measuring fluorescence intensity. Melittin (10 µM) was used to determine the 100% level of

membrane permeabilization.

Fig. 4. NSAID-induced hemolysis from erythrocytes.

Human erythrocytes were incubated in the presence of each of NSAIDs for 10 min at

30°C. Hemolysis was estimated by measuring the absorbance at 540 nm. Melittin (10 µM),

a membrane permeabilizing reagent, was used to determine the 100% level of hemolysis

(33) .

**Fig. 5.** Production of gastric lesions by NSAIDs.

Rats were intravenously administered with indomethacin or vehicle. After 1 h,

animals were administered orally with nabumetone, celecoxib, 6MNA or vehicle. After 6 h,

the stomach was removed and scored for hemorrhagic damage as described in Materials and

Methods section. Values are mean ± S.D. (n=5-6). **$P<0.01$; *$P<0.05$. n. d.; not

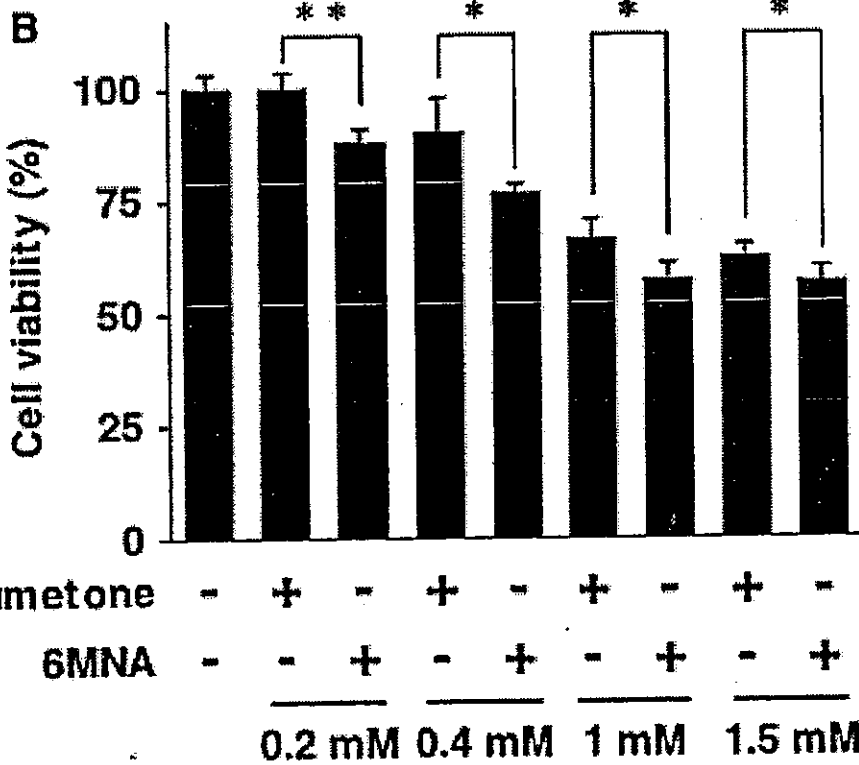detected.

Arai et al. Table 1.

