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The in vitro pharmacological profile of prucalopride, a novel enterokinetic compound

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Abstract

Prucalopride is a novel enterokinetic compound and is the first representative of the benzofuran class. We set out to establish its pharmacological profile in various receptor binding and organ bath experiments. Receptor binding data have demonstrated prucalopride's high affinity to both investigated 5-HT_4 receptor isoforms, with mean pK_i estimates of 8.60 and 8.10 for the human 5-HT_{4a} and 5-HT_{4b} receptor, respectively. From the 50 other binding assays investigated in this study only the human D_4 receptor (pK_i 5.63), the mouse 5-HT_3 receptor (pK_i 5.41) and the human σ_1 (pK_i 5.43) have shown measurable affinity, resulting in at least 290-fold selectivity for the 5-HT_4 receptor. Classical organ bath experiments were done using isolated tissues from the rat, guinea-pig and dog gastrointestinal tract, using various protocols. Prucalopride was a 5-HT_4 receptor agonist in the guinea-pig colon, as it induced contractions ($pEC_{50} = 7.48 \pm 0.06$; insensitive to a 5-HT_{2A} or 5-HT_3 receptor antagonist, but inhibited by a 5-HT_4 receptor antagonist) as well as the facilitation of electrical stimulation-induced noncholinergic contractions (blocked by a 5-HT_4 receptor antagonist). Furthermore, it caused relaxation of a rat oesophagus preparation ($pEC_{50} = 7.81 \pm 0.17$), in a 5-HT_4 receptor antagonist sensitive manner. Prucalopride did not cause relevant inhibition of 5-HT_{2A}, 5-HT_{2B}, or 5-HT_3, motilin or cholecystokinin (CCK_1) receptor-mediated contractions, nor nicotinic or muscarinic acetylcholine receptor-mediated contractions, up to 10 μM. It is concluded that prucalopride is a potent, selective and specific 5-HT_4 receptor agonist. As it is intended for treatment of intestinal motility disorders, it is important to note that prucalopride is devoid of anti-cholinergic, anticholinesterase or nonspecific inhibitory activity and does not antagonise 5-HT_{2A}, 5-HT_{2B} and 5-HT_3 receptors or motilin or CCK_1 receptors. © 2001 Published by Elsevier Science B.V.

Keywords: Prucalopride; Enterokinetic; 5-HT_4 receptor

1. Introduction

Abnormal motility of the gastrointestinal tract, such as in gastroparesis, ileus and various forms of constipation, may lead to complaints, which can be severe and socially and economically debilitating. Upper gastrointestinal tract motility disturbances can be treated with prokinetics such as metoclopramide, domperidone and cisapride. The mechanism of action of these compounds has been suggested to be peripheral dopamine D_2 receptor antagonism (domperidone and metoclopramide) or 5-HT_4 receptor agonism (metoclopramide, cisapride) (Briejer et al., 1995). However, these compounds seem less effective in the treatment of lower intestinal motility disturbances, e.g. constipation, although some efficacy has been reported for cisapride in a subset of patients (Müller-Lissner, 1987; Longo and Vernava, 1993). This effect of cisapride suggests that stimulation of 5-HT_4 receptors may be a means to influence colonic motility. Indeed, 5-HT_4 receptor-mediated responses have been demonstrated in the human isolated colon (Tam et al., 1994; McLean and Coupar, 1996; Prins et al., 2000a). However, cisapride is a 5-HT_3 and 5-HT_{2A} receptor antagonist as well. Both actions may be associated with constipation (Briejer et al., 1995), thus possibly counteracting the promotility effects of 5-HT_4 receptor stimulation on the colon. Clearly, a more selective 5-HT_4 receptor agonist could have more pronounced effects on colonic

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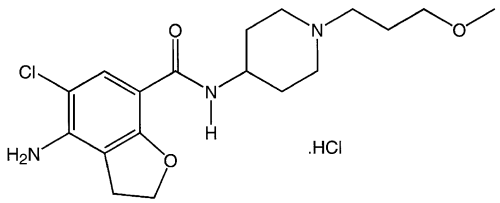

Fig. 1. Structural formula of prucalopride (4-amino-5-chloro-2,3-dihydro-*N*-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide monohydrochloride).

motility, and prove valuable for the treatment of slow transit constipation and post-operative atony.

Prucalopride (4-amino-5-chloro-2,3-dihydro-*N*-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofuran carboxamide monohydrochloride; Fig. 1) is a novel enterokinetic compound. In dogs, it induces colonic giant migrating contractions (the main drive behind distal propulsion of colonic contents; Karaus and Sarna, 1987) and a general change in colonic contractile motility patterns (Briejer et al., 1997c). Furthermore, it evokes defecation in cats as well as in human healthy volunteers and it accelerates gastric emptying in dogs (Briejer et al., 1997a,b) and humans (Poen et al., 1997; Vandeplassche et al., 1997; Emmanuel et al., 1998) and colonic transit in healthy volunteers (Bouras et al., 1999). The compound is currently in clinical trials for the treatment of idiopathic chronic constipation (Coremans et al., 1999) and post-operative ileus.

This paper describes the pharmacological profile of prucalopride. Prucalopride's high specificity for 5-HT_4 receptors, as determined in receptor binding studies, was confirmed in organ bath experiments that focused especially on pharmacological properties that are known to be important for modulation of gastrointestinal motility.

2. Materials and methods

2.1. Receptor binding studies

2.1.1. Tissue and cell membrane preparations for radioligand binding

Prucalopride was investigated for in vitro binding using membrane preparations of animal tissue or using membranes of cell lines transjected with cloned human (h) receptors. As tissue sources brain, peripheral organs, blood of animal or human origin or permanent cell lines were used. The procedures for membrane preparations were described previously (Lesage et al., 1998). The original references to the various receptor binding models are found in Lesage et al. (1998).

The culture medium consisted of DMEM supplemented with heat-inactivated dialysed calf serum (dCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and the cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Unless otherwise stated the receptor expression was promoted by addition of 5 mM sodium butyrate for 24 h before membrane preparation.

Cell membrane preparation from mammalian lines transiently or stably expressing 5-HT receptors were prepared from cells cultured on 150-mm Petri dishes in their standard media supplemented with animal sera (dCS or fetal calf serum = FCS) and antibiotics (see above). The plates were washed twice with ice-cold phosphate buffered saline (PBS), the cells were scraped from plates in 50 mM Tris/HCl, pH 7.4 and harvested by centrifugation for 10 min at 16,000 × *g* at 4 °C. The pellet was resuspended in 5 mM Tris/HCl, pH 7.4, homogenized with an Ultra Turrax homogenizer and membranes were collected by high-speed centrifugation (25,000 × *g* at 4 °C). The preparation was kept on ice during the entire procedure and the final pellet resuspended in 50 mM Tris/HCl, pH 7.4 at a concentration of approximately 1 mg protein/ml and stored in aliquots at −70 °C. The precise protein concentration was determined, after thawing and rehomogenization, on the day of the binding experiment using the Bradford protein assay (Bio Rad) and bovine serum albumin (BSA) as standard protein.

2.1.2. Binding assay conditions

Membrane fractions from cells or from tissue homogenates were incubated with a radioactively ([^{3}H]- or [^{125}I]-) labelled ligand with high affinity for a particular receptor. Specific receptor binding of the radioligand was distinguished from the nonspecific labelling by addition of an excess of an unlabelled compound, known to compete with the radioligand for binding to the receptor sites. The remaining nonspecific labelling was subtracted from total binding values obtained without the competitor. Filtration and counting conditions are described below.

