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VB20B7, a Novel 5-HT-ergic Agent with Gastrokinetic Activity. I. Interaction with 5-HT_3 and 5-HT_4 Receptors

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Abstract

This study describes the in-vitro interaction of the gastrokinetic agent 2[1-(4-piperonyl)piperazinyl]benzothiazole (VB20B7) with the 5-hydroxytryptamine 5-HT_3 and 5-HT_4 receptor subtypes, using functional as well as radioligand binding studies. The benzamide derivative cisapride was used as a comparison.

In radioligand binding assays VB20B7 showed, like cisapride, a weak affinity at 5-HT_3 receptors from rat cerebral cortex. The new compound lacked any affinity at other 5-HT receptors or at dopaminergic D_2 receptors, whereas cisapride showed high affinity for the 5-HT_4 receptors from guinea-pig hippocampus and moderate affinity at dopaminergic D_2 receptors. In the non-stimulated guinea-pig ileum, the concentration-response curves to the specific 5-HT_3 agonist 2-Me-5-HT and to 5-HT were shifted to the right by VB20B7. In the rat oesophagus tunica muscularis mucosae preparation (TMM), VB20B7 was evaluated for its activity at 5-HT_4 receptors. VB20B7 behaved as a 5-HT_4 receptor agonist, inducing a concentration-dependent relaxation of the preparation precontracted with carbachol. In this preparation, VB20B7 and cisapride were able to stimulate adenylate cyclase activity, an effect probably mediated through activation of 5-HT_4 receptors, as can be inferred from the blockade by the 5-HT_4 antagonist, tropisetron, of the enhanced cAMP formation. However, consistent with the lack of affinity at central 5-HT_4 receptors, VB20B7 did not stimulate cAMP formation in guinea-pig hippocampal slices. VB20B7 also caused an increase in the twitch response of the transmurally stimulated guinea-pig ileum, although at a concentration higher than cisapride. This effect was blocked by desensitization of the 5-HT_4 receptor with 5-MeOT and also by the 5-HT_4 receptor antagonist tropisetron. Both VB20B7 and cisapride increased the K^+-evoked acetylcholine release in this preparation.

The results show that VB20B7 possesses affinity for 5-HT_4 receptors located in the rat TMM and guinea-pig ileum preparations, but is devoid of affinity at central 5-HT_4 receptors. In addition, VB20B7 shows low to moderate affinity at both central and peripheral (enteric) 5-HT_3 receptors The interaction of VB20B7 with the peripheral 5-HT_4 and 5-HT_3 receptors may be relevant for the gastrokinetic effects of the new compound.

No less than fourteen 5-HT receptor subtypes have been identified (Zifa & Fillion 1992; Hoyer et al 1994). 5-HT_3 and 5-HT_4 receptor subtypes have been implicated in some of the gastrointestinal effects described for 5-HT. 5-HT_3 receptor is unique among 5-HT receptors in forming directly a ligand-gated ion channel and hence, not being a member of the G-protein receptor 'superfamily'. 5-HT_3 receptors are located exclusively in neurons and are present in both the peripheral and central nervous system (Wallis 1989; Laporte et al 1992). In the gastrointestinal tract, 5-HT_3 receptor activation modulates both secretion (Furman & Waton 1989) and motility (Costall & Naylor 1990) in animal models. With respect to gastrointestinal motility, 5-HT_3 receptor antagonists generally facilitate motor activity although this may depend on the degree of basal tone (Costall & Naylor 1990). Some 5-HT_3 receptor antagonists, such as MDL 72222 (Fozard 1984), ondansetron (Butler et al 1988), granisetron (Sanger & Nelson 1989) and YMY060 (Miyata et al 1991) have been reported to be useful in chemotherapy-induced emesis related to 5-HT_3 receptor activation (Miner et al 1987). In addition, some 5-HT_3 receptor antagonists enhance gastric emptying and facilitate gastrointestinal motility (Nemeth & Gullikson 1989; Craig & Clarke 1991). However, the gastroprokinetic activity is not clearly related to 5-HT_3 receptor antagonism because some potent 5-HT_3 antagonists are devoid of this effect (Cohen et al 1990; King et al 1993).

Prokinetic benzamides show both 5-HT_3 antagonist and 5-HT_4 receptor agonist properties. 5-HT_4 receptor activation stimulates adenylyl cyclase activity in rodent neuronal tissue (Shenker et al 1983; Weiss et al 1986), induces contraction of guinea-pig small intestine (Buchheit et al 1985; Craig & Clarke 1989) and relaxation of rat oesophageal smooth muscle (Reeves et al 1991) and modifies the activity of cardiac muscle both in-vitro and in-vivo (Kaumann et al 1990). In the periphery, 5-HT_4 receptors appear to be involved in alimentary tract pathology. 5-HT_4 receptor agonists, like cisapride, are useful in therapeutics for their gastrokinetic activity.

The aim of the present study was to examine the action of VB20B7, 2[1-(4-piperonyl)piperazinyl]benzothiazole (Fig. 1), a new gastrokinetic agent (Monge et al 1994), on 5-HT receptors, especially on the 5-HT_3 and 5-HT_4 subtypes. The benzamide derivative cisapride was used as a comparison.

