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Differential effects of CB₁ neutral antagonists and inverse agonists on gastrointestinal motility in mice

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Abstract

Background—Cannabinoid type 1 (CB₁) receptors are involved in the regulation of gastrointestinal (GI) motility and secretion. Our aim was to characterize the roles of the CB₁ receptor on GI motility and secretion *in vitro* and *in vivo* by using different classes of CB₁ receptor antagonists.

Methods—Immunohistochemistry was used to examine the localization of CB₁ receptor in the mouse ileum and colon. Organ bath experiments on mouse ileum and *in vivo* motility testing comprising upper GI transit, colonic expulsion, and whole gut transit were performed to characterize the effects of the inverse agonist/antagonist AM251 and the neutral antagonist AM4113. As a marker of secretory function we measured short circuit current *in vitro* using Ussing chambers and stool fluid content *in vivo* in mouse colon. We also assessed colonic epithelial permeability *in vitro* using FITC-labelled inulin.

Key Results—*In vivo*, the inverse agonist AM251 increased upper GI transit and whole gut transit, but it had no effect on colonic expulsion. By contrast, the neutral antagonist AM4113 increased upper GI transit, but unexpectedly reduced both colonic expulsion and whole gut transit at high, but not lower doses.

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Author Contributions

MAS, WKM & KAS designed the research, MAS, MB, CH, CMK & MD performed the research and analyzed data, VKV, BL, KM & AM contributed essential reagents and MAS, MB, WKM & KAS wrote the paper.

Competing Interests

The authors declare they have no competing interests.

Conclusions & Inferences—CB₁ receptors regulate small intestinal and colonic motility, but not GI secretion under physiological conditions. CB₁ inverse agonists and CB₁ neutral antagonists have different effects on intestinal motility. The ability of the neutral antagonist not to affect whole gut transit may be important for the future development of CB₁ receptor antagonists as therapeutic agents.

Keywords

cannabinoid-1 receptor; inverse agonist/antagonist; intestinal motility; ion transport

INTRODUCTION

Pharmacological activation of cannabinoid receptors reduces motility *in vitro* and *in vivo*^{1,2}. This has been extensively documented for the CB₁ receptor, whereas the role of the CB₂ receptor in regulation of GI motility is less well understood and remains to be fully resolved³⁻⁹. Cannabinoid receptors are part of the endocannabinoid system (ECS), which consists of endogenous ligands, as well as the biosynthetic and degradative enzymes for these ligands.¹⁰ The ECS is involved in the regulation of GI motility.^{3,11} Both *in vitro* and *in vivo* studies suggest that treatment with a CB₁ receptor inverse agonist/antagonist under physiological conditions results in the opposite effects observed to that of treatment with a CB₁ receptor agonist; increased contractility or motility of the gut.^{6,12-15} These effects of CB₁ receptor inverse agonists/antagonists were shown in animals under normal conditions and in models of diarrhea or ileus.^{5,16-18} Interestingly, data from clinical trials of CB₁ receptor inverse agonists/antagonists suggest that these findings hold true in humans as well, since nausea, vomiting and diarrhea were amongst the major dose-related side-effects observed in patients treated with rimonabant and taranabant^{19,20}.

Our knowledge of the involvement of the CB₁ receptor in GI physiology is largely based on data using CB₁ receptor selective inverse agonists/antagonists like rimonabant (SR141716A), AM251, AM281 and LY320135²¹ or from conventional receptor knockout mice. Besides competitive antagonism at the CB₁ receptor with the endogenous endocannabinoids, these compounds display inverse agonist activity, shifting a constitutively active CB₁ receptor from the “on” state to the inactive “off” state.^{22,23} It is not possible *in vivo* to discriminate between inverse agonism at the receptor or the blockade of endogenously released endocannabinoids acting at a constitutively active receptor. We sought to understand which of the GI actions of the CB₁ receptor antagonists are due to their inverse agonist activity by utilizing a novel CB₁ receptor specific antagonist with no inverse agonist actions.

