The Anthocyanidins Cyanidin and Delphinidin Are Potent Inhibitors of the Epidermal Growth-Factor Receptor

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The aglycons of the most abundant anthocyanins in food, cyanidin (cy) and delphinidin (del), were found to inhibit the growth of human tumor cells in vitro in the micromolar range, whereas malvidin (mv), a typical anthocyanidin in grapes, was less active. The aglycons preferentially inhibited the growth of the human vulva carcinoma cell line A431, overexpressing the epidermal growth-factor receptor (EGFR). The glycosides cyanidin-3-β-D-galactoside (cy-3-gal, idaein) and malvidin-3-β-D-glucoside (mv-3-glc, oenin) did not affect tumor cell growth up to 100 μM. The tyrosine kinase activity of the EGFR, isolated from A431 cells, was potently inhibited by cy and del. Mv and the glycosides cy-3-gal and mv-3-glc were inactive up to 100 μM. In intact cells the influence of anthocyanin treatment on downstream signaling cascades was investigated by measuring the phosphorylation of the transcription factor Elk-1. A431 cells were transiently transfected with a luciferase reporter gene construct whose expression is controlled by MAP kinase pathway dependent phosphorylation of a GAL4−Elk-1 fusion protein. We found that cy and del inhibited the activation of the GAL4−Elk-1 fusion protein in the concentration range where growth inhibition was observed. Thus, the anthocyanidins cy and del are potent inhibitors of the EGFR, shutting off downstream signaling cascades. These effects might contribute substantially to the growth-inhibitory properties of these natural food constituents.

Keywords: *Anthocyanins; cyanidin; delphinidin; malvidin; idaein; oenin; sulforhodamine B assay; EGFR; Elk-1*

INTRODUCTION

Anthocyanins are widely spread in foods of plant origin. Depending on their pH and the presence of chelating metal ions, anthocyanins are intensely colored in blue, violet, or red, contributing substantially to the coloring of a multitude of foods, such as berries, grapes, or cherries. Glycosides of the aglycons cyanidin (cy) and delphinidin (del) represent the most abundant anthocyanins in plants (Table 1). In grapes and grape products substantial amounts of malvidin glycosides are also found (*1*). Depending on nutrition customs, an intake of several mg per day of anthocyanins can be expected. In the U.S. the daily intake in humans has been estimated to be as much as 180−215 mg/day (*2*). Extracts with high anthocyanin content, such as those from bilberry or elderberry, are commercially available. If used according to recommended food supplement usage, they might substantially enhance the daily intake of these compounds. Anthocyanins have been associated with potentially beneficial effects on various diseases, such as diabetic retinopathy and various microcircular deficiencies, as well as having anti-inflammatory and chemoprotective properties (*3*). However, despite the relatively high intake in humans, information on potential cellular effects of these compounds is scarce.

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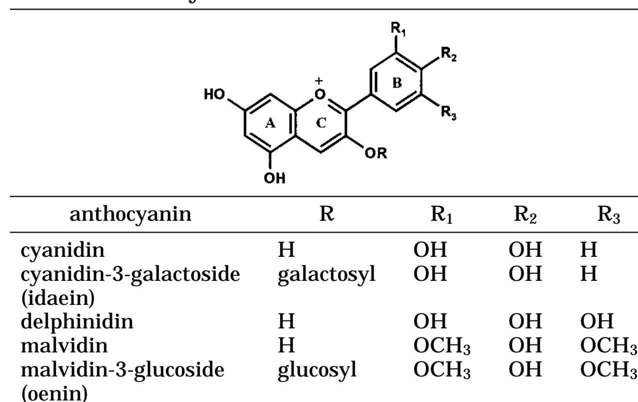
Table 1. Anthocyanin Structure



anthocyanin	R	R_1	R_2	R_3
cyanidin	H	OH	OH	H
cyanidin-3-galactoside (idaein)	galactosyl	OH	OH	H
delphinidin	H	OH	OH	OH
malvidin	H	OCH_3	OH	OCH_3
malvidin-3-glucoside (oenin)	glucosyl	OCH_3	OH	OCH_3

Anthocyanins have been reported to possess antioxidant properties in vitro (*3*−*5*). In this study, we investigated the influence of anthocyanins on the growth of human tumor cells in vitro. Because the structurally related (−)-epigallocatechin-3-gallate (EGCG) from green tea (Figure 1) has been reported earlier to inhibit the protein tyrosine kinase (PTK) activity of the epidermal growth-factor receptor (EGFR), we investigated whether anthocyanins also target the EGFR. Enzyme assays were performed using human EGFR purified from cells of the human vulva carcinoma A431. However, no sufficient information about cellular uptake and metabolism of anthocyanins is available to indicate whether the effects observed on isolated enzyme preparations might also be relevant to intact cells. Therefore, we