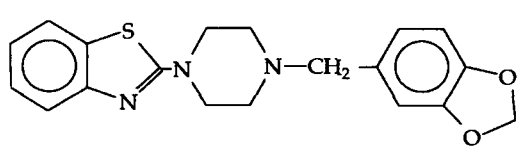

FIG. 1. Chemical structure of VB20B7 (2-[1-(4-piperonyl) piperazinyl] benzothiazole).

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Materials and Methods

Binding to several neurotransmitter receptors

Binding to 5-HT$_1$ receptors. Binding to the [^{3}H] 5-HT-labelled 5-HT$_1$ receptor class from rat cortical membranes was performed using a previously described procedure (Peroutka et al 1979). The tissue was homogenized in 20 vol of 50 mM Tris-HCl and centrifuged at 45 000 g for 10 min. The supernatant was discarded and the pellet was rehomogenized in the same buffer. The tissue homogenate was then incubated at 37°C for 10 min before being subjected to a second 10-min centrifugation. The final pellet was resuspended in 100 vol of buffer containing 0·1% ascorbic acid and 10 μM pargyline. The final mixture consisted of 0·1 mL of 0·3 nM [^{3}H] 5-HT (30 Ci mmol^{-1}, NEN), 0·1 mL of buffer or varying concentrations of test compounds and 0·8 mL of tissue suspension. Following incubation at 37°C for 10 min, the tubes were rapidly filtered and washed twice with 5 mL of buffer. Radioactivity was determined by liquid scintillation counting.

Binding to 5-HT$_{2A}$ receptors. Binding to 5-HT$_{2A}$ receptors was studied as described by Leysen et al (1982) with some modifications. Frontal cortex tissue obtained from male Wistar rats (180–220 g) was homogenized in 20 vol of ice-cold Tris-HCl buffer (50 mM, pH 7·4, 4°C). The homogenate was washed by centrifugation (40 000 g, 10 min) and the pellet suspended in 10 vol of cold buffer and centrifuged again. The final pellet was suspended in 400 mL of buffer Tris-HCl 50 mM. Displacement assays consisted of 25 μL of [^{3}H] ketanserin (0·5 nM, NEN), 25 μL of displacing drug and 500 μL of tissue. The incubation (37°C, 15 min) was terminated by rapid filtering through Whatman GF/C filters. Unlabelled standard drug was cyproheptadine.

Binding to 5-HT$_3$ receptors. Binding of [^{3}H] BRL 43694 to 5-HT$_3$ receptors from rat cerebral cortex homogenates was performed according to a previously described method (Nelson & Thomas 1989). Whole cerebral cortex tissue was obtained from male Wistar rats (180–220 g) and homogenized in 10 vol of ice-cold HEPES buffer (50 mM, pH 7·5). The homogenate was centrifuged at 50 000 g for 10 min and the pellet washed and centrifuged two additional times. The final pellet was suspended in 10 vol of HEPES buffer. Displacement studies were performed with 1 nM [^{3}H] BRL 43694 (final concentration) and 8 different concentrations of test compounds. The incubation (23°C for 30 min) was terminated by rapid filtering through GF/C filters using a Brandel Cell Harvester. The filters were rinsed immediately and measured by liquid scintillation counting.

Binding to 5-HT$_4$ receptors. Binding of [^{3}H] GR 113808 to 5-HT$_4$ receptors from guinea-pig hippocampus was performed as previously described (Grossman et al 1993). Tissue obtained from male albino guinea-pigs (300–400 g) was homogenized in 15 volumes HEPES buffer (50 mM, pH 7·4, 4°C). The homogenate was centrifuged at 48 000 g for 10 min. The final pellet was resuspended in 10 vol of HEPES buffer. Displacement assays were performed with 400 μL of [^{3}H] GR 113808 (0·1 nM, NEN), 200 μL of displacing drug and 100 μL of tissue. Samples, run in triplicate, were incubated at 37°C for 20 min. The incubation was terminated by rapid filtering through Whatman GF/C filters using a Brandel Cell Harvester. Filters were rinsed and measured by liquid scintillation counting.

Binding to D$_2$ receptors. Binding of [^{3}H] spiroperidol (Amersham) to dopamine D$_2$ receptors from rat striatum was performed as described (Leysen et al 1978) with small modifications. Striata were homogenized in 50 mM Tris-HCl buffer (pH 7·7) and centrifuged at 40 000 g for 15 min. The pellet was washed twice and resuspended in 100 vol of the same buffer containing 120 mM NaCl and 5 mM KCl. The incubation tubes contained 0·2 mL of the tissue suspension, 25 μL of the labelled ligand (0·1 nM) and 25 μL of varying concentrations of test compounds. Samples, run in triplicate, were incubated at 37°C for 10 min, filtered and rinsed 4 times with the same buffer.

