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Suppression of the Kinase Activity of Receptor Tyrosine Kinases by Anthocyanin-Rich Mixtures Extracted from Bilberries and Grapes

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Two standardized anthocyanin-rich mixtures were investigated for their ability to inhibit the receptor tyrosine kinases (RTKs) EGFR, ErbB2, ErbB3, VEGFR-2, and VEGFR-3. Both mixtures reduced the kinase activity of recombinant kinase domains of each RTK at concentrations ≤12.9 μg/mL, with preferential inhibition of VEGFR-2 and EGFR (≤3.4 μg/mL). Similarly, ligand-induced autophosphorylation of these RTKs in human vulva carcinoma or porcine aortic endothelial cells was suppressed by both mixtures, with ErbB3 and VEGFR-3 being preferentially inhibited. Anthocyanin-rich extracts completely abrogated VEGFR-3 phosphorylation at concentrations of ≥50 μg/mL. These results indicate that anthocyanin-rich mixtures can inhibit RTKs with low specificity. The rank order of inhibitory efficacy against the tested RTKs in intact cells was VEGFR-3 ≫ VEGFR-2 > ErbB3 > EGFR > ErbB2. Considering the important role of RTKs in carcinogenesis, their inhibition by anthocyanin-rich mixtures suggests that they may serve as biomarkers of the pharmacological efficacy of anthocyanins in future chemoprevention experiments and in clinical intervention studies.

KEYWORDS: Polyphenol; ErbB-receptor family; VEGFR; chemoprevention

INTRODUCTION

Dietary habits have been associated with the risk of development of many different types of cancer. Epidemiological studies indicate that colorectal cancer incidence in particular might be decreased by an enhanced dietary intake of flavonoids present in fruits and vegetables. Recently, an anthocyanin-rich bilberry (*Vaccinium myrtillius*) extract was found to significantly decrease the intestinal adenoma burden in the ApcMin(−/−) mouse model (*1*). The bilberry extract mirtocyan contains a mixture of 15 anthocyanins, intensely colored flavonoids found ubiquitously in fruits and vegetables. Notably, the dietary intake of anthocyanins is higher than that of any other flavonoid, with an average estimated daily intake of 12.5 mg per person in the USA (*2*). Anthocyanins and their aglycons, the anthocyanidins, have been associated with a multitude of biochemical effects germane to anticarcinogenesis, including the inhibition of tumor cell growth, antioxidative properties and suppression of the activity of receptor tyrosine kinases (RTKs) (*3*−*7*). RTKs are membrane-spanning cell surface receptors with cytoplasmic tyrosine kinase activity and are thought to play a crucial role in carcinogenesis and tumor progression (*8*−*11*). Important examples of RTKs are the ErbB-family, including the epidermal growth factor (EGFR, ErbB1) and ErbB2−4 receptors, insulin-like growth factor receptor 1 (IGFR1), and the vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3. ErbB and IGF receptors are overexpressed in many tumor types, resulting in changes in cellular signaling involved in cell growth and differentiation (*12*−*14*). VEGF receptors are key modulators of angiogenesis and lymphangiogenesis (*15*−*17*). Inhibition of the activity of these receptors results in the suppression of downstream oncogenic signaling cascades including those engaging mitogen-activated protein kinase and phosphatidylinositol-3-kinase (*3*, *15*, *18*, *19*).

Hitherto, modulation of RTK activity by anthocyanidins and anthocyanins, respectively has been observed exclusively with defined purified compounds such as delphinidin, cyanidin, and

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cyanidin-3-galactoside (*3, 19*). Increasingly, clinical intervention studies that have a cancer chemopreventive intent focus on complex mixtures of anthocyanins, exemplified by bilberry extract (*20*) and freeze-dried black raspberries (*21*). In light of these considerations, we tested the hypothesis that mixtures of anthocyanins can inhibit the activity of RTKs, as has been observed for isolated anthocyanin species. Two commercially available standardized anthocyanin-rich mixtures that each contain 15 anthocyanins were investigated, namely, mirtocyan from bilberries and oenocyanin from grapes. Inhibition of RTK activity was assessed using a recombinant kinase activity assay in a cell-free system and by determining ligand-induced autophosphorylation in A431 vulva carcinoma cells and in porcine aortic endothelial cells, ectopically expressing either VEGFR-2 or VEGFR-3. Furthermore, modulation of RTKs by the anthocyanin-rich mixtures was compared with their ability to modulate cell growth.

MATERIALS AND METHODS

Chemicals. Mirtocyan (formerly mirtoselect), a standardized extract of bilberries, and oenocyanin E163, an extract of red grape pomace and waste product of red wine and grape juice production, were kindly provided by Indena SpA (Milan, Italy). Mirtocyan is generated by Indena and oenocyanin by Bagnarese SpA (Bagnara di Romagna, Italy). Mirtocyan is prepared by an industrial proprietary process ensuring constant and reproducible anthocyanin composition (36%, w/w). Anthocyanin constituents of mirtocyan and their approximate relative abundance (% in brackets, by HPLC analysis) compared to total anthocyanins (=100%) are delphinidin-3-galactoside (16%), -3-glucoside (14%) and -3-arabinoside (12%), cyanidin-3-galactoside (10%), -3-glucoside (11%), and -3-arabinoside (8%), petunidin-3-galactoside (3%), -3-glucoside (8%), and −3-arabinoside (2%), peonidin-3-galactoside (1%), -3-glucoside (4%), and -3-arabinoside (1%), malvidin-3-galactoside (3%), -3-glucoside (5%), and -3-arabinoside (2%) (Indena datasheet). The other constituents of mirtocyan are polyphenols other than anthocyanins (phenolic acids, flavonols, proanthocyanidins ∼18%), carbohydrates (∼20%), aliphatic alcohols (∼9%), fats (∼0.04%), nitrogen compounds (∼1%), and ash (∼0.7%) with the remaining ∼15% undefined.

