

Disparate effects of non-steroidal anti-inflammatory drugs on apoptosis in guinea-pig gastric mucous cells: inhibition of basal apoptosis by diclofenac

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1 Non-steroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in gastrointestinal cancer cell lines. Similar actions on normal gastric epithelial cells could contribute to NSAID gastropathy. The present work therefore compared the actions of diclofenac, ibuprofen, indomethacin, and the cyclo-oxygenase-2 selective inhibitor, NS-398, on a primary culture of guinea-pig gastric mucous epithelial cells.

2 Cell number was assessed by staining with crystal violet. Apoptotic activity was determined by condensation and fragmentation of nuclei and by assay of caspase-3-like activity. Necrosis was evaluated from release of cellular enzymes.

3 Ibuprofen (250 μ M for 24 h) promoted cell loss, and apoptosis, under both basal conditions and when apoptosis was increased by 25 μ M N-Hexanoyl-D-sphingosine (C₆-ceramide).

4 Diclofenac (250 μ M for 24 h) reduced the proportion of apoptotic nuclei from 5.2 to 2.1%, and caused inhibition of caspase-3-like activity, without causing necrosis under basal conditions. No such reduction in apoptotic activity was evident in the presence of 25 μ M C₆-ceramide.

5 The inhibitory effect of diclofenac on basal caspase-3-like activity was also exhibited by the structurally similar mefenamic and flufenamic acids (1–250 μ M), but not by niflumic acid.

6 Inhibition of superoxide production by the cells increased caspase-3-like activity, but the inhibitory action of diclofenac on caspase activity remained. Diclofenac did not affect superoxide production.

7 Diclofenac inhibited caspase-3-like activity in cell homogenates and also inhibited human recombinant caspase-3.

8 In conclusion, NSAIDs vary in their effect on apoptotic activity in a primary culture of guinea-pig gastric mucous epithelial cells, and the inhibitory effect of diclofenac on basal apoptosis could involve an action on caspase activity.

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Abbreviations: NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; TNF- α , tumour necrosis factor alpha; N-Hexanoyl-D-sphingosine, C₆-ceramide

Introduction

In the gastrointestinal tract the size of the mucosal cell population is determined by the balance between cell gain from proliferation and cell loss from apoptosis (programmed cell death) and necrosis (cell lysis). Apoptosis occurs close to the proliferative zone and at the surface of the epithelium (Hall *et al.*, 1994). Too much apoptosis can lead to tissue atrophy or potentially to the induction of a compensatory hyper-proliferative response.

Non-steroidal anti-inflammatory drugs (NSAIDs) damage the gastric mucosa (Wallace, 1997; Hawkey, 2000). Inhibition of prostaglandin biosynthesis by NSAIDs, with associated reductions in mucus and bicarbonate secretion, and of blood flow, is very probably of major importance in the acute action of NSAIDs. In animal models a tumour necrosis factor α (TNF- α) mediated recruitment of neutrophils to the

capillary endothelium (Santucci *et al.*, 1995) is an important mediator of NSAID damage. Neutrophils may release damaging reactive oxygen intermediates, and obstruct blood flow. Direct topical actions of NSAIDs on gastric surface epithelial cells have also been suggested (see Wallace, 1997).

Administration of NSAIDs to rats as a bolus causes apoptosis by a mechanism that involves TNF- α (Fiorucci *et al.*, 1999). Furthermore apoptosis is increased in the stomach of human patients taking NSAIDs (Zhu *et al.*, 1998). Increased apoptosis could contribute to either the gastric atrophy or the hyperplasia seen with NSAIDs (Taha *et al.*, 1992), the final result depending on whether or not there is a compensatory proliferative response. Experiments with transformed cell lines (Lu *et al.*, 1995; Chan *et al.*, 1998; Simmons *et al.*, 1999; Kusuhara *et al.*, 1999; Zhu *et al.*, 1999) have indicated that NSAIDs can induce apoptosis by a direct action on cells. Furthermore, flurbiprofen increased apoptosis in a primary culture of guinea-pig gastric epithelial cells

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(Johal & Hanson, 2000) showing that such a target for NSAIDs could be present in non-transformed cells. The guinea-pig cell preparation consists of at least 90% of gastric mucous epithelial cells (Byrne & Hanson, 1998; Teshima *et al.*, 1998), and exhibits spontaneous apoptosis that is dependent on the presence of serum and protein synthesis, and which may represent the normal process of turnover of gastric surface mucous cells (Johal & Hanson, 2000; Tsutsumi *et al.*, 2000). Nuclear fragmentation and condensation and increased activity of the effector proteolytic enzyme, caspase 3 (Song & Steller, 2000), can be used as indicators of apoptosis, while necrosis can be estimated from the early release of enzymes such as lactate dehydrogenase and acid phosphatase.

The aim of the present work was to examine three commonly used NSAIDs, which do not exhibit major selectivity as inhibitors of cyclo-oxygenases-1 and -2 (Warner *et al.*, 1999) and the selective cyclo-oxygenase-2 inhibitor NS-398 (Warner *et al.*, 1999) for direct effects on apoptosis in primary cultures of guinea-pig gastric epithelial cells. Cyclo-oxygenase-2 selective agents exhibit considerably less gastro-toxicity than conventional non-selective NSAIDs (Whittle, 2000), but have nevertheless been shown to increase apoptosis in colon cancer cell lines (Elder *et al.*, 2000). Contrary to the findings obtained with transformed cell lines (e.g. Simmons *et al.*, 1999), we find that there are major differences between the NSAIDs in their effect on apoptotic activity in the primary culture of gastric epithelial cells.

Methods

Animals

Male Dunkin-Hartley guinea-pigs of 200–300 g body weight were obtained from Charles River, Margate, Kent U.K. and were fed on SDS Economy guinea-pig diet supplied by Lillico, Betchworth, Surrey, U.K.

Materials

RPMI 1640 medium, Hanks-buffered salt solution without phenol red, foetal calf serum, antibiotics and amphotericin B were from Life Technologies, Paisley U.K. Pronase E (70,000 PU units per g) was purchased from Merck, Lutterworth, U.K. [³H]-thymidine was from Amersham Pharmacia, Little Chalfont, U.K. Recombinant murine tumour necrosis factor- α (TNF- α , 6×10^7 units mg⁻¹) was from Roche, Lewes, U.K. Other reagents, including activated human recombinant caspase-3 with a C-terminal histidine tag on the 13.5 kDa subunit, were from Sigma, Poole, U.K.

