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The Selective Cyclooxygenase-2 Inhibitor Nimesulide Prevents *Helicobacter pylori*-Associated Gastric Cancer Development in a Mouse Model

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ABSTRACT

Purpose: *Helicobacter pylori* infection can lead to gastric cancer, and cyclooxygenase-2 (COX-2) is overexpressed in the stomach during *H. pylori* infection. Therefore, we investigated whether nonsteroidal anti-inflammatory drugs might protect against this form of cancer. Specifically, we examined the chemopreventive effect of the COX-2 inhibitor nimesulide on *H. pylori*-associated gastric carcinogenesis in mice.

Experimental Design: C57BL/6 mice were treated with the carcinogen *N*-methyl-*N*-nitrosourea (MNU) and/or *H. pylori*. To determine the effect of COX-2 inhibition, nimesulide was mixed with feed pellets and administered for the duration of the experiment. All of the mice were sacrificed 50 weeks after the start of the experiment. Histopathology, immunohistochemistry, and Western blotting for COX-2, Bax and Bcl-2 were performed in stomach tissues. *In vitro* experiments with the human gastric cancer cell line AGS were also performed to identify mechanisms underlying cancer chemoprevention by nimesulide.

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Results: Gastric tumors developed in 68.8% of mice that were given both MNU and *H. pylori*, whereas less than 10% developed gastric tumors when given either MNU or *H. pylori* alone. These findings indicate that *H. pylori* promotes carcinogen-induced gastric tumorigenesis. In mice treated with both MNU and *H. pylori*, nimesulide administration substantially reduced *H. pylori*-associated gastric tumorigenesis, whereas substantial inductions of apoptosis were observed. *In vitro* studies demonstrated that nimesulide and *H. pylori* when combined acted synergistically to induce more apoptosis than either alone.

Conclusions: Our data show that nimesulide prevents *H. pylori*-associated gastric carcinogenesis, and suggest that COX-2 may be a target for chemoprevention of gastric cancer.

INTRODUCTION

Helicobacter pylori causes chronic active gastritis and peptic ulcer disease, and is linked with gastric adenocarcinomas, including gastric mucosa-associated lymphoid tissue lymphoma (1). On the basis of epidemiologic data, WHO/IARC classified *H. pylori* as a group 1 carcinogen (2). Generally, gastric adenocarcinoma develops through a multistep process from normal gastric mucosa to chronic active gastritis, to gastric atrophy and intestinal metaplasia, and finally to dysplasia and neoplasia (3), and it has been postulated that *H. pylori* plays a causative role in the early phases of this malignant progression (4, 5). However, debate still exists as to whether *H. pylori* is really a carcinogen or a cancer promoter, and whether eradication of *H. pylori* is beneficial to people free of gastric tumors (6–9).

A possible explanation for the link between *H. pylori* infection and gastric carcinogenesis is that *H. pylori* infection raised cyclooxygenase-2 (COX-2) mRNA/protein levels, and stimulated release of prostaglandin E₂ in *H. pylori*-associated premalignant and malignant gastric lesions (10–16). There is strong evidence that COX-2 is causally involved in gastrointestinal cancer (17–22). In addition, growth of colon polyps was retarded or blocked by either administration of nonsteroidal anti-inflammatory drugs (NSAIDs; refs. 23–25) or targeted deletion of the COX-2 gene (26). Because induction of COX-2 expression has been shown to play an important role in neoplastic transformation in the large intestine, it is our hypothesis that COX-2 is involved in *H. pylori*-associated gastric cancer development, and that NSAID administration can prevent or retard this process.

It has been shown that *H. pylori* increased release of prostaglandin E₂ and COX-2, which were overexpressed in most metaplastic and adenomatous tissues, as well as in gastric adenocarcinoma (27). Despite these observations, it remains unknown as to whether COX-2 may be a target for chemoprevention of *H. pylori*-associated gastric carcinogenesis. In the

present study, we used an *H. pylori*-associated gastric cancer mouse model (28, 29) to investigate the preventive effects of the selective COX-2 inhibitor nimesulide, and we studied the mechanisms underlying nimesulide-induced chemoprevention with AGS human gastric cancer cell line.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. The mice were maintained in an accredited Korea FDA animal facility in accordance with the AAALAC International Animal Care policies (Accredited Unit-Korea Food and Drug Administration: Unit Number-000996). All of the mice were given a standard pellet chow diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) *ad libitum* and were maintained in specific pathogen-free conditions.

Chemicals and Bacteria. *N*-methyl-*N*-nitrosourea (MNU; Sigma Chemical Co., St. Louis, MO) solutions were freshly prepared twice a week by dissolving 200 ppm MNU in distilled water. When indicated, mice were given the 200-ppm MNU solution *ad libitum* in light-shielded bottles in place of drinking water. Mouse-adapted *H. pylori* (SS1) were inoculated on *Brucella* agar plates (Becton Dickinson, Cockeysville, MD) containing 10% heat-inactivated fetal bovine serum and Skirrow medium (Difco, Detroit, MI). They were kept at 37°C under microaerobic conditions with GasPak jars (Difco) and Campy-Paks (Becton Dickinson). After 24 hours of fasting, a 0.1-mL suspension of *H. pylori* containing 1×10^9 colony-forming units (CFU)/mL was administered by intragastric intubation.

Study Design. The experimental design is illustrated in Fig. 1. Mice were randomized into five groups. Animals of groups 2, 3, and 5 were given MNU. One week after completion of MNU administration, mice in groups 3 and 5 were inoculated with *H. pylori* three times every other day. Mice in groups 1 and 4 were inoculated with *H. pylori* three times every other day (no MNU treatment). Animals of groups 4 and 5 were then given a CRF-1 diet (Oriental Yeast Co. Ltd.) containing 200 ppm nimesulide (Choongwae Pharmaceutical Research Institute, Suwon, Korea). Animals of groups 1, 2, and 3 were maintained on basal

CRF-1 diets throughout the experiment. Mice were sacrificed 38 weeks after infection, making a total of 50 weeks of treatment.

