on the market. Because most NSAID prodrugs do not possess any significant specificity for COX-2, these prodrugs may become very important as NSAIDs, considering the potential risk for cardiovascular thrombotic events of selective COX-2 inhibitors.

NSAIDs have a direct cytotoxic effect on gastrointestinal mucosal cells (13, 14) and we recently demonstrated that NSAIDs induce both necrosis and apoptosis in cultured gastric mucosal cells in a manner independent of COX inhibition (15-17). We also found that NSAIDs cause membrane permeabilization, which in turn is implicated in their direct cytotoxicity; that is, liposomal membranes are directly permeabilized by NSAIDs at concentrations closely related to those which result in cytotoxicity (18). Furthermore, we recently suggested that the combined effect of COX inhibition and the direct cytotoxic effect of NSAIDs (direct cell damage) on the gastric mucosa induces the production of gastric lesions (19). Therefore, the direct cytotoxicity of individual NSAIDs is a key factor to be determined in assessing their harmfulness on the gastric mucosa.

Since the direct cytotoxicity of NSAID prodrugs has not been studied at all, we examined here the direct cytotoxicity of nabumetone which, along with its active metabolite 6-methoxy-2-naphthylacetic acid (6MNA), was found to not harm the gastrointestinal mucosa in clinical studies on humans and in animal models (20, 21). Compared to indomethacin and celecoxib, both nabumetone and 6MNA showed very low activities for inducing necrosis, apoptosis and membrane permeabilization. Furthermore, in combination with the intravenous administration of a low dose of indomethacin (conditions under which gastric mucosal COX activity is completely inhibited), the oral administration of nabumetone

did not result in the production of gastric lesions, which is in contrast to results obtained following the oral administration of celecoxib. Based on these observations, we consider that the low direct cytotoxicity of nabumetone will render its use safe on the gastrointestinal mucosa *in vivo*.

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) was from Sigma Co. Nabumetone and 6MNA were kindly gifted from Sanwa Kagaku Kenkyusho Co. Indomethacin was from Wako Co. Celecoxib was from LKT Laboratories Inc. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. The ELISA kit for PGE_2 quantitation was from Cayman Chemical 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 of NSAIDs.** Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (22, 23). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3% v/v FBS, 100 U/ml ampicillin 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 (22, 24).

NSAIDs were dissolved in DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium.

We used MTT assay for monitoring cell viability. Cells were incubated for 2 h with MTT solution at a final concentration of 0.5 mg/ml. Isopropanol and hydrochloric acid were added to the culture medium at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined spectrophotometrically using a reference wavelength of 630 nm (25).

Gastric Damage Assay. Gastric damage assays were performed as described previously (19). Rats (24 h fasted) were administered orally with NSAIDs in 1% methylcellulose in a volume of 5 ml/kg. In some experiments, indomethacin in PBS was administered intravenously 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. Determination of PGE_2 levels at the gastric mucosa was done by ELISA as previously described (26).

Assay for Erythrocyte Hemolysis. Hemolysis in erythrocytes were monitored as described (18). Human erythrocytes were washed twice with buffer A (5 mM HEPES/NaOH (pH 7.4) and 150 mM NaCl) and then suspended in fresh buffer A at a final concentration of 0.5%

hematocrit (5 x 10^7 cells/ml). After incubation with NSAIDs for 10 min at 30°C, hemolysis was estimated by measuring the absorbance at 540 nm.

Membrane Permeability Assay. Membrane permeability assays were performed as described previously (18). Liposomes were prepared using the reversed-phase evaporation method. 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 phosphate 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 □l of this suspension was incubated at 30°C for 10 min in the presence of the NSAID under investigation. The release of calcein from liposomes (the amount of calcein outside the liposomes) was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm), because the calcein fluoresces very weakly when at high concentrations (when calcein is trapped in liposomes) due to self-quenching.

Statistical Analyses. All values are expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test 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 $P<0.05$.

RESULTS AND DISCUSSION

Necrosis- and Apoptosis-inducing Activities of Nabumetone and 6MNA. We previously reported that NSAIDs induced 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 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 (15, 18, 19). Nabumetone and 6MNA were tested here for their ability to induce necrosis and apoptosis. Consistent with previous reports (15, 18, 19), 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, nabumetone and 6MNA decreased cell viability to a much lesser extent under the same experimental conditions (Fig. 1A), with the necrosis- and apoptosis-inducing effects of nabumetone being slightly but significantly lower than those of 6MNA (Fig. 1B). We confirmed that cell death highlighted in Fig. 1 was mediated by necrosis given that no accompanying apoptotic DNA fragmentation or chromatin condensation were evident (data not shown).