The anthocyanin content of oenocyanin E 163 is 22% (w/w). It contains the following 5 anthocyanins with their approximate relative abundance in percentage of total anthocyanins (=100%) in parentheses: delphinidin-3-glucoside (7%), cyanidin-3-glucoside (16%), petunidin-3-glucoside (12%), peonidin-3-glucoside (20%), and malvidin-3-glucoside (40%) (Bagnarese, datasheet). Minor anthocyanin constituents, which altogether amount to 5% of total anthocyanins, are delphinidin-3-glucoside acetate, cyanidin-3-glucoside acetate, petunidin-3-glucoside acetate, peonidin-3-glucoside acetate, malvidin-3-glucoside acetate, delphinidin-glucoside *p*-cumarate, cyanidin-3-glucoside *p*-cumarate, petunidin-3-glucoside *p*-cumarate, peonidin-3-glucoside p-cumarate, and malvidin-3-glucoside *p*-cumarate. The other constituents of oenocyanin E 163 are polyphenols other than anthocyanins (flavan-3-ols, i.e., catechins and procyanidins ∼12%), carbohydrates (∼25%), organic acids (∼14%), aliphatic alcohols (∼9%), nitrogen compounds (∼1%), and ash (∼1%) with the remaining ∼16% undefined. The identity of the major anthocyanins in either mixture was confirmed by HPLC-mass spectrometric analysis (*1*).

The EGFR-specific inhibitor tyrphostin AG1478 and the ErbB2-specific inhibitor tyrphostin AG879 were purchased from Sigma-Aldrich (Taufkirchen, Germany). For all assays, anthocyanin-rich solutions were freshly prepared in DMSO prior to the experiment and were not used as stored stock solutions. All compounds and mixtures were dissolved in DMSO with a maximum final concentration in the different test systems of 1% (v/v).

Cell Culture. Human vulva carcinoma A431 cells were cultured in minimum essential medium (MEM; Sigma, Taufkirchen, Germany) containing L-glutamine (4.5 g/L). The production, characterization, and culture of porcine aortic endothelial (PAE) cells and their VEGFR-2 and VEGFR-3 transfectants have been described previously (*22*). Cell culture media were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator (37 °C, 5% CO_2).

Sulforhodamine B (SRB) Assay. The assay was performed according to a modified method of Skehan et al. (*23*). Briefly, cells were seeded into 24-well plates and allowed to grow for 48 h prior to treatment. Cells were incubated with the anthocyanin-rich mixture for 72 h in serum-containing medium in the presence of catalase (100 U/mL) to prevent the formation of hydrogen peroxide in the medium. Incubation was stopped by addition of trichloroacetic acid (50% v/v solution). After 1 h at 4 °C, plates were washed four times with water. The dried plates were stained with a 0.4% (w/v) solution of SRB. The dye was eluted with Tris-buffer (10 mM, pH 10.5) and quantified photometrically (570 nm). Cytotoxicity was determined as percent survival, determined by the number of treated over control cells × 100% [T/C].

In Vitro Kinase Assays. The tyrosine kinase substrate (poly-Glu,Tyr)$^{4:1}$ (MW: 20.000−50.000) was diluted in 100 mM sodium bicarbonate, pH 9.6 at a concentration of 0.2 mg/mL, and used to coat 96-well microtiter plates by incubation overnight with 100 μL solution per well. The substrate solution was removed, the plates washed twice in TBS buffer (10 mM Tris-HCl, pH 8.1, 100 mM NaCl), and blocked by incubation with 5% (w/v) BSA/TBS for at least 30 min. Again, the plates were washed twice with TBS buffer. A reaction mix was prepared by combining the test compound dissolved in 10% DMSO with 25 μL of 4 × kinase dilution buffer (200 mM HEPES, 100 mM NaCl, 80 μM Na_3VO_4, and 0.04% BSA) containing recombinant GST-kinase (glutathione *S*-transferase), and 25 μL of 160 μM ATP (diluted in 40 mM $MnCl_2$). GST kinases (ProQinase, Freiburg, Germany) were used in the following amounts: EGFR, 50 ng/well; ErbB2, 200 ng/well; IGF1R, 50 ng/well; VEGFR-2, 300 ng/well; VEGFR-3 50 ng/well. Phosphorylation was allowed to proceed at 30 °C for 90 min. To stop the reaction, 30 mM EDTA solution (50 μL/well) was added, and the plates washed twice with 0.05% (v/v) Tween 20/TBS buffer. Peroxidase-conjugated antiphosphotyrosine antibodies (PY20-hrpo; BD Transduction Laboratories; 0.1 μg/mL) were diluted in 0.05% (v/v) Tween 20/TBS buffer (supplemented with 0.5% w/v BSA, 0.025% w/v nonfat dried milk powder, and 100 μM Na_3VO_4) and added to the wells. After incubation for 1 h at 37 °C, the antibody solution was removed, and the plates were washed 3 times with TBS buffer. The peroxidase reaction was initiated by addition of ABTS substrate. Absorbance was measured photometrically at 405 nm. All data points were performed in triplicate.