AM4113 is a novel CB₁ receptor antagonist without inverse agonist activity and is a so called neutral antagonist.²⁴ It is a pyrazole analog structurally related to AM251 and rimonabant. The aim of this study was to compare effects of the inverse agonist/antagonist AM251 and the neutral antagonist AM4113 on GI motility and secretion *in vitro* and *in vivo*. Using the neutral antagonist in this context is valuable in order to further characterize the physiological involvement of the ECS in the regulation of GI functions and to establish how the CB₁ receptor is involved in these physiological processes. AM4113, like the CB₁ receptor inverse agonists/antagonists, reduces food intake and body weight.^{24,25} However, we have shown that unlike AM251, AM4113 does not exacerbate emesis and others have shown that it does not have the same potential to cause nausea.²⁴⁻²⁶

METHODS

Animals

6-7 week old male C57BL/6N mice (20-26 g) and male CB₁^{-/-} mice (18-24 g) on a CB57BL/6N background as well as their wildtype littermates were used in the studies. The C57BL/6 mice were obtained from Charles River and the CB₁^{-/-} mice²⁷ were bred from heterozygous mice at the University of Calgary mouse breeding facility. Mice were housed at a constant temperature (22°C) and a photoperiod (12:12-h light-dark cycle) in sawdust-containing plastic cages with access to standard Laboratory Chow and tap water *ad libitum*. The mice were used after 1 week of habituation. Animal use for these studies was approved by the University of Calgary Animal Care Committee and the experiments were performed in accordance with institutional animal ethics committee guidelines that follow the guidelines established by the Canadian Council on Animal Care.

Immunohistochemistry

For preparation of whole mounts, segments of mouse ileum and colon (n=8) were opened along the mesenteric border, stretched, and pinned mucosal-side-up on a Sylgard-coated petri dish. For preparation of cross-sections, the lumen of the ileum and colon (n=16) was rinsed with PBS and stapled without stretching to cardboard. Tissues were fixed overnight with Zamboni's fixative (2% paraformaldehyde, 15% picric acid; pH 7.4) at 4°C and then rinsed for 3 × 10 min in phosphate buffered saline (PBS; pH 7.4).

Whole mounts were prepared by dissection with forceps under a dissecting microscope. Cross-sections of intact intestine were cyroprotected in PBS containing 20% sucrose at 4°C for several hours or overnight. Specimens were embedded in optimum cutting temperature compound (OCT; Tissue-Tek, Sakura Finetechnical Co. Ltd., Tokyo, Japan) and cryostat-sectioned at 14µm prior to thaw mounting onto poly-D-lysine coated slides. Whole mounts or sections were washed for 3 × 10 min in PBS containing Triton X-100 (0.1%) and incubated with goat anti-CB₁ C-terminal antibody (K. Mackie; 1:500)^{28,29} for 48 h at 4°C. The tissue was then washed for 3 × 10 min in PBS and incubated with the secondary antibody donkey anti-goat conjugated to CY3 (Jackson ImmunoResearch; 1:100) for 60 min at room temperature, followed by washing (3 × 10 min in PBS) and mounting in bicarbonate-buffered glycerol (pH 8.6). Tissues were examined using a Zeiss Axioplan microscope. Photographs were taken using a digital imaging system consisting of a digital camera (Sensys Photometrics, Tucson, AZ, USA) and image analysis software (V for Windows; Digital Optics, Auckland, New Zealand).

Isolated smooth muscle strips

Male mice were killed by cervical dislocation. Segments of the distal ileum were removed and submerged in ice-cold oxygenated Krebs solution (mM): NaCl 115, KCl 8.0, KH₂PO₄ 2.0, NaHCO₃ 25, MgCl₂ 2.4, CaCl₂ 1.3, and glucose 10. Luminal contents were gently flushed. Up to four segments of full-thickness strips, 1.5 cm each, were used from each animal. All experiments lasted less than 3 h and each preparation was used for a single experiment only.

The preparations were slipped through two platinum electrodes, which were 1 cm apart and placed in separate organ baths (25 mL; 37°C; oxygenated with 95% O₂ / 5% CO₂). Using a silk thread one end of the preparations was attached to the bottom of the organ bath, while the other end was connected to an isometric force transducer (Harvard Apparatus, model 50-7905, Kent, UK). 0.5 g tension was applied and the preparations were allowed to equilibrate for 30 min. Changes in tension were amplified by a transducer amplifier

(Harvard Apparatus, model 50-7970, Kent, UK), relayed to a bioelectric amplifier (Hewlett-Packard, model 8811A, Mississauga, Ontario, Canada).