Similar results to the above were obtained when apoptosis was induced. Treatment of cells for 16 h with indomethacin or celecoxib decreased cell viability in a dose-dependent manner (Fig. 2A), which is also consistent with previous reports (15, 18, 19). Nabumetone

and 6MNA showed very low activities for decreasing cell viability under these conditions (Fig. 2A), and nabumetone was again slightly but significantly less damaging than 6MNA (Fig. 2B). Because cell death as highlighted in Fig. 2 was accompanied by apoptotic DNA fragmentation and chromatin condensation (data not shown), it is most likely to have been mediated by apoptosis. Overall, the results in Figs. 1 and 2 show that nabumetone and 6MNA induce necrosis and apoptosis to a lesser extent than do indomethacin and celecoxib. Furthermore, although the metabolic conversion of nabumetone to 6MNA drastically increases the inhibition of COX activity, this conversion does not seem to be associated with a similar increase in direct cytotoxicity.

Membrane Permeabilization Activities of Nabumetone and 6MNA. The ability of nabumetone and 6MNA 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 and thus lower the calcein concentration (18). As shown in Fig. 3, indomethacin and celecoxib increased the calcein fluorescence in a dose-dependent manner, which is consistent with previous findings (18). Nabumetone and 6MNA also increased the calcein fluorescence, suggesting that they caused membrane permeabilization; however, as the concentrations of nabumetone and 6MNA required for membrane permeabilization were much higher than those of indomethacin and celecoxib, their abilities

to permeabilize membranes were thus very weak. The results shown in Fig. 3 suggest that the low direct cytotoxicity of nabumetone and 6MNA is due to their low membrane permeabilizing effects. When nabumetone and 6MNA were compared, 6MNA had a higher membrane permeabilizing effect than nabumetone (Fig. 3), which is consistent with the results describing their direct cytotoxicity (Figs. 1 and 2).

We also examined the membrane permeabilization activities of NSAIDs by measuring hemolysis. As shown in Fig. 4, results similar to calcein release (Fig. 3) were obtained, suggesting that NSAIDs cause membrane permeabilization not only in liposomes but also in cells. Membrane permeabilization (Figs. 3 and 4) was observed at relatively higher concentrations of NSAIDs than those required for decrease in cell viability (Figs. 1 and 2), being consistent with our previous report (18). This may be due to the difference in assay conditions.

Activities of Nabumetone and 6MNA for Production of Gastric Lesions. As described in the Introduction, we recently found that gastric lesions develop in a manner that depends both on intravenously administered low doses of indomethacin and on orally administered cytotoxic NSAIDs, such as celecoxib (19). Using this model, the ability of nabumetone to produce gastric lesions was compared to that of celecoxib. As shown in Fig. 5, in the absence of prior intravenous administration of indomethacin, the oral administration of either nabumetone or celecoxib did not clearly produce gastric lesions, which is consistent with previous results (27, 28). Oral administration of either nabumetone or celecoxib did not

significantly reduce the level of PGE_2 (Table 1). The intravenous administration of a low dose (5 mg/kg) of indomethacin alone also did not produce gastric lesions (Fig. 5), but did bring about a reduction of more than 90% in the level of PGE_2 (Table 1). A combination of the oral administration of celecoxib and the intravenous administration of indomethacin clearly gave rise to the production of gastric lesions (Fig. 5) as previously reported (19). In contrast, gastric lesions were not so evident when the oral administration of nabumetone and the intravenous administration of indomethacin were used in combination (Fig. 5). Oral administration of 6MNA produced a little but significant gastric lesions both in the presence or absence of intravenous administration of indomethacin (Fig. 6). This may be due to that oral administration of 6MNA, itself, significantly reduced the level of PGE_2 (Table 1). We consider from the results presented in Fig. 5 that nabumetone also has a low level of direct cytotoxicity *in vivo*.

