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Anthocyanidins Inhibit Migration of Glioblastoma Cells: Structure-Activity Relationship and Involvement of the Plasminolytic System

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Abstract Complete resection of malignant glioblastomas is usually impossible because of diffuse and widespread invasion of tumor cells, and complementary approaches need to be developed in order to improve the efficacy of current treatments. Consumption of fruits and berries has been associated with decreased risk of developing cancer and there is great interest in the use of molecules from dietary origin to improve anticancer therapies. In this work, we report that the aglycons of the most abundant anthocyanins in fruits, cyanidin (Cy), delphinidin (Dp), and petunidin (Pt), act as potent inhibitors of glioblastoma cell migration. Dp clearly exhibited the highest inhibitory potency, this effect being related to the ortho-dihydroxyphenyl structure on the B-ring and the presence of a free hydroxyl group at position 3. Dp decreases the expression of both urokinase-type plasminogen activator receptor (uPAR) and the low-density lipoprotein receptor-related protein (LRP), acting at the transcriptional levels. In addition, Dp upregulated urokinase-type plasminogen activator (uPA) and downregulated the plasminogen activator inhibitor-1 (PAI-1) but decreased, in a concentration-dependent manner, the uPA-dependent conversion of plasminogen to plasmin, indicating that the upregulation of uPA observed with these compounds was not associated with induction of the plasminolytic activity. Overall, these results demonstrate that Dp, Pt, and Cy affect plasminogen activation, thus leading to the inhibition of glioblastoma cell migration and therefore they may be helpful for the development of new strategies for cancer prevention and therapy. J. Cell. Biochem. 100: 100–111, 2007.

Key words: anthocyanidins; cell invasion; cell migration; glioblastoma; plasminogen activator

Glioblastoma [World Health Organization (WHO) Grade IV] is the most common and most malignant tumor of the human nervous system [Ohgaki and Kleihues, 2005]. Despite progress in surgery and adjuvant therapy, glioblastoma patients still have a very poor prognosis due to incomplete resection and resistance to radio- and chemotherapy [VandenBerg, 1992; Demuth and Berens, 2004; Lemke, 2004]. The dismal prognosis associated with glioblastomas is largely due to the fact that these tumors are characterized by a highly invasive phenotype with diffuse infiltration into the regions of the normal brain, which limits the effectiveness

Abbreviations used: BCS, bovine calf serum; Cy, cyanidin; Dp, delphinidin; Dp 3-glu, delphinidin 3-*O*-beta-glucopyranoside; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; U-87, human glioblastoma cells; IGF-1, insulin-like growth factor-1; LRP, lipoprotein receptor-related protein; Mv, malvidin; PA, plasminogen activators; PAI-1, plasminogen activator inhibitor type 1; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; SF/HGF, scatter factor/hepatocyte growth factor; S1P, sphingosine-1-phosphate; TGF-β1, transforming growth factor-beta 1; uPA(R), urokinase-type plasminogen activator (receptor); VEGF, vascular endothelial growth factor.

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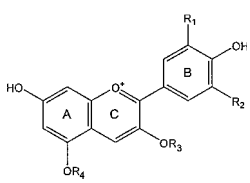
of current treatments [Matsuzawa et al., 1996]. Novel therapeutic approaches are therefore needed to prolong survival. Nutraceutical intervention is one such approach that has been investigated for application with different types of tumors, including brain tumors [Rooprai et al., 2003]. This approach uses naturally occurring agents, especially those that can be included in the diet [Hong and Sporn, 1997; Surh, 2003]. Epidemiological evidence suggests that fruits and vegetables are beneficial to human health and may have a role in cancer prevention [Block et al., 1992; Steinmetz and Potter, 1996; Greenwald et al., 2001]. The mechanisms responsible for these effects are not known although there is accumulating evidence that they are linked to the presence of polyphenolic compounds in various foods.

Anthocyans (i.e., anthocyanins and their aglycons, anthocyanidins) belong to the flavonoid group of polyphenols and are widely distributed in colored fruits and vegetables such as berries, red grapes, purple sweet potatoes, and red cabbages [Harborne, 1988]. In plants, they are present exclusively as glycosides (anthocyanins) having glucose, galactose, rhamnose, xylose, or arabinose attached to the aglycon nucleus, and comprise more than 500 compounds differing largely by the glycoside structures [Harborne, 1988]. Anthocyanidins exhibit different hydroxyl or methoxyl substitutions in their basic structure but are limited to a few main structural variants such that cyanidin (Cy), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), peonidin (Pn), and petunidin (Pt) (Table I), represent the aglycons of most anthocyanins in plants. Depending on nutritional custom, the daily intake of these compounds in humans is estimated to be a few hundred milligrams per day [Hollman and Katan, 1999]. They are absorbed by stomach [Passamonti et al., 2003] and intestinal cells [Talavera et al., 2004] and are rapidly detected in plasma in vivo [Mazza et al., 2002] suggesting that they are bioavailable to exert their biological effects. Consumption of anthocyanins has been shown to reduce the risk of coronary heart disease [Dell'Agli et al., 2004] and cancer [Hou, 2003]. Moreover, it has been reported that anthocyanins reduce the incidence of esophageal tumor [Kresty et al., 2001], reduce infiltration of macrophages in hemangioma [Atalay et al., 2003], and inhibit cell transformation [Xue et al., 2001], indicating potential cancer chemopreventive activity. While several studies indicate that anthocyanins exhibit strong scavenging of free radicals and antioxidant activities [Hou, 2003; Zheng and Wang, 2003], little is known about their antitumor activities. A few studies have shown that some of these compounds can inhibit the growth of human tumor cell lines [Meiers et al., 2001; Kang et al., 2003; Seeram et al., 2003; Marko et al., 2004; Zhang et al., 2005] and the molecular mechanisms involved in this effect is being increasingly understood. For example, it was demonstrated that the chemopreventive effects of grape seed proanthocyanidins are mediated by upregulating the antiapoptotic gene Bcl-2 and downregulating proapoptotic genes, *c-myc* and *p53* genes [Joshi et al., 2001].

TABLE I. Structure of Anthocyanidins



Anthocyanidin	R_1	R_2
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Malvidin (Mv)	OCH_3	OCH_3
Pelargonidin (Pg)	H	H
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OCH_3	OH

R_3, R_4, H or sugar moiety.

