Inhibition of Monoacylglycerol Lipase Attenuates Nonsteroidal Anti-Inflammatory Drug-Induced Gastric Hemorrhages in Mice

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used analgesics, but can cause gastric and esophageal hemorrhages, erosion, and ulceration. The endogenous cannabinoid (endocannabinoid; eCB) system possesses several potential targets to reduce gastric inflammatory states, including cannabinoid receptor type 1 (CB_1), cannabinoid receptor type 2 (CB_2), and enzymes that regulate the eCB ligands 2-arachidonoylglycerol (2-AG) and N-arachidonoyl ethanolamine (anandamide; AEA). In the presented study, we tested whether 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184), a selective inhibitor of the primary catabolic enzyme of 2-AG, monoacylglycerol lipase (MAGL), would protect against NSAID-induced gastric damage. Food-deprived mice administered the nonselective cyclooxygenase inhibitor diclofenac sodium displayed gastric hemorrhages and increases in proinflammatory cytokines. JZL184, the proton pump inhibitor omepra- zole (positive control), or the primary constituent of marijuana, Δ^9-tetrahydrocannabinol (THC), significantly prevented diclofe- nac-induced gastric hemorrhages. JZL184 also increased stomach levels of 2-AG, but had no effect on AEA, arachidonic acid, or the prostaglandins E_2 and D_2. MAGL inhibition fully blocked di- clofenac-induced increases in gastric levels of proinflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor α, and granulocyte colony-stimulating factor, as well as IL-10. Pharma- cological inhibition or genetic deletion of CB_1 or CB_2 revealed that the gastroprotective effects of JZL184 and THC were mediated via CB_1. The antihemorrhagic effects of JZL184 persisted with repeated administration, indicating a lack of tolerance. These data indicate that increasing 2-AG protects against gastric damage induced by NSAIDs, and its primary catabolic enzyme MAGL offers a promising target for the development of analgesic thera- peutics possessing gastroprotective properties.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most commonly used classes of analgesics, are responsi-

ble for thousands of hospitalizations each year (Singh, 1998) because of their ulcerogenic side effects. To combat this damage to the stomach tissues, many chronic NSAID users are also prescribed proton pump inhibitors (PPIs), which reduce the production of stomach acid. Unfortunately, chronic use of PPIs is also associated with adverse side effects, including increased risk of gastric polyps and can- cer, bone fractures, and bacterial infections, as well as vitamin deficiencies (Lodato et al., 2010). Although long-

term prospective follow-up studies are lacking, investigation of alternative treatments for NSAID-induced gastrointestinal tissue damage is warranted.

Cannabis sativa and various cannabis extracts have been used for thousands of years to treat pain and other ailments (Kogan and Mechoulam, 2007). Empirical research on the analgesic effects of cannabis dates back to the 19th century (Dixon, 1899). Similar to NSAIDs, cannabinoids possess anti-inflammatory and analgesic properties. However, abuse and dependence potential, coupled with undesirable side effects including cognitive deficits, motor disturbances, and psychomimetic effects, dampen enthusiasm for their clinical development as therapeutic analgesics. On the other hand, a rapidly growing body of research focuses on targeting the endogenous cannabinoid (endocannabinoid; eCB) system for the development of new analgesics (Schlosburg et al., 2009).

Two cannabinoid receptors have been identified and cloned: CB_1 and CB_2. CB_1 is expressed throughout the body and is believed to be responsible for the central, psychoactive effects of cannabinoid agonists (Herkenham et al., 1991). CB_2 is expressed predominantly on immune cells in the periphery and seems to play a general anti-inflammatory role. In addition, CB_2 has been identified in primed microglia (Cabral et al., 2008) and labeled at low levels in neurons in the brainstem (Van Sickle et al., 2005). Several endogenous ligands of these receptors have been identified, and the best characterized are N-arachidonoyl ethanolamine (anandamide; AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). AEA and 2-AG are regulated in vivo by the catabolic enzymes fatty acid amide hydrolase (FAAH) (Cravatt et al., 2001) and monoacylglycerol lipase (MAGL) (Blankman et al., 2007), respectively. Injections of eCBs generally lack efficacy in pain and inflammation models because of their rapid in vivo degradation. Alternatively, inhibiting FAAH and MAGL represents a viable strategy to elevate eCBs for therapeutic gain.

Although brain levels of 2-AG are more than 100-fold higher than those of AEA, the study of MAGL inhibition has lagged behind that of FAAH inhibition because of a lack of selective MAGL inhibitors, until the development of 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184) (Long et al., 2009a,c), which is approximately 500-fold more selective in inhibiting MAGL than FAAH in vitro. Moreover, systemic administration of this selective MAGL inhibitor increased 2-AG, but not AEA, levels in whole brain and elicited CB_1-mediated analgesic, hypothermic, and locomotor suppressant effects (Long et al., 2009a).

FAAH(−/−) or wild-type mice treated with the FAAH inhibitor [3-(3-carbamoylphenyl)phenyl]-N-cyclohexylcarbamate (URB597) are protected from the ulcerogenic effects caused by high doses of the nonselective cyclooxygenase inhibitor diclofenac sodium (Naidu et al., 2009). However, it is unknown whether 2-AG possesses gastroprotective properties. Thus, the present study tested whether MAGL inhibition protects against NSAID-induced gastric lesions. Gastric hemorrhages were induced with the NSAID diclofenac sodium (Naidu et al., 2009). The antihemorrhagic effects of JZL184 were compared with the actions of the proton pump inhibitor omeprazole (Omep), as well as Δ^9-tetrahydrocannabinol (THC), the primary constituent responsible for marijuana's cannabimimetic effects. Although JZL184 elevates 2-AG in brain, it is unclear whether it also increases this eCB in stomach. Moreover, the observation that JZL184 reduces free arachidonic acid (AA) in brain suggests the possibility that decreases in AA metabolites, including prostaglandins, could contribute to the pharmacological effects of this MAGL inhibitor. Accordingly, we measured eCBs, free arachidonic acid, and prostaglandins from the stomachs of mice that were treated with vehicle or JZL184 before receiving a high dose of diclofenac or vehicle. To test the anti-inflammatory effects of MAGL inhibition, we quantified cytokine levels in the stomachs of mice treated with diclofenac in the presence and absence of JZL184. In addition, we used complementary genetic and pharmacological approaches to test whether CB_1 or CB_2 mediate the gastroprotective effects of JZL184. Finally, to address further the possible therapeutic potential of endocannabinoid-modulating compounds, we tested whether repeated administration of either JZL184 or the long-lasting FAAH inhibitor N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl) pyridin-2-yloxy)benzyl)piperidine-1-carboxamide (PF-3845) (Ahn et al., 2009) would continue to provide gastroprotective effects.