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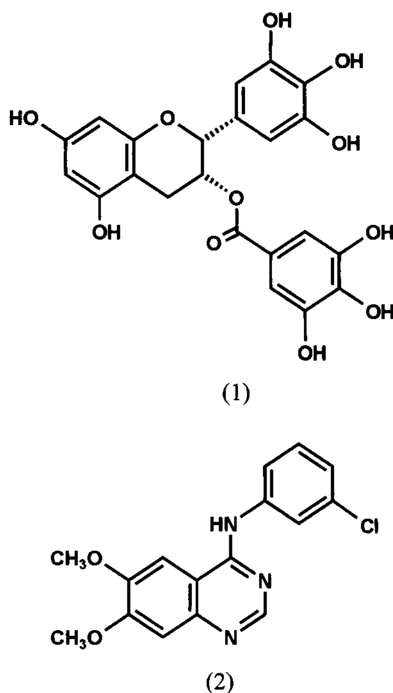

Figure 1. (1) (−)-Epigallocatechin-3-gallate, (2) Tyrphostin AG1478.

additionally addressed whether, in tumor cells, signaling cascades downstream of the EGFR (such as the MAP kinase pathway) are triggered by anthocyanin treatment. The transcription factor Elk-1 represents one of the major targets of the growth-factor-activated MAP kinase (*6*). The known EGFR inhibitor tyrphostin AG1478 has been reported to inhibit the phosphorylation of Elk-1 (*7*). Using a similar reporter gene approach we investigated whether treatment of A431 cells with anthocyanins affects the phosphorylation of Elk-1 in human tumor cells. To compare the effects of anthocyanins to those of the structurally related catechins, EGCG was used as a positive control in all assays.

MATERIALS AND METHODS

Chemicals. The anthocyanins were obtained from Roth (Karlsruhe, Germany). For all assays freshly prepared solutions of the compounds were used. Purity and stability of the anthocyanidins were tested by HPLC with UV detection (540 nm), using a LiChrospher 100 RP18 column (250 mm/4 mm/5μm, Merck Eurolab GmbH, Darmstadt, Germany) and A, H_2O/formic acid (90:10), B, MeOH + 0.1% HCl, mixed A/B, 60:40 as mobile phase. The anthocyanidins were dissolved in DMSO for assays and diluted with B to an adequate concentration for HPLC analysis. The anthocyanidins were tested for stability over a period of 24 h and showed no decay within this period when stored at −20 °C. Tyrphostin AG1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline] was purchased from Alexis Deutschland GmbH (Grünberg, Germany) and poly (Glu/Tyr) 4:1 was purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). (−)-Epigallocatechin-3-gallate was a generous gift of Novartis (Basel, Switzerland).

Cell Culture. The human vulva carcinoma cell line A431 (*8*) was cultured in MEM medium containing L-glutamine (4 mM) and the large cell lung tumor xenograft cell line LXFL529L (*9*) was cultured in RPMI 1640 medium, both with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin in humidified incubators (37 °C, 5% CO_2). Cell culture medium and supplements were obtained from Gibco Life Technologies (Karlsruhe, Germany). Cells were routinely tested for the absence of mycoplasm contamination.

Sulforhodamine B Assay. Effects on cell growth were determined according to the method of Skehan et al. (*10*). Briefly, cells were seeded into 24-well plates and allowed to grow for 24 h before treatment. Thereafter, cells were incubated with the respective drug for 3 days in serum-containing medium. Incubation was stopped by addition of trichloroacetic acid (50% solution). After 1 h at 4°C, plates were washed four times with water. The dried plates were stained with a 0.4% solution of sulforhodamine B. The dye was eluted with Tris buffer (10 mM, pH 10.5) and quantified photometrically at 570 nm. Cytotoxicity was determined as percent survival, determined by the number of treated cells over control cells × 100 (% T/C).