Invasion of tumor cells into normal tissue is thought to be a multifactorial process, consisting of cell–cell and cell–extracellular matrix (ECM) interactions as well as the biochemical processes required for active cell movement. Migration of cancer cells is one of the key factors responsible for cancer metastasis. Glioblastoma cell migration can be mediated, at least in part, through inflammation-associated cytokines [Murphy et al., 1995], plasminogen activators (PA) [Zhang et al., 2000] and through integrin receptors [Demuth and Berens, 2004]. The process of plasminogen activation in a healthy organism is controlled through the availability of PAs, localized activation, and interaction with specific inhibitors (PAIs). At the cell surface, urokinase-type plasminogen activator (uPA) binds to its specific receptor (urokinase-type plasminogen activator receptor (uPAR)), then binds its inhibitor plasminogen activator

inhibitor-1 (PAI-1), localized in the matrix, and the complex is internalized by endocytic receptors such as the low-density lipoprotein receptor-related protein (LRP) [Orth et al., 1994; Conese et al., 1995]. Recent evidence suggests that the uPA/uPAR system plays a role in the regulation of cell–matrix interactions such as cell adhesion and migration [Irigoyen et al., 1999]. Furthermore, uPAR seems to control a promigratory signaling system, and high expression of this receptor is associated with invasiveness of various tumors including glioblastoma [Andreasen et al., 2000; Mori et al., 2000].

The present study characterizes the effects of the six representative kinds of anthocyanidins (Cy, Dp, Mv, Pg, Pn, and Pt), and a Dp glycosylated form, delphinidin 3-*O*-beta-glucopyranoside (Dp 3-glu), on the migration of human glioblastoma cells (U-87). Our results show that the ortho-dihydroxyphenyl on the B-ring is critical for their inhibitory actions on glioblastoma cell migration. Furthermore, the present study indicates that these compounds exert their inhibitory actions by affecting the [uPAR · uPA · PAI-1 · LRP] plasminolytic system.

MATERIALS AND METHODS

Materials

Cell culture media were obtained from Life Technologies (Burlington, ON) and serum was purchased from Hyclone Laboratories (Logan, UT). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Anthocyanidins (Cy, Dp, Mv, Pg, Pn, Pt) and anthocyanin (Dp 3-glu) were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The anti-β-LRP (5A6 clone) monoclonal antibody was from Research Diagnostics, Inc. (Flanders, NJ). The anti-uPAR (#3937), -uPA, and -PAI-1 monoclonal antibodies were from American Diagnostica (Greenwich, CT). Anti-ERK-1/2 (extracellular-signal-regulated kinase 1 and 2) (K-23) polyclonal antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho-p44/42 MAPK (Thr 202/Tyr 204) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences (Boston, MA). Human recombinant vascular endothelial growth factor (VEGF; isoform 165) was produced and purified as described [Labrecque et al., 2005]. Epidermal growth factor (EGF) was purchased from BD Biosciences Discovery Labware (Bedford, MA). Insulin-like growth factor-1 (IGF-1), scatter factor/hepatocyte growth factor (SF/HGF), and transforming growth factor-beta 1 (TGF-β1) were from R&D Systems, Inc. (Aurora, OH). Sphingosine-1-phosphate (S1P) was obtained from Sigma (St-Louis, MO). Micro bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich, Canada.

Cell Culture

The human glioblastoma cell line U-87 was purchased from the American Tissue Culture Collection and maintained in modified Eagle's medium (MEM) supplemented with 1 mM sodium pyruvate and containing 10% bovine calf serum (BCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37°C under a humidified 95–5% (v/v) mixture of air and CO_2.

Migration Assays

Transwells (8-μm pore size; Costar, Cambridge, MA) were precoated with 0.15% gelatin/phosphate-buffered saline (PBS) by adding 600/100 μl in the lower/upper chambers for 24 h at 4°C. The Transwells were then washed with PBS and assembled into 24-well plates. The upper chamber of each Transwell was filled with 100 μl of cells (1.0×10^6 cells/ml) and cells were allowed to adhere for 30 min. The fresh MEM medium (0.5% BCS) containing vehicle (EtOH) or the drug solution (anthocyanins) was placed in the lower wells. One hundred microliters of twofold concentrated drug solution prepared in serum-free medium was loaded into each of the upper wells. The plate was placed at 37°C in 5% CO_2/95% air for 4 h. For growth factor-induced migration assays, the monolayers were pretreated for 2 h by adding 100 μl of twofold concentrated drug solution prepared in serum-free medium into the upper chamber and 600 μl of the drug solution into the lower chamber. Migration was initiated by adding S1P (1 μM) or

50 ng/ml of EGF, IGF-1, SF/HGF, TGF-β1, or VEGF to the lower chamber for 3 h. For all these experiments, cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% Crystal Violet/20% (v/v) methanol. The migration was quantitated using computer-assisted imaging and data are expressed as the average density of migrated cells per four fields (magnification 50×).

[^{3}H]-Thymidine Incorporation

Cells were seeded in culture wells (24-well plates) in 2 ml of MEM containing 10% BCS at a seeding density of 3×10^4 cells/well. Cells were allowed to reach ~50% confluence and were then exposed to fresh medium containing 0.2% BCS to induce a quiescent state in the cells. After 24 h, cells were incubated with medium (1% BCS) containing (or lacking) anthocyanidins (25 μM) for 16 h. Then the medium was removed and replaced with fresh medium containing 1% BCS. The experiments were initiated by adding 1 μCi/ml [^{3}H]-thymidine (Amersham Biosciences, UK) for 4 h at 37°C in 5% CO_2/95% air. Afterwards, cells were washed two times with PBS (37°C), fixed in EtOH/acetic acid (3:1), washed once with deionized H_2O and then three times in trichloracetic acid (10%) for 10, 5, and 5 min. After removal of unincorporated [^{3}H]-thymidine, the cells were incubated in perchloric acid (2 N) for three thermal cycles (60°C for 30 min, 20°C for 10 min, 75°C for 30 min) and analyzed by liquid scintillation counting.

Matrigel Invasion Assay

Cells were plated on Matrigel-coated cell-culture inserts in Transwell chambers as follows. Matrigel (Becton Dickinson Labware) was diluted with cold PBS and 0.5 mg/ml was applied to each filter, which were allowed to dry overnight in a laminar flow hood and were then incubated at 37°C for 30 min. The Transwells were washed with PBS before cell passage. Cells were trypsinized, centrifuged, and resuspended at 1×10^6 cells/ml in serum-free medium and added to the upper chamber. After a 24-h incubation period with various concentrations of Dp at 37°C in 5% CO_2/95% air, cells which had passed through to filters to the opposite side of the Transwell were fixed, stained, and quantitated as described above.