Materials and Methods

Animals. Subjects consisted of male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) that were approximately 10 weeks of age at the beginning of the study. In addition, male and female CB_1(−/−) and CB_2(−/−) mice, as well as CB_1(+/+) and CB_2 (+/+) littermate control mice, were obtained from the Center Transgenic Colony at Virginia Commonwealth University. CB_1(−/−) and CB_2(−/−) mice were backcrossed onto a C57BL/6J background for 13 and 6 generations, respectively. Mice were housed in a temperature- (20–22°C) and humidity-controlled, Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, with ad libitum access to food and water. Subjects weighed approximately 25 g and were housed four to six per cage maintained on a 12-h light/dark cycle. Mice were randomly assigned to treatment groups, although experiments using genetically altered mice also used a block design to evenly distribute male and female mice across conditions. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Induction of Gastric Lesions. The experimental procedure was based on published reports (Sánchez et al., 2002; Naidu et al., 2009). Mice were weighed, then food-deprived for 24 h with free access to water. Because mice are coprophagic, a wire mesh barrier was inserted into the cage to elevate the mice approximately 3 cm above the cage bedding. On the day of the hemorrhage induction, the mice were weighed, administered diclofenac (100 mg/kg p.o.) or vehicle, and returned to the cage. After 6 h, the mice were humanely euthanized via rapid cervical dislocation and their stomachs were harvested, photographed, and snap-frozen in liquid nitrogen. Stomachs were stored at −80°C until assayed for eCBs, prostaglandins, or cytokines as described below.

Gastric Hemorrhage Scoring. Gastric hemorrhages were quantified based on previously reported methods (Liu et al., 1998). Six hours after diclofenac treatment, stomachs were harvested, cut along the greater curvature, and rinsed with normal saline. The tissue was then placed on a lighted stage and photographed, along with a 1-mm reference, using a Canon EOS Rebel XS digital camera with a Canon 250D close-up lens (Adorama Inc., New York, NY). Images were renamed, to remove treatment information, and analyzed using Adobe Photoshop (version 7.0; Adobe Systems, Mountain View, CA). The length of each hemorrhage was marked with a 1-pixel-wide line and compared with the reference, such that the total hemorrhage score in millimeters for each stomach = pixels (hemorrhage)/pixels (reference). The experimenter scoring gastric hemorrhages was blind

to the treatment condition of each subject. This total gastric hemorrhage length measure has been reported to increase along with gastric myeloperoxidase activity, a marker of neutrophil activation, as well as neutrophil elastase and decreases in gastric blood flow (Liu et al., 1998).

Endocannabinoid and Prostaglandin Quantification. Metabolite levels were measured as described previously (Long et al., 2009b) using an Agilent Triple Quadrupole liquid chromatography/ mass spectrometer (Agilent Technologies, Santa Clara, CA). Tissues were weighed and dounce-homogenized in 6 ml of 1:1 (v/v) hexane/ ethyl acetate and 2 ml of phosphate-buffered saline containing internal standards for AEA, 2-AG, and a fatty acid (2 pmol d4-AEA, 0.5 nmol d5-2-AG, 10 nmol pentadecanoic acid). The mixture was vortexed and then centrifuged (1400g, 10 min). The organic layer was removed, evaporated under a stream of nitrogen, and resolubilized in 120 μl of chloroform. An aliquot of the extract (10 μl) was injected for analysis with an Agilent G6410B QQQ instrument. For liquid chromatography separation, mobile phase A consisted of 95:5 water/ methanol and mobile phase B consisted of 60:35:5 isopropanol/methanol/water. Formic acid (0.1%) or ammonium hydroxide (0.1%) was included to assist in ion formation in positive ionization and negative ionization modes, respectively. The flow rate for each run started at 0.1 ml/min with 0% B. At 5 min, the solvent was immediately changed to 60% B with a flow rate of 0.4 ml/min and increased linearly to 100% B over 10 min. This was followed by an isocratic gradient of 100% B for 5 min at 0.5 ml/min before equilibrating for 3 min at 0% B at 0.5 ml/min. The following mass spectrometry parameters were used to measure the indicated metabolites (precursor ion, product ion, collision energy in V): AEA (348, 62, 11), d4-AEA (352, 66, 11), 2-AG (379, 287, 8), d5-2-AG (384, 287, 8), PGE_2 (351, 271, 10), and PGD_2 (351, 271, 10). Arachidonic acid was measured by targeting the parent mass and quantified based on targeted measurements of pentadecanoic acid. PGE_2 and PGD_2 were quantified based on an external standard curve of the prostaglandin standards with pentadecanoic acid. Mass spectrometry analysis was performed with an electrospray ionization source. The drying gas temperature was 350°C, the drying gas flow rate was 11 L/min, and the nebulizer pressure was 35 psi. Lipids were quantified by measuring the area under the peak in comparison to the internal standards.

