Synergy between enzyme inhibitors of fatty acid amide hydrolase and cyclooxygenase in visceral nociception*

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Abbreviations: CB₁, cannabinoid receptor 1; CB₂ cannabinoid receptor 2; CNS, central nervous system; FAAH, fatty acid amide hydrolase, COX, cyclooxygenase; NSAIDs, Nonsteroidal anti-inflammatory drugs; URB597, 3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate

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Abstract

The present study investigated whether inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for anandamide catabolism, produces antinociception in the acetic acid-induced abdominal stretching model of visceral nociception. Genetic deletion or pharmacological inhibition of FAAH reduced acetic acid-induced abdominal stretching. FAAH-NS transgenic mice that express this enzyme exclusively on neurons displayed the antinociceptive phenotype, indicating the involvement of peripheral fatty acid amides. The CB₁ receptor antagonist, rimonabant, but not the CB₂ receptor antagonist, SR144528, blocked the antinociceptive phenotype of FAAH (-/-) mice, as well as the analgesic effects of URB597 or OL-135, respective irreversible and reversible FAAH inhibitors, administered to C57BL/6 mice. The opioid receptor antagonist, naltrexone, did not block the analgesic effects of either FAAH inhibitor. URB597 (ED₅₀ (95% CI) = 2.1 (1.5-2.9) mg kg⁻¹) and the nonselective COX inhibitor diclofenac sodium (ED₅₀ (95% CI) = 9.8 (8.2-11.7) mg kg⁻¹) dose-dependently inhibited acetic acid-induced abdominal stretching. Combinations of URB597 and diclofenac yielded synergistic analgesic interactions according to isobolographic analysis. Importantly, FAAH (-/-) mice, as well as URB597-treated mice displayed significant reductions in the severity of gastric irritation caused by diclofenac. URB597 lost its gastro-protective effects in CB₁ (-/-) mice, while it maintained its efficacy in CB₂ (-/-) mice; indicating a CB₁ mechanism of action. Taken together, the results of the present study suggest that FAAH represents a promising target for the treatment of visceral pain and combination of FAAH inhibitors and NSAIDs may have great utility to treat visceral pain, with reduced gastric toxicity.
Introduction

Visceral pain is a major cause of consulting in gastroenterology and the principal symptom of functional bowel disorders. This symptom is often associated with gut hypersensitivity to distension. The endogenous cannabinoid system possesses attractive targets for drugs that could potentially treat visceral and other types of pain. These targets include cannabinoid (i.e., CB₁ and CB₂) receptors, as well as fatty acid amide hydrolase (FAAH), the enzyme responsible for degradation of the endogenous cannabinoid, anandamide, and other fatty acid amides (FAAs) (Walker and Hohmann, 2005). Indeed, direct-acting cannabinoid receptor agonists, such as Δ⁹-tetrahydrocannabinol (THC), the primary psychoactive constituent of Cannabis sativa, as well as the irreversible FAAH inhibitor cyclohexylcarbamic acid 3-carbamoyl biphenyl-3-yl ester (URB597) inhibited visceral nociception, as assessed in the phenyl-p-quinone (PPQ) model (Haller et al., 2006). The antinociceptive effects of both of these compounds were blocked by the CB₁ receptor antagonist, rimonabant, indicating a CB₁ receptor mechanism of action.

Although direct-acting cannabinoid receptor agonists possess analgesic properties similar to that of opioids, but without respiratory depressant effects, their psychomimetic side effects have dampened enthusiasm for their development as therapeutic agents. In contrast, increasing endogenous cannabinoid levels by blocking FAAH represents an attractive alternate approach to elicit antinociception, but without eliciting cannabimimetic effects (Cravatt et al., 2001; Gobbi et al., 2005). Deletion of the FAAH gene leads to increased levels of anandamide, accompanied with CB₁ receptor-mediated hypoalgesic phenotypes in models of acute and inflammatory pain (Cravatt et al., 2001). Similarly, wild type mice treated with FAAH inhibitors, such as URB597 (Kathuria et al., 2003) or the reversible FAAH inhibitor, 1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenyl heptane (OL-135 (Lichtman et al., 2004a)) elicited hypoalgesic effects in acute models of pain.
that were accompanied with elevations of anandamide in the CNS. Additionally, these FAAH inhibitors reduced hypersensitivity to thermal and mechanical hypersensitivity in neuropathic pain models (Chang et al., 2006; Russo et al., 2007).

Nonsteroidal anti-inflammatory drugs (NSAIDs), the mainstays in acute and chronic pain management, produce their beneficial actions by inhibiting cyclooxygenases (COX): constitutive COX-1 and inducible COX-2 (Warner and Mitchell, 2004), which are responsible for the biosynthesis of the pro-inflammatory prostaglandins in the periphery (Vinegar et al., 1976) and CNS (Samad et al., 2001). However, the gastrointestinal adverse effects of nonselective COX inhibitors remain a major clinical concern (Wallace, 1996). On the other hand, FAAH deletion or inhibition leads to protective effects in animal models of colitis (Massa et al., 2004; Storr et al., 2008).

