Gastric Toxicity and Prostaglandin Content in Rats Dosed with Two Chemically Similar, Nonsteroidal Anti-Inflammatory Agents (43532)

ROBERT R. BROOKS,[1] THOMAS J. MOOREHEAD, AND SCHWE FANG PONG
Research and Product Development, Procter & Gamble Pharmaceuticals, Norwich, New York 13815

Abstract. **Two chemically similar nonsteroidal anti-inflammatory drugs, orpanoxin and F-1067, had almost identical potencies and efficacies as anti-inflammatory (rat paw edema) and analgesic (mouse writhing) agents, but differed markedly in gastrotoxicity. Orpanoxin alone aggravated stomach lesions in rats subjected to pylorus ligation and failed to protect stomachs of rats challenged with indomethacin. The compounds did not differ in their *in vitro* enzyme inhibition effects, both failing to inhibit 5- and 15-lipoxygenase and both inhibiting prostaglandin synthetase. Extraction of prostaglandins from the gastric mucosa of pylorus-ligated rats revealed, however, that the safer F-1067 depleted prostaglandin 6-keto-$F_{1\alpha}$ less and increased prostaglandin E_2 much more than did orpanoxin. A possible causality is suggested.** [P.S.E.B.M. 1993, Vol 202]

Gastrointestinal toxicity is a common adverse effect of nonsteroidal anti-inflammatory drugs (NSAID), including the large propionic acid group (1). Although sensitivity differs between species (e.g., dog > rat > monkey), most NSAID cause erythema, hemorrhage, and ulceration at some dose level and duration of dosing. Inhibition of cyclo-oxygenase and prostaglandin depletion at the tissue level seems to be the principle mechanism, since gastroprotection can be achieved by co-administration of a prostaglandin analog (2).

To further test the link between an NSAID's effect on prostaglandin synthesis and gastric toxicity, we have studied two chemically similar propionic acids, orpanoxin (3) and F-1067 (Fig. 1). Both are *para*-chlorophenylfuranyl propionic acids differing only in orpanoxin's α-carbon hydroxyl group. The two compounds were compared for *in vivo* analgesic activity in a mouse model and anti-inflammatory activity in a rat model. Comparison for gastric toxicity was done in three rat models of gastric irritation. Their potency as lipoxygenase and/or cyclo-oxygenase inhibitors was assessed with *in vitro* assays of potato tuber 5-lipoxygenase, soybean 15-lipoxygenase, and ram seminal vesicle prostaglandin synthetase. Finally, their effects on tissue levels of three prostaglandins were determined in rat stomachs.

[1] To whom requests for reprints should be addressed at Procter & Gamble Pharmaceuticals, P.O. Box 191, Norwich, NY 13815-0191.

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Materials and Methods

Chemicals. Orpanoxin and F-1067 were synthesized by Procter & Gamble Pharmaceuticals, Norwich, NY. Carrageenin, indomethacin, acetylcholine bromide, phenidone (Sigma Chemical Co., St. Louis, MO), methylcellulose (400 cps; Dow Chemical Co., Indianapolis, IN), and Metofane (Pittman Moore, Inc., Mundelein, IL) were purchased. Other reagents of laboratory grade were purchased.

Animals. Male Wistar or male or female (arachidonic acid metabolism work) Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA), Hilltop Lab Animals (Scottsdale, PA), or Blue Spruce Farms (Altamont, NY). Male CD-1 mice were from Charles River. All animals were maintained under standard conditions with food and water available *ad libitum* in USDA- and AAALAC-approved facilities. Experiments were conducted under a protocol approved by the site veterinarian and in compliance with the Animal Welfare Act.