Cytokine Quantification (Enzyme-Linked Immunosorbent Assay). Cytokines were measured using the Mouse Inflammatory Cytokines Multi-Analyte ELISArray kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Whole stomachs were homogenized in phosphate-buffered saline in the presence of 1× protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The supernatant from a 1000g centrifugation of the homogenate was then centrifuged at 100,000g for 40 min. The supernatant from this centrifugation (the mouse stomach soluble proteome) (100 μg) was used in the assay according to the manufacturer's instructions.

Drugs. The NSAID diclofenac sodium (100 mg/kg), the synthetic glucocorticoid dexamethasone (2 mg/kg), and the proton pump inhibitor omeprazole (20 mg/kg) were purchased from Sigma-Aldrich (St. Louis, MO). The CB_1 antagonist rimonabant [N- (piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl] (3 mg/kg), the CB_2 antagonist N-[(1S)-$endo$-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) (3 mg/kg), and THC (10 mg/kg) were obtained from the National Institute on Drug Abuse (Bethesda, MD). The MAGL inhibitor JZL184 (Long et al., 2009a) and the FAAH inhibitor PF-3845 (Ahn et al., 2009) were synthesized in the Cravatt laboratory as described previously, and 2-arachidonoylglycerol was synthesized as described previously (Han and Razdan, 1999). All drugs were dissolved in a vehicle consisting of ethanol, Alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18. All compounds were administered at a volume of 10 μl/g body mass intraperitoneally, with the exception of diclofenac, which was administered orally. All solutions were warmed to room temperature before injection.

Data Analyses. All data are reported as mean $\pm$ S.E.M. and were analyzed using one-way analysis of variance, with the exception of studies using genetic knockout mice, which were analyzed using two-way (genotype × drug treatment), between-subjects analysis of variance. Post hoc comparisons were made using the Student-Newman-Keuls test, with the exception of the JZL184 dose-response data, for which Dunnett's test was used to compare each dose with vehicle. All animals were included in the analyses. Differences were considered statistically significant at $p < 0.05$.

Results

The NSAID Diclofenac Induced Gastric Hemorrhages. As reported previously (Naidu et al., 2009), diclofenac (100 mg/kg p.o.) administration to food-deprived mice induced gastric inflammation, resulting in redness, spot ulcers, and hemorrhagic streaks 6 h after treatment. These gastric hemorrhagic streaks were quantified in each stomach. The plant-derived cannabinoid THC (10 mg/kg) significantly prevented diclofenac-induced hemorrhages ($F_{4,51} = 7.82$; $p < 0.0001$; Fig. 1A). Pretreatment with the CB_1 antagonist rimonabant (3 mg/kg) reversed this effect, but the CB_2 antagonist SR144528 had no effect. Likewise, the proton pump inhibitor omeprazole (20 mg/kg) reduced the incidence of hemorrhages ($F_{4,38} = 12.5$; $p < 0.0001$; Fig. 1B).
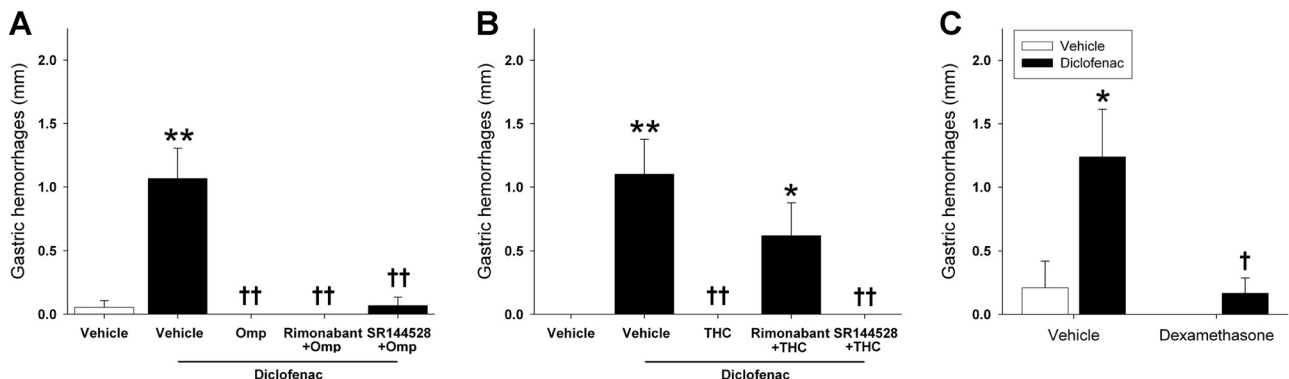
Fig. 1. Diclofenac induced gastric hemorrhages in mice. Mice were food-deprived for 24 h and then administered 100 mg/kg p.o. diclofenac sodium. Gastric hemorrhagic streaks were attenuated by a 2-h pretreatment with the proton pump inhibitor Omp (20 mg/kg) (A), the plant-derived cannabinoid THC (10 mg/kg) (B), or the synthetic glucocorticoid dexamethasone (2 mg/kg) (C). Pretreatment with the CB_1 antagonist rimonabant partially blocked the gastroprotective effects of THC, but the CB_2 antagonist SR144528 had no effect. Neither antagonist affected the gastroprotective effects of omeprazole. Data are expressed as mean $\pm$ S.E.M. ($n = 8$). ∗∗, $p < 0.01$; ∗, $p < 0.05$ versus vehicle treatment. †, $p < 0.05$ vs. diclofenac; ††, $p < 0.01$ versus diclofenac treatment.

