Cyanidin-3-glucoside, a Natural Product Derived from Blackberry, Exhibits Chemopreventive and Chemotherapeutic Activity*

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Epidemiological data suggest that consumption of fruits and vegetables has been associated with a lower incidence of cancer. Cyanidin-3-glucoside (C3G), a compound found in blackberry and other food products, was shown to possess chemopreventive and chemotherapeutic activity in the present study. In cultured JB6 cells, C3G was able to scavenge ultraviolet B-induced 'OH and O2 radicals. In vivo studies indicated that C3G treatment decreased the number of non-malignant and malignant skin tumors per mouse induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in 7,12-dimethylbenz[a]anthracene-initiated mouse skin. Pretreatment of JB6 cells with C3G inhibited UVB- and TPA-induced transactivation of NF-κB and AP-1 and expression of cyclooxygenase-2 and tumor necrosis factor-α. These inhibitory effects appear to be mediated through the inhibition of MAPK activity. C3G also blocked TPA-induced neoplastic transformation in JB6 cells. In addition, C3G inhibited proliferation of a human lung carcinoma cell line, A549. Animal studies showed that C3G reduced the size of A549 tumor xenograft growth and significantly inhibited metastasis in nude mice. Mechanistic studies indicated that C3G inhibited migration and invasion of A549 tumor cells. These finding demonstrate for the first time that a purified compound of anthocyanin inhibits tumor promotor-induced carcinogenesis and tumor metastasis in vivo.

Chemoprevention, the use of drugs or natural substances to retard or reverse the process of carcinogenesis, represents one of several promising strategies to reduce the development of cancer. Many naturally occurring substances present in the human diet have been identified as potential chemopreventive and/or chemotherapeutic agents (1–3). A recent report published by the American Institute for Cancer Research regarding dietary chemoprevention and/or chemotherapeutic agents (1–3). A recent report published by the American Institute for Cancer Research regarding dietary chemoprevention and/or chemotherapeutic agents (1–3).

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2 The abbreviations used are: UVB, ultraviolet B; C3G, cyanidin-3-glucoside; DMBA, 7,12-dimethylbenz[a]anthracene; AP-1, activator protein-1; COX-2, cyclooxygenase-2; TNFa, tumor necrosis factor α; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MKK4, MAP kinase kinase 4 (alternatively designated SEK1 or MKK4); TPA, 12-O-tetradecanoylphorbol-13-acetate; FBS, fetal bovine serum; ROS, reactive oxygen species; uPA, urokinase-type plasminogen activator; APAR, urokinase-type plasminogen activator receptor; CM-DCFDA, carbamoyl methyl dichlorofluorescein diacetate; PBS, phosphate-buffered saline; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate).

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catalyst in prostaglandin synthesis from arachidonic acid, contributes to the regulation of angiogenesis and metastasis (22, 23). Selective COX-2 inhibitors protect against the formation of tumors in animals, suggesting that prostaglandins, the products of COX-2 activity, substantially contribute to carcinogenesis (24–26). Overexpression of COX-2 is common in human lung cancer and seems to be associated with tumor progression, invasion, and metastasis. In experimental animal models, COX-2 has been shown to be involved in tumor angiogenesis, suggesting that COX-2 is a potential target for cancer therapy. Several in vivo studies have already shown that COX-2-specific inhibitors (celecoxib and rofecoxib) have antitumor activity, and clinical trials using COX-2 inhibitors are currently ongoing in patients with lung cancer (27, 28).

Reports focusing on mechanisms and in vivo data supporting the possible chemopreventive and chemotherapeutic effects of purified C3G are limited. In light of the important roles of NF-κB, AP-1, COX-2, TNFα, and MAPK activation in carcinogenesis, we investigated the potential ability of C3G to inhibit DMBA-TPA-induced skin papillomas in animal skin model, suppress proliferation of tumor cells, suppress tumor xenograft growth and metastasis in nude mice, down-regulate NF-κB, AP-1, and MAPK activities, down-regulate the expression of COX-2 and TNFα, and scavenge ROS generated in intact cells. The results of this investigation provide new insights into the mechanisms by which a natural antioxidant may control the production of ROS-induced activation of molecular signals involved in the initiation, promotion, and progression of cancer.