Histopathologic Examination. Immediately after sacrifice, mouse stomachs were opened along the greater curvature. The number, as well as the long diameter, of tumors in the stomach was measured. A record was kept of the size and number of tumors counted, with a diagnosis made after the final histopathologic examination. One half of the excised stomachs, including neoplastic nodules, were fixed in neutral-buffered 10% formalin and were cut into approximately six strips, which were processed by standard methods, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin (H&E). The remaining portions were quickly frozen in liquid nitrogen and stored at -70°C until analysis. Histologic classification was based on histopathologic and cytologic criteria proposed by Leininger and Jokinen (30). After histopathologic classification was done, tumor incidence and multiplicity was calculated.

Identification of *H. pylori* in Gastric Mucosa. To confirm *H. pylori* infection, we transferred samples ($\sim 3\text{-mm}^2$) of stomach mucosa from the greater curvature containing both fundic and pyloric glands to 1.0 mL of sterile 0.1 mol/L PBS; these were homogenized and plated on selective trypticase soy agar/5% sheep blood plates containing vancomycin (20 mg/mL), nalidixic acid (10 mg/mL), bacitracin (30 mg/mL) and amphotericin B (2 mg/mL) from Sigma Chemical Co., and grown for 3 to 5 days. Colonies were identified by characteristic Gram's stain morphology, and by urease, catalase, and oxidase activity. Another 3-mm² sample from the antrum was placed into the gel of a rapid urease test kit (CLO test, Ballard Medical Products, Draper, VT) and was left for 6 hours at room temperature to test for urease activity. The presence of *H. pylori* in the gastric pit was further confirmed by Warthin–Starry staining.

Immunohistochemistry for COX-2. Immunohistochemical identification of COX-2 expression was performed on replicate sections of stomach tissues. The sections were mounted on silanized slides (Dako, Glostrup, Denmark) and were dewaxed and rehydrated; endogenous peroxidase activity was quenched with hydrogen peroxide. After washing in dou-

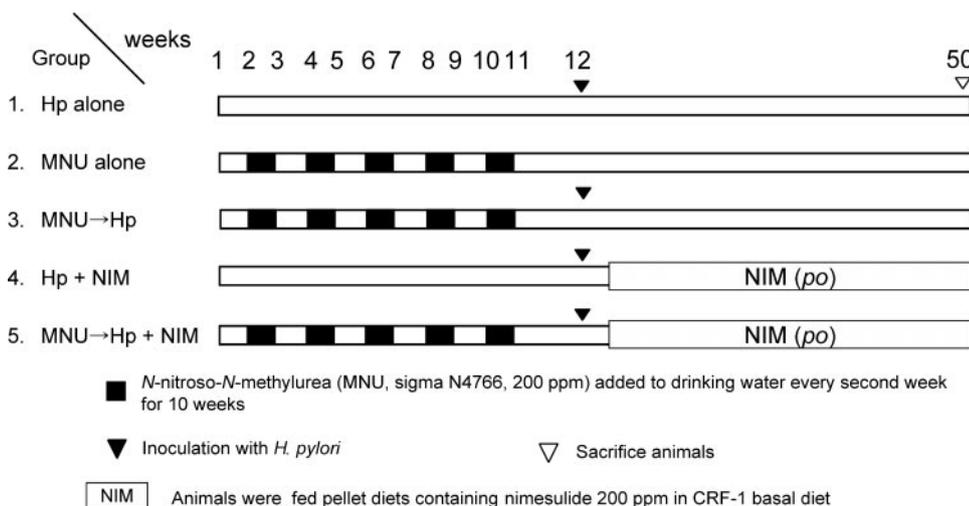


Fig. 1 The study design. Animals were randomized into five groups according to treatment: group 1, *H. pylori* alone (*Hp alone*); group 2, MNU alone; group 3, five weeks' MNU administration followed by *H. pylori* infection; group 4, *H. pylori* infection followed by continuous nimesulide via diet [*NIM (po)*]; and group 5, five weeks' MNU administration followed by *H. pylori* infection and nimesulide treatment [*NIM (po)*]. The mice were sacrificed 38 weeks after *H. pylori* infection.

ble-distilled water, sections were subjected to microwave antigen retrieval in 0.01 mol/L citric acid. The slides were incubated at 4°C overnight with rabbit anti-COX-2 polyclonal antibody (1:2,000; Cayman Chemical, Ann Arbor, MI). Immunoreaction complexes were detected with the avidin-biotin affinity system (Santa Cruz Biotechnology, Santa Cruz, CA) and were visualized with 3,3'-diaminobenzidine tetrahydrochloride (Zymed Laboratories Inc., San Francisco, CA) as the chromogen. The sections were counterstained with Mayer's hematoxylin and examined under a light microscope.

Western Blotting. The frozen stomach tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer [10 mmol/L Tris (pH 7.6), 1 mmol/L EDTA (pH 8.0), 100 mmol/L NaCl, 1 µg/mL aprotinin, and 100 µg/mL phenylmethylsulfonyl fluoride (all from Sigma Chemical Co.)]. Protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Extracted proteins (40 µg/lane) were resolved by SDS-PAGE and were transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with rabbit anti-COX-2 polyclonal antibody (Cayman Chemical), rabbit anti-Bax polyclonal antibody (Santa Cruz Biotechnology), mouse anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology). The membranes were then incubated for 45 minutes with secondary antibody (Santa Cruz Biotechnology). After incubation with the secondary antibody, blots were washed three times with PBS/0.1% Tween 20 and developed with a commercial chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom). Expression levels of protein were quantified with a Bio-Rad Imaging Densitometer system Model GS690 (Bio-Rad Laboratories) and the ratio of Bax to Bcl-2 was calculated.

The general procedure for Western immunoblot analyses of cultured AGS cells with antibodies against caspase-3 (Santa Cruz Biotechnology), (poly)ADP-ribose polymerase (PARP; Zymed Laboratories Inc.) were similar to the procedures described above. Cultured cells were washed twice with cold PBS on ice and harvested by scraping with a rubber scraper. Cells were sedimented by centrifugation at 4°C and resuspended in cell extraction buffer (50 mM/L PIPES/NaOH, 2 mM/L EDTA, 0.1% CHAPS, 5 mM/L DTT, 20 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL aprotinin, 1 µM/L phenylmethylsulfonyl fluoride).

