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Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist

(receptors/pancreas/gastrin)

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ABSTRACT **3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide (L-364,718) interacted in a competitive manner with rat pancreatic cholecystokinin (CCK) receptors as determined by Scatchard analysis of the specific binding of ^{125}I-labeled CCK. The affinity of L-364,718 for both pancreatic (IC_{50}, 81 pM) and gallbladder (IC_{50}, 45 pM) CCK receptors in radioligand binding assays greatly exceeded that of other reported nonpeptide CCK antagonists and was similar to that of CCK itself. *In vitro* functional studies utilizing CCK-induced contractions of the isolated guinea pig ileum and colon further demonstrated that L-364,718 acts as a competitive CCK antagonist, which lacks agonist activity and has a similar high affinity in these tissues (pA_2, 9.9). L-364,718 exhibited a very high selectivity for peripheral CCK receptors relative to brain CCK, gastrin, and various other peptide and nonpeptide receptors in both *in vitro* radioligand and isolated tissue assays. *In vivo*, low intravenous doses of L-364,718 (0.1 mg/kg) markedly antagonized the contractions of the guinea pig gallbladder produced by intravenous administration of CCK for at least 2 hr. Administered orally, L-364,718 (ED_{50}, 0.04 mg/kg) was highly effective as an antagonist of CCK-induced inhibition of gastric emptying in mice. The biochemical and pharmacological properties of L-364,718—namely, very high affinity and selectivity for peripheral CCK receptors, long-lasting *in vivo* efficacy, and oral bioavailability—makes this compound a powerful tool for investigating the physiological and pharmacological actions of CCK, and possibly its role in gastrointestinal disorders.**

Cholecystokinin (CCK) is a recognized hormone and proposed neurotransmitter that is found in gut and brain, predominantly as the octapeptide, CCK-8 (1). Activation of CCK receptors in peripheral tissues plays an important role in the control of pancreatic secretion, gallbladder contraction, and gut motility. Although the role of brain CCK receptors has not been established, they are generally considered as having different physical–chemical characteristics than peripheral CCK receptors (1, 2).

Recent interest in nonpeptide CCK antagonists has resulted in the development of several new agents. The first nonpeptide CCK antagonists—proglumide, dibutyryl cyclic GMP, and benzotript—exhibit very low *in vitro* potencies, which limits their *in vivo* utility (3). Furthermore, these agents lack a significant degree of selectivity for peripheral CCK receptors compared to brain CCK receptors or the closely related gastrin receptors, which mediate gastric acid secretion (4). Recently, asperlicin (4) and a proglumide derivative designated compound 16 (5) have been reported, which have improved potency and, for asperlicin, greater selectivity for peripheral CCK receptors than previous agents.

The present report describes the biochemical and pharmacological activities of a chemically novel, extremely potent nonpeptide CCK antagonist with very high selectivity for peripheral tissues. The compound, 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide (L-364,718) (6), has an affinity for peripheral CCK receptors that greatly exceeds that of asperlicin and compound 16 and is similar to that of CCK itself. The design, synthesis, and chemical structure of L-364,718 are described by Evans *et al.* (6) in an accompanying report.

METHODS

Receptor Binding Studies. ^{125}I-labeled CCK (^{125}I-CCK) binding assays were similar to those described by others for rat pancreas (7), guinea pig cerebral cortex (8, 9), and bovine gallbladder membranes (10), with the modifications noted below.

Membranes from rat (Sprague–Dawley) pancreas, guinea pig (Hartley) cerebral cortex, and bovine gallbladder were prepared by homogenization in 50–100 vol of 50 mM Tris·HCl (pH 7.4 at 37°C) using a Polytron (Brinkmann, PT 10, setting 4 for 10 sec for pancreas or brain and maximal speed for bovine gallbladder). Homogenates were centrifuged at 50,000 × *g* for 10 min, and the pellets were resuspended in the same buffer and centrifuged as described above. The resulting pellets were resuspended in 4000, 80, and 25 ml of binding assay buffer for each gram of original tissue wet weight of pancreas, brain, and gallbladder, respectively. ^{125}I-CCK-8 binding assay buffer contained 5 mM dithiothreitol, 50 mM Tris·HCl (pH 7.4 at 37°C), 5 mM $MgCl_2$, 2 mg of bovine serum albumin, and bacitracin at 0.14 mg/ml for pancreas; 10 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin at 0.25 mg/ml, and 130 mM NaCl (pH adjusted to 6.5 with NaOH) for brain; and 10 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin at 0.25 mg/ml, soybean trypsin inhibitors at 0.2 mg/ml, and 130 mM NaCl (pH 6.5) for bovine gallbladder.