A

B
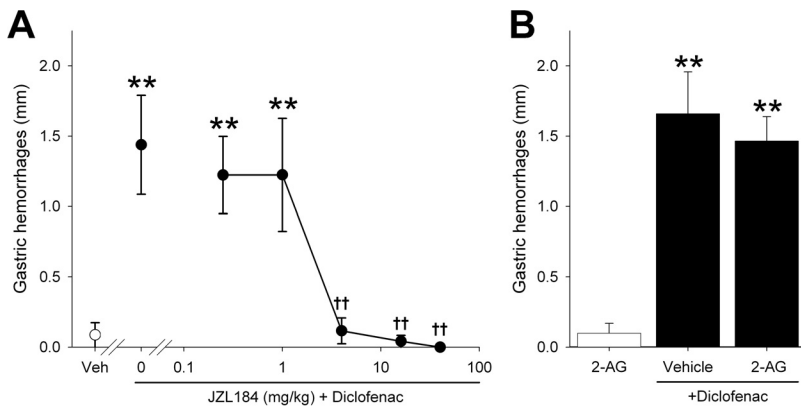
Fig. 2. Pretreatment with JZL184 prevented diclofenac-induced gastric hemorrhagic streaks, whereas exogenous 2-AG had no effect. Various doses of JZL184 (A) or 2-AG (50 mg/kg) (B) were administered intraperitoneally 2 h before diclofenac treatment. ○, vehicle treatment; ●, diclofenac treatment. Data are expressed as mean ± S.E.M. (n = 7–8). ∗∗, $p < 0.01$ versus vehicle treatment. ††, $p < 0.01$ versus diclofenac treatment.

Pretreatment with either antagonist had no effect on omeprazole treatment. An additional positive control, the synthetic glucocorticoid hormone dexamethasone (2 mg/kg), blocked diclofenac-induced hemorrhages ($F_{1,27}$ = 7.7; $p < 0.01$; Fig. 1C).

MAGL Inhibition Blocked NSAID-Induced Hemorrhages and Increased 2-AG in Stomach. Pretreatment with JZL184 attenuated diclofenac-induced gastric hemorrhagic streaks. Various doses of JZL184 (ranging from 0.25 to 40 mg/kg i.p.) were administered 2 h before diclofenac treatment. Overall, JZL184 significantly reduced the extent of diclofenac-induced hemorrhages ($F_{6,48}$ = 3.48; $p < 0.0001$; Fig. 2A). Post hoc analyses revealed that the gastroprotective effects of JZL184 occurred at doses of 4 mg/kg and higher. Exogenous 2-AG (50 mg/kg i.p.) administered 2 h before diclofenac treatment neither induced nor blocked NSAID-induced gastric hemorrhages ($F_{2,20}$ = 19.8; $p < 0.0001$; Fig. 2B).

MAGL inhibition increases central nervous system levels of 2-AG and decreases free arachidonic acid in the brain, but does not affect brain AEA (Kinsey et al., 2009; Long et al., 2009a). To determine whether MAGL inhibition produces a similar profile in stomach tissue, we assessed the effects of JZL184 (4 mg/kg i.p.) on these biomarkers from either stomachs subjected to diclofenac-induced gastric hemorrhages or control stomachs (Fig. 3). In whole stomach, JZL184 significantly increased levels of 2-AG ($F_{1,12}$ = 57.3; $p < 0.0001$; Fig. 4A), but had no effect on AEA ($p = 0.31$; Fig. 4B) or free arachidonic acid ($p = 0.41$; Fig. 4C), a metabolite of 2-AG and AEA and a precursor of prostaglandin synthesis. JZL184 had no effect on stomach levels of PGE_2 ($p = 0.19$) or PGD_2 ($p = 0.16$). Conversely, diclofenac sodium treatment significantly reduced levels of PGE_2 ($F_{1,12}$ = 46.8; $p < 0.0001$; Fig. 4D) and PGD_2 ($F_{1,12}$ = 53.6; $p < 0.0001$; Fig. 4E), but had no effect on 2-AG ($p = 0.11$), AEA ($p = 0.92$), or AA ($p = 0.45$).

