J. Pharm. Pharmacol. 1999, 51: 331–339
Received June 30, 1998
Accepted December 9, 1998

Inhibition by Leukotriene Inhibitors, and Calcium and Platelet-activating Factor Antagonists, of Acute Gastric and Intestinal Damage in Arthritic Rats and in Cholinomimetic-treated Mice

K. D. RAINSFORD

Division of Biomedical Sciences and Biomedical Research Centre, Sheffield Hallam University, Sheffield, S1 1WB, England, UK and Department of Biomedical Sciences, McMaster University Faculty of Health Sciences, Hamilton, Ontario, L8N 3Z5, Canada

Abstract

The leukotrienes, platelet activating factor and intracellular calcium have been implicated in the development of gastro-intestinal lesions induced by non-steroidal anti-inflammatory drugs (NSAIDs) but the relative significance of these inflammatory mediators in lesion formation has not been established in sensitive and specific models of gastro-intestinal ulceration. In the present study the effects of drugs affecting 5-lipoxygenase activity, the actions of platelet activating factor and intracellular calcium on the development of gastric and intestinal ulceration induced by NSAIDs were investigated in highly sensitive models of ulcerogenicity induced by treatment with either the cholinomimetic, acetyl-β-methyl choline chloride, in mice (gastric mucosal lesions) or adjuvant-induced polyarthritis in rats (gastric and intestinal mucosal lesions) as well as in normal mice (intestinal mucosal lesions).

The 5-lipoxygenase inhibitors, such as MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), given at doses shown to reduce the indomethacin-induced increase in mucosal leukotriene B_4 concentrations were found to partially prevent the development of gastric and intestinal lesion induced by indomethacin and gastric lesions from aspirin, but the same doses of MK-886 did not affect gastric lesions from diclofenac. Pretreatment with these inhibitors at both 3–5 h and 0–0·25 h was required to achieve protection against gastric mucosal damage from indomethacin. Immediate prior administration of platelet activating factor antagonists (e.g. WEB-2086) with the 5-lipoxygenase inhibitors did not affect gastric or intestinal lesions induced by indomethacin. The calcium antagonist, verapamil, was slightly protective against gastric and intestinal lesions induced by indomethacin. Gastric lesions were further prevented by combinations of a single dose of verapamil with a platelet activating factor antagonist but not combined with a 5-lipoxygenase inhibitor; other combinations of verapamil with lipoxygenase inhibitors or platelet-activating factor antagonists being without inhibitory effects on gastric or intestinal lesions compared with the drugs alone.

These results show that 5-lipoxygenase products and intracellular calcium play a major role in acute gastric and intestinal damage by the NSAIDs, but platelet-activating factor has little or no appreciable involvement.

Vascular injury as well as leucocyte adhesion to endothelia, infiltration and activation have now become recognized as important factors in the pathogenesis of gastro-intestinal ulceration induced by various necrotizing agents (Robins 1980; Rainsford 1983, 1986, 1987a,b, 1992; Szabo et al 1985, 1986; Rogers et al 1987; Guth 1987; Pihan et al 1988; Wallace & Granger, 1992; Gyömber et al 1996a,b). Production of lipid mediators of inflammation, principally the leukotrienes, occurs during gastric ulceration in rats induced by ethanol (Peskar et al 1986; Rogers et al 1987; Pihan et al 1988; Wallace 1990). Inhibitors of leukotriene production have variable protective effects on ethanol-induced

Correspondence: K. D. Rainsford, Division of Biomedical Sciences, School of Sciences and Mathematics, Sheffield Hallam University, Pond Street, Sheffield S1 1WB, UK.

gastric ulceration (Wallace & Whittle 1985; Peskar et al 1986; Boughton-Smith & Whittle 1988; Gyömber et al 1996b); this variability is possibly related to the mechanism of action of the drugs and the concentration, dose and timing of ethanol administration. Parenterally-administered peptido-leukotrienes induce gastric vasoconstriction (Whittle et al 1985), increase vascular permeability (Hua et al 1985; Pihan, et al 1988), promote mucosal barrier breakdown (Pendleton & Stavorski 1986), and stimulate the secretion of gastric acid (Schepp et al 1985) and pepsin (Pendleton & Stavorski 1986). Thus, peptido-leukotrienes as well as leucocyte-derived leukotriene B_4 (Wallace & Granger 1992) are prime candidates for mediating gastric mucosal damage.

Platelet activating factor also has ulcerogenic effects and has been proposed to be important in gastric ulcerogenesis from necrotizing agents (Wallace 1990). The inter-relationships between the metabolism of arachidonate and platelet-activating factor (Carlini et al 1985; Hogaboam et al 1992) and the activation of their metabolism by calcium (O'Flaherty 1988) suggests there are important interactions amongst all three of these mediators of potential importance in ulcerogenesis. Calcium antagonists have been shown to prevent ethanol- and indomethacin-induced gastric lesions in normal rats (Ghanayem et al 1987).

In earlier studies some inhibitors of 5-lipoxygenase and leukotriene antagonists were found to partially inhibit gastric mucosal lesions induced by some non-steroidal anti-inflammatory drugs (NSAIDs) in normal rats (Whittle et al 1985) and cholinomimetic-treated mice (Rainsford 1987a) suggesting that vasoconstrictor and leucoattractant leukotrienes could have a role in the development of gastric injury by these drugs. A nonspecific inhibitor of the 5-lipoxygenase pathway, nordihydroguaiaretic acid, which also has cyclo-oxygenase inhibitory and antioxidant activities, has not, however, been found to protect the gastric mucosa of normal rats when given with NSAIDs (Cho & Ogle 1987). An explanation of some of the variation in these results is that some of the 5-lipoxygenase inhibitors available at that time (mid-1980s) were relatively unspecific, their mechanism of action was not well understood, and they lacked the potency or specificity of action of the drugs now available (Gillard & Guindon 1987; Rouzer et al 1990). Moreover, the low sensitivity of the gastrointestinal tract of normal (i.e. non-inflamed) fasted rats employed in some studies is also a major limitation to accurate determination of the effects of inhibitors of lesion formation (Rainsford 1987b, 1992).

