Protection from diclofenac-induced small intestinal injury by the JNK inhibitor SP600125 in a mouse model of NSAID-associated enteropathy

Veronica Ramirez-Alcantara, Amanda LoGuidice, and Urs A. Boelsterli

University of Connecticut School of Pharmacy, Department of Pharmaceutical Sciences, Storrs, Connecticut

Submitted 10 June 2009; accepted in final form 24 August 2009

Ramirez-Alcantara V, LoGuidice A, Boelsterli UA. Protection from diclofenac-induced small intestinal injury by the JNK inhibitor SP600125 in a mouse model of NSAID-associated enteropathy. Am J Physiol Gastrointest Liver Physiol 297: G990–G998, 2009. First published August 27, 2009; doi:10.1152/ajpgi.00219.2009.—Small intestinal ulceration, bleeding, and inflammation are major adverse effects associated with the use of diclofenac (DCF) or other nonsteroidal anti-inflammatory drugs (NSAIDs). The underlying mechanisms of DCF enteropathy are poorly understood, but there is increasing evidence that topical effects are involved. The aim of this study was to explore the role of c-Jun-N-terminal kinase (JNK) in DCF-induced enterocyte death because JNK not only regulates mitochondria-mediated apoptosis but also is a key node where many of the proximal stress signals converge. Male C57BL/6J mice were injected intraperitoneally with DCF or vehicle (Solutol HS-15), and the extent of small intestinal ulceration was determined. A single dose of DCF (60 mg/kg) produced numerous ulcers in the third and fourth quartiles of the jejunum and ileum, with maximal effects after 18 h and extensive recovery after 48 h. To study the molecular pathways leading to enterocyte injury, we isolated villi-enriched mucosal fractions from DCF-treated mice. Immunoblot studies with a phosphospecific JNK antibody revealed that JNK1/2 (p46) was activated at 6 h, leading to phosphorylation of the downstream target c-Jun. The levels of the JNK-regulated proapoptotic transcription factor C/EBP homologous protein (CHOP) were also increased after DCF. The selective JNK inhibitor SP600125 (30 mg/kg ip), given both 1 h before and 1 h after DCF, blocked JNK kinase activity and afforded significant protection against DCF enteropathy. In conclusion, these data demonstrate that the JNK pathway is critically involved in the pathogenesis of DCF-induced enteropathy and suggest a potential application of JNK inhibitors in the prevention of NSAID-induced enteropathy.

mitochondria; small intestinal ulceration; villi

ONE OF THE MAJOR ADVERSE EFFECTS OF nonsteroidal anti-inflammatory drug (NSAID) therapy is gastrointestinal injury. This results in ulceration and bleeding of the mucosa, inflammation, and, in rare cases, perforation (1, 5, 46). Because of its high morbidity and mortality, NSAID-associated gastrointestinal injury is a serious clinical challenge causing a major burden on the health care system. Specifically, in the U.S. the annual number of hospitalizations for serious NSAID-associated gastrointestinal complications is estimated to be higher than 100,000 patients, and ~16,500 deaths occur each year; the impact of these adverse drug reactions has therefore been termed a “silent epidemic” (46).

It has become increasingly clear that, besides the well-known lesions in the stomach, the small intestine is also a major target organ of NSAID-associated toxicity (9, 20). With the advent of novel imaging techniques including capsule endoscopy, it has become possible to fully appreciate the incidence and extent of small bowel injury. Unexpectedly, recent clinical studies revealed that as many as approximately two-thirds of both long-term (>3 mo) and short-term (>1 wk) NSAID users exhibited drug-induced lesions in the jejunum and ileum (13, 25). Unfortunately, to date there is no mechanism-based therapy or preventive treatment for drug-induced enteropathy available (17).

