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Inhibitory Effects of Nabumetone, a Cyclooxygenase-2 Inhibitor, and Esculetin, a Lipoxygenase Inhibitor, on *N*-Methyl-*N*-nitrosourea-induced Mammary Carcinogenesis in Rats

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We investigated the modifying effects of nabumetone, a relatively selective cyclooxygenase-2 inhibitor, and esculetin, a lipoxygenase inhibitor, on *N*-methyl-*N*-nitrosourea(MNU)-induced mammary carcinogenesis in female Sprague-Dawley rats. A total of 124 rats, 6 weeks old, were divided into 6 groups. At 50 days of age, groups 1, 2, and 3 were treated with MNU (50 mg/kg body weight) by subcutaneous injection. From the age of 8 weeks, groups 2 and 4 were given 0.03% nabumetone in the diet and groups 3 and 5 were given 0.03% esculetin in the diet. All rats were necropsied at the termination (25 weeks after the start of experiment). The incidence and multiplicity of neoplasms in group 2 were significantly smaller than those in group 1 ($P<0.005$ and $P<0.001$, respectively). The incidence of neoplasms in group 3 was also significantly smaller than that in group 1 ($P<0.05$). These results indicate that the intake of nabumetone or esculetin during the time corresponding to the post initiation phase has a chemopreventive effect on MNU-induced mammary carcinogenesis in rats.

Key words: Cyclooxygenase-2 — Lipoxygenase — Rat mammary carcinogenesis — Chemoprevention

It is reported that the levels of prostaglandins (PGs) are high in cancers of the breast, head, neck, lung, and colon.[1–7] Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX), which catalyzes the transformation of arachidonic acid to PGs, and have an inhibitory effect on carcinogenesis.[8–11] COX has two isozymes (COX-1 and COX-2). COX-1 is expressed in most tissues. The expression of COX-2 is induced by growth factor, tumors promoters and so on. Recent studies, including ours, indicated that selective COX-2 inhibitors can inhibit azoxymethane-induced aberrant crypt foci in rat colon.[12–14] However, the mechanism by which COX-2 inhibitors inhibit carcinogenesis is not well understood.[15–17]

Barnnett *et al.* compared the COX-1/2 inhibitory activities of an extensive array of NSAIDs using purified human enzymes expressed in baculovirus and demonstrated that nabumetone is a relatively selective COX-2 inhibitor.[18] Nabumetone, which is used to treat rheumatic arthritis in Japan and has little side effect on the gastrointestinal mucosa, is a prodrug of 6-methoxy-2-naphthylacetic acid and is more selective for COX-2 than indomethacin, sulindac or ibuprofen.[18–21]

Esculetin is a 5- and 12-lipoxygenase inhibitor and inhibits the production of leukotrienes and 5-hydroxyeicosatetraenoic acid through the lipoxygenase pathway, which is a part of the arachidonic acid cascade. It was reported that esculetin inhibited cell proliferation of the TMT-801 rat mammary tumor cell line,[22] cell proliferation of MDA-MB-231 breast cancer cells *in vitro* and mammary tumorigenesis[23] and tumor proliferation in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in female rats.[24] In this paper, we investigated the chemopreventive effects of nabumetone, a relatively selective COX-2 inhibitor, and esculetin, a lipoxygenase inhibitor, on *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague-Dawley (SD) rats.

MATERIALS AND METHODS

Animals and chemicals Weaning female SD rats were obtained from Japan SLC, Inc. (Shizuoka). MNU was purchased from Nacalai Tesque, Inc. (Kyoto). Nabumetone was purchased from Sigma Chemical Co. (St. Louis, MO). Esculetin purchased from Tokyo Kasei Chemical Co. (Tokyo). Basal diet (CE-2) was obtained from CLEA Japan, Inc. (Tokyo) and the experimental diets were mixed in our laboratory. All animals were housed in wire cages (3 or 4 rats/cage). They had free access to water and diet under controlled environmental conditions of humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C).

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Experimental procedure A total of 124 rats were randomized into 6 groups. At 50 days of age, rats in groups 1, 2, and 3 were given s.c. injections of MNU dissolved in saline (pH 5.0), at a dose rate of 50 mg/kg of body weight, according to the procedure of Thompson and Meeker.[25] Animals intended for vehicle treatment were s.c.-injected with equal volumes of saline. From one week after MNU or saline injection, groups 2 and 4 were given the diet containing 0.03% nabumetone and groups 3 and 5 were given the diet containing 0.03% esculetin, for 24 weeks. Groups 1 and 6 were given the basal diet throughout the experiment. From 5 weeks after the exposure to MNU, each rat was carefully checked for palpable mammary tumors (Fig. 1). The experiment was terminated at 25 weeks and all animals were killed for evaluation of the incidence and multiplicity of mammary tumors. At the termination, the number and size of mammary tumors were recorded. The mammary tissues with neoplasms were fixed in 10% buffered formalin and embedded in paraffin. Tumor volume was obtained by use of the formula $4/3\pi abc$, where a, b, and c are the three dimensions of the tumor measured at the termination.

