530-induced necrosis or to the cytoprotective effect of NCX 530 on celecoxib-induced necrosis.

NO stimulates guanylaté cyclase, resulting in an increase in cGMP. Since an increase in cGMP in cells is known to inhibit apoptosis via the inhibition of caspase-3 (35), it is possible that activation of guanylate cyclase by NCX 530 is responsible for its low cytotoxicity and cytoprotective effects. In order to test this possibility, the effect of an inhibitor of guanylate cyclase (ODQ) on cell death in the presence of NCX 530 was examined. Pre-treatment of cells with ODQ did not affect the cell viability after treatment with NCX 530 for 1 h (necrotic conditions) (Fig. 6A). Furthermore, pre-treatment of cells with ODQ did not alter the extent of cell death induced by celecoxib in the presence NCX 530 for 1 h (necrotic conditions) (Fig. 6B). On the other hand, when the incubation period was changed to 16 h (apoptotic conditions), pre-treatment of cells with ODQ decreased the cell viability following treatment with NCX 530 (apoptotic conditions) (Fig. 6C) and increased the level of cell death induced by celecoxib in the presence NCX 530 (apoptotic conditions) (Fig. 6D). This concentration of ODQ did not affect the cell viability by itself (data not shown), however, enough to almost completely inhibit guanylate cyclase, based on previous papers (36, 37). These results suggest that activation of guanylate cyclase by NCX 530 may play an important role in the low cytotoxic activity and the cytoprotective effect of NCX 530 for apoptosis, but not for necrosis.

Production of Gastric Lesions by NCX 530. The low cytotoxicity of NCX 530 suggests that it is less likely to produce gastric lesions *in vivo*. As shown in Fig. 7, orally administered NCX 530 (42.7 mg/kg) did not produce gastric lesions to any significant extent, whereas orally administered indomethacin (30 mg/kg) (equal molar) clearly produced gastric lesions. This finding is consistent with a previous report (17) and shows that, in relation to its effects on the gastric mucosa *in vivo*, NCX 530 is safe for use.

As described in the introduction section, we recently found that gastric lesions develop in a manner that depends on both intravenously administered low doses of indomethacin and orally administered cytotoxic COX-2 selective inhibitors, such as celecoxib (16). Using this model, the ability of NCX 530 and indomethacin to produce gastric lesions was tested when either of these compounds was used in combination with the oral administration of celecoxib. Here, NCX 530 and indomethacin were administered intraperitoneally. As shown in Fig. 8, the oral administration of celecoxib alone or the intraperitoneal administration of a low dose (5 mg/kg) of indomethacin alone did not produce gastric lesions to any significant extent; however, simultaneous administration of both of compounds clearly produced gastric lesions as previously reported (16). In contrast, gastric lesions were not produced when the oral administration of celecoxib and the intraperitoneal administration of NCX 530 were used in combination (Fig. 8). Furthermore, intraperitoneally administered NCX 530 suppressed the production of gastric lesions following the oral administration of celecoxib together with the intraperitoneal administration of indomethacin (Fig. 8).

We also examined the effect of the intraperitoneal administration of NCX 530 on the production of gastric lesions by other gastric irritants. As shown in Fig. 9A, NCX 530 administered in this way significantly decreased the ethanol-induced production of gastric lesions. In contrast, gastric lesions were clearly apparent when indomethacin was administered in place of NCX 530 (Fig. 9A). On the other hand, the intraperitoneal administration of NCX 530 did not affect the production of gastric lesions following the oral administration of high doses (30 mg/kg) of indomethacin (Fig. 9B). Therefore, NCX 530 can suppress the production of gastric lesions by some but not all gastric irritants.

DISCUSSION

In this study, the cytotoxicity of NCX 530, one of the new breed of NO-NSAIDs, was assessed. NCX 530 induced both necrosis and apoptosis in gastric mucosal cells in primary culture at much lower levels than did indomethacin. These results are apparently inconsistent with recently published results (38). This may be due to the difference in species of cells and NO-indomethacin; they used colon cancer cells and another NO-indomethacin (NCX 2121) (38). The cytotoxicity of an irritant is determined by both its own toxicity and its capacity to induce cellular stress responses, which in turn protect cells from the irritant. The low cytotoxicity of NCX 530, however, could not be explained by its own toxicity given that NCX 530 gave rise to a similar degree of membrane permeabilization as that seen for indomethacin. On the other hand, a cGMP-dependent cellular response could be involved in the low level induction of apoptosis by NCX 530, since an inhibitor of guanylate cyclase (ODQ) stimulated apoptosis in the presence of NCX 530.