2.1.3. Competition binding assay

To determine the receptor binding affinity of unlabelled prucalopride, the ligand was added at various concentrations (usually ranging from 10^{-10} to 10^{-5} M) to the incubation mixture. Following an incubation period necessary to reach equilibrium at a given temperature (Tables 1 and 2), the membranes were harvested by rapid filtration and filters were placed in counting vials, 2 ml of Ultima Gold MV were added and filter-bound radioactivity was counted after shaking and resting (> 6 h) in a Packard (Meriden, CT, USA) liquid scintillation counter.

2.1.4. Data analysis

Data from inhibition binding assays were automatically expressed as percent of total binding measured in the absence of test compound by a program routine on a Mac II personal computer and inhibition curves, plotting percent of total binding versus the log concentration of the test compound, were automatically generated. The sigmoidal inhibition curves were analysed by computerised

Table 1
Pharmacological profile of prucalopride (non-5-HT receptors)

Receptor (species)	Radioligand	pIC_{50} ± S.D.	n	K_i (nM)
Monoamine receptors				
hα_1-adrenergic	[^{3}H]prazosin	< 5	4	> 10 000
hα_{2A}-adrenergic	[^{3}H]rauwolscine	< 5	4	> 10 000
hα_{2B}-adrenergic	[^{3}H]rauwolscine	< 5	4	> 10 000
hα_{2C}-adrenergic	[^{3}H]rauwolscine	< 5	3	> 10 000
hβ_1-adrenergic	[^{125}I]iodocyanopindolol	< 5	3	> 10 000
hβ_2-adrenergic	[^{125}I]iodocyanopindolol	< 5	3	> 10 000
hβ_3-adrenergic,	[^{125}I]iodocyanopindolol	< 5	3	> 10 000
rDopamine-D_1	[^{3}H]SCH23390	< 5	5	> 10 000
hDopamine-D_{2L}	[^{3}H]spiperone	< 5	4	> 10 000
hDopamine-D_3	[^{125}I]iodosulpride	< 5	4	> 10 000
hDopamine-D_4	[^{3}H]spiperone	5.3 ± 0.1	3	2350
hH_1	[^{3}H]pyrilamine	< 5	4	> 10 000
r-muscarinic	[^{3}H]dexetimide	< 5	5	> 10 000
Opiate receptors				
hμ-opiate	[^{3}H]DAGO	< 5	3	> 10 000
hδ-opiate	[^{3}H]DPDPE	< 5	2	> 10 000
gpκ-opiate	[^{3}H]U69593	< 5	4	> 10 000
Peptide receptors				
GpNeurotensin	[^{3}H]neurotensin	< 5	1	> 10 000
rCCK-A	[^{3}H]CCK8	< 5	4	> 10 000
hCCK-B	[^{3}H]CCK8	< 5	3	> 10 000
HBradykinin-B_2	[^{3}H]bradykinin	< 5	4	> 10 000
HNeurokinin-NK_1	[^{3}H]substanceP	< 5	4	> 10 000
HNeurokinin-NK_2	[^{3}H]SR48968	< 5	4	> 10 000
HNeurokinin-NK_3	[^{3}H]SR142801	< 5	3	> 10 000
hVIP1	[^{125}I]VIP	< 5	4	> 10 000
Ion channels				
rCa^{2+}-channel	[^{3}H]nitrendipine	< 5	3	> 10 000
rNa^+-channel	[^{3}H]BTX	< 5	3	> 10 000
rNMDA	[^{3}H]MK801	< 5	5	> 10 000
rNMDA	[^{3}H]glycine	< 5	4	> 10 000
rAMPA	[^{3}H]AMPA	< 5	4	> 10 000
Transporters				
rDA-transporter	[^{3}H]WIN35428	< 5	3	> 10 000
h5-HT-transporter	[^{3}H]paroxetine	< 5	3	> 10 000
rNE-transporter	[^{3}H]nisoxetine	< 5	3	> 10 000
hGlycine-1-transporter	[^{14}C]glycine	< 5	3	> 10 000
hGlycine-2-transporter	[^{14}C]glycine	< 5	3	> 10 000
Other				
gpLeukotriene D_4	[^{3}H]LTD4	< 5	1	> 10 000
hTromboxane-A2	[^{3}H]SQ29548	< 5	2	> 10 000
rbPAF	[^{3}H]PAF	< 5	2	> 10 000
hσ1-sites	[^{3}H]haloperidol	5.3 ± 0.17	4	3680

curve-fitting, using non-linear regression analysis for one- or two-site curve-fitting (modifications of equations described by Oestreicher and Pinto (1987)).

The pIC_{50} ($-\log IC_{50}$)-values (IC_{50}: concentration inhibiting 50% of the specific radioligand binding or neurotransmitter uptake) were derived from individual curves. K_i-values were calculated according to the Cheng–Prusoff equation: $K_i = IC_{50}/[1 + C/K_D]$ where K_i is the equilibrium inhibition constant of the unlabelled compound, C the concentration of the radioligand used and K_D the equilibrium dissociation constant of the labelled compound. Graphs were prepared using the GraphPad program (GraphPad, San Diego, USA).

2.2. Organ bath experiments

2.2.1. General

Dunkin–Hartley guinea-pigs (approximately 400–600 g), Wistar rats (approximately 200 g), New Zealand rabbits (2.0–2.5 kg) and Beagle dogs (approximately 7.5–10 kg) of both sexes were used. The animals were housed according to the EEC Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (Official Journal of the European Communities, L358, Volume 29, 18 December 1986). The guinea-pigs, rabbits and rats had free access to water and standard chow. The dogs also had free access to water, and were fed a standard meal once a day (about 350 g).

The guinea-pigs were sacrificed by stunning and subsequent decapitation, the rabbits by stunning and subsequent exsanguination and the rats were sacrificed by CO_2 asphyxiation. The dogs were sacrificed by decerebration and successive exsanguination through the carotid artery.

2.2.2. Guinea-pig proximal colon

2.2.2.1. Preparation. The proximal colon was cleansed by rinsing it with De Jalon solution (composition in mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.8), and mesenteric tissue was removed. Starting approximately 1 cm distal from the caecocolonic junction, four segments of about 3 cm were cut, which were suspended in 20-ml organ baths containing gassed (95% O_2 and 5% CO_2)

Table 2
Pharmacological profile of prucalopride on 5-HT receptor subtypes

Receptor (species)	Radioligand	pIC_{50}	S.D.	n	K_i (nM)
h5-HT_{1A}	[^{3}H]8-OH-DPAT	< 5		3	> 10 000
h5-HT_{1B}	[^{3}H]alniditan	< 5		3	> 10 000
h5-HT_{1D}	[^{3}H]alniditan	< 5		4	> 10 000
h5-HT_{1E}	[^{3}H]5-HT	< 5		4	> 10 000
h5-HT_{1F}	[^{3}H]5-HT	< 5		3	> 10 000
h5-HT_{2A}	[^{125}I]R093274	< 5		4	> 10 000
h5-HT_{2B}	[^{3}H]5-HT	< 5		2	> 10 000
h5-HT_{2C}	[^{3}H]mesulergine	< 5		3	> 10 000
m5-HT_3	[^{3}H]GR65630	5.08	0.07	5	3822
h5-$HT_{4(b)}$	[^{3}H]5-HT	7.7	0.5	5	8
h5-$HT_{4(a)}$	[^{3}H]5-HT	8.3	0.09	2	2.5
h5-HT_{5A}	[^{3}H]5-CT	< 5		4	> 10 000
h5-HT_6	[^{3}H]5-HT	< 5		2	> 10 000
h5-HT_7	[^{3}H]5-HT	< 5		3	> 10 000

warm (37.5 °C) De Jalon solution, under a load of 2 g, for isotonic measurement of changes in longitudinal muscle length. For experiments in which electrical field stimulation was imposed, segments were set up in another fashion. The segments were set up in 100-ml organ baths between co-axial platinum-wire electrodes running parallel to the longitudinal axis of the segment, with one platinum electrode running in the luminal space of the segment. Changes in longitudinal muscle tension were assessed isometrically. A tension of 40 mN was imposed, and re-adjusted until this tension was maintained.