Ligand-Induced Autophosphorylation Assays and Western Blot Analysis. Cells (2×10^6) were seeded and allowed to grow for 48 h. Thereafter, cells were further cultivated under serum-reduced (1% FCS) conditions for 24 h and incubated with the anthocyanin-rich mixture for 45 min in serum-free medium. Receptors were stimulated with their respective ligand EGF (100 ng/mL) for EGFR and ErbB2, and heregulin (20 ng/mL) for ErbB3 for the final 15 min of incubation. Tyrphostin AG1478 is a selective EGFR tyrosine kinase inhibitor. The specific ErbB2 receptor inhibitor tyrphostin AG879 (10 μM) showed only a marginal inhibitory effect on the ErbB2 receptor phosphorylation in A431 cells, whereas an equimolar concentration of tyrphostin AG1478 and AG879 (5 μM) potently suppressed the phosphorylation of all tested ErbB-receptors and was therefore used in the Western Blot assays. A combination of these two inhibitors at fixed ratios of 1:1 resulted in a potent inhibition of the receptor phosphorylation as described previously (*24*). After stimulation with ligand, cells were lysed at 4 °C in 0.2 mL of RIPA buffer (50 mM Tris/HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% (v/v) Igepal, 1 mM PMSF, 1 mM sodium orthovanadate, and 2% (v/v) protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)). Thereafter, the lysate was homogenized thoroughly and subsequently centrifuged for 10 min (20000*g*, 4 °C). The proteins were separated by SDS−PAGE (7% polyacrylamide gel) and transferred onto a nitrocellulose membrane. Western blot analyses were performed using mouse/rabbit monoclonal antibodies against human EGFR, ErbB2, ErbB3, phospho-EGFR (Tyr 1173), phospho-ErbB2 (Tyr 1248) (Santa Cruz, Heidelberg, Germany), or phospho-ErbB3 (Tyr 1228) (Cell Signaling Technology, Beverly, MA, USA). α-Tubulin antibodies were employed as a loading control. Antimouse

or antirabbit IgG peroxidase conjugates (Santa Cruz, Heidelberg, Germany) were used as appropriate as secondary antibodies. A chemoluminescent signal was then obtained with Lumi-GLO (Cell Signaling Technology), which was analyzed using the LAS 3000 system and quantified with the AIDA Image Analyzer 3.52 software (Raytest, Straubenhardt, Germany). Arbitrary light units were plotted as test over control [%].

Ligand-induced VEGFR phosphorylation assays were performed as previously described (*25*). Briefly, PAE cells transfected with either human VEGFR-2 or murine VEGFR-3 were cultured in serum-free medium (supplemented with 0.2% BSA) for 16−24 h (PAE/VEGFR-3) or 48 h (PAE/VEGFR-2). After 30−60 min of preincubation with serum-free medium containing 1 mM Na_3VO_4 and the inhibitor at the appropriate concentration, the cells were stimulated at 37 °C for 5 min (VEGFR-3) or 8 min (VEGFR-2). ΔNΔC/VEGF-C/Cys152→Ser (VEGF-C-Cys), a mutant form of VEGF-C protein that specifically activates VEGFR-3 but not VEGFR-2, was added at 400 ng/mL to the PAE/VEGFR-3 cells. Human recombinant $VEGF_{165}$ (Reliatech, Braunschweig, Germany) was added at 30 ng/mL to the PAE/VEGFR-2 cells. After growth factor stimulation, the cells were lysed in RIPA the following buffer: buffer (30 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton-X 100, 0.5% (w/v) sodium desoxycholate, 1 mM phenylmethanesulfonyl fluoride, 0.1 U/mL aprotinin, and 5 mM Na_3VO_4). The lysates were immunoprecipitated with 8 μg anti-VEGFR-2 (C-1158, Santa Cruz) or anti-VEGFR-3 antibody (AF743, R&D Systems) and 80 μL of protein G-Sepharose (Amersham). Half the volume of each sample was loaded onto two SDS-gels and Western blotted. The blots were probed with antiphosphotyrosine Ig (PY20-hrpo, Becton Dickinson) or with specific antireceptor Ig (VEGFR-3: AFL4, eBioscience; VEGFR-2: AF357, R&D Systems) to control the loading of the samples.

RESULTS

Anthocyanin-Rich Extracts Inhibit the Kinase Activity of Recombinant Kinase Domains Derived from a Variety of RTKs. As certain purified forms of anthocyanins have been reported to affect RTK activity (*3*, *6*), we hypothesized that complex mixtures of anthocyanins such as those currently being used in clinical intervention studies might also have an inhibitory effect on RTK activity. To determine if this is the case, the protein tyrosine kinase activities of the RTKs VEGFR-3, VEGFR-2, EGFR, ErbB2, and IGF1R were measured in the presence of mirtocyan or oenocyanin in a cell-free system using recombinant kinase domains of these receptors. The anthocyanin-rich extracts inhibited the activity of all RTKs (**Figure 1A**,**B**). RTKs were more consistently inhibited by oenocyanin than by mirtocyan, even though the IC_{50} values for inhibition by both mixtures were within comparable concentration windows. At <5 μg/mL, oenocyanin reduced enzyme activities by approximately half or more. Mirtocyan and oenocyanin inhibited VEGFR-2 and EGFR kinases slightly more potently than the other RTKs, with IC_{50} values of 3.2 ± 0.4 and 0.7 ± 0.5 μg/mL, respectively, for VEGFR-2, and 3.4 ± 0.8 and 0.8 ± 0.2 μg/mL, respectively, for EGFR. Oenocyanin impeded VEGFR-2 activity maximally at 5 μg/mL, while at 10 μg/mL, inhibition seemed to be less potent, possibly indicating a biphasic inhibition mechanism.