MAGL Inhibition Blocked NSAID-Induced Increases in Stomach Proinflammatory Cytokines. Acute diclofenac treatment in food-deprived mice caused statistically significant increases in levels of the proinflammatory cytokines IL-1β ($F_{3,12}$ = 9.7; $p < 0.01$; Fig. 5A), IL-6 ($F_{3,12}$ = 43.7; $p < 0.0001$; Fig. 5B), TNF-α ($F_{3,12}$ = 20.5; $p < 0.0001$; Fig. 5C), and G-CSF ($F_{3,12}$ = 10.9; $p < 0.001$; Fig. 5D), as well as the anti-inflammatory cytokine IL-10 ($F_{3,12}$ = 15.5; $p < 0.001$;
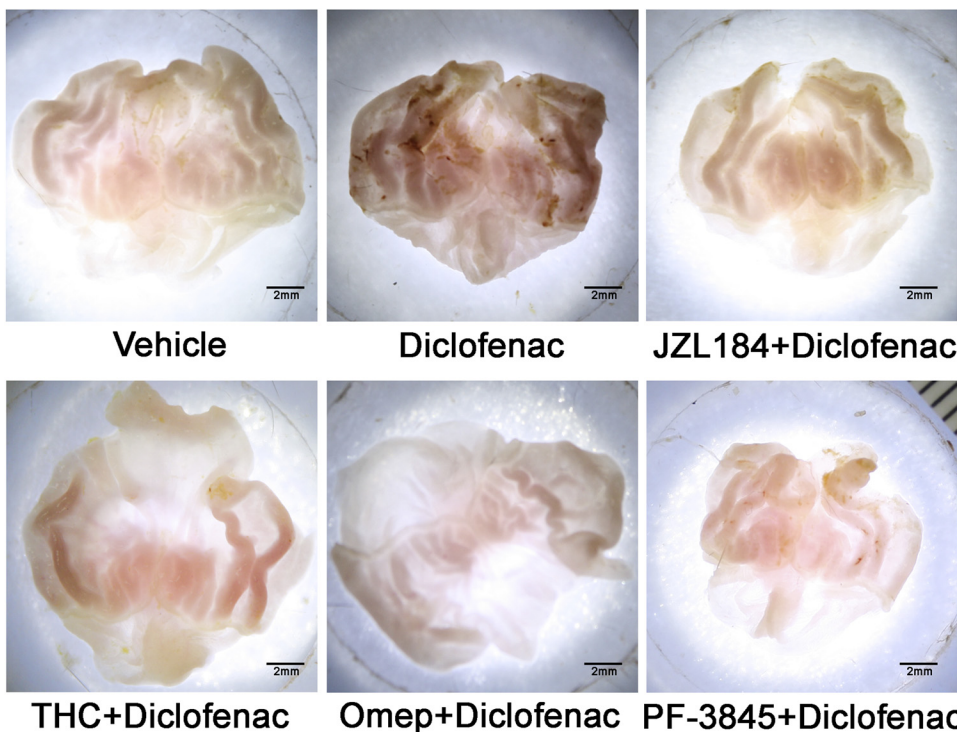
Fig. 3. Representative images of mouse stomachs. Mice were food deprived for 24 h, and then administered diclofenac sodium (100 mg/kg p.o.). Gastric hemorrhagic streaks were attenuated by pretreatment with the MAGL inhibitor JZL184 (4 mg/kg), the phytocannabinoid THC (10 mg/kg), the proton pump inhibitor omeprazole (Omep; 20 mg/kg), or the FAAH inhibitor PF-3845 (10 mg/kg).
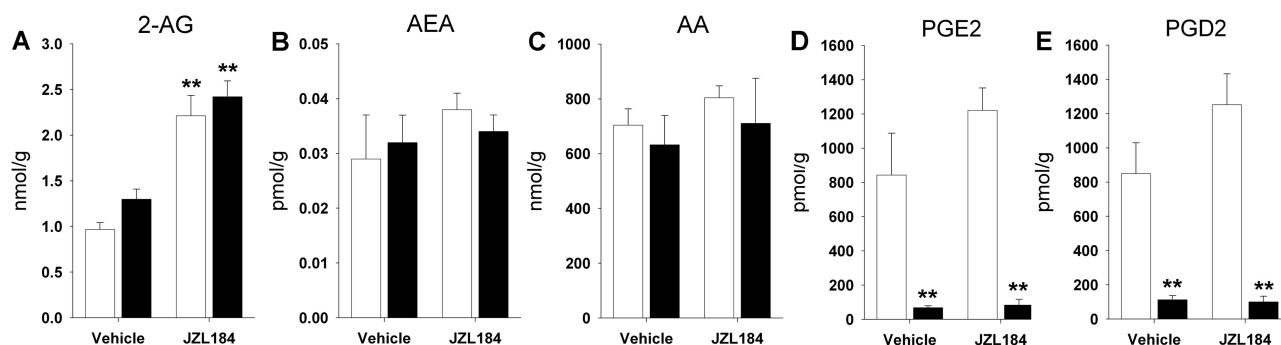
Fig. 4. MAGL inhibition increased 2-AG levels, and diclofenac inhibited prostaglandin levels, in stomach. To assess possible changes in endocannabinoid levels in stomach tissue, mice were fasted for 24 h, administered JZL184 (4 mg/kg i.p.) or vehicle, followed 2 h later by diclofenac (100 mg/kg p.o.) or vehicle, and stomachs were harvested 6 h later. In whole stomach, JZL184 significantly increased levels of 2-AG (A) but had no effect on levels of AEA (B), free AA (C), PGE_2 (D), or PGD_2 (E). Conversely, diclofenac sodium significantly reduced levels of PGE_2 and PGD_2, but had no effect 2-AG, AEA, or AA. Open bars, vehicle treatment; filled bars, diclofenac. Data are expressed as mean ± S.E.M. ($n = 4$). ∗∗, $p < 0.01$ versus vehicle/vehicle treatment.
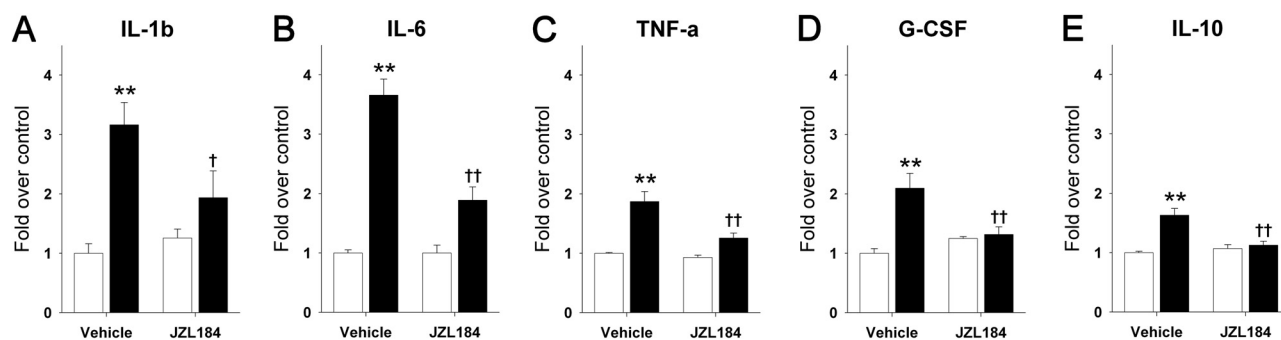
Fig. 5. MAGL inhibition blocked diclofenac-induced increases of proinflammatory cytokines in stomach. Mice were fasted for 24 h, administered JZL184 (4 mg/kg i.p.) or vehicle, followed 2 h later by diclofenac (100 mg/kg p.o.) or vehicle, and stomachs were harvested 6 h later. In whole stomach, diclofenac significantly increased levels of IL-1 (A), IL-6 (B), TNF-α (C), G-CSF (D), and IL-10 (E). Pretreatment with JZL184 blocked this increase in cytokines. Open bars, vehicle treatment; filled bars, diclofenac. Data are expressed as mean ± S.E.M. standardized to mean levels of vehicle-treated group ($n = 4$). ∗∗, $p < 0.01$ versus vehicle treatment. ††, $p < 0.01$ versus diclofenac treatment.