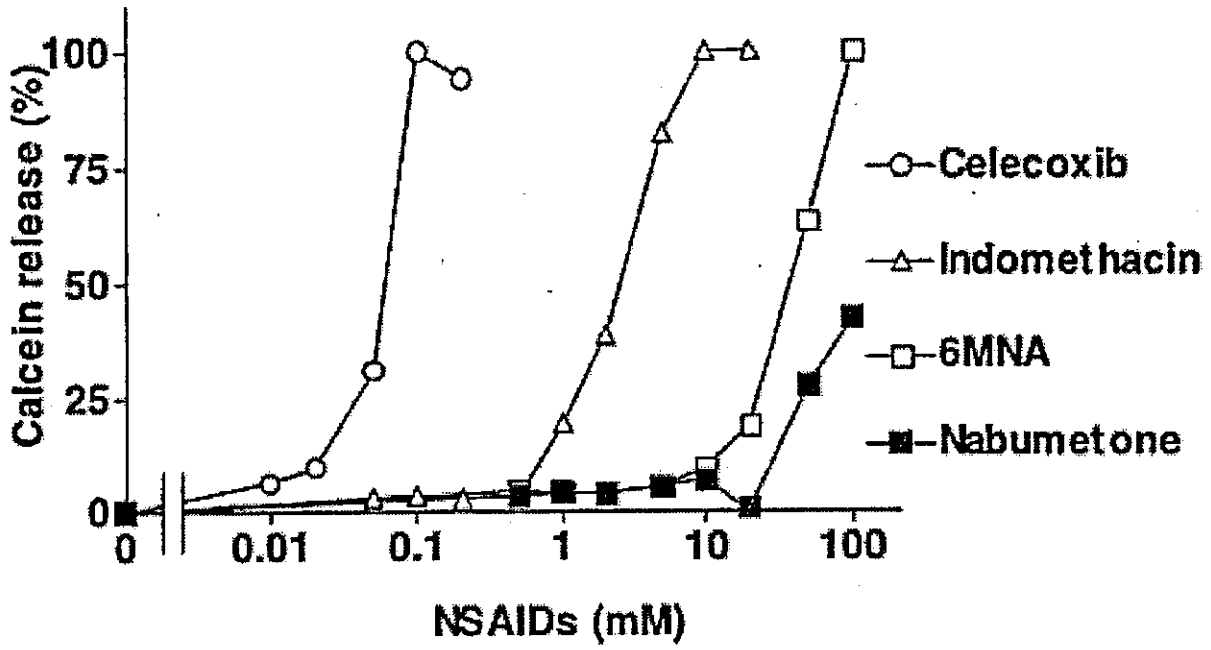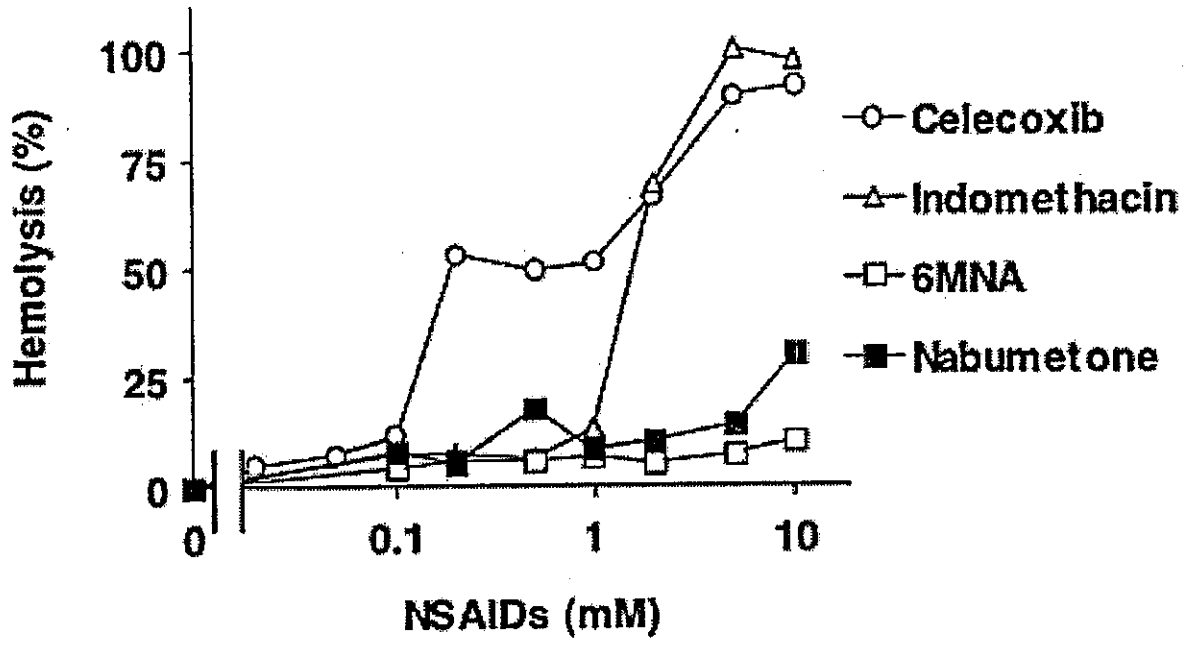| NSAIDs	Gastric PGE$_2$ (ng/g tissue)
Control	29.3 ± 2.6
5 mg/kg indomethacin i.v.	2.4 ± 0.4***