Anthocyanin-Rich Extracts Inhibit Ligand-Induced Autophosphorylation of ErbB and VEGFR Family Members. To determine whether the inhibitory properties of mirtocyan and oenocyanin translate into an inhibition of the intact receptor in its normal cellular context, effects of these extracts on the phosphorylation status of the ErbB receptors were determined in A431 cells that endogenously overexpress all three ErbB receptors. Western blot analysis was used to assess ligand-induced autophosphorylation of the receptor protein, which reflects receptor kinase activity. Incubation with

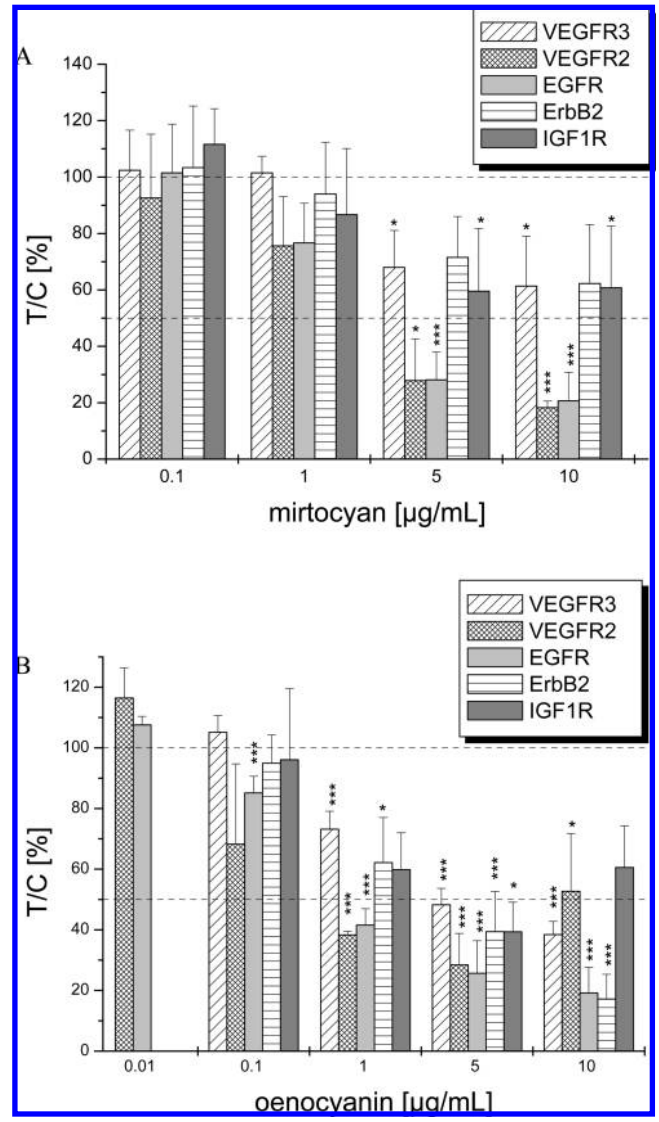

Figure 1. Effect of mirtocyan (**A**) and oenocyanin (**B**) on the activity of recombinant kinase domains derived from VEGFR-2, VEGFR-3, EGFR, ErbB2, and IGF1R. The phosphorylation of tyrosine residues of a peptide poly (Glu/Tyr) substrate was determined by ELISA using an antiphosphotyrosine antibody linked to peroxidase. The data presented are the mean ± SD of at least three independent experiments, each performed in triplicate. Statistical significance was calculated in relation to the lowest applied concentration (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

mirtocyan and oenocyanin afforded a concentration-dependent suppression of EGFR phosphorylation with IC_{50} values of 146 ± 9 and 121 ± 26 μg/mL, respectively (**Figure 2**). They also suppressed autophosphorylation of the ErbB2-receptor with IC_{50} values of 184 ± 31 and 154 ± 27 μg/mL, respectively (**Figure 3**). The phosphorylation of the heregulin-stimulated ErbB3-receptor was diminished in a similar fashion by both anthocyanin-rich mixtures at concentrations of ≥50 μg/mL, with 50% inhibition at 100 μg/mL (**Figure 4**). Thus, oenocyanin displayed slightly higher ErbB and EGFR kinase-inhibitory potency than mirtocyan. Both mixtures inhibited phosphorylation of the ErbB3-receptor slightly more potently than that of the EGFR and ErbB2-receptor. The rank order of sensitivity toward inhibitory potency by both mixtures was ErbB3 > EGFR > ErbB2.

The ability of anthocyanin-rich mixtures to affect ligand-induced autophosphorylation of VEGFR-2 and VEGFR-3 was