Rat oesophageal tunica muscularis mucosae (TMM) preparation

Effect of 5-HT agonists on the precontracted preparation. Male Wistar rats (200–300 g) were killed by decapitation and a 2-cm segment of intrathoracic oesophagus, proximal to the diafragma, was excised and placed in Tyrode's solution of the following composition (mM): NaCl (136), KCl (2·7), $CaCl_2$ (1·8), $MgCl_2$ (1·05), NaH_2PO_4 (0·42), $NaHCO_3$ (11·9), glucose (5·5), pH 7·4. The external muscularis propria, containing the outer longitudinal and circular muscle layers of the oesophagus was carefully removed to isolate the inner smooth muscle tube of the tunica muscularis mucosae as described by Baxter et al (1991). The strips were suspended in a 10-mL tissue bath containing Tyrode's solution at 37°C aerated with 95% O_2/5% CO_2, under 0·25 g tension and equilibrated for 60 min. In all assays, pargyline (10^{-4} M), cocaine (3×10^{-5} M), corticosterone (3×10^{-5} M) and methysergide (10^{-6} M) were included in the Tyrode's solution. Responses were recorded isometrically using UF-1 transducers coupled to a Panlab polygraph. Concentration-effect curves were obtained after contracting the rat oesophagus with carbachol (3×10^{-6} M). Only two concentration–effect curves to 5-HT were constructed per tissue since the third curve was not reproducible (Reeves et al 1991). Responses to the cumulative addition of agonists were expressed as percentage relaxation of the carbachol-induced tone. A complete concentration–effect curve to 5-HT was obtained with a maximum relaxation of approximately 80% of the initial contraction. The ability of drugs to relax oesophagus was expressed both in absolute terms, as EC50 relative to their individual maxima, and in terms of their relative potency versus 5-HT. Potency relative to 5-HT was calculated from experiments in which two concentration-effect curves were constructed in the same preparation, the first to 5-HT itself and the second to a test compound. The relative potency of agonists was then expressed as equipotent concentration ratios (ECR) measured at the 40% inhibition point (IC40) of the carbachol-induced contraction. ECR = IC40 test agonist/IC40 5-HT. The EC50 and confidence limits were estimated with Graph-Pad software. Intrinsic activities were calculated relative to the maximal response to 5-HT.

Stimulation of cAMP formation. Male Wistar rats (250–300 g) were used. Each experiment was performed with 8–10 oesophagi dissected free of the propria. The underlying tunica muscularis mucosae was cross-chopped (350 μm) as described

by Ford et al (1992). The slices were placed in Tyrode's solution and digested with collagenase (2·5 mg mL^{-1}) for 20 min. Subsequently, the slices were suspended for 30 min in a Tyrode's solution that contained cocaine (3×10^{-5} M), corticosterone (3×10^{-5} M), pargyline (10^{-4} M) and methysergide (10^{-6} M), and were incubated with [^{3}H] adenine (2 Ci mL^{-1}) for 40 min at 37°C. After washing with Tyrode's solution (3–4 times), 100-μL aliquots of stirred slices were pipetted into minivials containing 3-isobuthyl-1-methylxanthine (IBMX, 1 mM), and buffer (to 290 μL) and were shaken at 37°C for 30 min. Test agonist or vehicle (10 μL) was then added for 15 min and the reaction was terminated by addition of 30 μL ice-cold HCl (2·2 M). Approximately 2000 counts min^{-1} [^{14}C] cAMP was added to each assay tube to estimate recovery of the nucleotide. [^{3}H] cAMP formed was extracted and isolated on a single column of acidic alumina (degree 1) and dragged down with 4 mL of ammonium acetate (pH 7). Radioactivity was determined with a liquid scintillation counter for both ^{3}H and ^{14}C. [^{3}H]cAMP counts were normalized with the recovery in each column. Concentration-effect curves for 5-HT, VB20B7 and cisapride were expressed as percentage stimulation above basal levels. Moreover, the antagonism by tropisetron (10^{-5} and 10^{-4} M), added to the bath for 30 min, on cAMP formation was studied.

Stimulation of cAMP formation in slices from guinea-pig hippocampus

The method described by Lefebvre et al (1992) with some modifications was used. Aliquots of 50 μL of hippocampal slices (350 × 350 μm) were incubated for 10 min with different drugs in a total volume of 500 μL. The reaction was stopped with 500 μl of trichloroacetic acid (10%). After homogenizing and centrifuging the content of each tube at 10 000 g for 10 min, a 750-μL aliquot was taken from the supernatant for the determination of the cAMP levels by means of radioimmunoassay, using a commercial antibody (Amersham, RPA 509).

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

Guinea-pigs of either sex weighing 300–400 g were stunned by a blow to the head and bled. The ileum was excised, approximately 10 cm from the ileo-cecal junction, and longitudinal muscle strips with the myenteric plexus attached (LMMP) were prepared as described by Paton & Zar (1968). LMMP strips were suspended in a 10-mL organ bath containing Tyrode's solution (composition in mM: NaCl, 136; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 1·05; NaH_2PO_4, 0·42; $NaHCO_3$, 11·9; and glucose, 5·5; pH 7·4), aereated with 95% O_2/5% CO_2 and maintained at 37°C. Methysergide (10^{-6} M) was always present in the solution. Contractile responses were isometrically recorded with a resting tension of 0·5 g.

