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Pharmacological evaluation of IQM-95,333, a highly selective CCK_A receptor antagonist with anxiolytic-like activity in animal models

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1 The pyridopyrimidine derivative IQM-95,333 ((4a**S**,5**R**)-2-benzyl-5-[N$^{\alpha}$-*tert*-butoxicarbonyl)L-tryptophyl]amino-1,3dioxoperhydropyrido[1,2-*c*]pyrimidine), a new non-peptide antagonist of cholecystokinin type A (CCK_A) receptors, has been evaluated *in vitro* and *in vivo* in comparison with typical CCK_A and CCK_B receptor antagonists, such as devazepide, lorglumide, L-365,260 and PD-135,158.

2 IQM-95,333 displaced [^{3}H]-CCK-8S binding to CCK_A receptors from rat pancreas with a high potency in the nanomolar range. Conversely, the affinity of this new compound at brain CCK_B receptors was negligible ($IC_{50} > 10$ μM). IQM-95,333 was a more selective CCK_A receptor ligand than devazepide and other CCK_A receptor antagonists.

3 Like devazepide, IQM-95,333 was a more potent antagonist of CCK-8S- than of CCK-4-induced contraction of the longitudinal muscle from guinea-pig ileum, suggesting selective antagonism at CCK_A receptors.

4 IQM-95,333 and devazepide were also potent inhibitors of CCK-8S-stimulated amylase release from isolated pancreatic acini, a CCK_A receptor-mediated effect. The drug concentrations required (IC_{50}s around 20 nM) were higher than in binding studies to pancreas homogenates.

5 Low doses (50–100 μg kg^{-1}, i.p.) of IQM-95,333 and devazepide, without any intrinsic effect on food intake or locomotion, blocked the hypophagia and the hypolocomotion induced by systemic administration of CCK-8S, two effects associated with stimulation of peripheral CCK_A receptors.

6 IQM-95,333 showed an anxiolytic-like profile in the light/dark exploration test in mice over a wide dose range (10–5,000 μg kg^{-1}). Typical CCK_A and CCK_B antagonists, devazepide and L-365,260 respectively, were only effective within a more limited dose range.

7 In a classical conflict paradigm for the study of anxiolytic drugs, the punished-drinking test, IQM-95,333, devazepide and L-365,260 were effective within a narrow dose range. The dose-response curve for the three drugs was biphasic, suggesting that other mechanisms are operative at higher doses.

8 In conclusion, IQM-95,333 is a potent and selective CCK_A receptor antagonist both *in vitro* and *in vivo* with an anxiolytic-like activity in two different animal models, which can only be attributed to blockade of this CCK receptor subtype.

Keywords: Cholecystokinin (CCK); CCK_A receptors; CCK_B receptors; amylase release; anxiolytic drugs; food intake

Introduction

Receptors for the neuropeptide cholecystokinin (CCK) are divided into two subtypes, CCK_A, with higher affinity for the sulphated octapeptide (CCK-8S), and CCK_B/gastrin, whose specifics ligands are gastrin, the tetrapeptide CCK-4 and the octapeptide CCK-8 (Innis & Snyder, 1980; Moran *et al*., 1986). The CCK_A subtype is found mainly in the periphery, where it is associated with pancreatic enzyme secretion, gut motility and gallbladder contraction, and also in some discrete CNS regions (Hill *et al*., 1992; Patel & Spraggs, 1992). The CCK_B receptor is distributed throughout the CNS (Moran *et al*., 1986; Hill & Woodruff, 1990) and appears to be involved in the control of nociception (Watkins *et al*., 1983; Hill *et al*., 1987) and in the development of anxiety (Singh *et al*., 1991b).

A number of antagonists for both receptor subtypes are known. CCK_A antagonists include, among others, the benzodiazepines devazepide (Chang & Lotti, 1986) and FK-480 (Ito *et al*., 1994) and the amino acid derivatives lorglumide and loxiglumide (Makovec *et al*., 1985). Very recently, the pharmacological characteristics of the more selective CCK_A receptor antagonists, TP-680 (Akiyama *et al*., 1996) and T-0632 (Taniguchi *et al*., 1996), have been reported. CCK_B receptor antagonists include the analogue of devazepide, L-365,260 (Lotti & Chang, 1989), the dipeptoid PD-135,158 (Hughes *et al*., 1990) and other compounds, such as RP 73870 (Pendley *et al*., 1995) and LY 288513 (Rasmussen *et al*., 1993). CCK_A receptor antagonists may be useful for the treatment of acute and chronic pancreatitis, of appetite alterations and of gastrointestinal disorders, such as dyspepsia and irritable bowel syndrome (Wettstein *et al*., 1994). Some of these compounds, such as loxiglumide and FK-480 are already in phase II clinical trial for the treatment of pancreatitis or gastrointestinal disorders. Some CCK_B receptor antagonists, such as L-365,260, are also in phase II clinical trials for the treatment of anxiety and panic attacks (see reviews in Prous, 1995).

In spite of the predominant role suggested for the CCK_B receptor subtype in anxiety and of the induction of panic attacks in healthy volunteers by i.v. injection of CCK-4 (De Montigny, 1989; Bradwejn *et al*., 1991), an anxiolytic-like activity in some models has also been shown for the typical CCK_A antagonist, devazepide (Hendrie & Dourish, 1990; Hughes *et al*., 1990; Hendrie *et al*., 1993). Yet, devazepide is not devoid of affinity at CCK_B receptors and it has been sug-

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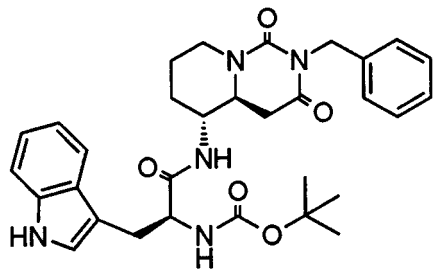

Figure 1 Chemical structure of IQM-95,333.

gested that the anxiolytic effect in animal models may be due to the CCK_B receptor blocking effect at high doses (Woodruff *et al*., 1991). It has also been suggested (Johnson & Rodgers, 1996) that possible effects of devazepide on spontaneous motor activity may result in false positives in some anxiety tests.