Isolation and culture of gastric mucous cells

The method has been described in detail previously (Byrne & Hanson, 1998). Gastric mucosa was minced with fine scissors, incubated with 45 ml of RPMI 1640 containing 2 g l⁻¹ bovine serum albumin (isolation medium) and 0.5 mg ml⁻¹ pronase for 20 min at 37°C, and then, after centrifugation, incubated with 45 ml of isolation medium containing 0.4 mg ml⁻¹ collagenase for 20 min. Cells were filtered through 150 μ m nylon mesh, washed in culture medium (RPMI 1640 containing 10% foetal calf serum, 100 u ml⁻¹

penicillin, 100 μ g ml⁻¹ streptomycin and 2.5 μ g ml⁻¹ of amphotericin B) and cultured on six- or twelve-well culture plates. The culture medium was renewed after 24 h, and again at 48 h, at which time some experiments were initiated by the inclusion of agents in the culture medium. For experiments involving shorter treatment periods a further change of medium was performed on the day on which cells were harvested after a total 72 h of culture. Agents were dissolved in dimethylsulphoxide, the concentration of which was never greater than 0.25% (v v⁻¹), and which was always the same in control and experimental wells. NS-398 was not used at concentrations higher than 100 μ M because of its limited solubility in culture medium.

Crystal violet assay

Cells were grown in 12-well plates. The culture medium was removed and 0.5 ml of crystal violet (0.4 g 100 ml⁻¹) in 30% (v v⁻¹) methanol was added to each well for 20 min at room temperature. After removal of the stain, wells were washed three times with 1 ml of water and then allowed to dry. Stain was released from cells by incubation with agitation for 30 min at room temperature with 400 μ l of sodium dodecyl sulphate (1 g 100 ml⁻¹) in each well. Absorbance of 150 μ l of the extracts was read at 570 nm on a plate reader.

Attached and detached cell protein

Medium was removed from the plates and centrifuged at 12,000 $\times g$ for 20 s. The pellet of detached cells was washed with 1 ml of 138 mM NaCl, 2.7 mM KCl, 10 mM phosphate pH 7.4 (PBS), and the pellet finally resuspended in 0.15 ml of PBS containing 1% (v v⁻¹) Triton X-100. The wells were washed with 1 ml of PBS, and attached cells were scraped off into 0.4 ml of PBS containing 1% (v v⁻¹) Triton X-100. Protein was measured by using bicinchoninic acid reagent with bovine serum albumin as standard (Redinbaugh & Turley, 1986).

Detection of apoptosis by staining with Hoechst 33258

Cells were removed from the culture plate by exposure to trypsin/EDTA, and were subjected to centrifugation for 20 s at 12,000 $\times g$. Cells were resuspended in 4% (w v⁻¹) paraformaldehyde in PBS for 10 min at room temperature, subjected to centrifugation at 12,000 $\times g$ for 10 s, resuspended in ethanol: water (4:1) and stored at 4°C. Cells were transferred to glass slides by use of a Shandon cytocentrifuge, and nuclei were stained with 8 μ g ml⁻¹ of Hoechst 33258. Slides were coded, and the proportion of apoptotic nuclei was determined by counting using a fluorescent microscope with apoptosis defined as the presence of two or more condensed bodies per nucleus.

Assay of caspase activity

Plates were placed on ice, the culture medium was removed and 1 ml of PBS was added to each well. The cells were removed from the plate with a cell-scraper, transferred to microfuge tubes, and were subjected to centrifugation for 20 s at 12,000 $\times g$. Pellets were rinsed with 1.0 ml of ice cold PBS and then were resuspended, by pipetting up and down 10

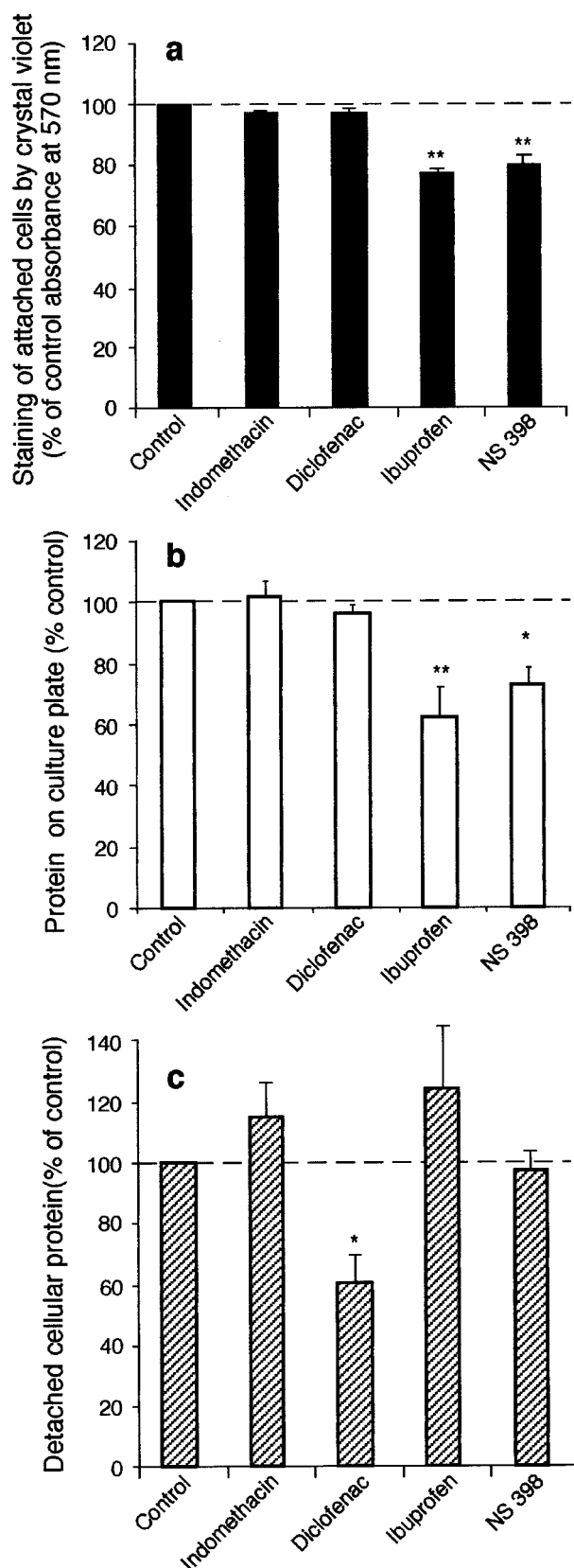


Figure 1 Effect of incubation with non-steroidal anti-inflammatory drugs (NSAIDs, 250 μM except for NS-398 at 100 μM) for 24 h on estimates of cell number. Staining of attached cells with crystal violet (a) has been normalized to the absorbance at 570 nm obtained with control cells, which was 1.162 ± 0.154 . In (b) data have been

times, in 110 μl of homogenization buffer (in mM) HEPES 100, NaCl 140, EDTA 1, (pH 7.4), containing phenylmethylsulphonyl fluoride 0.5, aprotinin 5 $\mu\text{g ml}^{-1}$, pepstatin 5 $\mu\text{g ml}^{-1}$ and leupeptin 10 $\mu\text{g ml}^{-1}$. Extracts were then frozen in liquid nitrogen and stored at -70°C . Before assay homogenates were thawed and refrozen twice, before a final thaw and centrifugation at $12,000 \times g$ for 20 min at 4°C .

