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Title page:

Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (SAB378), a peripherally restricted, cannabinoid CB_1/CB_2 receptor agonist inhibits gastrointestinal motility, but has no effect on experimental colitis in mice.

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Running title: GI effects of peripheral cannabinoid activation.

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Abbreviations: AM251, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; CB_1, cannabinoid 1 receptor; CB_2, cannabinoid 2 receptor; CNS, central nervous system; DSS, dextran sulphate sodium; GI, gastrointestinal; MPO,

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myeloperoxidase; SAB378, naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone; TNBS, 2,4,6-trinitrobenzene sulfonic acid; WIN55212-2 mesylate, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

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Abstract

The endocannabinoid system is involved in the regulation of gastrointestinal (GI) motility and inflammation. Using the peripherally restricted, cannabinoid CB_1/CB_2 receptor agonist naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (SAB378) we investigated the role of peripheral cannabinoid receptors in the regulation of GI motility and the development of colitis in mice. The actions of SAB378 on whole gut transit, upper GI transit, colonic propulsion and locomotor activity were investigated in C57BL/6N, CB_1 receptor knockout and CB_2 receptor knockout mice. The potential for SAB378 to modify inflammation was studied using dextran sulphate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) models of experimental colitis. SAB378 did not modify locomotor activity. SAB378 slowed all parameters of GI motility and these effects were significantly reduced by the CB_1 receptor antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide (AM251), but not by the CB_2 receptor antagonist 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (AM630). SAB378 did not inhibit GI transit or colonic propulsion in CB_1 receptor knockout mice, while its effects were observed in CB_2 receptor knockout mice. SAB378 did not reduce the degree of colitis induced by DSS or TNBS. The actions of SAB378 on GI motility are mediated by peripherally located CB_1 receptors. SAB378 was not effective against two models of experimental colitis which may indicate that peripheral cannabinoid receptor stimulation alone may not be sufficient to mediate anti-inflammatory effects of cannabinoids.

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Introduction

Cannabinoids are lipid mediators that activate cannabinoid (CB) receptors. Cannabinoids can be of exogenous (synthetic and *Cannabis sativa* derived compounds) or endogenous origin (endocannabinoids). Endocannabinoids, such as 2-arachidonoyl glycerol (2-AG) and anandamide, are produced on demand from the phospholipid precursors present in the cell membrane, and bind to CB_1 and/or CB_2 receptors (De Petrocellis and Di Marzo, 2009). Endocannabinoids are degraded by the enzymes fatty acid amide hydrolase and/or monoacylglycerol lipase (De Petrocellis and Di Marzo, 2009). Cannabinoid receptors are known to mediate a number of actions in the gastrointestinal (GI) tract including the stimulation of feeding and the inhibition of emesis, gastric secretion, gastroesophageal reflux and transepithelial ion transport (Izzo and Sharkey, 2010). In addition, cannabinoids are known to inhibit GI transit and to reduce the degree of intestinal inflammation in animal models of experimental colitis (Izzo and Sharkey, 2010). The development of cannabinoids as therapies for GI motility disorders and inflammatory bowel disease is hampered by their centrally mediated, psychoactive side effects. While studies have aimed to elucidate whether these cannabinoid effects are mediated through peripherally or centrally located cannabinoid receptors, a definitive answer has not been forthcoming due to a previous lack of pharmacological tools whose actions are restricted to the periphery. The GI tract is under hierarchical neural control with extrinsic autonomic nerves influencing the activity of enteric neurons located within the wall of the gut (Furness, 2006). As CB_1 receptors are located in the dorsal vagal complex, including the dorsal motor nucleus of the vagus (DMNX, (Van Sickle, et al., 2001) and in the myenteric plexus of the enteric nervous system (Coutts, et al., 2002; Kulkarni-Narla and Brown, 2000; Mascolo, et al., 2002; Pinto, et al., 2002; Van Sickle, et al., 2001) it follows that CB_1 activation in either of these

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regions could alter GI motility. Studies have attempted to elucidate, through the use of varying routes of drug administration and/or vagotomy or ganglionic blockade, the role that central versus peripheral CB receptor activation plays in the modulation of GI motility. Their findings revealed evidence which suggests there may be regional differences such that CB_1 receptors located in the vago-vagal circuitry mediate the actions in slowing gastric motility (Krowicki, et al., 1999) while mainly peripheral CB_1 receptors mediate the inhibitory actions on GI transit (Landi, et al., 2002), and that either central or peripheral CB_1 receptors can mediate the slowing of upper GI transit and colonic propulsion (Izzo, et al., 2000; Pinto, et al., 2002).

Experimental colitis is reduced by CB_1 and CB_2 receptor agonists (Kimball, et al., 2006; Engel, et al., 2008; Storr, et al., 2009) and by fatty acid amide hydrolase inhibitors (D'Argenio, et al., 2006; Storr, et al., 2008). There is little evidence to suggest whether cannabinoid-induced attenuation of colitis is mediated by peripheral and/or central receptors. Peripheral levels of CB_1 receptor expression (Izzo, et al., 2001; Massa, et al., 2004; Kimball, et al., 2006), CB_2 receptor mRNA (Storr, et al., 2009) and anandamide levels (D'Argenio, et al., 2006) are increased in the inflamed intestine suggesting peripheral receptors may be important in mediating the effects of cannabinoids. It has been shown, in a model of peritonitis, that cannabinoids mediate a protective effect via central CB_1, but not CB_2, receptors (Smith, et al., 2001).

Recently a novel CB_1/CB_2 receptor agonist (IC_{50} values of 15 ± 5 nM and 98 ± 7.6 nM at hCB_1 and hCB_2 receptors respectively), naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (SAB378), with restricted central actions has been described (Dziadulewicz, et al., 2007). SAB378 is antihyperalgesic and lacks centrally mediated side effects such as hypomotility in rats, nor does it accumulate in the CNS following twice daily oral doses over 5 days (Dziadulewicz, et al., 2007). Furthermore, SAB378 has been shown to have good oral

bioavailability in rats (Dziadulewicz, et al., 2007) dogs (Trevaskis, et al., 2009) and humans (Gardin, et al., 2009). We used this compound to study the role of peripheral cannabinoid receptors in the mediation of actions on GI motility and experimental colitis in mice. We used 2 well established models of experimental colitis, dextran sulphate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS). DSS-induced colitis is characterized by bloody diarrhea, epithelial ulceration and mucosal neutrophil infiltration whereas TNBS colitis is characterized by transmural inflammation, ulceration and neutrophil infiltration.

Using the peripherally restricted cannabinoid agonist SAB378, we report that cannabinoid-induced slowing of GI motility is mediated by peripheral CB_1 receptor activation while the protective actions of cannabinoids in experimental colitis may be dependent on central actions.