Selective CCK receptor antagonists are no doubt of interest in order to elucidate further the physiological roles of CCK. Clinical trials in progress will contribute to elucidate the real therapeutic interest of the CCK receptor antagonists. In this study we have described the pharmacological profile of (4a**S**,5**R**)-2-benzyl-5-[(N$^{\alpha}$-*tert*-butoxicarbonyl)L-tryptophyl]amino-1,3dioxoperhydropyrido[1,2-*c*]pyrimidine (IQM-95,333; Figure 1), a potent CCK_A receptor antagonist apparently devoid of any affinity at CCK_B receptors. The antagonist activity of this new compound was studied both *in vitro* and *in vivo*. Interestingly, anxiolytic-like properties of this new CCK_A antagonist were found in two different models of anxiety.

A preliminary account of the synthesis and pharmacological characteristics of IQM-95,333 has been presented elsewhere (González-Muñiz *et al*., 1996).

Methods

Animals

Male Swiss mice (20–25 g), Wistar rats (190–210 g) and Dunkin Hartley guinea-pigs (400–500 g) were used. Animals were kept in conditions of constant temperature (22±1°C), controlled lighting on a 12 h light/dark cycle with lights on at 08 h 00 min, and food and water *ad libitum* unless otherwise indicated. Behavioural experiments were always performed during the light period between 08 h 30 min and 14 h 30 min. Animals were never used more than once.

Receptor binding studies

CCK_A and CCK_B receptor binding assays were performed, in rat pancreas and cerebral cortex homogenates, respectively, according to the method described by Daugé *et al*. (1990) with minor modifications. Briefly, rat pancreas tissue was carefully cleaned and homogenized in 10 mM PIPES HCl buffer, pH 6.5, containing 30 mM $MgCl_2$ (15 ml g^{-1} wet tissue) and the homogenate was then centrifuged twice at 4°C for 10 min at 50,000 *g*. For displacement assays, pancreatic membranes (0.2 mg protein per tube) were incubated with 0.5 nM [^{3}H]-CCK-8S (sulphated cholecystokinin octapeptide) in PIPES HCl buffer, pH 6.5, containing 30 mM $MgCl_2$, 0.2 mg ml^{-1} bacitracin and 0.2 mg ml^{-1} SBTI (soybean trypsin inhibitor), for 120 min at 25°C. Rat brain cortex was homogenized in 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ (20 ml g^{-1} wet tissue) and the homogenate was centrifuged twice at 4°C for 35 min at 100,000 *g*. Brain membranes (0.45 mg protein per tube) were incubated with 1 nM [^{3}H]-CCK-8S in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 0.2 mg ml^{-1} bacitracin, for 60 min at 25°C. Final incubation volume was 0.5 ml in both cases. Non specific binding was determined with CCK-8S 1 μM as the cold displacer. The inhibition constants (K_i) were calculated by use of the equation of Cheng & Prusoff (1973) from the displacement curves analysed with the Receptor Fit Competition LUNDON programme.

Binding studies to central benzodiazepine, 5-hydroxytryptamine$_3$ (5-HT_3), neurotensin and neurokinin NK_1 receptors, as well as to Na^+ channels, ATP-sensitive K^+ channels and to the dihydropyridine binding site of Ca^{2+} channels were performed by use of previously described methods. The radioligands used were, respectively, [^{3}H]-flunitrazepam (Speth *et al*., 1979), [^{3}H]-BRL43694 (Hoyer & Neijt, 1988), [^{125}I]-neurotensin (Lugrin *et al*., 1991), [^{3}H]-substance P (De Felipe *et al*., 1989), [^{3}H]-saxitoxin (Catterall *et al*., 1979), [^{3}H]-glibenclamide (Angel & Bidet, 1991) and [^{3}H]-PN 200-110 (Lee *et al*., 1984).

Isolated longitudinal muscle-myenteric plexus (LMMP) preparation from guinea-pig ileum

Guinea-pigs were killed and bled. The ileum was excised approximately 10 cm from the ileo-caecal junction and longitudinal muscle strips with the myenteric plexus attached (LMMP) were prepared as described by Paton & Vizi (1969). LMMP strips were placed in a 10 ml organ bath containing Krebs bicarbonate buffer (composition in mM: NaCl 118.2, KCl 4.6, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 1.0) maintained at 37°C and aerated with 95% O_2 5% CO_2. Tissues were equilibrated for 30 min at 0.5 mg applied force and then stimulated with square wave pulses (0.1 Hz) of supramaximal voltage (15 V) and 0.7 ms duration to stabilize base-line force. Stimulation was then discontinued and tissues were challenged with KCl (47 mM) to determine initial maximum muscle contractility. After control responses to KCl had been obtained, noncumulative concentration-response curves to CCK-8S and CCK-4 were obtained by stepwise increases in concentration every 10 min; the preceding concentration was washed out and the tissue was exposed to the peptide for a period of 2 min. Concentration-response curves for each peptide were calculated as percentages of the initial KCl contraction. EC_{50} values were determined.

The effect of drugs on the contractile response to the peptides was expressed as pK_B values calculated according to the following equation (Furchgott, 1972):

$$pK_B = -\log(B)/(\text{dose–ratio} - 1)$$

where (B) is the concentration of the antagonist and dose-ratio is the quotient between ED_{50} of the agonist in the presence of the antagonist and control ED_{50}.

Amylase release

Dispersed rat pancreatic acini were prepared by using a modification of the technique of Jensen *et al*. (1982). The rat was decapitated and the pancreas was carefully cleaned. Tissue was injected with 1 ml of a solution of collagenase (type V, Sigma) at a concentration of 1 mg ml^{-1} (in distilled water) and subjected to the digestion step consisting of two 6-min incubations at 37°C. The tissue was washed three times in buffer A (composition in mM: NaCl 140, KCl 4.87, $MgCl_2$ 1.13, $CaCl_2$ 1.1, glucose 10 and HEPES 10, pH = 7.4) after each incubation. The tissue obtained after the last incubation, was dispersed with the aid of a Pasteur pipette and the homogenate was centrifuged twice (100 *g*, 1 min, 4°C). The final pellet was resuspended in 100 ml of buffer B (NaCl 98 mM, KCl 6 mM, NaH_2PO_4 2.5 mM, $CaCl_2$ 0.5 mM, theophylline 5 mM, glucose 11.4 mM, L-glutamine 2 mM, L-glutamic acid 5 mM, fumaric acid 5 mM, piruvate acid 5 mM, SBTI (soybean trypsin inhibitor) 0.01%, BSA (bovine serum albumin) 1%, essential amino acid mixture 1% and essential vitamin mixture 1%).