Analysis of ERK-1/2 Phophorylation

Cells grown to 80–90% confluence were rendered quiescent by a 24-h incubation in serum-free medium containing either vehicle or the indicated concentrations of Dp. Cells were then stimulated with S1P (1 μM) or SF/HGF (50 ng/ml) for 10 or 15 min, respectively. After incubation, cells were washed once with ice-cold PBS (pH 7.4) containing 1 mM each of NaF and Na_3VO_4, and were incubated in the same medium for 1 h at 4°C. The cells were solubilized on ice in lysis buffer [150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) Nonidet P-40, 1% (v/v) Triton X-100] containing 1 mM each of NaF and Na_3VO_4. The cells were then scraped from the culture dishes and the resulting lysates were clarified by centrifugation at 10,000*g* for 10 min. Protein concentrations were determined using the BCA method (Pierce). Lysates (20 μg protein) were solubilized in Laemmli sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.00625% bromphenol blue], boiled for 4 min and resolved by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 10% gel). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (PerkinElmer Life Sciences), blocked overnight at 4°C with TBS-Tween-20 [147 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20] containing 2% (w/v) bovine serum albumin and incubated 1 h at room temperature with primary antibodies (anti-pERK, 1:1,000 dilution or -ERK, 1:5,000 dilution). Immunoreactive bands were revealed after 1 h incubation with HRP-conjugated anti-rabbit antibodies (1:40,000 dilution) and the signals were visualized with an ECL detection system.

Western Blot Analysis

Identical amounts of protein (15 μg) from conditioned media (20-fold concentrated) and lysates were solubilized in nonreducing sample buffer [62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.00625% bromphenol blue], resolved by SDS–PAGE (β-LRP, 9% acrylamide; uPAR, uPA, PAI-1, 10% acrylamide) and transferred to PVDF. The blocked membranes were then incubated overnight at 4°C with the indicated antibody (1:250 dilution), and then incubated with the anti-mouse HRP-conjugated secondary antibody (1:10,000 dilution) for 1 h at room

temperature. The immunoreactive bands were visualized by ECL.

Casein-Plasminogen Zymography

The activity of uPA protein secreted into the culture medium was assessed by casein/plasminogen zymography. Briefly, 24 h conditioned media was separated under nonreducing conditions by SDS–PAGE on a 7.5% acrylamide gel containing 1 mg/ml casein (Sigma, St. Louis, MO) and 10 mg/ml human plasminogen (Roche Diagnostics, Québec, Canada). Following electrophoresis, the gels were washed twice for 30 min in 2.5% (v/v) Triton X-100 at room temperature and rinsed five times with nanopure water. Gels were incubated at 37°C in casein-plasminogen buffer [100 mM Tris-HCl, pH 8.0, 50 mM EDTA] for 3 h. Plasminogen-dependent proteolysis was detected as a clear zone following staining with 0.1% (w/v) Coomassie blue R-250 in 10% acetic acid/40% methanol and destained in 7.5% acetic acid/30% methanol. Digested bands were quantified by scanning densitometry.

Total RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cells were plated in 100-mm plastic dishes until 90% confluency and treated either with vehicle or with the indicated concentrations of anthocyanidins in serum-free medium for 24 h. Then cell monolayers were washed with PBS and total RNA was isolated from cells using TRIzol reagent (Invitrogen, Burlington, ON) following the manufacturer's instructions. RNA was dissolved in diethyl polycarbonate (DEPC)-treated H_2O and quantitated at 260 nm. One microgram of total RNA isolated from cells was amplified with Superscript™ One-Step RT-PCR with Platinium® Taq from Invitrogen, using specific primers designed for human (uPAR, LRP, and β-Actin) sequences. The upstream and downstream primers used were as follows: for uPAR: 5′-ACCGAGGTTGTGTGTGGGTTAGAC-3′ and 5′-CAGGAAGTGGAAGGTGTCGTTG-3′ (expected product 306 bp); for LRP: 5′-AGAAGTAGCAGGACCAGAGGG-3′ and 5′-TCAGTACCCAGGCAGTTATGC-3′ (expected product 301 bp); for β-Actin: 5′-CACCTTCTACAATGAGCTGC-3′ and 5′-AGGCAAGCTCGTAGCTCTTCT-3′ (expected product 465 bp). For uPAR and LRP, the RT-PCR was carried out under the following conditions: 1 cycle (50°C, 20 min; 94°C, 2 min) for the reverse transcription, 40 cycles (94°C for 30 s; 60°C for 30 s; 72°C for 30 s) for the amplification, and 1 cycle (72°C, 5 min) for the final extension. For β-Actin, the RT-PCR conditions used were: 1 cycle (50°C, 30 min; 94°C, 2 min) for the reverse transcription, 40 cycles (94°C for 30 s; 50°C for 45 s; 72°C for 1 min) for the amplification, and 1 cycle (72°C, 7 min) for the final extension. Products were resolved on 1% (w/v) agarose gels and visualized by ethidium bromide.

Plasminolytic Activity Assay

The in vitro enzymatic activity of uPA was measured by colorimetric assay with or without anthocyanidins (5, 10, or 25 μM). The reaction was performed in a final volume of 200 μl in an incubation medium [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 50 mM $CaCl_2$] containing 25 nM plasminogen (Roche Diagnostics) and 15 μg of the chromogenic plasmin substrate D-Val-Leu-Arg *P*-Nitroanilide (VLK-pNA). The reaction was started by the addition of 10 ng uPA. In this assay, the VLK-pNA is cleaved by plasmin into *P*-nitraniline molecule, the former of which is measured at 405 nm over 2 h at 37°C using a SpectraMAX™ Plus microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

The data are represented as mean ± SEM and statistical analyses were made with Student's *t*-test using GraphPad Prism (San Diego).