Gastric Irritation Assays. Three tests were conducted to compare the relative gastric toxicity of orpanoxin and F-1067. While assessment of gastric lesions was not done blinded, this part of the work was carried

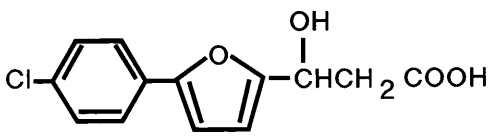
out by technicians at a contract facility who had no knowledge of the status of, or sponsor's interest in, the compounds. The risk of biased readings was further minimized by use of three separate rat models. Confirmatory assessment of gastric ulcerogenicity was done by in-house personnel in separate studies.

An inflammation-challenged rat model was prepared by implantating subcutaneously in the abdomen of Metofane-anesthetized animals (eight per dose level) a polyester sponge (40 mm × 18 mm × 6 mm) saturated with 1 ml of carrageenin solution (20 mg/ml in sterile saline). Immediately after surgery, at 6–8 hr, and at 21 hr after surgery, rats were dosed orally with orpanoxin, F-1067, or 0.25% (w/v) methylcellulose vehicle alone. Gastric lesions (areas of frank hemorrhage) were assessed in saline-rinsed organs visually at 24 hr after surgery.

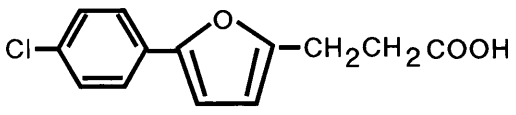
The pylorus-ligated rat model of Shay *et al.* (4) was used to assess intrinsic gastric irritation potential. Fasted rats (eight per dose level) weighing 140–180 g were anesthetized (Metofane). An abdominal midline incision afforded access to the pylorus, which was ligated. The incision was closed and orpanoxin or F-1067 in 0.25% (w/v) methylcellulose was administered in a volume of 10 ml/kg. Separate control rats received vehicle alone. Four hours after dosing, the rats were sacrificed with Metofane and the stomachs were examined for mucosal lesions.

An indomethacin-challenged rat model (5) was used to determine whether the compounds ameliorated or exacerbated NSAID-induced gastric lesions. Fasted rats (20 for vehicle, eight per dose of test article) weighing 160–325 g were dosed orally with 0.5% (w/v) methylcellulose alone or test article in methylcellulose at a volume of 10 ml/kg. Immediately thereafter, 30 mg/kg of indomethacin in the same vehicle and volume were administered orally. Four hours later, rats were anesthetized (Metofane) and stomachs were examined.

Anti-Inflammatory Assay. The standard carrageenin-induced rat paw edema model of Winter *et al.* (6) was used. Fasted rats (eight per dose level) weighing 170–225 g were dosed by gavage with orpanoxin, F-1067, or indomethacin suspended on the day of use in 0.5% (w/v) methylcellulose, or with methylcellulose alone (control group) 1 hr before administration of 0.1 ml of sterile 1% (w/v) carrageenin in distilled water into the left hindpaw (26-gauge needle). Prior to test article and 3 hr after carrageenin, the volume of the paw was determined by mercury displacement up to the anatomical hairline.



Orpanoxin



F-1067

Figure 1. Structures of orpanoxin and F-1067.

Analgesic Assay. The standard acetylcholine-induced writhing test similar to that of Pong *et al.* (7) was used. Fasted mice (10 per dose level) weighing 18–28 g were dosed with orpanoxin or F-1067 suspended in 0.5% methylcellulose. One or two hours later, the mice received 10 ml/kg of acetylcholine bromide, 0.55 mg/ml in 0.5% methylcellulose, administered intraperitoneally. The number of writhes over the next 10 min were recorded.

Enzymology. Potato tuber 5-lipoxygenase was purified to near homogeneity as described by Sekiya *et al.* (8) to produce a stock solution of 1.2 mg protein/ml. The assay was carried out at 30°C and pH 6.3 with 0.06 mg of enzyme (50-μl aliquot), 0.33 m*M* arachidonic acid, and test article in 50 μl of ethanol or ethanol alone in a final volume of 3.0 ml. Activity was measured with an oxygraph as consumption of oxygen from an air-saturated reaction mixture at 30°C. Test article was added to the reaction mixture 2 min before starting the reaction by the addition of arachidonic acid.