Twenty-five μl of supernatant was incubated at 37°C with 175 μl of assay buffer consisting of 100 mM HEPES (pH 7.4) and 20% (v/v) glycerol containing protease inhibitors at the same concentration as above, and the caspase substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin at a final concentration of 15 μM . Formation of fluorescent product was measured on a Victor multilable counter. Five mM dithiothreitol was added to the assay buffer to stabilize the activity of recombinant caspase-3, but dithiothreitol was not included in the assay of tissue homogenates since concentrations up to 20 mM had no effect on activity.

Lactate dehydrogenase activity

Samples were prepared as described for the protein assay, except that culture medium from wells with and without cells was retained after centrifugation and Triton X-100 added to give a final concentration of 1% (v/v). Lactate dehydrogenase activity was assayed immediately by mixing samples with 0.15 ml of 50 mM potassium phosphate buffer containing 4.4 mM pyruvate and 0.4 mM NADH and measuring the rate of change of absorbance at 340 nm on a plate reader. Results were corrected for lactate dehydrogenase present in the serum component of culture medium, and the activity in the medium was then expressed as a percentage of that in medium, detached cells and attached cells (total activity).

Acid phosphatase activity

Preparation of samples and analysis of results was as for lactate dehydrogenase. Enzyme activity was determined by the breakdown of p-nitrophenyl phosphate at pH 4.8 as described by Wong & Tepperman (1994).

Incorporation of [^3H]-thymidine

Two μCi of [^3H]-thymidine (25 Ci mmol^{-1}) was added to each well of a 12-well plate. After 24 h the medium was removed and the wells washed with 1 ml of ice-cold PBS. Cells were scraped into 0.75 ml of trichloroacetic acid (10 g 100 ml^{-1} (TCA)), and left for 20 min on ice. The contents of the wells, and 0.5 ml of TCA used to rinse the wells, were transferred to microfuge tubes, centrifuged at $12,000 \times g$ for 1 min, the pellet washed with TCA, and finally dissolved in 0.2 ml NaOH (0.3 M) containing 0.1 g 100 ml^{-1} sodium dodecylsulphate at 37°C for 4 h. Incorporation of [^3H]-

normalized to the attached cell protein in control wells, which was $77 \pm 7 \mu\text{g}$ per well. In (c) cell protein in the culture medium has been normalized to that in control incubations which was $12.4 \pm 1.9 \mu\text{g}$ per well. Data are means $\pm$ s.e. mean from respectively four and six cultures for the crystal violet and protein measurements. Data were analysed before normalization by ANOVA and Dunnett's test. * $P < 0.05$ and ** $P < 0.01$ for difference from control.

thymidine was determined by scintillation counting and was expressed as a function of cellular protein.

Production of superoxide

Wells were washed three times with 1 ml of PBS at 37°C. Hanks-buffered salt solution (0.75 ml) without phenol red and containing 80 μM cytochrome *c* from horse heart (Sigma C7752) was added to each well and the plate incubated at 37°C for 1 h. Superoxide dismutase from bovine erythrocytes (Sigma S2515) was included at 100 units per ml where appropriate. Reduction of cytochrome *c* was measured at 550 nm and an extinction coefficient of $21 \times 10^3 \text{ ml mmol}^{-1} \text{ cm}^{-1}$ used in the calculation.

Statistical analysis

Results are presented as means $\pm$ s.e.mean, with *n* equal to the number of separate cell cultures, and unless stated otherwise were subjected to analysis of variance, to remove variation between cell cultures, followed by either a Newman-Keuls or a Dunnett's multiple comparison test. Data on the percentage of apoptotic cells were arcsine-transformed before analysis.

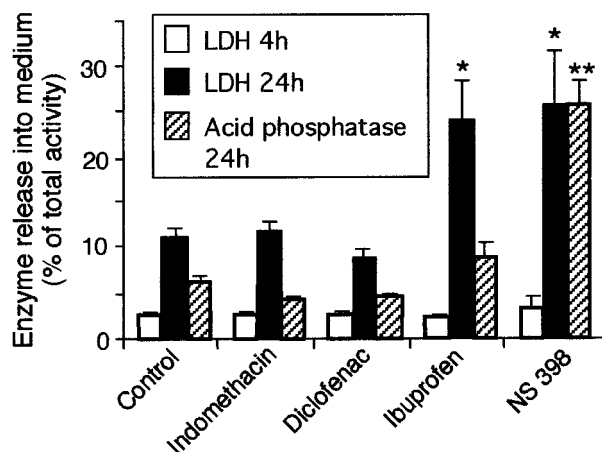


Figure 2 Effect of incubation with non-steroidal anti-inflammatory drugs (NSAIDs, 250 μM except for NS-398 at 100 μM) on release into the culture medium of lactate dehydrogenase (LDH) after 4 h (open bars, *n* = 4 cell batches) and 24 h (filled bars, *n* = 5 cell batches), and on release of acid phosphatase after 24 h (hatched bars, *n* = 3 cell batches). Results are means $\pm$ s.e.mean and are expressed as a percentage of total enzyme activity. Data were analysed by ANOVA and Dunnett's test. **P* < 0.05 and ***P* < 0.01 for difference from appropriate control.