Amylase release was measured by the procedure of Peikin *et al*. (1978). Samples (2 ml) of the acini suspension were placed in plastic tubes and incubated for 30 min at 37°C in atmosphere of pure O_2 with agitation (70 cycles min^{-1}).

Amylase activity was determined with the Amyl Kit Reagent (Boeringher Mannheim). Release (S) was calculated as the percentage of amylase activity in the acini that was released into extracellular medium during the incubation period. The percentage inhibition by drugs of amylase release elicited by a fixed CCK-8S concentration was calculated according to the formula:

$$\%I = [(S_{CCK} - S_C) - (S_T - S_C)/(S_{CCK} - S_C)] \times 100$$

Where S_C is control release (vehicle), S_{CCK} the release elicited by CCK-8S and S_T the release elicited by CCK-8S in the presence of increasing drug concentrations. Linear regression analysis was used in order to estimate the IC_{50} of the compounds on the dose-response curves.

Food intake in fasted rats

Studies with fasted rats were performed in order to measure the antagonism by drugs (devazepide or IQM-95,333, 10–100 $\mu g\ kg^{-1}$) of CCK-8S-induced hypophagia. Male Wistar rats (initial weight 200 g) were individually housed and previously adapted to a schedule of restricted access to food from 10 h 00 min to 13 h 00 min. After intake had stabilized (typically within 6–7 days), drug testing was initiated. Animals fasted for 24 h were given drug or vehicle 25 or 115 min before CCK-8S. The feeding test was started 5 min after the CCK-8S injection (8 $\mu g\ kg^{-1}$, i.p.). The amount of food (standard pellets, Purina) consumed in a 30 min period was measured (Hewson *et al*., 1988).

Feeding studies in satiated rats

The method of Cooper *et al*. (1990) was used. Animals were allowed free access to food and they were given a palatable diet daily. Diet was prepared fresh each day and consisted of sweetened condensed milk, 50% diluted in tap water, and ground Purina pellets. Rats, housed individually, were subjected to an adaptation period with the sweetened diet 3 h per day for 8 days. When the adaptation phase was finished, rats were given an i.p. injection of IQM-95,333 or devazepide (50–100 $\mu g\ kg^{-1}$) 10 min before a second injection of (+)-fenfluramine (1.5 mg kg^{-1}) or vehicle. Twenty min later, the amount of diet consumed in a 30 min period was measured.

Spontaneous locomotor activity

Hypomotility was induced by i.p. injection of CCK-8S, 20 $\mu g\ kg^{-1}$, 5 min before the test (Poncelet *et al*., 1993). IQM-95,333 and devazepide (10–100 $\mu g\ kg^{-1}$) were injected 25 min before CCK-8S. Five minutes after CCK-8S, the animals were placed in a black wooden open-topped box ($65 \times 65 \times 45$ cm high). Distance travelled in cm (locomotor activity) was recorded during a 30 min period divided into two sessions (15 min each) by using a digital Videomex-V-system (Columbus Inst. USA) working with the appropriate computer programme.

Light/dark exploration test in mice

This test was described by Crawley & Goodwin (1980). An open-topped rectangular box ($45 \times 27 \times 27$ cm high) divided into a black small (2/5) area under a red light and a large white (3/5) area brightly illuminated with an opening door located in the centre of the partition at floor level was used. The floor of each compartment was marked into 9 cm squares. Each mouse was placed individually in the centre of the white area and behaviour was recorded over a 5 min period. Two behavioural parameters were measured in the white area: the percentage of time spent and the number of line crossings. Treatment groups and vehicle controls were run on each day of testing. Mice were exposed to the test for 2 consecutive days, the first without any treatment and the second day after drug administration, 30 min before the test (Artaiz *et al*., 1995). IQM-95,333, the CCK receptor antagonists, devazepide and L-365,260, as well as diazepam were given at varying doses from 2 $\mu g\ kg^{-1}$ to 5 mg kg^{-1}. The percentage change in the time spent in the white compartment and in the number of line crossings after drug treatment were calculated.

Punished drinking in rats (Vogel test)

The experimental procedure was described by Vogel *et al*. (1971). The test chamber consisted of a $45 \times 20 \times 20$ cm plastic cage with a stainless steel grid floor. A drinking spout projected 3 cm through a hole in the top. A drinkometer circuit was connected between the drinking tube and the grid floor of the apparatus so the animal completed the circuit when it licked the tube. The number of licks and shocks was monitored with an anxiometer (mod. 102, Columbus Inst., U.S.A.). On the test day, 24 h water-deprived rats were placed in the test chamber and allowed to find the drinking tube and complete 20 licks before the application of a shock (0.1 mA, 2 s). A timer was automatically started at the end of the first shock and shocks were delivered every 20 licks for a 3-min period. Drugs (IQM-95,333, other CCK receptor antagonists and diazepam, 10 $\mu g\ kg^{-1}$ to 2 mg kg^{-1}) were given i.p. 30 min before the test.

Drugs and chemicals

IQM-95,333 was synthesized at the Institute of Medicinal Chemistry (Madrid, Spain). Devazepide, L-365,260 (3**R**(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′-(3-methylphenyl)urea), PD-135,158 (4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[[1,7,7-trimethyl-bicyclo[2.2.1] hept-2-yl)oxy] carbonyl] amino]propyl]amino]-1-phenylethyl] amino-4-oxo-[1**S**-1α,2β[**S***(**S***)] 4α]}-butanoate N-methyl-D-glucamine) and (+)-fenfluramine were a gift from Merck Sharp & Dohme, Parke Davis and Servier Laboratories respectively. CCK-8S and CCK-4 (sulphated cholecystokinin octapeptide and cholecystokinin tetrapeptide) were purchased from Bachem (Bubendorf, Switzerland) and lorglumide from R.B.I. (Natick, MA, U.S.A.). [^{3}H]-propionyl-CCK-8S (specific activity, 60–80 mCi $mmol^{-1}$) was from Amersham (Buckinghamshire, U.K). The other radioligands were: [^{3}H]-flunitrazepam, [^{3}H]-BRL 43694 (granisetron), [^{3}H]-PN 200-110 (isradepine), [^{3}H]-glibenclamide, [^{3}H]-substance P and [^{125}I]-neurotensin (DuPont NEN) and [^{3}H]-saxitoxin (Amersham). Amylase Kit Reagent was from Boeringher Mannheim (Germany). All other drugs and chemicals were from Sigma (St. Louis, MO, U.S.A.).