Soybean 15-lipoxygenase, activity 0.5–1 × 106 units/mg, was purchased from Sigma. One unit equals the oxidation of 0.12 μmol linoleic acid/min at pH 9.0 at 25°C. Assay conditions were as above with 1000 units of enzyme (50-μl aliquot of a 1/50 dilution) and 0.1 m*M* arachidonic acid at pH 8.0.

Prostaglandin synthetase was purified to near homogeneity from ram seminal vesicles by the procedure of Yamamoto (9) to produce a stock solution of 1.1 mg protein/ml. Similar to the procedure of van der Ouderaa and Buytenhek (10), assay conditions were as above with 0.11 mg of enzyme (100-μl aliquot), 4 μ*M* Mn protoporphyrin, and 0.1 m*M* arachidonic acid at pH 8.0.

Each assay was run at an enzyme concentration chosen to be within the range of linear dependence of reaction rate on enzyme concentration. A single preparation of enzyme and single-stock concentrations of test article were used for all assays. Activities were expressed in units of mmol of oxygen consumed per min per ml of enzyme, i.e., the stock enzyme or the 1/50 dilution of 15-lipoxygenase. Ethanol at the final concentration used to deliver test articles (50 μl/3 ml) did not affect enzyme activity. Five determinations of activity were made for each concentration of test article.

***In Vivo* Effects on Arachidonic Acid Metabolism.** The weighed fundus portion of stomachs from pylorus-ligated rats (see above) were minced and incubated in 1 ml of pH 7.4 Kreb's bicarbonate solution for 1 hr at 37°C under an atmosphere of 95% CO_2 and 5% O_2. H_3PO_4 (20 μl of 1/21 dilution) was added and prostaglandins were extracted with 4 ml of ethyl acetate. The organic phase was dried under nitrogen and stored at 4°C. The extract was redissolved in 0.5 ml of chloroform-ethyl acetate (85:15 v/v) and applied to a chloroform-equilibrated, 1-g LH-20 column. Vials were washed with an additional 0.5 ml of solvent, which was applied to the column. In sequence, the column was washed with 3.5 ml of chloroform-ethyl acetate, 10 ml of chloroform, and 2 ml of methanol. Prostaglandins, including 6-keto $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), $F_{2\alpha}$ ($PGF_{2\alpha}$), and E_2 (PGE_2), were eluted with an additional 1.5 ml of methanol, dried under nitrogen, and refrigerated.

Prostaglandins, dissolved in water-acetonitrile (70:30 v/v), were separated and quantified by a high-pressure liquid chromatography method, described in detail by Rydzik and colleagues (11), that separates all major prostaglandins and has a sensitivity in the nanogram range. Samples were applied to a Beckman C-18 5-micron 250 × 2-mm column, and eluted with a mobile phase of 2.5 m*M* H_3PO_4-acetonitrile (69:31 v/v) at a flow rate of 300 ml/min. Detection was done spectrophotometrically, at 190 nm. Authentic standards of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2 were run to assure system performance and adequate separation of the target lipids.

Treatment of Data. For gastric irritation assay data, mean lesion count per rat in test-article-treated groups was compared with the count in vehicle-treated groups by the Mann-Whitney nonparametric test. Other data from groups or samples treated with test articles was compared with the corresponding control group by a *t* test. A probability of <0.05 was considered significant.