Of relevance, co-administration of locally applied anandamide and COX inhibitors produced synergistic antinociceptive effects in the rat formalin test (Guindon et al., 2006) and also reduced mechanical and thermal hyperalgesia in the rat partial sciatic nerve ligation model (Guindon and Beaulieu, 2006). In addition, co-administration of the NSAID, ketorolac, and the mixed CB₁/CB₂ receptor agonist, WIN55,212-2, elicited additive analgesic effects in the acetic acid abdominal stretching test, a preclinical model of visceral nociception (Ulugol et al., 2006). However, the cannabimimetic properties of WIN55,212-2 that include hypomotility, catalepsy, and hypothermia, as well as THC-like subjective effects in the drug discrimination paradigm (Compton et al., 1992) limit the development of this compound for therapeutic applications. Conversely, FAAH blockade does not elicit any of these THC-like side effects (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a; Gobbi et al., 2005).
Thus, simultaneous inhibition of FAAH and COX might offer an attractive therapeutic approach that maintains analgesic efficacy, while minimizing untoward side effects associated with direct-acting cannabinoid receptor agonists, as well as the NSAIDs. Consequently, there were four goals of the present study. First, we examined whether FAAH (−/−) mice or wild type mice treated with inhibitors of FAAH would display decreased nociceptive behavior in the acetic acid-induced abdominal stretching test. Second, we sought to determine the receptor mechanism of action underlying the antinociceptive phenotype of FAAH-compromised mice. Mice were evaluated with the respective CB₁ and CB₂ receptor antagonists, rimonabant and SR144528. In addition, because the antinociceptive effects of FAAH inhibitors has been suggested to include an opioid receptor mechanism of action (Chang et al., 2006), we also evaluated whether naltrexone would block the antinociceptive effects of URB597 and OL-135. Third, we used an isobolographic approach to determine whether URB597 given in combination with diclofenac, a non-selective COX inhibitor, would elicit additive or synergistic antinociceptive effects. Fourth, we evaluated whether FAAH inhibition would offer gastro-protective effects against NSAID-induced gastric ulcers.
Methods

Subjects

The subjects consisted of male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), FAAH (-/-) mice backcrossed for at least 13 generations, CB1 (-/-) mice backcrossed for at least 13 generations, and CB2 (-/-) mice backcrossed for five generations onto a C57Bl/6J background. In addition, male and female FAAH-NS (nervous system FAAH restricted; (Cravatt et al., 2004)) mice backcrossed onto a C57Bl/6J background for at least 11 generations were used to determine whether the FAAH (-/-) antinociceptive phenotype was due to the deletion of this enzyme from neuronal tissue. All subjects weighed between 20 and 30 g and were housed four mice per cage in a temperature-controlled (20-22°C) facility, with food and water available ad libitum. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in concordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Drugs

Diclofenac sodium and naltrexone HCl were purchased from Sigma (St. Louis, MO). URB597 was purchased from Cayman Chemicals (Ann Arbor, MI). WIN55122-2 and ranitidine were purchased from Tocris Biosciences (Ellisville, MO). Rimonabant (CB1 receptor antagonist) and SR144528 (CB2 receptor antagonist) were obtained from NIDA (Rockville, MD). All the drugs were dissolved in a vehicle consisting of ethanol, alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18, with the exception of naltrexone, which was dissolved in 0.9% saline. Acetic acid (0.6%) was diluted in saline and administered via the intraperitoneal route of administration. All other drugs were administered through the s.c. route.
of administration, with the exception of diclofenac in the gastric tolerability test. The injection volume for acetic acid, as well as each drug was 10 µl g⁻¹ body weight.

**Acetic acid-induced stretching**

All animals were acclimated to laboratory environment for at least 2 h before testing. The acetic acid-induced stretching assay was carried out as previously described (Jain et al., 2002). In brief, each mouse was given an intraperitoneal injection of acetic acid. Beginning 3 min after the administration of acetic acid, the number of stretches (constriction of abdomen, turning of trunk (twist) and extension of the body and hind limbs) per mouse was counted for a 20-min period.

Diclofenac, WIN55,212-2, URB597, or OL-135 were given via the s.c. route of administration 60 min before acetic acid administration. In the antagonism studies, rimonabant (3 mg/kg) and SR144528 (3 mg/kg) were given 70 min before acetic acid, while naltrexone (1 mg/kg) was administered 30 min before acetic acid. Each of these doses and pretreatment times were based on previous reports from the literature and from previous studies from our laboratory.

