hemolysis was estimated by measuring the absorbance at 520 nm.

Statistical analyses

All values are expressed as the mean ± S.E.M. The Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively.

RESULTS AND DISCUSSION

Necrosis- and apoptosis-inducing activities of loxoprofen and loxoprofen-OH in primary culture of gastric mucosal cells

We previously reported that NSAIDs induce either necrosis or apoptosis depending on treatment conditions; short-term (1 h) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs (2.5 mM for indomethacin and 0.2 mM for celecoxib) and long-term (16-24 h) treatment of these cells with relatively low concentrations of NSAIDs (1 mM for indomethacin and 0.05 mM for celecoxib) induces necrosis and apoptosis, respectively.[18,19,24] Loxoprofen and loxoprofen-OH were tested here for their ability to induce necrosis and apoptosis. Consistent with previous reports,[18,19,24] cell viability decreased in a dose-dependent manner when guinea pig gastric mucosal cells in primary culture were treated with indomethacin or celecoxib for 1 h. In contrast, loxoprofen and loxoprofen-OH decreased cell viability to a much lesser extent under the same experimental conditions; cell viability of more than 60% was observed even with the highest concentration (20 mM) of loxoprofen and loxoprofen-OH (Fig. 1). We confirmed that cell death highlighted in Fig. 1 was mediated by necrosis given that no accompanying apoptotic DNA fragmentation or apoptotic chromatin condensation were evident (data not shown).

Similar results to the above were obtained when apoptosis was induced. Treatment of cells for 18 h with indomethacin or celecoxib decreased cell viability in a dose-

dependent manner (Fig. 2A), which is also consistent with previous reports.[18,19,24] Loxoprofen and loxoprofen-OH showed very low activities for decreasing cell viability under these conditions (Fig. 2A). Because cell death as highlighted in Fig. 2 was accompanied by apoptotic DNA fragmentation and apoptotic chromatin condensation (Fig. 2B and C), it is most likely to have been mediated by apoptosis. Overall, the results in Figs. 1 and 2 show that loxoprofen and loxoprofen-OH induce necrosis and apoptosis to a lesser extent than do indomethacin and celecoxib. Furthermore, although the metabolic conversion of loxoprofen to loxoprofen-OH drastically increases the inhibitory activity on COX, this conversion does not seem to be so apparently associated with a similar increase in direct cytotoxicity.

Membrane permeabilization activities of loxoprofen and loxoprofen-OH

The ability of loxoprofen and loxoprofen-OH to permeabilize the membranes of calcein-loaded liposomes was examined. Calcein fluoresces very weakly when at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by releasing calcein trapped within the liposomes.[18] As shown in Fig. 3A, indomethacin and celecoxib increased the calcein fluorescence in a dose-dependent manner, which is consistent with previous findings.[18] Loxoprofen and loxoprofen-OH also increased the calcein fluorescence, suggesting that they caused membrane permeabilization; however, as the concentrations of loxoprofen and loxoprofen-OH required for membrane

permeabilization were much higher than those of indomethacin and celecoxib, their abilities to permeabilize membranes were thus very weak.

Measurement of hemolysis is a standard method for testing the membrane permeabilization activities of drugs. As shown in Fig. 3B, all of the tested NSAIDs caused hemolysis of erythrocytes. The relative potency of each NSAID for hemolysis was approximately similar to that for permeabilization of calcein-loaded liposomes. Celecoxib showed the most potent activity for hemolysis, followed by indomethacin and both loxoprofen and loxoprofen-OH showed weak activity for hemolysis (Fig. 3B). Loxoprofen showed lower permeabilization activity than loxoprofen-OH on the hemolysis assay (Fig. 3B)

The results shown in Fig. 3 suggest that the low direct cytotoxicity of loxoprofen and loxoprofen-OH on gastric mucosal cells is due to their low membrane permeabilizing effects.

Cytotoxic effects of loxoprofen and loxoprofen-OH on gastric cancer cells

In addition to their anti-inflammatory effects, recent epidemiological studies have revealed that prolonged NSAID use reduces the risk of cancer, while preclinical and clinical studies have indicated that some NSAIDs are effective in the treatment and prevention of cancer.[35] The anti-tumorigenic activity of NSAIDs is believed to involve various mechanisms, including induction of apoptosis.[36,37] Thus, it is important to examine the apoptosis-

inducing ability of loxoprofen in cancer cells and we here used cultured AGS cells for this purpose.