2.2.2.2. 5-HT$_3$ receptor antagonism. After 30 min stabilisation, the strips were challenged with methacholine 3 μM. The buffer solution was supplemented with the 5-HT$_1$/5-HT$_2$ receptor antagonist methysergide (1 μM) and the 5-HT$_4$ receptor antagonist (1-butyl-4-piperidinyl)-methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate HCl (SB-204070; 10 nM), in order to block non-5-HT$_3$ receptor-mediated responses. The measurement was repeated after washing (i.e. replacing the bathing fluid twice) and 15 min stabilisation. This last response was taken as 100% contraction.

Of each guinea-pig, one segment was treated with solvent, and the other strips with granisetron or prucalopride. After a 15-min incubation period, non-cumulative concentration–response curves to 5-HT were established with a 0.5-log ascending concentration spacing. A 15-min dosing interval was used, with a washout (and re-addition of granisetron/prucalopride/solvent) as soon as the maximum effect to a dose of 5-HT was reached. Only one curve per segment was constructed.

2.2.2.3. 5-HT$_4$ receptor agonism—unstimulated colon. Indomethacin, 3 μM was included in the De Jalon solution in order to block prostanoid synthesis. After stabilization, the strips were challenged with carbachol 3 μM. The measurement was repeated after washing (i.e. replacing the bathing fluid twice) and 15 min stabilization. This last response was taken as 100% contraction.

Non-cumulative concentration–response curves to prucalopride were constructed with a 15-min dosing cycle. Prucalopride was applied in ascending order with a 0.5-log ascending concentration spacing. In a first series of experiments, ketanserin (0.3 μM), granisetron (1 μM), [1-[2-[(methylsulphonyl) amino] ethyl] - 4 - piperidinyl]methyl - 1 - methyl-1*H*-indole-3-carboxylate (GR113808; 30 nM) or solvent were applied 20 min before addition of the first concentration of prucalopride, and were re-added directly after each washout. In a second set of experiments, concentrations of GR113808 (1, 3 and 10 nM) or solvent were tested similarly. For each preparation, only one concentration–response curve was established. As four colon strips per guinea-pig were used, one was chosen randomly to serve as a control whereas the remaining three strips were subjected to antagonist treatment.

2.2.2.4. 5-HT$_4$ receptor agonism—electrical field-stimulated colon. The De Jalon solution contained atropine 0.3 μM, guanethidine 3 μM, indomethacin 3 μM, methysergide 1 μM and granisetron 1 μM, in order to study non-adrenergic, noncholinergic responses only, without involvement of 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ receptors. The preparations were left to stabilise for at least 30 min. A contraction was evoked with histamine (30 μM) to challenge the tissue. After a stable tone was attained, the segments were subjected to electrical field stimulation (9 V, 10-s trains every 5 min, 1.5 Hz). Immediately after at least five reproducible rebound contractions were obtained, GR113808 or solvent was administered. Immediately after another five rebound contractions, the agonist was given. Another five rebound contractions were induced. In a first series of six experiments, the agonist was 5-HT, in another set prucalopride. Responses (rebound contraction) were expressed as a percentage of the last 1.5-Hz-induced rebound contraction before addition of GR113808 or solvent.

2.2.2.5. Anti-cholinesterase, muscarinic M$_3$ receptor antagonistic and nonspecific inhibitory action. After 30 min stabilisation, the strips were challenged with 3 μM carbachol. After washout and 10 min stabilisation, this contraction was repeated. Then, 10 min later, a cumulative concentration–response curve was established to either carbachol or acetylcholine with a 0.5-log ascending concentration spacing. As soon as the maximum effect was attained, the strips were washed repeatedly, and stabilisation was allowed until the original length had restored to baseline levels. The maximum contraction of each strip was taken as 100% contraction for that preparation. Then, solvent or a concentration of prucalopride was added and left with the tissues for 15 min. Subsequently, the contraction to carbachol or acetylcholine was repeated. Per preparation, two curves were thus established. Of every four strips taken per animal, three were given a concentration of drug and one was treated with solvent.

2.2.3. Rat stomach fundus

2.2.3.1. Preparation. The stomach was excised and opened along the smaller curvature. The preparation was pinned out on a cork mat and the mucosa was removed by sharp dissection. From the greater curvature, four longitudinal strips were cut of approximately 2–3 mm wide. These strips were suspended in a 20-ml organ bath containing Tyrode solution (composition in mM: NaCl 137, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, KCl 2.7, MgCl 1.04, $CaCl_2$ 1.8, D-glucose 5.6), supplemented with indomethacin (3 μM), gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C, for isometric tension recording. A tension of 30 mN was imposed and the strips were allowed to equilibrate with re-adjustments of the tension, until a tension of 10 mN was maintained.

2.2.3.2. 5-HT_{2B} receptor antagonism. The strips were challenged with histamine 10 μM in a 10-min dosing cycle until the induced contraction yielded a reproducible amplitude. A cumulative concentration–response curve to 5-HT was constructed with a 0.5-log ascending concentration spacing. The maximum contraction to 5-HT per strip was taken as 100% contraction. After repeated washout and at least 15 min stabilisation, a concentration of prucalopride or its solvent were added and left with the tissue for at least 30 min. Then, a second cumulative concentration–response curve to 5-HT was constructed. Of four strips taken per animal, three received a different concentration of prucalopride and the fourth served as a control.

2.2.4. Rat oesophagus tunica muscularis mucosae

2.2.4.1. Preparation. The oesophagus was removed and the muscularis was carefully dissected. The remaining tunica muscularis mucosae was split in two by cutting along the circumference. Both mucosal preparations were individually mounted in 20-ml organ baths containing Krebs–Henseleit solution (composition in mM: NaCl 118, $NaHCO_3$ 25, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, D-glucose 10.1), gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C. Responses were measured isometrically. The preparation was carefully stretched until a tension of 5 mN was maintained and allowed to stabilise for at least 30 min.

2.2.4.2. 5-HT_4 receptor agonism. Carbachol (1 μM) was added in order to precontract the preparations. After a stable tone was obtained, a cumulative concentration–response curve to 5-HT was established with a 0.5-log ascending concentration spacing. The maximum relaxation as induced by 5-HT was taken as 100% relaxation. After repeated washout and stabilisation, this procedure was repeated, establishing a curve to 5-HT (control) or to prucalopride. In case the 5-HT_4 receptor antagonist GR113808 was tested against prucalopride, it was added immediately after wash-out of 5-HT of the first curve, and left with the tissue for the remainder of the experiment.

2.2.5. Dog distal colon

2.2.5.1. Preparation. A segment of about 5–10 cm of midcolon was quickly excised and put in Krebs solution. The luminal contents were washed out and the tissue was opened longitudinally by cutting along the mesenteric border. The mesenteric remains were carefully removed and the mucosa and submucosa were removed by sharp dissection. Longitudinal muscle strips of about 2–3 mm wide and 2 cm long were positioned into 20-ml organ baths containing warm (37 °C) carbogenated (95% O_2, 5% CO_2) Krebs solution supplemented with SB-204070 (10 nM) to inhibit any 5-HT_4 receptor-mediated responses (Prins et al., 1997). Changes in longitudinal muscle length were measured by means of isotonic transducers (2-g load).