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Methods:

Animals

Female C57BL/6N mice (17-25 g) and male CD1 mice (25-37 g) were purchased from Charles River (Montreal, Quebec, Canada). Two breeding pairs of heterozygous $CB_1^{+/-C57BL/6N}$ mice were obtained from Dr. B. Lutz (University Medical Center Mainz) and two breeding pairs of heterozygous $CB_2^{+/-C57BL/6}$ mice were obtained from Dr. N. Buckley (California State Polytechnical University, Pomona, CA) and bred in our facility to obtain $CB_1^{-/-C57BL/6N}$ and $CB_2^{-/-C57BL/6N}$ mice, respectively. Animals used in these studies were backcrossed from both heterozygous and homozygous breeding pairs to C57BL/6N for 6 generations and were used at the same age (female, $CB_1^{-/-}$: 8-16 weeks and $CB_2^{-/-}$: 6-15 weeks) and maintained under the same conditions as the C57BL/6N and CD1 mice. All $CB_1^{-/-}$ (Marsicano, et al., 2002) and $CB_2^{-/-}$ (Buckley, et al., 2000) mice were genotyped using established protocols and were confirmed as homozygous gene-deficient animals ($CB_1^{-/-C57BL/6N}$, $CB_2^{-/-C57BL/6N}$) prior to inclusion in the study. All mice were housed in plastic sawdust floor cages and allowed free access to tap water and standard laboratory chow, unless otherwise stated. All experimental procedures were approved by the University of Calgary Animal Care Committee and were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

Drugs

Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (SAB378) was synthesized and supplied by Novartis Pharmaceuticals (UK). The CB_1 receptor antagonist/inverse agonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide (AM251), the CB_2 receptor antagonist 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (AM630) and the CB_1/CB_2 receptor agonist (R)-(+)-[2,3-

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dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55212-2) were purchased from Tocris (Ellisville, MI, USA). All drugs were dissolved in a vehicle of 2 % DMSO, 1 % Tween 80 in physiological saline. Injections were administered intraperitoneally (i.p.) at 4 µl/g body weight. Dextran sulphate sodium (DSS; molecular weight 36,000 – 50,000) was purchased from MP Biomedicals (Solon, Ohio, USA). 2,4,6-trinitrobenzene sulfonic acid (TNBS) was purchased from Fluka (Switzerland).

Locomotor activity studies

Ambulatory locomotor activity was measured using an infrared beam activity monitor (Columbus Instruments, Columbus, OH, USA). Sequential breaking of the invisible infrared beams by movement of the mouse is recorded, by the monitor, as the ambulatory activity count. C57BL/6N mice were individually placed in the apparatus and the ambulatory count was recorded over a 10 min period. The activity apparatus was cleaned with Virkon spray between subjects. Mice underwent a locomotor activity trial approximately 4 h prior to the test. All experiments were started at 09:00. For the test, mice were injected i.p. with either vehicle or SAB378 (0.1 or 1.0 mg/kg) and 20 min later placed in the activity monitor where ambulatory activity was recorded for 10 min.

In vivo transit studies

Whole gut transit studies: 3 days prior to the experiment mice were individually housed. On the day of the experiment, mice were transferred to individual plastic cages without bedding and were left to acclimatize to the cage for 1 h. C57BL/6N mice were administered an i.p. injection of vehicle, AM251 (1.0 mg/kg) or AM630 (1.0 mg/kg) 20 min before receiving an i.p. injection of vehicle, WIN55212-2 (1.0 mg/kg) or SAB378 (0.1 or 1.0 mg/kg). 20 min later mice

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were gavaged (using a 3 cm, 20 G gavaging needle) with 200 µl of an Evans' blue (5 % Evans' blue, 5 % gum arabic) marker. Mice were returned to their individual cages (*ad libitum* access to food and water) and the latency to the detection of Evans' blue in the droppings was recorded. In further experiments CB_1 receptor knockout or CB_2 receptor knockout mice were injected i.p. with vehicle or SAB378 (0.1 or 1.0 mg/kg) and whole gut transit was measured as outlined above.

Upper GI transit studies: Mice were fasted for 10-14 h prior to the start of the experiment with *ad libitum* access to water. C57BL/6N mice were administered an i.p. injection of vehicle, AM251 (1.0 mg/kg) or AM630 (1.0 mg/kg) 20 min before receiving an i.p. injection of vehicle or SAB378 (0.1 or 1.0 mg/kg). 20 min later mice were gavaged (using a 3 cm, 20 G gavaging needle) with 200 µl of an Evans' blue (5 % Evans' blue, 5 % gum arabic) marker. 15 min later mice were killed via cervical dislocation and the intestine from the region of the pyloric sphincter to the ileo-caecal junction was removed. Without stretching the tissue the length of the intestine and distance travelled by the marker was recorded. In other experiments CB_1 receptor knockout mice were injected i.p. with vehicle or SAB378 (1.0 mg/kg) and CB_2 receptor knockout mice were injected i.p. with vehicle or SAB378 (0.1 or 1.0 mg/kg) and upper GI transit was measured as outlined above.

Colonic propulsion: Mice were lightly anesthetized with isoflurane before a 2.5 mm spherical glass bead was inserted 2 cm intrarectally. The latency to the expulsion of the bead was recorded. C57BL/6N mice were injected i.p. with vehicle, AM251 (1.0 mg/kg) or AM630 (1.0 mg/kg) 20 min before receiving an i.p. injection of vehicle, WIN55212-2 (1.0 mg/kg) or SAB378 (0.1 or 1.0 mg/kg). 20 min later colonic propulsion was recorded. In further experiments CB_1 receptor knockout or CB_2 receptor knockout mice were injected i.p. with vehicle or SAB378 (0.1 or 1.0 mg/kg) and colonic propulsion was measured as outlined above.

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Experimental colitis studies

DSS-induced colitis: Male CD1 mice were administered DSS (5 % DSS in drinking water, days 0-5) and body weight was recorded daily. On days 4-8 post-DSS initiation vehicle, WIN55212-2 (2.0 mg/kg) or SAB378 (0.1 and 1.0 mg/kg) was administered i.p. twice daily (9:00 and 17:00). On day 8 mice were killed via cervical dislocation. The colon was dissected and assessed for macroscopic evidence of colitis by a blinded investigator. Body weight score was calculated as the % weight loss from the initial body weight on day 0 (1 = 0-5 %, 2 = 5.1-10 %, 3 = 10.1-15 %, 4 > 15 %). Colon length score was calculated as a % of control colon length, 1 = 75-85 %, 2 = 65-74.9 % and 3 < 64.9 %. The presence (score = 1) or absence (score = 0) of erythema, fecal blood and diarrhoea was recorded. A total macroscopic damage score was calculated for each animal comprising, body weight score, colon length score, erythema score, fecal blood score, diarrhea score, length of inflamed colon as % of total length. Myeloperoxidase (MPO) activity was measured to assess neutrophil infiltration as has been previously described (Storr, et al., 2009).

TNBS-induced colitis: Male CD1 mice were intraperitoneally injected with SAB378 (0.1 and 1.0 mg/kg), WIN55212-2 (2 mg/kg) or vehicle. 1h later mice were lightly anesthetized with isoflurane before the intrarectal administration of TNBS (100µl of 40 mg/ml in 30% ethanol). Mice were injected with SAB378 (0.1 and 1.0 mg/kg), WIN55212-2 (2 mg/kg) or vehicle 8 h and 24 h after the initial drug injection. 3 days post-TNBS application mice were killed via cervical dislocation. The colon was dissected and assessed for macroscopic evidence of colitis by a blinded investigator. The length of the colon was measured and scored accordingly (> 8.1cm = 0, 7.1-8 cm = 1, < 7 cm = 2). The presence (score = 1) or absence (score = 0) of erythema, fecal blood and diarrhea was recorded. A total macroscopic damage score was calculated for each

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animal comprising, colon length score, length of inflamed colon (% total colon length), erythema score, fecal blood score, diarrhea score and length of ulcerated colon (cm). Myeloperoxidase (MPO) activity was measured as noted above. Tissue was also collected for microscopic analysis. Tissues were first fixed overnight in Zamboni's at 4°C then washed three times at 10 min intervals in phosphate-buffered saline. Tissues were cryo-protected in PBS-sucrose (20%) then embedded in optimal cutting temperature compound. Sections were cut (14 μm), using a cryostat, and stained with hematoxylin and eosin. A microscopic damage score of sections was determined, by two blinded investigators, based on the presence (score = 1) or absence (score = 0) of goblet cell depletion, the presence (score = 1) or absence (score = 0) of crypt abscesses, the destruction of normal architecture (normal = 1, moderate = 2, extensive =3), the extent of muscle thickening (normal = 1, moderate = 2, extensive =3) and the presence and degree of cellular infiltration (normal = 1, moderate = 2, transmural =3).