To study the antagonist effect of the drugs on the response evoked by 2-Me-5-HT or 5-HT, the compounds were added to the bath and left in contact with the tissue for 30 min before the second of two consecutive concentration-response curves to 2-Me-5-HT or 5-HT. In the case of the response to 5-HT, Tyrode's solution containing methysergide (10^{-6} M) included the 5-HT_4 receptor agonist 5-MeOT (10^{-5} M) to eliminate the first phase of the concentration-response curve, due to the stimulation of 5-HT_4 receptors. pA_2 values for all compounds were calculated using the following equation (MacKay 1978): $pA_2 = -\log$ [antagonist concentration] $+ \log$ [DR − 1], where DR represents the agonist dose ratio.

Electrically stimulated LMMP preparation. Longitudinal muscle strips were suspended in a 10-mL tissue bath containing a physiological solution (pH 7·4; 37°C; composition in mM: NaCl, 127; KCl, 3·8; $CaCl_2$, 2·6; $MgCl_2$, 1·2; NaH_2PO_4, 1.1; $NaHCO_3$, 25; NaH_2PO_4, (1·1), glucose, 10·8; and methysergide 0·001 mmol L^{-1}, and aereated continuously with 95% O_2/5% CO_2 under a 0·5-g tension. Electrical field stimulation (0·2 Hz, 1 ms, 60% maximal voltage) was delivered by platinum electrodes to evoke the cholinergically-mediated twitch response. When responses were stabilized, the effect of increasing concentrations of drugs on the basal twitch was tested. The effect of desensitization with 5-MeOT (5×10^{-7} M) and the antagonism by tropisetron (10^{-6} M), both added to the bath for 30 min, on the response to test compounds were also studied. The contractions were recorded isometrically using a TRI 110 transducer coupled to a LETICA 2006 polygraph. The response was referred to changes in the basal tone and in the amplitude of the twitch response. Drug-induced changes in twitch height were expressed as percentage of the control contraction taken as 100% response.

[^{3}H]Acetylcholine release. LMMP preparations were cut into 500-μm slices and washed out three times with Tyrode's solution containing hemicholinum-3 (10^{-6} M) and methysergide (10^{-6} M). After washout slices were incubated with [^{3}H] choline (7 μCi mL, Amersham) for 1 h at 37°C, continually gassed with 95% O_2/5% CO_2 and shaken. Subsequently, the tissue was washed out 3 times and placed in the chambers of a Brandel Superfusion 1000 apparatus. After a stabilization period of 1 h, samples were collected at 3-min intervals at a flow rate of 0·45 mL min^{-1}. At 12 min (S_1) and 45 min (S_2) after the beginning of the collection period, the slices were depolarized by changing the superfusion fluid for 6 min to a Tyrode's solution containing 50 mM KCl. All test drugs were added 15 min before S_2. The tritium content of the samples was measured by adding 3 mL of scintillation liquid (Biogreen Scharlau) to each sample, and radioactivity was determined by liquid scintillation spectrometry. Results, reported as S_2/S_1 ratio are expressed as mean ± s.e.m. and as a percentage increase over basal release.

Drugs

VB20B7 (2[1-(4-piperonyl)piperazinyl]benzothiazole) was synthesized at the Department of Organic Chemistry, CIFA, University of Navarra. Other drugs and chemicals were obtained from the sources indicated: cisapride, ondansetron (synthesized by Vita Laboratories, Spain); 2-methyl-5-HT, methysergide (RBI, USA); cocaine, corticosterone, hemicholinium-3, 5-HT, IBMX, 5-methoxytryptamine, pargyline (Sigma, USA); tropisetron (gift from Sandoz, Switzerland); [^{3}H]-adenine, [^{3}H]-cAMP, [^{3}H]-BRL 43694, [^{3}H]-choline, [^{3}H]-GR 113808, [^{3}H]-5-HT, [^{3}H]-ketanserin (NEN, USA); [^{3}H]-spiroperidol (Amersham, UK).

Results

Binding to several neurotransmitter receptors

VB20B7 was able to displace at concentrations below 10^{-6}M the binding of [^{3}H] BRL43694 to rat 5-HT$_3$ receptors from cerebral cortex homogenates. The displacing potency of VB20B7 was approximately the same as that of cisapride or metoclopramide whereas the 5-HT$_3$ receptor antagonist ondansetron was more potent by one order of magnitude (Table 1). Metoclopramide and cisapride, unlike VB20B7, also displaced the binding of [^{3}H]spiroperidol to D_2 dopamine receptors, although they were weaker than the typical antagonist haloperidol. VB20B7 did not show any affinity at 5-HT$_1$, 5-HT$_2$ or 5-HT$_4$ receptors, whereas cisapride displaced the binding of [^{3}H]GR 113808 to guinea-pig 5-HT$_4$ receptors from hippocampus homogenates with an IC50 of 5×10^{-8}M.

Rat oesophageal tunica muscularis mucosae *(TMM)*

Carbachol, 3×10^{-6}M, induced a tonic contraction with a non-significant decrease in tension after the 20-min equilibration period. 5-HT, the selective 5-HT$_4$ agonist 5-MeOT, VB20B7 and cisapride induced a concentration-dependent relaxation of the carbachol-induced tone (Fig. 2). The highest relaxation was reached with 5-HT 10^{-6}M or with cisapride and VB20B7, both at 10^{-5}M. The latter two drugs produced a slower maximum relaxation (Fig. 3). The pEC50 values for 5-HT, 5-MeOT, cisapride and VB20B7 are depicted in Table 2. The relative potency of VB20B7, expressed as ECR value, was lower than that of cisapride. To investigate whether this effect was mediated by 5-HT$_4$ receptors, VB20B7 was tested during the same experiment after a 100-min incubation period with 10 μM 5-MeOT, which abolished the relaxant effect of VB20B7 (data not shown).