2.2.5.2. 5-HT_{2A} receptor antagonism. The preparations were left to stabilise for at least half an hour. Consecutively, a contraction was evoked with carbachol (3 μM) to challenge the tissue. After repeated washout and 20 min stabilisation, this contraction was repeated. Then, contractions with 5-HT (3 μM) were evoked with a 10-min interval (washout after each dose), until these contractions were reproducible (usually after three to four times). The change in length due to the latter dose of 5-HT was taken as 100%. A concentration of prucalopride or its solvent was added to the organ bath and left to incubate for 15 min. Then, a non-cumulative concentration–response curve to 5-HT was constructed with a 10-min dosing interval, with a 0.5-log ascending concentration spacing. Prucalopride or solvent was re-added to the appropriate organ bath immediately after each washout. Only one curve per strip was constructed.

2.2.6. Guinea-pig ileum

2.2.6.1. Preparation. The distal small intestine was removed, the mesentery was dissected and the lumen washed by gentle flushing with Krebs solution. Four segments of approximately 4 cm were suspended in 100-ml organ baths under a load of 0.75 g (isotonic measurement of changes in longitudinal muscle length) containing Krebs solution in the presence of indomethacin (3 μM) and SB-204070 (30 nM).

2.2.6.2. Nicotinic acetylcholine receptor antagonism. First, after 20 min stabilization, the tissues were challenged twice with carbachol 3 μM. For each individual segment, the last contraction to carbachol was taken as 100% contraction. Then, prucalopride (1, 3 and 10 μM) or solvent was added. After 15 min, a concentration of nicotine was added to the bath fluid (contact time: 30 s, followed by immediate washout), establishing a non-cumulative concentration–response curve with a 15-min dosing cycle, with a 0.5-log ascending concentration spacing. Only one curve per segment was thus established.

2.2.7. Guinea-pig gall bladder

2.2.7.1. Preparation. The gall bladder was dissected, cut open lengthwise and its contents were removed. Then, two to three longitudinal strips were cut, which were placed in 20-ml organ baths containing carbogenated Krebs buffer of 37 °C for isotonic tension measurement (load: 0.75 g).

2.2.7.2. CCK_1 receptor antagonism. The tissues are challenged twice at 20-min intervals with carbachol 3 μM. Then, the CCK receptor antagonist, devazepide (1, 3 and 10 nM) or solvent, and in a second series of experiments,

prucalopride (1, 3 or 10 μM; in the presence of SB-204070 0.1 μM) or solvent was administered. Fifteen minutes later, a cumulative concentration–response curve to CCK-8 with a 0.5-log ascending concentration spacing was constructed. Only one curve per segment was established.

2.2.8. Rabbit duodenum

2.2.8.1. Preparation. The duodenum was removed and the lumen cleansed by gentle flushing, and the mesentery and adhering fatty tissue was dissected. Four segments of about 3 cm per rabbit were set-up in 20-ml organ baths containing Tyrode solution of 37 °C, gassed with carbogen, under a load of 1 g, for isotonic measurement of changes in longitudinal muscle length.

2.2.8.2. Motilin receptor antagonism. First, after 20 min stabilization (wash every 10 min), the strips were challenged with carbachol 3 μM. For each individual segment, the maximum contraction to carbachol is taken as 100%. After washout, solvent or prucalopride (1, 3 or 10 μM) were added immediately after washout of carbachol. After 10 min, the organ bath solution was refreshed and prucalopride or solvent were re-added. Again 10 min later (prucalopride/solvent had now been with the tissues for 20 min), cumulative concentration–response curves to [Leu^{13}]-motilin with a 0.5-log ascending concentration spacing were established. Only one curve to [Leu^{13}]-motilin per segment was established.

2.3. Data analysis

Concentration–response curves were analysed by fitting the data of individual curves to a four-parameter logistic function, assuming a single site interaction, with the aid of a computerized interactive procedure (Bowen and Jerman, 1995). This yielded a curve minimum, maximum, midpoint slope and $p\mathrm{EC}_{50}$ value per individual curve. The $p\mathrm{EC}_{50}$ values were subsequently used for Schild analysis, if the mean maximum effect or the mean mid-point slope was not altered significantly by antagonist treatment. If the slope of the Schild plot was different from unity, $p\mathrm{A}_2$ values were estimated using the Schild equation (Arunlakshana and Schild, 1959): $p\mathrm{A}_2 = \log\ (\mathrm{DR} - 1) - \log$ ([antagonist]) with DR being the dose ratio (EC_{50} in the presence of antagonist divided by EC_{50} in the absence of antagonist). Mean values were compared with Dunnett's method or Tukey–Kramer honestly significant difference (HSD) test, where appropriate (Jump 3.1 software, SAS Institute); a $P < 0.05$ was considered to reflect statistical significance.

2.4. Chemicals

2.4.1. Receptor binding studies

The Bradford protein assay was from BioRad (Nazarett Eke, Belgium). The liquid scintillation spectrometer and the scintillation fluid Ultima Gold MV were from Packard. Dulbecco's modified Eagle medium (DMEM) and cell culture sera were from Life Technologies (Gaithersburg, MD, USA). All other reagents were from Merck or Sigma (Belgium). The GraphPad Prism program was from GraphPad.

All compounds were dissolved and diluted in DMSO (except the indolamines, which were dissolved in water and protected from light throughout the experiment). The final DMSO concentration in the tests did not exceed 0.5% (vol/vol).

2.4.2. Organ bath experiments

The following chemicals were used (with their supplier between parentheses): acetylcholine chloride, indomethacin, CCK-8 (Sigma), 5-HT creatinine sulphate (Serva), methysergide maleate (Sandoz), carbachol chloride, atropine sulphate, histamine chloride, L-nicotine choloride (Janssen Chimica), methacholine chloride (Merck), guanethidine (Ciba-Geigy), devazepide (MSD Labs), [Leu^{13}]motilin (KW-5139; gift from Kyowa-Hakko), ketanserin tartrate, granisetron chloride, GR113808, SB-204070 and prucalopride (synthetised in-house). All chemicals were dissolved in distilled water, except for 5-HT, which was dissolved in distilled water supplemented with ascorbic acid (0.25 μM in the stock solution), and devazepide, which was dissolved in water acidified with tartaric acid + 10% cyclodextrine.

3. Results

3.1. Receptor binding studies

Prucalopride (Fig. 1) was tested as a competing ligand in binding tests on a wide range of receptors including classical monoamine and peptide receptors, ion channel binding sites, lipid derived factor and neurotransmitter transporter binding sites. The resulting $p\mathrm{IC}_{50}$ and K_i-values for non-serotonergic receptors are summarised in Table 1. Prucalopride exerted low binding affinity for dopamine-D_4 receptors measured in CHO cells expressing the cloned human receptor (K_i value = 2.3 μM). The compound showed also weak affinity for binding to human σ_1 binding sites expressed in Sf9 cells, measured with [^{3}H]haloperidol (K_i value = 3.7 μM). No interaction of the compound was found in the other binding tests when tested up to 10 μM ($K_i > 10{,}000$ nM).

In competition binding tests on 5-HT receptors (Table 2), prucalopride bound with high affinity to h5-HT$_{4(b)}$ receptors expressed in HEK 293 cells when labelled with [^{3}H]5-HT (K_i value = 8 nM). A comparable high affinity binding for prucalopride was found for the h5-HT$_{4(a)}$ receptor (K_i value = 2.5 nM). Both 5-HT$_4$ receptor isoforms were the only high affinity receptors for prucalopride. From all other 5-HT receptors tested, only the 5-HT$_3$

receptor labelled with [^{3}H]GR65630 in membranes of mouse N × G 108CC15 neuroblastoma cells displayed a weak affinity for prucalopride (K_i value = 3.8 μM). All other tested 5-HT receptors were not recognised by the compound (Table 2, K_i values > 10,000 nM). This was independent of the radioligands used (not shown). Therefore, also within the 5-HT receptor family prucalopride displayed a high specificity for the investigated 5-HT$_4$ receptor isoforms (at least 290-fold selectivity towards the hD$_4$ receptor).