Immunohistochemical staining Two sections (4 μm thick) of each tumor were obtained from paraffin blocks. One section was stained with hematoxylin-eosin for histological observation. Another section was subjected to immunohistochemical examination to check cell proliferation of tissues by using anti-proliferating cell nuclear antigen (PCNA) antibody.[26] Briefly, endogenous peroxidase activity was blocked by immersing the sections in methanol with 3% hydrogen peroxide for 5 min, and then the sections were rinsed three times with phosphate-buffered saline (PBS, pH 7.4). They were incubated in PBS with 1% bovine serum albumin (BSA) for 30 min at room temperature and then incubated with PCNA (DAKO, Denmark) at a 1:50 dilution in PBS-BSA for 1 h at room temperature. They were rinsed in PBS, and PCNA staining was done by the labeled streptavidin biotin (LSAB) method using an LSAB kit (DAKO, Carpinteria, CA). The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine in PBS. Finally, counterstaining was performed using Mayer's hematoxylin. We counted the PCNA-positive nuclei and the PCNA-negative nuclei in 10 high-magnification microscopic fields per slide, chosen at random, in 10 slides per group from groups 1–3. PCNA index was determined by measuring the number of PCNA-positive nuclei as a proportion of the total nuclei (approximately 10,000 nuclei).

Statistical analysis Data are presented as mean±SD, and Student's t test, Welch's method or the χ^2 test was used to determine the significance of differences between groups. The criterion of significance was taken as $P<0.05$.

RESULTS

No statistically significant differences in survival curves were observed. Body weight, liver weight and relative liver weight are summarized in Table I. The final body weight and liver weight of the rats in group 2 were significantly lower than those of rats in group 1 ($P<0.001$, $P<0.05$), but no significant difference in relative liver weight was found (Table I). We thought that this might reflect the weight of tumors in group 1, because the number of large tumors in rats of group 2 was less than that of rats in group 1. The liver weights of the rats in group 3 are significantly less than in group 1 ($P<0.05$), and those in groups 4 and 5 are significantly less than in group 6 ($P<0.05$) (Table I). There has been no previous report that nabumetone or esculetin reduced body weight or induced liver dysfunction, and the mechanism by which the liver weight of rats in groups 2–5 was decreased is unclear. In this study, the liver tissues of rats in each group were histopathologically normal.

The number of palpable tumors in each group is shown in Fig. 1. The first palpable mammary tumor in a rat of group 2 appeared 7 weeks after the start of the experiment

Table I. Final Body and Liver Weights

Group no.	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (%)
1	MNU alone	30	278±21.7[a]	14.1±2.23	5.05±0.68
2	MNU+0.03% nabumetone	31	261±15.0[b]	12.7±2.08[c]	4.93±0.70
3	MNU+0.03% esculetin	31	268±21.7	13.3±2.23[c]	4.96±0.65
4	0.03% nabumetone alone	11	266±12.3	12.8±1.08[d]	4.90±0.30
5	0.03% esculetin alone	10	274±16.0	12.4±2.15[d]	4.94±0.72
6	no treatment	11	280±25.2	14.5±1.80	5.03±0.45

a) Mean±SD.
b) Significant difference from group 1 by Student's t test ($P<0.001$).
c) Significant difference from group 1 by Student's t test ($P<0.05$).
d) Significant difference from group 6 by Student's t test ($P<0.05$).