The data was converted using a CED 1401 PLUS analogue to digital converter (Cambridge Electronic Design, Cambridge, UK.) and recorded using the Spike 3 software (Cambridge Electronic Design).

AM4113 (1-100nM), AM251 (1-100nM) and rimonabant (SR141716A, 1-100nM) were added to the organ bath and effects on spontaneous activity and on muscular contractions induced by bethanechol (10µM) were recorded.

Electrical field stimulation (EFS) was used according to two different regimens. One was to assess the effects of the antagonists at 4 different frequencies (2, 4, 8 and 16 Hz for 10 sec, 60 V, and 0.5 msec pulse duration) and the other was used continuously at a frequency of 8Hz (60 V, stimulus duration 0.5 msec, train duration 5 sec, with 100 sec interval) to assess the effects of the antagonists on WIN55,212-2 inhibition of contractility. EFS was applied by a Grass Stimulator (S88 stimulator, Grass, Quincy, MA, USA). Under these conditions, EFS resulted in stable contractions mediated via cholinergic and neurogenic mechanisms, as contractions were absent in presence of atropine (1µM) or tetrodotoxin (100nM). Drugs (AM4113: 1-100nM; AM251: 1-100nM; rimonabant 1-100nM) were added cumulatively into the organ bath and effects on the EFS-induced contractions were recorded. Each concentration was allowed to incubate for 20 min. Before adding drugs, the mean of 2 successive contractions at each frequency was defined as the control response. Contractions produced by each drug concentration were reported as the percentage of control response. In additional control experiments, the effects of the vehicle were also tested.

Upper gastrointestinal transit

After an overnight fast (water *ad libitum*) upper GI transit experiments were performed as described in detail by others^{30,31}. Briefly, 20 min after intraperitoneal (i.p.) administration of drugs (or vehicle) 0.2 ml of 5% Evans blue suspension in 5% gum arabic was given by gastric gavage. 15 min later animals were killed by cervical dislocation and the intestine was immediately removed. The distance travelled by the colored marker was measured in cm and expressed as a percentage of the total length of the small intestine from pylorus to cecum.

Colonic expulsion

Distal colonic expulsion was measured using the method of Broccardo *et al.*³². Briefly, 20, 120 and 220 min after i.p. administration of drugs (or vehicle), a plastic bead (2.5 mm) was inserted 3 cm into the distal colon of each mouse using a silicone pusher. The time to expulsion of the bead was determined for each animal at each of the three time points and then the mean of these individual trials was taken. A higher mean expulsion time indicates a greater inhibition of colonic expulsion.

Fecal water content

Fecal water content was assessed in mice following a modification of a method described by Izzo *et al.*¹² 48 h prior to the experiment mice were housed in individual cages with water *ad libitum* and they were preconditioned with overnight-fasting and vehicle injections. On the day of the experiment, animals were given drugs i.p. and immediately transferred to an empty cage (devoid of bedding). The stool-pellets discharged at 20, 120 and 220 min were collected and weighed immediately (wet weight). After drying (overnight at 50°C) the dry weight was determined. The ratio of wet to dry weight was calculated and used as a marker of stool fluid content. Control mice received vehicle treatment only.

Whole gut transit time

Mice were housed in individual cages 72 h prior to the experiment. On the day of the experiment, they were acclimated to an empty cage (devoid of bedding) for 1 h prior to drug treatment. Twenty min after i.p. administration of drugs (or vehicle) 0.2 ml of 5% Evans blue suspension in 5% gum arabic was given by gastric gavage. The time to the first blue bowel movement was measured in min and constituted the whole gut transit time.