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay. Apoptosis was visualized with terminal deoxynucleotidyl transferase (TdT) FragEL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA). The staining procedures were modified based on the manufacturer's recommendations. Briefly, after routine deparaffinization, rehydration, and washing in 1× PBS (pH 7.4), tissues were digested with proteinase K (20 mg/mL in 1× PBS) for 20 minutes at room temperature and were washed. After incubation in equilibration buffer for 10 minutes, sections were treated with terminal deoxynucleotidyltransferase (TdT) enzyme at 37°C for 1 hour.

Determination of Apoptotic Index. All of the slides were scored blindly three times without knowledge of the histologic findings. To determine the apoptotic index (AI) in each group, we first scanned Tdt-nick end labeling (TUNEL)-immunostained sections under low power magnification (×100) to

locate the apoptotic hot spots (areas with maximal TUNEL-positive cells). The AI at ×400 field was then scored by counting the number of TUNEL-positive cells. At least five hot spots in a section were selected to determine the average count. Data were expressed as a mean percentage of total cell numbers.

Cell Culture and Cell Viability Assay. The human gastric cancer cell line AGS was purchased from American Type Culture Collection (ATCC strain, Manassas, VA) and was maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT) in humidified environment at 37°C in 5% CO₂. To determine the cell growth rate, we seeded AGS cells into 24-well plate at 2 × 10⁵ cells/mL in triplicate, and we pretreated them with 5 µmol/L nimesulide or celecoxib (Pfizer Pharma, New York, NY) in 0.1% DMSO (Fisher Scientific, Pittsburgh, PA) for 24 hours; we then used *H. pylori* inoculation (2 × 10⁶ colony-forming units (CFU)/mL) for 24 hours. *H. pylori* filtrate was prepared by homogenizing the bacteria in distilled water, pelleting the bacteria by centrifugation, and then filtering through a 0.2 µm pore size filter (Gelman Sciences, Ann Arbor, MI). Nimesulide and celecoxib were added when the cells had 70 to 80% confluency. Cell numbers and their viability were determined by trypan blue exclusion assay.

Apoptotic Quantitation by Flow Cytometric Analysis. Apoptotic cells were quantified by staining with FITC-conjugated Annexin V (Clontech, Palo Alto, CA). Cells (1 × 10⁶) were collected at 72 hours for flow cytometric measurement and were stained with FITC-conjugated annexin V and propidium iodide as recommended by the manufacturer, and were then analyzed by flow cytometry with a FACScan (Becton Dickinson Facsort flow cytometer) with an argon laser set to excite at 488 nm. Propidium iodide (40 µg/100 µL PBS) was added to 1 × 10⁶ cells suspended in 800 µL of PBS, together with 100 µL of RNase A (1 µg/mL), and was incubated at 37°C for 30 minutes before flow cytometry analysis of 2 × 10⁴ cells. Red fluorescence of propidium-bound DNA was measured with a 630 nm-long bandpass filter.

Statistical Analyses. The data were analyzed with the JMP software package (version 4.0; SAS Institute, Cary, NC) on an IBM computer. Stomach tumor incidence data were analyzed with a χ^2 test. Other data were compared with the Dunnett *t* test after ANOVA analysis. For all comparisons, *P* values less than 5% (*P* < 0.05) were considered to be statistically significant.

RESULTS

Bacterial Colonization. At week 50, stomachs were removed, and all of the mice except those in group 2 (MNU alone) showed positive *H. pylori* colonization as determined by direct bacterial culture and rapid urease tests. The mean (*SEM*) number of CFU recovered from mice inoculated orally with *H. pylori* SS1 was 1.41 ± (0.35) × 10⁵/mg gastric tissue. Warthin–Starry staining showed numerous spiral bacteria in mucosal epithelium along the length of the gastric pits in both the antrum and the body of all of the animals inoculated with *H. pylori*.

***H. Pylori* Infection Promoted Carcinogen-Induced Gastric Tumorigenesis.** Mice were randomized into five groups according to treatment: group 1, *H. pylori* alone; group 2, MNU alone; group 3, five weeks' MNU administration followed by *H.*

Table 1 Incidence and multiplicity of glandular stomach tumors in mice

Group	Effective no. of mice*	No. of tumor-bearing mice (% incidence)	Tumor multiplicity†
1. Hp alone	13	0	0
2. MNU alone	10	1 (10.0)	0.10 ± 0.10
3. MNU→Hp	16	11 (68.8)‡	2.62 ± 0.36§
4. Hp + NIM	15	0	0
5. MNU→Hp + NIM	18	5 (27.8)¶	0.44 ± 0.12¶

Abbreviation: Hp, *H. pylori*.

* Living mice with *H. pylori* at the time of sacrifice, except in group 2 (MNU alone).

† Mean ± SEM.

‡ $P < 0.0001$ versus group 2.

§ $P < 0.01$ versus group 2.

¶ $P < 0.01$ versus group 3.

¶ $P < 0.05$ versus group 3.

pylori infection; group 4, *H. pylori* infection followed by continuous nimesulide via diet; and group 5, five weeks' MNU administration followed by *H. pylori* infection and nimesulide treatment (see Fig. 1). The mice were sacrificed 38 weeks after *H. pylori* infection. The mean body weight of each group was similar throughout all of the experiments. The incidence of tumors at sacrifice was 0% (0/13) in group 1, 10% (1/10) in group 2, and 68.8% (11/16) in group 3. Group 3 showed a significantly higher incidence of gastric tumor compared with groups 1 and 2 ($P < 0.0001$ for both comparisons), signifying that the mice were somewhat resistant to carcinogen-stimulated gastric tumorigenesis, and that *H. pylori* infection promoted, rather than initiated, gastric tumorigenesis. In addition to increased tumor incidence, tumor multiplicity was higher in group 3 than in group 2 (2.62 ± 0.36 versus 0.10 ± 0.10 ; $P < 0.01$; Table 1). Tumors were mostly formed in the pyloric mucosa adjacent to the fundic region. Macroscopically, the majority of tumor masses were polypoid, like type I stomach cancers in humans (Fig. 2A), and some were sessile.