The underlying mechanisms of NSAID-induced enteropathy are not fully understood, but a number of modes of action have been recognized (17, 21). For example, systemic inhibition by NSAIDs of both cyclooxygenase (COX)-1 and COX-2 (the therapeutic target) may be involved, similar to the situation in the stomach (38, 41). However, off-target effects have also been implicated in the pathogenesis of small intestinal injury, including direct topical effects of the drugs on enterocytes facing the lumen (39). This is primarily due to the relatively high (millimolar) concentrations of conjugated NSAIDs in the hepatobiliary tree following ATP-dependent transport of NSAID glucuronides into the bile canaliculi, from where the metabolites are transported into the small intestine where they are cleaved and the free parent drug reabsorbed (36, 42). In cell culture studies, NSAIDs have been shown to target mitochondria and to induce uncoupling of oxidative phosphorylation in a concentration-dependent manner (39). Furthermore, in gastric cells or hepatocytes, certain NSAIDs have been demonstrated to induce an endoplasmic reticulum (ER) stress response (43), causing increases in cytosolic (free) Ca2+ concentrations ([Ca2+]c) (24) and/or producing increased oxidant stress (24, 32). It is not clear to what extent reactive metabolites are involved in initiating or aggravating the injury (3, 36). Apart from these direct toxic effects of NSAIDs, indirect effects may also play a role in enterocytes. For example, because of the increased permeability of the gut, intestinal bacterial lipopolysaccharide (LPS) may activate Toll-like receptor-4 on macrophages, which in turn can lead to tumor necrosis factor-α (TNF)-mediated cell injury and activation and recruitment of other cells of the innate immune system (45).

In view of these apparently multiple modes of action involved in NSAID enteropathy, it becomes extremely difficult to point to a specific putative mechanism that could be targeted in search of a potential therapeutic intervention strategy. However, one common pathway that links most of these initial cellular stress signals with downstream events and with ensuing cell death is the activation of c-Jun-N-terminal kinase (JNK). For example, JNK is phosphorylated by apoptosis signal-regulating kinase 1 (Ask1) or other upstream mediators, which in turn are activated by multiple stressors including oxidative stress (from, e.g., drug-induced intracellular reactive oxygen species production, LPS signaling, TNF release), increased [Ca2+], or ER stress response signals (28), all con-
verging at this pivotal signaling node. Activation of JNK, which regulates a number of cellular functions including cell proliferation and immune reactions, is particularly important in the pathogenesis of tissue injury because it can result in mitochondria-mediated lethal cell injury through regulating pro- and antiapoptotic Bcl-2 family proteins, leading to mitochondrial permeabilization (15, 37). Currently little is known about a possible causal role of this pathway in mucosal injury of the small intestine induced by NSAIDs, despite the urgent need for preventive or therapeutic intervention. Therefore, the aim of this study was to explore the mechanistic role of JNK activation in mediating mucosal injury in a mouse model of diclofenac (DCF) enteropathy and to ascertain whether inhibition of JNK activation afforded protection against DCF-induced small intestinal ulceration.

MATERIALS AND METHODS

Chemicals. DCF sodium, nitrotetrazolium blue chloride (NBT), serine/threonine/tyrosine protein phosphatase inhibitors, acid and alkaline phosphatase inhibitors, protease inhibitors, and all other chemicals (unless indicated) were obtained from Sigma (St. Louis, MO). SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) was obtained from Biomol International (Plymouth Meeting, PA). Anti-JNK and anti-phospho-JNK antibodies were purchased from Cell Signaling (Danvers, MA); anti-phospho-c-Jun and anti-C/EBP homologous protein (CHOP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-e-Jun antibody was obtained from Calbiochem EMD Chemicals (Gibbstown, NJ). Solutol HS-15 was obtained from BASF Fine Chemical Division (Ludwigshafen, Germany). Phosphate-buffered saline (PBS) and 10% zinc formalin were obtained from Fisher Scientific (Kalamazoo, MI).

Animals. The study design and all protocols for animal care and handling were approved by the Institutional Animal Care and Use Committee of the University of Connecticut. Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and acclimatized for at least 1 wk before the experiments. The mice were kept on a 14:10-h light-dark cycle. Animals were 11–17 wk old at the time of experimentation. They received mouse chow (Teklad Global Rodent Diet, Harlan Laboratories, Boston, MA) and water ad libitum.

Drug administration. DCF was dissolved in a 7% (in PBS) Solutol HS-15 solution and administered intraperitoneally (30, 60, or 90 mg/kg) in a volume of 10 μl/g body wt. All animals were treated at 5 h before start of the dark cycle. SP600125, a potent and selective inhibitor of JNK, was dissolved in a 7% (in PBS) Solutol HS-15 solution and administered intraperitoneally (30 mg/kg) both 1 h prior to and 1 h after DCF administration.