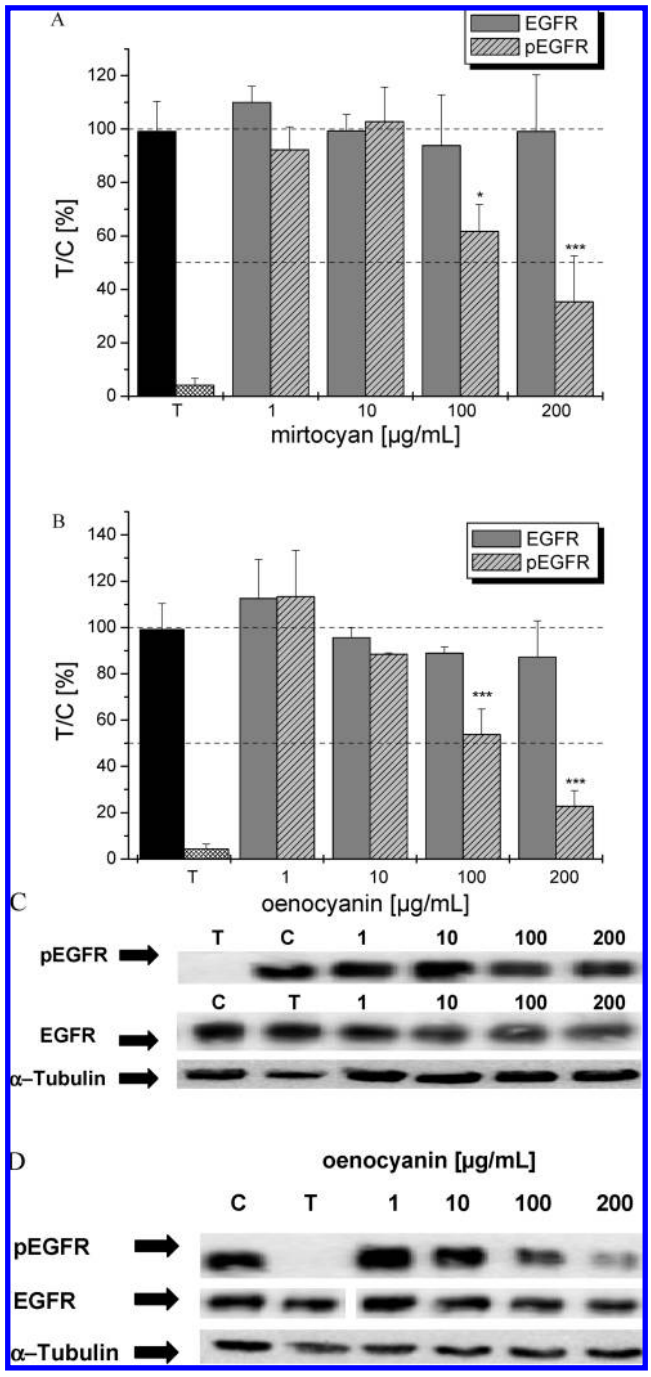

Figure 2. Effect of mirtocyan (**A**,**C**) and oenocyanin (**B**,**D**) on the phosphorylation of EGFR receptor protein expression in A431 cells. Cells were exposed to anthocyanin-rich extracts for 45 min, stimulated with EGF (100 ng/mL), and then protein analysis was performed by Western blot (40 μg total protein per lane). **C** and **D** are representative Western blots. Values, which are expressed as the percentage of solvent control stimulated by EGF (100 ng/mL), are the mean ± SD of 3 independent experiments. Statistical significance was calculated in relation to the lowest applied concentration (* $p < 0.05$; *** $p < 0.005$). C = solvent control, DMSO 1% final concentration; T = AG1478 + 879, specific inhibitors of EGFR and ErbB2 (each 5 μM).

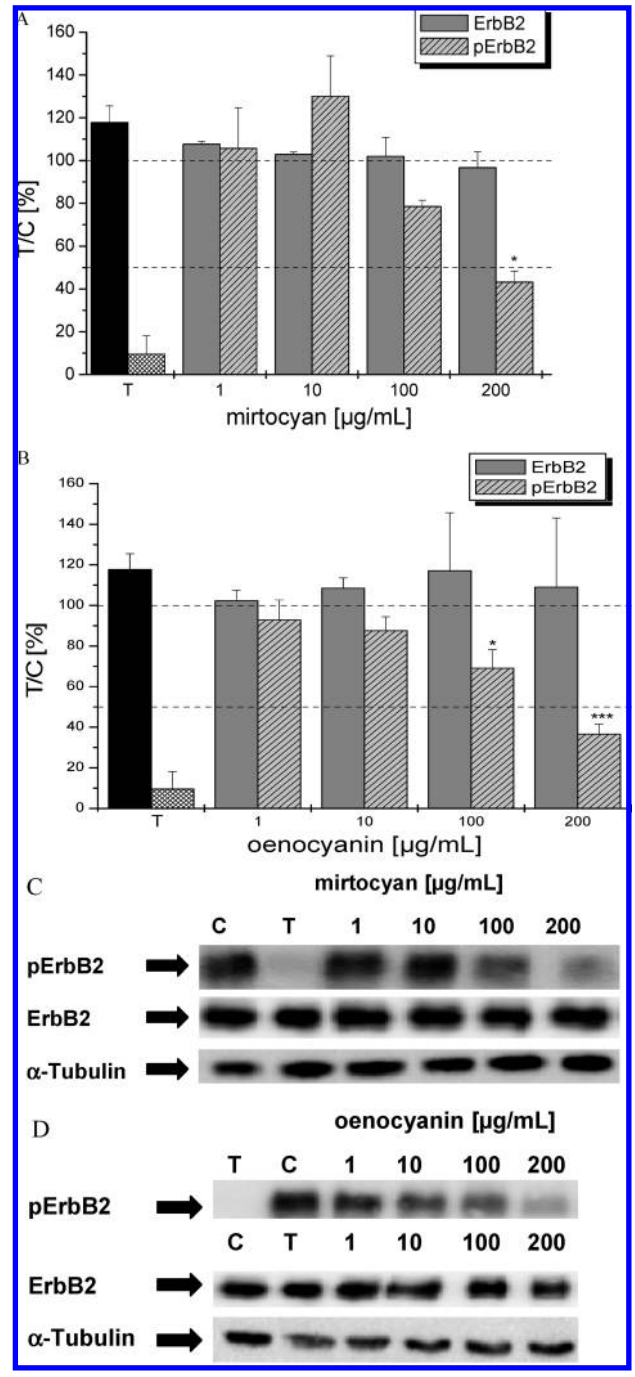

Figure 3. Effect of mirtocyan (**A**,**C**) or oenocyanin (**B**,**D**) on the phosphorylation of ErbB2 receptor protein expression in A431 cells. Cells were exposed to anthocyanin-rich extracts for 45 min and stimulated with EGF (100 ng/mL), and protein analysis was by Western blot. **C** and **D** are representative Western blots (40 μg total protein per lane). Values, which are expressed as the percentage of solvent control stimulated by EGF (100 ng/mL), are the mean ± SD of 3 independent experiments. Statistical significance was calculated in relation to the lowest applied concentration (* $p < 0.05$; *** $p < 0.005$); C = solvent control, DMSO 1% final concentration; T = AG1478 + 879, specific inhibitors of EGFR and ErbB2 (each 5 μM).