Nimesulide Significantly Suppressed *H. pylori*-Associated Gastric Carcinogenesis. The incidence of overall stomach tumors was lower in group 5 (MNU→*H. pylori* + nimesu-

lide) compared with group 3 [MNU→*H. pylori*; 27.8% (5/18) versus 68.8% (11/16); $P < 0.01$]. In addition, the multiplicity of gastric tumors in group 5 was lower than that in group 3 (0.44 ± 0.12 versus 2.62 ± 0.36 ; $P < 0.05$; Table 1). Although there was no significant difference in the incidence of gastric adenoma between groups 3 and 5 (Table 2), the incidence of gastric adenocarcinoma was markedly reduced in group 5 compared with group 3 [5.6% (1/18) versus 43.8% (7/16); $P < 0.01$; Table 2). These data indicate nimesulide inhibited either the development of *H. pylori*-associated gastric tumorigenesis or the process of gastric carcinogenesis.

Histologic Features of *H. pylori*-Associated Gastric Cancer. In group 1, the fundic and pyloric mucosa revealed chronic gastritis characterized by moderate-to-severe infiltration of lymphocytes, plasma cells, and a few neutrophils with foveolar hyperplasia (Fig. 3A); and in group 4, mild infiltration of inflammatory cells as well as epithelial hyperplasia was observed, but less than in group 1. The adenoma frequently noted in groups 2, 3, and 5 revealed irregular small compact glandular growths composed of pencil-like and hyperchromatic nuclei, and a few mitotic figures. Occasionally, superficial erosions covered with pinkish amorphous hemorrhagic debris were found on the surface of adenomas in group 3. However, there was no definite stromal invasion (Fig. 3B). Gastric adenocarcinoma, frequently found in group 3, showed an irregular glandular proliferation with solid pattern and angular structure indicative of early stromal invasion. The adenocarcinoma showed an atypical irregular glandular hyperplasia with cribriform appearance, which was composed of hyperchromatic atypical tumor cells (Fig. 2C and Fig. 3C). Small irregular atypical glands were observed infiltrating into the muscularis mucosa and submucosa (Fig. 3D).

Attenuated Expression of Gastric COX-2 in Nimesulide-Treated Mice. Immunohistochemistry revealed that COX-2 protein was expressed primarily in stromal cells of the lamina propria in gastric adenocarcinoma, and was characterized by a strong, intense cytoplasmic expression pattern (Fig. 4Aa and Ac). COX-2 was very strongly localized, not only in the area of gastritis but also in the stromal cells adjacent to erosion, displaying a spotty cytoplasmic staining pattern, and was limited to a small number of epithelial cells in gastric adenomas (Fig.

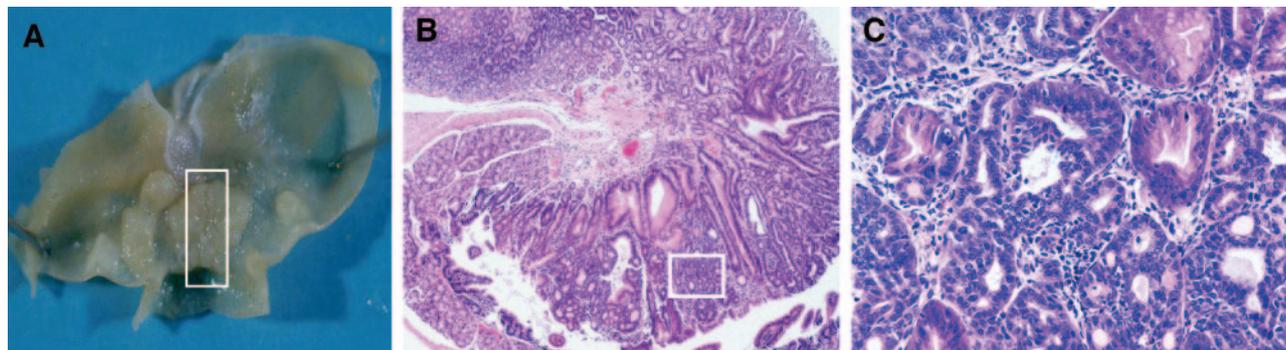


Fig. 2 Macroscopic (A) and microscopic (B and C) appearance of gastric cancer. A, multiple polypoid tumors developed in the stomach after MNU and *H. pylori* infection. B, the polypoid mass shows an intramucosal adenocarcinoma with foveolar epithelial hyperplasia in the antrum and pylorus ($\times 100$). C, the adenocarcinoma shows an atypical irregular glandular hyperplasia with cribriform appearance, which is composed of hyperchromatic atypical tumor cells ($\times 200$).

Table 2 Incidence of glandular stomach tumors: histopathologic findings

Group	Effective no. of mice	No. of tumor-bearing mice	Gastric adenoma (%)	Gastric adenocarcinoma (%)
1. Hp alone	13	0	0	0
2. MNU alone	10	1	1 (10)	0
3. MNU→Hp	16	11	4 (25.0)	7 (43.8)
4. Hp + NIM	15	0	0	0
5. MNU→Hp + NIM	18	5	4 (22.2)	1 (5.6)*

Abbreviation: Hp, *H. pylori*.

* $P < 0.01$ versus group 3.

4Ab). The overall intensity of COX-2 expression was lower in nimesulide-treated groups (groups 4 and 5; Fig. 4B and Ad) than in other groups (groups 1, 2, and 3; Fig. 4B and Ac). COX-2 levels in gastric mucosa homogenates were examined by Western blotting, and we found that group 3 mice had the highest level of homogenate COX-2, which was about 1.5-fold higher than that in group 2 (Fig. 4B). The amount of COX-2 protein in group 5 (MNU→*H. pylori* + nimesulide) homogenates was significantly lower than that in group 3 (MNU→*H. pylori*). The data indicate COX-2 expression in *H. pylori*-associated gastric cancer was significantly inhibited by nimesulide treatment.

A Ratio of Bax to Bcl-2 Was Increased in Nimesulide-Treated Mice. The mean expressions of proapoptotic Bax and antiapoptotic Bcl-2 were measured in mixed homogenates of stomach tissues according to each group. Compared with group 2 (MNU alone), the mean expression of Bax in group 1 (*H.*

pylori alone) was markedly increased, and the mean expression of Bcl-2 was rather decreased after *H. pylori* infection than in group 2, suggesting that significant apoptotic-prone tendency was induced after *H. pylori* infection. Interestingly, the expression of Bcl-2 in group 3 (MNU→*H. pylori*) was significantly increased, but the expression of Bax was attenuated, resulting in significant decreased in the mean ratio of Bax/Bcl-2 intensity. Significant increases in the expression of Bax were observed in groups treated with nimesulide (groups 4 and 5), signifying that the apoptotic actions of nimesulide might be the reason why carcinogenesis was ameliorated in animal groups treated with nimesulide (Fig. 4C).