15 mg/kg celecoxib p.o.	32.4 ± 11.8
60 mg/kg nabumetone p.o.	19.1 ± 9.6
60 mg/kg 6MNA p.o.	2.8 ± 0.6***
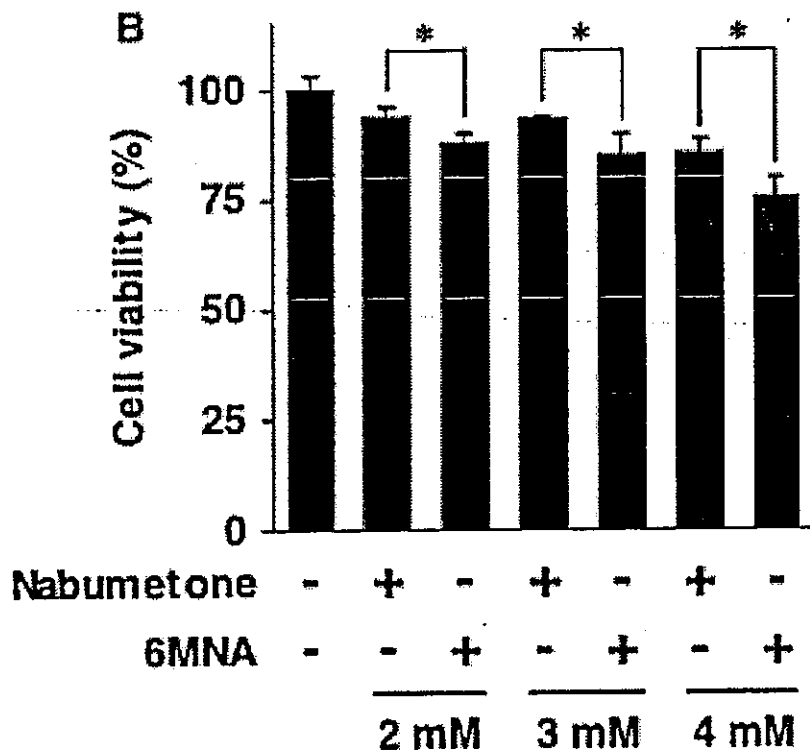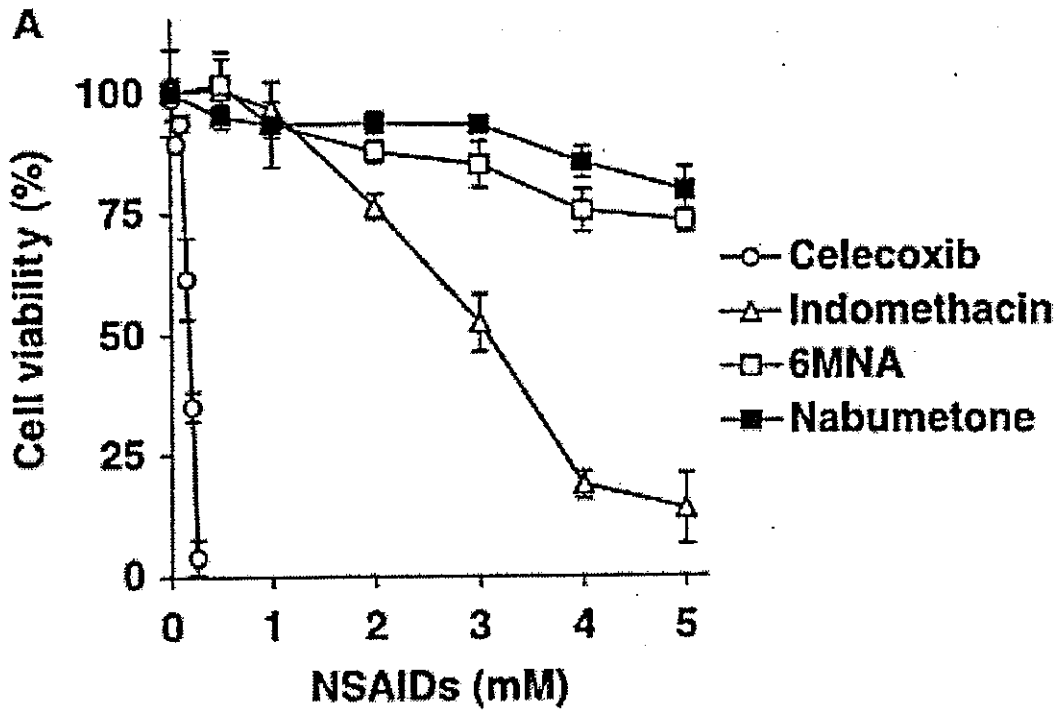
Arai *et al.* Fig. 1.