^{125}I Bolton–Hunter-labeled CCK-8 (11) purchased from New England Nuclear was used. Free and bound ^{125}I-CCK-8 were separated by filtration using Whatman G/F B glass fiber filters that were presoaked in 50 mM Tris·HCl (pH 7.4) containing bovine serum albumin (1 mg/ml). Immediately after the filtration, the filters were washed rapidly three times with 4 ml of Tris·HCl containing bovine serum albumin (0.1 mg/ml), and radioactivity was counted with a Beckman γ counter. Specific ^{125}I-CCK-8 binding was defined as the difference between total binding and nonspecific binding in the presence of 1 μM CCK. IC_{50} values were determined by regression analysis of displacement curves. Inhibitor dissociation constants (K_i) were calculated from the formula K_i =

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Abbreviations: CCK, cholecystokinin; ^{125}I-CCK, ^{125}I-labeled CCK; ^{125}I-gastrin, ^{125}I-labeled gastrin.

$(I)/(K_{d'}/K_{d-1})$ where I is the concentration of the inhibitor and K_d and $K_{d'}$ are the dissociation constants of ^{125}I-CCK-8 in the absence and presence of inhibitor, respectively.

^{125}I-labeled gastrin (^{125}I-gastrin) binding in guinea pig dispersed glands was determined according to Praissman *et al.* (12) with modifications described by Chang *et al.* (4).

All other radioligand binding assays were performed according to the following published methods using rat brain unless noted otherwise: [^{3}H]flunitrazepam (guinea pig brain) (13); [^{3}H]Ro 5-4864 (rat kidney) (14); [^{3}H]serotonin (15); [^{3}H]muscimol (16); [^{3}H]spiperone (17); [^{3}H]dihydroalprenolol (18); [^{3}H]quinuclidinylbenzilate (19); [^{3}H]ketanserin (20); [^{3}H]naloxone (21); [^{125}I]neuropeptide Y (22); [^{3}H]angiotensin II (bovine adrenal cortex) (23); [^{3}H]pyrilamine (guinea pig brain) (24); [^{3}H]prazosin (25); [^{3}H]pirenzepine (26); [^{3}H]rauwolscine (27).

Isolated Tissue Preparations. Segments of ileum and colon were taken from female guinea pigs (Duncan–Hartley; 250–400 g) and mounted in tissue baths for recording of longitudinal muscle contraction as described by Paton and Zar (28) and Zettler (29), respectively. Cumulative concentration–response curves to the agonists were determined alone and in the same tissues that had been treated for 20 min with various concentrations of L-364,718. The concentration of agonist causing 50% of the maximal contraction (EC_{50}) in the presence and absence of antagonist was determined by regression analysis. Control studies demonstrated that the volumes of L-364,718 vehicle (dimethyl sulfoxide) used (<0.1%) did not affect repetitive concentration–response curves or the maximal contractile responses to CCK or other agonists. The pA_2 values (±SEM) and slopes (±SEM) were obtained by regression analysis of Schild plots according to Arunlakshana and Schild (30).

For determination of acid secretion, the whole stomach was excised from CF_1 female mice (18–22 g), placed in tissue baths, and perfused by the methods described by Wan (31). The effluent of the perfused stomachs was collected at 15-min intervals, and the hydrogen ion concentration was determined by titration with 0.01 M NaOH to pH 7. The basal acid output and the increase in acid output produced by a submaximal concentration of pentagastrin (0.6 μM) before and 45 min after incubation with various concentrations of L-364,718 (0.1–1 μM) were determined. ED_{50} values and 95% confidence limits were determined on the basis of the percentage inhibition of control responses by regression analysis. Control studies demonstrated responses to pentagastrin to be reproducible in the same tissues before and after incubation with L-364,718 vehicle (dimethyl sulfoxide).

***In Vivo* CCK Antagonist Activity.** Female Duncan–Hartley guinea pigs (250–400 g) were anesthetized and prepared for recording of gallbladder contractions as described by Fara and Erde (32). Cumulative doses of CCK-8 were administered intravenously until a maximal contractile response was obtained. Fifty minutes later, L-364,718 (0.1 mg/kg; i.v.) in dimethyl sulfoxide vehicle was administered, and dose–response curves for CCK were again determined 10 min and 2 hr later. ED_{50} values and 95% confidence limits were determined by regression analysis. Control studies demonstrated the reproducibility of dose–response curves to CCK determined before and 10 min and 2 hr after the dimethyl sulfoxide vehicle (1 ml/kg).