Both COX-2 Inhibitor and *H. pylori* Acted Synergistically to Increase Apoptosis: A Possible Chemopreventive Mechanism of Nimesulide. On the basis of these observations of Fig. 4C, we measured the degree of apoptosis in

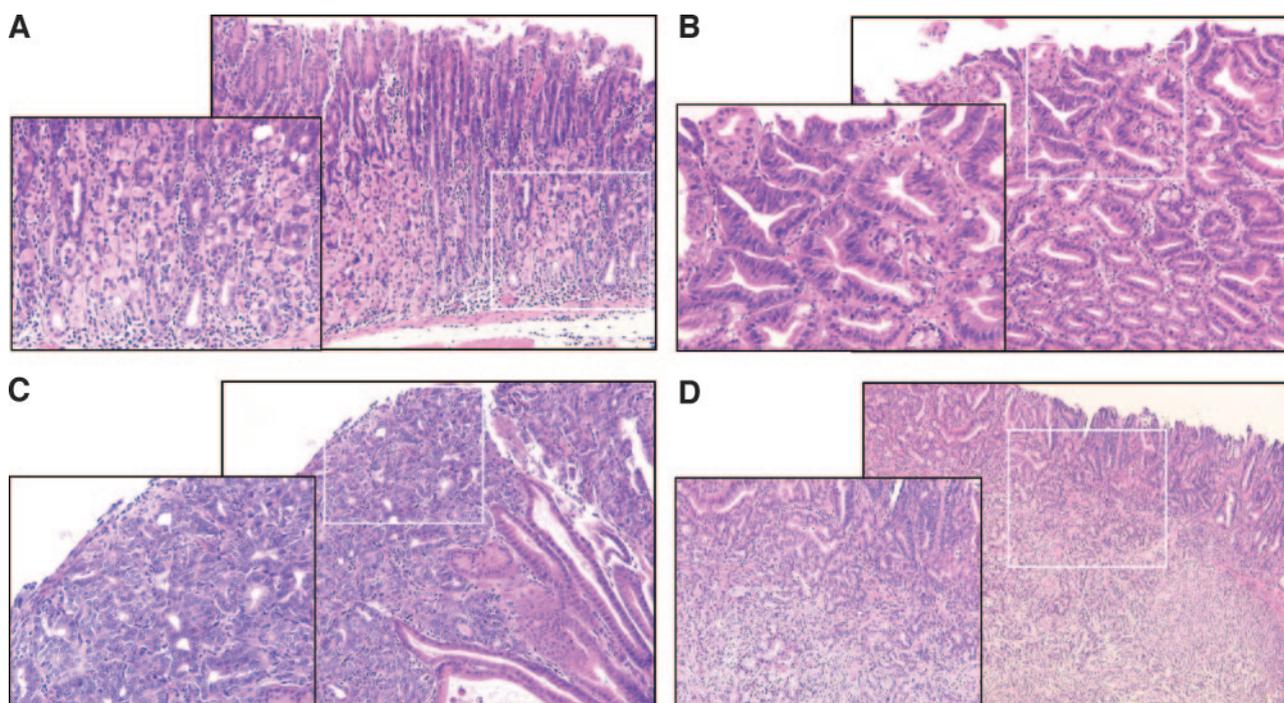


Fig. 3 Histopathology. A, fundic mucosa in group 1 revealed a patchy infiltration of chronic inflammatory cells with foveolar epithelial hyperplasia. B, adenoma in group 5 animals showed an irregular small glandular proliferation composed of high cellular, pencil-like, and hyperchromatic nuclei. C, adenocarcinoma in group 3 animals revealed a marked glandular proliferation with nuclear atypia and early stromal invasion. D, invasive adenocarcinoma in group 3 animals revealed small irregular carcinomatous glands infiltrating the muscularis mucosa and submucosa, accompanied by desmoplasia. $\times 100$; portion enlarged (on the left): $\times 200$.