Assessment of small intestinal ulceration. The treated mice were euthanized by CO2 inhalation. The abdomen and chest were rapidly opened by a midline incision, and blood was obtained by cardiac puncture. Blood was transferred to a separation tube containing clot activator and separating gel at the bottom, and serum was prepared by centrifugation and frozen at −80°C until use for chemistry analysis. A portion of the gastrointestinal tract (from stomach to cecum) was removed. The stomach was opened along the small curvature to and 1 h after DCF administration.

Western blotting. Because we found highest ALP activity in F2, we used this villus-enriched fraction to analyze the expression of proteins after DCF treatment. We used phosphatase inhibitors both in the EDTA solutions and lysis buffer because our study involved the analysis of phosphoproteins. The lysates were centrifuged at 20,000 g for 5 min at 4°C to remove debris. Protein content was determined by the BCA assay. Equal amounts of protein from each experimental group were reduced, denatured, and separated by SDS-PAGE. The resolved proteins were transferred to a nitrocellulose or polyvinylidine fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with anti-JNK (1:1,000 dilution), anti-phospho-JNK (1:1,000), anti-phospho-c-Jun (1:800), or anti-CHOP (1:1,000) antibodies. The bound antigen-antibody complexes were visualized after incubation with horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence detection system (Millipore, Billerica, MA). Equivalent protein loading among samples was tested by detection of β-actin protein.

Statistical analysis. Ulcer numbers and serum biochemistry values were expressed as means ± SD with a P value of ≤0.05 to indicate statistical significance. When normality of distribution failed, a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks was used followed by Dunn’s test for multiple comparison vs. the respective control group. When the normality test passed, a standard ANOVA was used followed by Dunnett’s test for multiple comparison vs. control group. For Western blot studies, samples from at least four animals per experimental group were evaluated. Data on densitometric analysis were expressed as means ± SD of fold changes compared with vehicle control. Statistical analysis was performed using the Kruskal-Wallis one-way ANOVA on ranks, using Dunn’s
test for multiple comparison vs. the respective control group. To evaluate the effects of DCF with or without JNK inhibitor, the t-test was applied (comparing two means only). A P value of \( \leq 0.05 \) was considered statistically significant.

**RESULTS**

Development of a mouse model of DCF enteropathy. Our first aim was to develop a mouse model of DCF enteropathy because, in contrast to rat models, detailed information on the toxic response to NSAIDs in the small intestine is limited in this species. Following administration of a single dose (30–90 mg/kg), DCF induced multiple ulcers and smaller erosions in the mouse small intestinal mucosa within less than 24 h. These ulcers were readily identified and quantitated by NBT-positive staining of the villi surrounding the focal mucosal lesions (Fig. 1). Gross pathological examination of the small intestine following an ulcerogenic dose of DCF typically revealed severe damage featuring multiple round or elongated ulcers localized primarily along the mesenteric side (Fig. 1A). Histopathological examination confirmed that the ulcers involved the entire mucosa, compromising the integrity of the submucosa and muscularis mucosae. The lesions were characterized by loss of villi and infiltration of inflammatory cells in the necrotic part of the ulcer. The villi adjacent to the ulcer were contracted, and necrosis of the epithelium extended to the base of the crypts. Similar to the tissue injury in rats (3), the lesions were most abundant in the third and fourth quartile of the small intestine, whereas the first quartile was free of any apparent ulceration and the second quartile exhibited few lesions only (Fig. 1B). The reason for this differential distribution along the jejunum and ileum has not been determined but could result from differential pH in these segments and/or different bacterial populations, or it could be determined by variations in the toxicodynamic response across the length of the small intestine. Because the mouse is a relatively new model for NSAID enteropathy, we first ran a time course study with a single...

