

# **A COX-2 inhibitor, nimesulide, inhibits chemically-induced rat tongue carcinogenesis through suppression of cell proliferation activity and COX-2 and iNOS expression**

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**Summary.** The modifying effects of a cyclooxygenase (cox)-2 selective inhibitor nimesulide on tongue carcinogenesis were investigated in male F344 rats initiated with 4-nitroquinoline-1-oxide (4-NQO). The cell proliferation activity measured by proliferating cell nuclear antigen (PCNA)-positive index and apoptotic index, and the immunohistochemical expression of COX-2, and inducible nitric oxide synthase (iNOS) in the tongue mucosa or neoplasms were also examined for mechanistic analysis of modifying effects of nimesulide on tongue carcinogenesis. All animals except those treated with nimesulide alone and untreated rats were given 20 ppm 4-NQO in drinking water for 8 weeks to induce tongue neoplasms. Starting 1 week after the cessation of 4-NQO exposure, rats given 4-NQO were fed the experimental diets containing nimesulide (100 and 400 ppm) for 22 weeks. At week 32, the incidence of tongue squamous cell carcinoma was significantly reduced by feeding of the diet containing 400 ppm nimesulide. Feeding of nimesulide significantly decreased polyamine content and PCNA-labeling index in tongue carcinoma. Apoptotic index in tongue carcinoma was increased by feeding of nimesulide. In addition, nimesulide feeding reduced COX-2 and iNOS expression in the tongue dysplasia and neoplasms. These results suggest that 400 ppm nimesulide in diet, when given during the promotion phase, exerts chemopreventive ability against 4-NQO-induced tongue tumorigenesis through inhibition of cell proliferation activity in conjunction with modification of COX-2 and iNOS expression of the target lesions.

**Key words:** Nimesulide, COX-2, Chemoprevention, Tongue carcinogenesis, iNOS

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## **Introduction**

It is well known that oral carcinoma, including tongue cancer, progresses from hyperplastic epithelial lesions through dysplasia to invasive carcinoma. The concept of "field cancerization" with molecular alterations can be applied to oral cavity tumorigenesis (Partridge et al., 1997). Such oral malignancy is a common neoplasm in Asia, the Pacific Islands, parts of Europe, and parts of Brazil (Parkin et al., 1993). Oral carcinoma is estimated to be the sixth most common cancer in the world. Despite recent surgical advances, the survival of patients with oral carcinoma remains poor: about 30-40% of patients with oral carcinoma have 5 years survival rate (Swango, 1996). The short survival time might mostly be caused due to late detection of this malignancy. Public awareness of oral carcinoma as compared with other cancers is low and this contributes to delays in diagnosis (Bhatti et al., 1995). An increase in the incidence has been reported in central and eastern Europe, especially among younger men (Macfarlane et al., 1994). Therefore, prevention of this malignancy is important.

Porteder et al. (1984) suggested that the alteration of cyclooxygenase (COX) and lipoxygenase (LOX) pathways of arachidonic acid (AA) metabolism involve in oral carcinogenesis. Two forms of COX have now been described: a constitutive enzyme COX-1 present in most cells and tissues, and an inducible isoenzyme COX-2 expressed in response to cytokines, growth factors, and other stimuli (Wakabayashi, 2000). Non-steroidal anti-inflammatory drugs (NSAIDs) are primarily recognized for their ability to inhibit specifically PG biosynthesis by inhibiting COX and a number of animal experiments indicated that NSAIDs exert inhibitory effects on cancer development in several organs (Wakabayashi, 2000), including tongue (Tanaka et al., 1989). Increased levels of PGs have been detected in malignant epithelial neoplasms of the head and neck

(Jung et al., 1985). Over-production of PGs could favor malignant growth (Milanovich et al., 1995; Sumitani et al., 2001). The discovery of COX-2 has provided the rationale for the development of novel anti-inflammatory agents with improved gastrointestinal tolerability compared with non-selective NSAIDs, which inhibit both COX-1 and COX-2 (Hawkey, 1999). Also, selective COX-2 inhibitors are suspected to be more effective and safer cancer chemopreventive agents than classical and non-selective NSAIDs (Wakabayashi, 2000). Recently, it has been reported that COX-2 is over-expressed in head and neck squamous cell carcinoma (Mestre et al., 1999). In addition, some carcinogens could upregulate COX-2 gene expression and PG production in cultured oral mucosal cells (Jeng et al., 2000). More recently, selective COX-2 inhibitors have been reported to inhibit COX-2 activity, proliferation activity, and PGE<sub>2</sub> production in oral cancer cell lines (Sumitani et al., 2001), and to suppress the growth of human head and neck squamous cell carcinoma xenografted in nude mice (Nishimura et al., 1999). These findings suggest that COX-2 inhibition may be a novel target for chemoprevention against oral cancer in addition to other organ cancers (Wakabayashi, 2000). In fact, recent reports have indicated possible chemopreventive effects of a selective COX-2 inhibitor nimesulide (4-nitro-2-phenoxymethanesulphonanilide, Fig. 1) (Bennett and Villa, 2000) on chemically-induced tongue (Shiotani et al., 2001), mammary gland (Nakatsugi et al., 2000), and urinary bladder carcinogenesis (Kitayama et al., 1999) in rats. Nimesulide, belonging to a class of compounds (sulfonanilides) that is unique among commercially available NSAIDs, appears to be a selective COX-2 inhibitor (Shah et al., 1999) with relatively low incidence of side effects (Rainsford, 1999). It exploits the enlarged binding site for establishing a number of

favorable contacts with the enzyme that lead to selective inhibition of COX-2 (Bennett, 1999; Garcia-Nieto et al., 1999).