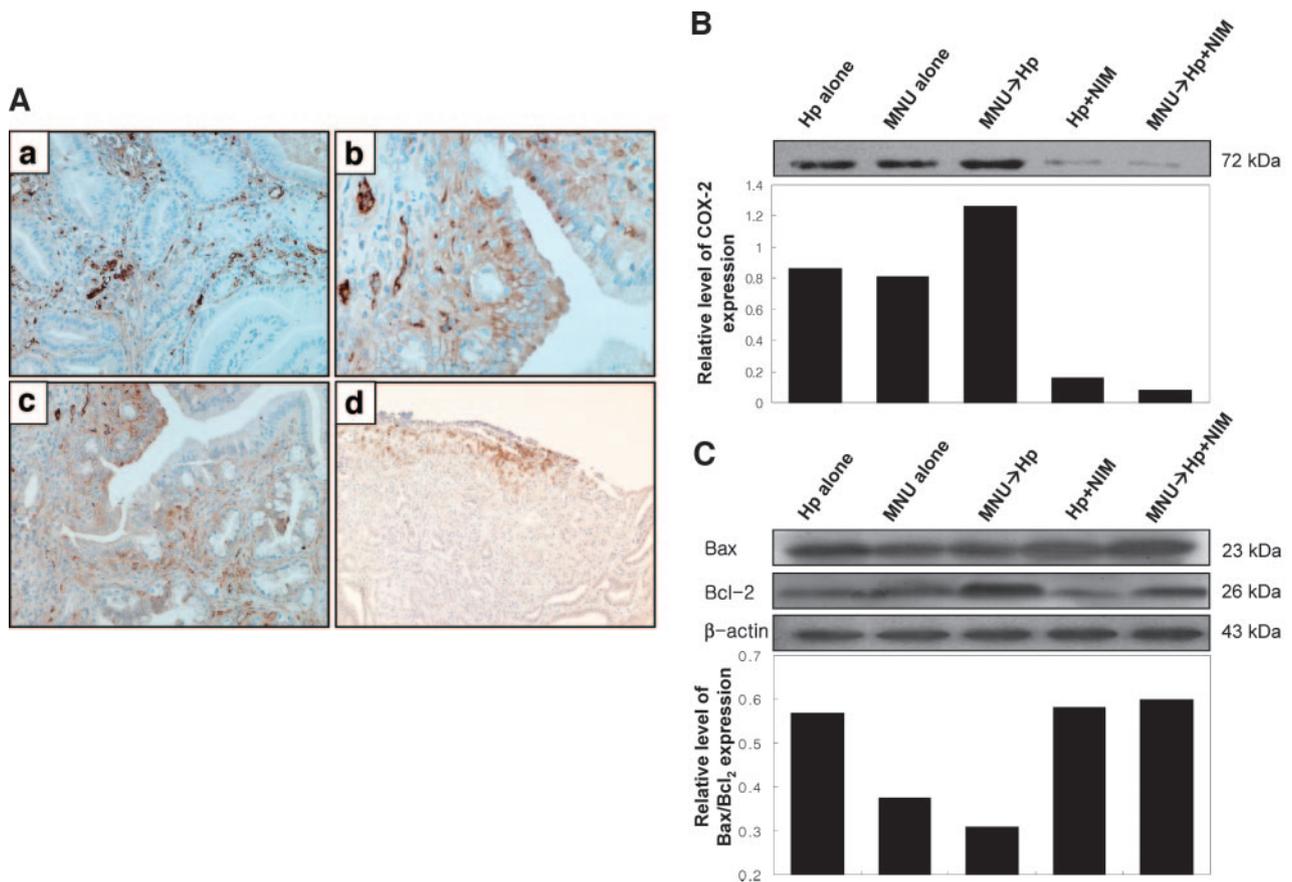


Fig. 4 Expression of COX-2, Bax, and Bcl-2. **A**, immunohistochemical staining for COX-2. In **Aa**, COX-2 expression was mainly confined to stromal cells composed of macrophages, lymphocytes, and interstitium in gastric adenocarcinoma. **Ab**, gastric adenoma epithelial cells expressing COX-2. In **Ac** and **Ad**, COX-2 expression was attenuated in nimesulide (NIM)-treated animals (**Ad**) compared with non-NIM-treated animals (**Ac**); $\times 200$. **B**, COX-2 protein expression levels in the experimental groups. Western blot analysis of COX-2 protein expression in stomach tissues (**Hp**, *H. pylori*; *kDa*, M_r in thousands). **C**, Bax and Bcl-2 expression levels in the experimental group. Bax and Bcl-2 expressions in stomach tissues were demonstrated according to group, and the ratio (Bax/Bcl₂) of the expression was depicted. *kDa*, M_r in thousands.

stomach tissues of each animal according to group with the TUNEL method. As shown in Fig. 5A, *H. pylori* infection increased the TUNEL staining of positive cells, but the apoptotic activities were markedly decreased in the portion of gastric adenoma. However, nimesulide administration increased TUNEL staining positive cells irrespective of gastric pathologies. Even in tumor tissues, significant apoptotic positive cells were noted in group treated with nimesulide, suggesting the active apoptotic activities might be the fundamental mechanisms of nimesulide on attenuated *H. pylori*-associated carcinogenesis. The mean changes of apoptotic index were shown in Fig. 5B.

When AGS cells were treated with 5 $\mu\text{mol/L}$ nimesulide (preferred COX-2 inhibitor) or 5 $\mu\text{mol/L}$ celecoxib (selective COX-2 inhibitor) or *H. pylori* (2×10^6 CFU), a significant decrease in cell survivals was observed with all three treatments, which was measured with cell counts (Fig. 6A). Significant attenuation in cell survivals was noted in cells treated with either celecoxib or nimesulide alone. These changes were more evident in cells cotreated with a COX-2 inhibitor and *H. pylori*, suggesting that cell death processes were more activated than

with either COX-2 inhibitor or *H. pylori* alone. All of these findings that the combination of COX-2 inhibitor and *H. pylori* provoked and augmented apoptosis were more evidenced with Western blot of caspase-3 and PARP (Fig. 6B) and with flow cytometry analyses (Fig. 6C). Active caspase-3, cleavage of PARP, and increased fractions of positive annexin V—observed after COX-2 inhibitor treatment—were more apparently increased after the cotreatment of COX-2 inhibitor and *H. pylori*. These *in vitro* experiments explain the similar mechanistic basis of the cancer prevention of nimesulide treatment in *H. pylori*-associated gastric tumorigenesis as observed in *in vivo* animal experiment. Attenuated development of gastric tumor in previous animal experiments could be achieved because both the COX-2 inhibitor and *H. pylori* acted synergistically to increase apoptosis, a possible chemopreventive mechanism of nimesulide.

DISCUSSION

The present study shows that *H. pylori*-associated gastric tumorigenesis and carcinogenesis was effectively suppressed by

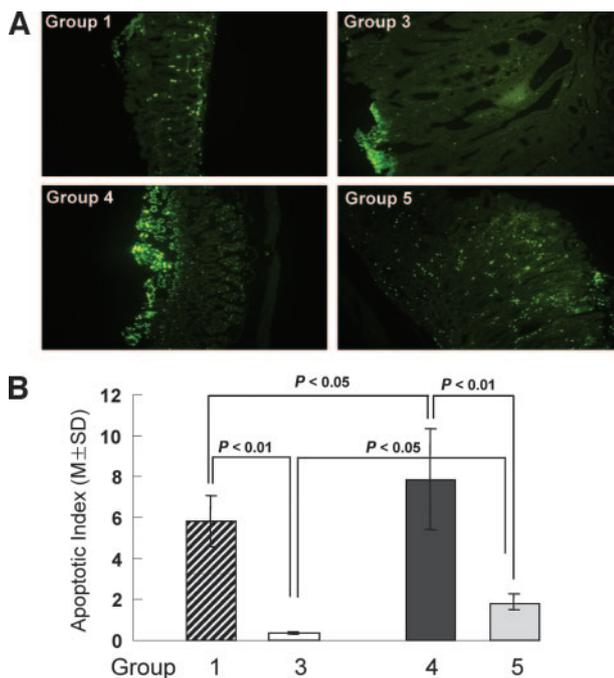


Fig. 5 TUNEL staining and apoptotic index. **A**, TUNEL staining positive cells were increased after *H. pylori* infection (Group 1), but the apoptotic activities were markedly decreased in gastric adenoma tissue (Group 3). However, nimesulide administration provoked increased apoptotic activities either in nontumor tissue of Group 4 or in tumor tissue of Group 5. **B**, mean index ($M \pm SD$) of apoptosis is shown, resulting in statistically increased apoptotic index in group treated with nimesulide irrespective of the presence of gastric tumor.