Stimulation of cAMP formation in the TMM

VB20B7 stimulated the production of [^{3}H] cAMP in slices from rat oesophageal tunica muscularis mucosae (Fig. 4). The stimulation was concentration-dependent to a maximum of approximately 170% above basal levels. Potencies for 5-HT, cisapride and VB20B7 (expressed as pEC50) were $6{\cdot}70 \pm 0{\cdot}46$, $6{\cdot}50 \pm 0{\cdot}28$ and $6{\cdot}64 \pm 0{\cdot}29$ respectively.

Tropisetron ($10^{-5} - 10^{-4}$M) inhibited in a concentration-dependent manner the formation of cAMP induced by 5-HT, cisapride or VB20B7 (10^{-5}M) in slices from rat oesophagus. The results obtained are listed in Table 3. At the higher concentration tested, tropisetron fully or almost fully blocked 5-HT or VB20B7 effect respectively whereas the action of cisapride was only inhibited by approximately 70%.

Stimulation of cAMP formation in slices from guinea-pig hippocampus

5-HT and cisapride, 10^{-5}M each, were able to enhance the cyclic nucleotide levels (149 ± 13 and 153 ± 16% stimulation over basal levels respectively, $n = 6$). The same concentration of VB20B7 did not significant stimulate cAMP formation (110 ± 12% enhancement over basal levels, $n = 6$).

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

After incubation with VB20B7, cisapride or the 5-HT$_3$ receptor antagonist ondansetron, the concentration-response curve to the 5-HT$_3$ agonist 2-Me-5-HT ($5 \times 10^{-6} - 10^{-4}$) in the non-stimulated LMMP isolated preparation was displaced to the right. The pA_2 value for VB20B7 against 2-Me-5-HT was in the same range of ondansetron, whereas cisapride was by more than two orders of magnitude more potent than either VB20B7 or ondansetron (Table 4). The concentration-response curve ($10^{-6} - 10^{-4}$M) to 5-HT in presence of 5-MeOT (10^{-5}M) was displaced to the right after incubation with test compounds. pA_2 values for VB20B7 and cisapride were 5·77 and 7·86 respectively (Table 4).

Cisapride and VB20B7 were examined for their ability to enhance the neurogenic twitch response in the field-stimulated LMMP preparation from guinea-pig ileum. Cisapride produced a biphasic effect, with an increase of the twitch response at concentrations up to 1 μM, while the response tended to decrease at higher concentrations. VB20B7 was able to enhance the twitch response, and produced the highest response, about 50%, when added at a concentration of 5×10^{-5}M (Fig. 5). This concentration of VB20B7 was selected to determine whether or not tropisetron (a 5-HT$_4$ receptor antagonist at high concentrations) was able to modify the response. The results obtained, depicted in Table 5, show that tropisetron (10^{-6}M) inhibited the contraction elicited by VB20B7 (5×10^{-5}M) by 52%. In order to confirm the possible involvement of 5-HT$_4$ receptors in the response to VB20B7 in this preparation, a previous receptor desensitization by incubation with 5-MeOT (5×10^{-7}M) was carried out. The effect of VB20B7 (5×10^{-5}M) was then inhibited by 75% (Table 5). However, previous desensitization with 2-Me-5-HT did not affect the response to VB20B7 (data not shown).

Table 1. Displacement of radioligand binding (IC50, M) to different 5-HT receptor subtypes and to D_2 dopamine receptors.

Drug	5-HT$_1$ [^{3}H]5-HT	5-HT$_2$ [^{3}H]ketanserin	5-HT$_3$ [^{3}H]BRL 43694	5-HT$_4$ [^{3}H]GR 113808	D_2 [^{3}H]spiroperidol
VB20B7	$> 10^{-5}$	$> 10^{-5}$	$9{\cdot}5 \times 10^{-7}$	$> 10^{-5}$	$> 10^{-5}$
Cisapride	—	—	$9{\cdot}0 \times 10^{-7}$	5×10^{-8}	$6{\cdot}8 \times 10^{-7}$
Metoclopramide	—	—	$6{\cdot}1 \times 10^{-7}$	—	$5{\cdot}0 \times 10^{-7}$
Ondansetron	$> 10^{-5}$	—	$4{\cdot}6 \times 10^{-8}$	—	—
5-HT	$6{\cdot}3 \times 10^{-9}$	—	—	—	—
Cyproheptadine	—	$2{\cdot}0 \times 10^{-9}$	—	—	—
Haloperidol	—	—	—	—	$7{\cdot}7 \times 10^{-8}$

Results are means of 2–4 experiments with 6–8 concentrations of drugs in triplicate. The source of tissue was the following: 5-HT$_1$ and 5-HT$_2$ binding: rat frontal cortex; 5-HT$_3$ binding: rat cerebral cortex; 5-HT$_4$ binding: guinea-pig hippocampus; D_2 binding: rat striatum.

