Jpn. J. Cancer Res. **91**, 686–691, July 2000

Suppression by Flavonoids of Cyclooxygenase-2 Promoter-dependent Transcriptional Activity in Colon Cancer Cells: Structure-Activity Relationship

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Cyclooxygenase-2 (COX-2) plays an important role in carcinogenesis. Investigation of the suppressive action of twelve flavonoids of different chemical classes on the transcriptional activity of the *COX-2* gene in human colon cancer DLD-1 cells using a reporter gene assay have revealed quercetin to be the most potent suppressor of COX-2 transcription (IC_{50}=10.5 μM), while catechin and epicatechin showed weak activity (IC_{50}=415.3 μM). Flavonoids have three heterocyclic rings as a common structure. A structure-activity study indicated that the number of hydroxyl groups on the B ring and an oxo group at the 4-position of the C ring are important in the suppression of COX-2 transcriptional activity. A low electron density of the oxygen atom in the hydroxyl group of the A ring was also important. Further examination of the role of the hydroxyl group in the A ring showed that bromination of resacetophenone to give 3,5-dibromo-2,4-dihydroxyacetophenone resulted in a 6.8-fold increase in potency for suppressing COX-2 promoter activity. These results provide a basis for the design of improved suppressors of COX-2 transcriptional activity.

Key words: Cyclooxygenase-2 — Colon cancer — Reporter gene assay — Flavonoids — Electron density

Flavonoids, polyphenolic compounds that are ubiquitously present in foods of plant origin, have long been recognized to possess antiallergic, anti-inflammatory, antiviral, antiproliferative and anticarcinogenic properties.[1,2] Physically, they are characterized by two phenyl rings connected by a pyran ring or a similar structure of three carbons. These rings are referred to as A, B and C, respectively. Flavonoids are classified into flavonols, flavones, catechins, flavanones, anthocyanidins, dihydrochalcone and isoflavones, based on variations in ring C. Several recent studies have demonstrated that, depending on their structure, flavonoids may be potent inhibitors of several enzymes, including tyrosine kinase, protein kinase C, and phosphatidylinositol 3-kinase,[3,4] which are all involved in regulation of cyclooxygenase-2 (COX-2) expression. However, the inhibitory effects of flavonoids on COX-2 expression have yet to be investigated in detail.

COX, a key enzyme in the biosynthetic pathway leading to the formation of prostaglandins, has two isoforms. COX-1 is constitutively expressed to maintain physiological functions, while COX-2 is an inducible enzyme that is upregulated during inflammation and colorectal tumor formation.[5–8] Many epidemiological and animal experiments have demonstrated that inhibition of COX-2 activity reduces the risk of colon carcinogenesis.[9–12] Thus, COX-2-selective inhibitors have potential as chemopreventive agents against development of cancer in the colon and other organs. It is also likely that agents that can suppress COX-2 expression at the gene level may be equally advantageous.

As documented in a previous paper,[13] we have constructed a β-galactosidase (β-gal) reporter gene system to test the effects of compounds on COX-2 transcriptional activity in human colon cancer cells, and found that three flavonoids, genistein, kaempferol and quercetin, suppress COX-2 transcriptional activity. In the present study, using these and an additional nine flavonoids, the relationships between structure and suppression of COX-2 transcriptional activities were examined, and an analysis of the electron density of hydroxyl groups of flavonoids was performed. Our results suggest that low electron density of oxygen at the 5,7-positions of the A ring, the number of hydroxyl groups on the B ring, and an oxo group at the 4-position of the C ring are important aspects of flavonoid structure that affect the suppression of COX-2 transcriptional activity.

MATERIALS AND METHODS

Chemicals (+)-Catechin, (−)-epicatechin, myricetin and transforming growth factor α (TGFα) were obtained from Sigma Chemical Co. (St. Louis, MO). Eriodictyol, fisetin, kaempferol, luteolin and rhamnetin were from Extrasyn-

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these (Genay, France). Genistein was from Fujicco Co., Ltd. (Kobe). Phloretine, quercetin and resacetophenone were from Wako Pure Chemical Ind., Ltd. (Osaka). (−)-Epigallocatechin was from Kurita Water Ind. (Tokyo).