the selective COX-2 inhibitor nimesulide. COX-2 protein was highly expressed in stromal cells of *H. pylori*-associated tumor tissues, which expression levels substantially decreased after nimesulide administration. In addition, significant inductions of apoptosis were observed. The results of *in vitro* experiments suggested that nimesulide and *H. pylori* synergistically operated to induce more apoptosis in AGS cells. These findings indicate that the inhibition of COX-2 activity might be a novel target for the treatment and prevention of *H. pylori*-associated gastric cancers, which are similar to colon cancers.

Cancer is the result of a multistep molecular and cellular process. Chemoprevention might be a better way to manage cancer rather than the current chemotherapy with cytotoxic agents in every aspect of efficacy, compliance, and feasibility. For this purpose, nontoxic nutrients or pharmacological compounds that might protect against the development and progression of mutant clones into malignant cells have been used in experimental and clinical studies (31). Nimesulide, a preferred selective inhibitor of COX-2 belonging to the sulfonamide class, has been used clinically as an anti-inflammatory drug with less ulcerogenic effects in the gastrointestinal tract than classical NSAIDs. It is regarded as a good candidate agent in chemopreventive trials against colon and breast cancers (32). A large body of recent epidemiologic data suggests regular NSAID use can reduce both the incidence of, and mortality from, colorectal cancer and other solid tumors such as breast cancer (19, 23–25, 33).

It has been postulated that the protective effects of NSAIDs are mediated through the inhibition of cyclooxygenase enzymes, COX-1 and COX-2 (19, 22). COX-2 leads to increased synthesis of prostaglandins in both inflammatory and malignant tissues, and may support the development and progression of human malignancy. Recently, a more direct causal link between COX-2 expression and malignancy was shown in studies of COX-2-overexpressing transgenic mice (34), which displayed mammary gland hyperplasia and transformation in breast tissue overexpressing COX-2. Similarly, COX-2 “knockout” mice showed reduced intestinal polyp development (26), again supporting a causative role of COX-2 in tumorigenesis. Studies of human colorectal tumors revealed that COX-2 is overexpressed in more than 80% of carcinomas and in at least 50% of premalignant adenomas (22). Furthermore, COX-2 inhibitors effectively reduced colorectal tumors induced by azoxymethane treatment in rats, and spontaneous colorectal tumors in *Apc*^{-/-} *Min* mice (24, 35). On the basis of preclinical profiles that COX-2 was highly expressed in polyp tissues obtained from patients with familial adenomatous polyposis (FAP), celecoxib entered a phase III randomized trial in 77 FAP patients. After 6 months of 400-mg intake twice daily, celecoxib caused a 28% reduction in polyp burden in the rectum (25).

Several studies have shown enhanced COX-2 expression in gastric cancer tissues (18, 27). Overexpression of COX-2 was a property shared by both intestinal and diffuse gastric carcinomas. It seems that COX-2 might play an important role during the early stage of gastric carcinogenesis (16, 27). *H. pylori* infection is a major risk factor for gastric carcinoma. *H. pylori* up-regulated COX-2 mRNA expression and stimulated release of prostaglandin E₂ in a gastric cancer cell line (10), as well as in the gastric mucosa of animal models and in humans (12, 14). Therefore, high levels of *H. pylori* infection might up-regulate COX-2 expression, which, in turn, could lead to the development of gastric carcinogenesis (27). Experimentally, Xiao *et al.* (14) demonstrated that COX-2 was directly involved in hyperplastic changes in mice infected with *H. pylori*. Consistent with the data from the above-mentioned studies, the present findings strongly support the contention that pharmacological inhibition of COX-2 overexpression may be useful against *H. pylori*-associated gastric cancer development and progression.

H. pylori and NSAIDs are known to be risk factors in the pathogenesis of gastric ulcers through apparently different mechanisms, reciprocally or independently. *H. pylori* infection may induce strong mucosal inflammation, stimulate cytokine release, and provoke apoptosis. *H. pylori* had been known to be responsible for significant apoptosis, by which gastric ulcerations or gastric atrophy (significant apoptotic cell death of gastric stem cells or parietal cells) can be developed. For this, several cytotoxins of CagA or VacA, ammonia generated from urease actions, chemokines, or cytokines like interleukin (IL)-8, interferon- γ , IL-12, tumor necrosis factor α , oxidative stress, and HNP (helicobacter neutrophil-activating peptide), and so forth, had been known to be responsible for apoptosis after *H. pylori* infection. In contrast, NSAIDs may inhibit mucosal prostaglandin synthesis, leading to a weakening in the gastric mucosal barrier, and impaired resistance to acid injury. Therefore, one might expect increased incidence of gastric mucosa damage in the presence of these two risk factors (36). However,