Ion transport

Whole thickness segments of mouse colon, taken from the mid-distal region of the colon, were mounted in modified Ussing chambers (0.38 cm² opening). Both sides were bathed in a modified Krebs solution (mM): 115 NaCl, 2.0 KH₂PO₄, 2.4 MgCl₂, 25.0 NaHCO₃, 8.0 KCl, 1.3 CaCl₂ containing 10 mM glucose (serosal side) or 10 mM mannitol (luminal side). Two tissue segments were used per mouse; one was used as a vehicle control, the other exposed to either AM251 or AM4113 (1 μM). Segments receiving vehicle or drug were alternated to eliminate possible differences in ion transport responses between the mid and distal regions of the colon. Tissues were studied under short-circuited conditions in which the voltage was clamped to 0 mV using a WPI EVC-4000 voltage clamp (World Precision Instruments, Sarasota, FL, USA). Tissues were unclamped at the beginning and end of each experiment to record open PD values for the calculation of tissue conductance (G_t). After baseline short-circuit current (I_{SC}) was established (15–30 min), either drug or an equal volume of vehicle (100% ethanol) was added to the serosal side of the tissue. The final concentration of ethanol in the bathing solution never exceeded 0.1% (v/v). 10 min following drug addition, tissues were exposed to electrical field stimulation at 1, 2.5, 5 and 10 Hz (pulse duration: 500 μs, stimulus duration: 5 sec, stimulus strength: 50 V) to assess neurally evoked secretory responses. I_{SC} was allowed to return to baseline following each stimulation. Tissues were then exposed to tetrodotoxin (1 μM, serosal addition) in order to reduce neural contributions to subsequent ion transport responses. Carbachol was added 10 min later (60 μM, serosal addition) and, finally, following a return to a steady baseline I_{SC} , forskolin was added (10 μM, serosal addition). For each challenge, the peak change in I_{SC} (ΔI_{SC}) was measured.

FITC-inulin flux

In some experiments, tissues mounted in Ussing chambers were exposed mucosally to FITC-labelled inulin, 1 mg/ml, to assess paracellular permeability. Aliquots of serosal buffer were sampled every 30 min and assayed for fluorescein fluorescence (wavelength 521 nm) in a VICTOR³ fluorescence plate reader (Perkin-Elmer, Waltham, MA). Data were expressed as a percentage of baseline fluorescence obtained at time zero.

Statistics

Student's t-test was used to compare single treatment means with control means and ANOVA followed by Dunnet's post-hoc test was used for analysis of multiple treatment means (SigmaStat, Jandel Scientific, San Rafael, CA, USA). P values < 0.05 were considered significant. Data represent the mean ± SEM of experiments in n mice.

Drugs

AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazolone-3-carboxamide) was obtained from Tocris (Tocris, Ellisville, USA). AM4113 was synthesized in the laboratory of Alex Makriyannis at the Center for Drug Discovery, Northeastern University, USA. AM4113 is a pyrazole-3-carboxamide analog of AM251 with a very similar molecular weight. AM251 and AM4113 were dissolved in a solution of dimethyl sulfoxide [DMSO] 2% and Tween 80 1% in saline (0.9%). For ion transport

studies, drugs were dissolved in 100% ethanol, as described above. Evans blue was obtained from Sigma-Aldrich (Oakville, ON, Canada). None of the vehicles used had effects on the observed parameters *in vitro* or *in vivo*.

RESULTS

Immunohistochemistry

In order to establish the localization of CB₁ receptor in the mouse GI tract we performed immunohistochemistry using an antibody that we confirmed does not react with CB₁ receptors in CB₁ receptor-knockout mice (data not shown). Sections of the mouse ileum and mouse colon were examined and revealed intense CB₁ immunoreactivity in neural elements throughout the wall of the gut. CB₁ immunoreactivity was localized to enteric neurons and nerve fibres of both the myenteric and submucosal plexus and nerve fibres in the mucosa. No immunoreactivity was detected on the epithelium (Fig. 1).

Influence of CB₁ receptor antagonists on ileal contractility *in vitro*

None of the antagonists tested, AM251 (1-100nM), rimonabant (1-100nM) or AM4113 (1-100nM) had an effect on the basal tone or spontaneous activity of mouse ileal smooth muscle or on contractions induced by bethanechol (10μM, data not shown; n=6). Electrical field stimulation was used to assess the effect of these compounds on nerve-mediated contractility of the ileum. These contractions were completely blocked by tetrodotoxin (100nM) and virtually abolished by atropine (1μM) (data not shown).