Aside from the well-known causative role of NSAIDS in gastroduodenal damage, the small and large intestinal ulceration from some NSAIDs is also a major clinical problem (Bjarnason 1988). The role of the leukotrienes, platelet-activating factor and calcium in the development of NSAID induced intestinal damage does not appear to have been extensively explored. Thus, these mediators or regulators of mucosal functions could be important in the pathogenesis of both gastric and intestinal damage from NSAIDs. Hence, it was decided in the present studies, to examine the effects of some novel potent and selective 5-lipoxygenase inhibitors, peptido-leukotriene and platelet-activating factor antagonists as well as a calcium antagonist given alone or in combination with one another on the development of both gastric and intestinal ulceration induced in a sensitive ulcer model of rodents induced using NSAIDs.

Methods

Gastric ulcer assays

Two types of assay were performed, one in arthritic rats and the other in cholinomimetic-stimulated mice. In both models the number and area or severity of lesions from NSAIDs is markedly increased compared with that in normal fasted, and otherwise untreated animals, although the mechanisms for this increased sensitivity are different (Shriver et al 1977; Rainsford 1978, 1987c). Thus, in the arthritic rats a variety of systemic responses (including defective drug metabolism) and decreased mucosal resistance (e.g. reduced mucus synthesis) appear responsible for the enhanced mucosal sensitivity to NSAIDs in both the stomach and intestine (Rainsford 1987b). In the cholinominetic-treated mouse model, however, the stimulus to produce acid and pepsin from intraperitoneal injection of acetyl-β-methylcholine (bethanechol) chloride is considered to underlie the enhanced ulcerogenic effects of the NSAIDs specifically in the stomach (Rainsford 1987c).

Male Swiss or CFI mice were housed in groups of 3–5 in all wire-mesh cages (to prevent coprophagy) and fasted overnight but allowed free access to water until the start of the experiment whereupon water was withdrawn. An oral dose (in 0·5 mL H_2O) of either the 5-lipoxygenase inhibitors, MK-886 (Rouzer et al 1990) or L-656,224 (Belanger et al 1987) Merck Frosst Institute for Therapeutic Research, Pointe-Claire Dorval, Quebec, Canada) (Figure 1), the PAF antagonists, WEB-2086 or WEB-2170 (Boehringer-Ingelheim, Ingelheim,

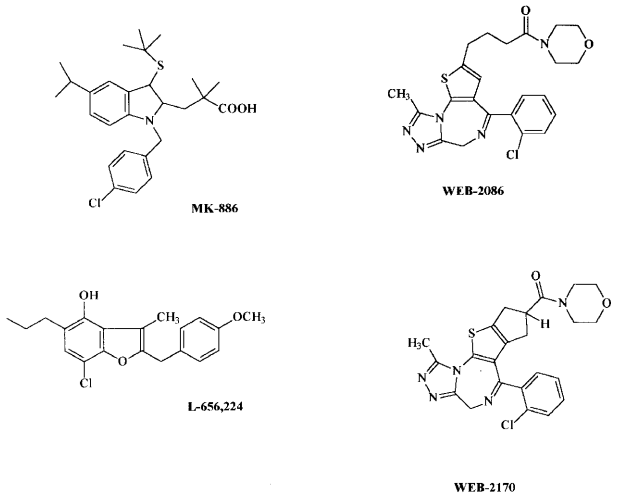

Figure 1. Chemical structures of the 5-lipoxygenase inhibitors, MK-886 and L-656,224, and the platelet-activating factor antagonists, WEB-2086 and WEB-2170.

Germany) (Figure 1; Casals-Stenzel et al 1987; Meade & Heuer 1990), or the calcium antagonist, verapamil (Sigma, St Louis, MO, USA), was given followed (in the case of the 5-lipoxygenase inhibitor-treated group) 0–5 h later by another dose of the inhibitor, or 0·5 mL H_2O, and either immediately or 0·25 h later by either subcutaneous indomethacin ($30\,mg\,kg^{-1}$, prepared as a sodium salt from Na_2HCO_3), subcutaneous diclofenac sodium ($30\,mg\,kg^{-1}$ in saline), or oral aspirin ($150\,mg\,kg^{-1}$ in 1 mL H_2O), with intraperitoneal acetyl-β-methyl choline chloride (Sigma, St. Louis, MO, $5\,mg\,kg^{-1}$ in 0·5 mL saline). The orally-administered NSAIDs were prepared as aqueous suspensions finely homogenized (Rainsford 1978) immediately before administration. The animals were killed by CO_2 asphyxiation either 2 h (aspirin) or 3 h (indomethacin, diclofenac) later (these being times of peak ulcerogenicity). The stomach was removed and contents washed free with saline. The number and area of haemorrhagic mucosal lesions was then determined as described previously (Rainsford 1987c).

Induction of arthritis in rats

Arthritis was induced in female SPF Lewis rats, (Charles River, Canada; 120–160 g starting body weight) by a subcutaneous injection of 0·5 mg heat-killed *Mycobacterium butyricum* (Difco) in 0·05 mL squalane (Sigma) in the dorsal region of the base of the tail. The animals were maintained under SPF conditions and their physical state and body weights were monitored at least twice weekly until front and hind limb joint and subplantar swelling was fully manifest indicating the disease was developed (about 14–16 days post-injection). Those animals with swelling in at least both hind limbs were selected, then housed singularly in all-wire mesh cages to prevent coprophagy, and fasted overnight with free access to water. The next day they were treated at times stated in the Results with 1 mL oral doses of either one of the 5-lipoxygenase inhibitors, calcium, or platelet-activation factor antagonists (as above), or 1 mL H_2O then dosed again at stated times with a single subcutaneous dose of aspirin (1 mL, $150\,mg\,kg^{-1}$ in water), or indomethacin (1 mL, $30\,mg\,kg^{-1}$ in sterile saline, freshly prepared as the Na_2HCO_3 salt). Control animals were given H_2O orally or saline subcutaneously alone. Groups of 5 animals each were killed (as above) at either 2 h (aspirin) or 4 h (indomethacin) later, the stomach dissected and contents washed free with saline. The number and area of haemorrhagic mucosal lesions was determined as previously described (Rainsford 1987c).