Results

Gastric Irritation. In inflammation-challenged normal rats whose stomachs were not subjected to a direct irritation, neither phenidone, orpanoxin, nor F-1067 were particularly ulcerogenic as compared with indomethacin (Table I). In such rats challenged with subcutaneous peritoneal carrageenin (sponge), 4 mg/kg of indomethacin significantly increased lesions per rat to 4.25, compared with none in rats treated with methylcellulose vehicle. Only the highest 200-mg/kg dose of F-1067 caused a significant increase to 2.88 lesions per rat, whereas the same dose of orpanoxin increased lesion count nonsignificantly to 1.5 per rat (Table I). Enumeration of leukocytes in the 24-hr sponge exudate

Table I. Comparison of Ulcerogenic Effects of Oral Orpanoxin, F-1067, and Other NSAID in Several Rat Models[a]

Model	Oral treatment	Dose (mg/kg)	*n*	Lesions per rat
Inflammation-challenged	0.25% Methylcellulose	10 ml/kg	8	0
	Indomethacin	4	8	4.25[b]
	Phenidone	50	8	1.12
	Orpanoxin	50	8	0
		100	8	0
		200	8	1.5
	F-1067	50	8	0
		100	8	0
		200	8	2.88[b]
Pylorus-ligated	0.25% Methylcellulose	10 ml/kg	8	0.38
	Orpanoxin	100	12	1.67
		200	12	7.5[b]
		400	12	5.08[b]
	F-1067	100	8	0.75
		200	8	0.5
		400	8	0.25
Indomethacin-challenged	0.25% Methylcellulose	10 ml/kg	20	9.85
	Orpanoxin	100	10	10.7
		200	10	11.4
		400	10	9.9
	F-1067	100	10	4.4[b]
		200	10	0.4[b]
		400	10	0.4[b]

[a] Inflammation-challenged rats were impregnated subcutaneously with a polyester sponge containing 20 mg of carrageenin, and dosed orally at 0, 6–8, and 21 hr after implantation, and visually evaluated for gastric lesions (frank hemorrhage) at 24 hr. Indomethacin-challenged rats were dosed immediately before oral administration of 30 mg/kg of indomethacin and evaluated for gastric lesions 4 hr later. Pylorus-ligated rats were dosed immediately after surgery and evaluated for gastric lesions 4 hr later.

[b] Significantly different from corresponding vehicle-treated group by Mann-Whitney test at $P < 0.05$.

of these rats did reveal a difference in the two compounds. F-1067 suppressed leukocyte migration to a greater extent than did orpanoxin. At 50-, 100-, and 200-mg/kg doses by mouth, cell counts were decreased, compared with vehicle-treated controls, by 2.9%, 22.3%, and 31.0% for orpanoxin-treated rats, and by 39.1%, 36.0%, and 80.6%, respectively, for F-1067-treated rats. The decrease at the 200-mg/kg dose of F-1067 was significant ($P < 0.02$). In pylorus-ligated rats, however, orpanoxin was more ulcerogenic than F-1067. Orpanoxin significantly increased mean lesion count 20-fold and 13-fold in comparison with the vehicle-treated group at the 200- and 400-mg/kg doses, respectively. F-1067 at the same doses did not cause increased lesion incidence compared with the methylcellulose-dosed animals. Further evidence of the greater ulcerogenic potential of orpanoxin was seen in rats challenged with indomethacin. Orpanoxin, 100, 200, and 400 mg/kg, was unable to protect rats from indomethacin-induced lesions, whereas F-1067 at all three doses significantly reduced lesion incidence (Table I).

Analgesic Action. Despite the difference in gastric irritation profile, orpanoxin and F-1067 were equally effective and potent as analgesic agents in the standard mouse acetylcholine-induced writhing assay (Fig. 2). At 1 hr after dosing, F-1067 had a nonsignificantly greater potency, but at 2 hr after dosing, the protection-versus-dose curves of the two compounds were almost superimposable.

Anti-Inflammatory Action. In the standard carrageenin-induced paw edema test in the rat, orpanoxin and F-1067 were equally effective and potent in reducing paw swelling as a function of oral dose (Fig. 3). Both compounds were less potent than indomethacin in this test, but had a similar efficacy (40% inhibition of edema).