3.2. Organ bath experiments

3.2.1. Guinea-pig proximal colon

3.2.1.1. 5-HT$_3$ receptor antagonism. 5-HT induced contractions from 3 μM onwards (Fig. 2). The contractions had a rapid onset and were not maintained. The concentration–response curve to 5-HT was steep, with a pEC_{50} = 5.4 ± 0.1 (Fig. 2). Granisetron (1 μM), a selective 5-HT$_3$ receptor antagonist, blocked the contractions to 5-HT (Fig. 2). This demonstrates that, under the applied conditions, the contraction to 5-HT is entirely due to 5-HT$_3$ receptor stimulation. Prucalopride (1 and 3 μM) did not significantly affect the curve at the tested concentrations. At 10 μM, prucalopride tended to inhibit the 5-HT-induced contractions, but this inhibition was not significant (pEC_{50} = 5.2 ± 0.04; maximum = 48 ± 4% versus 56 ± 3% for control) (Fig. 2).

3.2.1.2. 5-HT$_4$ receptor agonism—unstimulated colon. Prucalopride induced contractions in a concentration-dependent manner (Fig. 3; pEC_{50} = 7.5 ± 0.1; n = 4). Neither ketanserin (0.3 μM), nor granisetron (1 μM) affected the contractions to prucalopride, yielding curves coinciding with that of prucalopride alone (Fig. 3). GR113808 (30 nM), however, blocked the response to prucalopride up to 0.3 μM. At higher concentrations, prucalopride surmounted the blockade of GR113808, and it induced even larger contractions at supramicromolar concentrations (Fig. 3).

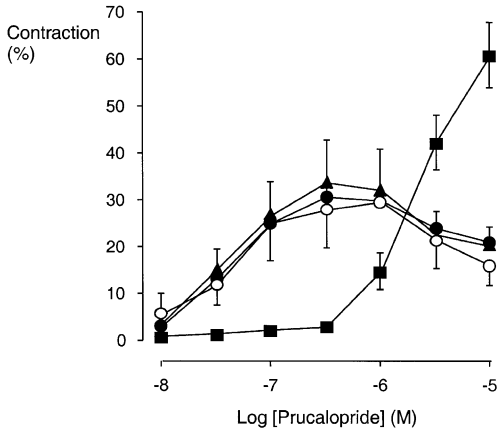

Fig. 3. Concentration–response curve to prucalopride in the absence (○) or presence of: ketanserin 0.3 μM (5-HT$_{2A}$ receptor antagonist) (●), granisetron 1 μM (5-HT$_3$ receptor antagonist) (▲), and GR113808 30 nM (5-HT$_4$ receptor antagonist) (■). Contractions to prucalopride were expressed as a percentage of contractions induced by carbachol (3 μM). Guinea-pig proximal colon, longitudinal muscle; means ± S.E.M.; n = 6.

In a second set of experiments, GR113808 was tested at 1, 3 and 10 nM. GR113808 caused a concentration-dependent, surmountable inhibition of the prucalopride-induced contractions (pEC_{50} control = 7.4 ± 0.1; n = 6), shifting the concentration–response curve rightward (Fig. 4). Further analysis revealed a slope of the Schild plot of 1.5. Therefore, it was chosen to calculate apparent pA_2 values for GR113808 using the Schild equation, yielding for 1, 3 and 10 nM GR113808, respectively, 8.7 ± 0.2, 9.0 ± 0.1 and 9.4 ± 0.2 (n = 6).

3.2.1.3. 5-HT$_4$ receptor agonism—electrical field-stimulated colon. 5-HT (1 μM; Fig. 5) as well as prucalopride (1 μM; Fig. 6) significantly amplified the rebound contraction after electrical field stimulation. GR113808 (30 nM)

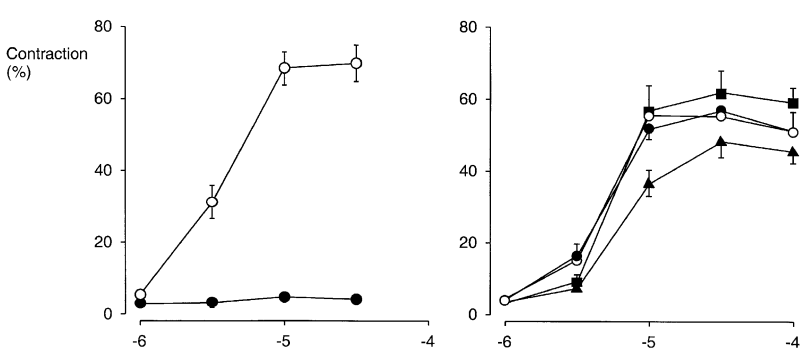

Fig. 2. Left panel: Concentration–response curve to 5-HT in the absence (○) or presence (●) of granisetron (1 μM). Right panel: Concentration–response curve to 5-HT in the absence (○) or presence of prucalopride: 1 μM (●), 3 μM (■) and 10 μM (▲). Experiments were done in the presence of methysergide (1 μM; preventing interactions of 5-HT with 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-ht$_5$, 5-HT$_6$, 5-HT$_7$ receptors) and SB-204070 (10 nM; selective 5-HT$_4$ receptor antagonist) in order to prevent any effects due to stimulation of other 5-HT receptors than 5-HT$_3$ receptors. Contractions to 5-HT were expressed as a percentage of contractions due to methacholine (3 μM). Guinea-pig proximal colon, longitudinal muscle; means ± S.E.M.; n = 13–14 (left panel); n = 6 (right panel).

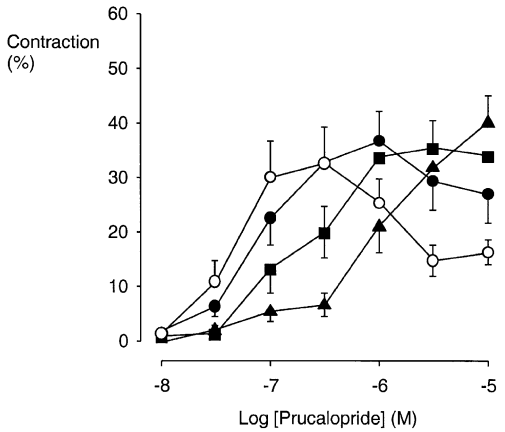

Fig. 4. Concentration–response curve to prucalopride in the absence (○) or presence of GR113808: 1 nM (●), 3 nM (■), and 10 nM (▲). Contractions to prucalopride were expressed as a percentage of contractions induced by carbachol (3 μM). Guinea-pig proximal colon, longitudinal muscle; means ± S.E.M.; $n = 8$.

completely prevented the 5-HT- and prucalopride-induced amplifications. When given alone, GR113808 did not affect the mean amplitude of the rebound contractions induced by electrical field stimulation (Figs. 5 and 6).

3.2.1.4. Anti-cholinesterase, muscarinic M_3 receptor antagonistic and nonspecific inhibitory action. Acetylcholine induced contractions from 3 nM onwards, yielding a monophasic concentration–response curve with a pEC_{50} of 7.0 ± 0.1 ($n = 24$) for the first series of curves (results not shown). The acetylcholine curves were reproducible, as reflected by the coinciding pEC_{50} and maximum effect values in the solvent group ($pEC_{50} = 6.9 \pm 0.1$ and 6.9 ± 0.2; maximum = $100 \pm 0\%$ and $104 \pm 4\%$; $n = 6$; results not shown). Prucalopride at 1, 3 and 10 μM, did not affect the concentration–response curve to acetylcholine (results not shown), the pEC_{50} value at 10 μM prucalopride being 7.0 ± 0.1.