Intestinal lesion assays

Male Sprague-Dawley, male Long-Evans or female SPF Lewis rats, in which the arthritis was induced as above (150–250 g body weight; Charles River, Canada) or male Swiss mice (30–50 g body weight; Charles River, Canada) were randomly allocated to treatment groups and given a single subcutaneous dose of indomethacin ($10\,mg\,kg^{-1}$ (rats) or $15\,mg\,kg^{-1}$ (mice)). The 5-lipoxygenase inhibitors, the platelet-activating factor antagonists or the calcium antagonist, verapamil, were given either up to 5 h before the dose of indomethacin or concurrently. At termination, the entire gastrointestinal tract was removed, opened and the lumenal contents washed free with water. The number of small intestinal lesions and their area were measured with the aid of a visual and hand magnifying lens (7×) to which the graticule was inserted (Verdick Scale Lupe, London, UK).

Mucosal leukotriene B_4 assay in rats

Groups of 5 fasted (20 h) male Wistar rats (180–220 g body weight) were dosed with either: $30\,mg\,kg^{-1}$ indomethacin in 1 mL saline subcutaneously and 1 mL H_2O orally; $30\,mg\,kg^{-1}$ indomethacin in 1 mL saline subcutaneously and $100\,mg\,kg^{-1}$ MK-886 in 1 mL H_2O orally; or 2 mL saline subcutaneously and 1 mL H_2O orally. The animals were killed 1 h later by CO_2 asphyxiation, fundic mucosal rapidly excised ($\leqslant 40$ s) washed and freeze clamped between liquid nitrogen cooled tongs. The eicosanoid fraction was extracted, purified and subject to HPLC assay for leukotriene B_4 as described previously (Rainsford et al 1995).

Approval for these studies was given by the McMaster Animals Research Ethics Board based on the principles of the Canadian Council for Animal Care.

Results

Gastric mucosal damage

In cholinomimetic-treated mice, oral administration of MK-886 at both 4·0 and 0·25 h prior to indomethacin (30 mg kg^{-1}, s.c.) or aspirin (150 mg kg^{-1}, p.o.) significantly ($P < 0·05$; Mann Whitney *U*-test) reduced the development of gastric mucosal lesions from these NSAIDs in a dose-related manner but did not affect the injury from diclofenac (Table 1). However, the protective effect of MK-886 was not evident when given as a single dose either 0·25 h (Table 2) or 4·0 h (data not shown) previously. MK-886 or L-656,224 given orally at both 4·0 h and 0·25 h previously reduced the gastric lesions from 15 mg kg^{-1} indomethacin but not when the inhibitors were given at only one of these times (data not shown).

In arthritic rats MK-886 or L-656,224 100 mg kg^{-1} given at both −3·0 or −5·0 h as well as at 0 h significantly reduced the gastric mucosal lesions from indomethacin (30 mg kg^{-1}, s.c.) in a dose-related manner while lower doses of these 5-lipoxygenase inhibitors were not effective (Table 3). However, administration of the single dose of these inhibitors at −3·0 or 0 h, respectively did not reduce gastric lesions induced by indomethacin (Table 5). The same effect of MK-886 in protecting the gastric mucosa against lesions induced by indomethacin or aspirin was achieved when this 5-lipoxygenase inhibitor was given at −5·0 or −3·0 h and at −0·25 h or 0 h as observed above when the inhibitors were given at −4·0 and 0·25 h in both mice and arthritic rats (data not shown). Likewise, doubling the dose of the 5-lipoxygenase inhibitors given as a single dose at any of these times did not

Table 3. Effects of oral treatment with the 5-lipoxygenase inhibitor, MK-886, on the development of gastric lesions induced by indomethacin (30 mg kg^{-1}, s.c.) given for 3 h to arthritic rats. MK-886 was given 5 and 0 h before indomethacin.

Dose MK-886 (mg kg^{-1})	Number of lesions	Area of lesions (mm^2)
0	38·9 ± 7·1	155·6 ± 28·3
10	49·5 ± 10·6	159·1 ± 19·4
50	28·3 ± 12·4	91·9 ± 12·4*
100	1·8 ± 0·0*	1·8 ± 0·0*

Means ± s.e.m. n = 5–6. *$P < 0·05$ compared with control (zero dose) (Mann-Whitney *U*-test).

Table 1. Effects of prior (− 4·0 and −0.25 h) oral administration of MK-886 on gastric lesions induced by NSAIDs in bethanechol-treated mice.

NSAID treatment	Dose of MK-886 (mg kg^{-1})	Number of lesions	Area of lesions (mm^2)
Aspirin (150 mg kg^{-1}, p.o.)	0	18·4 ± 0·0	58·7 ± 6·0
	10	21·5 ± 2·3*	9·0 ± 0·0*
	30	3·8 ± 3·8*	13·5 ± 0·0*
Diclofenac Na (30 mg kg^{-1}, s.c.)	0	8·2 ± 1·5	14·8 ± 3·7
	5	11·9 ± 4·5	21·6 ± 8·9
	30	7·4 ± 2·6	7·4 ± 2·6
Indomethacin (30 mg kg^{-1}, s.c.)	0	22·3 ± 3·0	35·7 ± 6·7
	5	8·9 ± 4·5	21·6 ± 9·0
	10	20·9 ± 2·2	28·7 ± 5·2
	30	1·5 ± 0·0*	1·4 ± 0·0*

Means ± s.e.m. n = 5. *$P < 0·05$ compared with controls (zero dose). (Mann-Whitney *U*-test).

Table 2. Effects of a single dose of MK-886 (100 mg kg^{-1}) given 0·25 h before NSAID treatment on gastric lesions induced by NSAIDs in bethanechol-treated mice.