CCK-8S and CCK-4 were stored as frozen aliquots and diluted to a suitable concentration before each experiment. For binding studies, IQM-95,333, L-365,260, devazepide, PD-135,158 and lorglumide were dissolved in dimethylsulphoxide and stored under the same conditions as CCK. In *in vivo* experiments, the compounds were prepared fresh in a saline solution (0.9% NaCl solution with one or two drops of Tween 80). The volume of administration varied between 0.5–1 ml 100 g^{-1} body weight.

Results

Binding of [^{3}H]-CCK-8S to rat pancreas and cerebral cortex homogenates

The affinity constants (K_d) obtained in Scatchard analysis for [^{3}H]-CCK-8S binding to CCK_A or CCK_B receptors were 0.47 and 1.1 nM, respectively. IQM-95,333, like devazepide, produced a concentration-dependent inhibition of radioligand binding to CCK_A receptors in the rat pancreas. The K_i and Hill coefficients (n_H) values for CCK-8S, IQM-95,333 and selected CCK_A and CCK_B receptor antagonists are depicted in Table 1.

Table 1 Inhibition of [^{3}H]-CCK-8S binding to rat pancreas and cerebral cortex homogenates

	Pancreas		*Cerebral cortex*		*Selectivity cerebral cortex/pancreas*
	K_i (nM)	n_H	K_i (nM)	n_H	
CCK-8S	0.52±0.04	0.91±0.05	2.8±0.15	0.88±0.09	5
IQM-95,333	0.62±0.05	0.99±0.07	>5,000		>8,000
Devazepide	0.3±0.03	0.95±0.04	342±67	0.99±0.08	1,000
Lorglumide	27.7±5.3	0.86±0.06	2,007±80		70
L-365,260	294±25	0.86±0.12	14.7±1.3	1.12±0.18	0.05
PD-135,158	647±73	0.84±0.02	5.9±0.5	0.98±0.09	0.009

Drug displacement studies were performed with seven concentrations in duplicate. Data represent the mean±s.e.mean of 4–6 experiments. K_i: inhibition constant; n_H: Hill coefficient.

The calculated K_i values for IQM-95,333 and devazepide at pancreatic CCK_A receptors were 0.6 and 0.3 nM, respectively, with n_H very close to unity. The affinity of lorglumide and especially of the typical CCK_B receptor antagonists, L-365,260 and PD-135,158, at pancreatic CCK_A receptors was much lower. Drug competition studies in membrane preparations from rat cerebral cortex showed that devazepide displayed a moderate affinity at CCK_B receptors (K_i=342 nM) whereas the affinity of L-365,260 and PD-135,158 at brain receptors was higher. IQM-95,333, 10 μM, only displaced specific [^{3}H]-CCK-8S binding to brain CCK receptors by approximately 20% and it was not considered of interest to test higher concentrations. By considering the ratio $K_i CCK_B/K_i CCK_A$ as an index of selectivity, it was found that devazepide was approximately 1,000 fold more selective at peripheral than at brain CCK receptors. IQM-95,333 was a more selective CCK_A receptor ligand by a factor higher than 8,000.

Other receptor binding studies

IC_{50} values for IQM-95,333 were higher than 10 μM in binding studies to central benzodiazepine, 5-HT_3, neurokinin NK_1 and neurotensin receptors and also in binding to Na^+ channels, Ca^{2+} channels (dihydropyridine sites) and to ATP-sensitive K^+ channels. By use of the higher concentration (10 μM), an appreciable inhibition of specific radioligand binding was only observed in binding to central benzodiazepine receptors and to Ca^{2+} channels, where a percentage inhibition of 35 and 27%, respectively (mean of 3 experiments in duplicate) was found. The IC_{50} values for diazepam and nitrendipine at these two sites were approximately 10 and 1.2 nM. In all other cases, no displacement of specific binding was observed with this high concentration of IQM-95,333.

Antagonism of the contractile response to CCK-8S and CCK-4 in the guinea-pig ileum

CCK-8S produced a transient fast contraction in the LMMP preparation, followed by a slower contraction with a tendency to plateau. CCK-4 produced only the fast initial contraction. Devazepide and IQM-95,333 were the more potent competitive antagonists of the response to CCK-8S, whereas L-365,260 and PD-135,158 were much more potent inhibitors of the response to CCK-4. The calculated pK_B values for these drugs are indicated in Table 2. It can be seen that IQM-95,333 was more selective than devazepide as an antagonist to CCK-8S-induced contraction.

Antagonism to CCK-8S-stimulated amylase release

The dose-response curve for α-amylase release from rat dispersed pancreatic acini by CCK-8S was clearly biphasic. The basal α-amylase secretion was 5.9%. A significant increase was observed with 10 pM CCK-8S, and a maximal effect was found with 0.5 nM (14% of total amylase content). With concentrations higher than 1 nM a lower amylase secretion was obtained.

Table 2 Antagonism of CCK-8S or CCK-4 in the isolated longitudinal muscle myenteric plexus preparation from guinea-pig ileum

	pK_B	
	CCK-8S	*CCK-4*
Devazepide	8.5 (8.4–8.6)	6.3 (6.1–6.4)
L-365,260	5.5 (5.3–5.6)	8.3 (7.6–8.7)
PD-135,158	5.1 (4.7–5.4)	8.1 (7.9–8.3)
IQM-95,333	8.4 (8.1–8.8)	5.7 (5.2–6.1)

Single point analysis. Values are means and 95% confidence limits in parentheses of 4–6 experiments.