As shown in Fig. 4A, each NSAID induced apoptosis in a dose dependent manner in AGS cells and loxoprofen and loxoprofen-OH showed less activity for inducing apoptosis than indomethacin and celecoxib. We confirmed that cell death observed in Fig. 4A is mediated by apoptosis, because it was accompanied by apoptotic DNA fragmentation and apoptotic chromatin condensation (Fig. 4B and C). Comparing to data in primary culture of gastric mucosal cells (Fig. 2), loxoprofen and loxoprofen-OH induced apoptosis more potently in AGS cells. The ED_{70} values of NSAIDs for apoptosis (concentrations of NSAIDs required for 70% cell viability by apoptosis) of loxoprofen and loxoprofen-OH were lower in AGS cells than in primary culture of gastric mucosal cells (Table 1). On the other hand, the ED_{70} values for apoptosis of indomethacin and celecoxib were nearly indistinguishable between AGS cells and primary culture of gastric mucosal cells (Table 1). Although the underlying mechanism is unknown at present, this character of loxoprofen and loxoprofen-OH may be clinically beneficial for their application as anti-tumor drugs.

We also examined the effect of NSAIDs on the COX activity in cultured AGS cells. As shown in Fig. 4D, each of all NSAIDs tested decreased the amount of PGE_2 in the culture medium, in other words, inhibited COX activity in a dose dependent manner.

In summary, we show here that loxoprofen and loxoprofen-OH have a very low level of direct cytotoxicity on gastric mucosal cells *in vitro*. As described above, it is well known that loxoprofen is clinically safe on gastric mucosa compared to other NSAIDs such

as indomethacin.[25,26] We propose here that the low direct cytotoxicity of loxoprofen make it less harmful on the gastric mucosa for clinical use.

As described above, we have suggested that both COX inhibition (decrease in gastric level of PGE_2) and gastric mucosal cell death are required for the formation of NSAID-induced gastric lesions *in vivo* (Fig. 5).[20,24] Based on this idea, either NSAIDs without decreasing gastric level of PGE_2 or NSAIDs with lower cytotoxic effect should be safe NSAIDs on gastric mucosa. In other words, NSAIDs that have high cytotoxic effect on gastric mucosa and high ability to inhibit COX-1 expressed in gastric mucosa should have high risk for formation of gastric lesions. Indomethacin belongs to this type of NSAIDs and has relatively high risk for formation of gastric lesions clinically. Selective COX-2 inhibitors are relatively safe for gastric mucosa, because they have lower ability to inhibit COX-1 expressed in gastric mucosa, resulting in maintenance of gastric level of PGE_2. However, potential risk for cardiovascular thrombotic events is concern. Thus, we propose that NSAIDs with lower cytotoxic effect likely to be therapeutically beneficial NSAIDs in terms of gastrointestinal and cardiovascular safety. We are now synthesizing derivatives of loxoprofen to obtain more safe NSAIDs on gastric mucosa.

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FIGURE AND TABLE LEGENDS

Table. 1. NSAID concentrations required for apoptosis.

ED_{70} values of NSAIDs for apoptosis (concentrations of NSAIDs required for 70% cell viability by apoptosis) in primary culture of gastric mucosal cells and in AGS cells were calculated based on results provided in Fig. 1 and Fig. 4, respectively.

Fig. 1. Necrosis induced by NSAIDs in primary culture of gastric mucosal cells.

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 1 h. Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3).

Fig. 2. Apoptosis induced by NSAIDs in primary culture of gastric mucosal cells.

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 18 h. Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3) (A). Apoptotic DNA fragmentation (B) and chromatin condensation (C) were monitored as described in materials and methods.

Fig. 3. Membrane permeabilization by NSAIDs.

Calcein-loaded liposomes were incubated for 10 min at 30°C with indicated concentrations of NSAIDs. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization (A). Rat erythrocytes were incubated in the presence of each of NSAIDs for 10 min at 30°C. Hemolysis was estimated by measuring the absorbance at 520 nm (B).

Fig. 4. Apoptosis induced by NSAIDs in AGS cells.

AGS cells were incubated with indicated concentrations of NSAIDs for 24 h (A-C) or 4 h (D). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3) (A). Apoptotic DNA fragmentation (B) and chromatin condensation (C) were monitored as described in materials and methods. The arachidonic acid (50 μM at final) was added and cells were incubated for 15 min. The amount of PGE_2 in culture medium was determined by EIA and results were shown as inhibition of COX synthesis (D).