RESULTS

Anthocyanidins Inhibit U-87 Cell Migration

We investigated the effects of six different anthocyanidins (Cy, Dp, Mv, Pg, Pn, Pt) (Table I), as well as the glycosylated form Dp 3-glu, on U-87 cell motility by using Transwell culture chambers in which the membranes were precoated with 0.15% gelatin/PBS. Treatment of U-87 cells with 25 μM of the compounds for 4 h affected cell migration compared to the control treatment (Fig. 1A); migration was strongly inhibited by Dp (83% inhibition), Cy (48%), and Pt (48%). Pn, Dp 3-glu, Pn, and Mv were much less potent inhibitors of migration (23%, 15%, 9%, and 8%, respectively). These results suggest that the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins and the presence of a free hydroxyl group at position 3 is essential

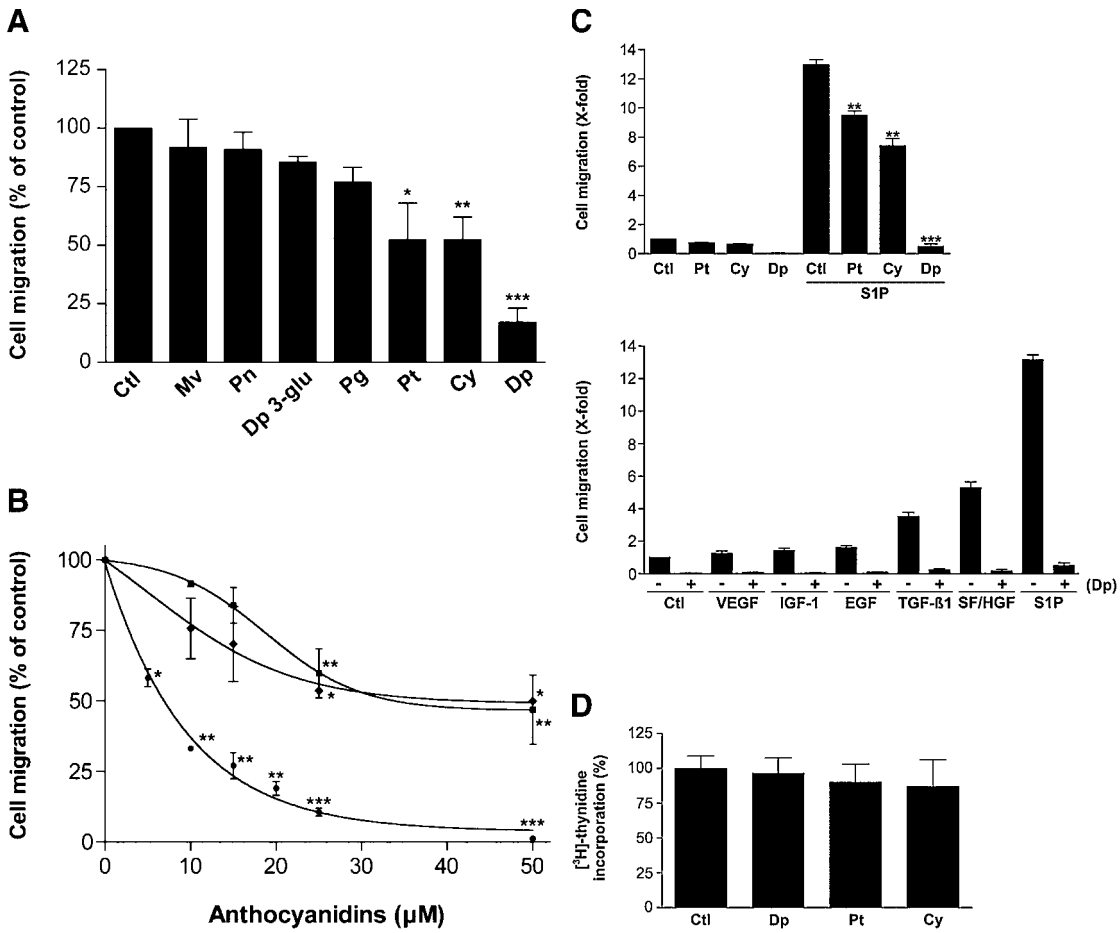


Fig. 1. Anthocyanidins inhibit U-87 cell migration. **A**: U-87 cells were treated for 4 h with 25 μM anthocyanidins in MEM, 0.5% BCS or (**B**) with various concentrations of Cy (◆), Dp (●), or Pt (■) (5, 10, 15, 20, 25 or 50 μM). **C**: U-87 cells were pretreated with Cy, Dp, or Pt (25 μM) for 2 h and migration was initiated by adding 1 μM of S1P (**top panel**) or 50 ng/ml of VEGF, IGF-1, EGF, TGF-β1, or SF/HGF (**bottom panel**) for 3 h. Migration was quantified by counting the cells that crossed the membrane to the lower side of the filter with optical microscopy at magnification 50×. The number of cells that migrated was compared to that observed with untreated cells. Values are mean of three independent experiments (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. control alone). **D**: U-87 cells (3×10^4) were seeded into wells of a 24-well plate. After 24 h, cells were treated with or without 25 μM of each anthocyanidin for 16 h. Then 1 μCi/ml [^{3}H]-thymidine was added and the cells incubated for 4 h. The incorporation of [^{3}H]-thymidine into trichloroacetic acid insoluble material was measured by a liquid scintillation counter. Values are mean of two representative, independent experiments.

for their inhibitory effects. As shown in Figure 1B, we observed a complete inhibition of glioblastoma cell migration by Dp, whereas Cy and Pt were less potent, confirming that Dp is the most potent anthocyanidin inhibitor. Dp caused a concentration-dependent inhibition of U-87 cell migration with an IC_{50} of 7.0 μM.

Several studies have shown that a bioactive lipid, S1P, potently stimulates motility for a wide variety of cell types, including glioma cell lines [Van Brocklyn et al., 2003]. Thus, we evaluated the effect of Dp, Cy, and Pt on U-87 cells migration induced by S1P. Briefly, U-87 cells were allowed to adhere to gelatin-coated Transwells and were incubated for 2 h with 25 μM of the compounds before the addition of S1P to the lower chamber. Dp, and, to a lesser extent, Cy and Pt inhibited S1P-induced cell migration compared to the control treatment (3.8%, 57.1%, and 73.2% inhibition, respectively) (Fig. 1C, top panel). To better understand the inhibitory action of the most potent inhibitor Dp on the chemotactic motility of U-87 cells, we examined its effect on U-87 cell migration induced by other growth factors which are known to be expressed in glioblastomas, including EGF, IGF-1, SF/HGF, TGF-β1, and VEGF [Hamel and Westphal, 2000]. The

result shows that all growth factors stimulated cell migration, and the effects were consistently induced more potently by S1P than by any of the growth factors analyzed. S1P induced an ~13-fold increase in motility compared to the control treatment (Fig. 1C, bottom panel). SF/HGF showed the second strongest effect (~fivefold stimulation) followed by TGF-1β (~threefold induction). Interestingly, Dp inhibited the stimulation of chemotactic migration by all of these growth factors. Moreover, Dp inhibited the basal migration of U-87 cells (Fig. 1C, bottom panel). As shown in Figure 1D, Dp, Pt, and Cy did not affect cell proliferation, as determined by [^{3}H]-thymidine incorporation into U-87 cells, confirming that the anticancer effect of anthocyanidins is specifically related to their inhibitory effects on U-87 cell motility. Taken together, these results clearly indicate that Dp is a potent inhibitor of U-87 cell migration.