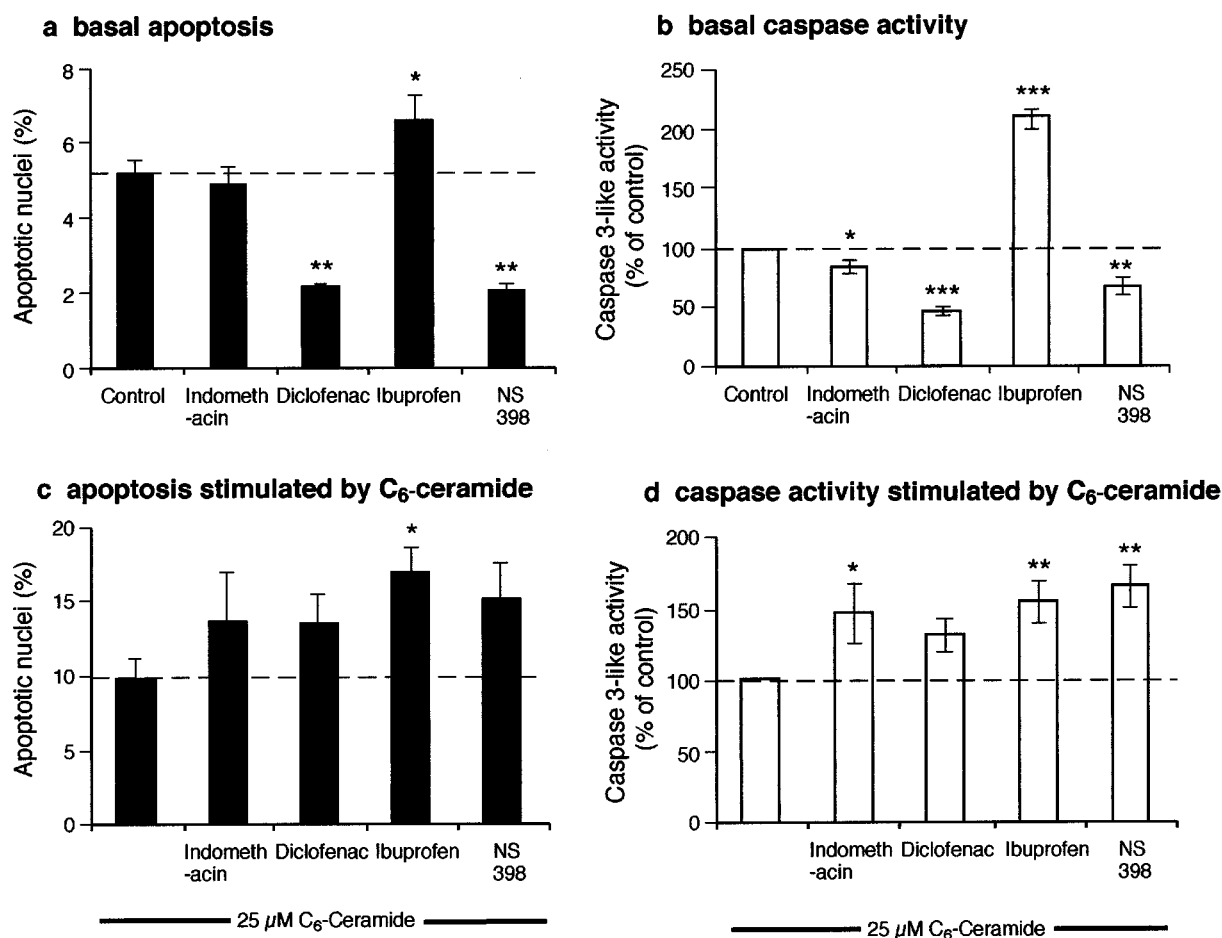


Figure 3 Effect of incubation with non-steroidal anti-inflammatory drugs (NSAIDs, 250 μM except for NS-398 at 100 μM) for 24 h on the proportion of apoptotic nuclei (a,c) and on caspase-3-like activity (b,d) in attached cells. Results are means $\pm$ s.e.mean for basal conditions in (a) and (b), *n* = 6 and *n* = 5 respectively, and in the presence of 25 μM C₆-ceramide in (c) and (d), *n* = 4 in both cases. The control caspase activity used for normalization in (b) was 199 ± 25 , and in (d), $498 \pm 147 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$. Data were analysed by ANOVA and Dunnett's test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 for difference from control or from 25 μM C₆-ceramide alone.

Results

Effect of incubation with NSAIDs for 24 h on the number of cells

Both ibuprofen (250 μM) and NS-398 (100 μM) reduced the number of viable cells attached to the culture plate as estimated by staining with crystal violet, while 250 μM indomethacin or diclofenac were without effect (Figure 1a). A similar pattern of results was obtained if attached cell protein was used as an indicator of cell number (Figure 1b). Reductions in attached cells were not reflected in significantly increased numbers of cells in suspension as indicated by measurement of detached cell protein (Figure 1c). Indeed the only difference from control was a reduction in this measurement in response to diclofenac.

Effect of incubation with NSAIDs on release of enzyme activity from cells

None of the NSAIDs affected the release of lactate dehydrogenase after 4 h of incubation. Ibuprofen (250 μM) increased lactate dehydrogenase release after 24 h and NS-398 (100 μM) increased release of both lactate dehydrogenase and acid phosphatase after this time interval (Figure 2).

Effect of incubation with NSAIDs for 24 h on basal apoptotic activity

The proportion of apoptotic nuclei in cells attached to the culture plate was increased slightly, but significantly by ibuprofen (250 μM) and decreased in response to diclofenac (250 μM) and NS-398 (100 μM) (Figure 3a). These changes in apoptotic activity were accompanied by changes, in the

appropriate direction, of caspase-3-like activity (Figure 3b). Caspase-3-like activity is elevated in cells which have been detached from the culture plate with the majority undergoing apoptosis (Johal & Hanson, 2000). Effects of NSAIDs on caspase activity ($\text{pmol min}^{-1} \text{mg protein}^{-1}$) in detached cells were (control result versus that with NSAID, n = number of preparations, P value for paired t -test for effect of NSAID): 250 μM indomethacin: 1733 ± 466 vs 1439 ± 445 , $n = 4$, $P < 0.05$; 250 μM diclofenac: 1614 ± 411 vs 1154 ± 359 , $n = 5$, $P < 0.05$; 250 μM ibuprofen: 1159 ± 307 vs 2048 ± 301 , $n = 6$, $P < 0.05$; 100 μM NS-398: 2045 ± 530 vs 821 ± 275 , $n = 4$, $P < 0.05$.

Effect of incubation with NSAIDs for 24 h on stimulated apoptotic activity

Since basal apoptotic activity in attached cells was low (Figure 3a), we investigated the effect of $\text{TNF-}\alpha$, which increases apoptosis in suspensions of guinea-pig chief cells (Fiorucci *et al.*, 1996). The proportion of apoptotic nuclei in cells treated for 24 h with 20 ng ml^{-1} $\text{TNF-}\alpha$ was 3.1 ± 0.22 compared with 3.4 ± 0.68 in control cells (no effect by paired t -test, $n = 3$). However 25 μM C_6 -ceramide, which is produced by $\text{TNF-}\alpha$ (Hannun & Luberto, 2000), and which is elevated in gastric cells infected with *Helicobacter pylori* (Masamune *et al.*, 1999), increased the percentage of apoptotic nuclei to 9.7 ± 1.5 from 6.7 ± 1.0 in controls ($P < 0.05$ by paired t -test, $n = 4$), and elevated caspase-3-like activity to 497 ± 147 $\text{pmol min}^{-1} \text{mg protein}^{-1}$ from 277 ± 89 $\text{pmol min}^{-1} \text{mg protein}^{-1}$ in controls ($P < 0.05$). In the presence of 25 μM C_6 -ceramide no NSAID reduced the percentage of apoptotic cells although ibuprofen was the only one to produce a significant stimulation above that with ceramide alone (Figure 3c). All NSAIDs stimulated caspase activity above that found with ceramide alone except diclofenac which had no effect (Figure 3d). Results for cell