Fig. 5. A model for production of gastric lesions by NSAIDs.

We have proposed that both COX inhibition (decrease in gastric level of PGE_2) and gastric mucosal cell death are required for the formation of NSAID-induced gastric lesions *in vivo*.[20,24] This idea can explain the safety of selective COX-2 inhibitors, such as celecoxib, and loxoprofen on gastric mucosa, because they have lower ability to decrease gastric level of PGE_2 and to induce gastric mucosal cell death, respectively.

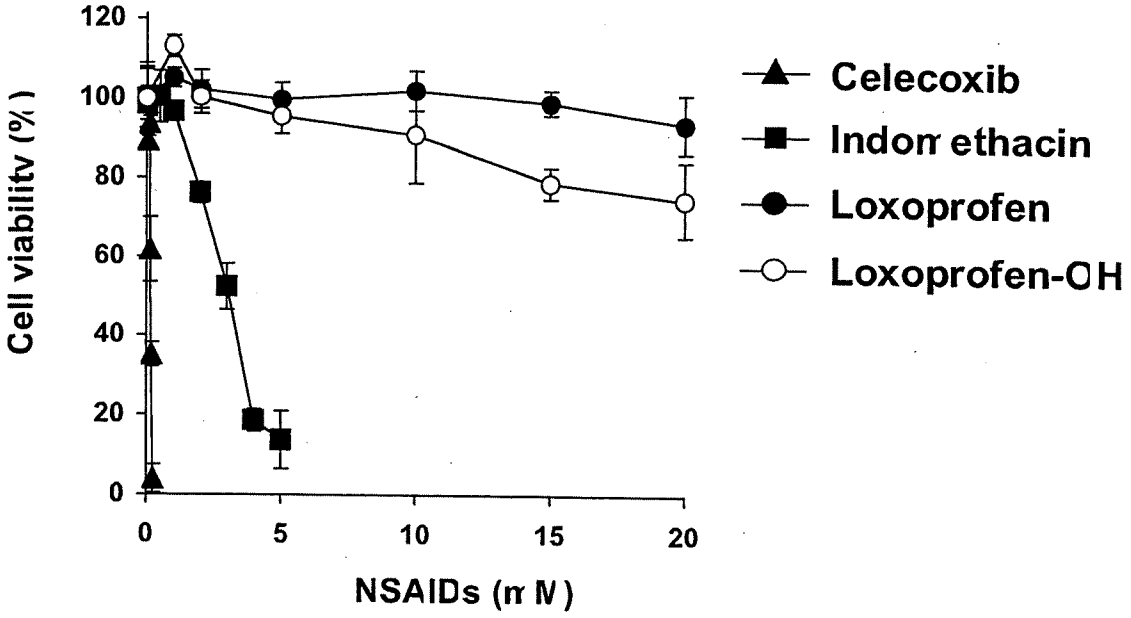
120
100
80
60
40
20
0
Cell viability (%)
0
5
10
15
20
NSAIDs (mM)
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH

Fig. 1

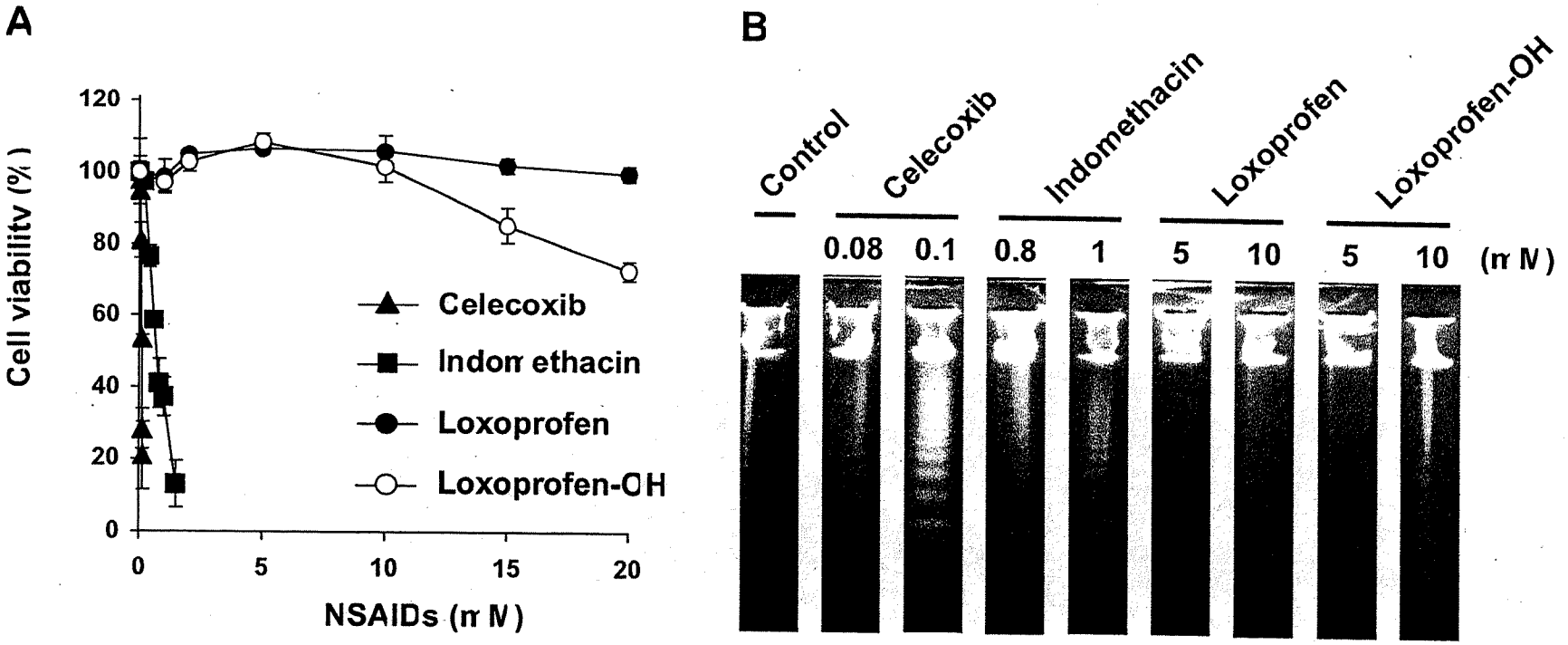
A
Cell viability (%)
120
100
80
60
40
20
0
0
5
10
15
20
NSAIDs (mM)
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH
B
Control
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH
0.08
0.1
0.8
1
5
10
5
10
(mM)

Fig. 2

C

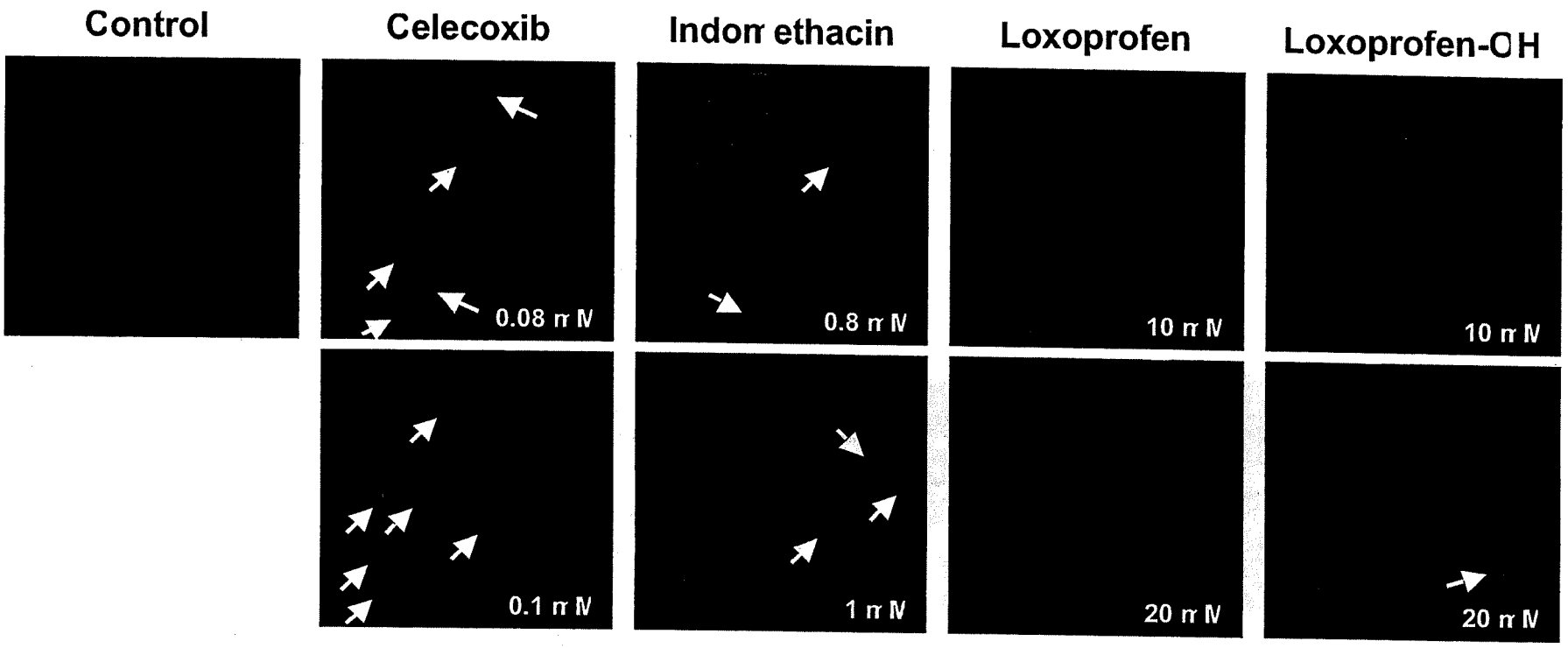
Control
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH
0.08 mM
0.8 mM
10 mM
10 mM
0.1 mM
1 mM
20 mM
20 mM