NSAID treatment		Number of lesions	Area of lesions (mm^2)
Aspirin (150 mg kg^{-1}, p.o.)	Control	18·2 ± 0·5	58·3 ± 5·2
	Dosed (2 h)	16·1 ± 4·7	43·8 ± 10·9
Diclofenac Na (30 mg kg^{-1}, s.c.)	Control	7·8 ± 1·0	10·4 ± 4·2
	Dosed (3 h)	9·3 ± 2·0	8·9 ± 7·3
Indomethacin (30 mg kg^{-1}, s.c.)	Control	9·4 ± 2·6	37·5 ± 2·6
	Dosed (3 h)	20·8 ± 4·2	74·5 ± 4·7

Means ± s.d. n = 4 or 5. No statistically significant differences were observed (Mann-Whitney U-test, $P > 0·05$) comparing control with MK-886 treated groups.

Table 4. Effects of oral treatment with the 5-lipoxygenase inhibitors, MK-886 and L-656,224 on the development of gastric lesions induced by indomethacin (30 mg kg^{-1}, p.o.) given for 3 h to arthritic rats. MK-866 or L-656,224 was given 3 and 0 h before indomethacin.

NSAID treatment	Dose (mg kg^{-1})	Number of lesions	Area of lesions (mm^2)
Control		38·9 ± 19·4	155·5 ± 27·8
MK-886	10	43·7 ± 1·4	138·9 ± 5·5
	50	30·5 ± 9·7	170·8 ± 22·2
	100	0·0 ± 0·0*	0·0 ± 0·0*
L-656,224	50	27·8 ± 9·7	108·3 ± 26·4*
	100	0·0 ± 0·0*	0·0 ± 0·0*

Means ± s.e.m. n = 5–6. *$P < 0·05$ compared with control (zero dose) (Mann-Whitney *U*-test).

Table 5. Effects of single oral doses of MK-886 given 3 h before indomethacin (30 mg kg^{-1}, s.c.) on the development of gastric lesions induced in arthritic rats.

Dose MK-886 (mg kg^{-1})	Number of lesions	Area of lesions (mm^2)
0	39·2 ± 7·1	157·1 ± 28·6
10	28·6 ± 21·4	121·4 ± 89·2
50	46·4 ± 8·9	242·8 ± 78·6
100	28·6 ± 7·1	100·0 ± 53·6

Means ± s.e.m. n = 4–5. No statistically significant effects were evident with any of the treatments (Mann-Whitney U-test, $P > 0·05$).

significantly reduce the gastric damage from indomethacin (Table 5).

The concentration of leukotriene B_4 in the fundic mucosa 1 h following treatment with subcutaneous indomethacin (30 mg kg^{-1}) was 19·5 ± 6·3 ng (mg protein)$^{-1}$ and was significantly increased (Student's *t*-test, n = 5 per group, $P \leqslant 0·05$) 4·5-fold compared with that in control mucosa (4·34 ± 2·8 ng mg^{-1}). The combined treatment with MK-886 100 mg kg^{-1} with indomethacin 30 mg kg^{-1}, resulted in leukotriene B_4 concentrations of 6·8 ± 1·3 ng mg^{-1}, this being a statistically significant reduction (Student's *t*-test, $P < 0·5$) of 77% compared with indomethacin-treated animals but not significant compared with control values.

Oral administration of verapamil 50 mg kg^{-1} significantly reduced indomethacin-induced lesions in arthritic rats (Table 6). A single oral dose of L-656,224 with verapamil did not further reduce the lesions from indomethacin (data not shown). However, oral administration of the platelet activating factor antagonist WEB-2170 (50 mg kg^{-1}), while not affecting lesions from indomethacin given alone did reduce the lesions when given in combination with verapamil (50 mg kg^{-1}, p.o.) (Table 6). Neither combination of a single dose of either L-656,224 or

Table 6. Effects of single oral doses of the calcium antagonist, verapamil (50 mg kg^{-1}), the 5-lipoxygenase inhibitor, L-656,224 (50 mg kg^{-1}), the platelet-activating factor antagonist, WEB-2170 (50 mg kg^{-1}), or the combination of verapamil (50 mg kg^{-1}) and WEB-2170 (50 mg kg^{-1}) on the development of gastric lesions 3 h after indomethacin (30 mg kg^{-1} s.c.) given simultaneously with the inhibitors to arthritic rats.

Treatment	Area of lesions (mm^2)
Control	201·9 ± 15·0
Verapamil	120·3 ± 30·1*
L-656,224	242·8 ± 8·6
WEB-2170	174·0 ± 23·6
Verapamil + WEB-2170	14·0 ± 11·8*

Mean ± s.e.m. *$P < 0·05$ compared with control (indomethacin alone) (Mann-Whitney *U*-test).

MK-886 (50 mg kg^{-1}, p.o.) with the PAF antagonists, WEB-2086 or WEB-2170, reduced the number and severity of gastric lesions in either mice or rats from aspirin (150 mg kg^{-1}, p.o.) or indomethacin (30 mg kg^{-1}, s.c.) compared with these NSAIDs alone (data not shown).

Small intestinal ulcers

Prior (−5·0 h) and concurrent oral treatment of mice with the 5-lipoxygenase inhibitors, MK-886 or L-656,224, produced a dose-related reduction in small intestinal lesions induced by 15 mg kg^{-1} indomethacin give subcutaneously after 20 h treatment in mice (Table 7). The dose of these inhibitors required for statistically significant reduction (Mann Whitney *U*-test) in lesion areas was $\geqslant$100 mg kg^{-1} for both MK-886 and L-656,224; higher doses did not lead to any further reduction in lesions. Likewise prior (−3·0 h) and concurrent treatment with MK-886 markedly reduced the numbers of lesions from indomethacin when this inhibitor was dosed orally at $\geqslant$25 mg kg^{-1} and the area of intestinal lesions with $\geqslant$25 mg kg^{-1} MK-886 orally in arthritic rats (Table 8). However, even at the highest doses there was no further reduction in lesions. Prior (−5·0 h or 3 h) and concurrent treatment with 100 mg kg^{-1} MK-886 also prevented small intestinal damage induced by indomethacin in normal (i.e. non-arthritic, Sprague-Dawley or Long-Evans male rats, data not shown). Single oral doses (at 0 h or at 5·0 h) of either MK-886 or L-656,224 (50 mg kg^{-1}) did not produce any significant reduction in the number or areas of mucosal lesions in normal or arthritic rats by 10 mg kg^{-1} oral doses of indomethacin or when this NSAID was given subcutaneously (data not shown). The mucosal lesions from indomethacin were not totally abolished upon treatment with various doses at various times with the 5-lipoxygenase inhibitors.