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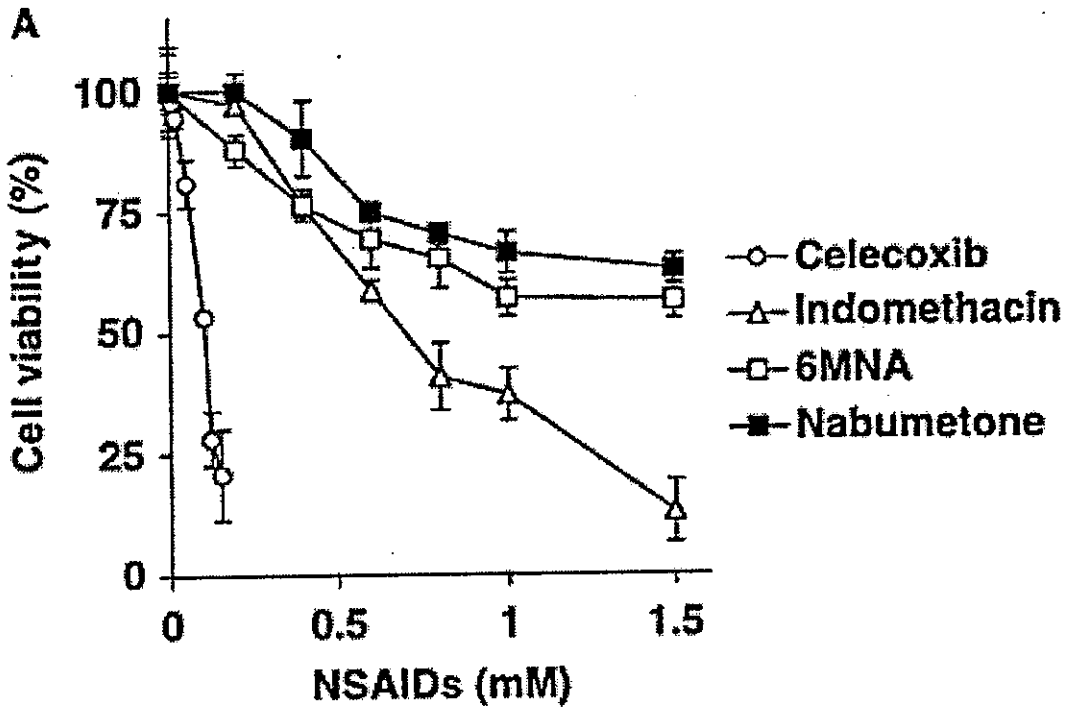
Arai *et al.* Fig. 3.

