiments clearly appeared as being involved in the recognition of the pyridopyrimidines, did not interact with the antagonist molecule. On the other hand, mutagenesis results supported that residues Ile329 and Ile352 take part of the hydrophobic pocket for the N-2 aryl substituent, in agreement with our initial model. It is interesting to note that mutants on these residues can distinguish between pyridopyrimidine derivatives with different groups at position 2, as discussed in the next section.

To account for the residues of the receptor showed to be involved in the binding, the orientation of the ligand into the CCK1R was modified, keeping the location of the N-2 group and relocating the rest of the molecule. An interaction with the Asn333, a crucial residue for the binding, was also maintained. In model I (Figure 3) the Asn333 side chain was H-bonded to the carbonyl at position 1 of the pyridopyrimidine core; however, H-

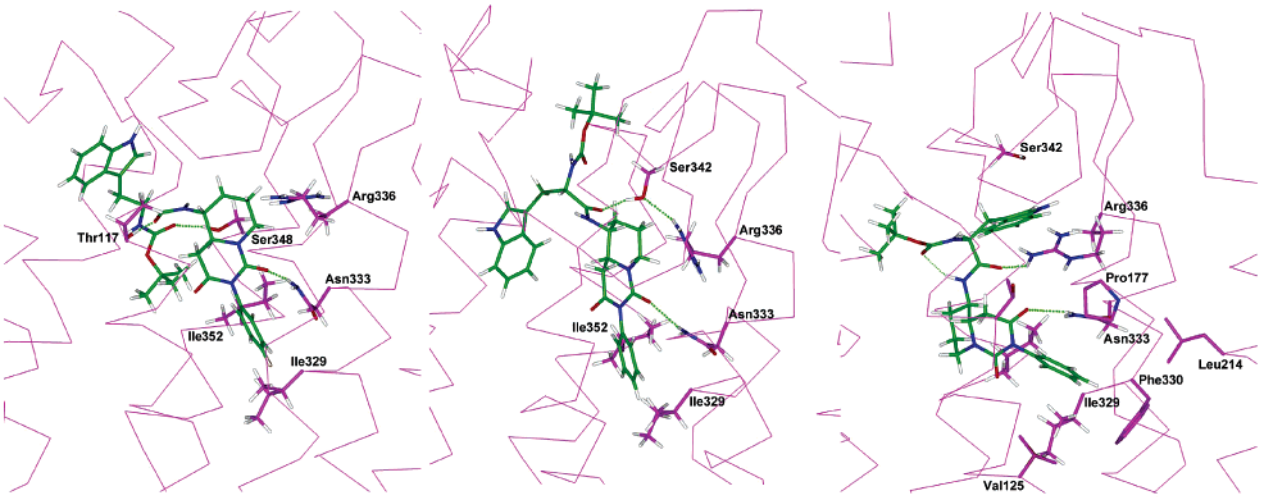



**Figure 3.** Simplified representation of the [pyridopyrimidine-CCK1R] **2** complex: model I, model $II_A$, and model $II_B$. For clarity, the view shows only the CCK1R amino acid side chains identified by mutagenesis studies in the interaction with the antagonist. In model $II_B$, the heavy atoms of the side chains surrounding the binding pocket of the substituent at position 2 of the pyridopyrimidine skeleton are also shown.

bonding with the carbonyl at position 3 will also locate the 2-aryl group at its hydrophobic pocket. Therefore, in a first instance, we set to establish which of the two carbonyl groups of the central pyridopyrimidine scaffold was involved in the interaction with the Asn333. This provided us with two alternative models, model $II_A$ and model $II_B$ (Figure 3), which presented two different binding orientations. In both models, the central scaffold and the substituent at position 2 are situated in the receptor transmembrane regions, whereas the Boc-Trp moiety neared the entrance of the CCK1R pocket. Model $II_A$ showed two H-bonds between the ligand and the CCK1R, one between the Asn333 side chain and the carbonyl at position 1, and the other involving the hydroxyl group of Ser342 and the carbonyl group of the Trp residue. Moreover, intramolecular H-bonding between the Ser342 and Arg336 side chain functional groups was also observed. Model $II_B$ showed one intramolecular H-bond and two intermolecular H-bonds, the latter involved two residues of the receptor located near the top of helix VI. The primary amide group of the Asn333 is H-bonded to the carbonyl at position 3 of the pyridopyrimidine core, while the guanidinium of Arg336 is implicated in a H-bond with the carbonyl group of the Trp moiety. From these results, both models are in agreement with the essential role of Asn333 in the binding and could also justify the contribution of Arg336 either by forming a direct H-bond with the ligand or by helping in positioning the Ser342 adequately.