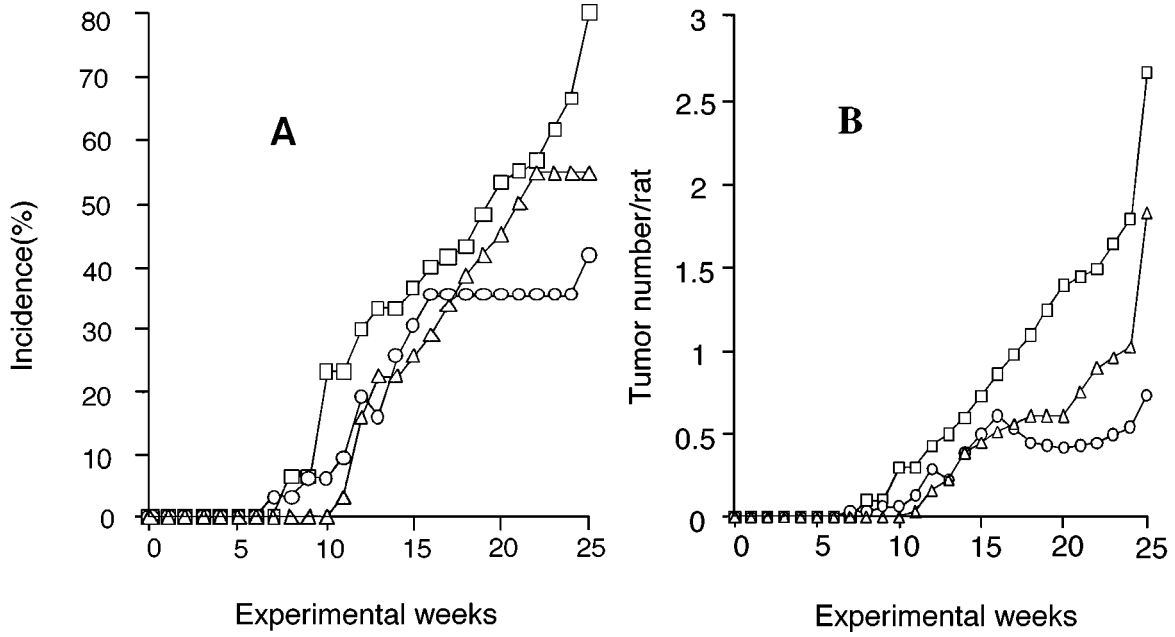

Fig. 1. Effect of dietary supplementation of nabumetone or esculetin on MNU-induced mammary tumors. A, The incidence of mammary tumors; B, The multiplicity of mammary tumors. □ MNU alone, ○ MNU+nabumetone, △ MNU+esculetin.

Table II. Incidence, Multiplicity and PCNA Labeling Index of Mammary Tumors

Group no.	Treatment	No. of rats	No. of tumor-bearing rats (%)	Total no. of tumors	Multiplicity	PCNA labeling index (%)	Tumor volume (cm^3)
1	MNU alone	30	24 (80)	80	2.67±2.55[a)]	41.1±7.1	1.0±2.2
2	MNU+0.03% nabumetone	31	13[b)] (42)	23	0.74±1.31[c)]	35.0±12	1.3±2.7
3	MNU+0.03% esculetin	31	17[d)] (55)	57	1.84±2.12	37.6±4.8	0.7±1.0
4	0.03% nabumetone alone	11	0 (0)	0	0	—	—
5	0.03% esculetin alone	10	0 (0)	0	0	—	—
6	no treatment	11	0 (0)	0	0	—	—

a) Mean±SD.
b) Significant difference from group 1 by χ^2 test ($P<0.005$).
c) Significant difference from group 1 by Welch's method ($P<0.001$).
d) Significant difference from group 1 by χ^2 test ($P<0.05$).

(Fig. 1). Mammary tumors were palpated in two rats of group 1 at 8 weeks and in one rat of group 3 at 11 weeks. Compared to the control group, tumor onset was advanced 1 week in rats fed diet containing nabumetone and delayed 3 weeks in rats fed diet containing esculetin. At the 24th experimental week, the incidences of palpable tumors reached 67%, 36%, and 55% in groups 1–3, respectively.

The final incidence and multiplicity in each group are shown in Table II. The incidences of tumors were 80%, 42%, and 55% in groups 1–3, respectively (Table II). Those of groups 2 and 3 were significantly lower than that of group 1 ($P<0.005$ and $P<0.05$, respectively) (Table II). Furthermore, the multiplicity of tumors was significantly decreased by nabumetone ($P<0.001$) (Table II).

Values of the PCNA index were 41.1±7.1, 35.0±12.3, and 37.6±4.8 in groups 1–3, respectively. Although those of groups 2 and 3 were a little lower than that of group 1, the difference was not significant (Table II). Mammary tumors were not palpated in groups 4–6 during the experiment and were not detected at termination. The tumor volumes were 1.0±2.2, 1.3±2.7, and 0.7±1.0 cm^3 in

groups 1–3, respectively, and the differences were not significant (data not shown).