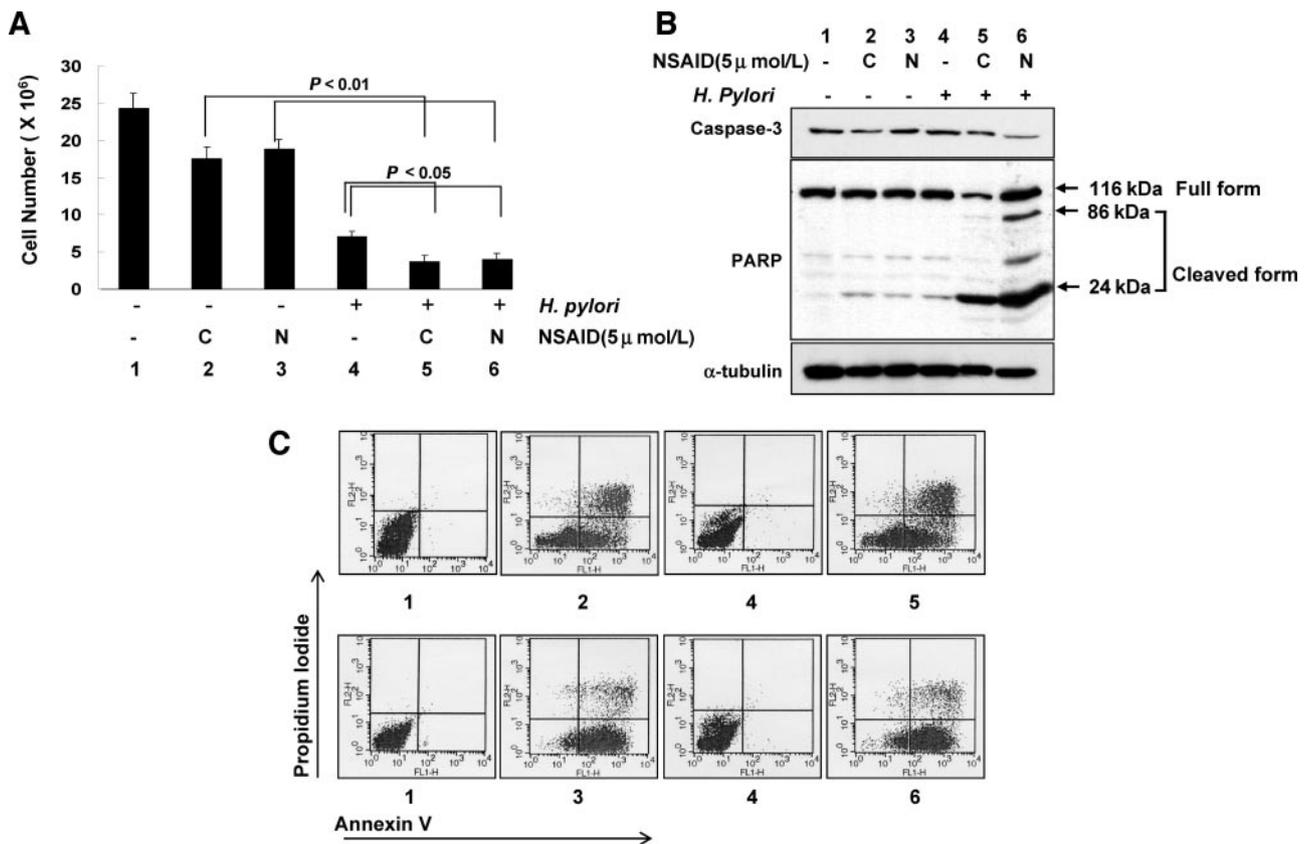


Fig. 6 *In vitro* experiments with AGS cells. **A**, cell survivals after each treatment analyzed by cell counts. Statistically significant decreases in cell survival were observed in AGS cells treated with both COX-2 inhibitor and *H. pylori* than were seen in cells treated with COX-2 inhibitor alone ($P < 0.01$) or *H. pylori* alone ($P < 0.05$). **B**, Western blot analysis of active form of caspase-3 and PARP. Significant decrease in the expression of active caspase-3 and cleavage of PARP were observed after both *H. pylori* and COX-2 inhibitor, signifying the occurrence of apoptotic cells death in cells treated with both COX-2 inhibitor and *H. pylori* infection. **C** stands for 5 μmol/L celecoxib, **N** stands for 5 μmol/L nimesulide. **C**, flow cytometry. Fluorescence-activated cell sorter analysis for annexin V, and propidium iodide staining of AGS cells. The percentages give the proportion of cells in the respective quadrant. Apoptosis was greatest in cells treated with both COX-2 inhibitor and *H. pylori*. **1**, control cells (AGS cells, gastric cancer cells); **2**, cells treated with celecoxib (5 μmol/L); **3**, cells treated with nimesulide (5 μmol/L); **4**, cells infected with *H. pylori* (2×10^6 CFU); **5**, cells treated with celecoxib (5 μmol/L) + *H. pylori* (2×10^6 CFU); **6**, cells treated with nimesulide (5 μmol/L) + *H. pylori* (2×10^6 CFU). (kDa, M_r in thousands.)

we found no evidence of any gastric mucosal damages in mice stomachs (group 4) assessed either by gross inspection or histologic evaluation, although the combination of COX-2 inhibitor and *H. pylori* did augment apoptosis in *in vitro* experiments (Fig. 6). The presence of *H. pylori* and coexisting gastritis has been shown to increase (37), have no effect (38), and even decrease (39), the risk of ulcer bleeding among patients ingesting aspirin or NSAIDs. In the present study, we found that despite long-term exposure to these two risk factors in groups 4 and 5, there was no increase in gastric inflammation or mucosal destruction compared with groups 1 and 2, which were exposed to a single risk factor. As for the explanations, we inferred that gastric defense mechanisms operated well in the groups exposed to both *H. pylori* and COX-2 inhibitor, and nimesulide administered to the *H. pylori*-infected mice must have blocked the effects of COX-2 on cellular proliferation, release of inflammatory mediators, and cell adhesion to matrix, which cause increased gastric inflammation (40). Moreover, *H. pylori*-alone provoked cellular apoptosis,

whereas NSAID-alone also contributed to apoptosis either by COX-2 inhibition or by direct activation of other cellular targets such as peroxisome proliferator-activated receptor γ observed in our present experiment (data not shown; ref. 41). *In vitro* experiments in this study showed the synergistic effects of cotreatment with COX-2 inhibitor and *H. pylori* on apoptosis and inhibition of proliferation. Recently, Kern *et al.* (42) suggested that hepatocarcinogenesis could be prevented by COX-2 inhibitors, based on the induction of apoptosis and the inhibition of proliferation; and data from Reddy *et al.* (19) strengthened the argument that selective COX-2 inhibitors possess chemopreventive activity against colon carcinogenesis.

In conclusion, we found that gastric tumorigenesis was significantly attenuated by long-term administration of the COX-2 inhibitor, nimesulide, in an *H. pylori*-associated gastric cancer mouse model, and we propose that selective COX-2 inhibitors may be clinically useful in protecting against gastric cancer development.

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