MATERIALS AND METHODS

Reagents—Eagle’s minimal essential medium was purchased from Whittaker Biosciences (Walkersville, MD). FBS, gentamicin, and L-glutamine were from Invitrogen. L-gluconic acid (0.01%) and methanol were from J.T. Baker (Phillipsburg, NJ). Acetone was from Sigma-Aldrich (St. Louis, MO). ABTS radical cation (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) test set (Randox Laboratories Ltd., UK). The principle of the assay depends on production of the radical cation ABTS+/+ in incubation medium containing the substrates H2O2 and peroxidase. ABTS+/+ has a blue-green color, which can be detected at 600 nm. The assays were calibrated against standards and expressed as micromoles per liter.

Cellular O2− and H2O2 Assay—Dihydroethidium and carboxymethyl dichlorofluorescein diacetate (CM-DCFDA) are specific dyes used for staining O2− and H2O2 produced by intact cells (31, 32). JB6 cells (2 × 10⁴/well) were seeded onto a glass coverslip in the bottom of a well of a 24-well plate for 24 h. The cells were pretreated with or without C3G for 30 min and exposed to UBV radiation (4 kJ/m²). At the end of the stimulation, dihydroethidium or CM-DCFDA were added at a final concentration of 5 μM for 30 min. After the incubation, the cells were washed with phosphate-buffered saline and mounted on coverslips. A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by using LSM software.

Assay of AP-1, NF-κB, COX-2, and TNFα Transcription/Promoter Activity—A confluent monolayer of JB6 cells, which contained an AP-1, NF-κB, COX-2, or TNFα luciferase-reporter plasmid, was trypsinized, and 5 × 10⁴ viable cells were seeded in each well of a 24-well plate (18, 29, 30). The cells were then pretreated with C3G for 1 h followed by exposure to TPA (20 ng/ml) or UBV radiation (4 kJ/m²) for 24 h. Then, the effects on AP-1, NF-κB, COX-2, and TNFα induction/expression were monitored. Luciferase activity was measured using the luciferase assay kit obtained from Promega as described previously (18).

Western Blot and Protein Kinase Phosphorylation Assay—Immunoblots for phosphorylation or expression of ERKs, JNKs, p38 kinase, MEK4, and COX-2 proteins were carried out as described in the protocol from New England Biolabs, using the specific antibodies against phosphorylated sites of ERKs, JNKs, p38 kinase, and MEK4. Antibody or non-phospho-specific antibodies provided in each assay kit were used to normalize the phosphorylation assay by using the same transferred membrane blot.

Animals and Two-stage Skin Carcinogenesis—Founder stocks for the mice (C57BL/6 crossed with DBA2) used in this study were obtained from the University of Minnesota (7). The mice were bred and housed in the West Virginia University Animal Facility. The mice were monitored free of specific pathogens housed in plastic filter-top cages on corncob bedding, provided autoclaved tap water and Prolab 3500 feed ad libitum. Both male and female mice (6–9 weeks old) were used in groups numbering 19 to 24. Dorsal skin of the mice was shaved. A single dose of 400 nmol of 7,12-dimethylbenz[a]anthracene (DMBA) dissolved in 300 μl of acetone was applied. Two weeks following initiation, the mice (except the negative control group) were promoted by dermal exposure to 17 nmol of TPA in 350 μl of acetone twice a week for 21 weeks. For the C3G-treated group, the dorsal skin was pretreated topically with C3G (3.5 μg/mouse) 30 min before each application of TPA. The negative control group was treated with acetone only. The incidence of papillomas was detected by palpation, and the number of papillomas appearing on each mouse was recorded once a week. At the end of the experiment, all the animals were sacrificed by intraperitoneal injection of pentobarbital (6.5 mg/mouse). For histopathology, the largest tumors were removed and fixed in freshly prepared 4% paraformaldehyde followed by paraffin embedding and subjected to pathologic evaluation.