Dose–response curves for URB597 and diclofenac were obtained using at least six animals at each dose. Mice were given s.c. injections of vehicle, diclofenac (3, 10, or 30 mg kg⁻¹), or URB597 (1, 3, or 10 mg kg⁻¹) and 60 min later were given an i.p. injection of acetic acid. Dose-response curves were also obtained after co-administration of URB597 and diclofenac (ED50 Mix) in fixed ratio combinations based on fractions of their respective ED50 values. The ratios of URB597 and diclofenac were 1:3, 1:1, and 3:1 (see Table 1 for specific doses of each drug). In the drug combination experiments, mice received a single injection containing both drugs.
Ulcer induction with diclofenac and scoring of gastric lesions

The mice were deprived of food for 18–24 h before the induction of ulcer, but had free access to water. On the day of the ulcer induction, the mice were given a single dose of 30 or 100 mg kg\(^{-1}\) diclofenac via gavage and left in their cages for 6 h. The experimental procedure was conducted according to a previous report in which the damage induced by diclofenac peaks 6 h after oral administration (Sanchez et al., 2002). Ranitidine, a histamine H\(_2\) receptor blocker and commonly used antiulcer agent, was used as a positive control. URB597 (10 mg kg\(^{-1}\)) or ranitidine (50 mg kg\(^{-1}\)) was administered s.c. 1 h before diclofenac.

At 6 h after gavage administration of diclofenac or vehicle, the mice were humanely euthanized by CO\(_2\) asphyxiation. The abdomen was then opened through a midline incision and the stomach was removed and opened along the greater curvature. After a gentle wash in PBS, the stomach was spread for subsequent analysis and photography. Each of the following indices was observed and scores were summed, as previously reported (Jain et al., 2002): red coloration (score = 0.5); spot ulcers (score = 1); hemorrhagic streaks (score = 1.5). If three or four ulcers were found, a value of 2 was added to the score. If five or more ulcers occurred, a value of 3 was added to the score. Thus, each stomach could get multiple scores and the ulceration index was the sum of its scores, with a maximum score of 6.

Statistical analysis

Results are presented as means ± S.E.M. The data were analyzed using two-way ANOVA, one-way ANOVA, or the student’s t-test, as indicated in the text. Dunnett’s test was used for post hoc analysis in the dose-response experiments. Additionally, planned comparisons were used to in the studies examining mechanism of action.
The ED<sub>50</sub> values with 95% confidence intervals (CI) were calculated using standard linear regression analysis of the dose–response curve for each drug alone or in combination. To determine the ED<sub>50</sub> values, the data were transformed to percent maximum possible effect (% MPE) using the following equation: % MPE = 100*(mean number of stretches in the control group - mean number of stretches in the test group) / mean number of stretches in the control group).

Isobolographic analysis was used to determine the nature of the drug interactions, as previously described (Tallarida, 2000). The dose of diclofenac required to elicit a 50% effect was plotted on the abscissa and the isoeffective dose of URB597 was plotted on the ordinate. The theoretical additive effect of the two drugs was represented by the straight line connecting the two points. If the experimentally determined data points and their confidence interval lie on this line, the drug effects are considered additive. If the points lie below this line, the interaction is considered to be super-additive (synergistic); however, if they lie above the line of additivity, the interaction is defined as sub-additive (antagonistic). To determine whether the interaction between two drugs was synergistic, additive or antagonistic, the theoretical additive ED<sub>50</sub> value of the two drugs combined (referred to as Z<sub>add</sub>) was calculated from the dose-response curves of each drug administered individually in which the combination is assumed to equal the sum of the individual effects of each drug. The experiment ED<sub>50</sub> value of the two drugs in combination (referred to as Z<sub>mix</sub>) in which the two drugs were summed at each concentration was then determined by linear regression. The statistical difference between Z<sub>add</sub> (the theoretical ED<sub>50</sub> value) and Z<sub>mix</sub> (the experimental ED<sub>50</sub> value) was analyzed using Fisher’s test. These calculations were performed using the program of Pharm Tools Pro (version 1.20, The McCary Group Inc.), based on Tallarida (2000). P values less than 0.05 were considered significant.
Results

Acetic acid-induced abdominal stretching

Intraperitoneal injection of 0.6% acetic acid in saline elicited a curving of the trunk and extension of limbs, which were scored as a stretch. Generally, the first stretch occurred between 2 and 5 min after the acetic acid injection in the control mice. Pretreatment with WIN55212-2 (2 mg kg\(^{-1}\)), a mixed CB\(_1\)/CB\(_2\) cannabinoid receptor agonist or diclofenac sodium (30 mg kg\(^{-1}\)), a non-selective COX inhibitor significantly reduced the acetic acid-induced abdominal stretches in mice as compared to vehicle controls, F(3,16)=156, p<0.001 (Fig.1). However, WIN55212-2 treated mice showed reductions in locomotor activity, one of many THC-like effects that has been described previously (Compton et al., 1992), thus making it difficult to delineate between antinociception and motor suppression. In contrast, none of the other drugs tested elicited any apparent alterations in behavioral activity.