Reactive oxygen metabolites involved oral carcinogenesis. Brennan et al. (2000a) reported the expression of iNOS in human oral dysplasia and carcinomas.

In the present study, we have examined chemopreventive efficacy of dietary nimesulide (100 and 400 ppm in diet) during the promotion phase on 4-nitroquinoline-1-oxide (4-NQO)-induced rat tongue carcinogenesis. Also, the modulatory effects of the compound on the proliferating cell nuclear antigen (PCNA)-index and the expression of COX-2 and iNOS were immunohistochemically investigated in the tongue lesions induced by 4-NQO, since a certain role of COX-2 (Jeng et al., 2000; Mestre et al., 1999; Nishimura et al., 1999) and iNOS (Brennan et al., 2000a,b; Chen and Lin, 2000) in head and neck cancer, including oral carcinoma, is suspected.

## Materials and methods

### *Animals, diets, and carcinogen*

Male F344 rats (Charles River Japan Inc., Kanagawa, Japan), 4 weeks old, were used. All animals were housed in wire cages (3 or 4 rats/cage) with free access to drinking water and basal diet, powdered CE-2 (345.2 Cal, CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (50±10%), light (12-h light/dark cycle) and temperature (23±2 °C). They were quarantined for 7 days and randomized into experimental and control groups. 4-NQO (CAS, 56-57-5; 98% pure) was obtained from Wako Pure Chemical Ind. (Osaka, Japan). Nimesulide was kindly provided by Helsinn Healthcare-SA (Pazzallo-Lugano, Switzerland). Experimental diets were prepared by mixing nimesulide at a concentration of 100 ppm or 400 ppm on weekly basis. The 4-NQO solution (20 ppm) was prepared every week. The experimental diets and 4-NQO solution were stored in a cold room until used. They were freely available during the study.

### *Experimental procedures*

A total of 67 male F344 rats were divided into 5 groups as shown in Fig. 2. Groups 1 through 3 were given 20 ppm 4-NQO in drinking water for 8 weeks. Groups 2 and 3 were fed diets mixed with 100 ppm and 400 ppm nimesulide, respectively, starting 1 week after cessation of 4-NQO treatment, and maintained on these diets for 22 weeks. Group 4 was given 400 ppm nimesulide-containing diet alone for 32 weeks. Group 5 served as an untreated control. Animals were weighed once weekly until they reached 9 weeks of age, and then every 4 weeks. The animals were monitored daily for their general health. The experiment was terminated at 32 weeks after the start, and all animals were sacrificed

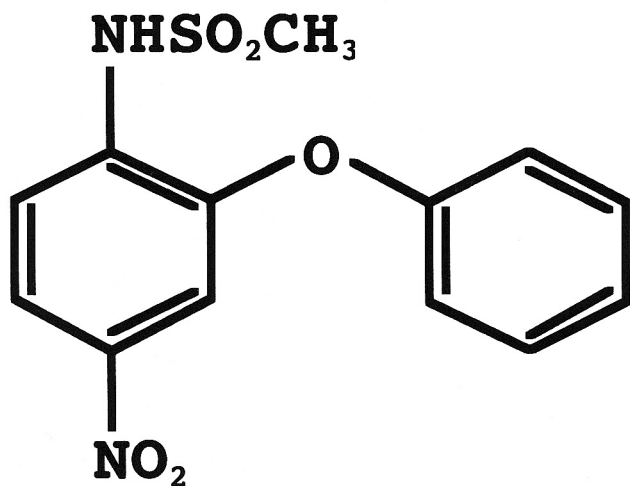


Fig. 1. Chemical structure of nimesulide.

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to assess the incidences of neoplastic and preneoplastic lesions in the tongue. At the termination of the study, complete autopsies were performed after the rats were killed by ether inhalation. At autopsy, all organs including tongue were carefully inspected for pathological lesions. The tongues were rapidly removed, rinsed with saline and cut into halves: one portion was used for histological examination and immunohistochemistry for PCNA, COX-2, iNOS, and apoptotic nuclei after fixing in 10% buffered formalin, and the other without fixing for tissue polyamine content. Tongue carcinoma was diagnosed according to the criteria described by Kramer et al. (1978).

### Polyamine assay

Tongue carcinomas developed in groups 1-3 and tongue mucosa without lesions from 3 rats each from groups 4 and 5 was scraped with a stainless steel disposable microtome bladed knife (S35, Feather Safety Razor Co., Ltd., Osaka), pooled and homogenized in 1.5 ml of homogenizing buffer (250 mmol sucrose, 50 mmol Tris-HCl, pH 7.4, containing 1 mmol dithiothreitol, 1 mmol EDTA and 0.4 mmol pyridoxal 5'-phosphate) using a Polytron. The homogenates were centrifuged at 15,000 rpm for 30 min at 4 °C. The resulting cytosol fraction was used for determination of tissue polyamine contents and protein. Tissue polyamine contents were determined as done in our previous study (Tanaka et al., 1997).