Delphinidin Inhibits U-87 Cell Invasion

Tumor metastasis comprises multiple steps, including increased motility and invasive capacity of tumor cells. We further evaluated the antimetastatic activity of the most active anthocyanidin, Dp, using the Transwell assay. We assessed the ability of U-87 cells to invade through a reconstituted basement membrane barrier (Matrigel) with or without Dp for 24 h. Similar to the migration assay results described above, Dp inhibited the invasion of Matrigel by U-87 cells in a concentration-dependent manner in the range of 5–50 μM, achieving a half maximal effect at 9.5 μM Dp (Fig. 2).

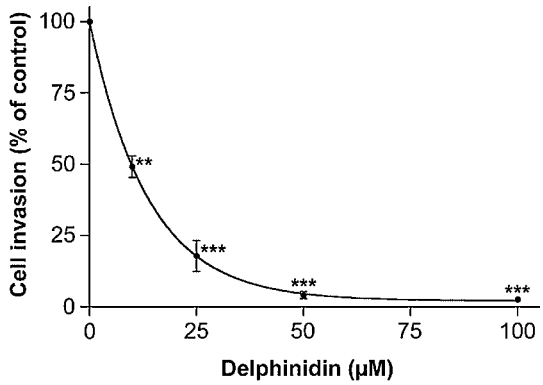


Fig. 2. Delphinidin inhibits U-87 cell invasion. U-87 cells were seeded in the upper well of a Boyden chamber coated with Matrigel to allow for invasion. Cells were treated with various concentrations of Dp (10, 25, 50, or 100 μM) and then allowed to invade for 24 h. The number of cells was quantified with optical microscopy at magnification 50×. The number of cells that crossed the membrane was compared to that observed with untreated cells. Values are mean of three independent experiments (**, $P < 0.01$; ***, $P < 0.001$ vs. control alone).

Delphinidin Does Not Affect the Tyrosine Phosphorylation State of ERK-1/2 Induced by S1P and SF/HGF

Since ERK-1/2 plays an important role in regulating cell motility [Howe et al., 2002], and recent evidence supports a role for anchorage-dependent activation of ERK by growth factors in regulating chemotactic migration [Cho and Klemke, 2000; Hauck et al., 2000], we examined whether Dp could have an effect on the induction of ERK-1/2 phosphorylation by the two most potent stimulators of glioblastoma cell migration, S1P and SF/HGF. Quiescent U-87 cells were incubated in serum-free medium in the presence or absence of Dp for 24 h and cells were then stimulated with 1 μM S1P or 50 ng/ml SF/HGF. As shown in Figure 3A, B (top panel), these factors caused a marked increase in the tyrosine phosphorylation state of ERK-1/2 in untreated U-87 cells. However, the increase in tyrosine-phosphorylated ERK-1/2 was not inhibited by Dp at 10 or 25 μM, and the total amount of ERK-1/2 was also unaffected by these treatments (Fig. 3A, B, bottom panel). These results demonstrate that the inhibitory action of

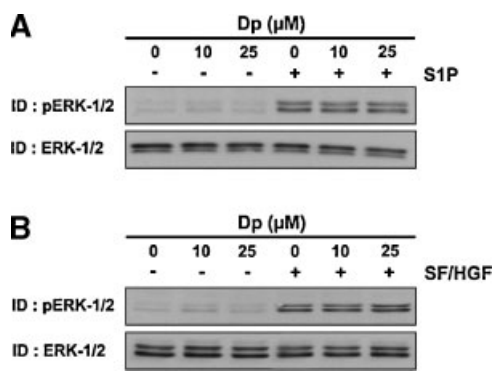


Fig. 3. Delphinidin did not affect ERK-1/2 phosphorylation induced by S1P and SF/HGF in U-87 cells. **A**: Quiescent U-87 cells were incubated in serum-free medium in the presence or absence of Dp (10 and 25 μM) and stimulated with 1 μM S1P for 10 min or (**B**) with 50 ng/ml SF/HGF for 15 min. Cells were lysed and equal amounts of proteins were separated by SDS–PAGE electrophoresis. The phosphorylated form of ERK-1/2 (**top panel**) and the effects of the treatments on the amount of ERK-1/2 (**bottom panel**) were visualized by immunoblotting using specific antibodies. Results are representative of two independent experiments.

Dp on cell migration and invasion was not related to the ERK signaling pathway.

Anthocyanidins Upregulate uPA Secretion and Downregulate uPAR, PAI-1, and LRP Protein Expression

To examine one possible mechanism by which Dp could inhibit in vitro U-87 cell movement, the effect of Dp on protein expression of the PA system was measured by Western blotting and RT-PCR analysis. U-87 cells were incubated in serum-free medium in the presence or absence of Dp, Cy, and Pt for 24 h. As shown in Figure 4A, treatment with these molecules downregulated uPAR and LRP protein expression in cell lysates whereas uPA and PAI-1 protein levels were unchanged as compared to control cells. The results show that Dp is the most potent inhibitor followed by Pt and Cy, respectively. Using an anthocyanidin concentration of 25 μM uPAR and LRP protein levels were reduced by 52% and 55% with Dp, by 28% and 21% with Pt, and by 17% and 6% with Cy (Fig. 4A). The differences in their inhibitory action of protein synthesis correlated with the inhibition observed for these compounds on the expression of uPAR and LRP mRNA. Figure 4B shows that Dp was the most effective anthocyanidin at downregulating uPAR mRNA levels. The expression of uPAR mRNA in cell lysates was completely inhibited at 25 μM by Dp compared to Pt-treated cells, which shows 55.6% inhibition. The Cy anthocyanidin had no significant effect. At the same concentration, the expression of LRP mRNA was less affected, Dp and Pt inhibited at 18.6% and 15.9%, respectively. We next tested the effects of anthocyanidins on uPA and PAI-1 protein expression in conditioned media. As shown in Figure 4C, all these compounds upregulated uPA and downregulated PAI-1 levels, this effect being stronger with Dp. This increased secretion of uPA following Dp treatment was confirmed by the marked enhancement of uPA activity as monitored by casein-plasminogen zymography (Fig. 4D).