tested in PAE cells (ectopically expressing either human VEGFR-2 or murine VEGFR-3, respectively). Again, Western blot analysis of the phosphorylation state of the receptors in response to the ligand was used as a measure of receptor kinase activity. Both anthocyanin-rich mixtures reduced the phosphorylation status of VEGFR-2 at ≥0.5 μg/mL, with approximately 50% inhibition at 50 μg/mL (**Figure 5A**,**B**). Anthocyanin-rich mixtures at this concentration completely abolished ligand-induced autophosphorylation of VEGFR-3.

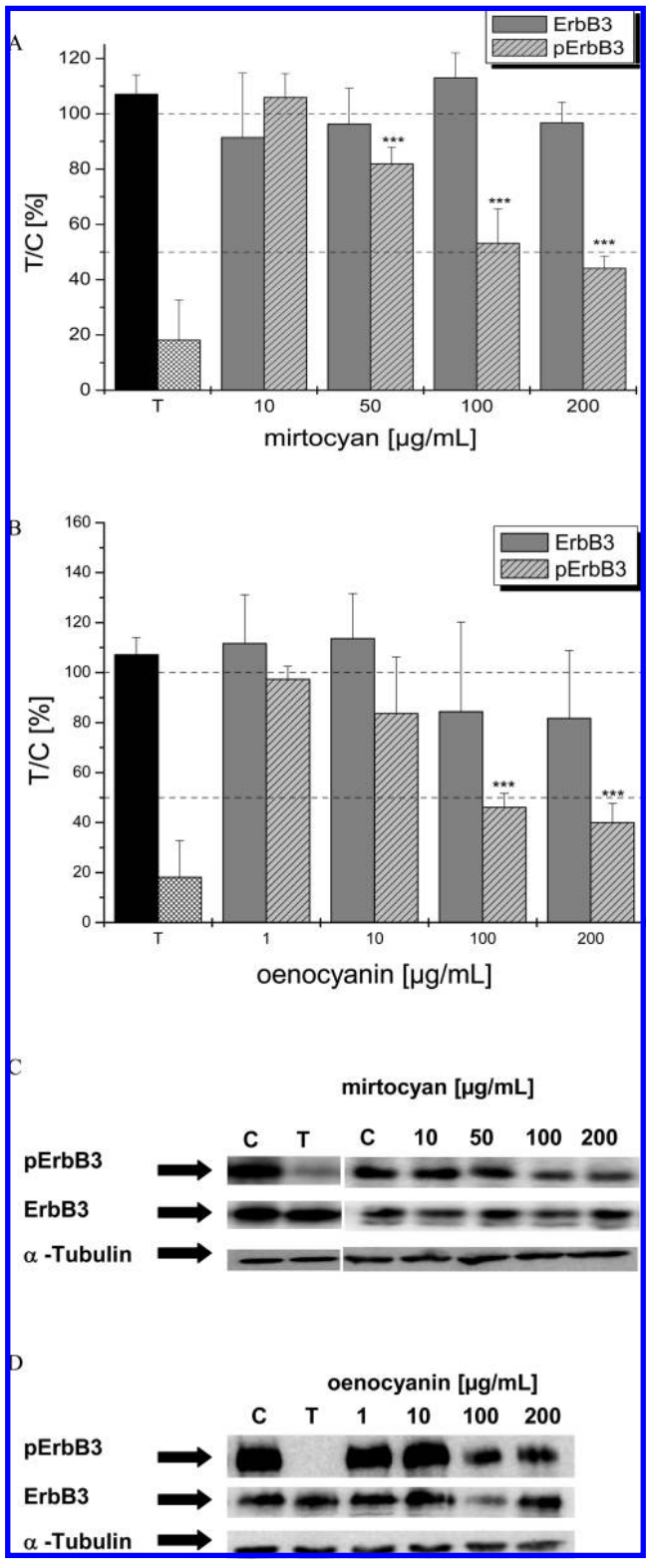

Figure 4. Effect of mirtocyan (**A**,**C**) or oenocyanin (**B**,**D**) on the phosphorylation of ErbB3 receptor protein expression in A431 cells. Cells were exposed to anthocyanin-rich extracts for 45 min and stimulated with heregulin (20 ng/mL), then protein analysis was performed by Western blot (40 μg total protein per lane). **C** and **D** are representative Western blots. Values, which are expressed as the percentage of solvent control stimulated by heregulin (20 ng/mL), are the mean ± SD of 3 independent experiments. Statistical significance was calculated in relation to the lowest applied concentration (* $p < 0.05$; *** $p < 0.005$); C = solvent control, DMSO 1% final concentration; T = AG1478 + 879, specific inhibitors of EGFR and ErbB2 (each 5 μM).