For determination of the oral activity of L-364,718, female Charles River (CF_1) mice weighing 18–21 g were used. Four dose levels of L-364,718 (range, 0.01–0.1 mg/kg) or vehicle (0.5% methylcellulose) was randomly administered by gastric intubation to groups of at least 20 mice 1 hr prior to CCK-8 (80 μg/kg; s.c.). Five minutes after administration of CCK-8, the mice were orally administered a charcoal meal consisting of 10% animal charcoal and 5% acacia blended with 1.0% methylcellulose. Gastric emptying was determined 5 min later on a blind basis by sacrificing the animal and visually observing for the presence of charcoal in the intestine. Under the present conditions, all of 300 untreated mice exhibited charcoal within the intestine, whereas gastric emptying was inhibited in 95% of 300 mice administered CCK-8 (80 μg/kg; s.c.). The dose of L-364,718 needed to antagonize CCK-8 inhibition of gastric emptying in 50% of mice (ED_{50}) was determined by probit analysis.

RESULTS AND DISCUSSION

L-364,718 was compared with the standard CCK antagonist, proglumide, as well as the more potent newer agents, asperlicin and compound 16, for its ability to displace the specific binding of ^{125}I-CCK-8 to CCK receptors in rat pancreatic tissue and bovine gallbladder. All agents inhibited the specific binding of CCK in a concentration-dependent manner and exhibited Hill slopes not significantly different from unity, indicating a single class of binding sites (33). In both tissues the concentration of L-364,718 causing half-maximal inhibition of binding (IC_{50}, 0.05–0.08 nM) was considerably less than the other antagonists examined (Table 1). The extremely high potency of L-364,718 as a peripheral CCK antagonist (discussed below) is exemplified by a receptor affinity comparable to the natural biological agonist of this receptor CCK-8 (IC_{50}, 0.06–0.17 nM) (Table 1).

The specific binding of ^{125}I-CCK to guinea pig brain tissues and of ^{125}I-gastrin to guinea pig gastric glands was inhibited by L-364,718 only at concentrations >3 orders of magnitude greater than its IC_{50} for inhibiting pancreatic or gallbladder ^{125}I-CCK binding. This result demonstrates that L-364,718 has a high degree of selectivity for the peripheral CCK receptor compared to the related brain CCK and gastrin receptor.

The specificity of L-364,718 for peripheral CCK receptors relative to other common peptide and nonpeptide receptors was further demonstrated by using several additional radioligand binding assays. The data in Table 2 demonstrate that, relative to its potency for peripheral CCK receptors, L-364,718 lacks significant interactions with (in order listed in

Table 1. IC_{50} (nM) of L-364,718 and other agents on specific binding of ^{125}I-CCK-8 and ^{125}I-gastrin in various tissues

Compound	^{125}I-CCK-8 in			^{125}I-gastrin in
	Rat pancreas*	Bovine gallbladder	Guinea pig brain	Guinea pig gastric gland
L-364,718	0.08 ± 0.02	0.05 ± 0.003	245 ± 97	300 ± 72
Compound 16	18 ± 1	7 ± 1	2230 ± 895	1900 ± 550
Asperlicin	184 ± 64	220 ± 11	>100,000	>100,000
Proglumide	250,000 ± 60,000	200,000 ± 42,000	800,000 ± 26,000	900,000 ± 20,000
CCK-8	0.17 ± 0.01	0.06 ± 0.01	0.39 ± 0.06	0.88 ± 0.21

Drug displacement studies were conducted with five or six concentrations of unlabeled drug, and IC_{50} values were determined by regression analysis. Values are the mean ± SEM of at least three determinations performed in triplicate.
*These values are somewhat lower than reported previously (4, 6), most likely as a result of the use of lower receptor concentrations and ^{125}I-CCK-8, rather than ^{125}I-CCK-33, as the ligand in the present studies.