Arai *et al.* Fig. 4.

Arai *et al.* Fig. 5.
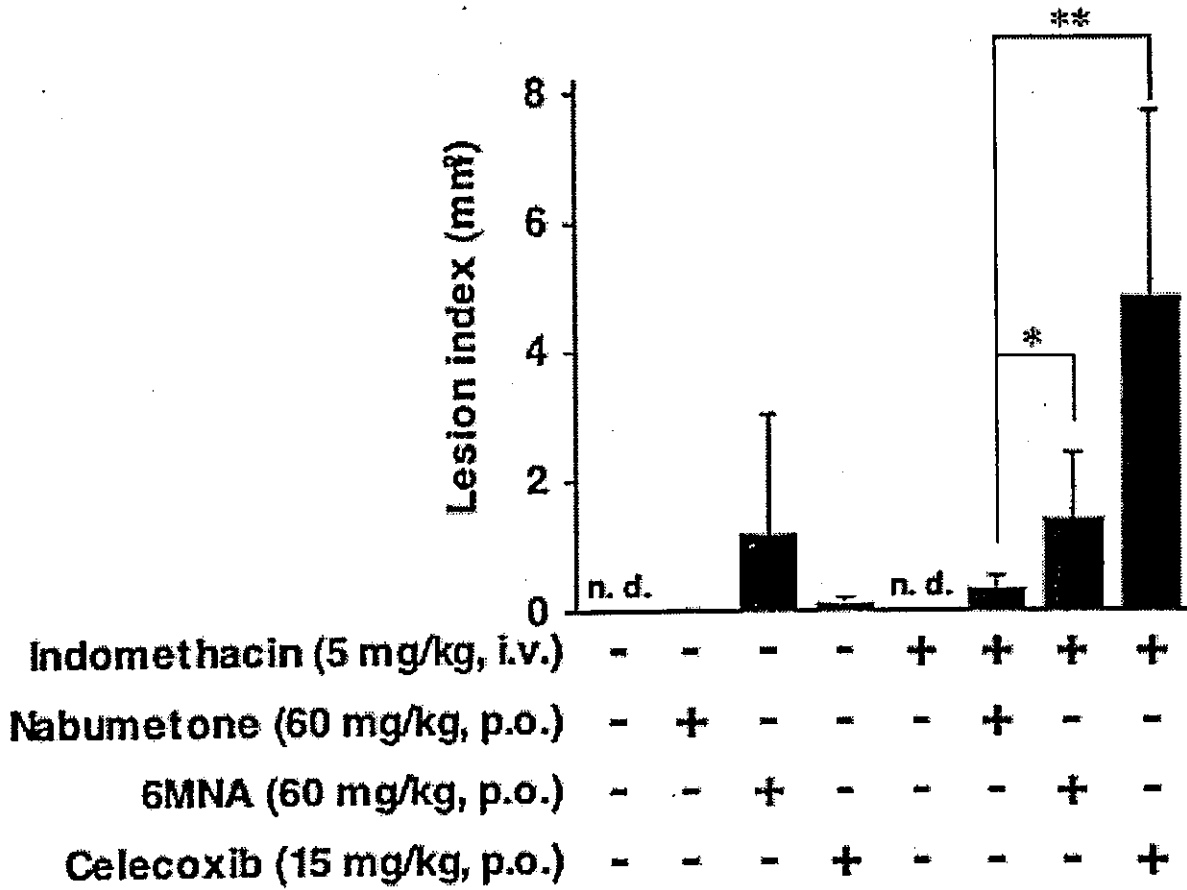
Low Direct Cytotoxicity and Cytoprotective Effects of

Nitric Oxide-Releasing Indomethacin

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Running head: Cytotoxicity of NO-NSAIDs

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Nitric oxide (NO)-releasing non-steroidal anti-inflammatory drugs (NSAIDs) have shown a marked reduction of gastrointestinal side effects and we here examined the cytotoxicity of NCX 530 (NO-indomethacin). Under conditions where indomethacin clearly induced both necrosis and apoptosis, NCX 530 induced neither. NCX 530 protected cells from celecoxib-induced necrosis and apoptosis. NCX 530 partially suppressed celecoxib-dependent membrane permeabilization and an inhibitor for guanylate cyclase suppressed the cytoprotective effect of NCX 530 against celecoxib. *In vivo*, NCX 530 alone produced fewer gastric lesions in rats than did indomethacin. A combination of the oral administration of celecoxib together with the intraperitoneal administration of indomethacin, but not of NCX 530, clearly resulted in the production of gastric lesions. The low direct cytotoxicity and the cytoprotective effect of NCX 530 observed *in vitro* may also act *in vivo*, thus ensuring that NCX 530 is safe for use on the gastric mucosa.

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Non-steroidal anti-inflammatory drugs (NSAIDs) are a very popular class of medicines that account for nearly 5% of all prescribed medications (1). The anti-inflammatory action of NSAIDs is mediated through their inhibition of cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the reverse side, NSAID use is associated with gastrointestinal complications (2), with about 15-30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (3-6). In the United States, about 16,500 people die per year as a result of NSAID-associated gastrointestinal complications (7).