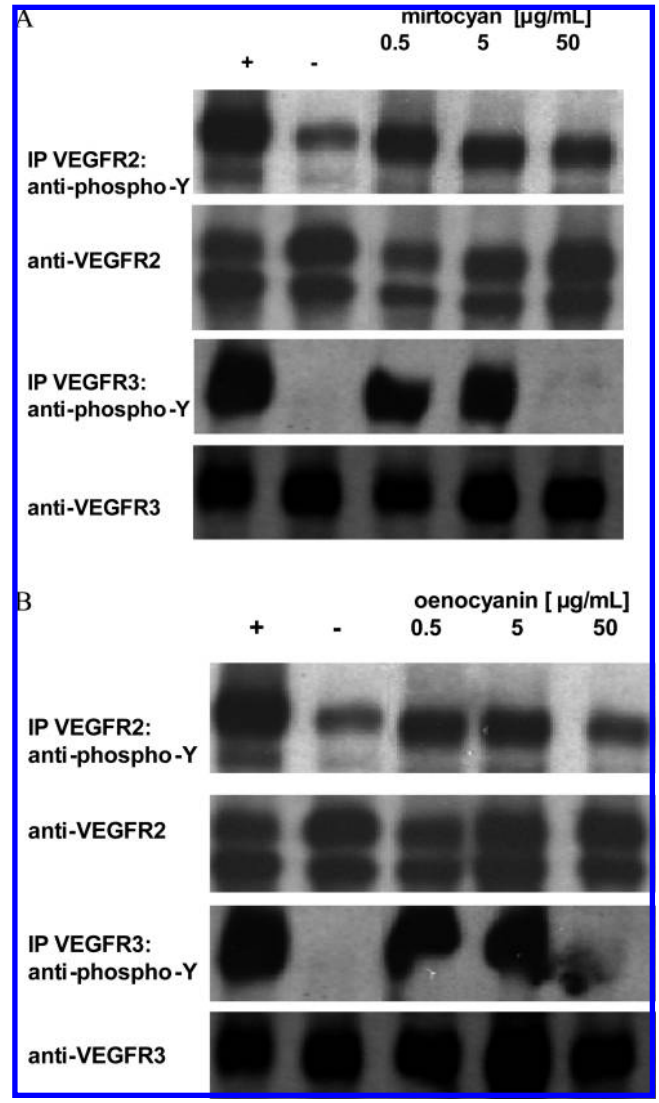

Figure 5. Effect of mirtocyan (**A**) or oenocyanin (**B**) on the phosphorylation of VEGFR-2 and VEGFR-3. Where indicated, PAE cells transfected to express either VEGFR-2 or VEGFR-3 were incubated with anthocyanin-rich extracts for 45 min and stimulated with either 400 ng/mL VEGF-C (VEGFR-3) for 5 min or 30 ng/mL VEGF (VEGFR-2) for 10 min. VEGFR proteins were then immunoprecipitated and Western blotted. Figures are representative of two Western blot analyses. C = solvent control (1% DMSO as final concentration), (+) = cells stimulated with ligand, and (−) = unstimulated cells.

RTK-Inhibitory Concentrations of Anthocyanin-Rich Extracts Have Only Marginal Effects on A431 Cell Growth. To determine whether the inhibition of RTK activity by anthocyanin-rich extracts affects cell proliferation or survival, the effect of mirtocyan and oenocyanin on cell growth was determined in A431 cells that had been incubated for 72 h with these anthocyanin-rich mixtures. Oenocyanin inhibited growth only marginally. At 400 μg/mL, it failed to affect cell growth, while at 500 μg/mL, cell numbers were only decreased by 23 ± 9%. Mirtocyan inhibited growth with an IC_{50} of 438 ± 48 μg/mL (data not shown), a concentration considerably higher than that required to achieve a similar inhibition of the RTK activity of ErbB family members in these cells.

DISCUSSION

Flavonoid-rich extracts and individual flavonoids are known to inhibit the activity of some RTKs (*3*, *6*, *18*, *19*, *26*, *27*). In

Table 1. Summary of the Inhibition by Mirtocyan and Oenocyanin of Protein Kinase Activity, as Assessed Using Recombinant Kinase Domains, and through Analysis of Ligand-Induced Autophosphorylation of ErbB and VEGFR Family Members[a]

compound	EGFR IC_{50} [μg/mL]	ErbB2 IC_{50} [μg/mL]	ErbB3 IC_{50} [μg/mL]	VEGFR-2 IC_{50} [μg/mL]	VEGFR-3 IC_{50} [μg/mL]	IGF1R IC_{50} [μg/mL]
mirtocyan cell free	3.4 ± 0.8	12.1 ± 3.6	n.t.	3.2 ± 0.4	12.7 ± 0.9	12.9 ± 3.6
mirtocyan cellular	146 ± 9	184 ± 31	$\sim$100	$\sim$50	$\ll$50	n.t.
oenocyanin cell free	0.8 ± 0.2	3.7 ± 2.3	n.t	0.7 ± 0.5	4.6 ± 2.0	3.6 ± 2.6
oenocyanin cellular	121 ± 26	154 ± 27	$\sim$100	$\sim$50	$\ll$50	n.t.

[a] n.t. not tested.

light of the importance of RTKs in the control of cell proliferation and survival as well as lymphangiogenesis and angiogenesis, this pharmacological property may conceivably contribute to the cancer chemopreventative action of certain flavonoids. The results presented above show for the first time that mixtures of anthocyanins extracted from bilberries or grapes inhibit the activity of a range of RTKs, both in a cell-free system and in living cells.