We also found that NCX 530 protects gastric mucosal cells from celecoxib-induced necrosis and apoptosis. This cytoprotective effect of NCX 530 involved both membrane permeabilization and a cGMP-dependent cellular stress response; NCX530 partially suppressed celecoxib-dependent membrane permeabilization and ODQ inhibited the NCX 530-dependent protection of cells from celecoxib-induced necrosis and apoptosis. However, the reason why NCX 530 protects cells from some irritants (celecoxib, ethanol), but not

others (indomethacin, hydrogen peroxide) is yet to be determined. Since the suppression by ODQ of the cytoprotective effect of NCX 530 was partial, other mechanisms may be involved in this cytoprotection. In addition to membrane permeabilization, stimulation of mucus synthesis by NCX530 may be involved in this cytoprotection as suggested previously (39).

We recently proposed that not only COX inhibition but also the direct cytotoxic effect of NSAIDs (direct cell damage at the gastric mucosa) is involved in the development of gastric lesions (16). On this basis, we proposed that NSAIDs that did not inhibit COX at the gastric mucosa or were without direct cytotoxic effects would not be capable of producing gastric lesions (16). Selective COX-2 inhibitors are NSAIDs that do not inhibit COX at the gastric mucosa, keeping in mind that the primary form of COX expressed at the gastric mucosa is COX-1. However, a recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events, which is caused by their specificity for COX-2 (40-46). As such, we proposed that NSAIDs without both specificity for COX-2 and direct cytotoxicity are safe for use from a viewpoint of the gastric mucosa and cardiovascular system and therefore have important advantages for clinical use (16). Based on results of this study, NCX 530 may belong to this category of NSAIDs.

A combination of the oral administration of celecoxib with the intraperitoneal administration of NCX 530 did not produce gastric lesions, which is different from the case of intraperitoneal administration of indomethacin. Furthermore, NCX 530 administered intraperitoneally suppressed the production of gastric lesions induced by ethanol or

celecoxib plus indomethacin. Since many factors can affect the production of gastric lesions *in vivo* (mucosal blood flow and gastric motility for example), a number of interpretations for this phenomenon are possible. However, we consider that the direct cytotoxicity of NSAIDs, or direct cell damage at the gastric mucosa by NSAIDs in other words, can explain this phenomenon. In gastric lesions produced by a combination of the oral administration of celecoxib with the intraperitoneal administration of indomethacin or NCX 530, the direct cell damage at gastric mucosa should occur on account of the orally administered celecoxib. As shown *in vitro*, NCX 530 may suppress celecoxib-induced cell death at the gastric mucosa, meaning that NCX 530 does not actually produce gastric lesions when administered in conjunction with the celecoxib. This idea can also be used to explain the NCX 530-dependent suppression of the production of gastric lesions by ethanol or celecoxib plus indomethacin, given that, *in vitro*, NCX 530 protected the gastric mucosal cells not only from celecoxib but also from ethanol. Furthermore, observations that NCX 530 did not protect gastric mucosal cells from indomethacin *in vitro* may explain why the production of gastric lesions by the oral administration of high doses of indomethacin was not suppressed by NCX 530 *in vivo*. However, in Fig. 8, NCX 530 almost completely inhibited the production of gastric lesions by celecoxib *in vivo*, whereas the effect of this drug on celecoxib-induced cell death is partial *in vitro* (Fig. 2A and 3A). Previous papers reported that NCX 530 stimulated mucosal blood flow and mucus synthesis and did not so clearly increase gastric motility and adhesion of neutrophil as indomethacin (17, 39). We consider that these phenomenon are involved in the safety of NCX 530 on gastric mucosa *in vivo*.

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FIGURE LEGENDS

Fig. 1. Necrosis and apoptosis induced by NCX 530 or indomethacin.

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NCX 530 or indomethacin for 1 h (A) (necrotic conditions) or 16 h (B, C) (apoptotic conditions). Cell viability was determined by the MTT method (A, B). Values are mean ± S.E.M. (n=3). ***P<0.001; **P<0.01. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (C).

Fig. 2. Effect of NCX 530 on apoptosis induced by various gastric irritants.

Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of NCX 530 for 1 h and further incubated with 0.1 mM celecoxib (A), 3% ethanol (B), 0.6 mM indomethacin (C) or 0.4 mM hydrogen peroxide (D) in the presence of indicated concentrations of NCX 530 for 16 h (apoptotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3). **P<0.001; **P<0.01; *P<0.05.

Fig. 3. Effect of NCX 530 on necrosis induced by various gastric irritants.

Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of NCX 530 for 1 h and further incubated with 0.18 mM celecoxib (A), 8% ethanol (B), 3 mM indomethacin (C) or 1 mM hydrogen peroxide (D) in the presence of

indicated concentrations of NCX 530 for 1 h (necrotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3). ***P<0.001; **P<0.01; *P<0.05.