Fig. 2

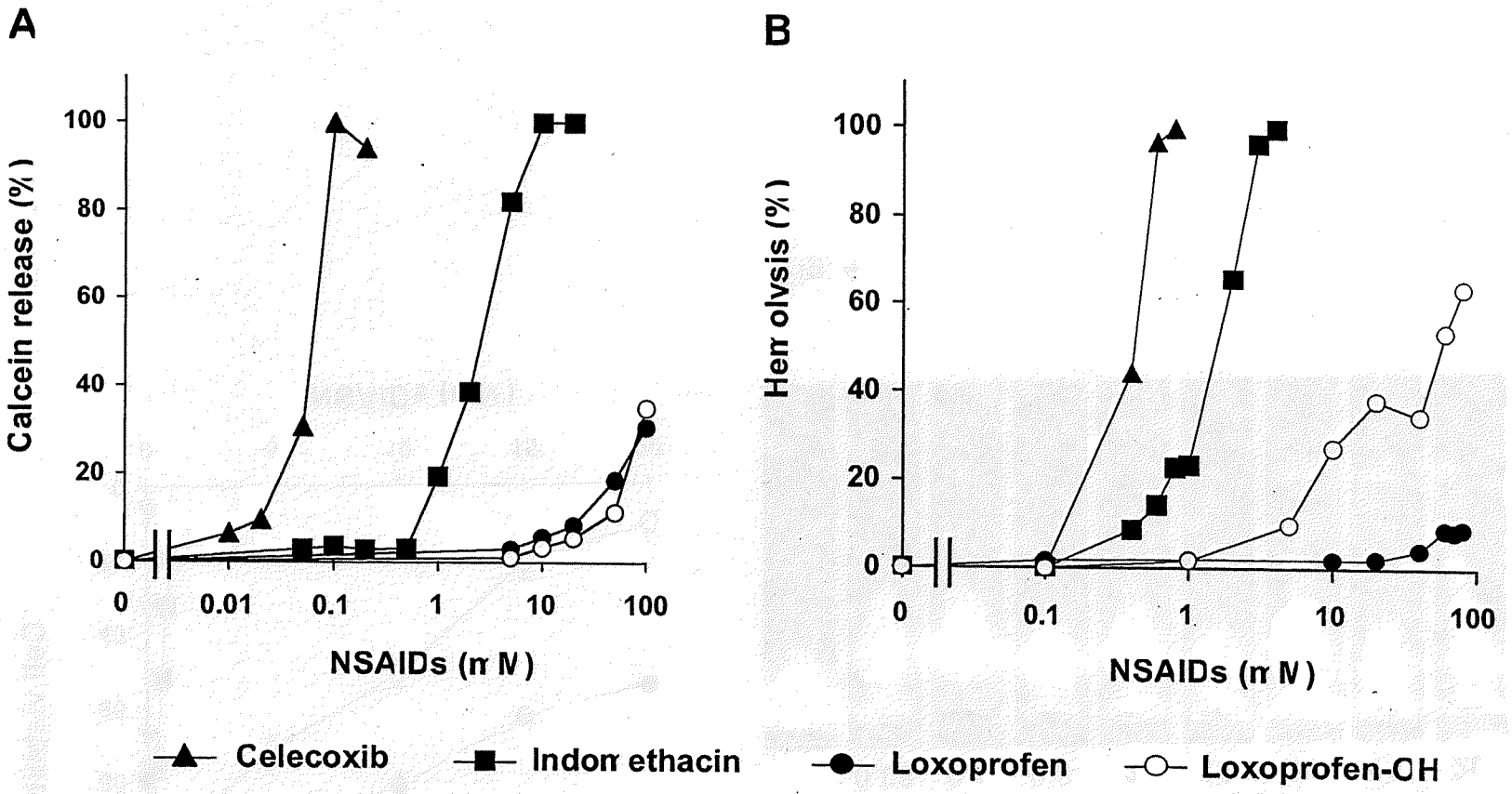
A
Calcein release (%)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
NSAIDs (μM)
B
Hemolysis (%)
100
80
60
40
20
0
0
0.1
1
10
100
NSAIDs (μM)
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH

Fig. 3

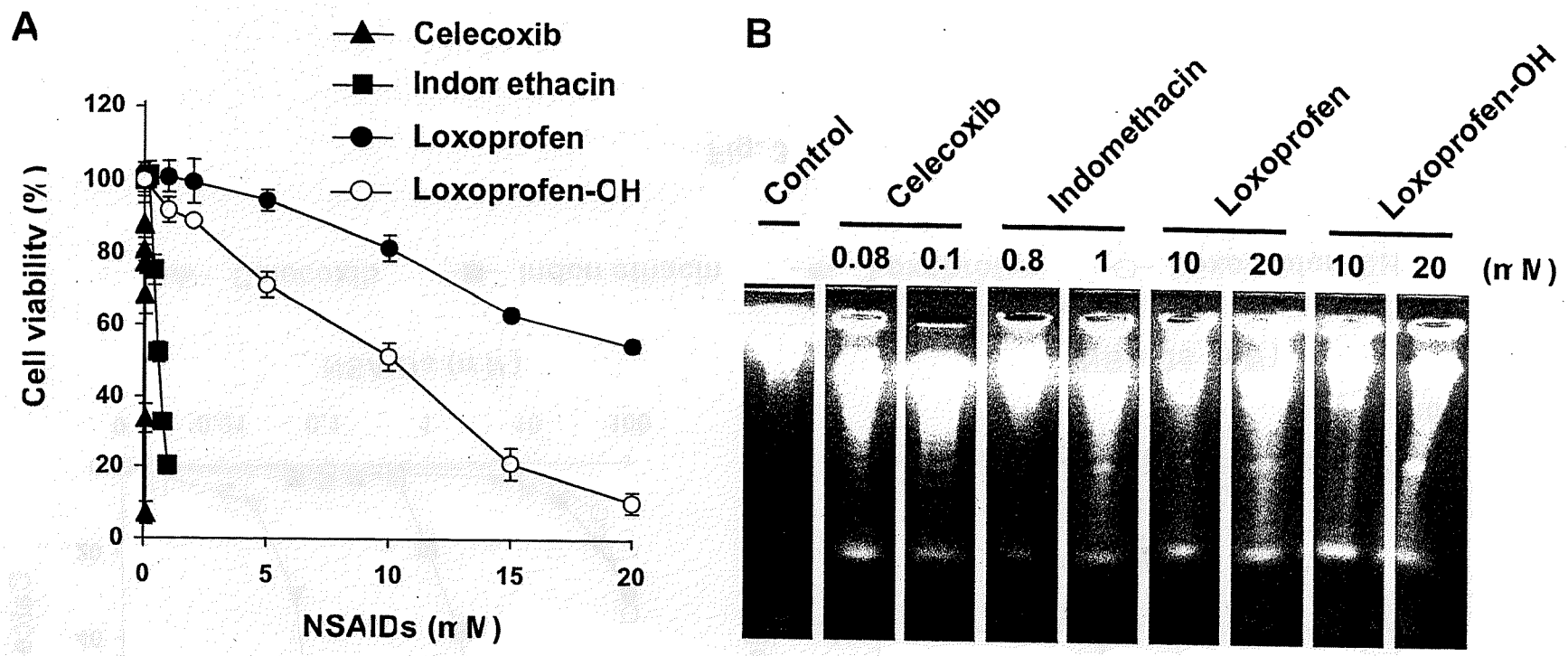
A
Celecoxib
Indom ethacin
Loxoprofen
Loxoprofen-OH
Cell viability (%)
120
100
80
60
40
20
0
0
5
10
15
20
NSAIDs (m M)
B
Control
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH
0.08
0.1
0.8
1
10
20
10
20
(m M)