The inhibition of COX by NSAIDs was previously thought to be the sole explanation for their associated gastrointestinal side effects (8), given that PGs have a strong protective effect on the gastrointestinal mucosa (9). However, since the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (10, 11), it was proposed that the induction of gastrointestinal lesions by NSAIDs involved additional mechanisms (12). It is well known that NSAIDs have a direct cytotoxic effect on gastrointestinal mucosal cells (12-14). We recently demonstrated that NSAIDs induce both necrosis and apoptosis in cultured gastric mucosal cells in a manner independent of COX inhibition (15, 16). Furthermore, we recently proposed that both COX inhibition and the direct cytotoxic effect of NSAIDs (direct cell damage) on the gastric mucosa are involved in the production of gastric lesions *in vivo*; gastric lesions develop in a

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manner that depends on both an intravenously administered low dose of indomethacin (inhibition of COX activity without direct gastric mucosal cell damage) and an orally administered cytotoxic COX-2-selective inhibitor, such as celecoxib (direct cell damage without inhibition of COX at the gastric mucosa) (16). Therefore, the direct cytotoxicity of individual NSAIDs is one of key factors to determine the safety of their use on the gastric mucosa.

Nitric oxide (NO)-releasing NSAIDs (NO-NSAIDs) are a newly developed group of NSAIDs, consisting of a traditional NSAID to which a group donating NO has been covalently attached via spacers. NO-NSAIDs show a marked reduction of gastrointestinal side effects but maintain, and in some cases extend, anti-inflammatory properties both in clinical studies on humans and in animal models (17-21). Further to this, given that the anti-thrombotic effect of NO-aspirin was reported to be superior to that of aspirin (22), much attention is now being paid to NO-NSAIDs as alternatives in treatment protocols.

Various mechanisms have been proposed for the gastrointestinal safety of NO-NSAIDs, such as inhibition of neutrophil adherence, promotion of mucosal blood flow and stimulation of mucin and bicarbonate secretion by NO (21, 23). In addition to these mechanisms, a lower cytotoxicity on gastric mucosal cells was reported for one NO-NSAID, NO-flurbiprofen, whose use resulted in reduced apoptosis compared to that seen with standard flurbiprofen (24). Furthermore, NO-flurbiprofen suppressed the extent of TNFα- or ceramide-induced apoptosis by inhibiting caspases *in vitro* (24, 25). However, necrosis induced by NO-NSAIDs and apoptosis induced by NO-NSAIDs other than NO-flurbiprofen

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are yet to have been studied. Furthermore, although more than two species of NSAIDs are usually used simultaneously in a clinical setting, the effect of NO-NSAIDs on cell death induced by other NSAIDs is also yet to be examined.

In this study, we used primary cultures of guinea pig gastric mucosal cells to examine the direct cytotoxicity of NCX 530 (NO-indomethacin), which according to one animal study (17) is safe from the perspective of the gastric mucosa. We also examined the effect of NCX 530 on cell death induced by other gastric-irritants, including NSAIDs. NCX 530 not only showed a lower propensity than indomethacin for inducing both necrosis and apoptosis, but also protected cells from necrosis and apoptosis induced by celecoxib or ethanol. *In vivo*, NCX 530 alone or in combination with orally administered celecoxib or ethanol resulted in the production of fewer gastric lesions than did indomethacin. Furthermore, NCX 530 suppressed the production of gastric lesions caused by other gastric irritants. These results concerning the safety of NCX 530 use *in vivo* are probably related to the low direct cytotoxicity and cytoprotective effects of NCX 530 observed *in vitro*.

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MATERIALS AND METHODS

Chemicals and Media. Fetal bovine serum (FBS) was from Gibco Co. 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), 1H-[1,2,4-]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and cycloheximide were from Sigma Co. Celecoxib was from LKT Laboratories Inc. Indomethacin and NCX 530 were kindly provided by NicOx S. A. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. Male Wistar rats weighing 160-200 g and male guinea pigs weighing 200-300 g were purchased from Shimizu Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

In vitro **Assay of Cytotoxicity and DNA Fragmentation.** Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (26, 27). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3% v/v FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% air and 37°C. After removing non-adherent cells, cells attached to the plate were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells (26, 28). NSAIDs were dissolved in DMSO and control experiments

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(without NSAIDs) were performed in the presence of same concentrations of DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium. Cell viability was determined by the MTT method.