To ascertain which of the two alternative models, $II_A$ or $II_B$, was more reliable, first, we thoroughly considered previous SAR studies. Although these studies have been obtained with rat CCK1R, we consider them valuable information to backup our model, as no sequence variation is seen between rat and human CCK1R within or near the binding site. In particular, we focused on those results concerning the 26-fold higher affinity of the (2*S*)-naphthyl atropoisomer **4**, $IC_{50}$(rat CCK1R) = 0.59 nM versus the (2*R*) atropoisomer, $IC_{50}$(rat CCK1R) = 15.4 nM.[17] Only in model $II_B$ is the additional phenyl ring of the naphthyl moiety positioned right through the hydrophobic pocket locating the substituent in position 2 of the pyridopyrimidine derivatives. Moreover, binding studies on several thio pyridopirimidine analogues showed that only the exchange of the carbonyl oxygen at position 1 by a sulfur atom led to a 30-fold increase in the affinity [compound **3**, $IC_{50}$(rat CCK1R) = 0.09 nM versus its regioisomer with a C=S group at position 3, $IC_{50}$(rat CCK1R) = 2.83 nM],[18] suggesting that the 1-CO and 1-CS groups are not directly implicated in intermolecular hydrogen bonds as in model $II_B$. Further support for model $II_B$ was provided by a last mutation, S342A, which did not change the potency of the pyridopyrimidine derivatives to inhibit the CCK-stimulated production of inositol phospate relative to the wild-type CCK1R, indicating that Ser342 is not involved in the binding site of the pyridopyrimidine derivatives.

## Discussion

The current study has shown the importance of an iterative cycle of mutagenesis-modeling studies to get an experimental based model of the complex between the CCK1R and the pyridopyrimidine antagonists. Thus, the first structural model, model I, essentially based on a single mutation (Asn333), was able to predict only the 2-aryl binding pocket, a groove that was then corroborated by Ile352 and Ile329 mutants (Table 2). It is interesting to note that the different influence of the I329A and I352A mutations on the ability of derivatives **2**−**4** to inhibit the CCK-induced production of inositol phosphates is in accordance with the volume/shape of the 2-substituent (naphthyl > benzyl > phenyl). Thus, the exchange of the bulkier Ile329 and Ile352 for Ala led to greater decreases in the inhibition of the pyridopyrimidine derivative **2**, which bears the smaller substituent at position 2, likely due to a greater loss of hydrophobic interactions. On the contrary, mutant

I329F had a higher effect on the bulkier 2-naphthyl pyridopyrimidine antagonist, probably due to steric hindrance. These data are pointing out the importance of optimal van der Waals interactions among the hydrophobic side chains within the CCK1R binding pocket and the N-2 group of the ligand.

Contrary to results on the mutations of residues around the N-2 binding pocket, the second set of mutation data were not in agreement with the position of the rest of the molecule in model I. Thus, new orientations of the molecules were tested, and two alternative models were built, models $II_A$ and $II_B$ (Figure 3). The discrimination between these two models was performed by additional mutagenesis and SAR studies, which backup model $II_B$ as the most likely one. Thus, only model $II_B$ can explain the ability of mutants I329A, I329F, and I352A to discriminate ligands with different substituents at C-2 (Table 2). In fact, the high affinity of the (2*S*) atropoisomer of the naphthyl derivative **4** could be due to correct placement of the naphthyl group within the hydrophobic cavity, situating the second phenyl ring of the naphthyl moiety pointing toward the Ile329 and Ile352 residues in model $II_B$. Moreover, rat CCK1R SAR data showing an increase in affinity of the thio derivative **3** versus its regioisomer (with a C=S at position 3) might be explained if the carbonyl oxygen exchanged by the sulfur atom is not involved in a direct H-bond within the complex, as in model $II_B$, since a H-bond with a thiocarbonyl group is weaker than with a carbonyl. The higher affinity of the thiocarbonyl compound **3** with respect to its carbonyl counterpart could be due to a decrease in the solvation energy in solution of the thiocarbonyl group. Further support for model $II_B$ was provided by mutant (S342A)-CCK1R, as already discussed.