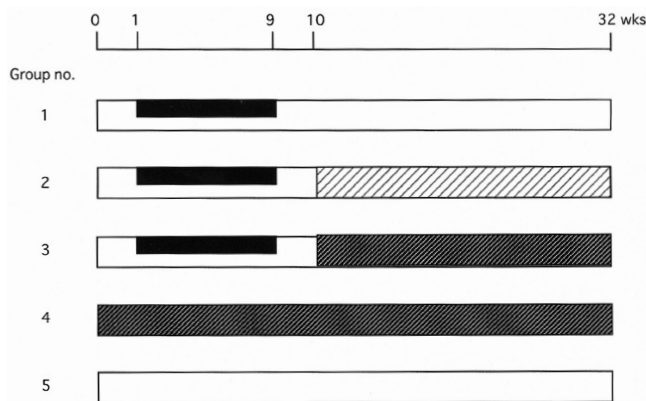
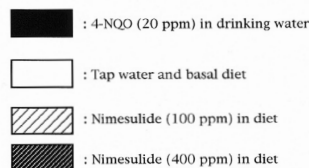


Fig. 2. Experimental protocol.



**Fig. 2.** Experimental protocol. Black rectangle: 4-NQO (20 ppm) in drinking water. White rectangle: Tap water and basal diet: Nimesulide (100 ppm) in diet: Nimesulide (400 ppm) in diet.

### Immunohistochemistry for PCNA, COX-2, iNOS, and apoptotic nuclei

In addition to hematoxylin and eosin staining for histopathological examination, four serial cross-sections of 3  $\mu$ m for immunohistochemistry of PCNA, COX-2, iNOS, and apoptotic nuclei were cut and mounted onto gelatin-coated glass slides. For the determination of PCNA-incorporated nuclei, the PCNA-immunohistochemistry was performed according to the method described by Watanabe et al. (1999). Apoptotic index was also evaluated by immunohistochemistry for single stranded DNA (ssDNA) (Watanabe et al., 1999). The immunohistochemistry was done using a stain system kit (DAKO LSAB 2 kit/HRP, DAKO Japan, Co., Ltd., Kyoto, Japan). A mouse monoclonal antibody against PCNA (1:50 dilution; PC10, DAKO Japan, Kyoto, Japan), a mouse monoclonal antibody against COX-2 (1:100 dilution, Transduction Laboratories, Lexington, KY), a mouse monoclonal antibody against iNOS (NOS2) (1:200 dilution, Transduction Laboratories), and a rabbit polyclonal antibody against ssDNA (1:300 dilution; DAKO Japan) were applied to the sections according to the manufacturer's protocol (DAKO LSAB 2 kit/HRP, DAKO Japan). All incubation steps were carried out for 15 min at 37 °C. The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride. Slides were lightly counterstained with Meyer's hematoxylin for 1 min, dehydrated, and coverslipped. Negative controls were performed by substituting the primary antibodies with non-immune mouse or rabbit serum. Slides were subsequently reviewed in a blind fashion. The PCNA and apoptotic indices were determined by counting the number of positive cells among at least 200 cells in the lesion, and were indicated as percentages. Each slide for COX-2 and iNOS was evaluated for intensity of immunoreactivity on a 0 to 4+ scale. The overall intensity of the staining reaction was scored, with 0 indicating no immunoreactivity and no positive cells, 1+ weak immunoreactivity and <10% of positive cells, 2+ mild immunoreactivity and 10-30% of positive cells, 3+ moderate immunoreactivity and 31-60% of positive cells, and 4+ strong immunoreactivity and 61-100% of positive cells.

### Statistical analysis

This was performed using the JMP software package (SAS Institute, Cary, NC). Where applicable, the data were analyzed using the chi-square test, Fisher's exact probability test or one-way ANOVA, followed by Bonferroni/Dunn post-hoc test, taking  $p < 0.05$  as the level of significance.

## Results

### General observation

The rats tolerated well the oral administration of 4-

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NQO and/or nimesulide feeding. As shown in Table 1, mean daily intake of food (g/rat) of all groups were comparable. During the study, no clinical signs of toxicity were present in any groups. Histologically, there were no pathological alterations suggesting toxicity of nimesulide in the liver, kidneys, heart, and lungs. The data on mean body, liver, and relative liver weights (g liver weight/100 g body weight) in all groups at sacrifice are also given in Table 1. The mean body weights of rats in all groups were comparable. The mean relative liver weight of group 3 (4-NQO → 400 ppm nimesulide) was significantly larger than that of group 1 ( $p < 0.02$ ).

### *Incidences of tongue preneoplastic lesions and neoplasms*

Tongue preneoplastic lesions (hyperplasia and dysplasia) and tumors (squamous cell papilloma and carcinoma) developed in the posterior tongue (dorsal region) of rats in groups 1-3. No preneoplastic or neoplastic lesions in any other organs, including tongue, were observed in groups 4 and 5. The incidences of these pathological lesions are summarized in Tables 2 and 3, respectively. As shown in Table 2, hyperplasia or various degrees of dysplasia with or without neoplasms

was observed in the tongue of rats in groups 1-3. The incidences of tongue squamous hyperplasia and dysplasia of rats in these groups were 100%. Among the various degrees of dysplasia, the incidence of severe dysplasia of rats in group 3 was significantly smaller than that of group 1 ( $p = 0.0343$ ). As indicated in Table 3, treatment of 4-NQO alone produced 48% incidence (13/27 rats) of tongue squamous cell carcinoma, while feeding of nimesulide after 4-NQO exposure reduced the incidence of this malignancy. The incidence (8%) of the tongue carcinoma in rats given 400 ppm nimesulide after 4-NQO exposure (group 3) was significantly lower than that of group 1 ( $p = 0.0173$ ). Also, the combined incidence of tongue neoplasms (papilloma + carcinoma) of this group was significantly smaller than group 1 ( $p = 0.0191$ ).