Tyrosine Kinase Assay. The EGFR was isolated from A431 cells and purified by affinity chromatography using wheat germ lectin agarose (Pharmacia Biotech, Uppsala, Sweden). 96-Well plates were coated by incubation overnight at 37 °C with 100 μL per well of 0.1 mg/mL of the tyrosine kinase substrate poly (Glu/Tyr) 4:1 sodium salt in PBS. Excess poly (Glu/Tyr) 4:1 was removed by aspiration, and the plates were washed with wash buffer (0.1% Tween 20 in PBS). The kinase reaction was initiated by adding 50 μL of ATP solution (50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 200 μM ATP) to 40 μL of purified membrane fraction. The test compound (10 μL, in 10% DMSO) was added to give a final DMSO concentration of 1%. Phosphorylation proceeded at 37 °C for 30 min. The kinase reaction was terminated by aspiration of the reaction mixture and the plate was washed with wash buffer (see above). Phosphorylated poly (Glu/Tyr) 4:1 was determined after 60 min of incubation with an antiphosphotyrosine−peroxidase conjugated antibody (Roche, Mannheim, Germany), 0.6 U/mL in 1% BSA/PBS, 75 μL per well, at 37 °C. The antibody was removed by aspiration, the plate was washed again with wash buffer, and the peroxidase reaction was started by addition of 100 μL ABTS per well. After incubation of the plate at 37 °C the absorbance was measured at 405 nm.

Reporter Gene Assay. *Transfection of A431 Cell Line.* Cells were plated in 24-well plates at a density of 3×10^5 cells in 1 mL of medium containing 10% FCS and incubated at 37 °C overnight. Thereafter, cells were held in serum-free medium for 24 h, and then they were transfected by adding 150 ng of pFR−Luc reporter plasmid and 2.5 ng of pFA2-Elk-1 fusion transactivator plasmid (Stratagene, La Jolla, CA) per well, together with 0.46 μL of FuGENE 6® (Roche, Mannheim, Germany) in 10 μL of serum-free medium (preequilibrated for 5 min). After the solution was gently mixed and incubated at room temperature for 15 min, cells were cultivated at 37 °C for a further 24 h.

Luciferase Assay. Cells were incubated at 37 °C for 0.5 h with substances or 1% DMSO prior to stimulation with 100 ng/mL of EGF. After 4.5 h the incubation was stopped by washing each well with 1 mL of PBS and treating it with lysis buffer (Promega, Mannheim, Germany). Lysates were transferred into 96-well plates and luciferase activity was measured using a luminometer.

RESULTS

Inhibition of Tumor Cell Growth. The growth-inhibitory potential of the anthocyanidins cy, del, and mv, as well as the glycosides cy-3-gal and mv-3-glc in comparison to EGCG was determined using the sulforhodamine B assay (Table 2). The aglycons cy and del potently inhibited the growth of the human large cell lung tumor xenograft cell line LXFL529L and, even more efficiently, the human vulva carcinoma cell line A431. Del exhibited growth-inhibitory potency similar to (−)-epigallocatechin-3-gallate (EGCG). Mv showed dose-dependent inhibition of A431 cells but was significantly less potent than cy and del. In contrast, the growth of LXFL529L cells remained completely unaffected by treatment with mv up to 100 μM. The

Table 2. Growth Inhibition in the Sulforhodamine B Assay[a]

substance	cell line	
	LXFL529L IC_{50} [μM]	A431 IC_{50} [μM]
cyanidin	73 ± 4	42 ± 1
delphinidin	33 ± 3	18 ± 2
malvidin	>100	61 ± 7
cyanidin-3-galactoside	>100	>100
malvidin-3-glucoside	>100	>100
(−)-epigallocatechin-3-gallate	32 ± 2	21 ± 1

[a] Cells were incubated for 3 days with the respective compound. Growth inhibition was calculated as survival of treated cells over control cells (treated with the vehicle 0.1% DMSO) × 100 [T/C %]. IC_{50} values were calculated by linear regression (at least three points). The values given are the mean IC_{50} ± SD of at least three independent experiments, each done in quadruplicate.