Mouse Model Study for Tumor Xenograft Growth and Metastasis—Male nu/nu homozygous nude mice (Crl: Nu-Foxn1nu, 8 weeks old)
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The mice were grouped in 10 mice per group and housed in autoclaved plastic filter-top cages and were provided with autoclaved tap water and Prolab 3500 feed ad libitum. Human lung carcinoma cells, A549, were harvested from tissue culture and subsequently injected (2 × 10⁶ cells/flank) in both the right and left flanks of each mouse to initiate tumor growth. Two days after cell implantation, the mice were treated intraperitoneal with either PBS or C3G dissolved in PBS (9.5 mg/kg, 3 times/week). Once tumor xenografts started growing, their sizes were determined by measuring tumor size with calipers twice weekly in two dimensions throughout the study. The tumor volume was calculated by the formula, \( V = \frac{1}{2} \times L_1 \times L_2^2 \), where \( L_1 \) is the long diameter and \( L_2 \) is the short diameter. The tumor volume \((\text{mm}^3)\) is represented as the mean of 10 mice in each group.

**Transwell Cell Migration Assay**—The assay was performed according to the methods described previously (31). The cells were serum-starved overnight, and the Transwells were coated with enhanced chemiluminescence cell attachment matrix (Upstate Biotechnology) at 20 \( \mu \text{g/ml} \). The top chambers of the Transwells were loaded with 0.2 ml of cells (4 × 10⁴ cells/ml) in serum-free media, and the bottom chambers contained 0.6 ml of Dulbecco’s modified Eagle’s medium with 0.5% FCS. The cells were incubated in Transwells at 37 °C in 5% CO₂ in the presence or absence of C3G for 12 h. Migrating cells were fixed and stained with 0.1% crystal violet, followed by dye elution. The microplate reader was used to measure the optical density of the eluted solutions to determine the migration values.

**Wound Healing Assay**—The wound healing assays were conducted according to the methods described previously (32). A549 cells were grown on coverslips to 100% confluent monolayer and then scratched to form a 100-μm “wound” using sterile pipette tips. The cells were then cultured in the presence or absence of C3G in serum-free media for 12 h and fixed on coverslips with 4% formalin. The images were recorded using an Olympus photomicroscope.

**Cell Invasion Assay**—The cell invasion assays were performed according to the methods described previously (32). A549 cells were cultured in serum-free media overnight. The cells (5 × 10⁴ cells) were loaded on pre-coated Matrigel 24-well invasion chambers (BD Biosciences) in the presence or absence of C3G. Then 0.5 ml of 5% fetal calf serum medium was added to the wells of the plate to serve as the chemoattractant for the cells. The Matrigel invasion chambers were incubated for 18 h. The invading cells were fixed with 10% formalin, stained with Harris modified hematoxylin (Fisher), and analyzed according to manufacturer’s instructions.

**Statistics**—Data presented are the means ± S.E. of \( n \) assays as noted in the figure legends. Significant differences were determined using the Student’s \( t \) test. Significance was set at \( p \leq 0.05 \).

**RESULTS**

**Total Antioxidant Capacity of C3G**—ROS have been known to be associated with many diseases. ROS are associated not only with initiation, but also with promotion and progression in the multistage carcinogenesis mode (33, 34). In light of the important role of ROS in tumor promoter-induced AP-1 activation, transformation, and progression, we measured the total antioxidant capacity of C3G using the Randox reagent set. As show in Fig. 1A, C3G displayed a stronger antioxidant activity than that of ascorbic acid over the same concentration range, suggesting that C3G can effectively scavenge ROS.

**Scavenging of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) Generation by C3G**—To confirm the ROS-scavenging activity of C3G, the effect of C3G on UVB-induced \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) generation was analyzed using intracellular staining by CM-H₂DCFDA and dihydroethidium. CM-H₂-DCFDA is a specific fluorescent dye for \( \text{H}_2\text{O}_2 \), and dihydroethidium is specific for \( \text{O}_2^- \). JB6 cells were pretreated with or without C3G for 30 min and then exposed to UVB. After staining by dihydroethidium or CM-DCFDA, the images were captured with a laser scanning confocal microscope. The bright greenish areas in the cells represent oxidized CM-H₂-DCFDA, and the bright reddish spots represent oxidized dihydroethidium showing the intracellular localization of \( \text{H}_2\text{O}_2 \) (B) and \( \text{O}_2^- \) (C), respectively.