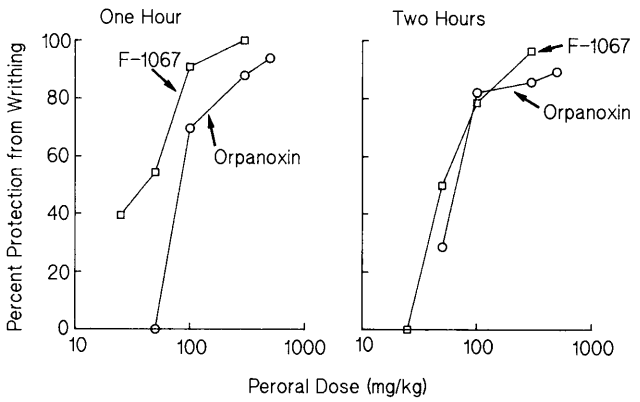


Figure 2. Equal analgesic efficacy of oral orpanoxin and F-1067 in the mouse acetylcholine-induced writhing test. Groups of 10 male mice, dosed with 0.5% methylcellulose vehicle or test article 1 or 2 hr previously, were observed for writhing responses for 10 min after an intraperitoneal dose of 5.5 mg/kg of acetylcholine bromide. Vehicle-treated mice exhibited 33 and 28 writhes in the 1-hr and 2-hr tests, respectively.

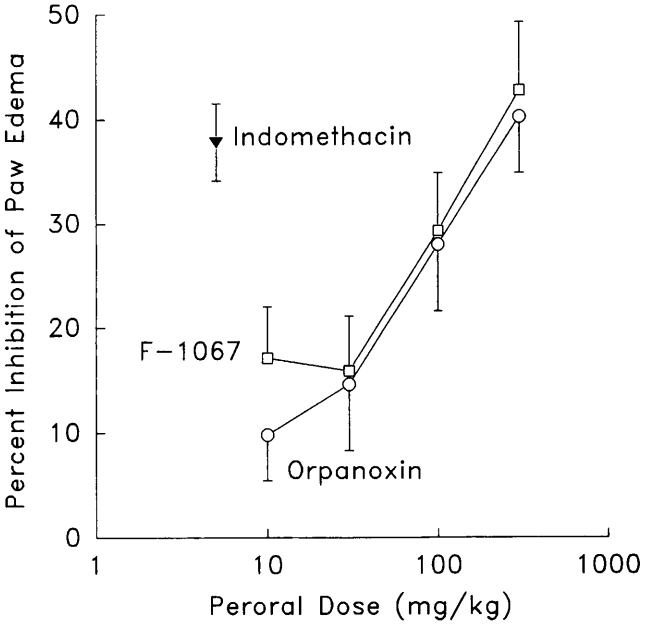


Figure 3. Equal anti-inflammatory efficacy of oral orpanoxin and F-1067 in the rat carrageenin-induced paw edema model. Groups of eight fasted male rats were dosed with 0.25% methylcellulose, orpanoxin, F-1067, or indomethacin 1 hr before receiving a subplantar injection of 0.5 mg of carrageenin in saline. The paw response was measured by immersion 3 hr later.

Effects on Arachidonic Acid Metabolism Enzymes *In Vitro*. A comparison was made of inhibitory effects of the compounds on arachidonic acid pathway enzymes *in vitro*. Only at the highest tested concentrations of 6000 m*M* and 1200 m*M*, respectively, did orpanoxin and F-1067 inhibit 5-lipoxygenase (Fig. 4). Neither benoxaprofen nor indomethacin were inhibitors of this enzyme. A similar profile of inactivity was seen with 15-lipoxygenase: only the highest 1200-m*M* concentration of F-1067 had a significant inhibitory effect. For prostaglandin synthetase, indomethacin was a potent inhibitor. Orpanoxin, F-1067, and benoxaprofen stimulated prostaglandin synthetase at 100 m*M* and then strongly inhibited the reaction at higher concentrations. The cause and significance of the stimulation are unknown. As inhibitors, these compounds were two to three orders of magnitude less potent than indomethacin. The potency of orpanoxin agrees with that reported previously for *in vitro* inhibition of the bovine seminal vesicle enzyme (12).