Fig. 5E). Pretreatment with JZL184 significantly blocked diclofenac-induced elevations of each of these cytokines.

CB₁ Mediates the Gastroprotective Effects of JZL184. In the next series of experiments, we used complementary pharmacological and genetic approaches to determine whether cannabinoid receptors mediate the gastroprotective effects of JZL184. The CB₁ antagonist rimonabant (3 mg/kg), or the CB₂ receptor antagonist SR144528 (3 mg/kg), was administered 10 min before JZL184 (4 mg/kg i.p.). As with the above experiments, JZL184 significantly reduced diclofenac-induced gastric hemorrhages ($F_{3,28} = 10.1$; $p < 0.0001$; Fig. 6A). Post hoc analyses revealed that rimonabant completely blocked the antihemorrhagic effects of JZL184, whereas SR144528 had no effect, indicating that CB₁, and not CB₂, mediated the gastroprotective effects of MAGL inhibition via JZL184. When administered alone, neither rimonabant (1.6 ± 0.4 mm) nor SR144528 (1.5 ± 0.3 mm) affected gastric hemorrhages.
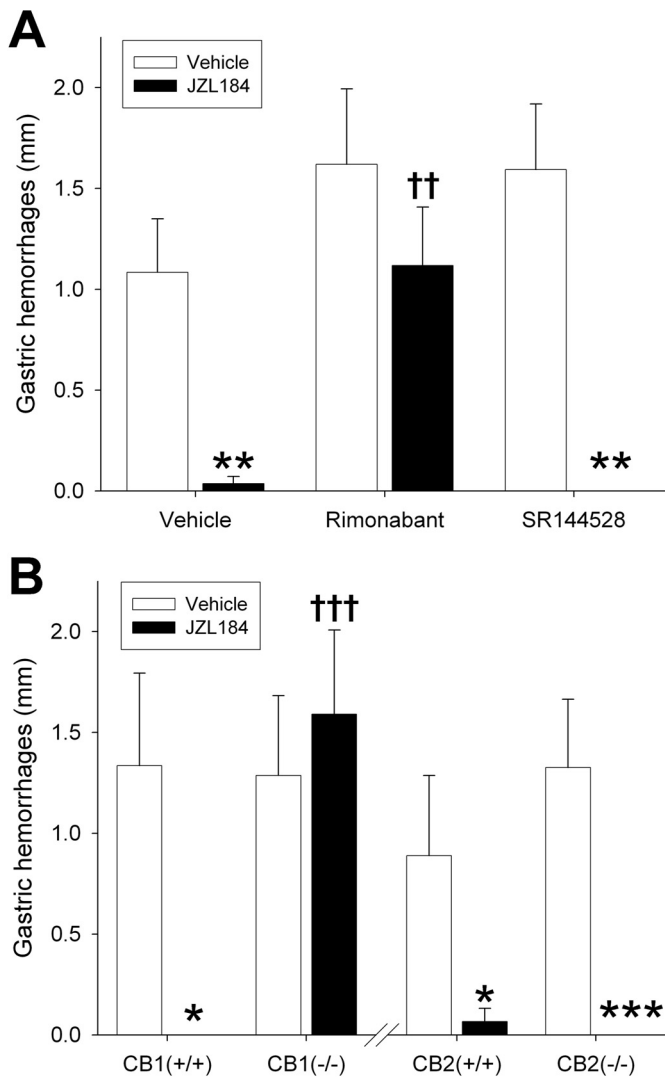
Diclofenac induced gastric hemorrhages in both CB₁(−/−) and CB₂(−/−) mice with comparable severity to their wild-type littermates (Fig. 6B), indicating that endocannabinoids do not tonically modulate NSAID-induced hemorrhages. Although JZL184 did not block hemorrhages in CB₁(−/−) mice, it maintained efficacy in CB₂(−/−) mice, indicating that the gastroprotective effects of JZL184 occur via a CB₁-specific mechanism of action. In the CB₁(−/−) experiment, there was a significant interaction between genotype and MAGL inhibition ($F_{1,27} = 5.01$; $p < 0.05$), as well as a main effect of genotype ($F_{1,27} = 4.44$; $p < 0.05$). In the CB₂(−/−) experi-

ment, there was a main effect of MAGL inhibition ($F_{1,25} = 17.3$; $p < 0.001$), but no interaction between genotype and MAGL inhibition ($p = 0.34$).

Antihemorrhagic Effects of MAGL and FAAH Inhibition Persist after Repeated Treatment. To test whether prolonged blockade of MAGL leads to tolerance and loss of gastroprotective effects, we tested the effects of JZL184 after chronic administration on diclofenac-induced hemorrhagic streaks. For comparison, we evaluated the impact of repeated administration of the FAAH inhibitor PF-3845 on diclofenac-induced hemorrhages. Mice were treated for 5 consecutive days with JZL184, PF-3845, or vehicle. On day 5, the mice were fasted for 22 h, then administered JZL184, PF-3845, or vehicle, followed 2 h later by diclofenac. Mice treated acutely with JZL184 or PF-3845 showed marked reductions in hemorrhagic streaks ($F_{5,42} = 5.74$; $p < 0.001$; Fig. 7). Repeated administration of either PF-3845 or JZL184 did not reduce these gastroprotective effects, indicating a lack of tolerance to each enzyme inhibitor.