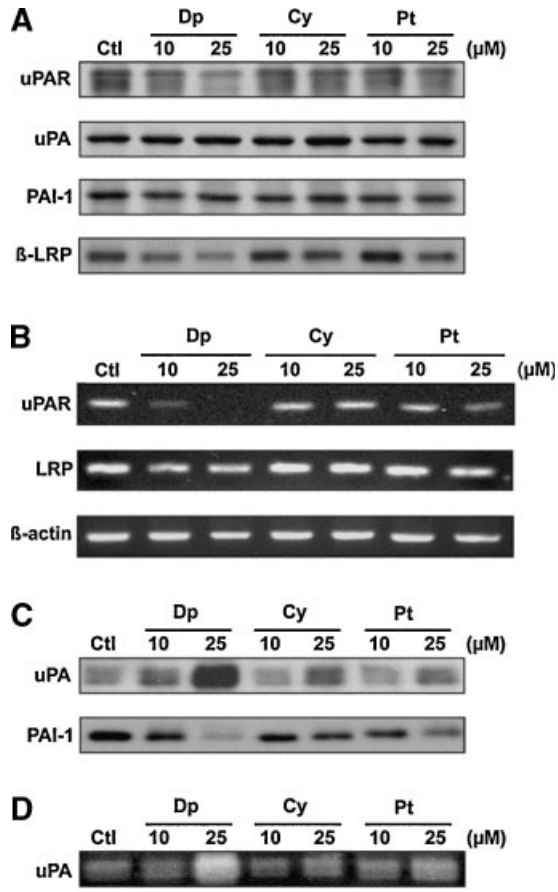


Fig. 4. Delphinidin, cyanidin and petunidin upregulate uPA secretion and downregulate uPAR, PAI-1, and LRP protein expression. Quiescent U-87 cells were incubated in serum-free medium in the presence or absence of anthocyanidins (10 and 25 μM) for 24 h. To analyze the expression of uPAR, uPA, PAI-1, and LRP, proteins from lysates (**A**–**B**) or conditioned media (**C**–**D**) were resolved by SDS–PAGE (A, C) or by casein-plasminogen zymography (D) whereas total RNA (B) was isolated and gene products were amplified by RT-PCR as described in Materials and Methods. Immunodetection, RT-PCR, and zymography obtained from a representative experiment are shown.

Anthocyanidins Inhibit uPA-Dependent Conversion of Plasminogen to Plasmin

Since the anthocyanidins affected the protein expression of uPA and its secretion into conditioned media, we examined the possibility that anthocyanidins might directly affect uPA activities by monitoring the effects of these compounds on the activities of this enzyme toward a synthetic substrate. As shown in Figure 5, the anthocyanidins decreased uPA-dependent conversion of plasminogen to plasmin in a concentration-dependent manner in the range of 5–25 μM. Moreover, these data confirm that Dp is the most potent inhibitor of the plasmin activity followed by Cy and Pt, respectively. Overall, these results indicate that, although the expression and secretion of uPA were upregulated in conditioned media from Dp-treated cells, these effects were not associated with induction of the plasminolytic activity.

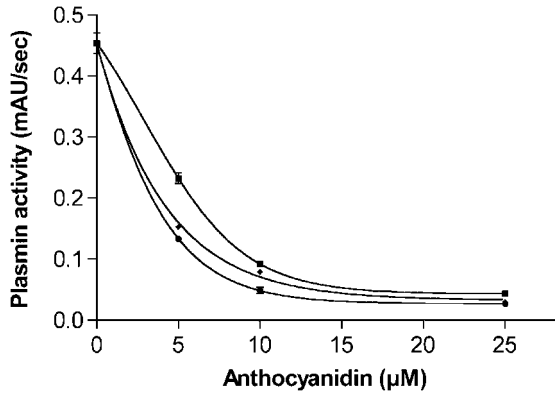


Fig. 5. Delphinidin, cyanidin and petunidin inhibit uPA-dependent conversion of plasminogen to plasmin. Plasminogen activation by uPA was monitored by measuring amidolytic activity of the plasmin generated by activation of plasminogen in the presence or absence of various concentrations of Cy (◆), Dp (●), or Pt (■) (5, 10, or 25 μM). Values are mean of two representative, independent experiments ($P < 0.001$ vs. control alone).

DISCUSSION

Brain tumors are one of the leading causes of death among young children and adults. Glioblastoma remains one of the most challenging solid cancers to treat due to its highly proliferative, angiogenic, and invasive nature. The highly invasive phenotype of malignant glioblastomas means that patients have a poor prognosis, even when being treated with multidisciplinary strategies including surgery, radiotherapy, and chemotherapy [Shapiro, 1999]. The present data demonstrate that three anthocyanidins, Dp, Pt, and Cy, potently inhibit migration of glioblastoma cells. It is noteworthy that the number of hydroxyl groups on the B-ring might be associated with their actions. Anthocyanidins that contain a single hydroxyl group on the B-ring such as Pg, Pn, and Mv showed no significant inhibitory effect. Pt and Cy, with two hydroxyl groups on the B-ring, showed stronger inhibition and Dp, with three hydroxyl groups, demonstrated the best inhibitory effect. Furthermore, a free hydroxyl group at position 3 also seems to be essential for potent inhibition since the presence of sugar residues in this position, in Dp 3-glu, eliminated the inhibitory properties of Dp. Thus Dp, a compound with four hydroxyl groups (3, 3′, 4′, and 5′) (Table I), clearly exhibited the highest inhibitory potency. Although the mechanisms underlying these structural requirements remain to be established, these results nevertheless identify Dp as a useful model with which to investigate the mechanism involved in the inhibition of U-87 cell migration by dietary-derived anthocyanidin.

In the present study, we first observed that the inhibitory effect of Dp on U-87 cell migration was neither related to the ERK signaling pathway, nor to decreased DNA synthesis, suggesting that Dp inhibits glioblastoma cell migration by other molecular mechanisms. With regard to other molecular mechanisms involved in glioblastomas, recent findings suggest that the uPA system is causally involved at multiple steps in cancer progression. In particular, uPA has been implicated in remodeling of the ECM, enhancing both cell proliferation and migration, and modulating cell adhesion [Choong and Nadesapillai, 2003; Reuning et al., 2003]. Recently, it was suggested that the inhibitory effects of two mulberry anthocyanins, cyanidin 3-glucoside and cyanidin 3-rutinoside, on the invasion and motility of A549 human lung carcinoma cells were related to the inhibition of uPA and MMP-2 activities and to the induction of the expression of PAI and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) [Chen et al., 2006]. Furthermore, it was demonstrated that Dp inhibited the MMP-2 and MMP-9 activities of human fibrosarcoma HT-1080 cells, an effect that may be responsible, at least in part, for the inhibition of tumor cell invasiveness by the molecule [Nagase et al., 1998].