**Inhibition of AP-1, NF-κB, COX-2, and TNFα Activation/Expression by C3G**—Previous studies have shown that AP-1, NF-κB, COX-2, and TNFα play an important role in carcinogenesis (7, 20, 21, 28). We tested the effects of C3G on UVB- and TPA-induced transactivation of AP-1 and NF-κB, and expression of COX-2 and TNFα using a reporter gene assay in JB6 cells. The results indicated that pretreatment of the cells with various concentrations of C3G produced a dose-dependent...
A decrease in AP-1, NF-κB, COX-2, and TNFα activity/expression induced by either UVB irradiation or TPA (Fig. 2, A–D). The induction of COX-2 expression was confirmed by Western blot analysis (Fig. 2C, bottom panel). The inhibitory actions of C3G were not caused by cytotoxicity, because the concentration range that inhibited AP-1, NF-κB, COX-2, and TNFα activity/expression did not affect cell proliferation as measured by the electric cell-substrate impedance sensor assay (data not shown).
C3G Blocks UVB- and TPA-induced p38, ERK, and JNK Phosphorylation—Because C3G showed an inhibitory effect on AP-1, NF-κB, COX-2, and TNFα activity/expression, we further investigated the effects of C3G on MAPK pathways. MAPK pathways, including ERK, JNK, and p38 kinase, are involved in up-regulation of AP-1 and NF-κB activity and subsequence expression of TNFα and COX-2 by increasing the abundance of the components and/or altering the phosphorylation of their subunits (35, 36). The results indicated that C3G suppressed UVB- and TPA-induced phosphorylation of p38, ERK, and JNK (Fig. 3A), as well as the upstream regulator, MKK4 (Fig. 3B), in a dose-dependent manner. These results suggest that the mechanism by which C3G inhibits AP-1 transactivation may involve early inhibition of ERK, JNK, and p38 signaling cascades.

C3G Inhibits TPA-induced JB6 Cell Transformation—Earlier studies have indicated that AP-1 and/or NF-κB activation is required in neoplastic transformation and tumorigenesis in JB6 cells (19, 20). We thus tested the effect of C3G on TPA-induced cell transformation using the soft agar assay. The result is shown in Fig. 4A. TPA-induced cell transformation was significantly inhibited by C3G at the concentration range from 10 to 40 μM. At the concentration of 80 μM C3G, the TPA-induced transformation was completely abolished.

Inhibition of Papillomagenesis and Malignant Transformation by C3G—To further study the antitumorigenic activity of C3G in vivo, we evaluated the effect of C3G on the two-stage mouse skin tumor model in which DMBA was used as initiator and TPA as promoter (37, 38). Fourteen days following DMBA initiation, the dorsal skin of the mice was exposed to TPA in the presence or absence of C3G three times per week to cause promotion. The results show that treatment of the animals with C3G decreased the number of tumors per mouse at all exposure times (Fig. 4B). Significant differences were observed 17 weeks after TPA promotion. The maximum number of papillomas after 20 weeks of TPA exposure in the C3G-treated mice was 1.59 ± 0.65 per mouse compared with 3.57 ± 1.06 papillomas per mouse in the positive control group, indicating a greater than 53% inhibition of papillomagenesis by C3G. In addition to the difference in the numbers of tumors, the size of tumors was smaller in the C3G-treated group. Two representative mice from each group showing the greatest number of papillomas are shown in Fig. 4C. Tumors in the positive control group grew rapidly and were well vascularized, whereas tumors in the C3G-treated group appeared growth-arrested and desiccated. After 22 weeks, there were four tumors greater than 4–5 mm in diameter in the TPA-treated group, whereas no large tumors were found in the C3G plus TPA-treated group. Histological analysis indicated that those large tumors were squamous cell carcinomas (data not shown). These data suggest that C3G plays an important role in preventing malignant conversion.