Fig. 4. Membrane permeabilization by NSAIDs.

Calcein-loaded liposomes were incubated for 10 min at 30°C with indicated concentrations of each NSAID (A) or 0.1 mM celecoxib plus indicated concentrations of NCX530 (B). 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 (47).

Fig. 5. Effect of cycloheximide on cell viability in the presence of NCX 530.

Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of cycloheximide for 1 h. Cells were further incubated with 2 mM NCX 530 and indicated concentrations of cycloheximide for 1 h (A) (necrotic conditions). Cells were pre-incubated with indicated concentrations of cycloheximide and 1 mM NCX 530 for 1 h. Cells were further incubated with 1 mM NCX 530, 0.18 mM celecoxib and indicated concentrations of cycloheximide for 1 h (B) (necrotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3).

Fig. 6. Effect of ODQ on cell viability in the presence of NCX 530.

Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of ODQ for 1 h. Cells were further incubated with indicated concentrations of NCX 530 and ODQ (A, C). Cells were pre-incubated with indicated concentrations of ODQ and 1 mM NCX 530 for 1 h. Cells were further incubated indicated concentrations of ODQ, NCX530 and celecoxib (B, D). Incubation was performed for 1 h (A, B) (necrotic conditions) or for 16 h (C, D) (apoptotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3). ***P<0.001; *P<0.05.

Fig. 7. Production of gastric lesions by NCX 530 or indomethacin.

Rats were orally administered with NCX 530 or indomethacin as indicated. After 6 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=5 - 6). **P<0.01. n. d.; not detected

Fig. 8. Production of gastric lesions by NCX 530 or indomethacin in combination with celecoxib.

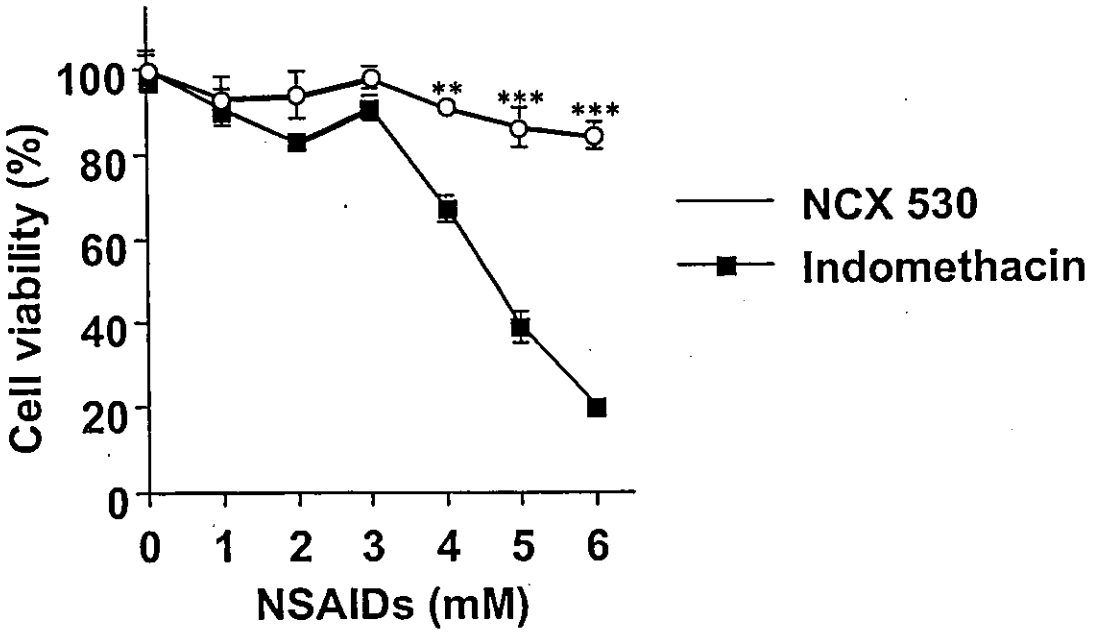
Rats were intraperitoneally administered with 5 mg/kg indomethacin or 7.1 mg/kg NCX 530 or vehicle. After 1 h, animals were administered orally with 15 mg/ml celecoxib or vehicle. After 6 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=5 - 6). **P<0.01; *P<0.05. n. d.; not detected

Fig. 9. Effect of NCX 530 on production of gastric lesions by other gastric irritants.

Rats were intraperitoneally administered with 7.1 mg/kg NCX 530 or 5 mg/kg indomethacin or vehicle. After 1 h, animals were administered orally with ethanol (A) or 30 mg/kg indomethacin (B) or vehicle. After 6 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=5 - 6). *P<0.05. n. d.; not detected

Tomisato et al. Fig. 1

A



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