During migration of several cell types, expression of uPA, uPAR, and PAI-1 is upregulated [Pepper et al., 1993; Andreasen et al., 1997; Chazaud et al., 2000]. Results with many different human cancers have shown that high levels of uPA, uPAR, and PAI-1 in tumors are correlated with poor patient prognosis [Andreasen et al., 1997]. Although the role of LRP in tumor progression remains unclear at the present time, several studies have shown a correlation between LRP expression and human cancer cell invasion and metastasis [Li et al., 1998; Huang et al., 2000]. Under physiological conditions, uPA bound to uPAR catalyzes the conversion of plasminogen to plasmin, which in turn degrades the ECM and facilitates cell migration [Chazaud et al., 2000]. The binding of PAI-1 to uPA inactivates uPA, triggers the recruitment of uPAR by LRP and the formation of a quaternary complex [uPAR · uPA · PAI-1 · LRP] for endocytosis [Chazaud et al., 2000; Czekay et al., 2001]. Subsequently, intracellular degradation of

the complex occurs with recycling of uPAR and LRP back to the cell surface [Nykjaer et al., 1997]. Thus, any interference with the [uPAR · uPA · PAI-1 · LRP] complex formation induced a marked decrease in cell migration [Chazaud et al., 2000]. Several lines of evidence suggest that the effects of Dp on U-87 cell migration and invasion involve the uPAR/LRP plasminolytic system. Dp decreased PAI-1 levels, as well as uPAR and LRP expression, while increasing uPA levels. These results suggest that less uPA · PAI-1 complex are formed and internalized by uPAR/LRP for degradation in lysosomes and thus more uPA is accumulated in the cell microenvironment. Moreover, Dp downregulated uPAR protein expression at the transcriptional level, also suggesting that less uPAR was available to bind uPA. This is consistent with another study that showed that polyphenolic compounds of red wine (i.e., catechin, epicatechin, quercetin, and resveratrol) increase the expression of uPA in human endothelial cells, which would reduce the risk for thrombotic events, coronary heart disease, and atherothrombotic complications of myocardial infarction by promoting fibrinolysis [Abou-Agag et al., 2001]. However, this upregulation of uPA observed in our work was not associated with an induction of the plasminolytic activity, indicating that the plasminogen activation system at the cell surface is strongly affected by Dp. Thus, given the important role of plasmin generation in tumor metastasis [Andreasen et al., 2000], a dietary-derived molecule like Dp that targets the formation of plasmin to the cell surface and acts on cell migration and invasion might be expected to affect cancer cell invasion and metastasis.

In conclusion Dp and, to a lesser extent, Cy and Pt seem to interfere in the clearance of the uPA · PAI-1 complex, the expression of uPAR and LRP, and plasmin generation which in turn leads to reduced invasiveness of glioblastoma cells. The fact that anthocyanins can cross the blood-brain barrier and are taken up by brain tissue [Andres-Lacueva et al., 2005; Passamonti et al., 2005] suggest that inclusion of foods rich in Dp to the diet may improve the efficacy of current therapeutic approaches of glioblastomas.

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REFERENCES

Abou-Agag LH, Aikens ML, Tabengwa EM, Benza RL, Shows SR, Grenett HE, Booyse FM. 2001. Polyphyenolics increase t-PA and u-PA gene transcription in cultured human endothelial cells. Alcohol Clin Exp Res 25:155–162.

Andreasen PA, Kjoller L, Christensen L, Duffy MJ. 1997. The urokinase-type plasminogen activator system in cancer metastasis: A review. Int J Cancer 72:1–22.

Andreasen PA, Egelund R, Petersen HH. 2000. The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci 57:25–40.

Andres-Lacueva C, Shukitt-Hale B, Galli RL, Jauregui O, Lamuela-Raventos RM, Joseph JA. 2005. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. Nutr Neurosci 8:111–120.

Atalay M, Gordillo G, Roy S, Rovin B, Bagchi D, Bagchi M, Sen CK. 2003. Anti-angiogenic property of edible berry in a model of hemangioma. FEBS Lett 544:252–257.

Block G, Patterson B, Subar A. 1992. Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. Nutr Cancer 18:1–29.

Chazaud B, Bonavaud S, Plonquet A, Pouchelet M, Gherardi RK, Barlovatz-Meimon G. 2000. Involvement of the [uPAR:uPA:PAI-1:LRP] complex in human myogenic cell motility. Exp Cell Res 258:237–244.

Chen PN, Chu SC, Chiou HL, Kuo WH, Chiang CL, Hsieh YS. 2006. Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. Cancer Lett 235:248–259.

Cho SY, Klemke RL. 2000. Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. J Cell Biol 149:223–236.

Choong PF, Nadesapillai AP. 2003. Urokinase plasminogen activator system: A multifunctional role in tumor progression and metastasis. Clin Orthop Relat Res XX: S46–S58.

Conese M, Nykjaer A, Petersen CM, Cremona O, Pardi R, Andreasen PA, Gliemann J, Christensen EI, Blasi F. 1995. alpha-2 Macroglobulin receptor/Ldl receptor-related protein(Lrp)-dependent internalization of the urokinase receptor. J Cell Biol 131:1609–1622.

Czekay RP, Kuemmel TA, Orlando RA, Farquhar MG. 2001. Direct binding of occupied urokinase receptor (uPAR) to LDL receptor-related protein is required for endocytosis of uPAR and regulation of cell surface urokinase activity. Mol Biol Cell 12:1467–1479.

Dell'Agli M, Busciala A, Bosisio E. 2004. Vascular effects of wine polyphenols. Cardiovasc Res 63:593–602.

Demuth T, Berens ME. 2004. Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 70: 217–228.

Greenwald P, Clifford CK, Milner JA. 2001. Diet and cancer prevention. Eur J Cancer 37:948–965.

Hamel W, Westphal M. 2000. Growth factors in gliomas revisited. Acta Neurochir (Wien) 142:113–137 (discussion 137–138).