Apoptotic DNA fragmentation was monitored as previously described (29). Cells were collected using a rubber policeman and suspended in 50 μl of lysis buffer, consisting of 50 mM Tris-HCl (pH7.8), 10 mM EDTA, and 0.5% sodium-N-lauroylsarcosinate. Proteinase K was added to a final concentration of 1 mg/ml, and the lysate incubated at 50°C for 2 h. RNaseA was then added to a final concentration of 0.5 mg/ml and incubated at 50°C for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide.

Gastric Damage Assay. Gastric damage assays were performed as described previously (16). Rats (24 h fasted) were administered orally with ethanol or NSAIDs in 1% methylcellulose in a volume of 5 ml/kg. In some experiments, indomethacin or NCX 530 in 1% methylcellulose was administered intraperitoneally 1 h before the oral administration. Six hours after the oral administration, the rats were anesthetized and the stomach was removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. The score involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

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Membrane Permeability Assay. Liposomes were prepared using the reversed-phase evaporation method (30, 31). Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1 : 2, v/v), dried, and dissolved in 1.5 ml of diethyl ether. This was followed by the addition of 1 ml of 100 mM calcein-NaOH (pH 7.4). The mixture was sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure at 25°C. The resulting suspension of liposome was centrifuged and washed twice with fresh buffer A (10 mM potassium buffer, containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 ml buffer A. A 0.3 ml aliquot of this suspension was diluted with 19.7 ml of buffer A, following which 400 μl of this suspension was incubated at 30°C for 10 min in the presence of the NSAID under investigation. The final PC concentration was 30 μM. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Statistical Analyses. All values are expressed as the mean ± standard error (S.E.M.). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used for evaluation of differences between the groups. A Student's t-test for unpaired results was performed for the evaluation of differences between two groups. Differences were considered to be significant for values of $P<0.05$.

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RESULTS

Comparison of Necrosis- and Apoptosis-inducing Activities of NCX 530 and Indomethacin. We previously reported that short-term (1 h) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs and long-term (16 h) treatment of these cells with relatively low concentrations of NSAIDs induces necrosis and apoptosis, respectively (15). The results of experiments in which guinea pig gastric mucosal cells in primary culture were treated with NCX 530 or indomethacin for 1 h are shown in Fig. 1A. Indomethacin decreased cell viability in a dose-dependent manner, while NCX 530 did not affect cell viability at concentrations lower than 5 mM. We confirmed that cell death induced by indomethacin (Fig. 1A) was mediated by necrosis as no accompanying apoptotic DNA fragmentation or chromatin condensation was evident (data not shown). The findings of experiments in which cells were treated with NCX 530 or indomethacin for 16 h are summarized in Fig. 1B. Again, indomethacin but not NCX 530 induced cell death. Because cell death under these conditions was accompanied by apoptotic DNA fragmentation (Fig. 1C), it is most likely to have been mediated by apoptosis. Overall, the results in Fig. 1 show that NCX 530 had a lower capacity than indomethacin for inducing necrosis and apoptosis.

Effect of NCX 530 on Cell Death Induced by Other Gastric Irritants. We next examined the cytoprotective effect of NCX 530 by pre-treating gastric mucosal cells with NCX 530

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for 1 h (pre-incubation step) and then with one of several of gastric irritants (celecoxib, ethanol, indomethacin or hydrogen peroxide) for 16 h (apoptotic conditions) (Fig. 2) or 1 h (Fig. 3) (necrotic conditions) (incubation step). As shown in Fig. 2A, treatment of cells with 0.1 mM celecoxib for 16 h induced cell death which could be partially suppressed by the pre-treatment of cells with NCX 530. A similar cytoprotective effect of NCX 530 was observed for cell death induced by exposure of cells to 3% ethanol for 16 h (Fig. 2B). Under these conditions (Fig. 2), the cell death was mediated by apoptosis given that apoptotic DNA fragmentation and chromatin condensation were also present (data not shown).