Discussion

Since the 19th century, when Felix Hoffman first produced acetylsalicylic acid (aspirin), patients have complained about gastrointestinal irritation when taking NSAIDs (Lanas, 2009). Indeed, the primary undesirable side effect and source of noncompliance of NSAID use is dyspepsia. The role of the eCB system in modulating gut motility, secretion, and in-

A

B

Fig. 6. JZL184 blocks diclofenac-induced hemorrhages via a CB$_1$-mediated mechanism of action. Gastric hemorrhagic streaks were attenuated by a 2-h pretreatment with JZL184 (4 mg/kg). A, this antihemorrhagic effect was blocked by the CB$_1$ antagonist rimonabant (3 mg/kg), whereas the CB$_2$ antagonist SR144528 (3 mg/kg) had no effect. Neither rimonabant nor SR144528 affected hemorrhages on its own ($n = 8$). B, genetic deletion of CB$_1$ prevented the gastroprotective effects of MAGL inhibition with JZL184, whereas genetic deletion of CB$_2$ had no effect. Diclofenac-induced gastric hemorrhages were assessed in CB$_1$($-/-$) versus CB$_1$($+/+$) littermates ($n = 5$–8) and CB$_2$($-/-$) versus CB$_2$($+/+$) littermates ($n = 5$–9). All mice received diclofenac sodium treatment (100 mg/kg). Open bars, vehicle treatment; filled bars, JZL184 treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus vehicle treatment. ††, $p < 0.01$ versus JZL184 treatment. †††, $p < 0.001$ versus CB$_1$($+/+$) diclofenac treatment.

flammation is a topic of a growing body of research (Izzo and Sharkey, 2010); however, less is known about their gastroprotective properties. AEA has been reported to have gastroprotective properties (Shujaa et al., 2009), and blockade of its chief catabolic enzyme, FAAH, reduces NSAID-induced ulcers (Naidu et al., 2009). In the present study, we used the highly selective MAGL inhibitor JZL184 to investigate whether elevating 2-AG protects against the development of NSAID-induced gastric hemorrhages. JZL184, the phytocannabinoid THC, dexamethasone, and the proton pump inhibitor omeprazole significantly reduced the formation of gastric hemorrhagic streaks induced by the NSAID diclofenac so-
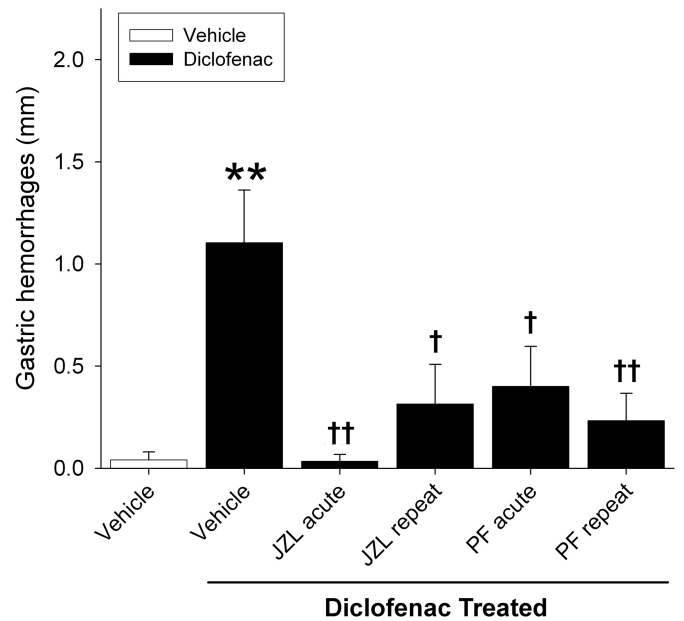
Fig. 7. The antihemorrhagic effects of MAGL or FAAH inhibition persist after repeated administration. Repeated administration of either JZL184 (JZL; 4 mg/kg) or PF-3845 (PF; 10 mg/kg) did not result in tolerance to the antihemorrhagic effects of either inhibitor. Open bar, vehicle treatment; filled bars, diclofenac. Data are expressed as mean ± S.E.M. ($n = 8$). **, $p < 0.01$ versus vehicle treatment. †, $p < 0.05$; ††, $p < 0.01$ versus diclofenac treatment.

dium. Moreover, JZL184 completely prevented diclofenac-induced increases of proinflammatory cytokines in stomach. The gastroprotective effects of MAGL inhibition and THC were blocked by rimonabant pretreatment, whereas the CB$_2$ antagonist SR144528 had no effect, indicating a CB$_1$ mechanism of action.

The results of the present study are consistent with the notion that JZL184 protects against NSAID-induced gastric lesions by elevating endogenous levels of 2-AG in the stomach through CB$_1$-mediated actions. In brain, 2-AG levels are two orders of magnitude higher than those of AEA, and JZL184 increases 2-AG another 10-fold (Long et al., 2009a,c), but here we demonstrated that stomach 2-AG levels are almost five orders of magnitude higher than those of AEA, but JZL184 elevates gastric levels of 2-AG only approximately 2-fold. Likewise, other studies have found that cannabinoids reduce stress- or NSAID-induced gastric lesions in rodents through a CB$_1$ mechanism. For example, the synthetic cannabinoid agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN55,212-2) attenuates stress-induced gastric pathology in rats, but the inactive enantiomer [(3*S*)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone monomethanesulfonate (WIN55,212-3) has no effect (Germanò et al., 2001). The gastroprotective effects of WIN55,212-2 were blocked by rimonabant pretreatment, but not by SR144528, indicating a CB$_1$-mediated mechanism of action (Germanò et al., 2001). The CB$_1$-selective agonist arachidonyl-2'-chloroethylamide (ACEA) attenuated aspirin-induced gastric ulcers in rats (Rutkowska and Fereniec-Goltbiewska, 2006), and the CB$_1$ antagonist *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) blocked the gastroprotective effects of exogenously administered AEA treatment in the rat water

immersion with restraint model of stress-induced gastric lesions (Dembiński et al., 2006). These findings taken together suggest that CB_1 activation is gastroprotective against NSAID-induced ulcers.