### *Polyamine content, PCNA-labeling index, and apoptotic index in tongue squamous cell carcinoma*

As summarized in Table 4, polyamine levels of squamous cell carcinoma of group 2 was significantly lower than group 1 ( $p < 0.05$ ). PCNA labeling index of squamous cell carcinoma developed in group 2 was significantly smaller than group 1 ( $p < 0.001$ ). Apoptotic

**Table 1.** Body, liver and relative liver weights, and food intake.

| GROUP no. | TREATMENT                    | No. OF RATS EXAMINED | BODY WEIGHT (g)     | LIVER WEIGHT (g) | RELATIVE LIVER WEIGHT (liver weight/100 g body weight) | MEAN DAILY INTAKE OF FOOD (g/rat) |
|-----------|------------------------------|----------------------|---------------------|------------------|--|-----------------------------------|
| 1         | 4-NQO                        | 27                   | 336±32 <sup>a</sup> | 11.8±1.3         | 3.52±0.17  | 15.43                             |
| 2         | 4-NQO→<br>100 ppm nimesulide | 12                   | 332±17              | 11.6±1.0         | 3.49±0.19  | 15.25                             |
| 3         | 4-NQO→<br>400 ppm nimesulide | 12                   | 329±24              | 12.9±0.8         | 3.94±0.36 <sup>b</sup>                                 | 15.60                             |
| 4         | 400 ppm nimesulide           | 8                    | 322±28              | 10.9±1.8         | 3.36±0.31  | 14.35                             |
| 5         | None                         | 8                    | 336±24              | 11.9±1.6         | 3.54±0.26  | 16.19                             |

<sup>a</sup>: mean±SD; <sup>b</sup>: significantly different from group 1 ( $p < 0.02$ ).

**Table 2.** Incidence of tongue hyperplasia, and dysplasia.

| GROUP no. | TREATMENT                    | No. OF RATS EXAMINED | HYPERPLASIA     | DYSPLASIA       |                 |                |                            |
|-----------|------------------------------|----------------------|-----------------|-----------------|-----------------|----------------|----------------------------|
|           |                              |                      |                 | Total           | Mild            | Moderate       | Severe                     |
| 1         | 4-NQO                        | 27                   | 27/27<br>(100%) | 27/27<br>(100%) | 24/27<br>(89%)  | 22/27<br>(81%) | 21/27<br>(78%)             |
| 2         | 4-NQO→<br>100 ppm nimesulide | 12                   | 12/12<br>(100%) | 12/12<br>(100%) | 12/12<br>(100%) | 11/12<br>(92%) | 6/12<br>(50%)              |
| 3         | 4-NQO→400<br>ppm nimesulide  | 12                   | 12/12<br>(100%) | 12/12<br>(100%) | 11/12<br>(92%)  | 10/12<br>(83%) | 5/12 <sup>a</sup><br>(42%) |
| 4         | 400 ppm<br>nimesulide        | 8                    | 0/8<br>(0%)     | 0/8<br>(0%)     | 0/8<br>(0%)     | 0/8<br>(0%)    | 0/8<br>(0%)                |
| 5         | None                         | 8                    | 0/8<br>(0%)     | 0/8<br>(0%)     | 0/8<br>(0%)     | 0/8<br>(0%)    | 0/8<br>(0%)                |

<sup>a</sup>: significantly different from group 1 by Fisher's exact probability test ( $p = 0.0343$ ).



### Inhibition of tongue carcinogenesis by nimesulide

index of group 2 was greater than group 1 without statistical significance, The polyamine content and indices of PCNA and apoptosis in group 3 were much lower and higher than group 1, respectively, but statistic analysis could not be done because only one carcinoma

developed in group 3.

#### Expression of COX-2 and iNOS in various tongue lesions

COX-2 and iNOS expression in various lesions is summarized in Table 5. In the positive cases of

**Table 3.** Incidence of tongue neoplasms.

| GROUP no. | TREATMENT                     | No. OF RATS EXAMINED | INCIDENCE OF TONGUE EOPLASMS |                         |                           |
|-----------|-------------------------------|----------------------|------------------------------|-------------------------|---------------------------|
|           |                               |                      | Total                        | Squamous cell papilloma | Squamous cell carcinoma   |
| 1         | 4-NQO                         | 27                   | 18/27<br>(67%)               | 10/27<br>(37%)          | 13/27<br>(48%)            |
| 2         | 4-NQO →<br>100 ppm nimesulide | 12                   | 7/12<br>(58%)                | 5/12<br>(42%)           | 5/12<br>(42%)             |
| 3         | 4-NQO →<br>400 ppm nimesulide | 12                   | 3/12<br>(25%) <sup>a</sup>   | 2/12<br>(17%)           | 1/12<br>(8%) <sup>b</sup> |
| 4         | 400 ppm nimesulide            | 8                    | 0/8<br>(0%)                  | 0/8<br>(0%)             | 0/8<br>(0%)               |
| 5         | None                          | 8                    | 0/8<br>(0%)                  | 0/8<br>(0%)             | 0/8<br>(0%)               |

<sup>a,b</sup>: significantly different from group 1 by Fisher's exact probability test (<sup>a</sup>p=0.0191 and <sup>b</sup>p=0.0173).