Fig. 1. Characterization of diclofenac (DCF)-induced small intestinal ulcers in C57BL/6J mice. A: mice were administered a single dose of DCF (60 mg/kg ip) and euthanized 18 h later. An opened segment of quartile 3 of the small intestine revealed ulcers on the mesenteric side of the mucosa. Villi surrounding the ulcers appeared dark blue after NBT staining, whereas the necrotic tissue remained in the center of the ulcer. Bottom: histological section (hematoxylin and eosin (H&E) staining) from the ulcer in the top panel (arrow). The mucosa overlying the mesentery appears severely damaged with focal destruction and necrosis of villi, extending to the submucosa and muscularis mucosae. Note the abundant infiltration of inflammatory cells. B: quantitative analysis and distribution of ulcers across quartiles 1 through 4 of the mouse small intestine. Mice were treated with a single dose of DCF (60 mg/kg ip), and the mucosa was analyzed at the indicated time points. Each point represents mean ± SD for 4–7 animals/group. C: time course of small intestinal ulceration after treatment of mice with a single dose of DCF (60 mg/kg ip). Each point represents mean ± SD for 4–7 animals. D: dose-dependent development of ulcerative damage in the small intestine. Mice were treated with a single intraperitoneal dose of DCF and euthanized after 18 h. Each column represents mean ± SD for 5–8 animals/group. *P < 0.05 vs. vehicle control.
ulcerogenic dose of DCF. Following intraperitoneal administration of 60 mg/kg DCF, mice developed multiple ulcerations in the small intestinal mucosa that became apparent as early as at 6 h postdose (Fig. 1C). The maximal effects were observed after 18 h, when the damage was severe with ulcers becoming confluent. Although the number of ulcers at 18 h was not significantly higher than that at 6 h, the average size of the ulcers was much greater. At 48 h postdose, all the lesions had been repaired, and the small intestinal mucosa appeared indistinguishable from vehicle control animals, except for small scars in the mucosa, indicating that extensive tissue regeneration had occurred after a single dose of DCF (not shown). The development of small intestinal ulceration was dose dependent (Fig. 1D). We chose a standard dose of 60 mg/kg for all subsequent experiments because higher doses did not significantly increase the number and extent of lesions but did increase the risk of lethality. This standardized dose, which is approximately two to three times higher than the therapeutic dose when based on body surface (rather than on mg/kg-based comparison between mice and humans), is high but necessary to study protective mechanisms.

Similar to the rat model (34), a single dose of DCF significantly decreased the plasma activity of ALP in a time- and dose-dependent manner (Fig. 2). The degree of ALP activity changes paralleled the pathological manifestation of ulceration, making ALP a valuable biomarker of small intestinal injury in the DCF mouse model. The mechanisms underlying the decrease in serum ALP activity has not been elucidated; however, it has recently been reported that ALP is involved in the metabolism and detoxication of bacterial LPS (14), where it prevents leakage of LPS into the bloodstream. In line with this, it has been demonstrated that following administration of an ulcerogenic dose of indomethacin to rats there was an ~50% reduction in ALP activity in the brush border compared with vehicle controls (39). The primary function of intestinal ALP has been suggested to be the detoxication of LPS, which is a substrate for ALP (14). Thus the attenuated serum activity could be due to inactivation of ALP by LPS during the NSAID-induced increases in intestinal permeabilization. Furthermore, total serum protein levels (another marker of small intestinal injury) were also consistently decreased following DCF exposure (Fig. 2). After an ulcerogenic dose (60 mg/kg ip), the total protein levels were decreased by 12% at 6 h postdose and the loss of serum protein further progressed, attaining 21% decrease at 18 h. The underlying mechanism likely involves loss of protein due to increased intestinal permeability and bleeding. At 48 h postdose, both serum ALP activity and total protein levels had returned to normal control values (not shown), confirming the morphological data and indicating that these serum markers of injury are reversible and paralleling the pathological changes.

Characterization of isolated small intestinal villi. Enterocytes lining the villi are the primary target of NSAIDs in the small intestine (3). Therefore, to study the signaling pathways leading to cell injury, we isolated the small intestinal mucosa and prepared a fraction enriched in villi from the third and fourth quartile. The original method produced “footprint”-like structures (8) (Fig. 3A), which we characterized by high specific expression of both aminopeptidase N (villi) and defensin (crypts, not shown). Here, by use of a modified method, subsequent fractionation yielded suspensions of tissue highly enriched in villi or crypts (Fig. 3, B and C). The F2 fraction (see MATERIALS AND METHODS) exhibited a 47% higher specific activity of ALP activity (villus brush border biomarker) than F3 and was used for all subsequent experiments.