Effect of C3G on Proliferation of Human Lung Cancer Cells In Vitro—To further investigate the antitumor activity of C3G, the effects of C3G on proliferation of A549 cells, a human lung carcinoma cell line, were evaluated by using an electric cell-substrate impedance sensor assay. As shown in Fig. 5A, C3G dramatically inhibited proliferation of A549 in a dose-dependent manner. At the concentration of 40 μM, the growth of tumor cells was completely blocked.

Inhibitory Effect of C3G on Tumor Growth in Mice—We further tested the in vivo effect of C3G on xenograft growth of A549 cells in athymic male nude mice. A549 cells were injected subcutaneously in both right and left flanks of each mouse to initiate tumor growth. Ani-
mals were treated intraperitoneally with either PBS or C3G (9.5 mg/kg) three times per week beginning 2 days after the tumor cell implantation. The cell colonies were scored by a computerized image analyzer. *, significant increase from untreated negative control. **, significant decrease from the positive control. 8, skin tumors were generated by DMBA and TPA treatment as described under “Materials and Methods.” The number of papillomas was recorded weekly. The results are presented as the mean of papillomas per mouse ± S.E. of 21–26 mice. *, a significant difference in the number of papillomas between the positive control group and the C3G treated group (p ≤ 0.05).

C3G Suppresses Tumor Cell Metastasis in Mice—The effect of C3G on tumor metastasis dissemination into other organs was evaluated by macroscopic and microscopic examination of tissue sections. Pathological analysis indicated that the mice in the positive control group showed a large subcutaneous tumor mass at the site of injection, and the tumors invaded through the abdominal wall and extended into the abdominal cavity, resulting in peritoneal carcinomatosis (Fig. 6A). Multiple small tumor nodules were observed on the peritoneal surface of the abdominal wall (Fig. 6A). The tumor also was extensively involved with the mesenteric fat with malignant ascites (Fig. 6B). Tumor involvement was not only present on the surface but also within the parenchyma of organs, such as liver, kidney, pancreas, and perigastric lymph nodes (Table 1). In contrast, the C3G-treated mice showed much less tumor involvement of the abdominal cavity, although a smaller subcutaneous tumor mass at the injection site was observed. Fewer tumor nodules were observed in the abdominal cavity and the mesenteric fat. There was no tumor detected in organ parenchyma in the C3G-treated mice. Microscopically, positive control mice show a large subcutaneous tumor mass at the site of injection (Fig. 6C), whereas the injection site of the C3G-treated mice showed collections of macrophages with pig-

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Effect of C3G on TPA-induced cell transformation and tumorigenesis in DMBA/TPA-treated mice. A, JB6 P<sup>−</sup> cells (1 × 10⁴) were exposed to TPA (20 μg/ml) with or without the indicated concentrations of C3G on soft agar medium for 14 days. The cell colonies were scored by a computerized image analyzer. *, significant increase from untreated negative control. **, significant decrease from the positive control. B, skin tumors were generated by DMBA and TPA treatment as described under “Materials and Methods.” The number of papillomas was recorded weekly. The results are presented as the mean of papillomas per mouse ± S.E. of 21–26 mice. *, a significant difference in the number of papillomas between the positive control group and the C3G treated group (p ≤ 0.05). C, external appearance of tumors. Two mice from the positive control group (left) and C3G-treated group (right) showing the greatest number of tumors are shown. The arrow indicates a tumor >5 mm in diameter.
ment, consistent with phagocytosis of pigment derived from the C3G compound. There were viable tumor cells in the deep skeletal muscle distant from the subcutaneous injection site. These results suggest that subcutaneous administrations of C3G not only inhibited A549 cells xenograft growth but also prevented metastasis.

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Inhibitory Effects of C3G on Cancer Cell Migration and Invasion—Because cancer cell migration and invasion are the key events in metastasis, we further tested the effects of C3G on cell migration and invasion in vitro. Our results showed that C3G significantly blocked A549 cell migration in both wound healing assays (Fig. 7A) and Transwell assays (Fig. 7B). The potency of inhibition for cell migration was 25% at 40 μM of C3G and 70% at 80 μM of C3G in the Transwell assays (Fig. 7B). C3G was also able to inhibit cell invasion in a dose-dependent manner, 57% at 40 μM and 85% at 80 μM, as measured by Matrigel invasion assays (Fig. 7C). These results are consistent with the in vivo animal study, demonstrating that C3G may have the ability to inhibit cancer cell migration and invasion and prevent metastasis.