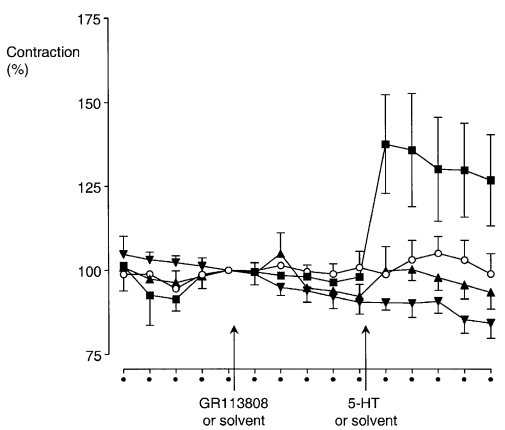

Fig. 5. Effects of 5-HT (1 μM) (■) on electrical field stimulation-induced noncholinergic rebound contractions of the guinea-pig proximal colon. GR113808 (30 nM) prevented the amplification to 5-HT (▲), whereas the antagonist, when given alone (▼), had no effect [cfr. no treatment (○)]. The experiments were done in the presence of 5-HT_1, 5-HT_2 and 5 HT_3 receptor blockers, atropine and guanethidine. Responses were expressed as a percentage of the 1.5 Hz contraction before GR113808 or solvent. Guinea-pig proximal colon, longitudinal muscle; mean ± S.E.M.; $n = 6$.

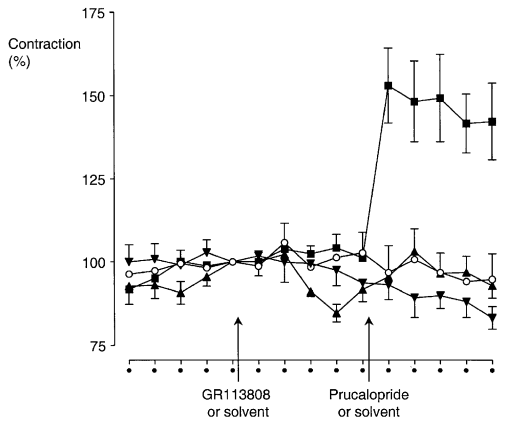

Fig. 6. Effects of prucalopride (1 μM) (■) on electrical field stimulation-induced noncholinergic rebound contractions of the guinea-pig proximal colon. GR113808 (30 nM) prevented the amplification to prucalopride (▲), whereas the antagonist, when given alone (▼), had no effect [cfr. no treatment (○)]. The experiments were done in the presence of 5-HT_1, 5 HT_2 and 5-HT_3 receptor blockers, atropine and guanethidine. Responses were expressed as a percentage of the 1.5 Hz contraction before GR113808 or solvent. Guinea-pig proximal colon, longitudinal muscle; mean ± S.E.M.; $n = 6$.

Carbachol induced contractions from 3 nM onwards, yielding a concentration–response curve with a pEC_{50} of 7.0 ± 0.04 ($n = 22$) for the first series of curves (results not shown). The carbachol curves were reproducible, as reflected by the coinciding pEC_{50} and maximum values in the solvent group ($pEC_{50} = 7.0 \pm 0.1$ and 7.0 ± 0.1; maximum = $100 \pm 0\%$ and $102 \pm 5\%$; $n = 5$; results not shown). Prucalopride at 1, 3 and 10 μM, did not affect the concentration–response curve to carbachol, the pEC_{50} value at 10 μM prucalopride being 7.0 ± 0.1 ($n = 5$; results not shown).

3.2.2. Rat stomach fundus

3.2.2.1. 5-HT_{2B} receptor antagonism. 5-HT induced contractions from 0.3 nM onwards, yielding a sigmoidal concentration–response curve ($pEC_{50} = 7.9 \pm 0.1$). This curve was reproducible in the same tissue ($pEC_{50} = 7.9 \pm 0.1$; maximum = $107 \pm 6\%$). At a concentration of 1 μM, prucalopride did not significantly affect the concentration–response curve to 5-HT ($pEC_{50} = 7.7 \pm 0.2$), but at 3 and 10 μM, it tended to shift the curve to 5-HT rightward (in a surmountable manner: at 3 μM calculated maximum = $103 \pm 4\%$; at 10 μM calculated maximum = 106 ± 3) (Fig. 7). The degree of rightward shift was however not statistically significant nor concentration-dependent, as the shift due to 3 and 10 μM

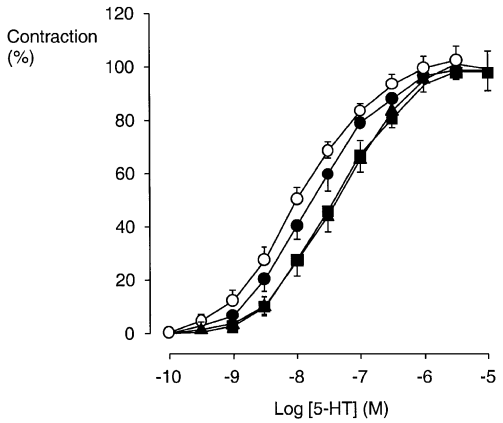

Fig. 7. Concentration–response curves to 5-HT in the absence (○) and presence of prucalopride: 1 μM (●), 3 μM (■) and 10 μM (▲). Contractions were expressed as a percentage of the maximum contraction to 5-HT before treatment. Experiments were done in the presence of indomethacin (3 μM). Rat stomach fundus, longitudinal muscle; mean ± S.E.M.; $n = 7–8$.

prucalopride was similar (3 μM: $pEC_{50} = 7.5 \pm 0.2$; 10 μM: $pEC_{50} = 7.3 \pm 0.1$; Fig. 7).

3.2.3. Rat oesophagus tunica muscularis mucosae

3.2.3.1. 5-HT$_4$ receptor agonism. Prucalopride induced relaxation of the rat oesophagus preparation, yielding a monophasic concentration–response curve (Fig. 8; $pEC_{50} = 7.8 \pm 0.2$, intrinsic activity = 0.45; $n = 6$). Prucalopride induced relaxation with a slower rate than 5-HT. GR113808 dose-dependently inhibited the prucalopride-induced relaxations, which resulted in a rightward shift of the concentration–response curves without depression of the maximum (surmountable antagonism: maximum effect after 10 nM GR113808 = 42 ± 4% versus control = 45 ± 9%). Further analysis revealed a shallow slope of 0.6 for the Schild plot, indicating the nature of the antagonism was non-competitive. Therefore, the Schild equation was used to estimate apparent pA_2-values, yielding 9.4 ± 0.3, 9.3 ± 0.2 and 9.0 ± 0.3 for the 1, 3 and 10 nM-induced shifts, respectively.

3.2.4. Dog distal colon

3.2.4.1. 5-HT$_{2A}$ receptor antagonism. 5-HT induced contractions from 3 nM onwards, with a maximum at 3 μM, yielding a sigmoidal curve ($pEC_{50} = 7.1 \pm 0.1$; $n = 6$). Up to 3 μM, prucalopride did not affect the curve to 5-HT. However, at 10 μM prucalopride caused a significant rightward displacement of the curve to 5-HT ($pEC_{50} = 6.6 \pm 0.1$) without a depression of the maximum effect (102 ± 3% versus 106 ± 5% for the control), indicative of competitive antagonism (Fig. 9). The pA_2 estimated from this displacement was 5.3 ± 0.1 ($n = 6$).