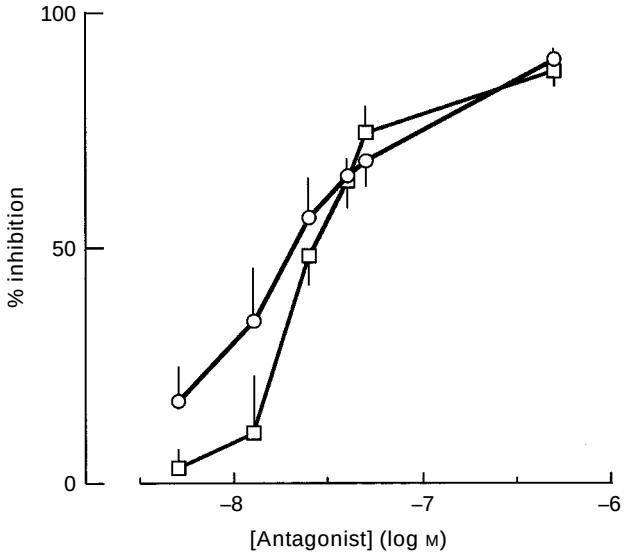

Figure 2 Inhibition by IQM-95,333 (○) and devazepide (□) of amylase release stimulated by CCK-8S (0.5 nM) in dispersed pancreatic acini. Data represent the mean and vertical lines s.e.mean of 5–9 separate experiments in duplicate.

On the basis of this curve, a CCK-8S concentration of 0.5 nM was chosen for the inhibition assays.

Neither the solvent (0.1% dimethylsulphoxide) nor any of the products tested had any effect by themselves on amylase release. Both IQM-95,333 and devazepide caused a concentration-dependent inhibition of CCK-8S-stimulated amylase secretion (Figure 2). The IC_{50} s, calculated by linear regression analysis, were 21.3 nM for IQM-95,333 and 25.4 nM for devazepide.

Reversion of hypophagia induced by CCK-8S

Following vehicle injections, the food consumption in control rats was about 6 g in 30 min (Figure 3). The administration of CCK-8S (8 μg kg^{-1}, i.p.) to food-deprived rats strongly reduced food intake, by 60–75%. Neither IQM-95,333 nor

devazepide (10–100 $\mu g\ kg^{-1}$, each) by themselves modified feeding behaviour, but they prevented the hypophagia induced by CCK when given 30 min before the test meal. The antagonism was significant with doses of both drugs from 50 to 100 $\mu g\ kg^{-1}$. In other experiments, IQM-95,333 and devazepide were given 2 h (instead of 30 min) before the test meal, i.e. 115 min before CCK-8S. At the doses tested (50–100 $\mu g\ kg^{-1}$) the antagonism to the anorexic effect of CCK-8S was still hightly significant (Table 3).

Antagonism of (+)-fenfluramine-induced anorexia

(+)-Fenfluramine, administered at a dose of 1.5 mg kg^{-1}, i.p., apparently devoid of any sedative effect, markedly reduced, by 75–80%, the palatable diet consumption in satiated rats. Devazepide and IQM-95,333 failed to antagonize (+)-fenfluramine-induced anorexia at doses (50–100 $\mu g\ kg^{-1}$, i.p.) effective in the previous hypophagia assays in food-deprived animals (Table 4).

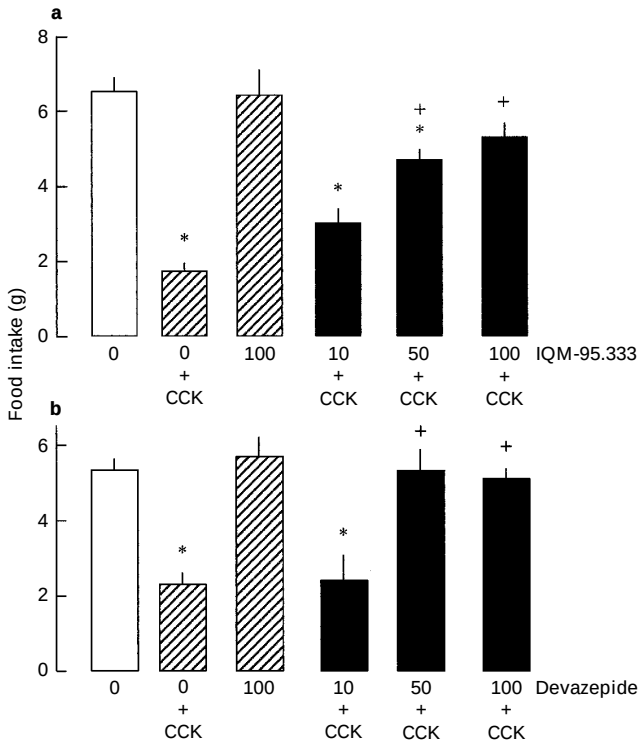

Figure 3 Effect of (a) IQM-95,333 and (b) devazepide on the hypophagia induced by CCK-8S in rats subjected to a food deprivation schedule. Antagonists (doses in $\mu g\ kg^{-1}$, i.p) given 25 min before CCK-8S (8 $\mu g\ kg^{-1}$). Food intake (mean ± s.e.mean of 8–10 animals), was measured 5 min after CCK-8S for 30 min. $^*P<0.01$ vs control; $^+P<0.01$ vs CCK (ANOVA followed by Tukey's test).

Table 3 Effect of IQM-95,333 and devazepide given 2 h before the test meal on the hypophagia induced by CCK-8S in rats subjected to a food deprivation schedule

Treatment ($\mu g\ kg^{-1}$, i.p.)	*Food Intake* (g)
Vehicle	6.0 ± 0.9
CCK-8S (8)	2.1 ± 0.4*
Devazepide(50) + CCK-8S	5.1 ± 0.4$^+$
Devazepide(100) + CCK-8S	5.6 ± 0.7$^+$
IQM-95,333(50) + CCK-8S	4.3 ± 0.3*$^+$
IQM-95,333(100) + CCK-8S	4.9 ± 0.2$^+$

Antagonists were given 115 min before CCK-8S. Food intake (mean ± s.e.mean of 8–10 animals) was measured 5 min after CCK-8S for 30 min. $^*P<0.05$ vs control; $^+P<0.05$ vs CCK-8S (ANOVA followed by the Tukey test).