**Table 4.** Polyamine content and indices of PCNA and apoptosis in tongue squamous cell carcinoma.

| GROUP no. | TREATMENT                  | SQUAMOUS CELL CARCINOMA           |                                 |                    |
|-----------|----------------------------|-----------------------------------|---------------------------------|--------------------|
|           |                            | polyamine level (nmol/mg protein) | PCNA-labeling index             | Apoptotic index    |
| 1         | 4-NQO alone                | 6.39±1.43 <sup>a</sup><br>(13)    | 48.70±4.55%<br>(13)             | 0.42±0.21%<br>(13) |
| 2         | 4-NQO → 100 ppm nimesulide | 4.86±1.11 <sup>b</sup><br>(5)     | 33.78±3.87% <sup>c</sup><br>(5) | 0.88±0.46%<br>(5)  |
| 3         | 4-NQO → 400 ppm nimesulide | 4.23<br>(1)                       | 28.45%<br>(1)                   | 2.26%<br>(1)       |
| 4         | 400 ppm nimesulide         | 3.35±0.50<br>(3)                  | 14±2%<br>(3)                    | ND                 |
| 5         | No treatment               | 3.38±0.41<br>(3)                  | 13±2%<br>(3)                    | ND                 |

Numbers in parentheses are numbers of lesions examined. Values in groups 4 and 5 were determined in non-lesional tongue epithelium. N: not determined. <sup>a</sup>: mean±SD. <sup>b,c</sup>: significantly different from group 1 (<sup>b</sup>p<0.05 and <sup>c</sup>p<0.001).

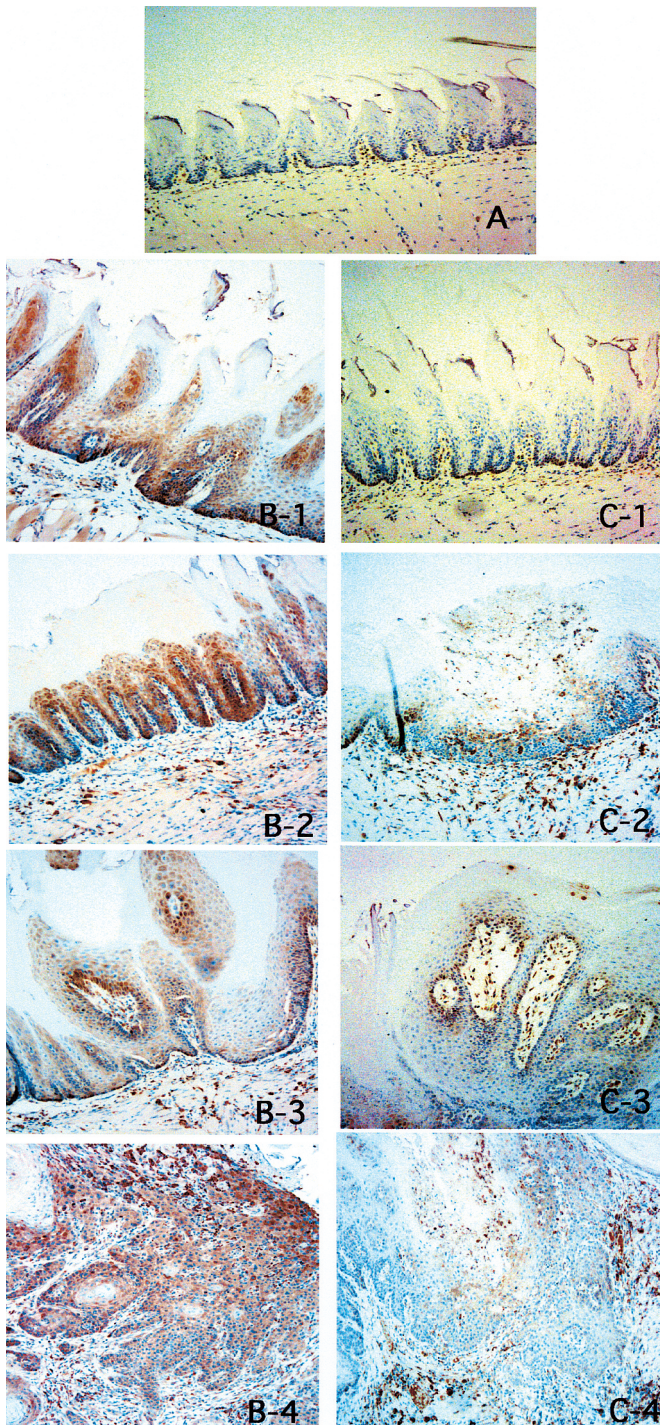
**Table 5.** Expression of COX-2 and iNOS in various lesions.