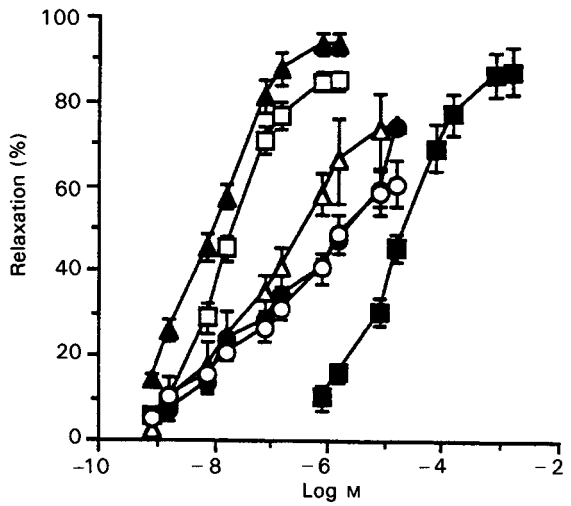

FIG. 2. Cumulative concentration–effect curves of VB20B7 and other 5-HTergic agents in rat oesophageal tunica muscularis mucosae precontracted with carbachol. (□) 5-HT, (■) 2-Me-5-HT, (▲) 5-MeOT, (△) cisapride, (●) VB20B7. Data are means ± s.e.m. from 4–8 experiments.

[^{3}H]Acetylcholine release. Stimulation with a high K^+ concentration, 50 mM, caused [^{3}H]ACh release from LMMP slices into the superfusate. In control experiments the S_2/S_1 ratio was 0·96 ± 0·09 (n = 8). 5-MeOT enhanced [^{3}H]ACh release in a concentration-dependent manner in a range of $10^{-7} - 10^{-4}$ M (data not shown). VB20B7 produced a linear increase in [^{3}H]ACh release at concentrations between $10^{-5} - 10^{-4}$ M. At 5×10^{-5} M S_2/S_1 ratio was 1·67 ± 0·21 (n = 6), that is, an approximate 74% increase in [^{3}H]ACh release. Cisapride also enhanced the tritium efflux by approximately 48% at a concentration of 5×10^{-7} M. However, in close analogy to the effects found in the electrically-stimulated guinea-pig ileum, a 10^{-5} M concentration of cisapride, did not produce a significant releasing effect.

Discussion

This study combines receptor binding studies in central tissues with functional responses in guinea-pig isolated small intestine and tunica muscularis mucosae of rat oesophagus to characterize the interaction of VB20B7 with central and peripheral 5-HT_3 and 5-HT_4 receptors. The results show that VB20B7 is a

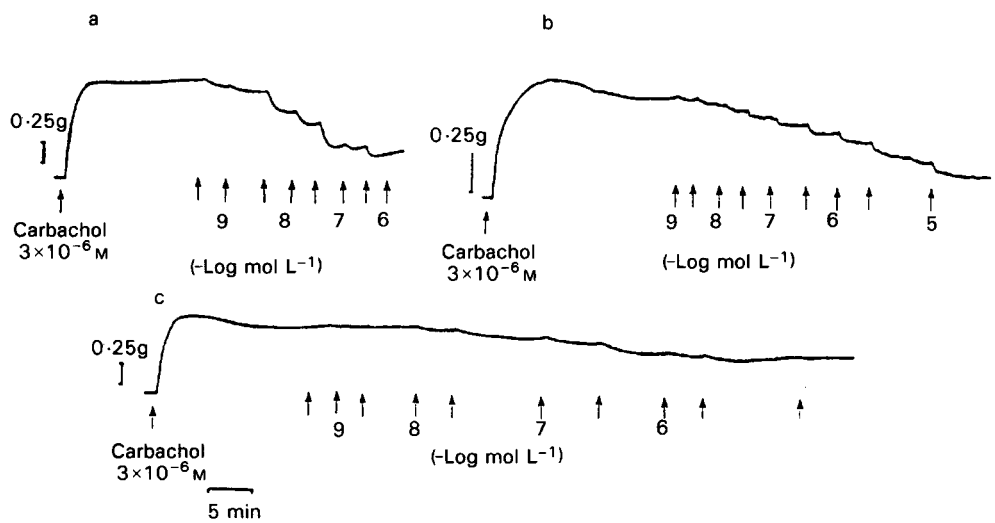

FIG. 3. Cumulative concentration–effect curves to (a) 5-HT (5×10^{-10} M – 10^{-6} M), (b) VB20B7 (10^{-9} M – 10^{-5} M) and (c) cisapride (5×10^{-10} M – 10^{-5} M) in rat oesophageal tunica muscularis mucosae precontracted with carbachol (3×10^{-6} M).

Table 2. Agonist potency in the rat oesophageal tunica muscularis mucosae.

Agonist	pEC50 (95% CL)	n	Intrinsic activity	ECR
5-HT	7·91 (7·85–7·96)	31	1·00	1
5-MeOT	8·08 (7·99–8·17)	4	1·20	0·26
2-Methyl-5-HT	4·90 (4·78–5·04)	8	1·00	643
Cisapride	7·23 (6·86–7·06)	8	0·92	7
VB20B7	6·58 (6·28–6·89)	8	0·91	22
Metoclopramide	6·98 (6·80–7·18)	7	0·83	6·8

ECR=IC40 test agonist/IC40 5-HT. These values were limited to experiments in which concentration-effect curves for both agonists were obtained in the same preparation.