JNK (p46) is activated after DCF. To explore a possible role of JNK signaling in the intestinal ulcerative damage induced by DCF, we first determined the levels of immunoreactive phospho-JNK (p-JNK) in lysates of isolated small intestinal mucosa in Western blots at various time points following administration of DCF. Both JNK1 and JNK2 can exist either as the full-length 54-kDa protein (p54) or as a COOH-terminally truncated form of 46-kDa (p46) (10). We found that the truncated form (p46) of p-JNK became increased at 3 h

---

*Fig. 2. Clinical-chemical biomarkers of small intestinal ulceration induced by DCF (60 mg/kg ip). Mice were euthanized at various time points, and serum levels of total protein and alkaline phosphatase (ALP) activity were determined. Each point represents mean ± SD for 4–7 animals. *P < 0.05 vs. 0 h (protein); #P < 0.05 vs. 0 h (ALP).

*Fig. 3. Morphological characterization of villus-enriched isolated mucosa. A: footprint-like structures isolated from mouse small intestine (quartiles 3 and 4) consisting of both villi and crypts. B: representative micrograph showing a villus from the F2 fraction. C: a crypt from the F3 fraction. Original magnification was ×200 (B) or ×400 (C).
post-DCF in two of four mice and that it was further increased at 18 h in all treated mice (Fig. 4A). Densitometric analysis (mean of 4 mice per group) revealed that p-JNK (p46) was significantly increased at 6 h, exhibiting even higher significance levels at 18 h postdose (3.4-fold increase over basal levels) (Fig. 4B). To ascertain that the increased levels of p-JNK (p46) were not simply a consequence of a higher abundance of inactive JNK, we determined the levels of nonphosphorylated p46. We did not find any significant alterations compared with the apparent JNK expression levels in vehicle control mice at the corresponding time points. Collectively, these findings suggest that JNK (p46) is activated prior to the appearance of any pathological changes and could therefore be involved in the signaling pathways leading to DCF-induced enteropathy.

To determine whether activation of JNK caused the phosphorylation of downstream protein targets, we assessed by immunoblotting the steady-state levels of phospho-c-Jun (p-c-Jun) at an early time point (6 h post-DCF). c-Jun is part of the transcription factor AP-1 (activator protein-1) and involved in the regulation of early response genes. We found indeed a strong p-c-Jun signal (Fig. 4C), confirming that DCF enhanced the kinase activity of p-JNK.

Administration of the JNK inhibitor, SP600125 protects from DCF enteropathy. DCF-induced stimulation of JNK phosphorylation and activation of its downstream target c-Jun suggested a role for JNK in the pathogenesis of ulcers.

**Fig. 5. Effects of the JNK inhibitor SP600125 on the extent and distribution of small intestinal ulcers induced by DCF.** Mice were administered a single dose of DCF (60 mg/kg ip) with or without SP600125 (30 mg/kg ip, given both 1 h before and 1 h after DCF). The mice were euthanized at 18 h post-DCF. Note that in the presence of SP600125 the ulcerative damage was almost completely abolished. A: total ulcer number. Each column represents mean ± SD for 4–6 animals/group; *P < 0.05 vs. DCF alone. B: distribution of ulcers across quartiles 1 through 4 of the small intestine. Each point represents mean ± SD for 4–6 animals/group. *P < 0.05 vs. DCF + SP600125.
further explore a possible causal role of JNK in DCF enteropathy, we used the cell-permeable, ATP-competitive small-molecule JNK inhibitor SP600125 (4). The inhibitor or its vehicle (Solutol HS-15, 7% in PBS) was administered intraperitoneally at a dose of 30 mg/kg both 1 h before and 1 h after DCF (60 mg/kg).

To ascertain that the JNK inhibitor was effective in blocking the downstream signaling, we first determined the immunoreactive levels of p-c-Jun. We found that SP600125 greatly attenuated the levels of immunoreactive p-c-Jun (Fig. 4C). This is in line with the concept that SP600125 decreases JNK activity, whereas it does not interfere with JNK phosphorylation itself (26). Next, we analyzed the effects of the JNK inhibitor on DCF-induced ulcer formation. We found that SP600125 afforded significant protection from ulceration induced by DCF; both the number (Fig. 5) and size (Fig. 6) of ulcers and erosions were greatly reduced compared with DCF alone controls. Specifically, the number of ulcers was reduced by 93% (Fig. 5A) with minor damage observed only in the third quartile (Fig. 5B). Gross examination of the small intestine stained with NBT revealed that the mucosa after combined DCF/SP600125 treatment had a vehicle control-like appearance except for a few shallow, small ulcers (Fig. 6, A–C) and a low number of erosions that did not exhibit loss of villi (not included in the ulcer count). Histopathological evaluation of the few small ulcers confirmed that the degree of severity was mild, featuring minor loss of villi and without compromising the integrity of the submucosa and muscularis mucosae and a reduced number of infiltrated neutrophils (Fig. 6D). The erosions in the DCF/SP600125-treated mice were only superficial changes in the mucosa without affecting crypts, submucosa, or muscularis mucosae, and no inflammation was observed (Fig. 6E). Taken together, the data clearly indicate that the JNK inhibitor afforded full protection from DCF enteropathy, and they suggest that JNK may play a key role in the cellular signaling leading to NSAID-induced small intestinal injury.