| GROUP no. | TREATMENT                  | NORMAL                         |                   | HYPERPLASIA                    |                                | DYSPLASIA                      |                                | SQUAMOUS CELL PAPILOMA |                   | SUAMOUS CELL CARCINOMA |                               |
|-----------|----------------------------|--------------------------------|-------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|------------------------|-------------------|------------------------|-------------------------------|
|           |                            | COX-2                          | iNOS              | COX-2                          | iNOS                           | COX-2                          | iNOS                           | COX-2                  | iNOS              | COX-2                  | iNOS                          |
| 1         | 4-NQO alone                | 0.85±0.53 <sup>a</sup><br>(27) | 0.68±0.75<br>(27) | 2.80±0.66<br>(27)              | 1.52±0.34<br>(27)              | 3.93±0.43<br>(27)              | 2.17±0.68<br>(27)              | 3.27±0.76<br>(10)      | 1.92±0.76<br>(10) | 3.57±0.77<br>(13)      | 2.84±0.53<br>(13)             |
| 2         | 4-NQO → 100 ppm nimesulide | 0.73±0.75<br>(12)              | 0.67±0.53<br>(12) | 2.20±0.33 <sup>b</sup><br>(12) | 1.40±0.52<br>(12)              | 3.00±0.45 <sup>c</sup><br>(12) | 2.02±0.33<br>(12)              | 2.83±0.55<br>(5)       | 1.77±0.75<br>(5)  | 3.41±0.38<br>(5)       | 2.08±0.15 <sup>d</sup><br>(5) |
| 3         | 4-NQO → 400 ppm nimesulide | 0.52±0.42<br>(12)              | 0.58±0.13<br>(12) | 2.13±0.51 <sup>e</sup><br>(12) | 1.14±0.22 <sup>f</sup><br>(12) | 2.63±0.84 <sup>g</sup><br>(12) | 1.38±0.91 <sup>e</sup><br>(12) | 2.60±0.28<br>(2)       | 1.58±0.31<br>(2)  | 2.36<br>(1)            | 1.37<br>(1)                   |

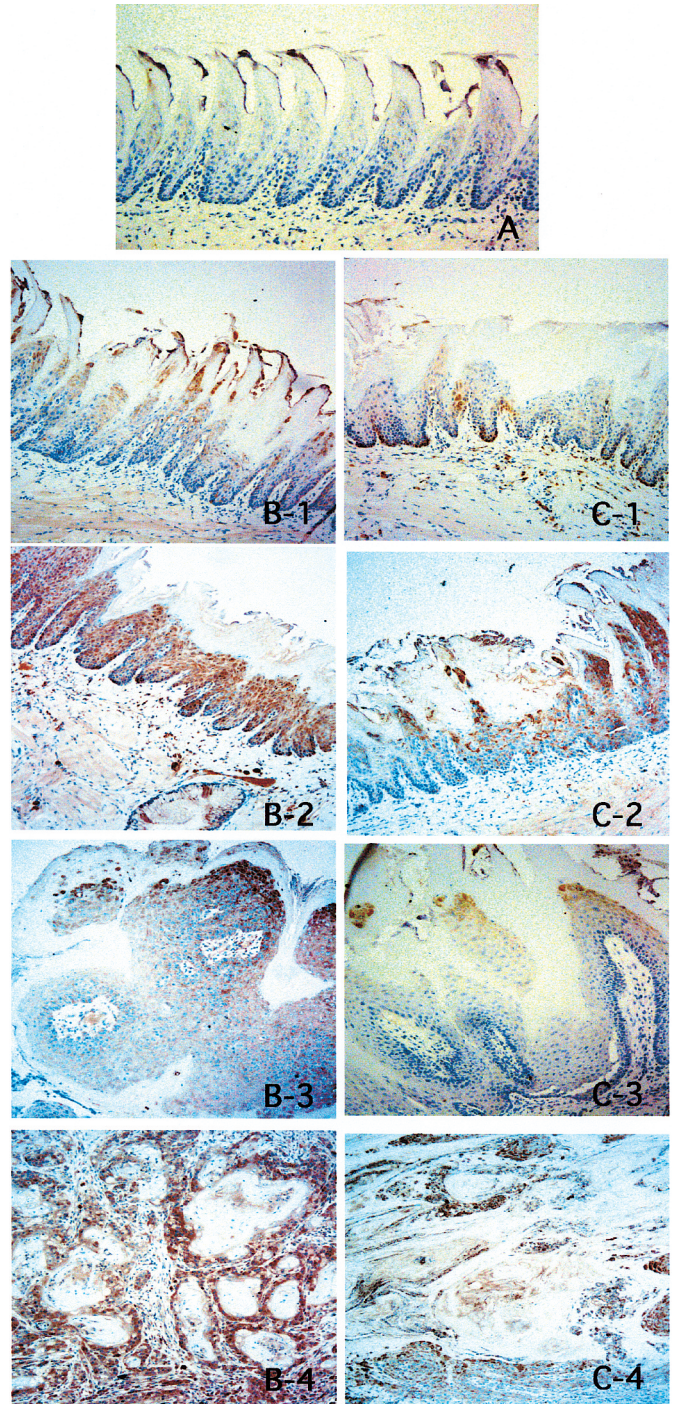
Numbers in parentheses are numbers of lesions or mucosa examined. <sup>a</sup>: mean±SD. <sup>b-g</sup>: significantly different from group 1 (<sup>b</sup>p<0.01, <sup>c</sup>p<0.001, <sup>d</sup>p<0.005, <sup>e</sup>p<0.05, <sup>f</sup>p<0.02, and <sup>g</sup>p<0.002).