Both anthocyanin-rich mixtures were more potent as inhibitors of recombinant kinase domain activity as determined using in vitro kinase assays than as inhibitors of ligand-induced receptor autophosphorylation in intact cells. In the in vitro kinase assays, oenocyanin at <5 μg/mL significantly decreased the kinase activities of VEGFR-2, VEGFR-3, EGFR, ErbB2, and IGF1R. Both mixtures inhibited kinases associated with EGFR and VEGFR-2 more than those linked to ErbB2, VEGFR-3, and IGF1R. The results obtained using the intact receptor expressed in living cells differ somewhat from those obtained in the cell-free in vitro kinase assays (**Table 1**). Careful analysis allows delineation of subtle but intriguing differences between both the experimental systems and the anthocyanin-rich mixtures. Inhibition of receptor autophosphorylation in intact cells required the presence of anthocyanins at 10−50 times higher concentrations than was the case for the inhibition of recombinant kinase activity under cell-free conditions. This discrepancy may be caused by poor access of the glycosides to intracellular kinase domains consequent to their limited cellular uptake. The kinase-inhibitory potency of mirtocyan in the cell-free system was about a third of that of oenocyanin, while in living cells both mixtures inhibited ligand-induced receptor autophosphorylation at broadly similar concentrations. The preferential inhibition of EGFR and VEGFR-2 kinases observed in the cell-free system was not seen in intact cells. Instead, ligand-induced autophosphorylation of ErbB3 and VEGFR-3 was more susceptible than that of the other receptors toward inhibition by anthocyanins. Even though anthocyanin-rich extracts inhibited the autophosphorylation of ErbB3 slightly more potently than the autophosphorylation of the other ErbB family members, inhibition exerted by the mixtures lacked marked ErbB receptor specificity.

The effect of the mixtures on VEGFR phosphorylation status was investigated because of the important role of the VEGFR system in the regulation of angiogenesis and lymphangiogenesis (*15*−*17*). In the cellular context, both extracts suppressed VEGFR-3 phosphorylation at 50 μg/mL, whereas VEGFR-2 phosphorylation was only decreased by $\sim$50%. While this result needs confirmation in other cell types, a difference of this type intimates relatively selective inhibition of VEGFR-3 phosphorylation by anthocyanin-rich extracts, possibly hinting at a putative usefulness in the prevention of lymphatic metastasis (*28*−*30*). Polyphenolic phytochemicals that have previously been shown to inhibit VEGFR include the anthocyanidin delphinidin (*6*), ellagic acid from fruits and vegetables (*31*), and the green tea constituents epigallocatechin-3-gallate, epigallocatechin, and catechin-3-gallate (*32*). To our knowledge, inhibition of VEGFR-3 phosphorylation by phytochemicals has hitherto not been reported.

In contrast to their considerable receptor kinase inhibitory potency, the anthocyanin-rich mixtures were only marginally growth-inhibitory in A431 cells, which exhibit strong endogenous expression of EGFR, ErbB2, and ErbB3. Cell growth-inhibitory capabilities of anthocyanins have been amply documented (*7*, *19*, *33*, *34*). The relatively weak effects of the anthocyanin-rich extracts on cell growth observed here may be related, at least in part, to their possibly limited stability in the cell culture medium (*35*, *36*). Anthocyanin degradation may have been an issue in these experiments even though special care was taken to ameliorate it by inclusion of catalase (100 U/mL) into the culture medium. Catalase reduces hydrogen peroxide, which can be generated by anthocyanidins in the cell culture medium, with the genuine potential of enhanced cytotoxicity (*36*). Another possibility is that in addition to ErbB family activity in these cells, other molecular pathways not affected by anthocyanins that promote cell growth and survival are able to compensate at least in part the inhibition of ErbB RTK activity. In this case, concentrations of anthocyanin-rich mixtures that inhibit ErbB RTK activity would not be expected to have such a pronounced effect on cell growth and survival as they do on RTK activity.

It is impossible to even tentatively allocate the inhibitory activities observed to individual components of the mixtures. Both anthocyanin-rich mixtures contain further polyphenolic substances as well as as-yet unidentified constituents (cf. Materials and Methods). Especially catechins, flavonols, and procyanidins, which are present in amounts lower than those of anthocyanins in the anthocyanin-rich extracts, are known to inhibit different RTKs (*18*, *24*, *26*, *27*, *31*). It is therefore possible that these substances at least partially contribute to the inhibitory effects of the anthocyanin-rich extracts. Nevertheless, the differential anthocyanin composition of the two mixtures employed here may well have been responsible for, or contributed to, some of the differences in the biological activity reported. Among the major constituents of the mixtures, only one, cyanidin-3-glucoside, occurs identically in both mixtures. Other major constituents of mirtocyan are delphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, and cyanidin-3-galactoside, while the major anthocyanins in oenocyanin, in addition to cyanidin-3-glucoside, are the 3-glucosides of petunidin, peonidin, and malvidin.

To evaluate the possible physiological effects of the anthocyanin-rich extracts in vivo, their relatively low bioavailability must be taken into account (*37*, *38*). Anthocyanin blood levels in humans of 10^{-8} to 10^{-7} M have been reported (*37*). In the urine of healthy volunteers, anthocyanins also present in mirtocyan were detected by HPLC (*39*). In mirtocyan-treated mice, quantifiable levels of anthocyanins were found in the intestinal mucosa and urine (*1*). However, the systemic concentrations might be underestimated because of unsatisfying overall recovery rates. Therefore, it is possible that local con-

centrations especially in the gastrointestinal tract might be achievable in the RTK inhibitory range.

In conclusion, anthocyanin-rich mixtures from bilberries and grapes displayed broad spectrum receptor protein tyrosine kinase-inhibitory activity. In intact cells, ErbB3 and VEGFR-3 seemed especially sensitive to inhibition by anthocyanin-rich extracts. These results hint at the possibility that the activity of certain RTKs may be exploitable as biomarkers of the pharmacological efficacy of anthocyanin-rich extracts in rodent intervention studies and ultimately in clinical intervention studies.

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