Electrical field stimulation of the ileum caused twitch contractions that were stable over 3 h. AM251 at doses up to 100nM had no significant effect on these contractions (Fig. 2A). This contrasts with the effects of rimonabant which concentration-dependently increased contractions (1-100nM) at stimulation frequencies of 4-16Hz (Fig. 2B). The neutral CB₁ receptor antagonist AM4113 at concentrations up to 100nM had no effect on ileal contractility (Fig. 2C). We confirmed CB₁ antagonism by assessing their ability to reverse the inhibitory effects of the non-selective cannabinoid agonist WIN55,212-2 on EFS-induced contractility (measured at 8Hz). WIN55,212-2 (100nM) significantly reduced contractility, this action was reversed in the presence of Rimonabant, AM251 and AM4113 (all 100nM) (Fig. 2D).

Upper gastrointestinal transit

Rimonabant has already been shown to dose-dependently enhance upper GI transit in mice³³, consistent with the *in vitro* findings described above. We compared AM251 and AM4113 and found that both these compounds also enhanced upper GI transit, despite not enhancing contractility. AM251 (0.5 - 1 mg/kg) and AM4113 (0.1 - 1 mg/kg) significantly increased upper GI transit in a dose-dependent manner (Fig. 3A & 3B). AM4113 (0.1 mg/kg) and AM251 (0.5 mg/kg) at doses that did not significantly increase upper GI transit, reversed the WIN55,212-2 (1 mg/kg) induced inhibition of upper GI transit indicating receptor blockade at these concentrations (Fig. 3C). Neither AM251 (1 mg/kg) nor AM4113 (2 mg/kg) at doses that were effective in enhancing upper GI transit in wild type mice had a significant effect on upper GI transit in CB₁^{-/-} mice (Fig. 3D). This rules out a non-specific action at a site other than the CB₁ receptor.

Colonic expulsion and fecal water content

AM251 (1-2 mg/kg) did not significantly alter the rate of colonic bead expulsion (Fig. 4A). Additionally, a high dose of AM251 (5mg/kg) also did not delay colonic expulsion (287 ± 64 sec; n=7). AM4113 (1-2 mg/kg) dose-dependently slowed expulsion of beads from the colon (Fig. 4B). AM4113 (1 mg/kg) and AM251 (2 mg/kg) at doses that did not

significantly alter colonic expulsion, completely blocked the effect of WIN55,212-2 (3 mg/kg) (Fig. 4C).

We assessed the fecal water content in animals treated with AM251 and AM4113. Neither the inverse agonist/antagonist AM251 (1 mg/kg), nor the neutral CB₁ antagonist AM4113 (1 mg/kg), altered fecal water content (Fig. 4D).

Whole-gut transit

Since we observed an increase in small intestinal motility induced by AM251 and AM4113 *in vivo*, despite seeing no effect on contractility in some cases, we wished to assess the passage of food through the entire gut, as this is influenced by gastric, small and large intestinal motility, as well as secretion. Consistent with the actions of rimonabant, the inverse agonist/antagonist AM251 (1 and 5 mg/kg) speeds up transit by about 50% (Fig. 5). In contrast, the neutral antagonist AM4113 (5 mg/kg) did not alter whole gut transit time *in vivo*, and a lower dose (1 mg/kg) slowed whole gut transit (Fig. 5).

Effects of AM251 and AM4113 on colonic epithelial ion transport and permeability

Colonic tissue responded to EFS with frequency-dependent increases in I_{SC} that were not affected by either AM251 or AM4113 (Fig. 6A & 6B). Similarly, no significant differences were observed between drug- and vehicle-treated groups with respect to both carbachol- and forskolin-induced I_{SC} responses (Fig. 6C). It should also be noted that, on their own, neither AM251 nor AM4113 produced a change in I_{SC} when added to the Ussing chambers (data not shown).

Fluorescein fluorescence in the serosal chamber increased over time, but neither drug appeared to affect the epithelial paracellular movement of FITC-labelled inulin in the colon (data not shown). Tissue conductance (G_t) was not significantly affected by either AM compound (data not shown).