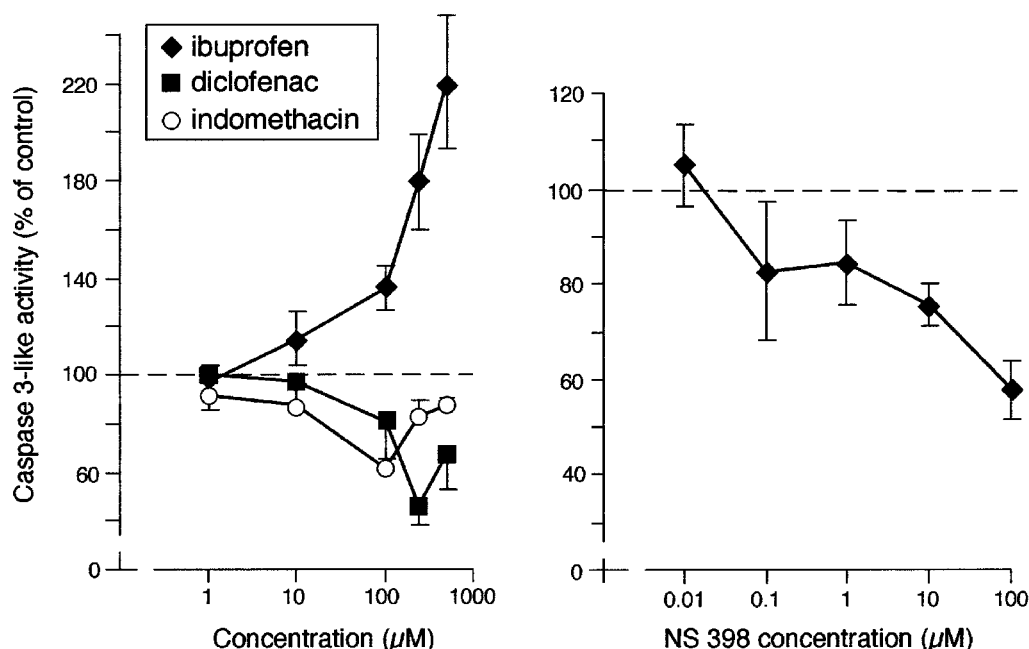


Figure 4 Effect of the concentration of non-steroidal anti-inflammatory drug (NSAID) on caspase-3-like activity in attached cells after 24 h of exposure. Results are means $\pm$ s.e.mean. The significance of any effect of NSAID concentration on caspase activity was assessed by analysis of variance with the following results (number of cell cultures): ibuprofen, $P < 0.001$ (5); diclofenac, $P < 0.01$ (4); indomethacin, $P > 0.05$ not significant; NS-398, $P < 0.05$ (3).

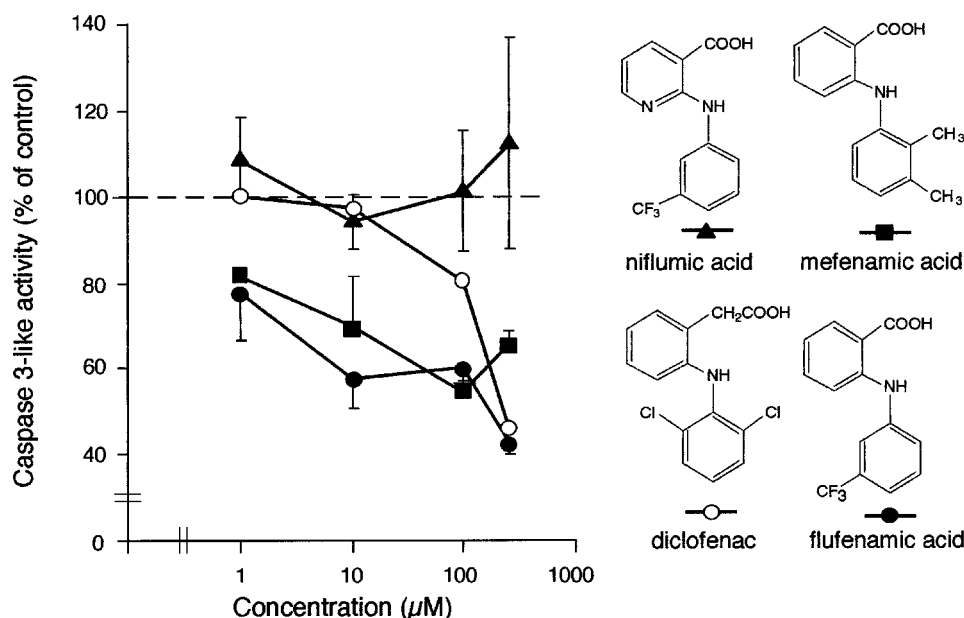


Figure 5 Effect of the concentration of non-steroidal anti-inflammatory drugs (NSAIDs) structurally related to diclofenac on caspase-3-like activity in attached cells after 24 h of exposure. Results are means $\pm$ s.e.mean, and data for diclofenac are taken from Figure 4. The significance of any effect of NSAID concentration on caspase activity was assessed by analysis of variance with the following results (three cell cultures in each case): niflumic acid, $P > 0.05$ not significant; mefenamic acid, $P < 0.05$; flufenamic acid, $P < 0.01$.

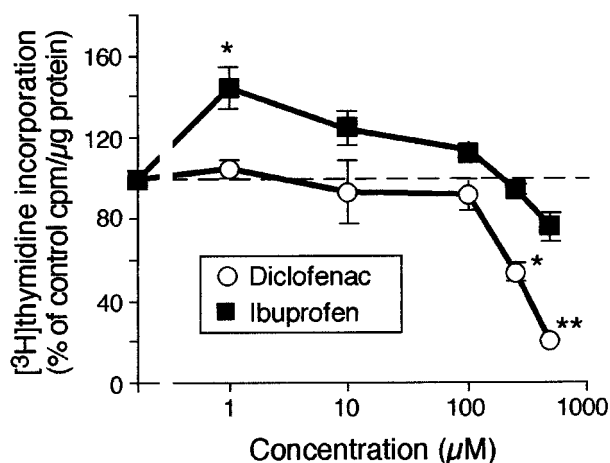


Figure 6 Effect of the concentration of ibuprofen (filled squares) and diclofenac (open circles) on incorporation of $[^3\text{H}]$ -thymidine into cellular DNA during 24 h of exposure. Results are means $\pm$ s.e.mean, for four cultures for diclofenac and three for ibuprofen. The control $[^3\text{H}]$ -thymidine incorporation used for normalization was 1761 ± 377 c.p.m. per μg protein. The significance of any effect of NSAID concentration was assessed by analysis of variance followed by a Dunnett's test for comparison with control: * $P < 0.05$, ** $P < 0.01$.