Effects on Gastric Prostaglandins. When the gastric fundi of the pylorus-ligated rats used to assess gastric lesion incidence were assayed for three products of prostaglandin synthetase, namely 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2, a difference in the two chemicals was revealed (Table II). Orpanoxin treatment significantly and dose-dependently suppressed formation of 6-keto-$PGF_{1\alpha}$. At the highest dose of 400 mg/kg, 6-keto-$PGF_{1\alpha}$ concentration was decreased over 90%. F-1067 diminished 6-keto-$PGF_{1\alpha}$ nonsignificantly and to a lesser

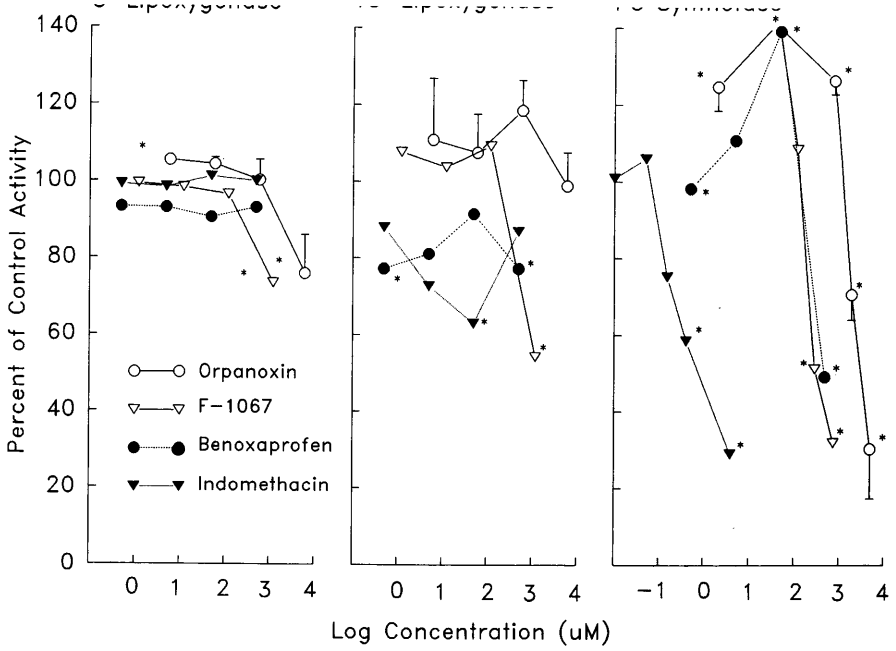


Figure 4. Inhibition of potato tuber 5-lipoxygenase, soybean 15-lipoxygenase, and ram seminal vesicle prostaglandin synthetase *in vitro* by orpanoxin, F-1067, benoxaprofen, and indomethacin. Purified enzyme was exposed to the test article 2 min before addition of arachidonic acid. Plotted are the mean percentage of the corresponding control reaction initial rate of oxygen consumption per minute per milliliter of enzyme, based on five replicate reactions. Standard deviation bars, representative of experimental variance, are shown for the orpanoxin reactions. Asterisks indicate points that are significantly different from the control reaction rate.

Table II. Effects of Oral Orpanoxin and F-1067 on Prostaglandin Levels in Gastric Fundi of Pylorus-Ligated Rats[a]

Treatment	Dose (mg/kg)	Mean ± SE pg/mg wet wt (% of control)		
		6-Keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGF_2
Methylcellulose	10 ml/kg	893 ± 159 (100)	41 ± 13 (100)	92 ± 44 (100)
Orpanoxin	100	385 ± 64[b] (43)	175 ± 53[b] (427)	638 ± 136[b] (693)
	200	280 ± 136[b] (31)	50 ± 26 (122)	375 ± 133 (408)
	400	86 ± 44 (10)	117 ± 44 (285)	334 ± 86[b] (383)
F-1067	100	1139 ± 337 (127)	48 ± 11 (117)	1249 ± 295[b] (1357)
	200	701 ± 93 (78)	59 ± 13 (144)	1739 ± 260[b] (1890)
	400	685 ± 104 (77)	185 ± 59[b] (451)	1733 ± 207[b] (1884)