DISCUSSION

CB₁ receptors were first identified in 1988³⁴ and since then, there has been a wealth of evidence supporting their involvement in GI motility. CB₁ receptor activation by specific CB₁ receptor agonists was shown to reduce cholinergic contraction in various species *in vitro*³⁵⁻³⁹. *In vivo* CB₁ agonists attenuate upper GI transit and slow down colonic expulsion^{3,12,15,17,40,41}. Since these actions are blocked by CB₁ antagonists and are absent in CB₁^{-/-} mice, the CB₁ receptor has been confirmed as the major CB receptor mediating these effects. In contrast to these consistent pharmacological reports, the involvement of the ECS and specifically the CB₁ receptor in the regulation of motility under physiological and pathophysiological conditions is less well defined. The regulation of GI motility by the ECS is suggested by (i) levels of endocannabinoids in the gut that can activate receptors⁴⁰ (ii) by the ability of antagonists/inverse agonists to alter motility,²³ (iii) altered motility in CB₁ receptor gene-deficient mice^{3,15} and (iv) by the activity of inhibitors of endocannabinoid degradation.^{42,43} There are some contradictory results in these studies, but overall the evidence is supportive of the involvement of the ECS and CB₁ receptors in the control of GI motility. In order to strengthen this evidence further and to develop potential novel therapeutic approaches for the treatment of GI related diseases, such as irritable bowel syndrome, we investigated an alternative approach to studies of the role of the CB₁ receptor in GI function by utilizing a CB₁ receptor specific neutral antagonist.

Neutral antagonists do not alter receptor signalling when there is constitutive receptor activity. These contrast with the actions of CB₁ antagonists/inverse agonists which can work in two ways: (i) they can act as antagonists and block the actions of endogenously released

endocannabinoids or (ii) they can have an independent inverse agonist activity on constitutively active receptors. In tissues or whole animals it is not possible to distinguish these two possibilities as the extent of constitutive receptor activity is not known and likely varies considerably in different cells. Recently, a neutral CB₁ receptor antagonist, AM4113, was developed that allows us to separate antagonism of the receptor without inverse agonism.²⁴⁻²⁶ We compared this compound with the well known inverse agonists/antagonists AM251 and rimonabant, in order to clarify the physiological involvement of the ECS in GI function.

We first investigated these compounds *in vitro* alone and found that despite AM251 and rimonabant being structurally related, they possessed markedly different actions. Rimonabant enhanced electrically stimulated contractility in a concentration-dependent manner, with its greatest effect at a stimulation frequency of 4Hz. In contrast, the inverse agonist/antagonist AM251 had no effect on contractility over the same concentration range, and neither did AM4113. Since all the compounds were completely effective at reversing the actions of WIN55,212-2, it suggests that their ability to act as antagonists is separable from other actions. Rimonabant has previously been found to enhance contractility in the guinea pig ileum,^{13,37} but interestingly it did not alter acetylcholine release.¹³ In the mouse colon, rimonabant enhanced tonic and phasic activity at high concentrations⁴⁴ whereas it did not alter nonadrenergic, noncholinergic contractions⁴⁵ and neither did it alter spontaneous contractile activity in the ileum⁴⁶, as we have also shown in this study. Finally, rimonabant was not shown to alter contractility in the human small or large intestine.^{47,48} The other compounds had not previously been examined, but on the basis of their effects it seems neither inverse agonism nor antagonism of endogenous tonically released endocannabinoids can completely account for the enhanced contractility observed with rimonabant *in vitro*. Since neither basal activity nor bethanechol induced contractions were altered by any of the compounds, we believe the site of action of rimonabant is on the enteric nervous system and not on the smooth muscle, as others have suggested.^{35,37-39,45}

We next extended our findings to examine the effects of AM251 and AM4113 *in vivo* on upper GI transit. Both compounds increased upper GI transit in a dose-dependent manner as has previously been shown for rimonabant in both rats and mice^{49,50}, and for AM251 in rats.⁶ We confirmed that both compounds completely blocked the effects of the CB₁ agonist WIN 55,212-2 and that effects of the antagonists were not present in CB₁^{-/-} mice, confirming that these are CB₁ receptor mediated actions. As the effect of the neutral antagonist AM4113 cannot be explained by inverse agonist activity and it mirrors what is observed in the CB₁^{-/-} mice, these results strongly support the idea that under physiological conditions *in vivo* endogenous cannabinoid tone is importantly involved in the regulation of upper GI transit. Upper GI transit evaluated by the method used is a measure of gastric emptying and small intestinal transit, and interestingly rimonabant was shown to increase gastric emptying in mice.⁵¹