Table 7. Effects of oral administration of MK-886 or L-656,224 given 5·0 and 0 h before indomethacin (15 mg kg^{-1}, s.c.) on the development of intestinal lesions in the small intestine of mice 20 h after the NSAID.

Treatment	Dose (mg kg^{-1})	Number of lesions	Area of lesions (mm^2)
MK-886	0	12·8 ± 4·5	51·6 ± 8·9
	10	8·1 ± 2·3	23·0 ± 7·5
	100	7·7 ± 2·1	7·5 ± 2·3*
	200	8·2 ± 2·9	7·0 ± 2·8*
L-656,224	0	19·9 ± 4·7	33·8 ± 10·8
	10	13·3 ± 2·7	30·9 ± 7·5
	100	7·7 ± 1·4*	14·5 ± 8·4*
	200	6·1 ± 2·1*	7·7 ± 2·8*

Means ± s.e.m. n = 5–6. *$P < 0·05$ compared with control (zero dose) (Mann-Whitney *U*-test).

Table 8. Effects of oral administration of MK-886 given 3·0 and 0 h before indomethacin (10 mg kg^{-1}, s.c.) on the development of lesions in the small intestine of arthritic rats 20 h after dosing with the NSAID.

Dose (mg kg^{-1})	Number of lesions	Area of lesions (mm^2)
0	54·9 ± 8·2	167·6 ± 53·6
10	33·0 ± 1·4	140·1 ± 5·5
25	19·2 ± 9·6*	38·5 ± 20·6*
100	15·1 ± 11·0*	22·0 ± 33·0*

Means ± s.e.m. n = 5–6. *$P < 0·05$ compared with control (zero dose) (Mann-Whitney *U*-test).

Table 9. Effects of single oral doses (50 mg kg^{-1}) of the platelet-activating antagonist, WEB-2086, with or without the 5-lipoxygenase inhibitor, L-656,224, on the mucosal lesions induced in the small intestine by indomethacin (10 mg kg^{-1}).

Treatment	Number of lesions	Area of lesions (mm^2)
Control	75·5 ± 15·1	207·1 ± 42·9
WEB-2086 (50 mg kg^{-1})	65·6 ± 10·1	176·8 ± 40·4
L-656,224 (50 mg kg^{-1})	65·6 ± 5·0	222·2 ± 30·3
WEB-2086 + L-656,224	98·5 ± 10·1	358·6 ± 50·5

Means ± s.e.m. n = 5. No statistically significant effects were evident with any of the treatments (Mann-Whitney *U*-test, $P > 0·05$).

The platelet activating factor antagonist, WEB-2086, (50 mg kg^{-1}) given orally alone, failed to affect the development of intestinal ulcers induced by indomethacin (10 mg kg^{-1}, p.o., Table 9). Co-administration of this antagonist with the 5-lipoxygenase inhibitor, L-656,224 at 0 h was without any effect. However, combination of the platelet-activating factor antagonist, WEB-2170, with the calcium antagonist, verapamil, both given at 0 h markedly reduced the intestinal damage from indomethacin (10 mg kg^{-1}, s.c.), this effect being greater than that observed with verapamil alone (Table 10). Co-administration of verapamil with MK-886 (50 mg kg^{-1} each, p.o.) did not affect lesions from indomethacin (10 mg kg^{-1}, s.c.) compared with the effects of the former two drugs alone (data not shown).

Table 10. Effects of oral administration (50 mg kg^{-1}) of the platelet-activating factor antagonist, WEB-2170, with or without the calcium antagonist, verapamil, on the mucosal lesions induced in the small intestine by indomethacin (10 mg kg^{-1}, p.o.) in arthritic rats 20 h after dosing with the NSAID.

Treatment	Number of lesions	Area of lesions (mm^2)
Control	50·0 ± 3·33	200·0 ± 15·0
WEB-2170 (50 mg kg^{-1})	36·7 ± 3·33	170·0 ± 23·3
Verapamil (50 mg kg^{-1})	45·0 ± 3·33	116·7 ± 30·0
WEB-2170 + verapamil	3·33 ± 3·33*	13·3 ± 10·0*

Means ± s.e.m. n = 5. *$P < 0·05$ compared with control (Mann-Whitney *U*-test).

Discussion

The results show that the pre-treatment together with a second dose at 0 h of 5-lipoxygenase inhibitors, MK-886 and L-656,224, produced a dose-related reduction in both gastric and small intestinal mucosal lesions induced by indomethacin or gastric lesions from aspirin in standard, highly sensitive models of NSAID-induced acute gastrointestinal mucosal injury in rodents. It appears that the protection by these inhibitors is only evident when they are given 3·0 to 5·0 h before as well as at the same time of dosing with the NSAID. One possible reason for this might be that sufficient concentrations of these inhibitors may be required in mucosal cells for inhibitory effects of these 5-lipoxygenase inhibitors to be effective. However, in the present studies it was found that leukotriene B_4 concentrations were markedly increased in the fundic mucosa of rats which received indomethacin compared with controls thus supporting the concept that indomethacin diverts arachidonic acid through the 5-lipoxygenase pathway as a consequence of cyclooxygenase inhibition (Rainsford 1987a), while MK-886 reduced this effect of indomethacin 1 h after oral administration of 100 mg kg^{-1} of this inhibitor. It appears that by 1 h following a single dose of MK-886 it has achieved its pharmacological effects in the gastric mucosa. In other studies oral doses of MK-886 were found to achieve pharmacological effects. Thus, a single oral dose of 10 mg kg^{-1} MK-886 was found to reduce the