Although the mechanisms underlying the gastroprotective effects are unknown, one possibility is decreased gastric acid secretion. Gastric acid secretion by parietal cells in the gastric fundus can be stimulated neuronally or hormonally, by histamine (paracrine) or gastrin (endocrine) (Schubert, 2008). Although the synthetic cannabinoid agonists (6aR)-$trans$- 3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo [b,d]pyran-9-methanol (HU-210) and WIN55,212-2 had no effect on basal stomach acid production in rats, each compound blocked pentagastrin-induced acid secretion (Coruzzi et al., 1999; Adami et al., 2002). However, neither compound affected histamine-induced acid production, suggesting a systemic endocrine mechanism of acid modulation, not direct tissue stimulation (Adami et al., 2002). Vagotomy blunted the acid-reducing effect of HU-210, indicating the involvement of peripheral cholinergic pathways and vagal afferents (Adami et al., 2002). These data are supported by a study in which intravenous, but not intracerebroventricular, administration of either HU-210 or WIN55,212-2 significantly reduced pentagastrin-induced acid production (Adami et al., 2004). Future studies will determine whether MAGL inhibition reduces basal or pentagastrin-induced gastric acid secretion as well as reduces NSAID-induced ulcers through this type of mechanism.

It is also possible that JZL184 produces its antiulcerogenic activity via one or more acid-independent pathways. For example, central (intracerebroventricular) or peripheral (intravenous) administration of WIN55,212-2, as well as anandamide and its biologically stable analog methanandamide (Shujaa et al., 2009), attenuated acute ethanol-induced gastric mucosal damage, an acid-independent model (Glavin and Szabo, 1993). Alternatively, MAGL inhibition may increase blood flow in the stomach, thereby increasing oxygenation of gastric endothelial cells and decreasing infiltration of neutrophils. One mechanism through which this effect could occur is an increase in levels of heme oxygenase-1, which reduces oxidative stress in gastric endothelial cells. The PPIs omeprazole and lansoprazole up-regulate heme oxygenase-1, resulting in acid-independent gastroprotective effects (Becker et al., 2006). Likewise, the transient receptor potential cation channel V1 receptor agonist capsaicin causes vasodilatation in GI smooth muscle (Holzer et al., 1989). Transient receptor potential cation channel V1 also binds the eCB anandamide, but not 2-AG (Schlosburg et al., 2009).

The antihemorrhagic effects of MAGL inhibition in the present studies occurred via CB_1, which is present in the central nervous system as well as in the stomach. For example, CB_1 is colocalized to cholinergic neurons innervating the gastric mucosal endothelium of the fundus, corpus, and antrum of the rat stomach (Adami et al., 2002). Likewise, CB_1 has been identified in ganglia adjacent to the gastric endothelium along both the greater and lesser curvatures of the mouse stomach (Casu et al., 2003). Thus, it is possible that increased levels of 2-AG act centrally and/or locally in the stomach to attenuate diclofenac-induced gastric hemorrhages. Finally, in contrast to rodents, human gastric parietal cells express CB_1 (Pazos et al., 2008). Thus, there may be species-specific mechanisms through which cannabinoids inhibit NSAID-induced gastric tissue damage.

NSAIDs damage the gastrointestinal tract through multiple mechanisms, grossly grouped together as topical and systemic causes (Schubert, 2008). This drug class is believed to cause gastric pathology through the reduction of prostaglandin biosynthesis in stomach via cyclooxygenase inhibition, which leads to increased stomach acid production and decreased mucosal secretion (Musumba et al., 2009). Because JZL184 also reduces free arachidonic acid levels in brain, another goal of the present study was to determine whether it produces the same effect in stomach as well as whether it reduces prostaglandin levels in stomach. In addition, we evaluated gastric eCB levels to determine whether MAGL regulates 2-AG levels in stomach. The observation that JZL184 did not affect levels of free arachidonic acid, PGE_2, or PGD_2 in the stomachs of mice treated with high doses of diclofenac or vehicle suggests that MAGL inhibition protects against NSAID-induced gastric ulcers through a mechanism other than the prostaglandins. Diclofenac-induced increases in proinflammatory cytokine levels were blocked by JZL184 pretreatment. However, as mentioned above, further studies measuring the effects of MAGL inhibition on gastric acid secretion in vivo are important for delineating the underlying mechanisms by which JZL184 produces gastroprotection.

As shown previously (Naidu et al., 2009), FAAH($-/-$) mice or wild-type mice treated with the FAAH inhibitor URB597 showed a significantly attenuated response to diclofenac-induced gastric hemorrhages. In addition to being more selective for FAAH than URB597, PF-3845 is longer lasting, inhibiting FAAH for at least 24 h (Ahn et al., 2009). Because repeated administration of high doses of JZL184 leads to down-regulation and desensitization of CB_1, along with tolerance to its analgesic effects (Schlosburg et al., 2010), we evaluated whether prolonged administration of FAAH or MAGL inhibitors would retain their antiulcerogenic actions. This apparent disparity between the present and previous (Schlosburg et al., 2010) studies may be caused by different end point measures (antiulcerogenic effects versus antinociceptive effects) or the dose of JZL184 used. Whereas Schlosburg et al. (2010) used a 40 mg/kg dose, the present study administered 4 mg/kg JZL184. It is noteworthy that, in both studies, tolerance did not develop to FAAH inhibition via PF-3845.

The present data indicate that inhibiting degradation of the endogenous cannabinoids AEA and 2-AG attenuates NSAID-induced gastric endothelial damage. These findings, coupled with the synergistic antinociceptive interactions between the NSAIDs and FAAH inhibition (Fowler et al., 2009; Naidu et al., 2009), support the idea that FAAH and MAGL are promising targets for the development of analgesic therapeutics that not only lack adverse gastrointestinal side effects, but also protect against NSAID-induced gastric ulcers.

Authorship Contributions

Participated in research design: Kinsey, Nomura, Cravatt, Grider, and Lichtman.

Conducted experiments: Kinsey, Nomura, and O'Neal.

Contributed new reagents or analytic tools: Long and Mahadevan.

Performed data analysis: Kinsey and Nomura.

Wrote or contributed to the writing of the manuscript: Kinsey, Nomura, O'Neal, and Lichtman.

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