3.2.5. Guinea-pig ileum

3.2.5.1. Nicotinic cholinoceptor antagonism. Nicotine induced contractions from 3 μM onwards (Fig. 10). The contractions had a rapid onset and were not maintained. The concentration–response curve to nicotine was steep, with a $pEC_{50} = 5.3 \pm 0.1$ ($n = 8$; Fig. 10). Prucalopride (1, 3 and 10 μM) slightly enhanced the contractions to nicotine in a seemingly dose-dependent fashion (maximum = 82 ± 5% in the presence of prucalopride 10 μM versus 65 ± 2% for control; $n = 8$; Fig. 10). Prucalopride, however, did not affect the midpoint location of the curve

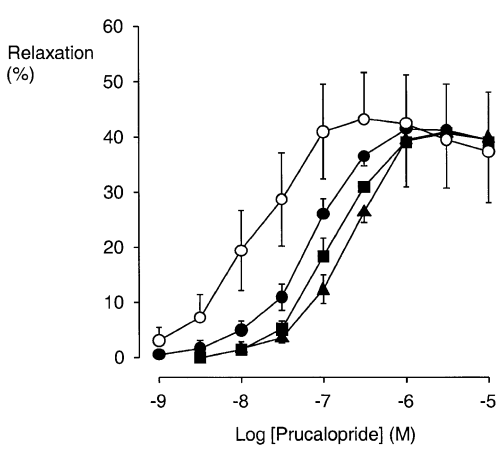

Fig. 8. Concentration–response curves to prucalopride in the absence (○) or presence of GR113808: 1 nM (●), 3 nM (■), and 10 nM (▲), in strips precontracted with carbachol (1 μM). The relaxations were expressed as a percentage of maximum relaxations induced by 5-HT. Rat oesophagus tunica muscularis mucosae; mean ± S.E.M.; $n = 6$.

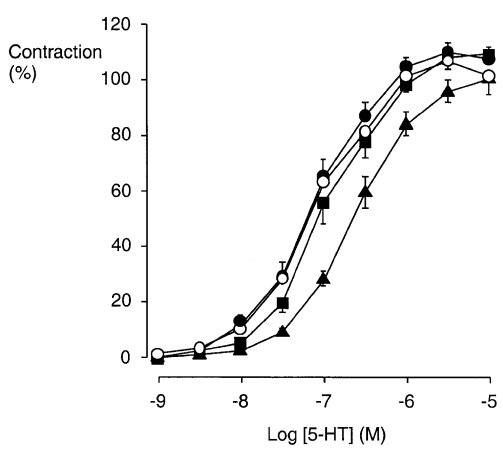

Fig. 9. Concentration–response curves to 5-HT in the absence (○) or presence of prucalopride: 1 μM (●), 3 μM (■), and 10 μM (▲). Experiments were done in the presence of the 5-HT$_4$ receptor antagonist SB-204070 (10 nM). The contractions were expressed as a percentage of maximum contractions induced by 5-HT. Dog distal colon, longitudinal muscle; mean ± S.E.M.; $n = 6$.

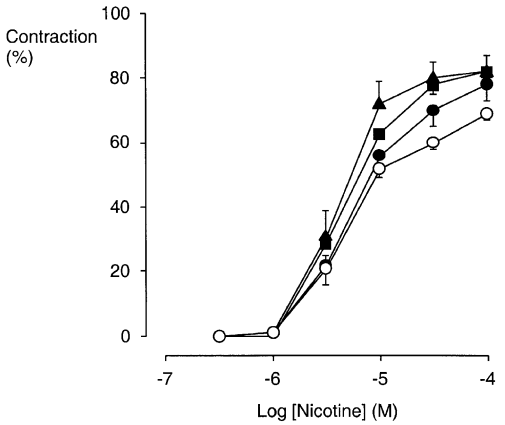

Fig. 10. Concentration–response curves to nicotine in the absence (○) or presence of prucalopride: 1 μM (●), 3 μM (■), and 10 μM (▲). Experiments were done in the presence of the 5-HT_4 receptor antagonist SB-204070 (30 nM) and indomethacin (1 μM). The contractions were expressed as a percentage of contractions induced by carbachol (3 μM). Guinea-pig ileum, longitudinal muscle; mean ± S.E.M.; $n = 8$.

($pEC_{50} = 5.4 \pm 0.1$ in the presence of prucalopride 10 μM; $n = 8$).

3.2.6. Guinea-pig / rabbit gallbladder

3.2.6.1. CCK_1 receptor antagonism. CCK-8 induced contractions from 3 nM onwards, yielding a sigmoidal concentration–response curve with a pEC_{50} of 6.1 ± 0.1 ($n = 6$; results not shown). Devazepide (1, 3 and 10 nM) inhibited the CCK-8-induced contractions, displacing the curve of CCK-8 rightward (all observations $n = 6$; results not shown). In the presence of the highest concentration of devazepide (10 nM), the maximum effect to CCK-8 was not yet attained at the highest concentration of CCK-8 tested (3 μM). Therefore, the Schild equation was used to estimate pA_2 values; these were $pA_2 = 8.9 \pm 0.4$ for devazepide 1 nM, and $pA_2 = 9.2 \pm 0.3$ for devazepide 3 nM. Prucalopride, up to 10 μM, did not affect the contractions to CCK-8, yielding concentration–response curves coinciding with the control curve (in the presence of prucalopride 10 μM: $pEC_{50} = 6.5 \pm 0.1$, maximum is 140 ± 10% versus $pEC_{50} = 6.7 \pm 0.1$; 126 ± 11% for the control curve; $n = 6$, data not shown).

3.2.7. Rabbit duodenum

3.2.7.1. Motilin receptor antagonism. [Leu^{13}]motilin induced contractions and a reduction of the spontaneous movements of the duodenal muscle from 0.3 nM onwards, yielding a sigmoidal concentration–response curve with a pEC_{50} of 8.3 ± 0.1 ($n = 6$; results not shown). Prucalopride, up to 10 μM, did not affect the contractions to [Leu^{13}]motilin, yielding concentration–response curves coinciding with the control curve (results not shown; in the presence of prucalopride 10 μM: $pEC_{50} = 8.3 \pm 0.2$, maximum is 97 ± 4% versus 98 ± 5% for the control curve; both $n = 6$).

4. Discussion

In our detailed receptor binding profile, prucalopride showed very high specificity for 5-HT_4 receptors. Based on the comparison of K_i-values, prucalopride had 290- and 460-fold higher affinity for the h5-$HT_{4(b)}$ receptor over the hD_4 receptor labelled with [^{3}H]spiperone and the hσ1-site labelled with [^{3}H]haloperidol, respectively. For the h5-$HT_{4(a)}$ receptor the selectivity was 780- and 1500-fold over the hD_4 receptor and the hσ1-site, respectively. Within the 5-HT receptor family the selectivity was 480- and 1500-fold over the m5-HT_3 receptor measured on the h5-$HT_{4(b)}$ and h5-$HT_{4(a)}$ isoform, respectively. This is to our knowledge the highest selectivity reported for 5-HT_4 agonists. SDZ-HTF919, another high affinity 5-HT_4 agonist (Appel-Dingemanse et al., 1999), has been found in our hands to be only 50-fold selective over the h5-HT_{1D} and 44-fold selective over the h5-HT_{1A} receptors.

The high affinity K_D value of [^{3}H]prucalopride for both tested h5-HT_4 receptor splice variants is supported by results from competition binding studies (where similar K_i values were found) and from direct binding measurements with [^{3}H]prucalopride on membranes from tissue samples (Bonaventure et al., 2000). In a recent study we showed that [^{3}H]prucalopride can be a suitable radioligand to localise and investigate the G-protein coupled 5-HT_4 receptor fraction on human brain sections and membranes (Bonaventure et al., 2000). Indeed, due to its high selectivity and low nonspecific binding [^{3}H]prucalopride is an ideal selective 5-HT_4 agonist radioligand.