Statistical analysis

Data are expressed as the mean ± S.E.M. and analyzed using either an unpaired t-test, one-way analysis of variance (ANOVA) or two-way ANOVA (with time as the repeated measure) followed by Bonferroni's post hoc test as appropriate. $P < 0.05$ was considered significant.

Results:

Locomotor activity studies:

The action of SAB378 on locomotor activity in mice was examined in order to confirm that it did not have actions typical of centrally acting cannabinoid agonists (Dziadulewicz, et al., 2007), which would be to reduce locomotor activity (Herkenham, 1992). SAB378 at doses of 0.1 and 1.0 mg/kg had no effect on ambulatory motor activity in mice (Figure 1), with the number of beam breaks in SAB378 treated mice being comparable to that seen in the vehicle treated controls.

Whole gut transit assay:

Experiments were carried out to examine the action of SAB378 on whole gut transit. The centrally active CB_1/CB_2 receptor agonist WIN55212-2 was used as a reference compound to show established effects of CB_1 receptor activation on transit. WIN55212-2 delayed whole gut transit and this effect was blocked by the CB_1 receptor antagonist AM251, but not by the CB_2 receptor antagonist AM630 (Figure 2A). SAB378 slowed whole gut transit (Figure 2A). In mice treated with AM630 before SAB378 the inhibitory effect was still observed, however, AM251 significantly reduced the action of SAB378 on transit (Figure 2A).

To further confirm that the action of SAB378 was mediated through CB_1 receptors, whole gut transit assays were carried out in CB_1 receptor knockout and in CB_2 receptor knockout mice. While 0.1 mg/kg SAB378 slowed whole gut transit to some degree in CB_1 knockout mice (transit was 49.9 ± 12.0 % slower than in vehicle treated CB_1 knockout mice), this was not to the same magnitude as in C57BL/6N mice (transit was 106.9 ± 8.0 % slower than in vehicle treated C57BL/6N mice). 1.0 mg/kg SAB378 has no effect on whole gut transit in CB_1 knockout mice (Figure 2B). SAB378 had a significant effect on whole gut transit in CB_2 receptor knockout mice

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(Figure 2C). Interestingly, the inhibitory effect of SAB378 (1.0 mg/kg) on whole gut transit was much greater in C57BL/6N mice than in CB_2 receptor knockout mice. Whole gut transit in C57BL/6N mice was 174.7 ± 11.9 % slower than in vehicle treated mice while in CB_2 receptor knockout mice transit was only 64.7 ± 3.4 % slower than in vehicle treated mice.

Upper GI transit assay:

In order to determine whether a specific region of the GI tract was being inhibited by SAB378 contributing to an overall slowing of whole gut transit, its action on upper GI transit was investigated. SAB378 (1.0 mg/kg) slowed transit of the upper GI tract in a manner that was significantly decreased by AM251 but not AM630 (Figure 3A). In CB_1 receptor knockout mice upper GI transit in mice treated with SAB378 (1.0 mg/kg) was comparable to that observed in vehicle treated controls (Figure 3B). SAB378 inhibited upper GI transit in CB_2 knockout mice (Figure 3C).

Colonic propulsion assay:

To further characterize the action of SAB378 on GI transit its effect on colonic propulsion was examined. The positive control WIN55212-2 slowed colonic propulsion (Figure 4A). This effect was blocked by AM251 but not by AM630 (Figure 4A). SAB378 (1.0 mg/kg) inhibited colonic propulsion in a manner that was not blocked by AM630 and was significantly reduced by AM251 (Figure 4A). In CB_1 receptor knockout mice SAB378 did not modify colonic propulsion (Figure 4B). The lower dose of 0.1 mg/kg SAB378 had no effect on propulsion in CB_2 receptor knockout mice, while SAB378 at a dose of 1.0 mg/kg slowed colonic propulsion in these mice (Figure 4C). However, the inhibitory effect of SAB378 (1.0 mg/kg) on colonic propulsion was significantly greater ($p < 0.01$) in C57BL/6N mice than in CB_2 receptor knockout mice. Propulsion in C57BL/6N mice was 491.6 ± 103.9 % slower than in vehicle treated mice

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while in CB_2 receptor knockout mice transit was only 104.0 ± 45.8 % slower than in vehicle treated mice.

DSS-induced colitis:

DSS was used to induce colitis in mice. Body weight loss is an indicator that colitis has been established, and vehicle treated control animals showed an 11.3 % loss in body weight from their starting weight on day 0 (Figure 5A). WIN55212-2 blocked the effect of DSS on body weight change such that by day 6 those mice did not lose further body weight. In fact DSS mice treated with WIN55212-2 were significantly heavier than their vehicle treated counterparts on days 7 and 8 (Figure 5A). SAB378 at the lower dose of 0.1 mg/kg induced a greater body weight loss than the controls while the body weight of mice treated with 1.0 mg/kg SAB378 was comparable to that in the vehicle control mice (Figure 5A).

The total macroscopic colonic damage score in DSS mice treated with WIN55212-2 was significantly reduced compared to the vehicle controls (Figure 5B). While there was a trend for WIN55,2122 to reduce MPO levels in DSS administered mice, indicating a less severe inflammatory response to DSS, this failed to reach significance ($p > 0.05$; Figure 5C). SAB378 did not modify the macroscopic colonic damage score or MPO activity in DSS mice compared to controls (Figure 5B and 5C).

TNBS-induced colitis:

To further assess the effect of SAB378 in colitis we carried out investigations in TNBS-induced colitis. In vehicle treated mice TNBS induced colitis (Figure 6). WIN55212-2 significantly reduced body weight loss (Figure 6A), total macroscopic and microscopic colonic damage score (Figure 6B and D) and demonstrated a trend to reduce MPO activity (Figure 6C) in TNBS-induced colitis. SAB378 (0.1 mg/kg) had no effect on these parameters of TNBS-induced

colitis compared to vehicle controls (Figure 6). SAB378 (1.0 mg/kg) demonstrated a non-significant trend to reduce body weight loss (Figure 6A), macroscopic and microscopic damage scores (Figure 6B and D) and MPO activity (Figure 6D) compared to vehicle treated controls.

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Discussion:

We report the actions of a peripherally restricted cannabinoid receptor agonist, SAB378, on gastrointestinal motility and in experimental models of colitis. This mixed CB_1/CB_2 receptor agonist inhibited gastrointestinal transit however, SAB378 had no effect on the degree of inflammation induced in the DSS or TNBS model of colitis. WIN55212-2, which is a centrally active CB_1/CB_2 receptor agonist (Compton, et al., 1992), inhibited both GI motility and attenuated colitis.

It has been shown that orally administered SAB378 does not induce typical cannabinoid-mediated CNS effects such as catalepsy, at doses and time points which are anti-hyperalgesic (Dziadulewicz, et al., 2007). This, along with pharmacokinetic studies showing a high affinity for plasma protein thus limiting the passage of SAB378 across the blood brain barrier, suggest that this compound is peripherally restricted (Dziadulewicz, et al., 2007). We confirmed this property in the present study by demonstrating that SAB378, when administered intraperitoneally, does not significantly reduce locomotor activity in mice. The induction of hypomotility by cannabinoid agonists (Little, et al., 1988) is believed to be a centrally mediated event due to the high density of CB_1 receptors in the basal ganglia, and it is likely that activation of these receptors results in impaired locomotor activity (Herkenham, 1992).