Synthesis of 3,5-dibromo-2,4-dihydroxyacetophenone 2,4-Dihydroxyacetophenone (0.15 g) was suspended in carbon tetrachloride (10 ml), and *N*-bromosuccinimide (0.39 g) was added. The reaction mixture was stirred and heated in a water bath (40°C) for 4 h. The solvent was removed under reduced pressure, and the residue was recrystallized from methanol once to give 3,5-dibromo-2,4-dihydroxyacetophenone (BHAP) (0.13 g) as a colorless material. Positive-ion EI-MS (*m*/*z*): 312, 310, 308 (M^+). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ; 2.60 (3H, s, CH_3), 8.12 (1H, s, H−6), 11.05 (1H, brs, 2−OH).

Calculation of electron density of oxygen atom The electron densities of the oxygen atoms of the hydroxyl groups at the 5 and 7 positions of flavonols and those of the corresponding hydroxyl groups of resacetophenone and 3,5-dibromo-2,4-dihydroxyacetophenone were calculated by means of the semi-empirical quantum mechanical method AM-1 using MOPAC ver 6.3. The initial geometry was constructed from standard bond lengths and angles, and then completely optimized using an algorithm in the MOPAC program.[14, 15]

Cell culture and analysis of cell viability Cells of the DLD-1 human colon adenocarcinoma cell line were obtained from the Health Science Research Resources Bank (Osaka) and maintained in RPMI1640 medium supplemented with 5% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and antibiotics (100 μg/ml of streptomycin and 100 units/ml of penicillin) at 37°C in 5% CO_2. Cells (2.0×10^4 cells/well in 100 μl) were plated in 96-well tissue culture dishes and precultured for 24 h before treatment with 100 ng/ml of TGFα and test reagents for 48 h.

Cell viability in each culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously reported.[13]

Reporter gene assay for COX-2 promoter-dependent transcriptional activity pB2-βGal-BSD and pCOX2/B2-βGal-BSD plasmid DNAs were constructed as reported earlier.[13] In brief, pB2-βGal-BSD is a basic vector for reporter gene assay that contains the *lacZ* gene and the blasticidin S deaminase (*BSD*) gene. pCOX2/B2-βGal-BSD was constructed by insertion of the 2078-nucleotide human *COX-2* gene promoter fragment stretching from −2046 to +32 relative to the transcription start site of the human *COX-2* gene into the upstream site of the *lacZ* gene of the pB2-βGal-BSD plasmid. The pB2-βGal-BSD, as well as the pCOX2/B2-βGal-BSD plasmid DNA, were separately transfected into DLD-1 cells, designated DLD-1/B2-βGal-BSD and DLD-1/COX2-B2-βGal-BSD, respectively. Transfected cells were selected in medium containing 20 μg/ml of blasticidin S hydrochloride (Kaken Pharmaceutical, Tokyo) and subcloned by limiting dilution. A subclone that contained an intact DNA fragment of the 2078-base-pair COX-2 promoter region and downstream *lacZ* gene in the genome DNA was used for