Spontaneous locomotor activity in rats

CCK-8S, 20 $\mu g\ kg^{-1}$, reduced by approximately 64% the spontaneous motor activity in rats (Figure 4). Both devazepide and IQM-95,333 at low doses (10–50 $\mu g\ kg^{-1}$) were able to counteract CCK-8S-induced hypomotility, devazepide being more potent in this regard. These low doses of the antagonists lacked any intrinsic effect on rat locomotion (not shown). A much higher dose of both drugs (0.5 mg kg^{-1}) was also devoid of any effect on locomotor activity (Figure 4).

Light/dark exploration test in mice

Control animals consistently spent less time in the white compartment on the second day of exposure to the test, whereas anxiolytic treatment resulted in time spent in the white compartment by an animal approaching or higher than 100% of its own control (Table 5). Diazepam induced an anxiolytic effect at the only dose used (1 mg kg^{-1}, i.p.). The CCK_B antagonist L-365,260 induced an anxiolytic-like effect at doses of 10–100 $\mu g\ kg^{-1}$ and the effect of the CCK_A antagonist, devazepide, was only significant at a dose of 100 $\mu g\ kg^{-1}$. In both cases, the dose-response curves had an inverted U shape. IQM-95,333 showed a marked anxiolytic profile within a wide dose range, between 10 $\mu g\ kg^{-1}$ and 5 mg kg^{-1} (Table 5). The higher doses of the three drugs used did not significantly modify spontaneous locomotor activity (not shown).

Punished drinking in rats (Vogel test)

L-365,260, devazepide and IQM-95,333 produced a bell-shaped dose-response curve in this test. With all three drugs, the anxiolytic effect was only significant within a narrow

Table 4 Effect of IQM-95,333 and devazepide on the anorexic effect of (+)-fenfluramine in satiated rats

Treatment ($\mu g\ kg^{-1}$, i.p.)	*Food intake* (g)
Vehicle	13.2 ± 3.0
(+)-Fenfluramine(1,500)	3.3 ± 1.5*
Devazepide(50) + (+)-fenfluramine	3.7 ± 1.3*
Devazepide(100) + (+)-fenfluramine	1.2 ± 0.3*
IQM-95,333(50) + (+)-fenfluramine	3.6 ± 1.0*
IQM-95,333(100) + (+)-fenfluramine	1.6 ± 0.9*

Rats received an i.p. injection of the antagonists 10 min before (+)-fenfluramine. Twenty min later the amount of palatable diet consumed (mean ± s.e.mean of 8 animals) was measured for 30 min. $^*P<0.001$ vs control (ANOVA followed by the Tukey test).

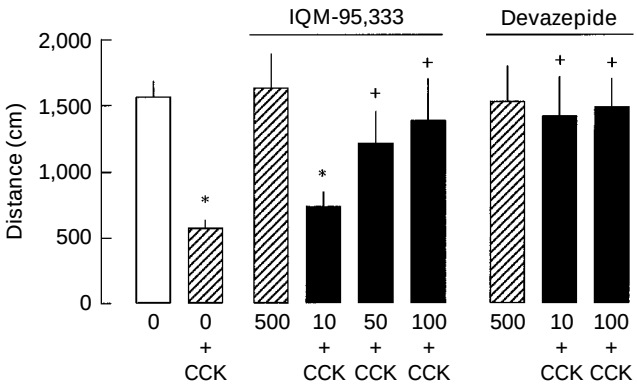

Figure 4 Effect of IQM-95,333 and devazepide on the hypomotility induced by CCK-8S in rats. Antagonists (doses in $\mu g\ kg^{-1}$, i.p.) given 25 min before CCK-8S (20 $\mu g\ kg^{-1}$). Spontaneous locomotor activity (mean ± s.e.mean of 8–10 animals) was measured 5 min after CCK-8S for 30 min. $^*P<0.01$ vs control; $^+P<0.01$ vs CCK (ANOVA followed by Tukey's test).

Table 5 Anxiolytic effect of CCK antagonists and diazepam in the two compartment test in mice

Drug	*Dose* ($\mu g\ kg^{-1}$, i.p.)	*Time in white area* (% change)	*Locomotion in white area* (% change)
IQM-95,333	Vehicle	48.8 ± 5.2	42.3 ± 4.1
	2	48.9 ± 12.2	59.9 ± 19.1
	10	99.4 ± 18.0*	77.5 ± 17.7*
	100	76.9 ± 7.7*	84.5 ± 8.7*
	1,000	69.0 ± 12.8*	101.3 ± 16.5*
	5,000	86.3 ± 16.8*	89.2 ± 17.3*
Devazepide	Vehicle	49.0 ± 5.2	62.2 ± 10.1
	2	52.8 ± 8.2	67.5 ± 14.3
	10	51.9 ± 5.3	49.9 ± 10.9
	100	93.9 ± 15.8*	105.9 ± 14.8*
	1,000	56.6 ± 10.4	81.7 ± 11.2
L-365,260	Vehicle	44.6 ± 4.7	48.0 ± 6.6
	2	43.1 ± 12.6	45.7 ± 8.8
	10	74.3 ± 12.6*	82.0 ± 14.4*
	100	66.7 ± 8.5*	80.5 ± 11.0*
	1,000	57.9 ± 11.9	58.7 ± 15.9
Diazepam	Vehicle	42.6 ± 7.6	45.2 ± 8.2
	1,000	80.1 ± 16.0*	82.7 ± 16.6*

Drugs were given i.p. 30 min before the second exposure to the test. Values (mean ± s.e.mean of 8–10 animals) show the % change produced by the drugs on the behavioural response recorded in the same animals 24 h before. Absolute values (means) for the time spent by vehicle-treated controls in white area on the first and second day were 111 and 51 s, respectively. The corresponding mean values for the locomotion of controls were 42 and 20 crossed lines. *$P<0.05$ vs corresponding vehicle-treated controls (ANOVA followed by Dunnett's test).