It is well established that cannabinoids exert a braking effect on physiological GI transit inhibiting gastric emptying and motility (Izzo, et al., 1999b; Krowicki, et al., 1999; Shook and Burks, 1989), upper GI transit (Colombo, et al., 1998; Izzo, et al., 1999a; Izzo, et al., 2000; Izzo, et al., 2001; Landi, et al., 2002; Mathison, et al., 2004; Shook and Burks, 1989) and colonic propulsion (Pinto, et al., 2002), through the activation of CB_1 receptors. In the current study, through the use of specific CB_1 and CB_2 receptor antagonists, we confirmed the CB_1-mediated

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inhibitory action of WIN55212-2 on GI tract motility and colonic propulsion. Furthermore we report that SAB378 also slows whole gut transit and upper GI transit in a CB_1 mediated manner. Although the lower dose of SAB378 (0.1 mg/kg) slowed whole gut transit in CB_1 receptor knockout mice this was not to the same degree as was observed in control C57BL/6N mice and the higher dose (1.0 mg/kg) was without transit effects in these mice. Furthermore, the CB_1 receptor antagonist AM251 completely reversed 0.1 mg/kg SAB378-induced inhibition of whole gut transit. SAB378 did not modify upper GI transit in CB_1 receptor knockout mice and AM251 reversed SAB378-induced inhibition of upper GI transit in C57BL/6N mice. The CB_2 receptor antagonist AM630 did not modify the SAB378-induced effect on whole gut transit or upper GI transit, and the inhibitory effect in both parameters was observed in CB_2 receptor knockout mice, suggesting that CB_2 receptors are not involved in these actions of SAB378. Under physiological conditions GI motility is not modified by CB_2 receptor agonists (Mathison, et al., 2004), nor is the inhibitory action of mixed CB_1/CB_2 receptor agonists blocked by CB_2 receptor antagonists (Izzo, et al., 1999b; Izzo, et al., 2000; Pinto, et al., 2002). Thus it was not surprising to observe in our study that SAB378 was not acting via CB_2 receptors to slow physiological upper GI transit.

In the current study, the inhibitory action of SAB378 on colonic propulsion was significantly reversed by AM251 but not by AM630, again suggesting that this action is CB_1 and not CB_2 receptor mediated. SAB378 did not inhibit colonic propulsion in CB_1 receptor deficient mice and while there was an inhibition of colonic propulsion in CB_2 receptor deficient mice following SAB378 administration, this was not to the same magnitude as was observed in control C57BL/6N mice. This suggests that activation of CB_1 is essential for the inhibitory action of SAB378 in colonic propulsion. Activation of the CB_2 receptor may not be necessary for SAB378-induced inhibition of colonic propulsion to be seen (as evidenced by pharmacological

blockade of the receptor having no effect on the SAB378 action). However, the presence of CB_2 receptors may be required for the full CB_1-mediated inhibitory effect to be revealed. To the best of our knowledge this is the first report of such an interaction between CB_1 and CB_2 receptors and it appears that, in the regions of the GI tract examined in these studies, this phenomenon is unique to the colon.

Whole gut transit was also inhibited by SAB378 in a CB_1 receptor-mediated manner, as determined through the use of cannabinoid receptor specific antagonists and cannabinoid receptor gene deficient mice. In CB_2 receptor knockout mice, SAB378 did not inhibit whole gut transit to the same degree as was observed in the C57BL/6N mice and this is likely due to SAB378 having a blunted effect on colonic propulsion in these mice.

Overall, in all regions examined, SAB378 inhibited gastrointestinal transit suggesting that cannabinoid-induced slowing of GI motility is mediated by peripherally located receptors. It has been suggested, via the systemic and/or central administration of cannabinoid agonists and antagonists, that mainly peripheral CB_1 receptors are involved in cannabinoid-induced modulation of gastrointestinal transit (Landi, et al., 2002). However, it has also been shown that the CB_1/CB_2 receptor agonist tetrahydrocannabinol (THC) inhibits gastric motility when applied to the dorsal surface of the medulla (Krowicki, et al., 1999) and that the inhibitory actions on gastric motility of systemically administered THC were blocked by vagotomy and by ganglionic blockade by hexamethonium suggesting the vago-vagal circuitry is the site of action of THC in this effect (Krowicki, et al., 1999). Intracerebroventricular (i.c.v.) administration of the CB_1/CB_2 receptor agonist WIN55212-2 inhibited upper GI transit in mice with a significantly lower ED50 than when injected i.p. suggesting that slowed transit induced by this agonist is mediated by central CB_1 receptors (Izzo, et al., 2000). However, these authors also found that despite

ganglionic blockade, i.p. injected cannabinoid agonists still slowed upper GI transit suggesting that peripheral CB_1 receptors mediate the inhibitory effect when systemically administered (Izzo, et al., 2000). Similarly, colonic propulsion in mice was slowed following either i.c.v. or i.p. administration of the CB_1 receptor agonist ACEA suggesting that activation of either central or peripheral receptors can induce an effect on the colon (Pinto, et al., 2002). We have confirmed, that the stimulation of peripheral CB_1 receptors by SAB378 inhibits motility of the whole gut and also of upper GI transit and colonic propulsion as separate elements, and that peripheral CB_2 receptors may play a role in the mediation of SAB378-induced inhibition of colonic propulsion.

In addition to its actions on GI physiology, the endocannabinoid system is known to exert anti-inflammatory actions in the GI tract. Despite this, it has not been determined whether the effects of cannabinoids on inflammation are mediated by central or peripheral cannabinoid receptors or if receptors in both regions play a part. As described above, CB_1 receptors are located in the CNS regions primarily involved in GI motor control and in the enteric nervous system and CB_2 receptors are similarly present in these regions (Duncan, et al., 2008; Van Sickle, et al., 2005). In experimental colitis a local response to the chemical insult (DSS and TNBS in these studies) is initiated resulting in the release of pro-inflammatory cytokines and mediators. Anti-inflammatory responses are also initiated in order to control the inflammatory response. The balance between the pro and anti-inflammatory responses is, in part, controlled by the CNS. This suggests that CB receptors located in the CNS or periphery could mediate the cannabinoid-induced anti-inflammatory actions. The present study is the first to demonstrate that the CB_1/CB_2 receptor agonist WIN55212-2 reduced the severity of DSS- and TNBS-induced colitis. Furthermore, SAB378 had no effect on the degree of colitis induced by DSS or TNBS suggesting that activation of peripheral cannabinoid receptors alone may not be sufficient to

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afford protection against either DSS- or TNBS-induced inflammation in the mouse colon. Studies investigating colitis following either chemical ablation of capsaicin-sensitive primary afferents or surgical vagotomy have revealed that these nerves play a protective role on DSS- and TNBS-induced colitis in rodents (Ghia, et al., 2007; McCafferty, et al., 1997). However, other groups have suggested that these neurons are essential for the induction of these types of experimental colitis in rats such that destruction of the nerves results in a less severe inflammatory outcome (Fujino, et al., 2004; Kihara, et al., 2003). It has been shown that cannabinoid agonists block the production of neutrophil chemoattractants and prevent the migration of neutrophils into the peritoneal cavity in a mouse model of peritonitis via central CB_1, and not CB_2, receptors (Smith, et al., 2001). The present study suggests that activation of central cannabinoid receptors may be required for the anti-inflammatory actions of cannabinoid agonists in both DSS and TNBS colitis. Whether the activation of only central receptors could protect against colitis or whether dual activation of receptors in both the CNS and periphery is required, remains to be determined. Our findings demonstrate that the higher dose of SAB378 (1.0 mg/kg) showed a non-significant trend to improve colitis which may suggest that peripheral CB receptors could play a role in the mediation of anti-inflammatory actions of cannabinoids. However, investigating this would prove problematic due to the inhibitory actions that higher doses of SAB378 would exert on GI transit. In the present study, daily administration of SAB378 (0.1 mg/kg) further reduced body weight in mice treated with DSS, while having no effect on the degree of inflammation in this model of colitis. Similarly, sub-chronic daily treatment of the cannabinoid agonist HU210 has been shown to reduce body weight, at doses that do not affect food intake, in rats (Giuliani, et al., 2000). The present study may have revealed a biphasic action of daily SAB378 administration on body weight in mice that is not related to the inflammatory

state of the GI tract, as the effect was not observed at a higher dose (1.0 mg/kg) nor was it observed when administered acutely during the TNBS colitis study.