Role of CHOP and mitochondria-mediated cell death. Recent studies have indicated that ER stress can activate JNK, which in turn leads to CHOP upregulation and apoptosis (23, 47). CHOP is a transcription factor that has been associated with mitochondria-mediated cell death. To ascertain whether CHOP may be involved in DCF enteropathy, we measured the time-dependent changes of CHOP protein in the isolated epithelial-enriched fraction of the small intestine following an ulcerogenic dose (60 mg/kg) of DCF (Fig. 7). We found a time-dependent increase in immunoreactive CHOP attaining a 2.1-fold increase at 18 h compared with the basal levels, but not at earlier time points. Because CHOP is positively regulated by JNK, these data could provide a link between DCF-induced JNK activation and enterocyte apoptosis. However, because CHOP activation is not an early event, it is more likely that CHOP-mediated pathways could aggravate the injury at an advanced stage rather than being the primary event.

DISCUSSION

The aim of this study was to evaluate the mechanistic role of JNK in DCF-induced small intestinal injury in the mouse. We found that JNK plays a pivotal role in the pathogenesis of DCF-induced small intestinal ulceration and that targeting JNK could become a potentially novel way for therapeutic intervention. These conclusions were based on a number of observations. First, a single ulcerogenic dose of DCF selectively increased the levels of one activated (phosphorylated) JNK

![Figure 6. Macroscopic and histopathological characterization of the cytoprotective effects of SP600125 against DCF-induced ulceration. A: close-up view of one of the few small intestinal ulcers induced by DCF (60 mg/kg ip) in the presence of SP600125. Note the small size of the circular shaped ulcer. B: opened segment of quartile 3 of the small intestine showing the mesenteric side of the mucosa from the same experimental group as in A. Note the “vehicle control-like” macroscopic appearance of the mucosa. C: close-up view of an erosion. Note that the central villi are retained in the minor lesion. D: histopathological analysis of the ulcer displayed in A, confirming the moderate mucosal damage on the mesenteric side. E: microscopic view of the erosion displayed in C showing the superficial injury confined to the mucosa; no evidence of inflammation was observed. H&E stain, original magnifications ×10 (B), ×30 (A and C), and ×100 (D and E).]
form (p46) prior to the development of ulcers. Furthermore, and importantly, we found that coadministration of the selective JNK inhibitor, SP600125, fully protected from DCF-induced small intestinal injury, indicating that JNK and its downstream signaling pathways are critical in mediating DCF-induced lethal cell injury in enterocytes.

JNK are a protein family of serine/threonine kinases, encoded by three genes: JNK1, JNK2, and JNK3. The gene products can be alternatively spliced to give α and β forms of proteins (p54 and p46) (10). JNK1 and JNK2 are ubiquitously expressed (including enterocytes), whereas JNK3 is present in the nervous system and cardiomyocytes and not expressed in the small intestine. The functions of the forms differ, but they are all activated by different forms of cellular stress, among which oxidative stress including reactive oxygen species generated in mitochondria and other cellular sources is a major activator (29, 37). Further downstream, JNK can phosphorylate a number of targets; one pathway is activation of c-Jun, a protein that is also expressed in murine enterocytes (18). Activation of c-Jun enables activation of AP-1 and plays a role in the regulation of the expression of a number of proinflammatory cytokines including TNF and IL-1β and the activation of the innate immune system (2, 12). On the other hand, JNK also activates other proteins, including Bcl-2 family members, and is therefore involved in the regulation of mitochondria-mediated cell death (11, 35). For example, JNK can phosphorylate (activate) Bax and other proapoptotic proteins, whereas at the same time it phosphorylates and thereby inactivates Bcl-XL and other antiapoptotic proteins (19). Because both mitochondria (39, 40) and the innate immune system (45) have been implicated in mediating NSAID-associated enteropathy, inhibition of JNK by a chemical inhibitor likely blocks multiple important downstream pathways.