In summary, we show here that nabumetone has a very low level of direct cytotoxicity on gastric mucosal cells *in vitro* and suggest that this is also the case *in vivo*. As described above, it is well known that nabumetone is experimentally and clinically safe and that its use is not as harmful to the gastric mucosa compared to other NSAIDs such as indomethacin and aspirin (20, 29). In addition to its inability to inhibit gastric mucosal COX activity soon after oral administration, its inhibitory effect on neutrophil functions was also recently suggested (30). We propose here that in addition to these mechanisms, the low direct cytotoxicity of nabumetone make it far less harmful on the gastric mucosa and therefore much safer for clinical use.

It is known that non-selective NSAIDs modulate the gastric acid secretion and inhibit bicarbonate secretion (31, 32). Although we did not examine the effect of nabumetone on these processes, it is possible that these process also involve the safety of this drug on gastric mucosa *in vivo*.

REFERENCES

1. Hawkey CJ: Nonsteroidal anti-inflammatory drug gastropathy. Gastroenterology 119: 521-535, 2000
2. Gabriel SE, Jaakkimainen, L, Bombardier, C: Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. Ann Intern Med 115: 787-796, 1991
3. Kurata JH,Abbey, DE: The effect of chronic aspirin use on duodenal and gastric ulcer hospitalizations. J Clin Gastroenterol 12: 260-266, 1990
4. Miller TA: Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. Am J Physiol 245: G601-623, 1983
5. Vane J: Towards a better aspirin. Nature 367: 215-216, 1994
6. Smith CJ, Zhang, Y, Koboldt, CM, Muhammad, J, Zweifel, BS, Shaffer, A, Talley, JJ, Masferrer, JL, Seibert, K, Isakson, PC: Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc Natl Acad Sci U S A 95: 13313-13318, 1998
7. Chan CC, Boyce, S, Brideau, C, Charleson, S, Cromlish, W, Ethier, D, Evans, J, Ford, HA, Forrest, MJ, Gauthier, JY, Gordon, R, Gresser, M, Guay, J, Kargman, S, Kennedy, B, Leblanc, Y, Leger, S, Mancini, J, O'Neill, GP, Ouellet, M, Patrick, D, Percival, MD, Perrier, H, Prasit, P, Rodger, I, et, al: Rofecoxib [Vioxx, MK-0966;

4-(4'-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. J Pharmacol Exp Ther 290: 551-560, 1999

8. FitzGerald GA,Patrono, C: The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med 345: 433-442, 2001

9. Mukherjee D, Nissen, SE, Topol, EJ: Risk of cardiovascular events associated with selective COX-2 inhibitors. Jama 286: 954-959, 2001

10. Mukherjee D: Selective cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. Biochem Pharmacol 63: 817-821, 2002

11. McAdam BF, Catella, LF, Mardini, IA, Kapoor, S, Lawson, JA, FitzGerald, GA: Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci U S A 96: 272-277, 1999

12. Belton O, Byrne, D, Kearney, D, Leahy, A, Fitzgerald, DJ: Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. Circulation 102: 840-845, 2000

13. Lichtenberger LM: Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited. Biochem Pharmacol 61: 631-637, 2001

14. Somasundaram S, Rafi, S, Hayllar, J, Sigthorsson, G, Jacob, M, Price, AB, Macpherson, A, Mahmod, T, Scott, D, Wrigglesworth, JM, Bjarnason, I: Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine. Gut 41: 344-353, 1997

15. Tomisato W, Tsutsumi, S, Rokutan, K, Tsuchiya, T, Mizushima, T: NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am J Physiol Gastrointest Liver Physiol 281: G1092-1100, 2001

16. Tomisato W, Tsutsumi, S, Hoshino, T, Hwang, HJ, Mio, M, Tsuchiya, T, Mizushima, T: Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. Biochem Pharmacol 67: 575-585, 2004

17. Tsutsumi S, Gotoh, T, Tomisato, W, Mima, S, Hoshino, T, Hwang, HJ, Takenaka, H, Tsuchiya, T, Mori, M, Mizushima, T: Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ 11: 1009-1016, 2004

18. Tomisato W, Tanaka, K, Katsu, T, Kakuta, H, Sasaki, K, Tsutsumi, S, Hoshino, T, Aburaya, M, Li, D, Tsuchiya, T, Suzuki, K, Yokomizo, K, Mizushima, T: Membrane permeabilization by non-steroidal anti-inflammatory drugs. Biochem Biophys Res Commun 323: 1032-1039, 2004