In conclusion, utilizing SAB378, a cannabinoid receptor agonist whose action is restricted to the periphery, we show that while the actions of cannabinoid receptor activation to slow GI motility may be peripherally mediated, and thus may be of therapeutic value, the anti-inflammatory effects of cannabinoid agonists on experimental colitis may require central cannabinoid receptor activation.

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Footnotes:

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Legends for Figures:

Figure 1 The effect of vehicle (veh; 4 % DMSO, 2 % Tween 80 in physiological saline) or SAB378 (0.1 or 1.0 mg/kg) on ambulatory locomotor activity, recorded for 10 min, in C57BL/6N mice. All injections were administered i.p. Bars represent the mean ± S.E.M., n = 8-11.

Figure 2 The effect of vehicle (veh; 4 % DMSO, 2 % Tween 80 in physiological saline), 1.0 mg/kg AM251 or 1.0 mg/kg AM630 administered 20 min prior to vehicle, WIN55212-2 (WIN; 1.0 mg/kg) or SAB378 (0.1 or 1.0 mg/kg) on whole gut transit in C57BL/6N mice **(A)**. The effect of vehicle or SAB378 (0.1 or 1.0 mg/kg) on whole gut transit in CB_1 **(B)** and CB_2 **(C)** knockout mice. All injections were administered i.p. Bars represent the mean ± S.E.M., n = 6-11. ** ($p < 0.01$) and *** ($p < 0.001$) denotes a significant difference to the vehicle treated control and ††† ($p < 0.001$) denotes a significant difference between indicated groups.

Figure 3 The effect of vehicle (veh; 4 % DMSO, 2 % Tween 80 in physiological saline), 1.0 mg/kg AM251 or 1.0 mg/kg AM630 administered 20 min prior to vehicle or SAB378 (0.1 or 1.0 mg/kg) on upper GI transit in C57BL/6N mice **(A)**. The effect of vehicle or SAB378 (1.0 mg/kg) on upper GI transit in CB_1 knockout mice **(B)** and the effect of vehicle or SAB378 (0.1 and 1.0 mg/kg) on upper GI transit in CB_2 knockout mice **(C)**. All injections were administered i.p. Bars represent the mean ± S.E.M., n = 4-8. * ($p < 0.05$) and *** ($p < 0.001$) denotes a significant difference to the vehicle treated control and †† ($p < 0.01$) denotes a significant difference between indicated groups.

Figure 4 The effect of vehicle (veh; 4 % DMSO, 2 % Tween 80 in physiological saline), 1.0 mg/kg AM251 or 1.0 mg/kg AM630 administered 20 min prior to vehicle, WIN55212-2 (WIN;

1.0 mg/kg) or SAB378 (0.1 or 1.0 mg/kg) on colonic propulsion in C57BL/6N mice **(A)**. The effect of vehicle or SAB378 (0.1 or 1.0 mg/kg) on colonic propulsion in CB_1 **(B)** and CB_2 **(C)** knockout mice. All injections were administered i.p. Bars represent the mean ± S.E.M., n = 6-10. * ($p < 0.05$) and ** ($p < 0.01$) denotes a significant difference to the vehicle treated control and † ($p < 0.05$) denotes a significant difference between indicated groups.

Figure 5 The effect of vehicle (veh; 4% DMSO, 2% Tween 80 in physiological saline), WIN55212-2 (WIN; 2.0 mg/kg) or SAB378 (0.1 or 1.0 mg/kg), administered twice daily i.p., on changes in body weight of CD1 mice following DSS administration (**A**; 5 % DSS in drinking water, day 0-5). Mice were killed on day 8 and the effect of vehicle, WIN55212-2 or SAB378 on total macroscopic damage score **(B)** and MPO activity **(C)** of the colon was measured. Data points or bars represent the mean ± S.E.M., n = 5-8. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) denotes a significant difference to the vehicle treated control.

Figure 6 The effect of vehicle (veh; 4% DMSO, 2% Tween 80 in physiological saline), WIN55212-2 (WIN; 2.0 mg/kg) or SAB378 (0.1 or 1.0 mg/kg), administered i.p., on changes in body weight of CD1 mice following TNBS administration (**A**). Mice were killed on day 3 post-TNBS administration and the effect of vehicle, WIN55212-2 or SAB378 on total macroscopic damage score **(B),** MPO activity **(C)** and microscopic damage score **(D)** of the colon was measured. Representative photomicrographs of hematoxylin and eosin staining in TNBS administered colon following vehicle, WIN55212-2 or SAB378 (0.1 and 1.0 mg/kg) treatment **(E)**. Scale bar represents 100 μm. Data points or bars represent the mean ± S.E.M., n = 5-8. * ($p < 0.05$) denotes a significant difference to the vehicle treated control.