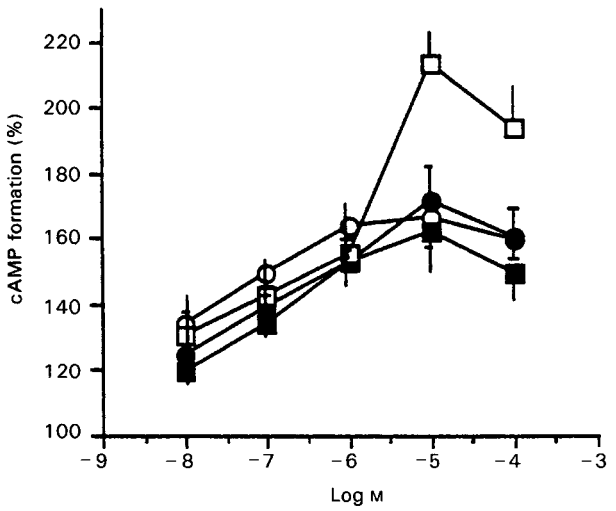

FIG. 4. Stimulation of cyclic AMP formation in slices from rat oesophageal tunica muscularis mucosae by VB20B7 and other 5-HT-ergic agents. (○) 5-HT, (●) cisapride, (■) metoclopramide, (□) VB20B7. Data are means ± s.e.m. from 5–8 experiments.

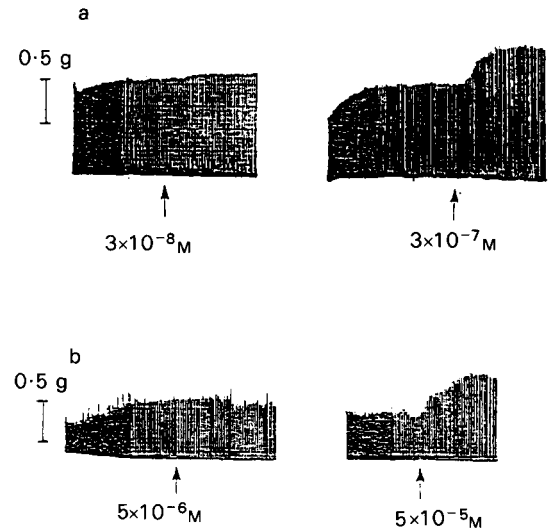

FIG. 5. Twitch responses obtained by transmural electrical stimulation of the LMMP preparation from guinea-pig ileum (0·2 Hz ms^{-1}; 60% of the maximal twitch). Effect of (a) cisapride and (b) VB20B7.

Table 3. Inhibition by tropisetron of cAMP formation induced by 5-HT, cisapride or VB20B7 (10^{-5} M), in rat oesophageal tunica muscularis mucosae.

Dose of tropisetron	% Formation of cAMP induced by		
(μM)	5-HT	Cisapride	VB20B7
10	58 ± 5*	49 ± 5*	60 + 5*
100	2 ± 0***	35 ± 4*	9 ± 1***

Data are means ± s.e.m. (n = 4; triplicate experiments) expressed as percentage inhibition. *$P < 0.05$, ***$P < 0.001$ (unpaired t-test).

Table 5. Contractile effect of VB20B7 (5×10^{-5} M) on the electrically-stimulated LMMP preparation of guinea-pig ileum with or without tropisetron or 5-MeOT.

	% Increase in twitch response
VB20B7 without tropisetron	78 ± 20
+Tropisetron (5×10^{-6} M)	33 ± 2*
VB20B7 without 5-MeOT	80 ± 3
+5-MeOT (5×10^{-7} M)	20 ± 6***

Data are means ± s.e.m., n = 4. *$P < 0.05$, ***$P < 0.001$ vs controls (paired t-test).

Table 4. Antagonism to 2-Me-5-HT and 5-HT in the non-stimulated LMMP preparation from guinea-pig ileum.

Drug	pA_2 2-Me-5-HT	5-HT
VB20B7	6·53	5·77
Cisapride	9·00	7·86
Ondansetron	6·90	—
Tropisetron	8·40	—

weak 5-HT$_3$ receptor antagonist and a peripheral 5-HT$_4$ receptor agonist. In keeping with this receptor profile, VB20B7 appears to be a gastrokinetic agent in rats and dogs (García-Garayoa et al 1996).

In radioligand binding studies to homogenates from rat cerebral cortex, VB20B7 was a weak displacer of [^{3}H]BRL43694 binding to 5-HT$_3$ receptors with a potency similar to cisapride and lower than that of the 5-HT$_3$ receptor antagonist ondansetron. VB20B7 lacked any affinity at other 5-HT receptors subtypes or, at variance with cisapride, at dopaminergic D_2 and 5-HT$_4$ receptors. The results on D_2 receptors obtained for cisapride are in good agreement with those previously reported (Karasawa et al 1990; Yoshida et al 1991; Wiseman & Faulds 1994).