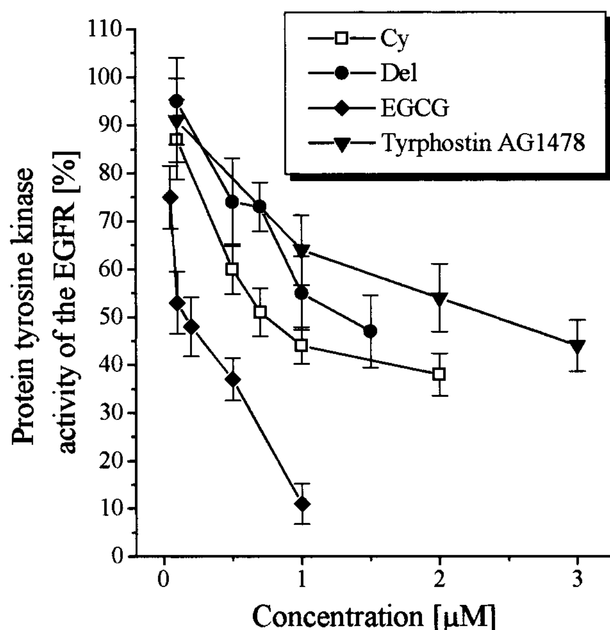

Figure 2. Inhibition of the tyrosine kinase activity of the EGFR. Phosphorylation of tyrosine residues of a peptide poly (Glu/Tyr) 4:1 was determined by ELISA using an antiphosphotyrosine antibody linked to POD. The data presented are the mean ± SD of three independent experiments, each performed in quadruplicate.

glycosides cy-3-gal and mv-3-glc had no inhibitory effect on either of the cell lines up to 100 μM.

Epidermal Growth-Factor Receptor. The EGFR was isolated from A431 cells by affinity chromatography. The influence of the compounds on the protein tyrosine kinase activity of the EGFR was determined by ELISA using a monoclonal antibody against phosphotyrosine. The known EGFR inhibitor tyrphostin AG1478 (Figure 1) was used as a positive control (*11* and *12*). EGCG (0.2 ± 0.02 μM) and the anthocyanidins cy (0.8 ± 0.2 μM) and del (1.3 ± 0.2 μM) were more potent inhibitors of the EGFR protein tyrosine kinase activity than tyrphostin AG1478 (Figure 2). In contrast, mv and the glycosides cy-3-gal and mv-3-glc were inactive up to 100 μM.

Phosphorylation of Elk-1. The activity of MAP kinase was measured as phosphorylation of the transcription factor Elk-1 using a reporter gene assay. A431 cells were transiently cotransfected with a plasmid encoding a fusion protein consisting of the DNA binding domain (dbd) of GAL4 and Elk-1 together with a plasmid containing the upstream activating domain (UAS) of GAL4 and the luciferase gene as a reporter. Phosphorylation of the fusion protein enables its binding to the UAS of GAL4 resulting in the expression of luciferase (Figure 3).

At low concentrations (5 μM) EGCG induced a slight but significant ($p < 0.05$) induction of luciferase activity, an effect which was reversed at concentrations ≥ 20 μM (Figure 4). Comparable inducing effects were observed with del at 10 μM. However, at 30 μM luciferase activity was already diminished to 50%. Cy and mv were less active than del and EGCG, also exhibiting dose-dependent inhibition of luciferase expression. No significant inhibition of luciferase expression was observed by treatment with cy-3-gal and mv-3-glc up to 100 μM. In contrast, cy-3-gal significantly enhanced luciferase activity at concentrations up to 20 μM. At higher concentrations, subsequent effects approached base levels (> 50 μM). Mv-3-glc exhibited slightly enhancing effects on luciferase expression (≤ 20 μM) with a continuous increase at higher concentrations (≤ 70 μM).

DISCUSSION

We here show that the aglycons of the most abundant anthocyanins cy and del inhibit the growth of human tumor cells, exemplified by A431 and LXFL529L. This agrees with inhibitory effects on the human colon tumor cell line HCT115 described earlier for the anthocyanins del and cy (*13*). Consistent with our findings, del was shown to possess the highest growth-inhibitory potency.

EGCG, the most abundant polyphenol in green tea, also is known to inhibit the growth of human tumor cells in vitro (*14*−*18*). The effect on the growth of A431 cells presented here is in good accordance with earlier reports (*19* and *20*). Furthermore, we found cells of the human large cell lung carcinoma LXFL529L to be less sensitive to the growth-inhibitory potential of EGCG. Because LXFL529L cells express significantly less EGFR than A431 cells (*21*) this might explain in part the observed differences in sensitivity. Of note, the anthocyanidins cy, del, and mv also inhibited the growth of A431 cells more potently than LXFL529L. However, mv was completely inactive up to 100 μM, whereas cy and del potently affected the protein tyrosine kinase activity of the EGFR (Figure 2). Thus, growth inhibition of A431 cells by mv is observed at concentrations where no effect on the protein tyrosine kinase activity of the EGFR is to be expected. This indicates that the higher sensitivity of A431 cells compared to LXFL529L cells may not necessarily be due to the higher expression of EGFR. However, as shown in the reporter gene assay, mv treatment inhibits the phosphorylation of Elk-1 downstream of the EGFR at growth-inhibitory concentrations in a dose-dependent manner (Figure 4). This leaves two options: first, mv might interfere with the EGFR without inhibiting the protein tyrosine kinase activity; and second, mv targets signaling elements downstream of the EGFR or upstream of Elk-1 independent from the EGFR.

