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Research Article

Comparison of delphinidin, quercetin and (–)-epigallocatechin-3-gallate as inhibitors of the EGFR and the ErbB2 receptor phosphorylation

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In the present study, delphinidin was found to suppress the phosphorylation of the epidermal growth factor receptor (EGFR) within human tumour cells (human colon carcinoma cell line (HT29), human vulva carcinoma cell line (A431)), albeit less effective than the flavonol quercetin. The higher potency of quercetin was also observed downstream on the level of the mitogen-activated protein kinase (MAPK) cascade. In addition, delphinidin, quercetin and (–)-epigallocatechin-3-gallate (EGCG) were found to suppress the phosphorylation of the ErbB2 receptor, with delphinidin exhibiting the strongest inhibitory properties. Their potency to suppress the ErbB2 receptor phosphorylation can be summarised as delphinidin > EGCG > quercetin. The effectiveness of delphinidin against the EGFR and the ErbB2 receptor was comparable, indicating a broader spectrum of activity against receptor tyrosine kinases. At low micromolar concentrations delphinidin showed some preference towards the ErbB2 receptor. In summary, quercetin and delphinidin appear to differ in their activity profile towards the ErbB receptor family members. Whereas quercetin was most effective against the EGFR, delphinidin exhibited some preference towards the ErbB2 receptor.

Keywords: A431 / Autophosphorylation / Cellular signalling / ERK / HT29

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1 Introduction

Dietary habits and lifestyle play an important role in carcinogenesis of many different tumour types. Epidemiological studies indicate that cancer incidence might be significantly modulated by an enhanced dietary intake of flavonoids with fruits and vegetables [1]. Malignant transformation is associated with changes in cellular signalling cascades conducting cell growth, differentiation and the induction of apoptosis [2–4]. One of the major intracellular signalling cascades involved in the control of cell proliferation is the extracellular signal-regulated/mitogen-activated protein kinase (ERK/MAPK) pathway [5–8]. Effective inhibition of the upstream located epidermal growth factor receptor (EGFR) activity is expected to suppress the activation of the subsequent MAPK cascade, leading to cell growth inhibition [7].

The EGFR and the ErbB2 receptor belong to the ErbB family of receptor tyrosine kinases (RTK). All family members (EGFR, ErbB2-4 receptors) have in common a glycosylated extracellular ligand-binding domain, a single hydrophobic transmembrane region and an intracellular domain with protein tyrosine kinase (PTK) activity [9–14]. The ErbB receptors exist as monomers, but form receptor dimers upon ligand binding. They can either generate homodimers or heterodimers with other receptor isoforms (*e.g.* EGFR-ErbB2) [10, 15]. A family of ligands, the EGF-related peptide growth factors, binds to the extracellular domain of the receptors, leading to the formation of both homo- and heterodimers. Dimerisation initiates the PTK activity of the receptor and triggers the autophosphorylation of specific tyrosine residues within the intracellular domain. This phosphorylation sequence is involved in the regulation of signalling cascades steering gene expression as the biological response to receptor activation [12, 16]. The EGFR is activated by EGF-like ligands, whereas the

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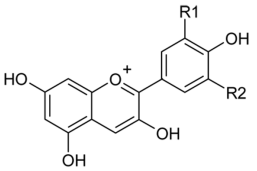
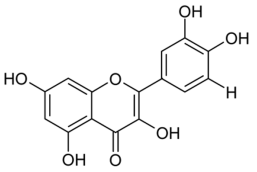
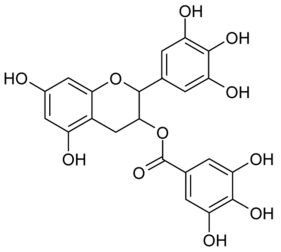
Abbreviations: A431, human vulva carcinoma cell line8; **EGCG,** (–)-epigallocatechin-3-gallate; **EGFR,** epidermal growth factor receptor; **ELK-1,** Ets like kinase 1; **ERK/MAPK,** extracellular-signal-regulated/mitogen-activated protein kinase; **HT29,** human colon carcinoma cell line; **PTK,** protein tyrosine kinase; **RTK,** receptor tyrosine kinase; **T/C,** test over control



Delphinidin: R1= R2= OH
Malvidin: R1= R2= OCH_3



Quercetin



(-)-Epigallocatechin-3-gallate (EGCG)

Figure 1. Structures of the selected flavonoids.

ErbB3 and the ErbB4 receptor bind preferentially neuregulins. No direct ligand of the ErbB2 receptor has been identified so far, indicating that the ErbB2 receptor acts predominantly as a partner for the formation of heterodimers, responding to several different ligands [9]. The transactivation by EGF-like ligands results in the formation of EGFR – ErbB2 heterodimers [17], whereas neuregulins induce the formation of EGFR – ErbB3-, ErbB2 – ErbB3- and ErbB2 – ErbB4 receptor combinations [15, 18, 19]. Heterodimers generate more potent signals than homodimers, and those containing the ErbB2 receptor possess particularly high ligand binding affinity and signalling potency compared to hetero- and homodimers without the ErbB2 receptor [20].

