Inhibition of Proliferation of Human Cancer Cells and Cyclooxygenase Enzymes by Anthocyanidins and Catechins

Navindra P. Seeram, Yanjun Zhang, and Muraleedharan G. Nair

Abstract: The widespread consumption of diets rich in anthocyanin and catechin content prompted the evaluation of their in vitro inhibitory effects on cyclooxygenase (COX) enzymes and on the proliferation of human cancer cell lines. Five anthocyanins consisting of cyanidin (1), delphinidin (2), pelargonidin (3), peonidin (4), and malvidin (5) were tested for COX-1 and -2 enzyme inhibitory activities at 40 

\[ \text{M concentrations} \]

Eleven catechins consisting of (+)-catechin (6), (–)-catechin (7), (±)-catechin (8), (–)-epicatechin (9), (–)-epicatechin gallate (10), (–)-epigallocatechin (11), (–)-gallocatechin (12), (–)-epicatechin gallate (13), (–)-gallocatechin gallate (14), (–)-epigallocatechin gallate (15), and (–)-gallocatechin gallate (16) were tested for inhibitory effects of COX-1 and -2 enzymes at 80 

\[ \text{M. Of the compounds tested, the galloyl derivatives of the catechins 11–15, cyanidin (1) and malvidin (5), showed the best COX inhibitory activities compared with the commercial anti-inflammatory drugs ibuprofen (at 10 

\[ \text{M), naproxen (at 10 

\[ \text{M), Vioxx® (at 1.67 ppm), and Celebrex™ (at 1.67 ppm).} \]

Inhibition of the proliferation of the human cancer cell lines MCF-7 (breast), SF-268 (central nervous system, CNS), HCT-116 (colon), and NCI-H460 (lung) was evaluated at concentrations between 100 and 6.25 

\[ \text{M compared with the commercial standard, adriamycin (doxorubicin)} \]

At 100-

\[ \text{M concentrations, anthocyanidins 1–5 and catechins 6–10 did not inhibit proliferation of the four cell lines. At 50-

\[ \text{M concentrations, catechins 12, 15, and 16 showed 95%, 100%, and 97% inhibition of breast cells, respectively. At 50-

\[ \text{M concentrations 12 and 16 were the most effective catechins against colon cells (85% and 93%, respectively) and lung cells (87% and 67%, respectively). CNS cells were the most sensitive of the test cell lines, and total growth inhibition was obtained with catechins 12 and 16 at 100-

\[ \text{M concentrations. Overall, only the galloyl derivatives of catechins 11–16 inhibited the proliferation of the cancer cell lines.} \]

Introduction

There is increasing evidence suggesting an association between cancer and COX-2 because prostaglandins (PGs) are mediators of inflammation, and chronic inflammation predisposes to carcinogenesis (1,2). The COX isozymes COX-1 and COX-2 are involved in the control of inflammatory reactions and catalyze the conversion of arachidonic acid to PGH2, the precursor of PGs (2,3). COX-1 is the constitutive form and is regarded as a housekeeping enzyme, whereas COX-2 is highly inducible by inflammatory stimuli (3). Inhibition of COX-1 may result in gastric ulceration, so there is a demand for compounds that selectively inhibit COX-2 with fewer side effects (3,4).

In recent years, there has been growing interest in identifying naturally occurring antimutagenic and anticarcinogenic agents, especially those that can be included in the diet (5,6). Studies have shown that dietary factors could play an important role in influencing mortality due to cancer (7). Reports have shown that a large number of food components and related compounds block different stages of the carcinogenic process in animal models, and some of these substances partially prevent or delay cancer formation in some high-risk human populations (8).

Studies suggest that flavonoids play an important role in the prevention of chronic diseases, such as cancer, arteriosclerosis, and allergies (9). Flavonoids are multifunctional bioactive compounds implicated with beneficial health effects due to their strong antioxidant activity for scavenging free radicals that are involved in cell damage and tumor promotion. Evidence has accumulated to suggest that flavonoids potently suppress tumor cell proliferation and may effectively work as chemopreventive agents against carcinogenesis in humans (9). Flavonoids are widely distributed in the plant kingdom and can be divided into major classes, including flavanols (catechins) and anthocyanidins and their glycosides, anthocyanins.