**DISCUSSION**

Chemoprevention, the use of drugs or natural substances to inhibit carcinogenesis, is an important and rapidly evolving aspect of cancer research that is providing a practical approach to identify potentially useful inhibitors of cancer development. Anthocyanins are a group of naturally occurring phenolic compounds related to the coloring of plants, flowers, and fruits and recently have been reported to be as potential cancer preventive agents (10). There is increasing interest in the pharmacological activity of anthocyanins, and C3G is a major member of anthocyanins (10, 39–41). Herein we report the chemopreventive and chemotherapeutic potential of C3G using in vitro and in vivo studies. The results from this study identify C3G as representing a novel compound with potent activity against a solid tumor cell growth and metastasis in vivo. Topical application of C3G prior to TPA application inhibits the formation of keratoacanthomas and squamous cell carcinomas in the mouse skin model. The molecular pathways involved in anticarcinogenesis by C3G were also investigated.

AP-1 and NF-κB are transcription factors that have been implicated in a wide range of cell biological events, including cell proliferation, inflammation, differentiation, metastasis, and apoptosis. Inhibition of AP-1 and NF-κB activation by a variety of agents has been shown to reduce neoplastic transformation (19). In vivo studies in transgenic mice indicate that AP-1 transactivation is required for tumor promotion (20). The blockade of TPA-induced cell transformation and tumorigenesis in the mouse skin model by C3G might be through the inhibition of AP-1 and NF-κB activity. Therefore, the inhibitory effects of C3G on AP-1 and NF-κB activation noted in this study suggest a potential beneficial role in preventing carcinogenesis in vivo.

MAPKs, including ERK, JNK, and p38 MAPK, are activated in
response to environmental stresses and growth factors. Studies indicate that ERK, JNK, and p38 kinase are key molecules activated in response to oxidant injury. Both UVB and TPA can induce ROS generation in the cells (36, 42–44). AP-1 is a downstream target of these three MAPKs. We found that C3G could scavenge UVB-induced ROS in JB6 cells and inhibit UVB- and TPA-induced phosphorylation of ERK, JNK, and p38 kinase. These observations suggest that these inhibitory effects on AP-1 and MAPK activation with C3G may be due to its antioxidant properties.

Angiogenesis is essential for tumor growth in vivo (45). Angiogenesis is a complex process where several proteins and enzymatic pathways converge. COX-2 contributes to the regulation of angiogenesis by various genes, including platelet-derived growth factor and transforming growth factor-β (46). Overexpression of COX-2 is common in non-small cell lung cancer and seems to be associated with tumor progression, invasion, and metastasis (47). Several in vivo studies have already shown that COX-2-specific inhibitors (celecoxib and rofecoxib) have antitumor activity (27, 48). The inhibitory effect of C3G on COX-2 expression may contribute to the suppression of human lung carcinoma growth.

Tumor invasion and metastasis are multistep and complex processes that include cell division and proliferation, proteolytic digestion of the

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<th>Treatment</th>
<th>Number of mice</th>
<th>Peritoneal metastasis</th>
<th>Other parenchyma organs (liver, kidney, pancreas) metastasis</th>
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<tr>
<td>PBS</td>
<td>8 (80)*</td>
<td>4 (40)</td>
<td></td>
</tr>
<tr>
<td>C3G</td>
<td>2 (20)</td>
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* Numbers in parentheses are percent.
extracellular matrix, cell migration through the basement membranes to reach the circulatory system, and the remigration and growth of tumors at metastatic sites (50–52). AP-1 and NF-κB, COX-2, and TNFα, through its ROS scavenger capacity. These data suggest that C3G may function as a potential anticancer agent with little cytotoxicity to normal tissue. Thus, C3G merits further investigation as a cancer therapeutic and preventive agent in humans. These studies establish a promising area of investigating in understanding the molecular mechanisms responsible for the beneficial effects of phytochemicals on human health.

Acknowledgment—We thank Dr. Peilin Zhang for expert assistance with pathology.

REFERENCES

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