Fig. 4

194

C

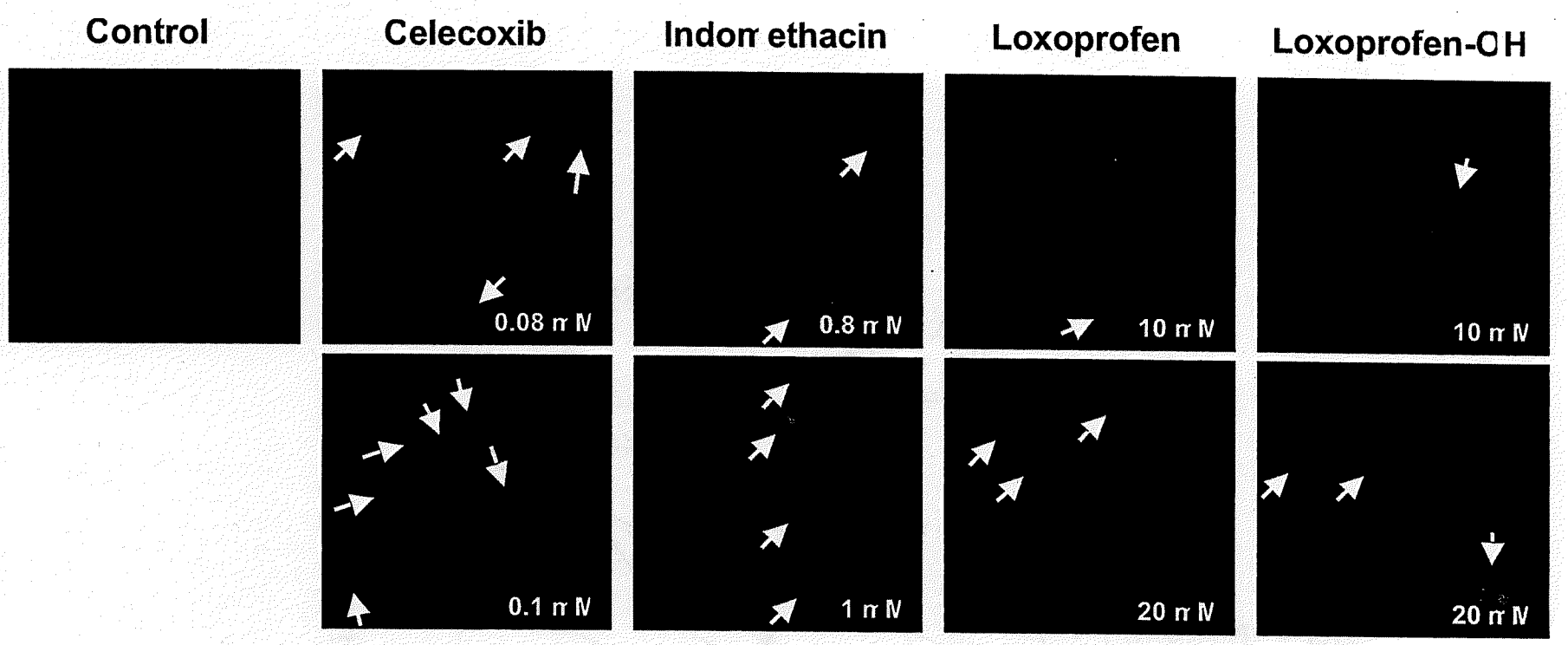
Control
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH
0.08 mM
0.8 mM
10 mM
10 mM
0.1 mM
1 mM
20 mM
20 mM