Catechins, although widely distributed in plants, are of highest yield in tea (Camellia sinensis) leaves, constituting up to 30% dry weight (10). Tea is one of the most popular beverages in the world and has attracted significant attention because of its reported health benefits, in particular, as an antioxidant but also as an anticarcinogenic and antiarteriosclerotic agent, attributed to its high catechin and flavonol content (10–12). Tea flavonoids or tea extracts are being increasingly added to foods and have gained considerable popularity in the consumer market due to their reported health benefits (11).
Anthocyanins are pigments primarily responsible for the attractive colors in fruits and vegetables and form substantial constituents of human diet. It has been estimated that humans consuming diets high in vegetables and fruits can intake 80–215 mg of anthocyanins daily (13). Anthocyanins are implicated with health benefits, and previous studies in our laboratory have shown that tart cherry anthocyanins exhibit in vitro antioxidant and anti-inflammatory activities comparable with over-the-counter, pain-relieving agents and nonsteroidal anti-inflammatory drugs (14,15). The extracellular and intracellular antioxidative potential of anthocyanins and anthocyanidins from fruits including chokeberry and elderberry has also been reported (16). Anthocyanins have also been reported to protect against DNA damage (17) and to induce apoptosis of human leukemia and human colon carcinoma cells in vitro (18).

Because anthocyanins and catechins are an integral part of the human diet, it is worthwhile to evaluate bioactivities that can support their health benefits. In this article, we report the evaluation of the COX and human tumor cell growth inhibitory activities of several commercially available anthocyanidins 1–5 (Fig. 1) and catechins 6–16 (Fig. 2).

Materials and Methods

Chemicals

Cyanidin (1) was prepared by acid hydrolysis of tart cherry anthocyanins as previously reported (15). Delphinidin (2) and pelargonidin (3) were prepared from their respective galactosides, isolated from fruits of dogwood (Cornus mas), similar to the preparation of cyanidin (19). Peonidin (4) and malvidin (5) were purchased from Chromadex™ (Laguna Hills, CA). (+)-Catechin (6), (−)-catechin (7), (±)-catechin (8), (±)-epicatechin (9), (−)-epicatechin (10), (−)-epigallocatechin (11), (−)-gallocatechin (12), (−)-epicatechin gallate (13), (−)-catechin gallate (14), (−)-epigallocatechin gallate (15), and (−)-gallocatechin gallate (16) were purchased from Sigma Chemical (St. Louis, MO). Ibuprofen and naproxen standards were purchased from Sigma Chemical. Celebrex™ capsules and Vioxx® tablets were physician’s professional samples provided by Dr. Subash Gupta (Sparrow Pain Center, Sparrow Hospital, Lansing, MI). Whole tablets or capsules of a known quantity of active ingredient were solubilized in dimethyl sulfoxide (DMSO). Fetal bovine serum (FBS) and Roswell Park Memorial Institute 1640 (RPMI 1640) medium were obtained from GibcoBRL (Grand Island, NY). Doxorubicin, DMSO, acetic acid, trichloroacetic acid (TCA), and sulforhodamine B (SRB) were purchased from Sigma Chemical.

Cell Lines

Human tumor cell lines MCF-7 (tumor origin: breast, histologic type: mammary adenocarcinoma, host strain: athymic nude), SF-268 (tumor origin: central nervous system, CNS, histologic type: glioblastoma, host strain: athymic nude), and NCI-H460 (tumor origin: lung, histologic type: large cell carcinoma, host strain: athymic nude) were purchased from the National Cancer Institute (NCI, Bethesda, MD) and are the three-cell line prescreen utilized by the Developmental Therapeutics Program of the NCI. HCT-116 (tumor origin: colon, histologic type: colon carcinoma) was purchased from the American Type Culture Collection (ATCC, Rockville, MD).

In Vitro COX-Enzyme Inhibitory Assay

Cyclooxygenase-1 (COX-1) enzyme was prepared from ram seminal vesicles (Oxford Biomedical Research, Inc., Oxford, MI). COX-2 enzyme was prepared from insect cell lysate. COX assays were performed by monitoring the initial rate of O2 uptake using an Instech micro oxygen chamber and electrode (Instech Laboratories, Plymouth Meeting, PA) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument, Inc., Yellow Springs, OH). The assay was conducted according to the previously reported procedure (15,20).