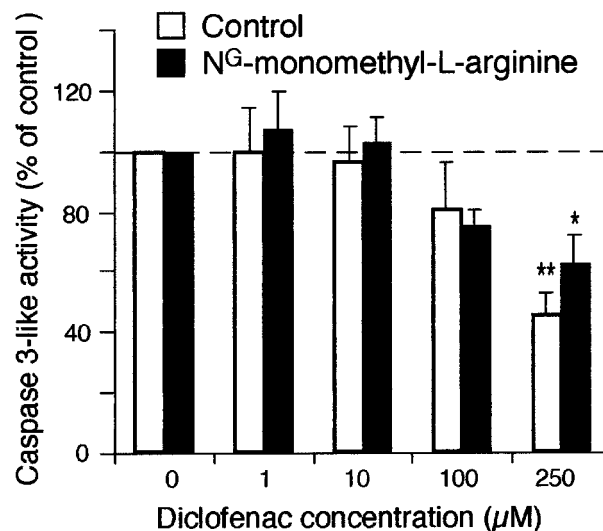


Figure 7 Effect of the concentration of diclofenac on caspase-3-like activity in the presence (filled bars) and absence (clear bars) of N^G -monomethyl-L-arginine (2 mM). Results are means $\pm$ s.e.mean of respectively four and five separate cell cultures and have been normalized to the activities with or without N^G -monomethyl-L-arginine which were: 181 ± 35 and 203 ± 56 pmol min^{-1} mg protein $^{-1}$ respectively. * $P < 0.05$, ** $P < 0.01$ for comparison with the appropriate control by ANOVA and Dunnett's test.

number, as assessed by the crystal violet assay, and expressed as a percentage of basal conditions were ($n=4$): ceramide, 75 ± 1.3 ; ceramide + 250 μM indomethacin, 65.4 ± 3.3 ; ceramide + 250 μM diclofenac, 64 ± 4.7 ; ceramide + 250 μM ibuprofen, 55.8 ± 1.6 ; ceramide + 100 μM NS-398, 61 ± 3.36 . All results, except that for indomethacin + ceramide were significantly different ($P < 0.05$) from ceramide alone by ANOVA and Dunnett's test.

Effect of exposure to different concentrations of NSAIDs for 24 h on basal caspase-3-like activity

The effects of ibuprofen, diclofenac and NS-398 were dose-related, and the differential actions of the NSAIDs on caspase activity were exhibited over a range of concentration (Figure 4). Exposure of cells for 24 h to NSAIDs structurally

related to diclofenac showed that mefenamic acid and flufenamic acid also decreased caspase-3-like activity but that niflumic acid did not (Figure 5).

Effect of time on inhibition of basal caspase-3-like activity by 250 μ M diclofenac

Cells were incubated with and without diclofenac for varying periods of time and caspase activity after incubation with diclofenac expressed as a percentage of that in control wells with the following results: 15 min, $98 \pm 3\%$ (4); 1 h, $67 \pm 3\%$ (4), $P < 0.01$ by paired *t*-test; 3 h, $60 \pm 6\%$ $P < 0.01$ by paired *t*-test; 6 h, $60 \pm 8\%$ (4) $P < 0.05$ by paired *t*-test; and 24 h, $46 \pm 7\%$ $P < 0.01$ by paired *t*-test.

Effect of ibuprofen and diclofenac on proliferative activity of the gastric mucous cells

Two hundred and fifty and 500 μ M diclofenac substantially reduced incorporation of [3 H]-thymidine into DNA over a 24 h period ($P < 0.05$ and < 0.01 respectively by ANOVA and Dunnett's test), while using the same analysis procedure, no significant effect of 250 or 500 μ M ibuprofen was detected (Figure 6).

Lack of involvement of nitrogen and oxygen radicals in the inhibitory action of diclofenac

Nitric oxide (NO) inhibits apoptosis in guinea-pig gastric mucous cells (Potter & Hanson, 2000). However, inclusion of the non-selective inhibitor of NO synthase, N^G-monomethyl-L-arginine, did not prevent the inhibitory effect of diclofenac on caspase-3-like activity (Figure 7). Guinea-pig gastric mucous cells produce superoxide in substantial amounts relative to activated neutrophils (Teshima *et al.*, 1998). Reduction of exogenous cytochrome *c* by the cultured cells was decreased by $87 \pm 4\%$ (6) by added superoxide dismutase, and superoxide production was therefore taken as cytochrome *c* reduction inhibitable by superoxide dismutase. Values obtained were (nmol h⁻¹ mg protein⁻¹, $n = 5$): control 25 ± 7 , 250 μ M diclofenac, 27 ± 6 ; 10 μ M diphenyleneiodonium (inhibitor targeting the flavoprotein component of NADPH oxidase), 5.9 ± 1.9 , and 250 μ M diclofenac plus 10 μ M diphenyleneiodonium, 4.8 ± 1.7 . Thus diclofenac did not inhibit superoxide production, which, however, was reduced by diphenyleneiodonium ($P < 0.05$ by ANOVA and Dunnett's test).

Caspase-3-like activity (pmol min⁻¹mg protein⁻¹) in cells incubated for 3 h with 10 μ M diphenyleneiodonium was 245 ± 39 , and with superoxide dismutase (100 u ml⁻¹) and catalase (100 u ml⁻¹) was 303 ± 91 , ($P < 0.001$ after ANOVA and Newman Keuls test versus control caspase-3-like activity of 135 ± 41 (all $n = 4$)). However, the inhibitory effect of 250 μ M diclofenac was not significantly altered being respectively: $66 \pm 5.6\%$, $67 \pm 9.7\%$ and $58 \pm 7.7\%$ under control conditions, with diphenyleneiodonium, and with catalase and superoxide dismutase (all $n = 4$).

Effect of diclofenac on caspase-3-like activity in homogenates, and on recombinant caspase-3

If homogenates prepared from control cells were incubated with diclofenac at 2.5 mM and then the homogenate added

to the assay system to give a final concentration of diclofenac in the assay of 300 μ M, then significant inhibition relative to control was found after 1, 2 and 3 h of incubation (Figure 8a). If diclofenac was added to active recombinant human caspase-3 to give a concentration of 2.5 mM and the mixture added to the assay system to give a concentration of 250 μ M, then in both the presence and absence of NSAID activity was linearly related to time but that with diclofenac was significantly lower (Figure 8b,c). Preincubation for 1 h with diclofenac before assay did not increase the inhibitory effect (paired *t*-test, Figure 8c). With diclofenac at concentrations (μ M) in the assay of 0.1, 1, 10, 100 and 250 the activity of caspase-3 (per cent control, $n = 4$) was : 98 ± 6 , 98 ± 4 , 96 ± 4 , 92 ± 9 and 84 ± 6 respectively.

Discussion

The main findings of this investigation are firstly that under basal conditions in primary cultures of guinea-pig gastric epithelial cells there are major differences between the actions of NSAIDs on apoptotic activity in contrast with the uniform stimulatory effects seen in transformed cell lines (Lu *et al.*, 1995; Chan *et al.*, 1998; Simmons *et al.*, 1999; Zhu *et al.*, 1999), and secondly that one NSAID, diclofenac, inhibits basal apoptosis with an effect on caspase-3 possibly contributing to this action.