plasma extravasation in passive anaphylaxis in rats when this drug was given 2 h before challenge (Fernandez-Gallardo et al 1992). A single dose of 30 mg kg^{-1} MK-886 for 5 h significantly reduced *Pseudomonas* lethality in mice (Vogels et al 1994). Similarly, an oral dose of 10 mg kg^{-1} MK-886 for 4 h reduced carrageenan raw oedema in rats (Braga da Motta et al 1994) and antigen-induced leukotriene-dependent bronchoconstriction at 2 to 4 h in guinea pigs (Howell et al 1994). In humans single oral doses of 250 to 750 mg MK-886 (equivalent to approximately 3·6–10 mg kg^{-1} in a 70-kg individual) have been found to reduce significantly leukotriene B_4 production in whole blood ex-vivo at 1 to 12 h after administration (Depre et al 1993), while the two lower doses block allergen-induced airway responses in atopic asthmatics at 1 to 7 h post-challenge (Friedman et al 1993). Thus, the pharmacological effects of MK-886 in inhibiting leukotriene B_4 production are convincingly established in the current studies and supported by other published studies in standard inflammatory models. Consequently, despite high doses of the drugs, clearly in excess of those required for inhibition of 5-lipoxygenase activity (Rouzer et al 1990; Depre et al 1993), overall total abolition of gastric intestinal lesions is not evident in all the experiments in mice or rats. It would appear, therefore, that enhanced 5-lipoxygenase activity is only a limited component of NSAID-induced gastrointestinal damage.

The lack of effects of MK-886 on diclofenac-induced gastric lesions may be related to the reported inhibitory effects of this NSAID on production of leukotrienes (Ku et al 1985, 1986; Liauw et al 1985), the added effects of no inhibition by the administration of MK-886 being apparently of no benefit.

Verapamil reduced the gastric lesions from indomethacin in arthritic rats and this is in accord with previous results in normal (i.e. non-inflamed) animals (Ghanayem et al 1987). The platelet-activating factor antagonists, WEB-2086 and WEB-2170, given alone did not affect either the gastric mucosal damage induced by indomethacin or aspirin, or the indomethacin-induced small intestinal damage. The doses of these PAF antagonists which were employed in the present studies are in excess of those reported to inhibit anaphylaxis in rats (Fernandez-Gallardo et al 1992) and other immuno-inflammatory reactions in rodents (Meade & Heuer 1990; Vogels et al 1994). The lack of effects of these specific and potent antagonists in gastric mucosal damage is in agreement with earlier results with the weaker platelet-activating factor antagonist, kadsurenone, which failed to affect aspirin-induced gastric damage in rats (Rainsford 1986) and the other platelet-activating factor antagonists, BN-52021 and BN-52063 in less sensitive ulcer models (Braquet et al 1987). Combination of platelet activating factor antagonists with a 5-lipoxygenase inhibitor did not confer any protection in the stomach compared with the latter two classes of drugs alone. However, combination of the PAF antagonists with the calcium antagonist, verapamil, did result in further reduction of the intestinal ulceration from indomethacin, but did not enhance the effects of the 5-lipoxygenase inhibitor, MK-886. These results suggest that platelet activating factor does not have a role in acute gastric mucosal damage from NSAIDs but does have a limited role in combination with alterations in intracellular calcium in the development of intestinal damage from indomethacin.

While calcium antagonists have been shown to reduce indomethacin-induced gastric mucosal lesions (Ghanayem et al 1987) no studies appear to have been reported on the effects of calcium antagonists on intestinal damage from NSAIDs. Since indomethacin-induced intestinal damage is partly reduced by verapamil alone but not when given with a 5-lipoxygenase inhibitor or platelet-activating factor antagonist, this indicates that alterations in intracellular calcium are only partly responsible for the development of this intestinal pathology.

NSAIDs have been shown to inhibit the smooth muscle contractile responses to exogenous calcium in the rat mesenteric vasculature (Stanton et al 1986). The metal ion chelator, ethylenediaminetetra-acetate (EDTA), which would be expected to chelate calcium has been shown to enhance the inward permeability of the rabbit gastric mucosa to hydrogen ions even though it did not affect the structural integrity of the mucosa (Chung et al 1970). Calcium ions are clearly important for maintenance of the surface permeability barrier and of the vascular constrictor responses. Alterations in calcium concentrations could, therefore, be important in the development of mucosal lesions in the gastrointestinal tract.

These results emphasize the importance of leukotrienes and other 5-lipoxygenase products together with calcium in the gastro-intestinal ulcerogenic actions of NSAIDs as well as the maintenance of mucosal integrity. The role of platelet-activating factor appears of less significance. The effects of the various inhibitors have been discriminated in more stringent ulcer models than have been employed previously thus making clear the relative roles of the leukotrienes, calcium and platelet activating factor as mediators of mucosal injury from NSAIDs.

Acknowledgements

The author thanks the Merck-Frosst Institute for Therapeutic Research (Pointe Claire-Dorval, Quebec, Canada) and Boehringer-Ingelheim, (Ingelheim, Germany and Burlington, Ontario, Canada) for generous donations of drugs used in these studies. We also thank Mr. S. Debski and Mrs. Veronica Rainsford-Koechli for valuable technical assistance, and Drs A.W. Ford-Hutchinson and J.W. Gillard, Merck Frosst Institute for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada, for advice and helpful discussions.