19. Tomisato W, Tsutsumi, S, Hoshino, T, Hwang, HJ, Mio, M, Tsuchiya, T, Mizushima, T: Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. Biochem Pharmacol 67: 575-585, 2004

20. Melarange R, Gentry, C, O'Connell, C, Blower, PR, Neil, C, Kelvin, AS, Toseland, CD: Anti-inflammatory and gastrointestinal effects of nabumetone or its active metabolite, 6MNA (6-methoxy-2-naphthylacetic acid): comparison with indomethacin. Agents Actions Spec No: C82-83, 1992

21. Bernhard GC: Worldwide safety experience with nabumetone. J Rheumatol Suppl 36: 48-57, 1992

22. Hirakawa T, Rokutan, K, Nikawa, T, Kishi, K: Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology 111: 345-357, 1996

23. Tomisato W, Takahashi, N, Komoto, C, Rokutan, K, Tsuchiya, T, Mizushima, T: Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. Dig Dis Sci 45: 1674-1679, 2000

24. Tomisato W, Hoshino, T, Tsutsumi, S, Tsuchiya, T, Mizushima, T: Maturation-associated increase in sensitivity of cultured guinea pig gastric pit cells to hydrogen peroxide. Dig Dis Sci 47: 2125-2133, 2002

25. Tsutsumi S, Tomisato, W, Takano, T, Rokutan, K, Tsuchiya, T, Mizushima, T: Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. Biochim Biophys Acta 1589: 168-180, 2002

26. Futaki N, Arai, I, Hamasaka, Y, Takahashi, S, Higuchi, S, Otomo, S: Selective inhibition of NS-398 on prostanoid production in inflamed tissue in rat carrageenan-air-pouch inflammation. J Pharm Pharmacol 45: 753-755, 1993

27. Spangler RS: Gastrointestinal damage demonstrated with nabumetone or etodolac in preclinical studies. Am J Med 95: 35S-39S, 1993

28. Laudanno OM, Cesolari, JA, Esnarriaga, J, Rista, L, Piombo, G, Maglione, C, Aramberry, L, Sambrano, J, Godoy, A, Rocaspana, A: Gastrointestinal damage induced by celecoxib and rofecoxib in rats. Dig Dis Sci 46: 779-784, 2001

29. Huang JQ, Sridhar, S, Chen, Y, Hunt, RH: Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. Gastroenterology 114: 1169-1179, 1998

30. Ishiwata Y, Okamoto, M, Yokochi, S, Hashimoto, H, Nakamura, T, Miyachi, A, Naito, Y, Yoshikawa, T: Non-steroidal anti-inflammatory drug, nabumetone, prevents indometacin-induced gastric damage via inhibition of neutrophil functions. J Pharm Pharmacol 55: 229-237, 2003

31. Mertz-Nielsen A, Hillingso, J, Bukhave, K, Rask-Madsen, J: Indomethacin decreases gastroduodenal mucosal bicarbonate secretion in humans. Scand J Gastroenterol 30: 1160-1165, 1995

32. Borrelli F,Tavares, IA: Effect of nimesulide on gastric acid secretion in the mouse stomach in vitro. Life Sci 72: 885-896, 2003

33. Katsu T, Kobayashi, H, Hirota, T, Fujita, Y, Sato, K, Nagai, U: Structure-activity relationship of gramicidin S analogues on membrane permeability. Biochim Biophys Acta 899: 159-170, 1987

FIGURE LEGENDS

Table. 1. Inhibition of gastric PGE_2 synthesis by NSAIDs..

Rats were intravenously (i.v.) or orally (p.o.) administered with indicted doses of NSAIDs. After 6 h (p.o.) or 7 h (i.v.), the level of PGE_2 in gastric mucosa was determined by ELISA. Values are mean ± S.E.M. (n=3). ***P<0.001.

Fig. 1. Necrosis induced by NSAIDs.

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.D. (n=3). *P<0.05.

Fig. 2. Apoptosis induced by NSAIDs.

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 16 h. Cell viability was determined by the MTT method. Values are mean ± S.D. (n=3). **P<0.01; *P<0.05.

Fig. 3. Membrane permeabilization by NSAIDs.

Calcein-loaded liposomes were incubated for 10 min at 30°C with indicated concentrations of each NSAID. 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.

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.

Table
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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***

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Arai *et al.* Fig. 1.