In animal models intragastric administration of NSAIDs causes release of TNF- α probably from macrophages (Fiorucci *et al.*, 1999). Effects of TNF- α on the primary culture system were therefore investigated. No change in apoptotic activity was detected. This result contrasts with that obtained using a similar concentration of TNF- α in freshly isolated guinea-pig chief cells. It is possible that the guinea-pig gastric epithelial cells had lost receptors for TNF- α during culture, but it should be remembered that many 'normal' cells e.g. hepatocytes do not show loss of viability to TNF- α (Leist *et al.*, 1994; Wallach, 1997), and that the trauma of isolation, or the lack of attachment to a substratum, could have sensitized the chief cells. However C₆-ceramide, which is produced in cells stimulated with TNF- α (Hannun & Luberto, 2000), and which is elevated in gastric cells incubated with *Helicobacter pylori* (Masamune *et al.*, 1999), elevated apoptotic activity and was used to test for effects of NSAIDs in cells in which apoptosis was stimulated above basal.

The NSAIDs could have affected the number of cells attached to the culture plate by causing apoptosis, necrosis, inducing detachment, or promoting proliferation. The relationship between numbers of cells on the culture plate and those in suspension is likely to be complex. Thus a reduction in cells on the plate over a period of 24 h because of apoptosis or necrosis will not necessarily be reflected in an increased number of cells in suspension as the dead cells may have been lost from the system. In two cases under basal conditions the number of cells on the plate was reduced. With ibuprofen the primary cause of cell loss was probably increased apoptotic activity as evidenced by an enhanced caspase-3-like activity and an increased percentage of attached cells showing condensed and fragmented nuclei. There was no evidence for ibuprofen causing major

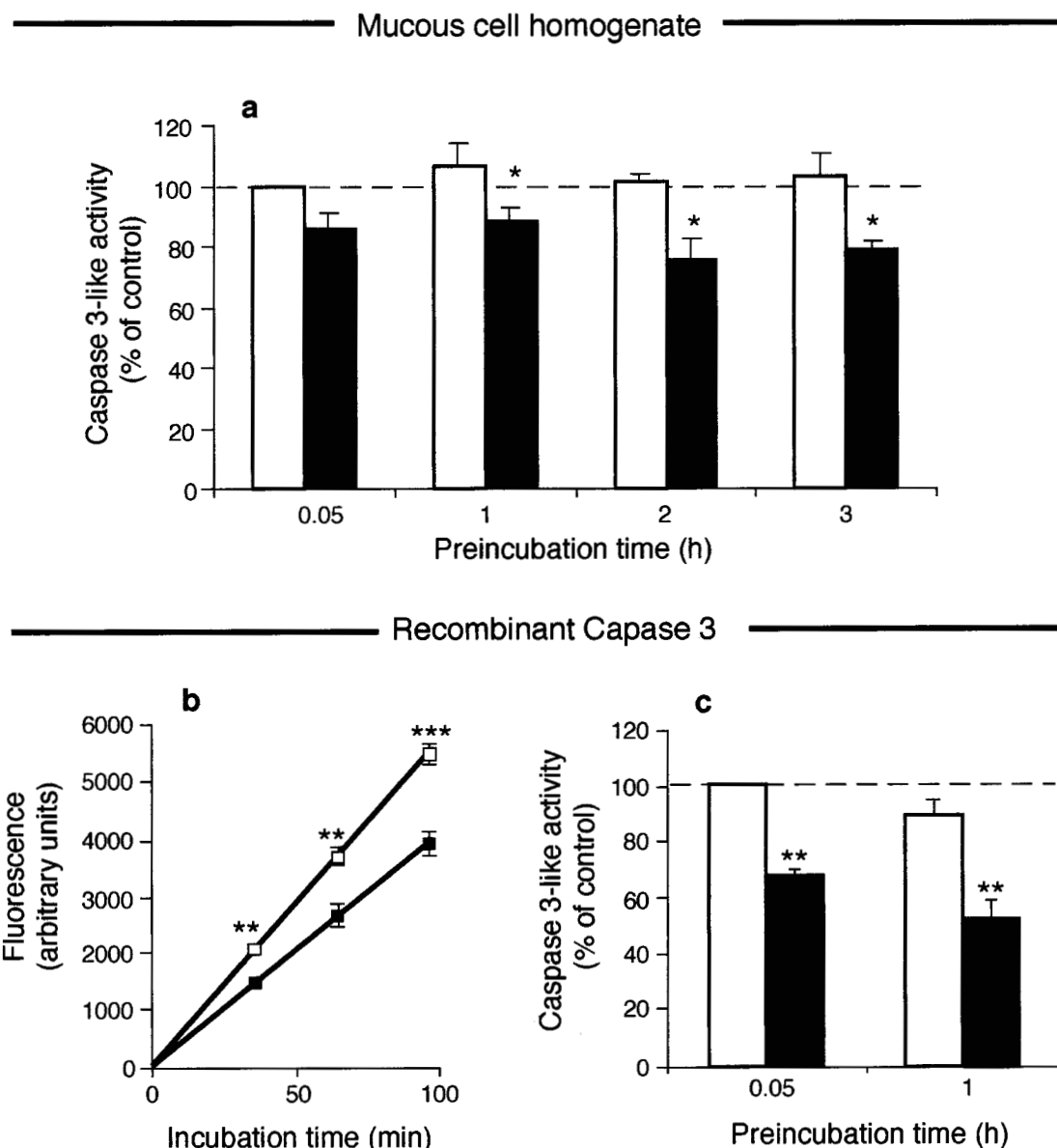


Figure 8 Effect of diclofenac (filled bars and symbols) on caspase-3-like activity in cell homogenates (a) and on recombinant caspase-3 (b,c). In (a) results are means $\pm$ s.e. for five separate homogenates, with the concentration of diclofenac at 2.5 mM during preincubation at 20°C and 300 μ M during the assay. Control homogenate activity used for normalization was 167 ± 31 pmol min^{-1} mg protein $^{-1}$. In (b,c) the concentration of diclofenac was 2.5 mM during preincubation and 250 μ M in the assay and the results are means $\pm$ s.e. mean for five repetitions in the absence (clear bars/open symbols) or presence of diclofenac (filled bars/symbols) with 50 ng of enzyme in each assay.

alterations in proliferative activity or primary cell detachment. The time course of lactate dehydrogenase release, elevated after 24 h, but not after 4 h, is most easily interpreted as indicating 'secondary-necrosis' i.e. enzyme release following on from primary apoptotic cell death. Ibuprofen, and the structurally similar flurbiprofen (Johal & Hanson, 2000) are the only NSAIDs in this primary culture which showed enhanced apoptosis under basal conditions in a manner similar to previous results with cell lines (Lu *et al.*, 1995; Chan *et al.*, 1998; Simmons *et al.*, 1999; Kusuhashi *et al.*, 1999; Zhu *et al.*, 1999). Ibuprofen also further enhanced apoptotic activity above that obtained with 25 μ M C₆-ceramide.