References

Belanger, P., Maycock, A., Guidon, Y., Bach, T., Dollob, A. L., Dufresne, C., Ford-Hutchinson, A. W., Gale, P. H., Hopple, S., Lau, C. K., Letts, L. G., Luell, S., McFarlane, C. A., MacIntyre, E., Meurer, R., Miller, D. K., Piechuta, H., Riendeau, D., Rokach , J., Rouzer, C., Scheigets, J. (1987) L-656,224 (7-chloro-2-[4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol): a novel, selective orally active 5-lipoxygenase inhibitor. Can. J. Physiol. Pharmacol. 65: 2441–2448

Bjarnason, I. (1988) Non-steroidal anti-inflammatory drug-induced small intestinal inflammation in man. In: Pounder R, (ed.) Recent Advances in Gastroenterology. Churchill-Livingstone, Edinburgh, pp 23–46

Boughton-Smith, N. K., Whittle, B. J. R. (1988) Failure of the inhibition of rat gastric mucosal 5-lipoxygenase by novel acetohydroxamic acids to prevent ethanol-induced damage. Br. J. Pharmacol. 95: 155–162

Braga da Motta, J. I., Cunha, F. Q., Vargaftig, B. B., Ferriera, S. H. (1994) Drug modulation of antigen-induced paw oedema in guinea-pigs: effects of lipopolysaccharide, tumour necrosis factor and leucocyte depletion. Br. J. Pharmacol. 112: 111–116

Braquet, P., Etienne, A., Mencia-Huerta, J. M., Clostre, F. (1987) Effect of the specific platelet activating factor antagonists, BN 52021 and BN 52063, on various experimental gastrointestinal ulcerations. Eur. J. Pharmacol. 150: 269–276

Carlini, C. R., Guimarães, J. A., Ribeiro, J. M. C. (1985) Platelet release reaction and aggregation induced by canatoxin, a convulsant protein: evidence for the involvement of the platelet lipoxygenase pathway. Br. J. Pharmacol. 84: 551–560

Casals-Stenzel, J., Muacevic, G., Weber, K.-H. (1987) Pharmacological actions of WEB 2086, a new specific antagonist of platelet activating factor. J. Pharmacol. Exp. Ther. 241: 974–981

Cho, C. H., Ogle, C. W. (1987) Potentiation of indomethacin-induced gastric ulcers in rats by nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase. Asia Pacific J. Pharmacol. 2: 49–52

Chung, R. S. K., Sum, P. T., Goldman, A., Silen, W. (1970) Effects of chelation of calcium on the gastric mucosal barrier. Gastroenterology 59: 200–207

Depre, M., Friedman, B., Tanaka, W., Van Hecken, A., Buntinx, A., DeSchepper, P. J. (1993) Biochemical activity, pharmacokinetics, and tolerability of MK-886, a leukotriene biosynthesis inhibitor; in humans. Clin. Pharmacol. Ther. 53: 602–607

Fernandez-Gallardo, S., Gijon, M. A., Garcia, C., Furio, V., Liu, F.-T., Crespo, M. S. (1992) The role of platelet-activating factor and peptidoleukotrienes in the vascular changes of rat passive anaphylaxis. Br. J. Pharmacol. 110: 119–105

Friedman, B. S., Bel, E. H., Buntinx, A., Tanaka, W., Han, Y.-H. R., Shingo, S., Spector, R., Sterk, P. (1993) Oral leukotriene inhibitor (MK-886) blocks allergen-induced airway responses. Am. Rev. Resp. Dis. 147: 839–844

Ghanayem, B. I., Matthews, H. B., Maronpot, R. R. (1987) Calcium channel blockers protect against ethanol- and indomethacin-induced gastric lesions in rats. Gastroenterology 92: 106–111

Gillard, J. W., Guindon, Y. (1987) The leukotrienes: prospects for therapy against a unique family of pathophysiological mediators. Drugs of the Future 12: 453–474

Guth, P. H. (1987) Vascular factors in gastric mucosal injury. In: Szabo, S., Pfeiffer C. J. (eds) Ulcer Disease: New Aspects of Pathogenesis and Pharmacology, CRC Press, Boca Raton, FL. pp 125–134

Gyömber, E., Vattay, P., Szabo, S., Rainsford, K. D. (1996a) Role of early vascular damage in the pathogenesis of gastric mucosal haemorrhagic lesions induced by indomethacin in rats. Int. J. Exp. Path. 77: 1–6

Gyömber, E., Vattay, P., Szabo, S., Rainsford, K. D. (1996b) Effect of lipoxygenase inhibitors and leukotriene antagonists on acute and chronic gastric mucosal haemorrhagic lesions in ulcer models in the rat. J. Gastroenterol. Hepatol. 11: 922–927

Hogaboam, C. M., Donigi-Gale, D., Shoupe, T. S., Bissonette, E. Y., Befus, A. D., Wallace, J. L. (1992) Platelet activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds. Br. J. Pharmacol. 105: 87–92

Howell, R. E., Sickels, B. D., Woeppel, S. L., Jenkins, L. P., Rubin, E. B., Weichman, B. M. (1994) Leukotrienes mediate antigen-induced airway hyper-reactivity in guinea pigs. J. Pharmacol. Exp. Ther. 268: 353–358

Hua, H. Y., Dahlen, S. E., Lundberg, J. M., Hammerström, S., Hedqvist, P. (1985) Leukotrienes C_4, D_4 and E_4 cause widespread and extensive plasma extravasation in the guinea pig. Naunyn-Schmiedeberg's Arch. Pharmacol. 330: 136–141

Ku, E. C., Lee, W., Kothari, H. V., Kimble, E. F., Liauw, L. (1985) The effects of diclofenac sodium on arachidonic acid metabolism. Semin. Arth. Rheum. 15 (Suppl. 1): 36–41

Ku, E. C., Lee, W., Kothari, H. V., Scholer, D. W. (1986) Effect of diclofenac sodium on the arachidonic acid cascade. Am. J. Med. 80 (Suppl. 4B): 18–23

Liauw, L., Waiter, S., Lee, L., Ku, E. (1985) Effects of diclofenac on synovial eicosanoid product formation in arthritic patients. J. Clin. Pharmacol. 25: 456

Meade, C. J., Heuer, H. O. (1990) Hetrazepines as PAF antagonists. In: O'Flaherty J. T., Ramwell P. W. (eds) Platelet-Activating Antagonists. New Developments for Clinical Application. Portfolio Publishing pp 47–80