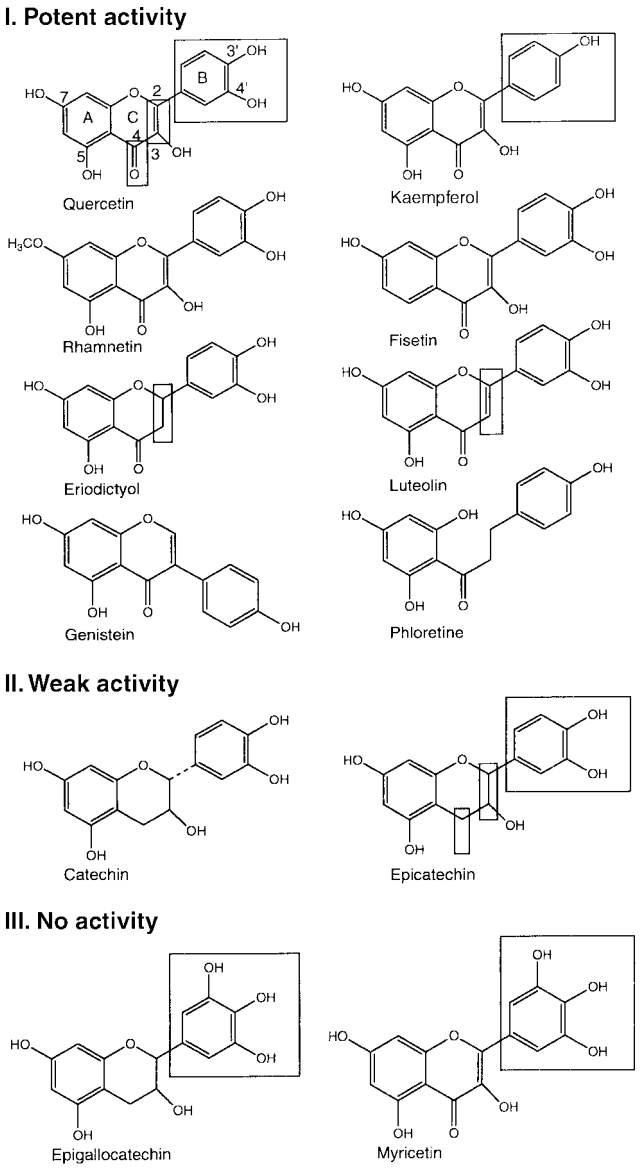

Fig. 1. Structures of the flavonoids examined. They were classified into three groups, I, II, and III, based on their suppressive effects. The A, B and C rings are indicated for quercetin. The structural moieties employed for assessment of structure-activity relationships are indicated by boxes; structural differences are evident between quercetin and epicatechin, between luteolin and eriodictyol and between the B ring of flavonols (myricetin, kaempferol and quercetin) and that of flavanols (epicatechin and epigallocatechin).

the present study, as described previously.[13] The total β-gal activities of DLD-1 cells in each well were determined by a colorimetric assay using *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The background β-gal activity was determined in untreated DLD-1/B2-βGal-BSD cells and subtracted from the β-gal assay values. In the present study, IC_{50} was defined as the concentration that caused a 50% decrease in TGFα-stimulated COX-2 transcriptional activity. The percentage β-gal activity for each treatment was calculated from data for triplicate wells. The values of β-gal activity were normalized for viable cell number, assessed by the MTT assay. All experiments were repeated three times. IC_{50} (n=3) values were plotted as means±SD.

RESULTS

Suppression of COX-2 transcriptional activity by flavonoid compounds Nine compounds belonging to six representative chemical classes of flavonoids were tested at various concentrations up to 500 μM with regard to their effects on COX-2 transcriptional activity (Fig. 1). Treatment of cells with 100 ng/ml TGFα for 48 h increased COX-2 transcriptional activity to 2.1 times the value in untreated DLD-1/COX2-B2-βGal-BSD cells. No significant decrease in cell viability was observed after a 48-h culture with TGFα and flavonoids at concentrations equivalent to their IC_{50} values. The suppressive effects of nine flavonoid compounds on COX-2 transcriptional activity are shown in Table I. Based on their ability to induce a 50% decrease in TGFα-stimulated COX-2 transcriptional activity, the flavonoids could be classified into three groups: potent, weak, and no suppression. Eriodictyol, fisetin, luteolin, phloretine and rhamnetin were potent suppressors of COX-2 transcriptional activity, with IC_{50} values of 18.6–52.5 μM. In contrast, catechin and epicatechin showed only very weak suppression of COX-2 transcriptional activity (IC_{50}=415.3 μM) and epigallocatechin and myricetin exhibited no suppression.

For comparison with the present data, the suppressive effects of three flavonoids, quercetin, genistein and kaempferol, on COX-2 transcriptional activity, which were previously reported,[13] are also given in Table I, and the structures of these compounds are included in Fig. 1. These three flavonoids are classified as potent suppressors; quercetin was found to be the most potent suppressor among the twelve compounds tested in the present and previous studies. Moreover, among the chemical classes in the present and previous studies, flavonols were all potent suppressors of the COX-2 transcriptional activity, except for myricetin. On the other hand, flavanols, which lack a 4-oxo group, were weak suppressors, except for epigallocatechin. Eriodictyol, a flavanone, genistein, an isoflavone, and phloretine, a dihydrocalcone, all possess a 4-oxo group and potently suppressed COX-2 transcriptional activity. Myricetin and epigallocatechin which have three hydroxyl groups on the B ring, showed no significant effects. Based on the comparison of quercetin and kaempferol, 3′,4′-OH groups on the B ring appear to enhance the suppressive effect on COX-2 transcriptional activity.