The other NSAID, which caused cell loss under basal conditions, was the cyclo-oxygenase-2 selective inhibitor NS-398. However, in this instance both indices of apoptotic activity were reduced by comparison with control, while release of both acid phosphatase and lactate dehydrogenase were elevated after 24 h compared with control. This profile would imply some form of slow necrotic cell death. It may be relevant that in L929 fibrosarcoma cells inhibition of caspase activity promoted necrotic cell death in response to TNF- α (Vercammen *et al.*, 1998). In colorectal adenoma and carcinoma cell lines incubated with a similar range of concentrations of NS-398, no increase in apoptosis of attached cells was found, but by contrast with the present

results NS-398 stimulated detachment and consequently apoptosis (Elder *et al.*, 2000). Interpretation of the action of NS-398 is further complicated by the stimulatory effect of NS-398 on apoptotic activity in the gastric cells in the presence of C₆-ceramide. Cyclo-oxygenase-2 selective inhibitors, including NS-398, show reduced gastric toxicity relative to non-selective NSAIDs (Futaki *et al.*, 1993; Whittle, 2000). It is unclear what relevance the present data with NS-398 have to the role of cyclo-oxygenase-2 selective inhibitors as antiinflammatory agents. Thus the concentration of NS-398 in the stomach after a dose appropriate to inhibit cyclo-oxygenase-2 is unknown. It is also uncertain whether NS-398, which has not been developed for use in humans, is representative of all cyclo-oxygenase-2 selective inhibitors, but investigation of this question is beyond the scope of the present investigation.

Under basal conditions, indomethacin did not show any major effects on cell number, indices of apoptosis or cell detachment of the guinea-pig gastric epithelial cells which is in marked contrast to the stimulation of apoptosis found in AGS gastric cancer cells (Zhu *et al.*, 1999) and in colon cancer cells (Chan *et al.*, 1998). However, it did stimulate caspase-3-like activity above that obtained with C₆-ceramide alone.

Diclofenac inhibited basal apoptotic activity, and by contrast with NS-398, this inhibition was not associated with any increase in enzyme release above control. Diclofenac also inhibited caspase-3-like activity when this was enhanced by detachment of cells under basal conditions, or in attached cells by inhibition of superoxide production. However, when caspase and apoptotic activity was stimulated by C₆-ceramide the inhibitory effect of diclofenac was lost. Although apoptosis was decreased under basal conditions there was no increase in cell number, which can be explained by the inhibition of proliferative activity by diclofenac. The smaller loss of cells into the medium (Figure 1c) may reflect the reduced cell 'turnover' in the preparation. Inhibition of proliferation by NSAIDs of colonic cell lines in well-established (Rigas & Shiff, 1999). Because the result for diclofenac exhibited the greatest contrast with data for cells lines, where stimulation of apoptosis is found (e.g. Simmons *et al.*, 1999, J774.2 macrophages; Kusuvara *et al.*, 1999, AGS cells), the possible mechanism of the inhibition of caspase-3-like activity was investigated further. Previous work has indicated that changes in caspase-3-like activity correlate well with the proportion of apoptotic cells attached to the culture plate (Johal & Hanson, 2000; Potter & Hanson, 2000).

The structure-activity relationship was probed by using fenemates. Mefenamic acid and flufenamic acid have respectively 2,3-dimethylphenyl amino and 3-trifluorophenyl amino substituents somewhat different from the equivalent region of diclofenac, yet were still active. By contrast, changing the ring with the carboxyl group from benzoic acid, to nicotinic acid as in niflumic acid, caused a loss of inhibitory activity. All four compounds are inhibitors of cyclo-oxygenases-1 and -2 (Warner *et al.*, 1999) so inhibition of either form of cyclo-oxygenase is unlikely to be involved in the inhibitory action of diclofenac. Flufenamic acid is an activator of peroxysome proliferator-activated receptor γ (PPAR γ), but this receptor is unlikely to be the target in the present work because indomethacin, which was essentially inactive, and ibuprofen, which stimulates apoptosis, also activate this receptor (Lehmann *et al.*, 1997).

An interesting feature of guinea-pig gastric epithelial cells, possibly distinctive relative to gastrointestinal cell lines, is their production of superoxide (Teshima *et al.*, 1998), which has been reported to inhibit apoptosis in these cells (Rokutan *et al.*, 1999). However, diclofenac did not affect superoxide production, and although inhibition of superoxide production, or its removal by superoxide dismutase and catalase, over a 3 h period increased caspase-3-like activity the inhibitory effect of diclofenac was unaltered. Thus, superoxide production was neither affected by diclofenac nor required for its inhibitory action. Similarly, although NO inhibits apoptosis in guinea-pig gastric epithelial cells (Johal & Hanson, 2000; Potter & Hanson, 2000), inhibition of NO synthase did not prevent the action of diclofenac.

Johal & Hanson (2000) found that the stimulatory effect of flurbiprofen on caspase-3-like activity required an incubation period longer than 6 h to become apparent, but the inhibitory effect of diclofenac was apparent within 1 h. This suggested that a direct interaction of diclofenac, or a metabolite (Tang *et al.*, 1999), on caspase activity might be possible. Indeed incubation of tissue homogenates, or recombinant human caspase-3, with diclofenac produced an inhibition of enzyme activity, which remained constant during the assay period. Whether an adequate concentration of diclofenac in the vicinity of caspase-3 was achieved within intact cells, or whether the action of diclofenac is somehow enhanced in the cellular environment is unclear. The loss of the inhibition of caspase-3-like activity in cells incubated with C₆-ceramide suggests that incubation with ceramide may modify some cellular component required for the inhibitory effect of diclofenac.

The stimulation of basal apoptosis with ibuprofen and inhibition with diclofenac were obtained with concentrations of 100 μ M and above. Plasma concentrations in humans, on a moderate dosage of ibuprofen and diclofenac, can reach 200 μ M (Davies, 1998) and 10 μ M (Davies & Anderson, 1997) respectively. The results with diclofenac may therefore be more relevant to potential topical effects of NSAIDs, but estimation of the appropriate concentration is complicated by the likelihood of binding of NSAIDs to protein (Lin *et al.*, 1987), and the possible accumulation of NSAIDs with carboxyl groups in surface epithelial cells, in the presence of acid *in vivo*, by 'ion trapping' (Hawkey, 2000). In conclusion, the potential of NSAIDs to contribute to gastropathy by increasing apoptosis through a direct effect on surface epithelial cells may not be uniform.

The growth inhibitory and pro-apoptotic activities of NSAIDs on cancer cells, which are all found within the concentration ranges used in this investigation, are of considerable interest because of the linkage between usage of NSAIDs and a reduction in the risk of colon and stomach cancer (Boland *et al.*, 2000). The target for the pro-apoptotic activity of NSAIDs remains to be clearly defined (Boland *et al.*, 2000). The lack of a comparable uniform effect of NSAIDs on apoptosis in the present work raises questions as to whether the same target is present in primary cultures of gastric mucous epithelial cells, or alternatively implies that some NSAIDs may have multiple sites of action on the apoptotic process.

Klairi Kavvada cultured the cells for the experiments with TNF- α .

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