Figure 1

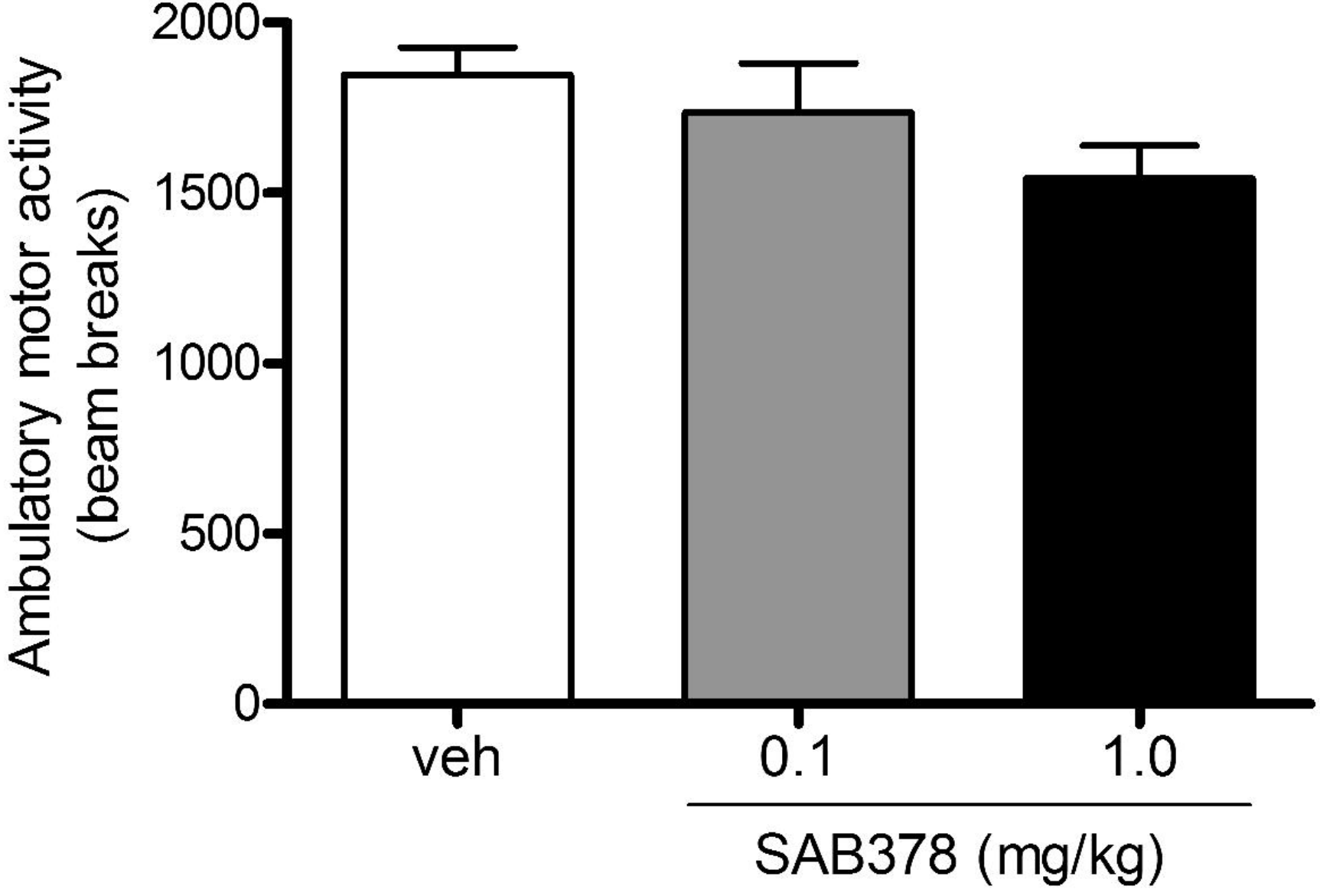

Ambulatory motor activity
(beam breaks)
2000
1500
1000
500
0
veh
0.1
1.0
SAB378 (mg/kg)

Figure 2

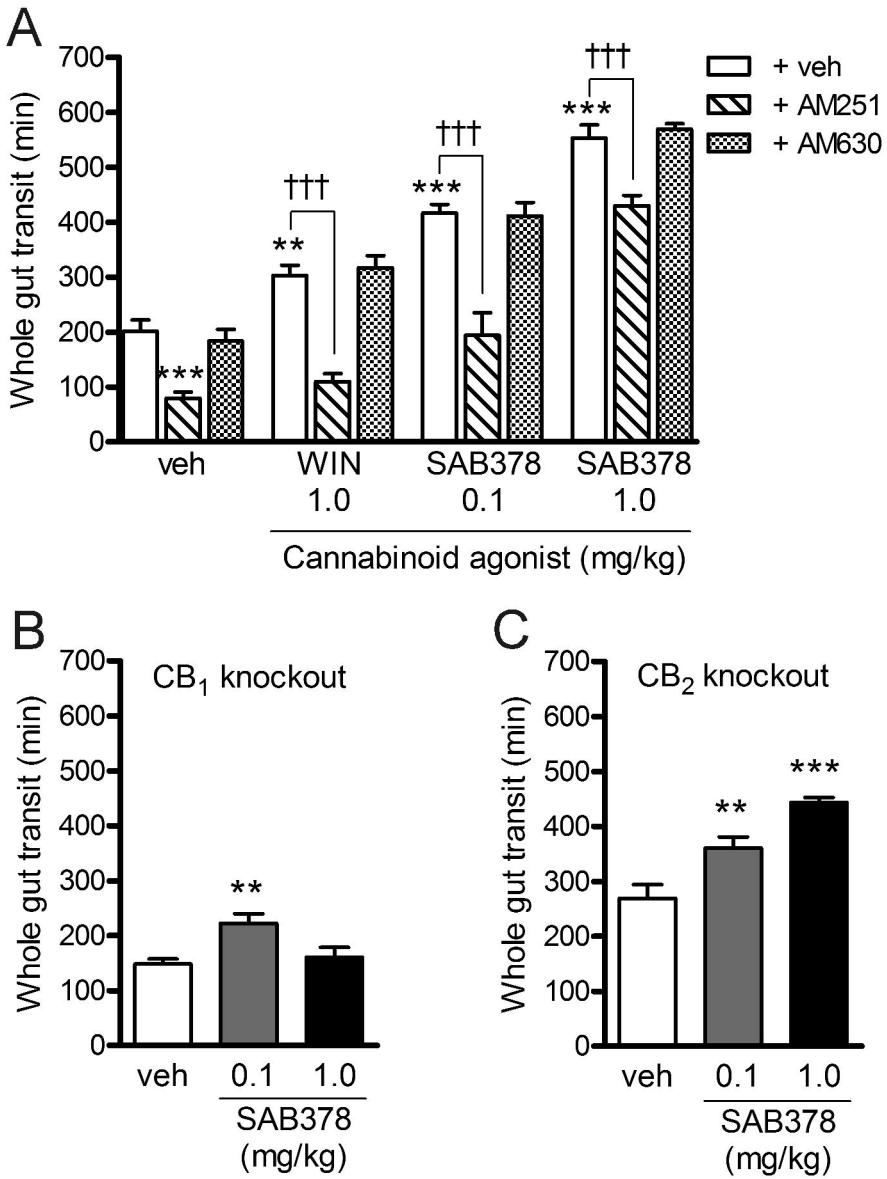

A
Whole gut transit (min)
700
600
500
400
300
200
100
0
+ veh
+ AM251
+ AM630
***
**
***
***
†††
†††
†††
veh
WIN
1.0
SAB378
0.1
SAB378
1.0
Cannabinoid agonist (mg/kg)
B
CB1 knockout
Whole gut transit (min)
**
veh
0.1
1.0
SAB378
(mg/kg)
C
CB2 knockout
Whole gut transit (min)
**
***
veh
0.1
1.0
SAB378
(mg/kg)