In isolated tissues prucalopride was an agonist on rat and guinea-pig 5-HT_4 receptors. In guinea-pig intestinal preparations, 5-HT_4 receptor stimulation causes facilitation of cholinergic (Kilbinger and Wolf, 1992) as well as noncholinergic (Kojima and Shimo, 1995) neurotransmission. In the current study prucalopride as well as 5-HT caused an amplification of the electrical field stimulation-induced noncholinergic excitatory responses in the guinea-pig colon, which was prevented by pre-incubation with the selective 5-HT_4 receptor antagonist GR113808 (Gale et al., 1994). These observations confirm the findings of Kojima and Shimo (1995), and, at the same time, are indicative for prucalopride being a 5-HT_4 receptor agonist. Furthermore, prucalopride contracted the guinea-pig colon. 5-HT contracts the colon by stimulation of 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors (Briejer et al., 1993). The prucalopride-induced contractions, however, were not affected by the selective 5-HT_{2A} receptor antagonist ketanserin (Van Nueten et al., 1981) or the selective 5-HT_3 receptor antagonist granisetron (Sanger and Nelson, 1989), but were strongly inhibited by GR113808. The estimated pA_2 val-

ues (8.7–9.4) were in good agreement with the reported affinity of GR113808 at guinea-pig 5-HT_4 receptors (pA_2 = 9.2–9.7; Gale et al., 1994). This confirms that prucalopride is a 5-HT_4 receptor agonist in the guinea-pig gut.

A robust assay for 5-HT_4 receptor-mediated responses is the rat oesophagus tunica muscularis mucosae (Baxter et al., 1991). In this tissue, 5-HT_4 receptors are on the smooth muscle mediating relaxation. The 5-HT_4 receptor antagonist GR113808 displaced the concentration–response curve of prucalopride in the rat oesophagus rightward in a surmountable fashion. The estimated pA_2 values (9.0–9.4) were in good agreement with the reported affinity of GR113808 at rat 5-HT_4 receptors (pA_2 = 9.0–9.4; Gale et al., 1994). Hence, also in the rat oesophagus tunica muscularis mucosae, prucalopride was a 5-HT_4 receptor agonist.

In the rat preparation, the Schild plot slope was significantly lower than unity, whereas in contrast, in the guinea-pig preparation the Schild plot slope was greater than unity. These conflicting results may reflect differences in interaction of prucalopride with guinea-pig and rat 5-HT_4 receptors. Indeed, small differences in amino acid sequence of the 5-HT_4 receptor between various animal species have been found, as well as splice variants of the 5-HT_4 receptor with possible different distributions. Alternatively, they may reflect differences in the ability of prucalopride to reach equilibrium conditions in both tissues due to a different micro-milieu.

Prucalopride also expresses selective 5-HT_4 receptor agonism in isolated tissues of higher animal species, such as dogs and humans. Studies from our laboratories revealed that prucalopride induced relaxation of circular smooth muscle of dog rectum (Prins et al., 1999) and human colon (Prins et al., 2000a) in a selective 5-HT_4 receptor antagonist-sensitive manner. Furthermore, prucalopride was found to stimulate excitatory 5-HT_4 receptors located on cholinergic nerves of longitudinal muscle of canine and human colon (Prins et al., 2000b). This latter action is associated with enhanced output of acetylcholine, which in turn, causes stimulation of contractile amplitudes. Thus, prucalopride can be considered a selective and efficacious 5-HT_4 receptor agonist in gastrointestinal tissues from rodents up to humans.

Antagonism at 5-HT_3 and possibly also 5-HT_{2A} receptors is associated with constipation (Briejer et al., 1995), and is thus not a desired property for a colon motility stimulant such as prucalopride. 5-HT_{2B} receptors, like 5-HT_{2A} receptors, are found on the smooth muscle of the gut in various animal species including man (Engel et al., 1984; Baxter et al., 1994; Schmuck et al., 1994; Borman and Burleigh, 1995; Kuemmerle et al., 1995; Prins et al., 1997). Thus, it is anticipated that antagonism at these receptors may also lead to the induction of constipation. Various experiments were designed to evaluate the effects of prucalopride as an antagonist on these receptors: the isolated canine colon longitudinal muscle for 5-HT_{2A} receptors (Prins et al., 1997), the rat fundus for 5-HT_{2B} receptors (Baxter et al., 1994) and the guinea-pig colon for 5-HT_3 receptors (Briejer et al., 1995), in the presence of appropriate antagonists. In these experiments prucalopride was devoid of agonistic or antagonistic effects on 5-HT_{2A}, 5-HT_{2B} and 5-HT_3 receptors. Prucalopride did not affect contractions of the guinea-pig colon to acetylcholine (nor to carbachol). Contractions to acetylcholine (and carbachol) of intestinal muscle are mediated primarily by muscarinic M_3 receptors (Eglen et al., 1996) (the nicotinic cholinoceptors rapidly desensitize and are thus masked in a cumulative dosing experiment). Prucalopride is therefore not a muscarinic M_3 cholinoceptor antagonist. This is important to ascertain, as acetylcholine is the main neurotransmitter of the motor neurones in the enteric nervous system, and muscarinic cholinoceptor antagonists such as atropine cause paralysis of motility. Cholinesterases are abundantly present in intestinal tissue (Ambache et al., 1971). In such tissues, cholinesterase inhibitors will thus cause a leftward shift (potentiation) of the concentration–response curve to acetylcholine. Cholinesterase inhibitors are known to enhance intestinal contractility both in vitro and in vivo (Meunier et al., 1979), but this motility is chaotic and noncoordinated. Therefore, anticholinesterase activity is a nondesired property. The lack of potentiating effects of prucalopride on cholinergic responses, however, indicate that it is devoid of anti-cholinesterase activity. Furthermore, these data indicate a lack of nonspecific inhibitory ("spasmolytic") effects on gastrointestinal muscle.

Acetylcholine is not only used by the enteric nerves for nerve–smooth muscle signalling via muscarinic cholinoceptors, but also to a large extent for neuron–neuron communication, utilising primarily nicotinic cholinoceptors. Thus, it is considered important to also assess whether prucalopride interferes with nicotinic cholinoceptors. Prucalopride, however, was devoid of any inhibitory action up to 10 μM.

Motilin is an intestinal hormone, which is thought to be the key signalling peptide for the regulation of the cyclic patterns of antral digestive motility, the so-called migrating motor complex (Itoh, 1997). More than one motilin receptor subtype may exist (Van Assche et al., 1997); however, no selective agonists or antagonists are known. Also prucalopride did not interfere up to 10 μM with motilin-induced contractions of the rabbit duodenum, a classical preparation for motilin responses. Hence, it is anticipated that prucalopride will not interfere in vivo with the migrating motor complex by affecting motilin receptor function.

Another important gastrointestinal hormone is CCK. This peptide is involved in the regulation of gastrointestinal and gallbladder motility (primarily CCK_1 receptor subtype) as well as gastric acid secretion (CCK_2 or gastrin receptor subtype) (Wank, 1995). Devazepide is a selective and potent CCK_1 receptor antagonist (reported pK_b = 9.98; Bishop et al., 1992). In the guinea-pig gallbladder

preparation it was a competitive antagonist with an estimated pA_2 of 8.9 and 9.2, thus confirming that a CCK_1 receptor system was studied. In this system, prucalopride caused no inhibition up to 10 μM. This indicates that prucalopride has no relevant affinity for CCK_1 receptors.

The organ bath data, together with these receptor binding data, suggest that prucalopride is one of the most specific and selective 5-HT_4 receptor agonists currently available, offering an explanation for its effect to accelerate gastrointestinal transit.

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