On the whole, model $II_B$ is supported by experimental data derived from both mutagenesis and SAR studies. Thus, the pyridopyrimidine antagonists bound to both extracellular and helical sequences. The main features of model $II_B$, beside the mentioned H-bonds with Asn333 and Arg336, include two well-defined cavities for the N-2 group and the pyridopyrimidine bicycle that share several residues, such as Gly122, Pro177, Ile352, Asn333, and Thr118. The N-2 aryl group is in the vicinity of a cluster of hydrophobic residues constituted by Val125, Leu214, Ile329, and Phe330, while the C-6 and C-7 methylene groups of the pyridopyrimidine core are located near the hydrophobic residues Leu46 and Leu50.

While the present work allows the mapping of the binding site of the central scaffold and the substituent at position 2, it does not accurately establish a defined position for the indol and Boc groups. These moieties are interacting with the upper part of the transmembrane helices and the extracelular loops. Their location has been tentatively assigned on the basis of the slight involvement of residue Phe107 (Table 2), and the data derived from molecular modeling studies of analogues in which the Boc-L-Trp residue has been replaced with Boc-D-Trp, Boc-L-αMeTrp, and Boc-L-Tpi (tetrahydro-β-carboline) (data not shown).[19] These studies suggested that the proposed orientation shown in Figure 3 (model $II_B$) is able to explain the differences in affinity of these derivatives. Nevertheless, further studies are required to establish the accurate location of the Boc and indole groups of the Trp moiety.

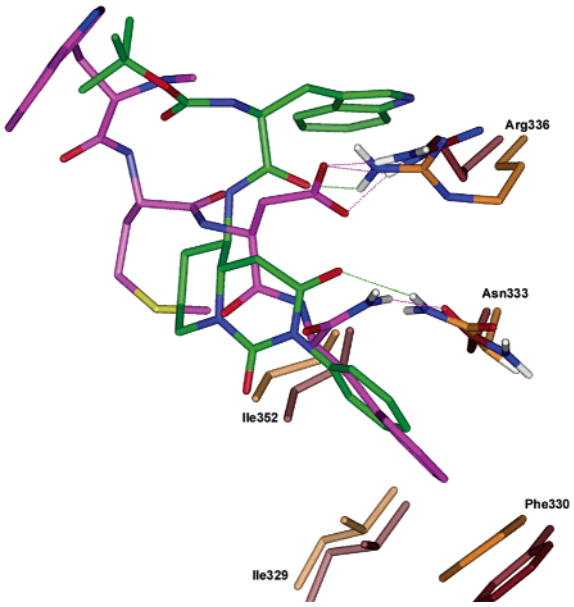



**Figure 4.** Superposition of the structure of pyridopyrimidine derivative **2** and the C-terminal tetrapeptide of CCK-9 binding site at CCK1R. For clarity only the amino acid side chains relevant for the discussion are shown. Green dashed line, H-bond between CCK1R and compound **2**; purple dashed line, H-bond between CCK1R and CCK-9.