Receptor mechanisms underlying the antinociceptive phenotype of FAAH-compromised mice

As shown in Fig 2A, FAAH (-/-) mice displayed a significant attenuation of acetic-induced nociception, t (10) = 5.0, p < 0.001. FAAH regulates endocannabinoid pathways in both the central nervous system and peripheral tissues, either of which could regulate nociception. Thus, we next evaluated acetic acid-induced abdominal stretching in transgenic mice that express FAAH exclusively in the nervous system (FAAH-NS mice; (Cravatt et al., 2004)). FAAH-NS mice retained antinociceptive phenotype, F(2,19)=10.858, p<0.001 (Fig. 2B), implicating the involvement of non-neuronal endogenous cannabinoids in this phenotype.

Rimonabant and SR144528 were used respectively to ascertain the involvement of CB\(_1\) and CB\(_2\) receptors in the antinociceptive phenotype exhibited by FAAH (-/-) mice. Rimonabant (3
mg kg\(^{-1}\)), but not SR144528 (3 mg kg\(^{-1}\)), significantly blocked the antinociceptive phenotype of FAAH (-/-) mice, F(2,33) = 6.1, p < 0.01 (Fig 2C). In contrast, these cannabinoid receptor antagonists administered alone to wild type mice did not alter the number of abdominal stretches compared to vehicle-treated control mice. These results suggest that the antinociceptive phenotype of FAAH (-/-) mice in the acetic acid model of visceral nociception is mediated through a CB\(_1\) cannabinoid receptor mechanism of action.

We next evaluated the effects of URB597, a well studied irreversible FAAH inhibitor (Kathuria et al., 2003) and OL-135, a reversible FAAH inhibitor (Boger et al., 2001), in the acetic acid stretching test. As shown in Figure 3A, URB597 (10 mg kg\(^{-1}\)) significantly reduced acetic acid-induced abdominal stretching, F(3,32)=14.8, p<0.001. Pretreatment with rimonabant (3 mg kg\(^{-1}\)), but not SR144528 (3 mg kg\(^{-1}\)), significantly blocked the antinociceptive effects of URB597. Similarly, administration of OL-135 (30 mg kg\(^{-1}\)) produced CB\(_1\) receptor mediated antinociceptive effects in the acetic acid model, F(3,20) = 19.4, p < 0.001 (Figure 3B). As in the case of URB597, pretreatment with rimonabant (3 mg kg\(^{-1}\)), but not SR144528 (3 mg kg\(^{-1}\)), significantly blocked the antinociceptive effects of OL-135. The antinociceptive effects of URB597 or OL-135 [main effect of FAAH inhibitor: F(2,30) = 68, p < 0.001] was not blocked by naltrexone (1 mg kg\(^{-1}\); see Figure 3C). Finally, diclofenac sodium (30 mg kg\(^{-1}\) s.c.) significantly attenuated acetic acid-induced nociception, F(3,16)=12, p<0.001. The failure of rimonabant pretreatment to reverse the antinociceptive effects of this drug, indicates that the underlying mechanism of action is independent of CB\(_1\)receptors (see Figure 3D). As previously shown in Figure 2C), rimonabant alone did not affect acetic acid-induced stretching.

\textit{Dose-response analysis of URB597 and diclofenac sodium alone and in combination.}
Figure 4A depicts the dose-response curves for the antinociceptive effects of URB597 [F(3,17)=23, \( p < 0.001 \)] and diclofenac [F(3,17)=47, \( p < 0.001 \)] alone in mice. The ED\(_{50}\) values and 95% CL for URB597 and diclofenac were 2.1 (1.5-2.8) mg kg\(^{-1}\) and 9.8 (8.2-11.7) mg kg\(^{-1}\), respectively. Shown in Figure 4B are the 1:3, 1:1, and 3:1 combinations of URB597 and diclofenac, with the dose of URB597 plotted on the abscissa. The dose-response curve of URB597 alone is plotted in this graph for comparison. The same data are also plotted in Figure 4C, with the dose of diclofenac plotted on the abscissa. The dose-response curve of diclofenac alone is included in this graph for comparison. The plots of the combination ED\(_{50}\) values for both fixed ratios (total dose) in relation to the ED\(_{50}\) values of the drugs alone are shown in Figure 4D. The isobologram suggests that a synergistic interaction occurs between URB597 and diclofenac, since the experimental points lie significantly below the line of additivity. This graphic display of synergism is confirmed mathematically by statistical analysis of the predicted additive ED\(_{50}\) values (\( Z_{\text{add}} \)) and experimentally derived ED\(_{50}\) values (\( Z_{\text{mix}} \)) shown in Table 1. At each ratio tested, the \( Z_{\text{mix}} \) value is significantly less than the \( Z_{\text{add}} \) value.