Table 2. Lack of activity of L-364,718 in various common peptide and nonpeptide radioligand binding assays

Radioligand	IC_{50}, nM
[^{3}H]Flunitrazepam	>100
[^{3}H]Ro 5-4864	>100
[^{3}H]Prazosin	>10
[^{3}H]Rauwolscine	>10*
[^{3}H]Serotonin	>10*
[^{3}H]Ketanserin	>10*
[^{3}H]Spiperone	>10*
[^{3}H]Dihydroalprenolol	>10*
[^{3}H]Pirenzepine	>10
[^{3}H]Quinuclidinylbenzilate	>10*
[^{3}H]Pyrilamine	>10
[^{3}H]Naloxone	>10
[^{125}I]Neuropeptide Y	>10
[^{3}H]Angiotensin II	>10

*Racemic L-364,718 was used in these studies rather than the most biologically active isomer (−)-L-364,718, which was used in all other studies in this paper.

Table 2) central or peripheral benzodiazepine, α_1- and α_2-adrenergic, β-adrenergic, serotonin, dopamine, M_1 and M_2 cholinergic, histamine, opiate, neuropeptide Y, or angiotensin II receptors (13–27). The lack of interaction of L-364,718 with classical central ([^{3}H]flunitrazepam) or peripheral ([^{3}H]Ro 5-4864) benzodiazepine receptors is of particular note, in view of the fact that L-364,718 chemically contains a benzodiazepine nucleus (6).

To determine whether L-364,718 interacts competitively or noncompetitively with peripheral CCK receptors, ^{125}I-CCK binding in pancreatic tissues in the presence and absence of L-364,718 (0.1 nM) was analyzed according to Scatchard (34). As shown in Fig. 1, L-364,718 reduced the slope but not the x intercept of the Scatchard plot, indicating a change in the dissociation constant (K_d) of CCK for the receptor without a change in the maximum number of receptors (B_{max}). The data suggest that L-364,718 interacts competitively with CCK receptors in pancreatic tissue with an inhibition constant (K_i) of 0.08 ± 0.02 nM. By comparison, the K_d for CCK in the same experiments was also 0.08 ± 0.01 nM, again indicating that L-364,718 and CCK-8 have a similar affinity for pancreatic CCK receptors (Fig. 1).

Functional *in vitro* studies using the isolated guinea pig ileum and colon were used to characterize the nature of the interaction of L-364,718 with peripheral CCK receptors. Unlike CCK-8, L-364,718 (10–30 nM) did not cause contractions in either tissue, indicating a lack of CCK agonist activity. However, L-364,718 (0.15–30 nM) antagonized the contractions produced by CCK-8 in the ileum and colon as a function of concentration. The antagonism was characterized by a parallel shift to the right of the CCK-8 concentration–response curves without significant reduction in the maximal contractile response. Schild plots of the data gave slopes not significantly different from unity, indicating competitive antagonism (Fig. 2). The pA_2 values for L-364,718 as an antagonist of CCK-8 in the guinea pig ileum and colon were 9.89 ± 0.18 and 9.93 ± 0.13, respectively. These pA_2 values are in good agreement with the K_i values for L-364,718 in displacing ^{125}I-CCK binding in pancreas ($-\log K_i$ = 10.16 ± 0.09) and gallbladder ($-\log K_i$ = 10.35 ± 0.03). The similar affinity for L-364,718 in these tissues strongly indicates that the CCK receptors in pancreas, gallbladder, ileum, and colon are similar.

L-364,718 also demonstrated specificity for antagonism of contractions in the guinea pig ileum produced by CCK compared with other contractile agonists of this tissue. Concentrations of L-364,718 (13 nM), which were ≈100 times its pA_2 value for antagonism of CCK-8, did not significantly affect the concentration–response curves or maximal contractile responses to acetylcholine, histamine, or pentagastrin. Furthermore, using another functional assay that measures acid output from the isolated mouse stomach, only high concentrations of L-364,718 (IC_{50}, 330 nM; 95% confidence limits, 290–380 nM), which correspond to its IC_{50} value for inhibition of ^{125}I-gastrin binding (Table 1), were effective in antagonizing pentagastrin-stimulated acid secretion.

The high potency of L-364,718 in antagonizing the action of CCK on peripheral tissues *in vitro* was also evident *in vivo* after intravenous or oral administration. L-364,718 (0.1 mg/kg), administered intravenously, was highly effective in antagonizing contractions of the gallbladder produced by intravenous CCK-8 in anesthetized guinea pigs (Fig. 3). By comparison, and in agreement with their lower potencies *in vitro*, proglumide (50 mg/kg; i.v.) was inactive and asperlicin (13 mg/kg; i.v.) was much less effective as a CCK antagonist in this assay (4).