The data from the sulforhodamine B assay indicate that for potent growth inhibition free hydroxy groups at the B ring as well as a free hydroxy group in 3 position seems to be essential. Sugar residues in position 3 completely eliminated the growth-inhibitory properties (Table 2). Del, a compound with four hydroxy groups (3, 3′,4′, and 5′), exhibited clearly the highest growth-inhibitory potency. A decrease in the number of hydroxy

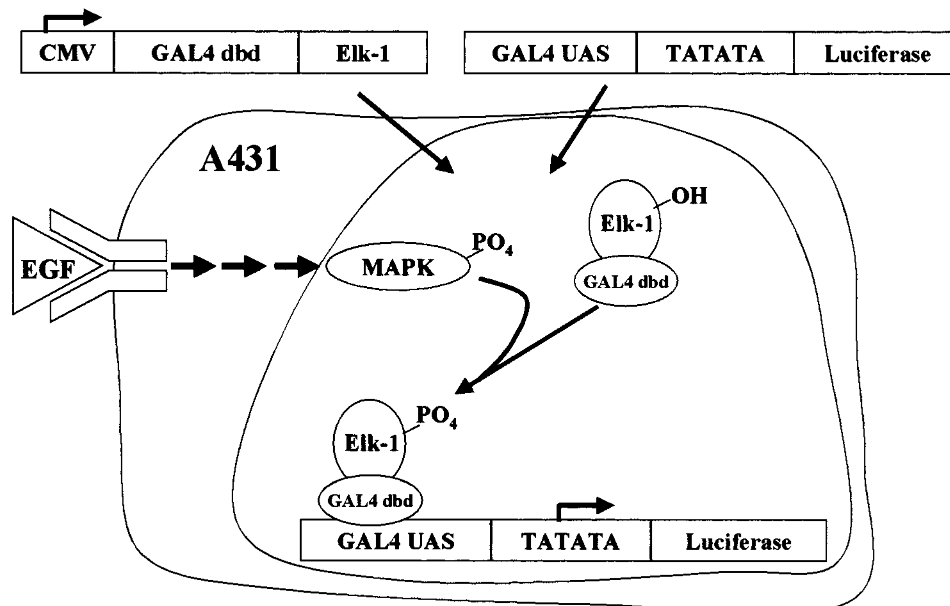

Figure 3. Scheme of the reporter gene assay for Elk-1 phosphorylation (Stratagene, La Jolla, CA). A431 human vulva carcinoma cells were transiently cotransfected with a plasmid encoding a fusion protein consisting of the DNA binding domain (dbd) of GAL4 and Elk-1 as well as a plasmid containing the upstream activating domain (UAS) of GAL4 and the luciferase gene as a reporter. MAPK, mitogen activated protein kinase; EGF, epidermal growth factor; GAL4, *trans*-acting transcriptional activator from yeast (*23*); Elk-1, transcription factor.