DISCUSSION

Since breast cancer is the most frequent major tumor in American women and an increasing cause of cancer deaths in Japanese women, it is important to investigate the chemoprevention of breast cancer.[27] Several researchers have investigated the inhibitory effect of nonsteroidal anti-inflammatory drugs (NSAIDs), i.e., the sulfone metabolite of sulindac,[28] indomethacin,[29] or flurbiprofen,[30] on mammary carcinogenesis in rats. However, Kitagawa and Noguchi found that piroxicam, one of the NSAIDs, had no inhibitory effect on DMBA-induced mammary carcinogenesis.[24] Usual NSAIDs inhibit both COX-1 and -2, and induce stomach ulcers and renal dysfunction as side effects. Recently, selective COX-2 inhibitors, which have few side effects, have attracted the attention of many researchers. In this study, we used nabumetone, a relatively selective COX-2 inhibitor. Our results indicate that nabumetone or esculetin can inhibit MNU-induced mammary carcinogenesis. To our knowledge, this is the first report to show that nabumetone inhibits MNU-induced mammary tumors in rats. Nabumetone has the ability to inhibit COX-1 and COX-2 and inhibits COX-2 more selectively than commonly used NSAIDs, such as indomethacin, ibuprofen, and sulindac.[18, 21] Further, nabumetone induces little gastrointestinal mucosal injury.[20, 21] At the termination, we could not find gastric ulcer or duodenal ulcer in any of the MNU-injected rats.

In this study, the incidence and multiplicity of mammary tumors in the group of rats given the diet containing nabumetone were reduced. The PCNA index of rats in group 2 was slightly reduced. Thus, we suggest that nabumetone inhibits MNU-induced mammary carcinogenesis. There seem to be three possible mechanisms by which nabumetone suppresses MNU-induced mammary carcinogenesis. Firstly, nabumetone may suppress cell proliferation of MNU-induced mammary tumors, because although mammary tumors of rats fed diet containing nabumetone occurred at the same time as those in rats fed the control diet, the incidence and multiplicity of rats given nabumetone were significantly lower than those of rats fed control diets and their PCNA index was also lower. Secondly, there may be a reduction of cells initiated with MNU via apoptosis. Thompson *et al.* demonstrated that sulindac sulfone, which lacks prostaglandin synthetase-inhibitory activity, inhibited MNU-induced mammary carcinogenesis in a dose-dependent manner and induced apoptosis in MCF-7 cells.[31] We also reported that NS-398, a selective COX-2 inhibitor, induced apoptosis and inhibited colon carcinogenesis in colorectal cancer cell lines.[32] Thirdly, mammary carcinogenesis may be associated with inflammation. Several researchers have investigated the link between carcinogenesis and inflammation.[33, 34] Inflammation seems to be associated with the promotion phase of carcinogenesis, because the metabolites of arachidonic acid which induce inflammation may be related to cell proliferation.[34, 35] We have also found an increase of cytokines and arachidonic acid cascade-related enzymes, phospholipase A_2 and COX-2, in rat colon carcinogenesis.[36, 37] Recently, Oshima *et al.* demonstrated that COX-2-selective inhibitors may be a novel class of therapeutic agents for colorectal polyposis and cancer by the use of $Apc^{\Delta 716}$ knockout mice given a novel COX-2-selective inhibitor MF tricyclic and by introducing a knockout mutation of the COX-2 gene.[38] Nakatsugi *et al.* demonstrated that nimesulide, a selective COX-2 inhibitor, suppressed intestinal polyp development in Min mice.[39] However, the mechanism by which COX-2 inhibitors suppress carcinogenesis is not clear.

Kitagawa and Noguchi investigated the effects of piroxicam, a COX inhibitor, and esculetin, a lipoxygenase inhibitor, on DMBA-induced mammary carcinogenesis in female rats and demonstrated that esculetin inhibited mammary tumorigenesis and tumor proliferation in rats.[24] We decided to compare the effects of nabumetone, a cyclooxygenase inhibitor, and esculetin, a lipoxygenase inhibitor. Though the carcinogen which we used, MNU, was different from the one they used, the results in this study were similar to theirs. Esculetin is a 5- and 12-lipoxygenase inhibitor and inhibits the production of leukotrienes and 5-hydroxyeicosatetraenoic acid through the lipoxygenase pathway. A recent study indicated that esculetin inhibits cell proliferation of the TMT-801 rat mammary tumor cell line[22] and the MDA-MB-231 breast cancer cell lines *in vitro*.[23] Lipoxygenase inhibitors are considered to have more potency as cell proliferation suppressors than cyclooxygenase inhibitors.[40, 41] This study indicates that esculetin may also suppress the incidence of MNU-induced mammary carcinogenesis and inhibit cell proliferation of MNU-induced mammary tomors. However, since the mechanism is still unclear, further study is needed, including work at the molecular level. In addition, according to our results, the combination of nabumetone and esculetin might inhibit mammary carcinogenesis more effectively than single use of either of them, because phospholipase A_2, which lies above COX and lipoxygenase in the arachidonic acid cascade, is increased in rat colon carcinogenesis.

In conclusion, our data suggest that nabumetone, a relatively selective COX-2 inhibitor, inhibits mammary carcinogenesis and may inhibit cell proliferation, and that esculetin, a lipoxygenase inhibitor, may also inhibit mammary carcinogenesis.

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