O'Flaherty, J. T. (1988) Biochemical interactions of platelet-activating factor with arachidonic acid. In: Curtis-Prior P. B. (ed.) Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids, Churchill-Livingstone, Edinburgh, pp 663–670

Pendleton, R. G., Stavorski , J. R. (1986) Evidence for differing leukotriene receptors in gastric mucosa. Eur. J. Pharmacol. 125: 297–299

Peskar, B. M., Lange, K., Hoppe, V., Peskar, B. A. (1986) Ethanol stimulates formation of leukotriene C_4 in the rat gastric mucosa. Prostaglandins 31: 283–293

Pihan, G., Rogers, C., Szabo, S. (1988) Vascular injury in acute gastric mucosal damage. Mediatory role of leukotrienes. Dig. Dis. Sci. 33: 625–632

Rainsford, K. D. (1978) The role of aspirin in gastric ulceration. Some factors involved in the development of gastric mucosal damage induced by aspirin in rats exposed to various stress conditions. Am. J. Dig. Dis. 23: 521–530

Rainsford, K. D. (1983) Microvascular injury during gastric mucosal damage by anti-inflammatory drugs in pigs and rats. Agents Actions 13: 457–460

Rainsford, K. D. (1986) Relative roles of leukotrienes and platelet activating factor in experimentally induced gastric ulceration. Pharmacol. Res. Commun. 18 (Suppl.): 209–215

Rainsford, K. D. (1987a) The effects of 5-lipoxygenase inhibitors and leukotriene antagonists on the development of gastric lesions induced by non-steroidal anti-inflammatory drugs in mice. Agents Actions 21: 316–319

Rainsford, K. D. (1987b) Mechanisms of gastric contrasted with intestinal damage by non-steroidal anti-inflammatory analgesic drugs. In: Rainsford, K. D., Velo, G. P. (eds) Side-effects of Anti-inflammatory Drugs, Volume 2. MTP Press, Lancaster, pp 3–26

Rainsford, K. D. (1987c) Gastric ulcerogenicity of non-steroidal anti-inflammatory drugs in mice with mucosa sensitized by cholinomimetic treatment. J. Pharm. Pharmacol. 39: 669–672

Rainsford, K. D. (1992) Mechanisms of gastrointestinal ulceration from non-steroidal anti-inflammatory drugs: a basis for use and development of protective agents. In: Rainsford K.D., and Velo G. P. (eds) Side Effects of Anti-inflammatory Drugs-3. Kluwer Academic Publishers, Lancaster, pp 97–114

Rainsford, K. D., Perkins, W. E., Stetsko, P. I. (1995) Chronic effects of misoprostol in combination with the NSAID, diclofenac, on the gastro-intestinal tract of pigs. Relation to diarrheagenic activity, leukocyte infiltration and mucosal leukotrienes. Dig. Dis. Sci. 40: 1435–1444

Robins, P. G. (1980) Ultrastructural observations on the pathogenesis of aspirin-induced gastric lesions. Br. J. Exp. Path. 61: 497–504

Rogers, C., Pihan, G., Raza, A., Szabo, S. (1987) Possible participation of leukotrienes (LT) in the pathogenesis of gastric mucosal lesions and vascular injury induced by ethanol, HCl, NaOH, or aspirin (ASA) in the rat. Effect of eicosapentaenoic acid (EPA) or a lipoxygenase inhibitor L-651,392. Gastroenterology 92: 1808

Rouzer, C. A., Ford-Hutchinson, A. W., Morton, H. E., Gillard, J. W. (1990) MK-886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. J. Biol. Chem. 265: 1436–1442

Schepp, W., Kath, D., Clatten, M. (1985) Leukotrienes C_4 and D_4 potentiate acid production by isolated rat parietal cells. Gastroenterology 97: 1420–1429

Shriver, D. A., Dove, P. A., White, C. B., Sandor, A., Rosenthale, M. E. (1977) A profile of the gastrointestinal toxicity of aspirin, indomethacin, oxaprozin, phenylbutazone and fentiazic in arthritic and normal rats. Toxicol. Appl. Pharmacol. 42: 75–83

Stanton, B. J., Couper, I. M., Burcher, E. (1986) The activity of non-steroidal anti-inflammatory drugs in the rat on mesenteric vasculature. J. Pharm. Pharmacol. 38: 674–678

Szabo, S., Trier, J. S., Brown, A., Schnoor, J. (1985) Early vascular injury and increased vascular permeability in gastric mucosal injury caused by ethanol in the rat. Gastroenterology 88: 228–236

Szabo, S., Pihan, G., Trier, J. S. (1986) Alterations in blood vessels during gastric injury and protection. Scand. J. Gastroenterol. 21 (Suppl.725): 92–96

Vogels, M. T. E., Hermsen, C. C., Huys, H. L. P. G., Eling, W. M. C., van der Meer, J. W. M. (1994) Roles of tumour necrosis factor alpha, granulocyte-macrophage colony-stimulating factor and arachidonic acid metabolites in interleukin-1-induced resistance to infection in neutropenic mice. Infect. Immun. 62: 2065–2070

Wallace, J. L. (1990) Lipid mediators of inflammation in gastric ulcer. Am. J. Physiol. 258: G1–G11

Wallace, J. L., Granger, D. N. (1992) Pathogenesis of NSAID gastropathy: are neutrophils the culprits Trends Pharmacol. Sci. 13: 129–131

Wallace, J. L., Whittle, B. J. R. (1985) Role of prostanoids in the protective actions of BW 755C on the gastric mucosa. Eur. J. Pharmacol. 115: 45–51

Whittle, B. J. R,. Oren-Wolman, N., Guth, P. H. (1985) Gastric vasoconstrictor actions of leukotrienes C_4, $PGF_{2\alpha}$ and thromboxane mimetic U-46619 on rat submucosal microcirculation in vivo. Am. J. Physiol. 248: G580–G586