Cancer Cell Growth Inhibitory Assay

Cells were routinely maintained as adherent cell cultures in RPMI 1640 medium supplemented with 10% FBS and 10 units of penicillin and 10 µg/ml of streptomycin at 37°C in a humidified air incubator containing 5% CO2. Cells were subcultured weekly and, according to their growth profiles, the optimal plating densities of each cell line were deter-
mined to ensure exponential growth throughout the experimental period. Cells were inoculated into 96-well microtiter plates in 100 µl at plating densities depending on the doubling time of individual cell lines. After cell inoculation, the plates were incubated for 24 h prior to addition of experimental drugs. Stock solutions of all compounds were in DMSO and diluted to the desired concentration in media prior to the assay. Additional four ½ log serial dilutions were made to provide a total of five drug concentrations plus control. The final concentration of DMSO (0.25%) showed no interference with the growth of the cell lines. Aliquots of 100 µl of the test compounds were added to the appropriate microtiter wells already containing 100 µl of medium, resulting in the desired final concentrations. Microtiter plates were incubated for 48 h after which the assay was terminated by the addition of cold TCA. Plates were incubated for 60 min at 4°C and then washed five times with distilled water and air-dried. SRB solution (100 µl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 30 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid. Bound stain was then solubilized with 10 mM trizma base (100 µl), the plates were shaken for 5 min on a gyratory shaker, and absorbance was read at 515 nm on an automated microplate reader (Model ELx800, Bio-Tek Instruments, Inc., Winooski, VT). The effects of the test compounds on the growth of human cancer cell lines were evaluated according to the procedure adopted from the NCT's in vitro anticancer drug screening

that uses the SRB assay to assess growth inhibition (21). The assay uses a dye (SRB) that binds electrostatically to macromolecular counterions (e.g., protein residues) in fixed cells, which can then be quantitatively extracted for measurement of optical density (21). Therefore, residual cell proteins are stained and compared with a control representing continuous growth of the particular tumor cell line, which can then be translated into a factor representing the inhibition of cell growth.

Statistics

The COX enzyme inhibitory assays were conducted in triplicate, and the data represent the mean ± SD. Statistical comparisons were made with Students t-test. All comparisons were made at the two-sided 0.05 significance level. The controls and test compounds in the cell growth inhibitory assay were tested in triplicate for each concentration and replicated three times for each cell line. These data were then subjected to multiple regression analysis using the general linear models procedure of SAS to develop least-squares polynomial equations describing the influence of polyphenol concentration on cell number. These equations were then used to iteratively calculate the concentration of polyphenols to plot growth curves.

Results

In COX enzyme inhibitory assays, the commercial anti-inflammatory drugs naproxen (10 µM), ibuprofen (10 µM), Celebrex (1.67 ppm), and Vioxx (1.67 ppm) showed 54.3% and 41.3%, 47.5% and 39.8%, 46.2% and 66.3%, and 23.8% and 88.1% COX-1 and COX-2 inhibition, respectively (Fig. 3A). Anthocyanidins (1–5) were evaluated at 40-µM concentrations because they showed higher COX-inhibitory activities compared with the catechins 6–16 in the preliminary assays. Catechins were evaluated at 80-µM concentrations for their COX activities.

Cyanidin (1), which has a 3′,4′-dihydroxyl B ring, was the most potent inhibitor of the COX-1 and -2 enzymes, with activities of 52.2% and 74.2%, respectively (Fig. 3B). However, there was a decrease in COX-1 and -2 inhibitory activities for delphinidin (2) (20.4% and 30.9%) and pelargonidin (3) (29.5% and 40.2%), which have 3′,4′,5′-trihydroxyl and 4′-hydroxyl groups in their B rings, respectively. Peonidin (4) and malvidin (5), which have methoxyl substituents in their B ring, showed 39.6% and 48.2% COX-1 and 49.1% and 64.8% COX-2 inhibitory activities, respectively (Fig. 3B).

Among catechins, cis-trans isomerization, epimerization, and racemization did not result in significant variations in COX activities. This was indicated by the COX-1 and -2 inhibitory activities for 6 (20.4% and 46.3%), 7 (45.7 and 41.2%), 8 (24.1% and 38.6%), 9 (40.7% and 26.8%), and 10 (57.6% and 47.9%; Fig. 3C). However, the galloyl derivatives of the catechins 11–16 showed greater inhibition of the

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Figure 2. Structure of catechins 6–16 that were studied for in vitro inhibition of COX-1 and -2 enzymes and growth of human tumor cell lines.
COX enzymes than catechins 6–10. COX-1 and -2 activities for 11 (55.7% and 62.5%), 12 (38.6% and 53.3%), 13 (66.3% and 59.2%), 14 (56.5% and 66.1%), 15 (40.4% and 49.3%), and 16 (54.9% and 53.5%) are shown in Fig. 3D.

Anthocyanidins 1–5 and catechins 6–16 were tested for their effects on the proliferation of MCF-7, SF-268, HCT-116, and NCI-H460 cancer cell lines at concentrations of 100, 50, 25, 12.5, and 6.25 µM according to the procedure adopted from the NCI’s in vitro anticancer drug screening that uses the SRB assay to assess growth inhibition (21). Adriamycin (doxorubicin) was used as a positive control and gave 74, 69, 82, and 83% inhibition of growth of breast, CNS, colon, and lung cells, respectively, at 0.6 × 10⁻⁵ M. Of all the compounds tested, only the galloylated derivatives of the catechins 11–16 inhibited the growth of the cancer cell lines. Growth inhibitory effects of 11–16 on breast, CNS, colon, and lung cells are shown in Figs. 4A–D, respectively. At 50-µM concentrations, total growth inhibition of SF-268 cells was obtained with gallocatechin (12) and gallocatechin gallate (16) (Fig. 4D).