Figure 3

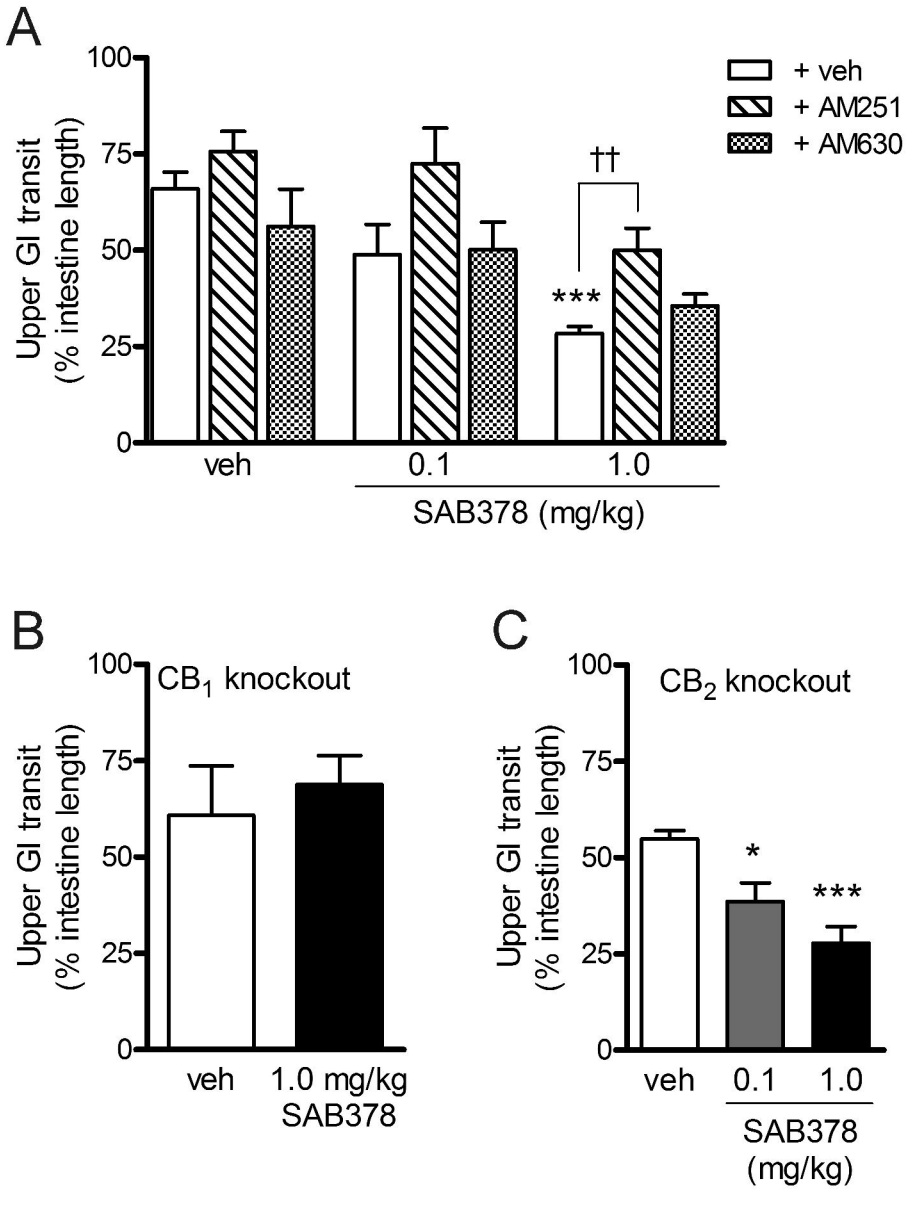

A
100
75
50
25
0
Upper GI transit
(% intestine length)
+ veh
+ AM251
+ AM630
††
***
veh
0.1
1.0
SAB378 (mg/kg)
B
CB1 knockout
Upper GI transit
(% intestine length)
veh
1.0 mg/kg
SAB378
C
CB2 knockout
Upper GI transit
(% intestine length)
*
***
veh
0.1
1.0
SAB378
(mg/kg)

Figure 4

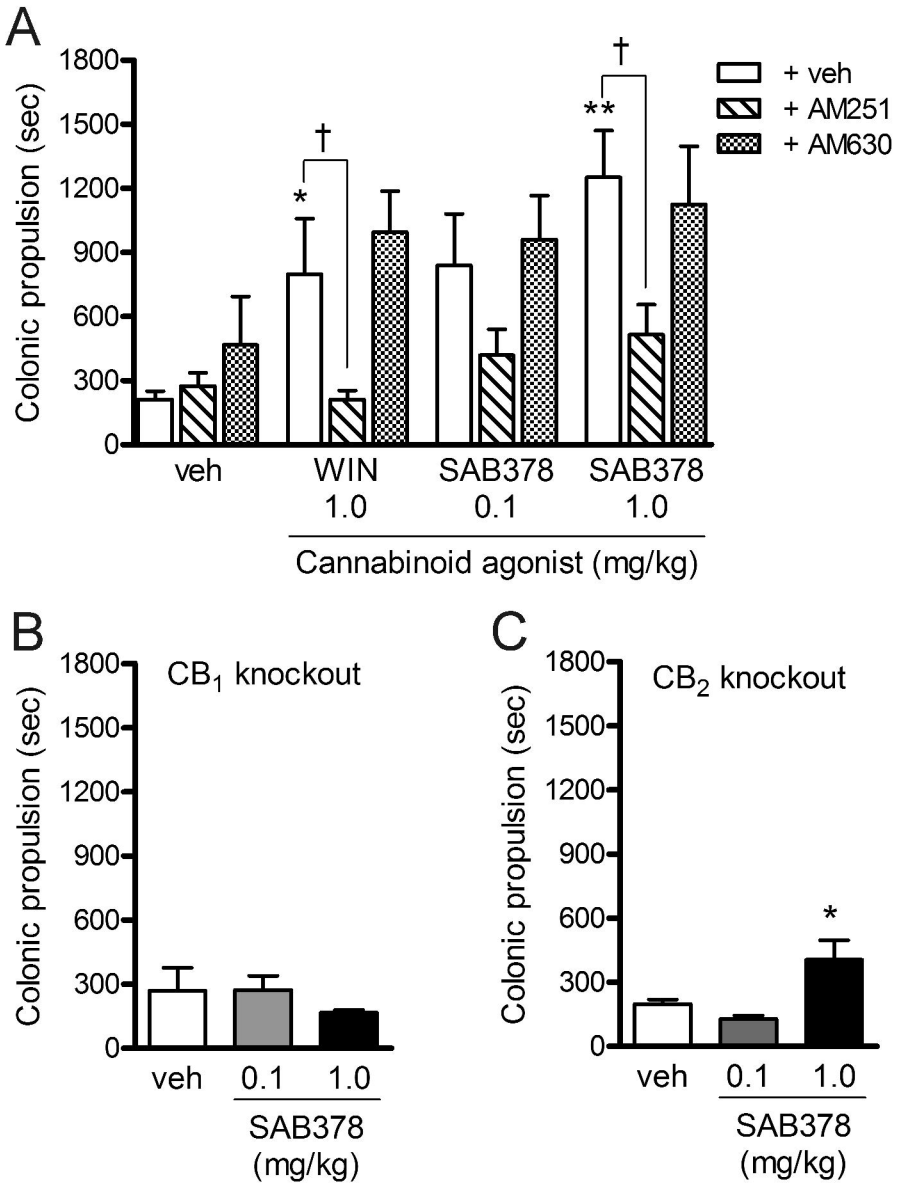

A
Colonic propulsion (sec)
1800
1500
1200
900
600
300
0
+ veh
+ AM251
+ AM630
*
**
†
†
veh
WIN 1.0
SAB378 0.1
SAB378 1.0
Cannabinoid agonist (mg/kg)
B
CB1 knockout
Colonic propulsion (sec)
veh
0.1
1.0
SAB378 (mg/kg)
C
CB2 knockout
Colonic propulsion (sec)
*
veh
0.1
1.0
SAB378 (mg/kg)