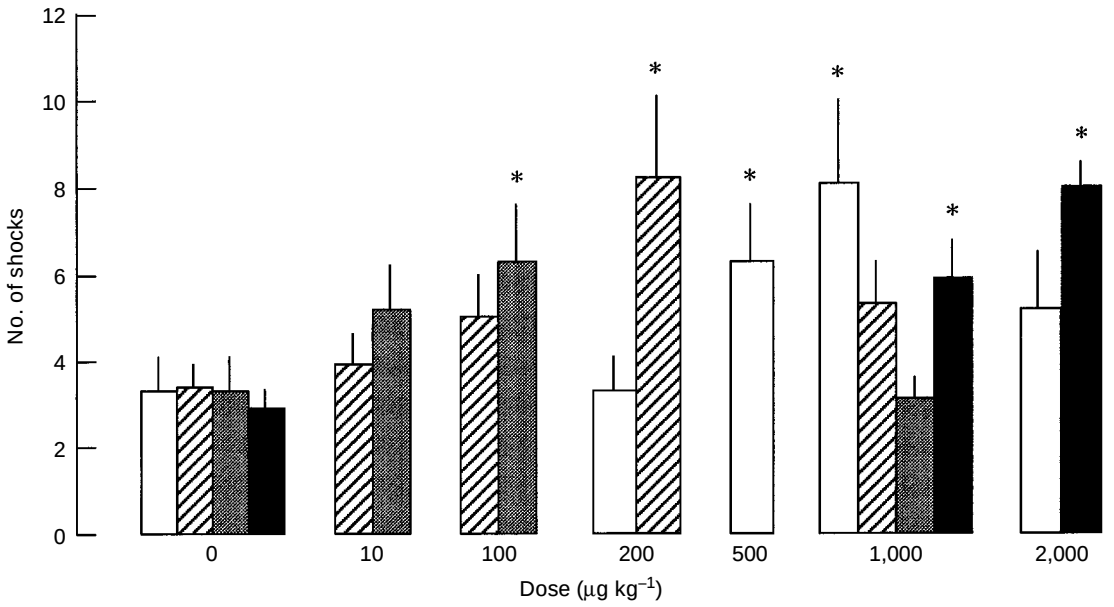

Figure 5 Effect of CCK antagonists and diazepam in the punished-drinking test in rats. Drugs given i.p. 30 min before the 3 min test. Values are the mean ± s.e.mean of 8–10 animals. Drugs: IQM-95,333 (open columns), devazepide (hatched columns), L-365,260 (stippled columns) and diazepam (solid columns). *$P<0.05$ vs corresponding controls (Mann-Whitney U-test).

dose-range and was not apparent after higher doses (Figure 5). The effective dose of L-365,260 (100 $\mu g\ kg^{-1}$) was lower than that of devazepide. IQM-95,333 increased punished drinking at doses higher than devazepide, 500–1,000 $\mu g\ kg^{-1}$.

Discussion

IQM-95,333 was a highly selective CCK_A receptor ligand, with negligible affinity at CCK_B receptors. Experiments in the guinea-pig isolated ileum longitudinal muscle showed that this new pyridopyrimidine derivative was as potent competitive antagonist of the contraction elicited by CCK-8S, with a lower effect on CCK-4, suggesting selective antagonism at CCK_A receptors. IQM-95,333 was also able to prevent amylase release from isolated pancreatic acini, another CCK_A receptor-mediated effect. *In vivo*, this new compound antagonized, like devazepide, the hypophagia and the hypolocomotion induced by CCK-8S. Interestingly, IQM-95,333 was effective in two different animal models of anxiety in rodents, in particular in the light/dark exploration test in mice, in which the anxiolytic-like effect was apparent over a wide dose-range.

In the present study, [^{3}H]-CCK-8S was used for labelling CCK receptors. The dissociation constants obtained for binding to CCK_A and CCK_B receptors were similar to those previously found (Daugé *et al.*, 1990). In other studies with radioiodinated peptides, the K_d for binding to CCK_A and

CCK_B receptors were slightly lower (e.g. Chang & Lotti, 1986; Kuwahara *et al*., 1993; see also review by Silvente-Poirot *et al*., 1993). In binding studies to rat pancreatic CCK_A receptors, IQM-95,333 showed a high affinity, in the nanomolar range, similar to the typical CCK_A antagonist devazepide and approximately 2–3 orders of magnitude higher than lorglumide or the CCK_B antagonists, L-365,260 and PD-135,158. The Hill coefficients for IQM-95,333 and devazepide were not different from unity, indicating a single binding site. Conversely, a high concentration of IQM-95,333 (10 μM) only produced a negligible displacement of [^{3}H]-CCK-8S binding to rat brain membranes. This new compound was thus a more selective CCK_A receptor ligand than many other CCK_A antagonists, including devazepide. In our hands, devazepide was approximately 1,000 fold more selective as a CCK_A receptor ligand. Other studies have found a selectivity from 170 (Hughes *et al*., 1990) to 3,000 (Chang & Lotti, 1986) and intermediate values have also been obtained (Hill & Woodruff, 1990; Akiyama *et al*., 1996; Taniguchi *et al*., 1996). The different species used (Kuwahara *et al*., 1993) and different preparations may account for these discrepancies in selectivity values.

Contractility studies in the LMMP preparation from guinea-pig ileum showed that, like devazepide, IQM-95,333 was a potent antagonist of CCK-8S. In the guinea-pig ileum, CCK-8S appears to contract the ileum, through interaction with a CCK_A receptor whereas CCK-4 acts mainly through a CCK_B/gastrin receptor (Lucaites *et al*., 1991; but see also Dal Forno *et al*., 1992). Accordingly, devazepide was approximately two orders of magnitude more potent as a CCK_A antagonist and IQM-95,333 was as potent as devazepide but still more selective. It should be noted that the pK_B values obtained against CCK-8S were not identical to the pK_i values of the binding assays to peripheral CCK receptors, although the rank of potency for the different drugs was identical. It is possible that CCK_A receptors are not entirely responsible for the contractile effect of CCK-8S in the guinea-pig ileum (Dal Forno *et al*., 1992).