Harborne JB. 1988. The flavonoids: Advances in research since 1980. London; New York: Chapman and Hall. p 621.
Hauck CR, Hsia DA, Schlaepfer DD. 2000. Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. J Biol Chem 275:41092–41099.
Hollman PC, Katan MB. 1999. Dietary flavonoids: Intake, health effects and bioavailability. Food Chem Toxicol 37:937–942.
Hong WK, Sporn MB. 1997. Recent advances in chemoprevention of cancer. Science 278:1073–1077.
Hou DX. 2003. Potential mechanisms of cancer chemoprevention by anthocyanins. Curr Mol Med 3:149–159.
Howe AK, Aplin AE, Juliano RL. 2002. Anchorage-dependent ERK signaling—Mechanisms and consequences. Curr Opin Genet Dev 12:30–35.
Huang H, Colella S, Kurrer M, Yonekawa Y, Kleihues P, Ohgaki H. 2000. Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays. Cancer Res 60: 6868–6874.
Irigoyen JP, Munoz-Canoves P, Montero L, Koziczak M, Nagamine Y. 1999. The plasminogen activator system: Biology and regulation. Cell Mol Life Sci 56:104–132.
Joshi SS, Kuszynski CA, Bagchi D. 2001. The cellular and molecular basis of health benefits of grape seed proanthocyanidin extract. Curr Pharm Biotechnol 2:187–200.
Kang SY, Seeram NP, Nair MG, Bourquin LD. 2003. Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. Cancer Lett 194:13–19.
Kresty LA, Morse MA, Morgan C, Carlton PS, Lu J, Gupta A, Blackwood M, Stoner GD. 2001. Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. Cancer Res 61:6112–6119.
Labrecque L, Lamy S, Chapus A, Mihoubi S, Durocher Y, Cass B, Bojanowski MW, Gingras D, Beliveau R. 2005. Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. Carcinogenesis 26:821–826.
Lemke DM. 2004. Epidemiology, diagnosis, and treatment of patients with metastatic cancer and high-grade gliomas of the central nervous system. J Infus Nurs 27: 263–269.
Li Y, Wood N, Grimsley P, Yellowlees D, Donnelly PK. 1998. In vitro invasiveness of human breast cancer cells is promoted by low density lipoprotein receptor-related protein. Invasion Metastasis 18:240–251.
Marko D, Puppel N, Tjaden Z, Jakobs S, Pahlke G. 2004. The substitution pattern of anthocyanidins affects different cellular signaling cascades regulating cell proliferation. Mol Nutr Food Res 48:318–325.
Matsuzawa K, Fukuyama K, Hubbard SL, Dirks PB, Rutka JT. 1996. Transfection of an invasive human astrocytoma cell line with a TIMP-1 cDNA: Modulation of astrocytoma invasive potential. J Neuropathol Exp Neurol 55:88–96.
Mazza G, Kay CD, Cottrell T, Holub BJ. 2002. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J Agric Food Chem 50:7731–7737.
Meiers S, Kemeny M, Weyand U, Gastpar R, von Angerer E, Marko D. 2001. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. J Agric Food Chem 49:958–962.
Mori T, Abe T, Wakabayashi Y, Hikawa T, Matsuo K, Yamada Y, Kuwano M, Hori S. 2000. Up-regulation of urokinase-type plasminogen activator and its receptor correlates with enhanced invasion activity of human glioma cells mediated by transforming growth factor-alpha or basic fibroblast growth factor. J Neurooncol 46: 115–123.
Murphy GM, Jr., Lee YL, Jia XC, Yu AC, Majewska A, Song Y, Schmidt K, Eng LF. 1995. Tumor necrosis factor-alpha and basic fibroblast growth factor decrease glial fibrillary acidic protein and its encoding mRNA in astrocyte cultures and glioblastoma cells. J Neurochem 65:2716–2724.
Nagase H, Sasaki K, Kito H, Haga A, Sato T. 1998. Inhibitory effect of delphinidin from Solanum melongena on human fibrosarcoma HT-1080 invasiveness in vitro. Planta Med 64:216–219.
Nykjaer A, Conese M, Christensen EI, Olson D, Cremona O, Gliemann J, Blasi F. 1997. Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. EMBO J 16:2610–2620.
Ohgaki H, Kleihues P. 2005. Epidemiology and etiology of gliomas. Acta Neuropathol (Berl) 109:93–108.
Orth K, Willnow T, Herz J, Gething MJ, Sambrook J. 1994. Low density lipoprotein receptor-related protein is necessary for the internalization of both tissue-type plasminogen activator-inhibitor complexes and free tissue-type plasminogen activator. J Biol Chem 269: 21117–21122.
Passamonti S, Vrhovsek U, Vanzo A, Mattivi F. 2003. The stomach as a site for anthocyanins absorption from food. FEBS Lett 544:210–213.
Passamonti S, Vrhovsek U, Vanzo A, Mattivi F. 2005. Fast access of some grape pigments to the brain. J Agric Food Chem 53:7029–7034.
Pepper MS, Sappino AP, Stocklin R, Montesano R, Orci L, Vassalli JD. 1993. Upregulation of urokinase receptor expression on migrating endothelial cells. J Cell Biol 122: 673–684.
Reuning U, Sperl S, Kopitz C, Kessler H, Kruger A, Schmitt M, Magdolen V. 2003. Urokinase-type plasminogen activator (uPA) and its receptor (uPAR): Development of antagonists of uPA/uPAR interaction and their effects in vitro and in vivo. Curr Pharm Des 9:1529–1543.
Rooprai HK, Christidou M, Pilkington GJ. 2003. The potential for strategies using micronutrients and heterocyclic drugs to treat invasive gliomas. Acta Neurochir (Wien) 145:683–690.
Seeram NP, Zhang Y, Nair MG. 2003. Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. Nutr Cancer 46:101–106.
Shapiro WR. 1999. Current therapy for brain tumors: Back to the future. Arch Neurol 56:429–432.
Steinmetz KA, Potter JD. 1996. Vegetables, fruit, and cancer prevention: A review. J Am Diet Assoc 96:1027–1039.
Surh YJ. 2003. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3:768–780.
Talavera S, Felgines C, Texier O, Besson C, Manach C, Lamaison JL, Remesy C. 2004. Anthocyanins are efficiently absorbed from the small intestine in rats. J Nutr 134:2275–2279.

Van Brocklyn JR, Young N, Roof R. 2003. Sphingosine-1-phosphate stimulates motility and invasiveness of human glioblastoma multiforme cells. Cancer Lett 199: 53–60.

VandenBerg SR. 1992. Current diagnostic concepts of astrocytic tumors. J Neuropathol Exp Neurol 51: 644–657.

Xue H, Aziz RM, Sun N, Cassady JM, Kamendulis LM, Xu Y, Stoner GD, Klaunig JE. 2001. Inhibition of cellular transformation by berry extracts. Carcinogenesis 22: 351–356.

Zhang X, Fei Z, Bu X, Zhen H, Zhang Z, Gu J, Chen Y. 2000. Expression and significance of urokinase type plasminogen activator gene in human brain gliomas. J Surg Oncol 74:90–94.

Zhang Y, Vareed SK, Nair MG. 2005. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. Life Sci 76:1465–1472.

Zheng W, Wang SY. 2003. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J Agric Food Chem 51:502–509.