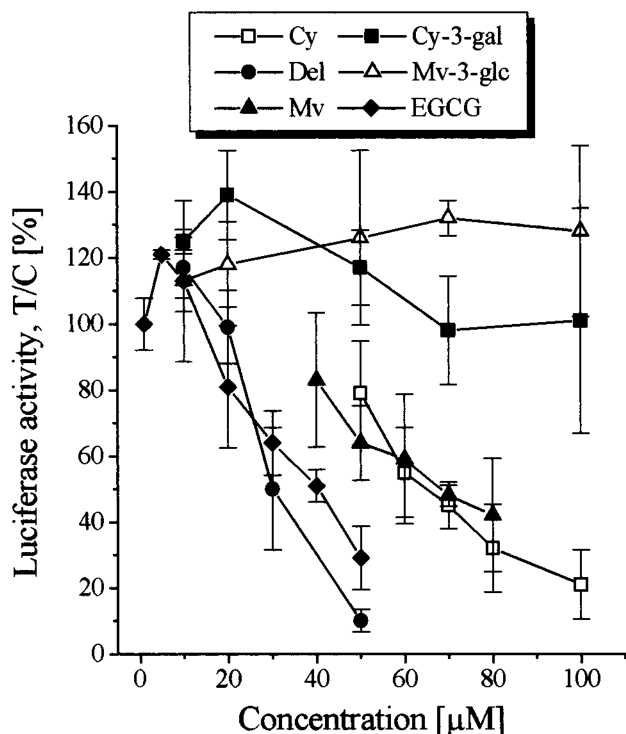

Figure 4. Inhibition of luciferase expression as a measure for the inhibition of Elk-1 phosphorylation. After transfection, the cells were cultivated for 24 h. Thereafter, incubation with the test compounds was started 30 min prior to stimulation with 100 ng/mL EGF and continued for 4.5 h. Luciferase activity was determined in three independent experiments. Data are presented as mean ± SD.

groups, as exemplified by cy, significantly diminished growth inhibition. Methoxy substituents in the 3′ and 5′-positions strongly reduced the growth-inhibitory properties.

EGCG has been reported to potently inhibit the protein tyrosine kinase activity of the EGFR (*22*). One of the proposed mechanisms is the inhibition of EGF binding to its receptor (*20*). Consistent with these earlier reports, we found strong inhibition of the PTK activity of the EGFR by EGCG in our test system. In comparison, the anthocyanidins cy and del showed inhibitory properties in the same concentration range. In contrast to the results from the sulforhodamine B assay, inhibition of the PTK activity of the EGFR was not enhanced by the additional hydroxy group of del compared to cy. Both compounds had almost similar inhibitory properties in the EGFR ELISA (Figure 2). Thus, free hydroxy groups in 3, 3′, and 4′ positions were sufficient for effective enzyme inhibition. A sugar residue in the 3 position or methoxy groups at the B ring (mv) completely abolished the enzyme inhibitory properties.

At growth-inhibitory concentrations, the anthocyanidins cy, del, and mv abrogated the expression of luciferase, indicative for inhibition of Elk-1 phosphorylation (Figure 4). Of note, treatment with cy and mv yielded almost identical effects with significant inhibition of luciferase expression at concentrations ≥ 50 μM. Consistent with the observed growth inhibition in the SRB assay, del and EGCG induced similar effects on the luciferase expression. However, these data indicate that at low, subcytotoxic concentration a slight induction of Elk-1 phosphorylation is induced, an effect which is completely reversed at higher concentrations. In accordance with the lack of growth-inhibitory properties, no significant inhibition of luciferase expression was observed by treatment with the glycosides cy-3-gal and mv-3-glc.

Of note, the concentration range for PTK inhibition was more than an order of magnitude lower compared to the substance concentrations inhibiting Elk-1 phosphorylation and cell growth. The discrepancy in substance concentration between effects observed in isolated enzyme preparations and cell culture might be due to cellular pharmakokinetics. Little is known about cellular uptake, subcellular distribution, metabolism, and elimination of anthocyanins. It also cannot be excluded that solubilization of the originally membrane-

bound EGFR, as required for determination of the PTK activity in vitro, modifies the conformation of the protein in terms of facilitating the binding of potential inhibitors, thus increasing the susceptibility. The different sequence in effectiveness between inhibition of cell growth and inhibition of the EGFR further indicates that possibly additional cellular targets might play a role for the observed cellular effects of that compounds. However, the results clearly show that in intact cells, treatment with anthocyanidins results in the effective shutting off of the MAP kinase pathway downstream of the EGFR, a crucial signaling event in the regulation of cell proliferation. Therefore, it is reasonable to assume that the inhibition of the EGFR at least contributes substantially to the growth-inhibitory properties of cy and del.

In summary, this study showed that the anthocyanidins cy and del are potent inhibitors of the EGFR. In terms of growth-inhibitory properties and its effects on Elk-1 phosphorylation as a measure for the MAP kinase pathway activity del is equivalent to the known chemopreventive agent EGCG. Cy is less active, but still inhibited the growth of the human tumor cell lines tested. Thus, anthocyanidins represent a promising class of compounds which might be interesting in the view of chemoprevention.

ABBREVIATIONS USED

Cy, cyanidin; del, delphinidin; mv, malvidin; cy-3-gal, cyanidin-3-galactoside; mv-3-glc, malvidin-3-glucoside; dbd, DNA binding domain; UAS, upstream activating sequence; EGFR, epidermal growth-factor receptor; MAP kinase, mitogen-activated protein kinase.

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This work is dedicated to G. Eisenbrand on the occasion of his 60th birthday.

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