As repeatedly shown (see reviews by Jensen *et al*., 1989; Silvente-Poirot *et al*., 1993; Williams & Blevins, 1993), after the interaction of CCK with cell surface CCK_A receptors in pancreatic acini, the stimulation of amylase release follows a biphasic concentration-response curve, with a stimulating phase that in the present study was observed with concentrations of CCK-8S between 0.01–0.5 nM. It is supposed that the increase and decrease in amylase secretion are due to occupancy of high and low affinity binding sites on the acinar cells, respectively (Jensen *et al*., 1989). The potency of IQM-95,333 and devazepide as inhibitors of CCK-8S-stimulated α-amylase release from pancreatic acini was approximately the same. However, it should be noted that the IC_{50} s were higher than in binding assays to CCK_A receptors from rat pancreas and correlate better with the studies in the guinea-pig ileum. This discrepancy has been noted previously in other studies (e.g. Taniguchi *et al*., 1996) and may be simply due to the different *in vitro* preparations (isolated acini vs whole pancreas homogenates).

Two typical behavioural effects induced by systemic CCK-8S injection, which are known to be selectively reversed by CCK_A antagonists, are satiety (Dourish *et al*., 1989; Khosla & Crawley 1988) and hypomotility (Khosla & Crawley 1988; Soar *et al*., 1989; O'Neill *et al*., 1991). These effects seem to be initiated at the peripheral level but may also include central mechanisms (Crawley *et al*., 1981; Reidelberger *et al*., 1994). Initially, assays with fasted rats were carried out to avoid the influence of endogenous CCK (Hewson *et al*., 1988). The degree of food-intake suppression produced by CCK-8S was similar to that found in other studies (Hewson *et al*., 1988; Eberle-Wang & Simansky, 1992; Poncelet *et al*., 1993). Devazepide and IQM-95,333 significantly blocked CCK-induced hypophagia, the former being slightly more potent. IQM-95,333 also reversed the hypolocomotion induced in rats by CCK-8S and devazepide was again more potent. It is not possible to deduce whether this reflects a lower *in vivo* effect of IQM-95,333 on CCK_A receptors or is rather a consequence of a more reduced bioavailability of this new compound. However, it should be noted, that the metabolic stability of IQM-95,333 appears to be acceptable since its pharmacological action was still significant 2 h after drug administration. This was also the case for devazepide which is considered to be CCK_A antagonist resistant to enzyme degradation (Chang & Lotti, 1986; Khosla & Crawley, 1988).

In the other feeding model with satiated animals, we were not able to replicate the findings of Cooper *et al*. (1990), since devazepide did not antagonize the suppression of feeding induced by (+)-fenfluramine. In our experiments, the dose of (+)-fenfluramine (1.5 mg kg^{-1}) was one half of that used by Cooper *et al*. (1990). In our strain of rats we found that higher (+)-fenfluramine doses produced a marked sedative effect. The different strain of rat used and also the different composition of the palatable diet may account for this discrepancy.

In an animal model predictive of anxiolytic activity, such as the light-dark exploration test (or two-compartment box) in mice, IQM-95,333 was effective over a wide dose range, from 10 μg kg^{-1} to 5 mg kg^{-1}, whereas other CCK receptor antagonists showed this effect within a more limited dose range. It is generally assumed that only CCK_B receptors are involved in the anxiogenic effect of CCK (Hughes *et al*., 1990; Costall *et al*., 1991; Rex *et al*., 1994), although an anxiolytic-like activity of the CCK_A receptor antagonist, devazepide has also been obtained in some studies (Daugé *et al*., 1989; Hendrie & Dourish, 1990; Hendrie *et al*., 1993) and in the two-compartment box the anxiolytic-like profile appears to be more clearly related to antagonism at CCK_A receptors (Hendrie *et al*., 1993). It is always possible that devazepide may also block CCK_B receptors at high doses (Hughes *et al*., 1990), but this should not be the case for IQM-95,333, which was virtually devoid of affinity at brain CCK receptors. This new compound showed a minimal or null affinity at central benzodiazepine or 5-HT_3 receptors, other sites which could account for an anxiolytic activity. According to the present data, it should be supposed that the effect found in the two compartment test is mediated through CCK_A receptor blockade.

In a typical conflict procedure, such as the punished-drinking test, IQM-95,333 and also the CCK_A and CCK_B receptor antagonists, devazepide and L-365,260, respectively, produced an anxiolytic effect within a narrow dose range. In all cases, the drug dose-response curve was biphasic suggesting that different mechanisms are operative at high doses. Other studies have previously addressed the effect of CCK receptor antagonists in conflict procedures, and limited effects or no activity have been found (Singh *et al*., 1991a; Charrier *et al*., 1995). It is possible that the low shock intensity used in the present study determines the increase in punished responses. The potency of IQM-95,333 was much lower in the present conflict paradigm in rats than in the two-compartment test in mice. The latter model is not based on an external aversive stimulus but on the innate rodent aversion to brightly illuminated spaces. Anxiety probably does not represent a unitary concept and animal models of anxiety in use for the predicition of the clinical effect of drugs acting through mechanisms other than benzodiazepines await further clinical validation. It should be noted that there is an analogy between the preclinical pharmacology of CCK-related anxiolytics and that of some 5-HT-related anxiolytics, such as the 5-HT_3 receptor antagonists, ondansetron and tropisetron, which are highly efficacious in the two-compartment test but only moderately active or not active at all in conflict procedures (for review see: Roca *et al*., 1995). Moreover, the dose-response curve for the anxiolytic-like effect of these two classes of compounds is generally biphasic, whereas the effect of the benzodiazepines is dose-related when sedation has still not been reached. This is probably a factor of concern in the clinical development of CCK-related anxiolytics and raises obvious doubts on the predictive value of some animal models of anxiety not invol-

ving a conflict situation. In this regard, the clinical effectiveness of CCK_B antagonists has not been until now unequivocally demonstrated (e.g. Bradwejn *et al*., 1995; Kramer *et al*., 1995). Yet, it is still conceivable that CCK receptor antagonists may be of interest in some but not all of the subgroups of human anxiety.

In summary, IQM-93,333 is a potent CCK_A receptor antagonist with a high *in vitro* selectivity at this CCK receptor subtype and with anxiolytic-like properties in two different animal models. The present results suggest that CCK_A receptors may be involved in the mediation of the anxiolytic effects of this new compound.

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