[a] Prostaglandins 6-keto-$F_{1\alpha}$, $F_{2\alpha}$, and E_2 were extracted with ethyl acetate from aqueous 1-hr incubates of gastric fundi of treated rats, separated by high pressure liquid chromatography, and detected spectrophotometrically.
[b] Significantly different from vehicle group value by *t* test ($P < 0.05$).

extent. Another difference between the two compounds was evident in their effects on PGE_2. Although both chemicals increased this prostaglandin, F-1067 had a far greater effect, elevating levels of PGE_2 dose dependently 13- to 19-fold over levels in vehicle-treated rats. Orpanoxin caused 3- to 6-fold increases. Both compounds tended to increase levels of $PGF_{2\alpha}$, but only the 4-fold increase at 100 mg/kg of orpanoxin and 400 mg/kg of F-1067 was statistically significant.

Discussion

F-1067 and orpanoxin are similar propionic acid NSAID in many respects. Chemically, they differ only in the substitution of a hydroxyl group in place of a hydrogen. They display equal potency as anti-inflammatory and analgesic drugs in animal models. Both compounds lack important lipoxygenase inhibition activity and both compounds are similarly potent as prostaglandin synthetase inhibitors, although less potent than indomethacin. Yet they differ markedly in their acute gastric toxicity in rats. The gastric toxicity of orpanoxin administered to rats for 2 weeks or longer was similar to its acute toxicity; the multidose effect of F-1067 on gastric lesions has not been determined. For this study, however, the compounds differ in toxicity at doses that show equal efficacy and potency in anti-inflammatory and analgesic tests.

The gastric toxicity of NSAID has been ascribed to

depletion, by enzyme inhibition, of gastric mucosal prostaglandins with a protective function, and to a direct damaging action at the mucosa (13). Aspirin and indomethacin, for example, although causing gastric mucosal damage in rats, decreased equally five different gastric prostaglandins (14).

Despite similar pharmacology, chemistry, and *in vitro* enzyme inhibition, orpanoxin and F-1067 did differ significantly in effects on specific gastric mucosal prostaglandins. The more gastrotoxic orpanoxin depleted 6-keto-$PGF_{1\alpha}$ and had less of a stimulatory effect on PGE_2 than did the less toxic F-1067. These differences in effects on prostaglandin production, possibly related to differences in gastric toxicity, may reflect differences in inhibition or stimulation of the enzymes of the arachidonic pathway, or may reflect differences in metabolism and pharmacokinetics. Information comparing the pharmacokinetics of the two compounds in rats is lacking. F-1067-induced increases in PGE_2 approaching 22-fold may be particularly significant, since there is evidence for a protective effect of E prostaglandins (15). The PGE_1 analog misoprostol has gastric acid and pepsin antisecretory action and protects from NSAID-gastric lesions in humans (16). The semisynthetic PGE_2 analog enprostil had similar clinical actions (17). Thus, the ability of F-1067 to augment synthesis of PGE_2 may be construed as evidence in favor of the role of the E prostaglandins in gastroprotection. These results invite further examination of whether gastric safety can be achieved by finding an NSAID with a particular pattern of prostaglandin depletion and augmentation. Dvornik and Lee (18) have provided evidence that etodolac's gastrointestinal safety profile may be related to the same two prostaglandins that are spared/augmented by F-1067. In rats, an anti-inflammatory dose of etodolac did not reduce gastric mucosal PGE_2 or 6-keto-$PGF_{1\alpha}$ as compared with the more toxic naproxen, piroxicam, and aspirin.

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