As shown in Fig. 3A, B, NCX 530 partially suppressed the cell death induced by treatment of cells with 0.18 mM celecoxib or 8% ethanol for 1 h. Cell death under these conditions was mediated by necrosis as apoptotic DNA fragmentation and chromatin condensation were not in evidence (data not shown). NCX 530 was included in both the pre-incubation and incubation steps of experiments whose results are detailed in Figs. 2 and 3. When NCX 530 was omitted in the pre-incubation step, its cytoprotective effect was similar to that when it was used in both the pre-incubation and incubation steps (data not shown). On the other hand, when NCX 530 was omitted from the incubation step (but included in the pre-incubation step), its cytoprotective effect was not as clear-cut. Thus, it seems that NCX 530 must be present simultaneously with celecoxib or ethanol in order to exert fully its cytoprotective effect.

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The cytoprotective effect of NCX 530 was not observed for all gastric irritants tested. For example, NCX 530 affected neither apoptosis (Fig. 2C, D) nor necrosis (Fig. 3C, D) induced by indomethacin or hydrogen peroxide. Similar results were obtained when NCX 530 was omitted in the pre-incubation or incubation steps (data not shown). Based on these findings, it appears that NCX 530 protects gastric mucosal cells from necrosis and apoptosis induced by celecoxib or ethanol but not by indomethacin or hydrogen peroxide.

Mechanism for the low cytotoxicity and cytoprotective effect of NCX 530. We recently found that NSAIDs cause membrane permeabilization, which in turn is implicated in their cytotoxicity (induction of necrosis and apoptosis); that is, NSAIDs directly permeabilize the membranes of liposomes, with concentrations of NSAIDs required for this effect being closely related to those which result in cytotoxicity (32). In further experiments, the ability of NCX 530 and indomethacin to permeabilize the membranes of calcein-loaded liposomes was compared. Calcein fluoresces very weakly 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 (30, 31). As shown in Fig. 4A, NCX 530 and indomethacin showed similar dose-response curves for the increase in calcein fluorescence, suggesting that their ability to cause membrane permeabilization is virtually indistinguishable. Therefore, it seems that the low cytotoxicity of NCX 530 cannot be explained on the basis of its membrane permeabilization activity. Celecoxib caused membrane permeabilization at very low concentrations, as found

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previously (32), which could be partially suppressed by NCX 530 (Fig. 4B) but not by

indomethacin (data not shown). On the other hand, NCX 530 did not affect the

indomethacin-dependent membrane permeabilization (data not shown). Therefore, the

cytoprotective effect of NCX 530 on celecoxib-induced cell death probably involves changes

to the membrane permeabilizing capacity of celecoxib. However, at present, it is unclear

why NCX 530 with membrane permeabilizing activity itself, protects membrane from

celecoxib but not from indomethacin.

NSAIDs have been shown stimulate the induction of some protective proteins such

as heat shock proteins (33) and endoplasmic reticulum chaperons (34). It is possible that

induction of protective proteins mediates the low cytotoxicity and the cytoprotective effect

of NCX 530. In order to test this possibility, we examined the effect of an inhibitor of

protein synthesis (cycloheximide) on the low cytotoxicity and cytoprotective effects of NCX

530. Pre-treatment of cells with cycloheximide did not affect cell viability after incubation

with NCX 530 for 1 h (necrotic conditions) (Fig. 5A). Furthermore, pre-treatment of cells

with cycloheximide did not affect the protective action of NCX 530 against celecoxib-

induced cell death (necrotic conditions) (Fig. 5B). This concentration of cycloheximide did

not affect the cell viability by itself but inhibited protein synthesis, the incorporation of

[^{35}S]methionine into acid-insoluble fractions to more than 90%. Since cycloheximide itself

inhibits apoptosis, we could not examine its effect on NCX 530-induced apoptosis or the

cytoprotective effect of NCX 530 on apoptosis. The results, however, suggest that proteins

newly synthesized in the presence of NCX 530 do not contribute to the low level of NCX

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