**FAAH (-/-) mice and URB597-treated wild type mice show gastro-protective effects**

Because one of the most common adverse effects associated with NSAID treatment is gastric ulcers, we evaluated the impact of FAAH deletion or inhibition on diclofenac-provoked gastric irritation. Diclofenac sodium treatment (100 mg kg\(^{-1}\), p.o.) caused a significant induction of gastric ulcers as compared to saline-treated mice in food restricted mice (Figure 6A). Pretreatment with the histamine H\(_2\) receptor antagonist, ranitidine (50 mg kg\(^{-1}\), s.c.), as a positive control, significantly reduced diclofenac-induced gastric ulcers, F(2,16) = 28, \( p < 0.001 \). Shown in Figure 5B are representative illustrations of stomachs from a mouse in each condition. Next,
we compared the effects of diclofenac (100 mg kg\(^{-1}\)) between FAAH (+/+) and (-/-) mice. As can be seen in Figure 5C, diclofenac provoked less gastric ulceration in FAAH (-/-) mice than in wild type mice, \(t(14) = 2.2, p < 0.05\).

In the final series of experiments, we evaluated the effects of URB597 on diclofenac-induced gastric ulcers. Subjects were treated with either vehicle or URB597 (10 mg kg\(^{-1}\), s.c.) 1 h before diclofenac (0, 30, or 100 mg kg\(^{-1}\), gavage). Two-way ANOVA revealed a significant interaction between URB597 and diclofenac, \(F(2,40) = 5.9, p < 0.01\) (Figure 6A). URB597 significantly reduced the severity of ulcers elicited by both concentrations of diclofenac. Stomachs from representative mice treated with vehicle or diclofenac (30 mg kg\(^{-1}\), s.c) and vehicle or URB597 are shown in Figure 6B. In order to examine whether the gastro-protective effects of URB597 are mediated through a cannabinoid receptor mechanism, we evaluated this drug in mice lacking CB\(_1\) or CB\(_2\) receptors. As illustrated in Figure 6C, URB597 no longer offered gastric protection in CB\(_1\) (-/-) mice \((p = 0.50)\), though it continued to reduce ulcer index scores in CB\(_1\) (+/+ ) mice \((p < 0.05)\). Finally, URB597 significantly reduced diclofenac-induced ulcers to an equal magnitude in CB\(_2\) (-/-) and (+/+ ) mice, \(F(1,20) = 18.9, p < 0.001\). In the absence of URB597, diclofenac elicited ulcers of a similar magnitude between CB\(_1\) (-/-) and (+/+ ) mice, as well as between CB\(_2\) (-/-) and (+/+ ) (see Figure 6C and D).
Discussion

The first goal of the present study was to test the hypothesis that FAAH is a viable target to reduce visceral nociception. Complementary approaches of pharmacological blockade and genetic deletion of FAAH significantly reduced acetic acid-induced abdominal stretches. These findings are consistent with those of Haller et al. (2006), who showed that URB597 reduced abdominal stretching in the PPQ model of visceral nociception. Previous research has also found that FAAH (-/-) mice, as well as mice treated with FAAH inhibitors possess elevated levels of anandamide in the CNS and periphery that reduce baseline pain thresholds to noxious thermal and chemical stimuli (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a). Additionally, FAAH-compromised mice have been shown to display anti-hyperalgesic effects in the carrageenan paw edema model (Lichtman et al., 2004b). Thus, FAAH blockade elicits hypoalgesic effects in a wide range of preclinical pain models.

It is noteworthy that FAAH (-/-) mice possess increased brain levels of fatty acid amides, including N-palmitoyl ethanolamine (PEA; (Cravatt et al., 2001)), as well as the N-acyl taurines (Saghatelian et al., 2006), any of which could contribute to antinociceptive phenotype. In particular, PEA has been well described to have anti-inflammatory actions (Mazzari et al., 1996; Conti et al., 2002; Lo Verme et al., 2004). Thus, it is plausible that elevated levels of this or other lipid signaling molecules, in addition to anandamide, may contribute to the anti-hyperalgesic phenotype observed in FAAH (-/-) mice or mice treated with FAAH inhibitors. However, anandamide is the only substrate of FAAH known to bind and activate cannabinoid receptors. Thus, the second objective of the present study was to determine whether cannabinoid receptors mediate the antinociceptive phenotype of FAAH-compromised mice. Notably, rimonabant, but not SR144528, blocked the antinociceptive phenotype of FAAH (-/-) mice, as well as OL-135- or
URB597-treated mice. In contrast to the results of Chang et al. (2006), who reported that naloxone blocked the anti-hyperalgesic effects of OL-135 in rat spinal nerve ligation and thermal injury models, we found no evidence of opioid receptor involvement in the antinociceptive effects of URB597 and OL-135 in the acetic model of visceral nociception. Thus, these results indicate that CB₁ receptors play a critical role in the antinociceptive effects of FAAH blockade.