The obtained model of docking is in agreement with our initial working hypothesis of an overlapping binding site for the C-terminal part of CCK and the pyridopyrimidine derivatives at the CCK1R (Figure 4). Thus, there are four residues of the CCK1R that are clearly demonstrated to be involved in the binding of both ligands, namely, Ile329, Asn333, Arg336, and Ile352. It is worth mentioning that although Asn333 is able to form a H-bond with CCK-9 and our derivatives, there is a change in the heteroatom involved in this H-bond, either the carbonyl or the amino group of its carboxamide moiety, respectively (Figure 4). The proposed model also shows that the environment surrounding the C-terminal Phe of $CCK^{23}$ is very similar to that occupied by the N-2 aryl group of the pyridopyrimidine ligands. Moreover, several residues of the binding site for Met/Nle[31] in $CCK^{23}$ are also forming the binding pocket for the C-6, C-7, and C-8 methylene groups of the pyridopyrimidine core, suggesting that the pyridopyrimidine skeleton is not just a scaffold for the correct positioning of the Phe and Trp side chains but also taking part in the binding. From the results presented here, it is evident that the pyridopyrimidine antagonists are sharing the binding site of the C-terminal end of the endogenous agonist CCK. This is not without precedents, since several reports show the existence of nonidentical but overlapping binding sites for agonists and antagonists in other rhodopsine-like GPCRs. These include GPCR that binds catecholamines, as dopamine[30] and α- and β-adrenergic receptors;[31−33] peptide receptors, like those for bradykinin,[34] neuropeptide Y,[35] and neurotensin;[36] and nucleotides receptors, such as adenosin receptors.[37,38]

Another interesting question raised by the current study is whether our antagonist binding-site is also overlapping the binding pocket of other non-peptide

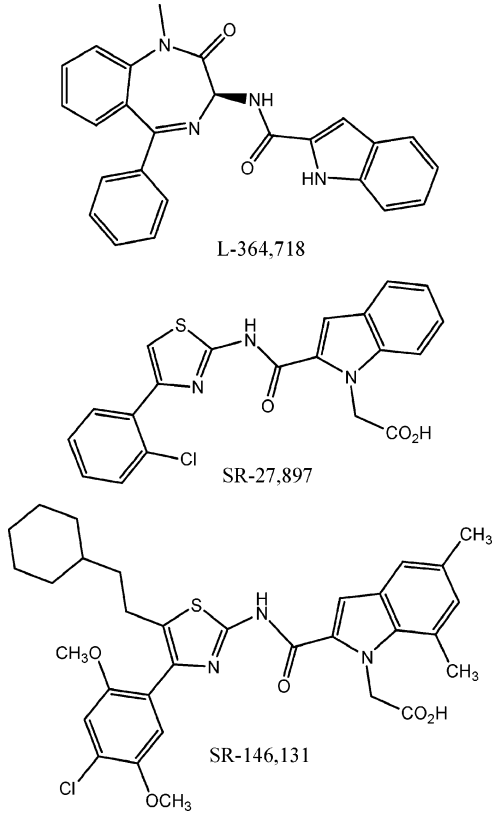



**Figure 5.** Non-peptide agonist and antagonist of CCK1R.

antagonists and agonists. The benzodiazepine L-364,-718 and the thiazole SR-27,897 are two well-known CCK1R non-peptide antagonists (Figure 5). Site-directed mutagenesis and molecular modeling studies on these antagonist pointed out that, like for our derivatives, Asn333 of the CCK1R plays a crucial role in their binding and has been proposed to form a H-bond with the central amide function of these non-peptide ligands.[22] Moreover, a detailed comparison of these nonstructurally related antagonists allows us to outline a common motif, namely an amide or carbonyl function able to interact with Asn333 through a H-bond, next to a hydrophobic group, that in general is an aromatic moiety. In addition, an adequate scaffold would be required to get an appropriate spatial orientation of these and other additional pharmacophore groups.