In the anesthetized guinea pig preparation, the CCK-8 dose–response curves were markedly displaced to the right when L-364,718 was administered either 10 min or 2 hr before CCK-8, indicating a long duration of action *in vivo* (Fig. 3). The shift in the CCK-8 dose–response curves produced by L-364,718 was parallel to the control curve and not associated with a reduction in the maximal contractile response of the

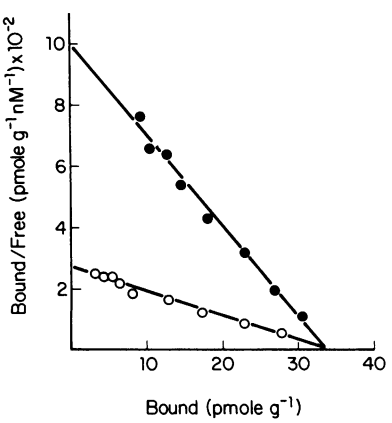

FIG. 1. Scatchard analysis of specific ^{125}I-CCK-8 binding in pancreatic membranes in the absence (●) and presence (○) of 0.1 nM L-364,718. Each point represents the mean of triplicate determinations. The lines were determined by regression analysis. This experiment was repeated six times. The mean K_d values in control and treated membranes were 0.076 ± 0.13 and 0.18 ± 0.01 nM, respectively. The B_{max} values in the presence of L-364,718 were 96% ± 5% of control.

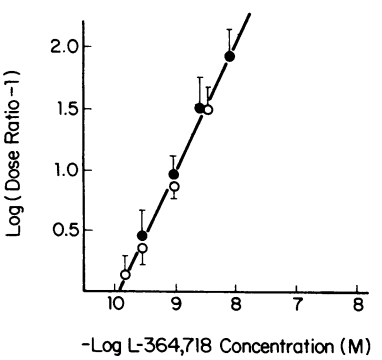

FIG. 2. Schild plots for L-364,718 as an antagonist of CCK-8 contractions in the isolated guinea pig ileum (●) and colon (○). The pA_2 values (x intercept) and slopes (●, 1.07 ± 0.35; ○, 1.05 ± 0.31) were determined by regression analysis. Each point represents the mean ± SEM of at least four tissues at each concentration.

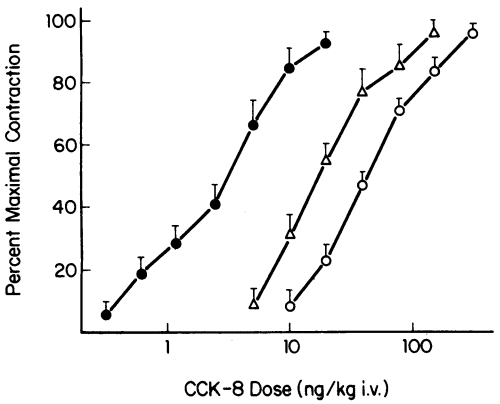

FIG. 3. Antagonism by intravenously administered L-364,718 (0.1 mg/kg) of gallbladder contractions produced by CCK-8 in anesthetized guinea pigs. The control ED_{50} value for CCK-8 (●) was 2.7 (2.2–3.3) ng/kg (i.v.). ED_{50} values for CCK-8 10 min (○) and 2 hr (△) after L-364,718 were 44.7 (38.8–51.5) and 19.5 (15.5–24.5) ng/kg (i.v.), respectively. Each point is the mean ± SEM of five animals.

gallbladder to CCK-8; this result is consistent with competitive antagonism of CCK receptors by L-364,718 *in vivo* as well as *in vitro* (see above).

L-364,718 was similarly highly effective in antagonizing CCK-8 when administered by the oral route. The compound antagonized the inhibition of gastric emptying produced by CCK-8 in conscious mice in a dose-related manner. The ED_{50} for L-364,718 in this assay was 0.04 mg/kg (p.o.) (95% confidence limits, 0.03–0.05 mg/kg).

The present studies demonstrate that L-364,718 is an extremely potent, competitive, and specific antagonist of peripheral CCK receptors in *in vitro* radioligand binding and functional isolated tissue assays. Furthermore, this compound is devoid of CCK-like agonist activity. This compound thus provides a powerful tool for investigating the physiological and pharmacological actions of CCK. The *in vivo* potency, oral activity, and long duration of action of L-364,718 indicate a potential application for this compound in exploring the diagnostic and therapeutic utilities of CCK antagonists in CCK-related disorders of the gastrointestinal system.

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