Although FAAH has been shown to metabolize a second endogenous cannabinoid, 2-arachidonoyl glycerol (2-AG), under certain in vitro conditions (Di Marzo et al., 1999), this enzyme does not appear to play a relevant role in regulating 2-AG (Blankman et al., 2007). Moreover, FAAH (-/-) mice do not display cannabinoid effects to exogenous administration of 2-AG, hydrolyze 2-AG in brain and liver homogenates at equivalent rates as wild type mice, and possess similar levels of 2-AG as wild type mice (Lichtman et al., 2002). Notwithstanding the fact that the present study did not quantify specific substrates of FAAH, our overall results are consistent with the hypothesis that anandamide is responsible for the antinociceptive phenotype of FAAH-compromised mice. Additionally, acetic acid elicited virtually the identical number of abdominal stretches in mice treated with rimonabant, SR144528, or vehicle, suggesting that endogenous cannabinoids do not tonically modulate basal nociceptive responses in this model.

Of note, there were fluctuations in nociceptive behavior elicited by i.p. acetic acid administration across the different experiments. Several factors that may account for this variability include the variations in the distribution of acetic acid within the peritoneal cavity, as well as individual differences among different lots of mice evaluated at different times, and the number of subcutaneous injections mice received before acetic acid.

The site of action of endocannabinoids in visceral nociception is not well understood. The reduction in pain behaviors seen in FAAH (-/-) mice persisted when FAAH was expressed
exclusively under the control of a neuron-specific promoter, suggesting that endocannabinoids were producing their effects through a peripheral site of action. These results are in accordance with other studies showing analgesic effects of cannabinoids after local administration (Guindon and Beaulieu, 2006; Guindon et al., 2006; Jhaveri et al., 2006).

The strategy of combining analgesics of different classes can facilitate patient compliance, simplify prescriptions, improve efficacy without increasing adverse effects, and decrease adverse effects without loss of efficacy (Raffa, 2001). Combination analgesic therapy is especially useful when the selected drugs have different mechanisms of action that provide additive or synergistic efficacy, reducing the required doses of the individual drugs compared with monotherapy and potentially limiting side effects. Accordingly, the third goal of the present study was to determine whether combined administration of a FAAH inhibitor and a COX inhibitor would produce additive or synergistic antinociceptive actions in the acetic acid model of visceral nociception. Using an isobolographic analysis, our results revealed a synergistic antinociceptive interaction between the FAAH inhibitor, URB597, and the NSAID, diclofenac sodium. Thus, administration of a combination of FAAH and COX inhibitors can reduce the amount of drugs required to produce analgesic effects.

However, the isobolographic approach does not provide insight into the mechanism of action underlying this interaction. Although the mechanism of action underlying the synergistic interaction between diclofenac and URB597 is beyond the scope of the present study, it may be related to pharmacokinetic and/or pharmacodynamic factors. The NSAIDs elicit analgesia by inhibiting COX, the enzyme responsible for the biosynthesis of prostaglandins (Vane, 1971). In contrast, the antinociceptive effects caused by FAAH blockade were likely caused by elevated levels of anandamide acting at CB$_1$ receptors. The combination of URB597 and diclofenac
would simultaneously block the production of prostaglandins in the viscera and activate CB₁ receptor mediated antinociceptive pathways. Additionally, several studies have found that a variety of NSAIDs, including indomethacin and ibuprofen, inhibit FAAH, particularly at low pH that occurs in inflamed tissues (Holt et al., 2001; Holt et al., 2007). However, NSAIDs only inhibit FAAH at considerably high concentrations with EC₅₀ values greater than 50 μM, though it should be noted that diclofenac has not been evaluated in FAAH activity assays. The failure of rimonabant to reverse the antinociceptive effect of diclofenac sodium rules out the involvement of CB₁ receptors for this NSAID. Conversely, other targets (e.g., CB₂, TRPV₁, or PPARα receptors) that are activated by substrates of FAAH may contribute to the interaction between these two drugs. Finally, the interactions are likely to involve peripheral, as well as central pathways. Although the NSAIDs act mainly through peripheral inhibition of COX, a central action has also been described (Miranda et al., 2003). Similarly, cannabinoids have central and peripheral sites of action (Walker and Hohmann, 2005; Agarwal et al., 2007). In future studies, it will be of value to assess the degree to which FAAH inhibitors and COX inhibitors given alone and in combination reduce the inflammation caused by acetic acid in the viscera. In addition, it will important to examine whether the synergistic interaction between diclofenac and URB597 extends to other pain models, as well as assess whether combinations of other FAAH inhibitors and other COX inhibitors produce similar effects.

A synergistic interaction for the antinociceptive effects of FAAH and COX inhibitors is highly appealing; however, this beneficial effect would be nullified, if combined administration of these drugs increases the magnitude of adverse effects. Gastric ulcers are the most frequently associated side effects with NSAID therapy and are generally related to dose. Therefore, the fourth objective of the present study was to determine whether pretreatment with URB597 would
significantly reduce NSAID-induced gastric ulcers. FAAH (-/-) mice, as well as URB597-treated wild type mice displayed a significant amelioration in the magnitude of gastric irritation caused by diclofenac given via gavage in fasted mice. These findings are consistent with studies reporting that genetic deletion or pharmacological blockade of FAAH makes mice resistant to experimentally-induced colitis (Massa et al., 2004; Storr et al., 2008). Our observations that CB₁ (-/-) mice, but not CB₂ (-/-) mice, were resistant to the gastro-protective effects of URB597 suggests that both the antinociceptive and gastro-protective effects caused by FAAH blockade are mediated by CB₁ receptors. These findings, taken together, indicate that the combination of URB597 and diclofenac sodium not only shows a synergistic antinociceptive effect, but also can significantly reduce a serious adverse effect of the NSAIDs.