Oxygen electron density of the resorcinol moiety To determine the relationship between the electron density of oxygen atoms in hydroxyl groups of the compounds and the inhibitory effects on COX-2 transcriptional activity, the electron densities of the 5- and 7-oxygens in the A ring of the 5 flavonoids, which are listed in Table I and Fig. 1, were calculated by the semi-empirical quantum mechanical method AM-1. The potent suppressor, quercetin, had a lower calculated electron density for the 7-oxygen than the weak suppressors, catechin and epicatechin. Among flavonoids with the same B ring structure and a resorcinol

Table I. Effects of Flavonoids on COX-2 Transcriptional Activity

Inhibition	Compound	IC_{50} (μM)	Cell viability in IC_{50} (%)	Chemical class
Potent	Quercetin[a]	10.5±0.7	100.7	Flavonol
	Rhamnetin	18.6±2.1	96.5	Flavonol
	Genistein[a]	20.7±1.4	88.3	Isoflavone
	Eriodictyol	22.0±0.2	88.8	Flavanone
	Luteolin	22.0±0.4	99.2	Flavone
	Kaempferol[a]	39.3±2.1	94.7	Flavonol
	Fisetin	47.9±2.9	87.6	Flavonol
	Phloretine	52.5±3.4	73.6	Dihydrocalcone
Weak	Catechin	415.3±25.4	78.8	Flavanol
	Epicatechin	415.3±17.0	79.5	Flavanol
None	Epigallocatechin	>500		Flavanol
	Myricetin	>500		Flavonol

a) The data for these three flavonoids were reported previously.[13]

Table II. Electron Densities of the 5- and 7-Oxygens in Flavonoids with a Resorcinol Moiety

Inhibition	Compound	Electron density	
		7-Oxygen	5-Oxygen
Potent	Quercetin	−0.2801	−0.2554
	Eriodictyol	−0.2814	−0.2490
	Luteolin	−0.2823	−0.2555
Weak	Catechin	−0.2933	−0.2790
	Epicatechin	−0.2938	−0.2801

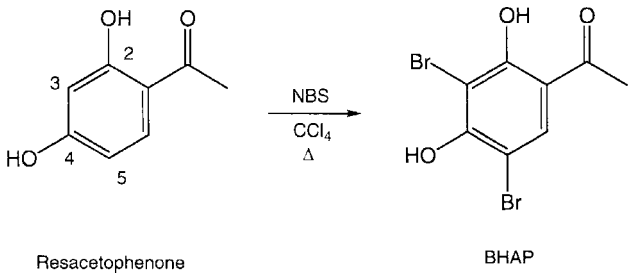

Fig. 2. Synthesis of BHAP from resacetophenone.

Table III. Electron Densities of Oxygens and IC_{50} Values of Resacetophenone and BHAP

Compound	Electron density		IC_{50} (μM)
	4-Oxygen	2-Oxygen	
BHAP	−0.2376	−0.2330	73.2±3.6
Resacetophenone	−0.2877	−0.3142	500.0±25.4

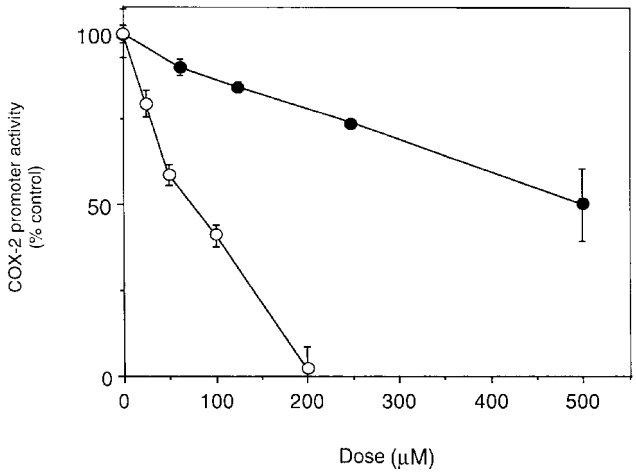

Fig. 3. Suppression of TGFα-stimulated COX-2 promoter-dependent transcriptional activity by resacetophenone and BHAP. DLD-1/COX-2-B2-βGal-BSD cells were treated with 100 ng/ml TGFα in the presence of the indicated concentrations of resacetophenone (●) and BHAP (○) for 48 h. The β-galactosidase activity in untreated negative control DLD-1/B2-βGal-BSD cells was set at 0, and that in TGFα-stimulated DLD-1/COX-2-B2-βGal-BSD cells was set at 100%. β-Galactosidase reporter gene activity in each treatment was normalized for cell viability. n=3; bars, SD.

moiety, an inverse correlation was observed between the electron density of the 7-oxygen and the suppression of COX-2 transcriptional activity (Table II). A similar correlation was observed for the electron density of the 5-oxygen.