By contrast, AM251 had no effect on colonic bead expulsion, but AM4113 slowed bead expulsion at the highest dose tested (2mg/kg). Again, both compounds completely reversed the effects of the CB₁ receptor agonist WIN 55,212-2 at doses that had no effect on bead expulsion. These data suggest that endocannabinoids act centrally or peripherally to modulate colonic function, either through slowing of motility or increasing the threshold for sensation of a colonic bead (or both). CB₁ receptors have been implicated in the regulation of visceral sensation,^{2,11} but in rats rimonabant alone did not alter visceral sensitivity.⁵² It remains to be determined the exact site of action of AM4113 and whether this effect on bead expulsion is central or peripheral.

Since normal colonic function requires adequate lubrication, but excess secretion results in diarrhea, we also examined stool water content in animals treated with both AM251 and AM4113. In neither case was this altered, in contrast to rats treated with rimonabant, which have highly fluid stools.¹² CB₁ receptor expression in the submucosal plexus of the guinea pig²⁹ has been previously reported, we now confirm CB₁ receptor expressions in mouse submucosal plexus. CB₁ receptors have also been described on the colonic epithelium in the human,⁵³ but this was not the case in the mouse, possibly indicating an important species difference that may be of functional significance.

Finally, we examined whole gut transit time to gain an impression of all elements of motility in a freely moving animal. Whereas AM251 enhanced whole gut transit, the neutral antagonist AM4113 did not, suggesting that inverse agonism is the feature of these compounds that leads to reduced whole gut transit time, which may translate into the symptoms of diarrhea in humans.^{19,20,54} This finding was not expected, because AM4113 enhanced transit along the small intestine, but is very exciting since it may allow this class of drug to be developed further for a range of indications without significant GI side-effects. Exactly why AM4113 did not speed up whole gut transit is not known, but may relate to pharmacodynamic or pharmacokinetic properties or to some aspect of lack of inverse agonism. In this regard, it is noteworthy that the inverse agonists rimonabant and taranabant both induce diarrhea and GI disturbances in humans.^{19,20,54} Whether reducing weight and cardiometabolic risk factors can also be achieved with a neutral CB₁ receptor antagonist in humans and whether the side-effect profile is more favorable remains to be established, but our data suggest, that a neutral antagonist may have a more favorable GI side-effect profile. Moreover, AM4113 has been examined in animal studies and found to reduce weight and food intake whilst have less nauseogenic potential, a feature that is favorable considering future human use.²⁴⁻²⁶

Interestingly, neither antagonist had an effect on fecal stool water content, suggesting that the increased whole gut transit in animals treated with AM251 is a motor and not a secretomotor effect. Nevertheless, we were interested whether the CB₁ antagonists altered other parameters of ion transport or barrier function, to address whether there may be a physiological regulation of these functions. Using various stimuli, including electrical field stimulation, carbachol and forskolin, which stimulate secretion indirectly via the enteric nervous system and directly at the epithelium, as shown previously,⁵⁵ we did not observe any effects of the CB₁ receptor antagonists on ion transport, tissue conductance or mucosal permeability. This lack of effect on secretion and barrier function suggests that the physiological role of the ECS may be limited to activation of CB₁ receptors that regulate GI motility and is not a general involvement of CB₁ receptors which are found throughout the enteric nervous system.

Our findings do not contradict previous reports in the guinea pig ileum, where pharmacological activation of CB₁ receptors using the potent CB receptor agonist WIN55,212-2 resulted in a reduction of secretion, which was not observable in the presence of the CB₁ antagonist rimonabant.²⁹ Comparable data were also reported for rat ileal secretion.⁵⁶ Both studies identified that CB₁ receptors reduce neuronally induced secretion, but not secretion induced by direct activation of the epithelium.^{29,56} Experiments with the antagonist alone were not reported in these studies. This pharmacological effect is also observable in larger mammals like pigs, where the CB₁ agonist HU 210 reduces secretion induced by the proinflammatory peptide kallidin⁵⁷. Therefore secretion in the gut can be pharmacologically reduced by drugs activating CB₁ receptors and our study adds that secretion seems not to be under physiological control by the ECS. This is supported by another study in mice, where cannabinoid drugs are involved in pathophysiological but not in physiological states.⁴³ The role of CB₁ receptors on neurons of the submucosal plexus

remains to be fully established. One possibility is that the CB₁ receptors are only involved in the regulation of secretion under pathophysiological conditions,⁴³ a hypothesis that needs to be addressed in the future.