*Inhibition of tongue carcinogenesis by nimesulide*



**Fig. 3.** Immunohistochemistry of COX-2 expression in various tongue lesions. **A.** Normal tongue mucosa. **B-1.** Hyperplasia. **B-2.** Dysplasia. **B-3.** Squamous cell papilloma. **B-4.** Squamous cell carcinoma developed in a rat from group 1. **C-1.** Hyperplasia. **C-2.** Dysplasia in a rat from group 2. **C-3.** Squamous cell papilloma. **C-4.** Squamous cell carcinoma developed in a rat from group 3. x 10



**Fig. 4.** Immunohistochemistry of iNOS expression in various tongue lesions. **A.** Normal tongue mucosa. **B-1.** Hyperplasia. **B-2.** Dysplasia. **B-3.** Squamous cell papilloma. **B-4.** Squamous cell carcinoma developed in rats from group 1. **C-1.** Hyperplasia. **C-2.** Dysplasia in a rat from group 2. **C-3.** Squamous cell papilloma. **C-4.** Squamous cell carcinoma developed in a rat from group 3. x 10



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expression for COX-2 and iNOS in non-lesional areas, dysplasia, and tongue neoplasms, the staining pattern was granular and localized to the cytoplasm. Very little immunoreactivity for COX-2 and iNOS was obtained in non-lesional tongue squamous epithelium in all groups. In the tongue epithelium that demonstrated histological evidence of inflammation in the submucosa, immunoreactivity was slightly higher than those lacking inflammation. Normal tongue epithelium (dorsal region) of rats in group 1 showed slight staining of COX-2 in the superficial layers of the epithelium and in parts of basal layer (Fig. 3A), as was also seen with iNOS (Fig. 4A). COX-2 expression of hyperplasia in these layers was more intensive than that of the normal epithelium (Fig. 3B-1), as was observed in iNOS expression (Fig. 4B-1). Intense cytosolic staining for COX-2 in severe dysplasia extended throughout all layers (Fig. 3B-2), as found for iNOS (Fig. 4B-2). COX-2 expression of squamous cell papilloma was noted basically in the basal layers (Fig. 3B-3), as seen in iNOS immunohistochemistry (Fig. 4B-3). In squamous cell carcinoma, positive staining for COX-2 was seen in the neoplastic cells (Fig. 3B-4) and iNOS (Fig. 4B-4). Different areas of the carcinoma stained with different intensity for COX-2 similar to that seen with iNOS. In group 1, COX-2 and iNOS expression increased steadily from normal through hyperplasia, dysplasia to squamous cell carcinoma, although the expression in squamous cell papilloma was relatively weak when compared with that in severe dysplasia (Fig. 3B, 1, 2, 3, and 4 for COX-2; Fig. 4B-1, 2, 3, and 4 for iNOS). This increase was significant (regression coefficient: COX-2 expression,  $r=0.762$ ,  $p<0.047$ ; iNOS,  $r=0.869$ ,  $p<0.020$ ). In groups 2 and 3, COX-2 and iNOS expression of various lesions was decreased when compared with that in group 1 (Fig. 3C-1, 2, 3, and 4 for COX-2; Fig. 4C-1, 2, 3, and 4 for iNOS). The differences on COX-2 expression of hyperplasia and dysplasia between group 1 and group 2 or 3 was statistical significant ( $p<0.01$ ,  $p<0.001$ ,  $p<0.05$  or  $p<0.002$ ). COX-2 expression of squamous cell carcinoma in group 2 was decreased when compared with group 1, but statistical significance was not found. iNOS expression of hyperplasia of group 3 and that of carcinoma in group 2 were significantly lower than that of group 1 ( $p<0.02$  and  $p<0.005$ , respectively). The correlation between the expression of COX-2 and iNOS of various tongue lesions was strong in groups 1 and 2 (correlation coefficient,  $r=0.983$ ,  $p<0.003$  in group 1 and  $r=0.940$ ,  $p<0.017$  in group 2), while that in group 3 (correlation coefficient,  $r=0.788$ ,  $p<0.112$ ) was not strong. In addition, the immunoreactive intensity of both enzymes in the invasive parts of squamous cell carcinoma was very strong.

### **Discussion**

Our results clearly indicate that dietary administration of nimesulide at a dose of 400 ppm during the promotion phase of 4-NQO-induced tongue

carcinogenesis significantly inhibited the occurrence of tongue squamous cell carcinoma. The suppressive effect of nimesulide on the development of tongue carcinoma was well correlated with the inhibition of cell proliferation activity and immunoreactivity of COX-2 and iNOS in the tongue with or without lesions. Previously, a significant inhibition of 4-NQO-induced rat tongue tumor development by other NSAIDs, piroxicam and indomethacin, which are inhibitors of both COX-1 and COX, was observed (Tanaka et al., 1989). However, COX and iNOS expression was not investigated in the study. Cancer inhibitory effects of 400 ppm nimesulide (83% inhibition) in this study was superior to that of piroxicam (56% inhibition) and indomethacin (79% inhibition) in our previous study (Tanaka et al., 1989). This is the first report on chemopreventive ability of a COX-2 inhibitor nimesulide in conjunction with suppression of COX-2 and iNOS expression and cell proliferation in 4-NQO-induced tongue tumorigenesis, although we did not analyze COX-2 and iNOS protein expression.