Figure 5

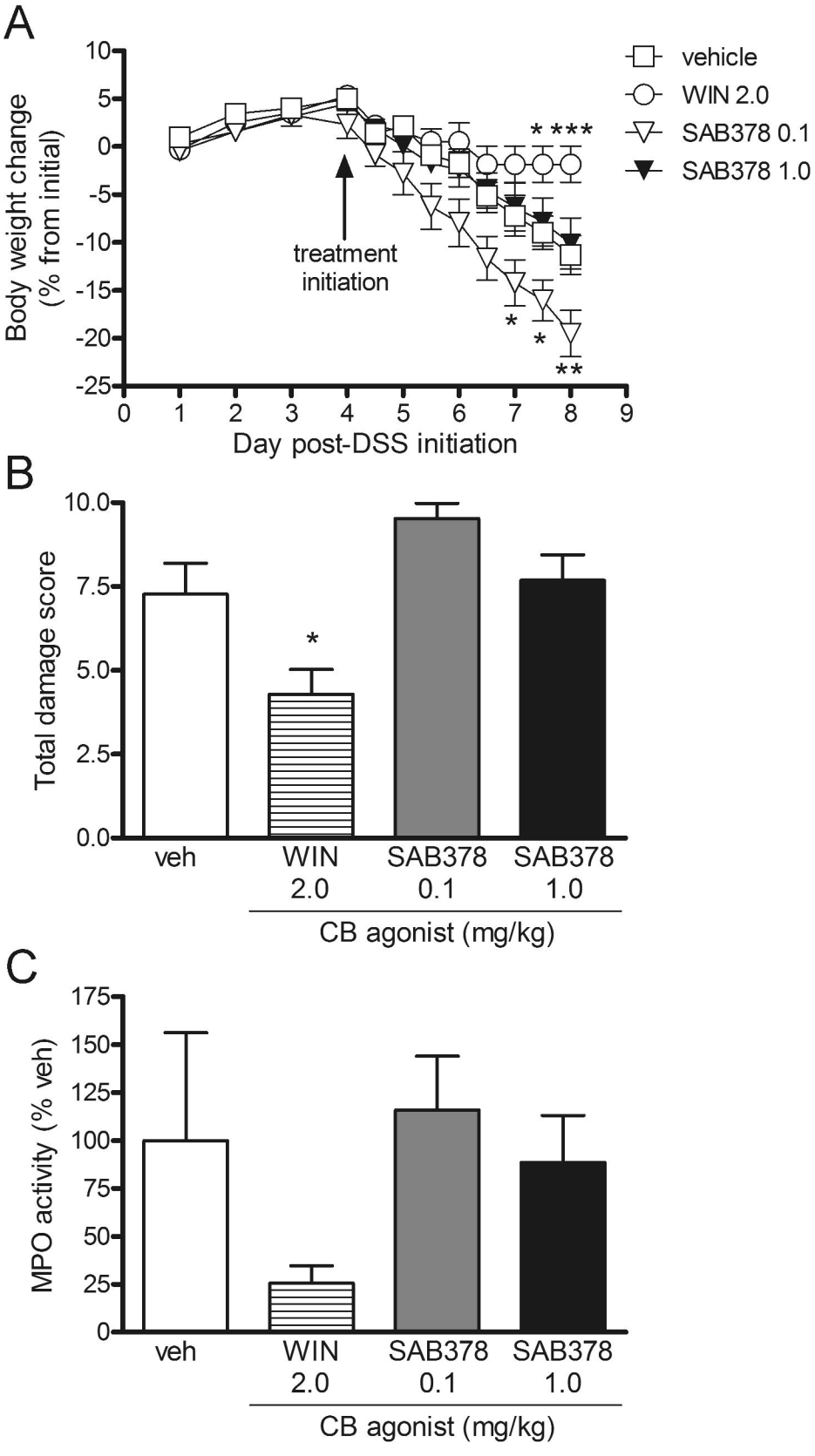
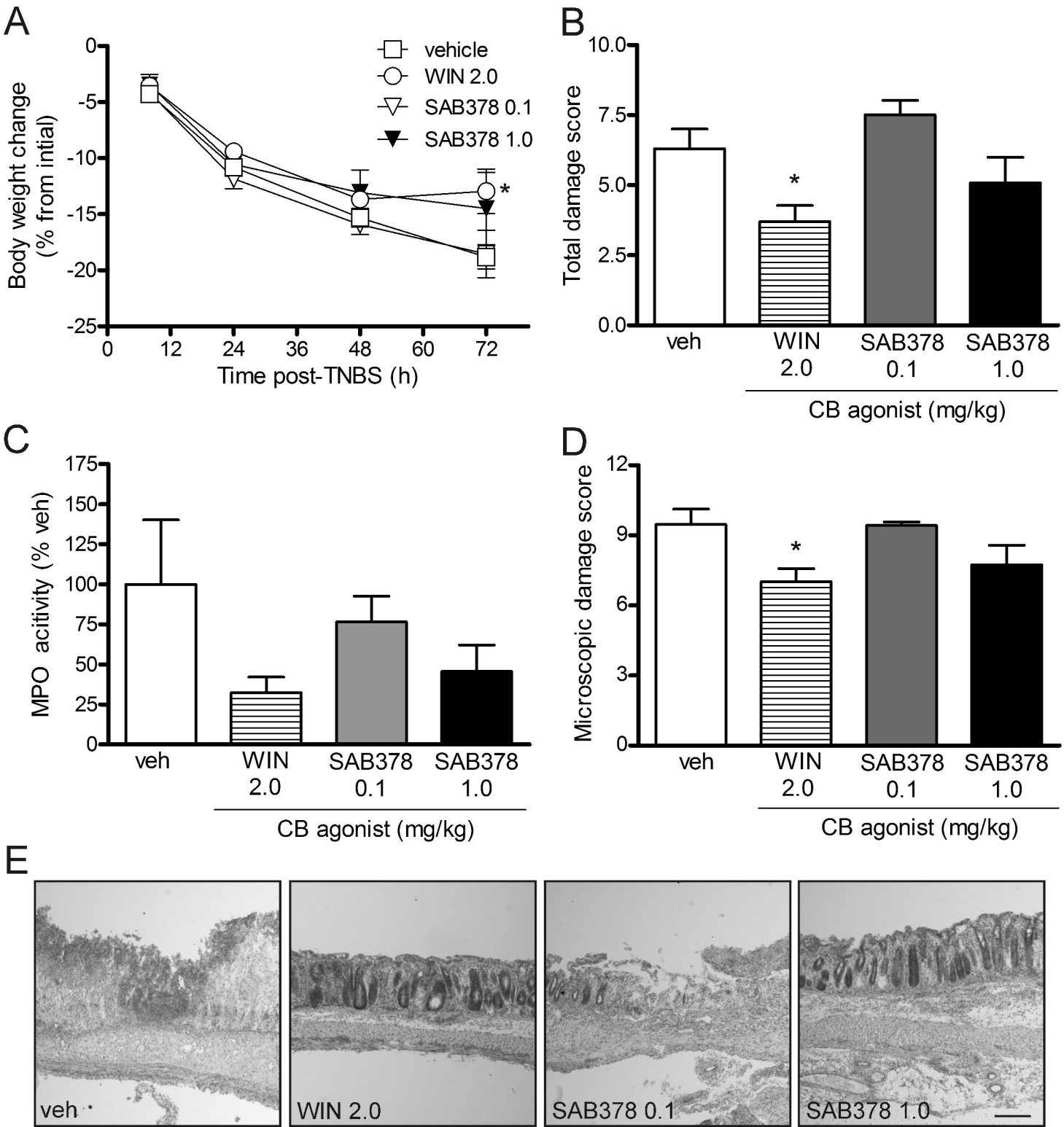

A
Body weight change (% from initial)
10
5
0
-5
-10
-15
-20
-25
0 1 2 3 4 5 6 7 8 9
Day post-DSS initiation
treatment initiation
vehicle
WIN 2.0
SAB378 0.1
SAB378 1.0
* *** * * **
B
Total damage score
10.0
7.5
5.0
2.5
0.0
*
veh
WIN 2.0
SAB378 0.1
SAB378 1.0
CB agonist (mg/kg)
C
MPO activity (% veh)
175
150
125
100
75
50
25
0
veh
WIN 2.0
SAB378 0.1
SAB378 1.0
CB agonist (mg/kg)

Figure 6


A
Body weight change (% from intial)
0
-5
-10
-15
-20
-25
0
12
24
36
48
60
72
Time post-TNBS (h)
vehicle
WIN 2.0
SAB378 0.1
SAB378 1.0
*
B
Total damage score
10.0
7.5
5.0
2.5
0.0
*
veh
WIN 2.0
SAB378 0.1
SAB378 1.0
CB agonist (mg/kg)
C
MPO acitivity (% veh)
175
150
125
100
75
50
25
0
veh
WIN 2.0
SAB378 0.1
SAB378 1.0
CB agonist (mg/kg)
D
Microscopic damage score
12
9
6
3
0
*
veh
WIN 2.0
SAB378 0.1
SAB378 1.0
CB agonist (mg/kg)
E
veh
WIN 2.0
SAB378 0.1
SAB378 1.0