In summary, we report that pharmacological inhibition or genetic deletion of FAAH produces CB₁ receptor mediated antinociceptive effects in the acetic acid model of visceral nociception. Co-administration of URB597 and diclofenac sodium elicited a synergistic antinociceptive effect in this assay. Moreover, FAAH (-/-) mice, as well as C57BL/6 mice treated with URB597 displayed a reduction in the caustic effects of diclofenac on the gastric mucosa. The observations that URB597 retained its efficacy in CB₂ (-/-) and was ineffective in CB₁ (-/-) mice indicates its gastro-protective effects were mediated through a CB₁ mechanism of action. In conclusion, the combination of FAAH inhibitors with NSAIDs may be particularly advantageous in maximizing antinociceptive effects, while minimizing gastric irritation.
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The authors dedicate this paper to their beloved mentor and world renowned pharmacologist, the late Professor Billy R. Martin, whose keen insight, wisdom, and advice were invaluable. The authors also thank Dr. S. Stevens Negus for his advice and guidance in analyzing the drug interaction experiments.
References


Footnotes

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Legends for Figures

Figure 1. Diclofenac sodium and WIN55212-2 pretreatment reduces acetic acid-induced visceral nociception. Intraperitoneal administration of 0.6% acetic acid elicited a significant increase in abdominal stretches in mice. Pretreatment with diclofenac sodium (30 mg kg\(^{-1}\), s.c.) or WIN55212-2 (2 mg kg\(^{-1}\), s.c.) attenuated the number of acetic acid-induced abdominal stretches compared to the vehicle-treated group. *** \(p < 0.001\) vs. vehicle-treated group; data depicted as means ± SEM., \(n = 6-8\) mice/group.

Figure 2. The FAAH (-/-) antinociceptive phenotype in the acetic acid model of visceral nociception is mediated through a CB\(_1\) receptor mechanism of action. (A) FAAH (-/-) mice exhibit less abdominal stretching than FAAH (+/+)) mice. \(n = 8\) mice /group. (B) FAAH-NS mice, which express FAAH only in neural tissue, show an equivalent reduction in acetic acid-induced abdominal stretches as global FAAH (-/-) mice. \(n = 5-9\) mice /group. (C) Pretreatment with rimonabant (SR1; 3 mg kg\(^{-1}\), s.c.), but not SR144528 (SR2; 3 mg kg\(^{-1}\), s.c.), prevented the FAAH antinociceptive phenotype in acetic acid-treated mice, \(n = 12\) mice/group. ** \(p < 0.01\) as compared to respective control groups; data depicted as means ± SEM.

Figure 3. Pharmacological inhibition of FAAH reduces acetic acid-induced visceral nociception through a CB\(_1\) receptor mechanism of action. (A) Pretreatment with URB597 (URB; 10 mg kg\(^{-1}\), s.c.), an irreversible FAAH inhibitor significantly reduced the number of abdominal stretches in acetic acid-treated mice. Pretreatment with rimonabant (SR1; 3 mg kg\(^{-1}\), s.c.), but not SR144528 (SR2; 3 mg kg\(^{-1}\), s.c.), prevented the antinociceptive effects of URB597. \(n = 9\) mice/group. (B) OL-135 (OL; 30 mg kg\(^{-1}\), s.c.), a reversible FAAH inhibitor significantly attenuated the number
of abdominal stretches in acetic acid-treated mice. Pretreatment with SR1 (3 mg kg\(^{-1}\), s.c.), but not SR2 (3 mg kg\(^{-1}\), s.c.), prevented the antinociceptive effects of OL-135. \(n = 6\) mice/group. **(C)** The antinociceptive effects of URB597 (10 mg kg\(^{-1}\)) or OL-135 (30 mg kg\(^{-1}\)) were not blocked by naltrexone (1 mg kg\(^{-1}\)). \(n = 6\) mice/group. **(D)** Pretreatment with SR1 (3 mg kg\(^{-1}\), s.c.) failed to block the antinociceptive effects of diclofenac sodium (30 mg kg\(^{-1}\), s.c.). \(n = 6\) mice/group. **\(p<0.01\); ***\(p<0.001\)** as compared to the appropriate control group; ##\(p < 0.01\) as compared to URB-treated group; ###\(p < 0.001\) as compared to OL-treated group. Data depicted as means \(\pm\) SEM.