Fig. 4

D

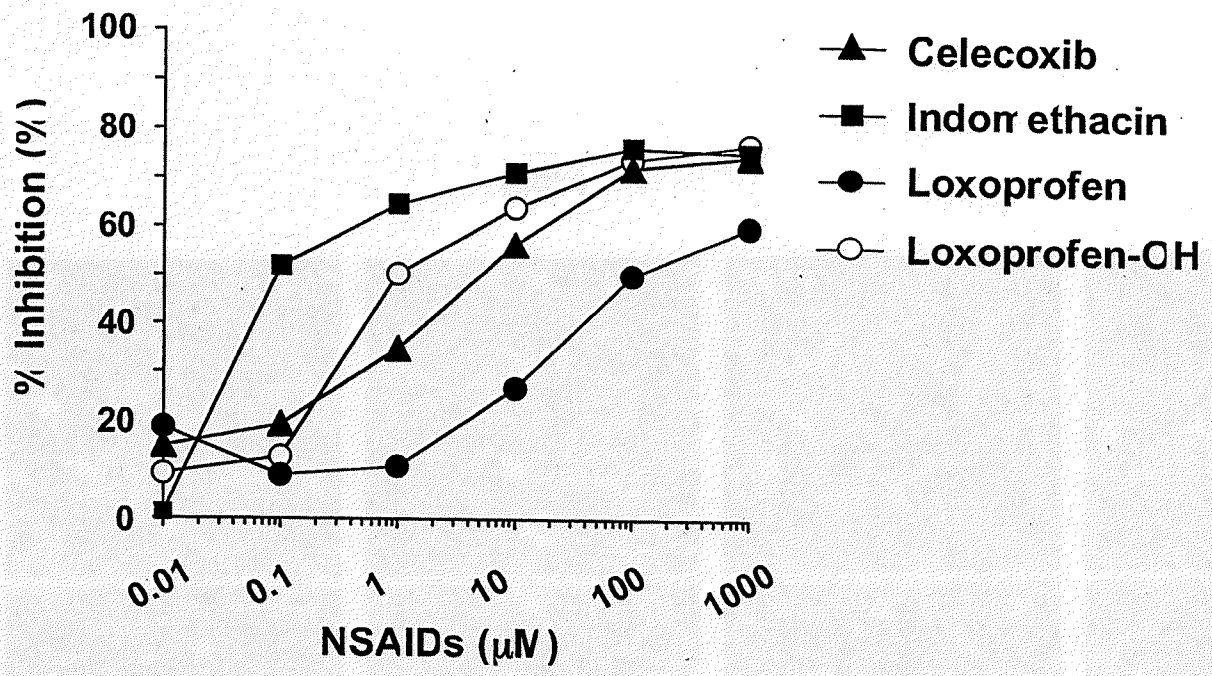
100
80
60
40
20
0
% Inhibition (%)
0.01
0.1
1
10
100
1000
NSAIDs (μM)
Celecoxib
Indomethacin
Loxoprofen
Loxoprofen-OH

Fig. 4

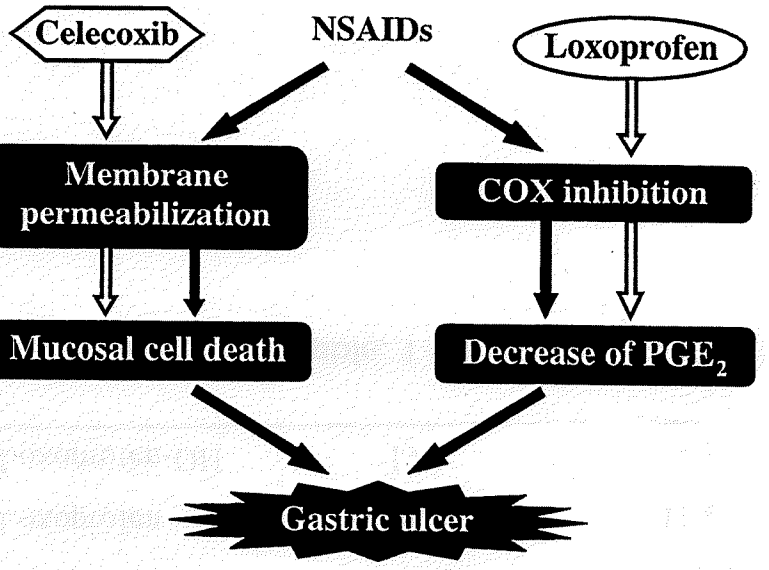
Celecoxib
NSAIDs
Loxoprofen
Membrane permeabilization
COX inhibition
Mucosal cell death
Decrease of PGE_2
Gastric ulcer

Fig. 5

NSAIDs	Primary cell (mM)	AGS cell (mM)
Celecoxib	0.06	0.05
Indomethacin	0.42	0.40
Loxoprofen	< 20	11.8
Loxoprofen-OH	19.1	4.3

Table. 1