Recently, Shiotani et al. (2001) found the protective action of nimesulide using a different 4-NQO-induced rat tongue tumorigenesis model. In their model, rats were initiated with three cycles of 4-NQO exposure for 12 weeks (25 ppm 4-NQO for the first 2 weeks, 30 ppm of 4-NQO for the next 2 weeks, and 35 ppm of 4-NQO for the last 8 weeks), and the rats were fed 150, 300 or 600 ppm nimesulide containing diet after 4-NQO treatment for 14 weeks. In their study, 150 or 300 ppm nimesulide in diet significantly reduced the development of tongue squamous cell carcinoma, but dietary feeding of 600 ppm nimesulide did not significantly affect the incidence and multiplicity of tongue cancer. Also, nimesulide feeding at each dose did not alter the incidence of tongue dysplasia. They did not investigate the effects of nimesulide on immunoreactivity of COX-2 of various tongue lesions. Our results indicated that dietary administration of 400 ppm, but not 100 ppm, nimesulide significantly reduced the incidences of tongue carcinoma and dysplasia. As reported by Shiotani et al. (2001), there was no clear dose-response between the dosage of nimesulide used and the incidence of tongue squamous cell carcinoma in the current study, although the PCNA-labeling index, COX-2 and iNOS expression were decreased in tongue carcinoma developed in rats given 100 ppm nimesulide after 4-NQO exposure. It is likely that 100 ppm nimesulide in diet is enough to suppress the growth of tongue carcinoma, but not to inhibit the development of tongue malignancy. Taken together, there may be the existence of certain limited effective doses, being between 150 and 400 ppm in diet, of cancer protective ability of nimesulide.

In the current study, nimesulide treatment (100 ppm and 400 ppm in diet) was given to rats for 22 weeks after 4-NQO exposure, and dietary nimesulide at 400 ppm suppressed development of both tongue dysplasia and carcinoma, as found in our previous study where rats

were given piroxicam (150 ppm in diet for 24 weeks) and indomethacin (10 ppm in drinking water for 24 weeks) after 4-NQO exposure (10 ppm in drinking water for 12 weeks) (Tanaka et al., 1989). The findings may suggest that nimesulide feeding accelerates the regression of tongue dysplasia and/or blocks the progression of dysplasia to squamous cell carcinoma, since nimesulide feeding reduced cell proliferation activity of the tongue epithelium.

Immunohistochemical over-expression of COX-2 and iNOS and their increased level of mRNA in oral cancer are reported (Chan et al., 1999; Brennan et al., 2000b; Chen and Lin, 2000). Certain carcinogens up-regulate COX-2 expression in oral squamous cells (Kelley et al., 1997; Jeng et al., 2000). Although the effect of NO on tumor growth is both complicated and multifaceted (Brennan et al., 1999), NO itself increases the production of PGE<sub>2</sub>, which in turn suppresses the NO-dependent tumor-killing macrophages (Orucevic and Lala, 1996). As reported Shiotani et al. (2001), COX-2 expression was increased with progression of the lesions, hyperplasia, dysplasia, and squamous cell carcinoma in the current study. Inhibition of such an increase by COX-2 inhibitors may lead to inhibition of carcinogenesis (Mestre et al., 1999; Wakabayashi, 2000). In the present study, we immunohistochemically observed iNOS expression in tongue hyperplasia, dysplasia and neoplasms, as reported in oral lesions in human (Brennan et al., 2000a,b) and rodents (Chen and Lin, 2000). The expression was strong in histopathologically progressed lesions, as found in COX-2 expression. In the current study, a merely COX-2 inhibitor nimesulide in diet decreased the immunohistochemical expression of COX-2 in the tongue lesions induced by 4-NQO. It is likely that iNOS is associated with the modulation of COX-2 activity in tongue carcinogenesis induced by 4-NQO, since NO enhances the activity and expression of COX-2 in a variety of cell types (Salvemini et al., 1995). A new cancer therapy by preventing angiogenesis, invasion, and metastasis through inhibition of NOS generation is proposed (Thomsen and Miles, 1998). Therefore, it is interesting to investigate the effect of nimesulide on angiogenesis during 4-NQO-induced tongue carcinogenesis (Gallo et al., 1998, 2001; Gavilanes et al., 1999).

Cell proliferation plays an important role in multistage carcinogenesis and involves multiple genetic alterations (Cohen and Ellwin, 1990; Moore and Tsuda, 1998). In the current study, nimesulide feeding significantly lowered the PCNA-labeling index in the tongue carcinoma, suggesting that nimesulide in diet could suppress the high-proliferative activity of cells initiated with a carcinogen and inhibit carcinogenesis. During the 4-NQO-induced carcinogenesis a significant increase in apoptosis-related gene expression (Bcl-2 and BAX) was reported (Nishimura, 1999). It is known that COX-2 inhibitors can induce apoptosis in neoplastic cells (Tsuji et al., 2001; Wakabayashi, 2000). In this

study, apoptotic index in the tongue squamous cell carcinoma was increased by feeding of nimesulide, suggesting that chemopreventive effects of nimesulide on the 4-NQO-induced rat tongue tumor model are due to its ability to induce apoptosis as well as to inhibit COX-2 expression. Thus, one of the mechanisms by which nimesulide exerts chemopreventive ability might be related to suppression of cell proliferation and induction of apoptosis.

In conclusion, our results suggest that dietary nimesulide during the promotion phase exerts a chemopreventive effect on 4-NQO-induced rat tongue carcinogenesis. This protective effect of nimesulide might relate to suppression of COX-2 and iNOS expression, induction of apoptosis, and/or the control of carcinogen-induced hyper-cell proliferation. Although we did not investigate the effects of nimesulide on angiogenesis, it may be possible that nimesulide inhibits the progression of tongue carcinogenesis by inhibition of iNOS and/or COX-2 expression. Additional works to investigate this possibility need to be done for the ultimate goal of our chemoprevention research to prevent and inhibit the development of oral cancer including tongue cancer in humans.

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