Although the pyridopyrimidine-based antagonist and CCK have overlapping binding sites, they differ in their functional activity. A possible explanation for the antagonist activity of the pyridopyrimidine derivatives might be the lack in these molecules of a chemical moiety that would mimic the N-terminal part of CCK-8. This hypothesis is very unlikely in the light of data indicating that the C-terminal tetrapeptide of CCK is an agonist on the CCK1R, despite its 1000-fold lower affinity than CCK-8. Furthermore, small non-peptide ligands such as SR-146,131 (Figure 5), which only share the C-terminal tripeptide binding site of CCK, are full agonists on the CCK1R.[23] Previous studies aimed at delineating the binding site of the non-peptide agonist SR-146,131 indicated that residues from transmembrane segments VI (Arg336, Asn333, Phe330, Ile329) and VII (Ile352, Leu356) are part of the SR-146,131 binding site. Moreover, the orientation of SR-146,131 within the binding grove is quite similar to that of the related antagonist SR-27,897.[24] However, main differences are found in their hydrophobic interactions within the binding grove. Although a clear understanding of the activation mechanism of the human CCK1R is still under intensive investigation, both SAR data and docking results with SR-146,131 and SR-27,897 strongly support that proper contacts within the deeper part of the CCK1R binding pocket are sufficient to cause activation of this receptor by small ligands. Concerning the activation mechanism of CCK1R by CCK, the correct positioning of the phenyl group of C-terminal Phe of CCK toward the Phe330 residue in CCK1R has been proposed to be critical.[23] This contact is maintained to some extent in the complex of CCK1R with our antagonists through a T-shape interaction between the N-2 aryl group and the side chain of Phe330 (Figure 4). Therefore, the activation of CCK1R does not appear to depend on a unique interaction within the binding pocket.

Finally, it is worth thinking that although the pyridopyrimidine derivatives present high affinity and selectivity toward CCK1R, they were designed on the basis of the C-terminal tetrapeptide CCK-4, an endogenous ligand selective for CCK2R. In this sense, the analysis of the 58 divergent amino acids of CCK2R versus CCK1R revealed that eight of them significatively shifted the affinity of several of the competitors tested.[20,39] In particular, when Val349 or Tyr350 of the CCK2R was changed to the corresponding residues in CCK1R, namely Ile329 and Phe330, there was an increase in the affinity for the CCK2R of the benzodiazepine-derived CCK1R antagonist L364,718. Thus, our results suggest that the key interaction of our derivatives with Ile329 and/or Phe330 might be responsible for their high selectivity for CCK1R versus CCK2R.

In conclusion, the combination of molecular modeling and pharmacological analysis of CCK1R mutants in an iterative cycle, along with SAR data, have allowed us to construct a molecular model of the [pyridopyrimidine·CCK1R] complex consistent with a large body of experimental data. This model has helped in a deeper understanding of the binding mode of these antagonists to CCK1R, providing indications of crucial interactions within the binding pocket, in particular, showing partial overlapping with the binding site for the endogenous agonist and other non-peptide ligands. The availability of a realistic model of [pyridopyrimidine·CCK1R] complex will prompt us to propose experimentally testable hypotheses regarding the molecular basis that governs the CCK1R subtype selectivity and functional activity. On the whole, all this knowledge will be a useful tool in the rational development of new potent and selective CCK-based drugs with potential therapeutic interest in feeding disorders.

## Experimental Section

**Chemicals**. The C-terminal nonapeptide analogue (Thr,-Nle)CCK-9 was synthesized as described previously.[40] Pyrido-[1,2-*c*]pyrimidine derivatives **1**−**5** were prepared as formerly described.[13,17,18]

**Site-Directed Mutagenesis and Transfection of COS-7 Cells.** All mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Quick-Change site-directed mutagenesis kit, Stratagene) using the human CCK1R cDNAs cloned into pRFENeo vector as template. The presence of the desired and the absence of undesired mutations were con-

firmed by automated sequencing of both cDNA strands (Applied Biosystem).

COS-7 cells ($1.5 \times 10^6$) were plated onto 10-cm culture dishes and grown in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum (complete medium) in a 5% $CO_2$ atmosphere at 37 °C. After overnight incubation, cells were transfected with 2 $\mu$g/plate of pRFENeo vectors containing the cDNA for the wild-type or mutated CCK1 receptors, using a modified DEAE−dextran method. Cells were transferred to 24-well plates at a density of 20 000−80 000 cells/well 24 h after transfection, depending on the transfected mutant and the experiment to be performed.