**Figure 4.** Co-administration of URB597 and diclofenac sodium elicits synergistic antinociceptive effects in the acid acetic model of visceral nociception. **(A)** URB597 (1-10 mg kg\(^{-1}\), s.c.) or diclofenac sodium (3-30 mg kg\(^{-1}\), s.c.) dose-dependently reduced the number acetic acid-induced abdominal stretches as compared to the control groups. **\(p < 0.01\), ***\(p < 0.001\)** as compared to the respective vehicle control groups. **(B)** Dose-effect curves for URB597 alone and in mixtures with diclofenac. **(C)** Dose-effect curves for diclofenac alone and in mixtures with URB597. **(D)** Isobologram showing the interactions between diclofenac sodium and URB597 in the mouse acetic acid-induced abdominal stretching test. The ED\(_{50}\) values for diclofenac and URB597 are depicted on the X- and Y-axes, respectively. The isobole of additivity is shown as a solid line drawn between the ED\(_{50}\) values of diclofenac and URB597, which connects the X- and Y-axes. The experimental ED\(_{50}\) values with 95% CI of mixtures of URB597 and diclofenac at the fixed-ratio combinations of 3:1, 1:1 and 1:3 were significantly below the theoretical isoboles of additivity (see Table 1 for statistics), indicating super-additive (synergistic) interactions. \(N = 6\) mice/group.
**Figure 5.** Diclofenac sodium (100 mg kg\(^{-1}\), p.o.) caused a significant induction of ulcers in mice fasted for 18-24 h. (A) The selective H\(_2\) receptor antagonist, ranitidine (50 mg/kg; s.c.) significantly attenuated diclofenac sodium-induced gastric ulcers. ***p < 0.001 compared to control and ranitidine groups; n = 6 mice/group. Data depicted as mean ulcer index ± SEM. (B) Representative stomachs from control (left; score = 0), diclofenac + vehicle (center; score = 6), and diclofenac + ranitidine (right; score = 1.5). (C) FAAH (-/-) mice display a significant attenuation in diclofenac sodium-induced gastric ulcers compared to FAAH (+/+) mice. *p < 0.05 as compared to FAAH (+/+) mice; n = 8 mice/group. Data depicted as mean ± SEM ulcer index.

**Figure 6.** URB597 attenuates diclofenac sodium-induced ulcers through a CB\(_1\) receptor mechanism of action. (A) Pretreatment with URB597 (10 mg kg\(^{-1}\), s.c.) significantly attenuated diclofenac sodium-induced (30 or 100 mg kg\(^{-1}\), gavage) gastric ulcers. (B) Representative stomachs from vehicle-vehicle (top left; score = 0), diclofenac (30 mg kg\(^{-1}\))-vehicle (top right; score = 6), vehicle-URB597 (bottom left; score = 0), and diclofenac (30 mg kg\(^{-1}\))-URB597 (bottom right; score = 3). (C) CB\(_1\) (-/-) mice were resistant to the gastro-protective effects of URB597 in mice treated with diclofenac sodium (100 mg kg\(^{-1}\), gavage). (D) URB597 ameliorated diclofenac sodium-induced (100 mg kg\(^{-1}\), gavage) gastric ulcers to a similar magnitude in CB\(_2\) (+/+) and (-/-) mice. *p < 0.05 or **p < 0.01 as compared to corresponding control mice; n = 6 mice/group. Data depicted as mean ± SEM ulcer index.
**Table 1:** Fixed combinations of diclofenac and URB597 produce synergistic analgesic effects in the acetic acid abdominal stretch assay. Predicted additive ED$_{50}$ values ($Z_{add}$) and experimentally determined ED$_{50}$ values ($Z_{mix}$) for mixtures of diclofenac and URB597 in mice. Doses for each drug in combination at the three different ratios are presented in the first column.

<table>
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<tr>
<th>Combination</th>
<th>Mixture (doses in mg kg$^{-1}$)</th>
<th>ED$_{50}$ mg kg$^{-1}$ s.c. (95% CL)</th>
<th>$Z_{add}$ (Theoretical)</th>
<th>$Z_{mix}$ (Experimental)</th>
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<tr>
<td>Diclofenac/URB597</td>
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<td>2.01 (1.68-5.49)*</td>
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<td></td>
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<td>2.45 / 1.5</td>
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<tr>
<td>Diclofenac/URB597</td>
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<td>5.91 (4.90-7.05)</td>
<td>2.16 (1.69-2.68)*</td>
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<td></td>
<td>15 / 1.0</td>
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The $Z_{\text{add}}$ and $Z_{\text{mix}}$ values reflect the total amount of both drugs combined in which diclofenac and URB597 was summed for each combination.

* $p < 0.05$ as compared to respective $Z_{\text{add}}$ (theoretical value) using the Fisher test
Figure 1

# Abdominal Stretches

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<th>WIN</th>
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Treatment

---

* *** indicates statistical significance.
Figure 2

(A) # Abdominal Stretches

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(C) # Abdominal Stretches

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** Indicates significant difference.
Figure 5

(A) Ulcer Index

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(B) Genotype

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(C) Ulcer Index

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