In conclusion, using a novel neutral CB₁ receptor antagonist we have confirmed and extended findings that the ECS is involved in the regulation of intestinal motility under physiological conditions. We found no evidence that it is involved in the control of secretomotor or barrier functions under normal physiological conditions, despite the presence of CB₁ receptors on enteric nerves throughout the wall of the gut. We also demonstrated markedly variable effects of CB₁ receptor antagonists on contractility assessed *in vitro* and found no evidence that the ECS regulates electrically-evoked cholinergic contractility in the mouse ileum under these conditions. We made the interesting finding that whole gut transit time was not reduced by the neutral CB₁ antagonist AM4113, suggesting that inverse agonism, but not CB₁ receptor antagonism, is the feature that underlies the reduced whole gut transit seen when these compounds are administered to humans and animals.^{19,20} This unexpected and interesting finding is of importance for the future development of CB₁ receptor antagonists as pharmacological agents for the treatment of a variety of metabolic and other disorders, as it may help to reduce their unwanted GI side-effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative micrographs of CB₁ immunoreactivity in cross-sections and whole mount preparations of ileum and colon from C57BL/6N male mice. Cross-sections of full thickness ileum (A) and colon (B). Whole mount preparations of the myenteric plexus of the ileum (C), colon (D) and submucosal plexus of the ileum (E) and colon (F). Note the dense innervation of the plexuses and extensive innervation of the mucosa. The epithelium was not labelled. Scale bars: 50µm.

Figure 2. Concentration-dependent effects of (A) AM251, (B) rimonabant, (C) AM4113 (all 1nM-100nM) on electrically-evoked contractions of the mouse ileum and (D) the effects of AM251, rimonabant and AM4113 prior to the addition of WIN55,212-2 on electrically-evoked contractions. Note the significant inhibitory effect of WIN55,212-2 is blocked by the presence of all the CB₁ receptor antagonists. ** $p < 0.01$; *** $p < 0.001$; $n = 6-9$ each point.

Figure 3.

(A) AM251 (0.5-1 mg/kg, n=8-12/group) and (B) AM4113 (0.1-1 mg/kg, n=5-6/group) increase upper GI transit of a marker dye *in vivo*. (C) AM4113 (0.1 mg/kg) and AM251 (0.5 mg/kg) completely block the inhibitory effects of the CB₁ agonist WIN55,212-2 (1 mg/kg, n=4-9/group). Note, the effects of AM4113 (2 mg/kg) and AM251 (1 mg/kg) are absent in the CB₁^{-/-} mouse (D, n=4-8/group), which has an enhanced upper GI transit compared to wild type control mice. * p<0.05; ** p < 0.01; *** p < 0.001.

Figure 4.

(A) AM251 (1-2 mg/kg, n=4-12/group) and (B) AM4113 (1-2 mg/kg, n=6-16/group) have no significant effect on colonic expulsion time *in vivo*. (C) AM4113 (1 mg/kg) and AM251 (2 mg/kg) completely block the inhibitory effect of the CB₁ agonist WIN55,212-2 (3 mg/kg, n=4-10/group). (D) Fecal water content is not altered by AM251 (1 mg/kg) or AM4113 (1 mg/kg) n=6-7/group. * p<0.05; ** p < 0.01; *** p < 0.001.

Figure 5. Effect of the inverse agonist/antagonist AM251 and the neutral antagonist AM4113 on whole gut transit time. (A) shows the whole gut transit time when the antagonists are given 1 mg/kg (n=5/group), and (B) when the antagonists are given 5 mg/kg (n=8-9/group). * $p < 0.05$; ** $p < 0.01$.

Figure 6.

Neither AM251 (A) nor AM4113 (B) affected I_{sc} responses to electrical field stimulation or (C) serosal forskolin (FSK, 10 μM) or carbachol (CCh, 60 μM). (V – vehicle; $n = 7$ mice in A, $n = 6$ mice in B, $n = 9-13$ mice in C).