**Receptor Binding Assay.** Approximately 24 h after the transfer of transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline (pH 6.95), 0.1% BSA and then incubated for 60 min at 37 °C in 0.5 mL of Dulbecco's Modified Eagle's Medium, 0.1% BSA with 71 pM [$^{125}$I]BH-(Thr,Nle)CCK-9 (specific activity 1600−2000 Ci/mmol), prepared as in ref 41, in the presence or in the absence of competing agonists or antagonists. The cells were washed twice with cold phosphate-buffered saline (pH 6.95) containing 2% BSA, and cell-associated radioligand was collected with 0.1 N NaOH added to each well. The radioactivity was directly counted in a gamma counter (Auto-Gamma, Packard, Downers Grove, IL). Nonspecific binding was determined in the presence of 1 $\mu$M [$^{125}$I]BH-(Thr,Nle)CCK-9. $K_d$ values were calculated from homologous competition binding experiments using Ligand software (Kell, Cambridge, UK). $K_i$ values were calculated using the nonlinear curve-fitting software GraphPad Prism (San Diego, CA).

**Inositol Phosphate Assay.** Approximately 24 h after the transfer to 24-wells plates and following overnight incubation in complete medium containing 2 $\mu$Ci/mL of *myo*-2-[$^3$H]inositol, (Amersham biosciences, Les Ulis, France), the transfected cells were washed with Dulbecco's Modified Eagle's Medium and then incubated for 30 min in 1 mL/well Dulbecco's Modified Eagle's Medium containing 20 mM LiCl at 37 °C. The cells were washed with PI buffer (phosphate-buffered saline containing 135 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA) at pH 7.45. The cells were then incubated for 60 min at 37 °C in 0.3 mL of PI buffer with or without ligands at various concentrations. The reaction was stopped by adding 1 mL of methanol/hydrochloric acid to each well and the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad, Hercules, CA) for the determination of inositol phosphates. The columns were washed twice with 3 mL of distilled water and twice more with 2 mL of 5 mM sodium tetraborate/60 mM sodium formate. The content of each column was eluted by addition of 2.5 mL of 1 M ammonium formate/100 mM formic acid. Then 0.5 mL of the eluted fraction was added to scintillant and $\beta$-radioactivity was counted.

**Computer Modeling of the CCK1R and Docking of Pyridopyrimidine-Based Ligands.** The model of the human CCK1R that was used in the current study was that previously described.[24] For the ligand, a conformational search was performed to consider low-energy conformations in the docking exercises. Docking of pyridopyrimidine-based ligands into CCK1R was achieved using manual preliminary positioning inside the receptor groove. The manual position was preferred as it will allow us to take into account previous and extensive SAR studies on this series of compounds, as well as mutagenesis studies that identified contacts points between the receptor and the ligand. Thus, the pyridopyrimidine compound **2** was positioned within the CCK1R on the basis of such data, together with the hypothesis of an overlapping binding site with that of CCK. The resulting complex was submitted to simulated annealing molecular dynamics calculations, and no explicit constraint functions were used to maintain the initial docking contacts during the simulation, whereas the backbone of the CCK1R was kept fixed. The simulated annealing strategy consisted of 100 cycles of slow cooling, each one leading to a low-energy conformation. Each loop begins by fixing the temperature at 400 K, followed by 1000 steps of molecular dynamics (MD). The temperature is then decreased in steps of 100 K every 10 000 steps, up till the temperature of the system corresponds approximately to 300 K. The final conformation obtained at the end of this process was refined using a conjugate gradient algorithm and, after storage, was used to start a new simulation at high temperature with a slow cooling stage. This procedure produced samples of 100 energy-minimized conformations. From these samples, the lowest energy structure that fulfill the requirement of the SAR and mutagenesis studies was chosen and submitted to a new simulated annealing calculation at 600 K, at described above, then to one at 800 K, and finally to one at 1000 K. From this last simulation the resulting [pyridopyrimidine•CCK1R] complex that best explains the mutagenesis data and fulfill the requirements of the SAR studies was selected and was further refined using molecular dynamics and energy minimization. This process was repeated for the different low-energy conformers of the ligand, and the models selected were compared and serve as the basis for suggesting further mutagenesis studies. The program package (Insight II, Discover, Homology, Biopolymer) from Molecular Simulations Inc. (San Diego, CA) was used for all the calculations.

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