In the isolated longitudinal muscle myenteric plexus (LMMP) preparation from guinea-pig ileum 5-HT produced a biphasic contractile curve by interacting with 5-HT$_4$ receptors (first phase) and 5-HT$_3$ receptors (second phase) (Craig et al 1990; Hagihara et al 1994). In this preparation, VB20B7 behaved as an antagonist to the contractions elicited by 5-HT or by the specific 5-HT$_3$ agonist 2-Me-5-HT but was weaker than either cisapride or the typical 5-HT$_3$ antagonist ondansetron. The affinities for 5-HT$_3$ receptors in radioligand binding were not the same as those found in LMMP from guinea-pig ileum for the different compounds tested. The lack of correlation with binding studies suggests the existence of possible species-dependent 5-HT$_3$ receptor subtypes (Richardson & Engel 1986; Kilpatrick et al 1990; Hagihara et al 1994; Wong et al 1995).

The more usual isolated preparation to quantify the actions of 5-HT$_4$ receptor agonists and antagonists is the isolated tunica muscularis mucosae (TMM) from rat oesophagus. In this preparation, both the potency and intrinsic activity for VB20B7 were in the range of other 5-HT$_4$ receptor agonists,

such as cisapride, and were one order of magnitude lower than those of the selective 5-HT_4 agonist 5-MeOT. The effect of VB20B7 in this preparation can be mediated by the activation of 5-HT_4 receptors, as the desensitization of the receptor with 5-MeOT abolished the response to VB20B7.

As previously suggested (Ford et al 1992; Moummi et al 1992), the activation of the 5-HT_4 receptor mediates relaxation of rat oesophageal tunica muscularis mucosae via cAMP generation. Biochemically, the second messenger coupling of 5-HT_4 receptors in the TMM was shown to be the same as that described in brain and in mammalian heart. Thus, 5-HT and cisapride have been described to stimulate cAMP production in TMM preparations, an effect blocked by tropisetron (Moummi et al 1992). In keeping with the above profile, VB20B7 markedly stimulated cAMP formation in slices from rat oesophageal tunica muscularis mucosae. The stimulation was concentration-dependent and was again in the range of 5-HT or cisapride (cf. Ford et al 1992). Furthermore, the 5-HT_4 antagonist tropisetron significantly inhibited the stimulation of cAMP formation induced by 5-HT, cisapride and VB20B7. However, in keeping with the lack of affinity of VB20B7 for central 5-HT_4 receptors, this compound was not able to stimulate cAMP formation in slices from guinea-pig hippocampus, at variance with other 5-HT_4 receptor agonists such as 5-HT and cisapride.

In the field-stimulated LMMP preparation, the gastrokinetic agent cisapride and the compound VB20B7 enhanced the magnitude of the electrically-induced contraction. As previously reported, cisapride at high concentrations tended to decrease the twitch response (Linnik et al 1991). The enhancement in the neurogenic twitch response, without change in the basal tone, has been related to 5-HT_4 receptor stimulation (Dumuis et al 1988; Craig & Clarke 1990). Moreover, desensitization of 5-HT_4 receptors with 5-MeOT, an approach previously used to characterize 5-HT_4 agonist (Schuurkes et al 1985; Rizzi et al 1992) attenuated the effect of VB20B7 on the electrically-stimulated LMMP preparation. The weak 5-HT_4 receptor antagonist tropisetron also reduced the response to VB20B7.

The prokinetic benzamides, such as cisapride and metoclopramide, are known to augment the electrically-induced release of acetylcholine in the guinea-pig ileum (Buchheit et al 1985; Kilbinger et al 1995). This effect has been related to the stimulation of 5-HT_4 receptors and may be responsible for the gastrokinetic activity of these compounds. In order to confirm that the stimulant effect of cisapride and VB20B7 on the gastrointestinal tract was 5-HT_4 receptor-mediated, the release of acetylcholine induced by both compounds was studied. The results obtained showed an increase in the release of [^{3}H]ACh, consonant with the increase obtained in the twitch response of the LMMP preparation.

The results obtained in rat oesophagus and in LMMP from guinea-pig ileum, where VB20B7 acted as a 5-HT_4 receptor agonist, do not correlate with the lack of affinity for 5-HT_4 receptors observed in binding studies to guinea-pig hippocampus or with the inability to stimulate cAMP formation in the same brain region. These differences may be due to tissue-dependent or species-dependent differences in 5-HT_4 receptors (Ford & Clarke 1993). According to the present results, it seems that VB20B7 is an agonist at 5-HT_4 receptors located in the enteric but not in the central nervous system. The differences found in agonist activity at central and peripheral tissues have led to the consideration of the possible existence of 5-HT_4 receptor subtypes (Kaumann et al 1991; Ford & Clarke 1993; McLean et al 1995).

In summary, it can be concluded that VB20B7 has affinity at peripheral 5-HT_4 receptors, as shown in functional studies in the TMM and field-stimulated LMMP preparations, but is devoid of affinity at central 5-HT_4 receptors. VB20B7 seems to be also a 5-HT_3 receptor antagonist in the LMMP preparation. These mechanisms may contribute to explain the gastrokinetic properties of VB20B7 in rats and dogs.

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