Effects of resacetophenone and its brominated homologue The calculated oxygen electron density of the resorcinol moiety suggested that 7-oxygen may play a role in suppressing COX-2 transcriptional activity. To determine whether this is the case, resacetophenone was brominated to reduce the electron density. The structure and synthetic pathway of 3,5-dibromo-2,4-dihydroxyacetophenone are illustrated in Fig. 2. As shown in Table III, BHAP has a lower electron density of the oxygens at positions 2 and 4 in the resorcinol moiety than resacetophenone. BHAP suppressed COX-2 transcriptional activity in a dose-dependent manner, and was 6.8 times more potent than resacetophenone (Fig. 3).

DISCUSSION

Ten of the twelve flavonoids tested in the present and previous studies suppressed COX-2 transcriptional activity in our reporter gene assay system. Among the twelve, epicatechin, epigallocatechin, genistein, luteolin and quercetin have been reported to have chemopreventive properties in several carcinogenesis systems.[16–20] Thus, the suppression of COX-2 expression by these flavonoids could be involved in the mechanism of cancer prevention.

Subclasses of flavonoids are based on variations in the heterocyclic C ring. Quercetin, a flavonol, and epicatechin, a flavanol, differ with regard to the state of saturation of the C2−C3 bond and the presence of a 4-oxo group (Fig. 1). The former strongly influences the molecular conformation. The structure-activity analysis of eriodictyol and luteolin suggested that the 2,3-double bond of the C ring has little effect on COX-2 transcriptional activity. Thus, these results suggest that the 4-oxo group plays an important role in suppressing COX-2 transcriptional activity. All flavonoids with a 4-oxo group, except for myricetin, were found to be potent suppressors of COX-2 transcriptional activity. On the other hand, compounds that lacked a 4-oxo group, catechin, epicatechin and epigallocatechin, exhibited weak or no activity.

The potent COX-2 suppressors, eriodictyol, fisetin, luteolin, quercetin and rhamnetin, have 3′,4′-OH groups in

their B ring. Compounds, such as epigallocatechin and myricetin, with three hydroxyl groups on the B ring did not suppress COX-2 transcriptional activity. Therefore, the number of hydroxyl groups on the B ring may be related to a molecular conformation that influences the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C, which are involved in the transcriptional activity of COX-2. Indeed, it has been reported that flavonoids which inhibit tyrosine kinase and protein kinase C have 3′,4′-OH groups on the B ring, and 5,7-OH groups on the A ring.[3, 4] Flavonoids with a 4-oxo group on the C ring have also been reported to be potent radical scavengers.[21] The results of the present study suggest that flavonoid structures which are essential for suppressing COX-2 expression are very similar to those required for the inhibition of tyrosine kinase and protein kinase C.

Moreover, crystallography has demonstrated that there is an intramolecular hydrogen bond between the 5-hydroxyl group and the 4-oxo group in quercetin.[22] Since a free 7-hydroxyl group in the A ring may be able to attack enzymes such as tyrosine kinases, we examined the electron density of the 7-oxygen. Our data suggest a possible inverse link between this parameter and the suppression of COX-2 transcriptional activity.

The present study of the structure-activity relationship has provided important information regarding the types of moieties that are involved in the suppression of COX-2 transcriptional activity. On the basis of our findings resacetophenone, which has a weak suppressive effect on COX-2 transcriptional activity, was brominated to reduce the electron density of the 2- and 4-oxygens in the resorcinol moiety. The resulting compound had a greatly enhanced suppressive effect on COX-2 transcriptional activity.

In conclusion, we propose that the structural requirements for the suppression of COX-2 transcriptional activity by flavonoids are the presence of a 4-oxo group in the C ring, low electron density in the 7-oxygen group in the A ring, and a 3′,4′-dihydroxyl structure in the B ring.

ACKNOWLEDGMENTS

We thank Prof. Yoshiteru Ida, Dr. Yasuaki Hirai and Dr. Tsuyoshi Miura (Dept. of Pharmacognosy and Phytochemistry, School of Pharmaceutical Sciences, Showa University) for their technical support in measuring the NMR spectra. This work was supported in part by a grant from the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan, and a Grant-in-Aid from the Ministry of Health and Welfare for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control. M. Mutoh and T. Enya are the recipients of Research Resident Fellowships from the Foundation for Promotion of Cancer Research.