We found indeed that the specific JNK inhibitor, SP600125 almost completely prevented ulcer formation following administration of a normally ulcerogenic dose (60 mg/kg) in mice. SP600125 is a small-molecule, ATP-competitive inhibitor with low selectivity for a specific JNK form (it inhibits both JNK1 and JNK2), but it is highly selective and has a >300-fold selectivity over the related MAPKs, ERK and p38 (4). SP600125 is an established tool to evaluate JNK-dependent processes in vivo or in vitro (7), including chemically induced injury to the gastrointestinal tract. For example, SP600125 protected from dextran sulfate sodium-induced colitis in mice (2), and the inhibitor prevented JNK-dependent gastric lesions induced by ethanol in rats (27). At the high dose (30 mg/kg ip) that we used, SP600125 has been shown to decrease JNK-regulated TNF plasma levels in mice treated with LPS (4). Although it is not yet clear to what extent TNF is causally involved in DCF enteropathy, it is, however, unlikely that the observed protection by SP600125 from DCF ulceration is solely due to an inhibitory effect on TNF, since JNK inhibition also prevents the activation of other pathways including mitochondrial permeabilization (19, 22, 33). Another downstream target that may be indirectly inhibited by SP600125 is COX-2; it has been shown that COX-2 is regulated by JNK and that the enzyme was downregulated by SP600125 following induction of COX-2 by LPS in murine macrophages (30). In enteropathy, loss of COX-2 activity, however, would promote rather than protect from damage; therefore, downregulation of COX-2 is unlikely to be the sole cause for the protection provided by SP600125. Another JNK-regulated pathway is the activation of the proapoptotic transcription factor CHOP. It has been shown that JNK activation during ER stress leads to increases in CHOP levels (23) and that inhibition of JNK by SP600125 attenuates CHOP expression (47), which is commensurate with our findings.

Although these downstream effects (mitochondria-mediated and/or innate immune system-mediated enterocyte cell demise and its protection by SP600125) can be explained by the key role of JNK, the proximal pathways leading to JNK activation in the first place are much less clear. Although DCF has been shown to cause early increases in [Ca\(^{2+}\)], and to enhance oxidant stress in other cell types such as hepatocytes (24), it is not clear whether similar changes are involved in enterocytes. Both increased [Ca\(^{2+}\)] and oxidant stress could lead to activation of Ask1-dependent signaling and JNK phosphorylation (31).

Alternatively, the high concentrations of the electrophilic and protein-reactive DCF acyl glucuronide and iso-glucuronides present in the gut, which have been shown to cause DCF adduct formation to enterocyte plasma membrane proteins (3, 6, 36, 44), could initiate some signaling events; however, a causal role of these protein adducts has never been proven. Once activated, JNK can in turn either phosphorylate Bcl-2 proteins that regulate mitochondria-mediated apoptosis, or, alternatively, translocate to mitochondria where JNK can directly trigger mitochondrial permeabilization (16).
In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of numbers of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West. 

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West.

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West.

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West.

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West.

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West.

In conclusion, this study demonstrates that specific JNK forms (p46) are activated in the small intestinal mucosa early following administration of an ulcerogenic dose of DCF in mice and that the highly selective JNK inhibitor SP600125 completely prevents the injury, implicating a mechanistic role of JNK in the pathogenesis of NSAID ulceration. These findings could lead to a potential therapeutic application of novel JNK inhibitors in drug-induced intestinal ulceration, since currently there is no mechanism-based treatment available to treat or prevent NSAID-induced enteropathy. Furthermore, the use of the generally intestine-safer selective COX-2 inhibitors (as opposed to the mixed COX-1/2 inhibitors like DCF, which have higher gastrointestinal liability) has been restricted in recent years after it was detected that some COX-2 inhibitors have a higher risk for cardiovascular disease; therefore the number of prescriptions for the traditional NSAIDs will likely be increasing. In light of the severity and the high incidence of non-steroidal anti-inflammatory drugs on the small and large intestine in the West.