Discussion

Catechins and anthocyanidins are biogenetically produced from a C-15 tetrahydroxylchalcone precursor, naringenin, in the pivotal step of flavonoid biosynthesis (22). These two classes of compounds differ structurally from other flavonoids by the absence of a 4-keto group in the pyran ring and the presence of a 3-hydroxyl group. These natural antioxidant polyphenols are ubiquitously present in foods of plant origin and belong to one of the groups of phytoceuticals. Phytoceuticals are compounds of plant origin that provide beneficial effects other than nutraceutical values. It is estimated that the intake of total flavonoids by humans consuming fruit and vegetable diets can reach up to 1 g/day, of which catechins and anthocyanins contribute to a substantial proportion (10). Little is known as to the metabolic fate of anthocyanins ingested in humans to correlate their beneficial pathophysiological properties in vivo as reported for in vitro experiments (13).
ingestion, anthocyanidins (aglycones) may be produced in vivo from their respective anthocyanins (glycosides) by hydrolysis with enzymes produced by intestinal bacteria, e.g., β-glucosidase (23).

We have previously reported that cyanidin exhibited superior COX-1 and -2 activities compared with the anthocyanins cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside isolated from tart cherries (14,15). These reports have resulted in an increased consumer demand for fresh and frozen cherries, cherry concentrate, dried cherries, and various other forms of processed cherry products for the relief of arthritis and gout-related pains.

The overall order of COX-1 and -2 inhibitory activities for the anthocyanidins was cyanidin (1) > malvidin (5) > peonidin (4) > pelargonidin (3) > delphinidin (2) (Fig. 3B). From a structure-activity relationship perspective, the number and position of hydroxyl groups in ring B of anthocyanidins were important determinants of COX enzyme inhibitory activities. The presence of a 3',4',5'-trihydroxyl B ring in anthocyanidins resulted in enhanced COX activities, as indicated by cyanidin (1), which was the most potent COX enzyme inhibitor. The 3',4'-dihydroxy substituted B ring in anthocyanidins appears to be the critical moiety for activity because COX inhibitory activities were significantly reduced for delphinidin (2) and pelargonidin (3), which have 3',4',5'-trihydroxyl and 4'-hydroxyl groups in their B rings, respectively. The substitution of methoxyl substituents on the B ring of anthocyanidins also considerably influenced COX inhibitory activities. Malvidin (5), which can be considered the 3',4'-dimethoxylated counterpart of delphinidin (2), showed COX inhibitory activities second only to cyanidin (1). Similarly, peonidin (4) inhibited the COX enzymes to a greater extent than delphinidin (2) and pelargonidin (3).

Epidemiological studies suggest that tea may possess a protective role in the prevention of certain human cancers (24). Although tea is a predominant source of catechins 6, 10, 11, 13, and 15, we have evaluated the bioactivities of several other related catechins (6–16) that are reported to be present in fruits and vegetables (25). Previous studies have shown that galloyl derivatives of catechins play an important role in determining biological activities such as apoptosis (26,27). It has been reported that catechins with a pyrogallol (3',4',5'-trihydroxy) moiety induced apoptosis of human lymphoma cells (27). This activity was enhanced by 3-O-galloyl esters of catechins, but catechins without a pyrogallol moiety lacked cancer preventative activity (27). In our COX enzyme inhibitory studies, the galloyl derivatives of the catechins 11–16 showed greater inhibition of the COX

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enzymes than catechins 6–10. Similarly, of all the catechins studied, only the galloyl derivatives of the catechins 11–16 inhibited the growth of human tumor cell lines. Catechins 11–16 were most effective against the growth of breast tumor cells and showed significant inhibitory activity at 25-µM concentrations. Interestingly, the CNS cells were relatively insensitive to the catechins, and inhibition of cell proliferation was only obtained at 100-µM concentrations. Cytotoxicity effects of tea catechins have been studied (28,29), and it has been reported that catechins induce apoptosis, resulting in the inhibition of proliferation of human cancer cells (30).

In conclusion, the COX inhibitory activities of anthocyanidins and catechins as well as their inhibition of the proliferation of human tumor cell lines suggest that these compounds can be further investigated for their health benefits. The use of these bioflavonoids as potential phytocuticals could be significant in the prevention of fatal diseases such as cancer and crippling inflammatory conditions such as arthritis and gout.

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