(Received February 25, 2000/Revised April 28, 2000/Accepted May 8, 2000)

REFERENCES

1) Middleton, E., Jr. and Kandaswami, C. Effect of flavonoids on immune and inflammatory cell functions. *Biochem. Pharmacol.*, **43**, 1167–1179 (1992).
2) Wattenberg, L. W. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.*, **52**, 2085S–2091S (1992).
3) Ferriola, P. C., Cody, V. and Middleton, E. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochem. Pharmacol.*, **38**, 1617–1624 (1989).
4) Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Remesy, C., Chap, H. and Payrastre, B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol.*, **53**, 1649–1657 (1997).
5) Loll, P. J. and Garavito, R. M. The isoforms of cyclooxygenase: structure and function. *Expert Opin. Invest. Drugs*, **3**, 1171–1180 (1994).
6) Herschman, H. R. Prostaglandin synthase 2. *Biochim. Biophys. Acta*, **1299**, 125–140 (1996).
7) Eberhart, C. E., Coffey, R. J., Radhike, A., Giardiello, F. M., Ferrenbach, S. and Dubois, R. N. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, **107**, 1183–1188 (1994).
8) Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.*, **55**, 3785–3789 (1995).
9) Elder, D. J. and Paraskeva, C. COX-2 inhibitors for colorectal cancer. *Nat. Med.*, **4**, 392–393 (1998).
10) Kawamori, T., Rao, C. V., Seibert, K. and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, **58**, 409–412 (1998).
11) Nakatsugi, S., Fukutake, M., Takahashi, M., Fukuda, K., Isoi, T., Taniguchi, Y., Sugimura, T. and Wakabayashi, K. Suppression of intestinal polyp development by nimesulide, a selective cyclooxygenase-2 inhibitor, in Min mice. *Jpn. J. Cancer Res.*, **88**, 1117–1120 (1997).
12) Fukutake, M., Nakatsugi, S., Isoi, T., Takahashi, M., Ohta, T., Mamiya, S., Taniguchi, Y., Sato, H., Fukuda, K., Sugimura, T. and Wakabayashi, K. Suppressive effect of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice. *Carcinogenesis*, **19**, 1939–1942 (1998).
13) Mutoh, M., Takahashi, M., Fukuda, K., Matsushita-Hibiya, Y., Mutoh, H., Sugimura, T. and Wakabayashi, K. Sup-

pression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. *Carcinogenesis*, **21**, 959–963 (2000).

14) Enya, T., Suzuki, H. and Hisamatsu, Y. Reaction of benzanthrone (7*H*-benz[*d*,*e*]anthracen-7-one) with nitrogen dioxide alone or admixture with ozone. Implications for the atmospheric formation of genotoxic 3-nitrobenzanthrone. *Bull. Chem. Soc. Jpn.*, **71**, 2221–2228 (1998).
15) Dewar, M. J. S., Zoebisch, E. G., Healy, E. F. and Stewart, J. J. P. AM-1: a new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.*, **107**, 3902–3909 (1985).
16) Matsumoto, N., Kohri, T., Okushio, K. and Hara, Y. Inhibitory effects of tea catechins, black tea extract and oolong tea extract on hepatocarcinogenesis in rat. *Jpn. J. Cancer Res.*, **87**, 1034–1038 (1996).
17) Steele, V. E., Pereira, M. A., Sigman, C. C. and Kellof, G. J. Cancer chemoprevention agent development strategies for genistein. *J. Nutr.*, **125**, 713S–716S (1995).
18) Pereira, M. A., Barnes, L. H., Rassman, V. L., Kelloff, G. V. and Steele, V. E. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. *Carcinogenesis*, **15**, 1049–1054 (1994).
19) Holland, M. B. and Roy, D. Estrone-induced cell proliferation and differentiation in the mammary gland of the female Noble rat. *Carcinogenesis*, **16**, 1955–1961 (1995).
20) Deschner, F. E., Ruperto, J., Wong, G. and Newmark, H. L. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis*, **12**, 1193–1196 (1991).
21) Miller, N. J. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *In* "Natural Antioxidants and Food Quality in Atherosclerosis and Cancer Prevention," ed. J. T. Kumpulainen and J. T. Salonen, pp. 256–259 (1996). The Royal Society of Chemistry, Cambridge.
22) Rossi, M., Rickles, L. F. and Halpin, W. A. The crystal molecular structure of quercetin: a biologically active and naturally occurring flavonoid. *Bioorg. Chem.*, **14**, 55–69 (1986).