The MAP kinases ERK1/2 represent central downstream signalling elements. The effective inhibition of the upstream located EGFR and ErbB2 receptors results in the suppression of ERK1/2 phosphorylation, leading to the inhibition of cell growth. Several food constituents have been reported to target the EGFR [5 – 7, 21, 22]. Within the class of flavonoids, the flavonol quercetin, the green tea constituent (–)-epigallocatechin-3-gallate (EGCG) and the anthocyanin aglycon delphinidin (Fig. 1) are among the most potent food constituents inhibiting the PTK activity of the EGFR, reported so far [5, 7, 23]. However, studies on the EGFR-inhibiting properties of delphinidin were so far limited to cell-free systems. Quercetin and EGCG have already been reported to affect the activity of the EGFR also in intact cells [4, 21, 23, 24]. In the present study, we addressed the question whether the inhibition of delphinidin on the EGFR activity is also of relevance within intact cells, measured as changes in the phosphorylation status of the receptor and the impact on the subsequent extracellular regulated MAP kinases ERK1/2. We further investigated whether the inhibitory properties of delphinidin, quercetin and EGCG, as flavonoids out of different structural classes, are specific for the EGFR or whether other related RTKs, as exemplified here for the ErbB2 receptor, are affected as well.

2 Materials and methods

2.1 Chemicals

Delphinidin, malvidin and EGCG were purchased from Extrasynthèse (Genay, France). Quercetin, tyrphostin AG1478 (AG1478) and tyrphostin AG879 (AG879) were received from Sigma (Taufkirchen, Germany). For all assays the compound solutions were freshly prepared, without the use of stored stock solutions. All compounds and mixtures were dissolved in DMSO with a final concentration in the different test systems of maximum 1%.

2.2 Cell culture

The human vulva carcinoma cell line (A431) was cultured in minimum essential medium (MEM) (Sigma, Taufkirchen, Germany) containing L-glutamine (4.5 g/L). The human colon carcinoma cell line (HT29) was cultivated in Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L glucose, without sodium-pyruvate; Invitrogen Life Technologies, Karlsruhe, Germany). Both cell culture media were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The cell lines were kept in humidified incubators (37°C, 5% CO_2).

2.3 Western blot analysis

A total of 1.2×10^6 HT29 cells or 2.2×10^6 A431 cells were seeded *per* Petri dish and allowed to grow for 48 h. Thereafter, cells were serum-reduced (1% FCS) for 24 h and incubated with the respective compound for 45 min in serum-free medium. The stimulation with EGF (100 ng/mL) was performed within the last 15 min of incubation. Cells were abraded at 4°C in 0.2 mL RIPA buffer (50 mM Tris/HCl, pH 7.4, 250 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 1% v/v Igepal; 1 mM PMSF, 1 mM sodium orthovanadate and 40 µL protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) freshly added to 2 mL RIPA buffer). Thereafter the cell lysate was homogenised thoroughly and subsequently centrifuged for 10 min

(20 000 × *g*, 4°C). The supernatant was separated by SDS-PAGE (7% polyacrylamide gel) and the proteins were transferred onto a nitrocellulose membrane. Detection was performed using rabbit polyclonal antibodies against the human EGFR/phospho EGFR (Tyr1173, 175 kDa; New England Biolabs, Frankfurt, Germany) and the ErbB2/phospho ErbB2 receptor (Tyr1248, 180 kDa, Santa Cruz Biotechnology, Heidelberg, Germany). An anti-rabbit IgG peroxidase conjugate (New England Biolabs, Frankfurt, Germany) was used as secondary antibody. The detection of the MAP kinases ERK1/2 (44/42 kDa) was carried out as described previously [7]. α-Tubulin (55 kDa, Santa Cruz Biotechnology, Heidelberg, Germany) was used as loading control. The respective chemoluminescent signals (Lumi-GLO, New England Biolabs, Frankfurt, Germany) were analysed using the LAS 3000 with the AIDA Image Analyzer 3.52 software for quantification (Raytest, Straubenhardt, Germany). Arbitrary light units were plotted as test over control (T/C %).

3 Results

The activity of both receptors (EGFR, ErbB2 receptor) and the MAP kinases (ERK1/2) were measured as changes in the phosphorylation status by Western blot analysis in human colon (HT29) or vulva (A431) carcinoma cells. HT29 cells, expressing substantial amounts of EGFR, were chosen as a model for colon cancer. AG1478 (10 μM), a selective EGFR tyrosine kinase inhibitor, was selected as positive control, inhibiting the EGFR phosphorylation in HT29 cells up to 90% (Fig. 2 A). Whereas the EGFR phosphorylation was found to be sensitive to EGF-stimulation in HT29 cells, the phosphorylation status of the ErbB2 receptor in these cells was not modulated. Therefore, A431, known to overexpress all members of the ErbB receptor family, were used to study the effects of polyphenols on the ErbB2 receptor activity. The specific ErbB2 receptor inhibitor AG879 showed only a marginal inhibitory effect (15% at 10 μM) on the ErbB2 receptor phosphorylation in A431 cells, whereas an equimolar concentration of AG1478 and AG879 suppressed the ErbB2 receptor phosphorylation up to 45% (Fig. 2 B). A combination of these two inhibitors at fixed ratios of 1:1 resulted in a potent inhibition of the ErbB2 receptor phosphorylation up to 60% (Fig. 2 B).

3.1 Delphinidin

Treatment of HT29 cells with delphinidin resulted in a concentration-dependent suppression of EGFR phosphorylation with an IC_{50}-value of 54 ± 11 μM (Fig. 3). Also in A431 cells, delphinidin was found to diminish the phosphorylation of the EGFR (IC_{50} = 71 ± 32 μM, Fig. 2 A). The endogenous level of the receptor was not modulated significantly. Moreover, a concentration-dependent

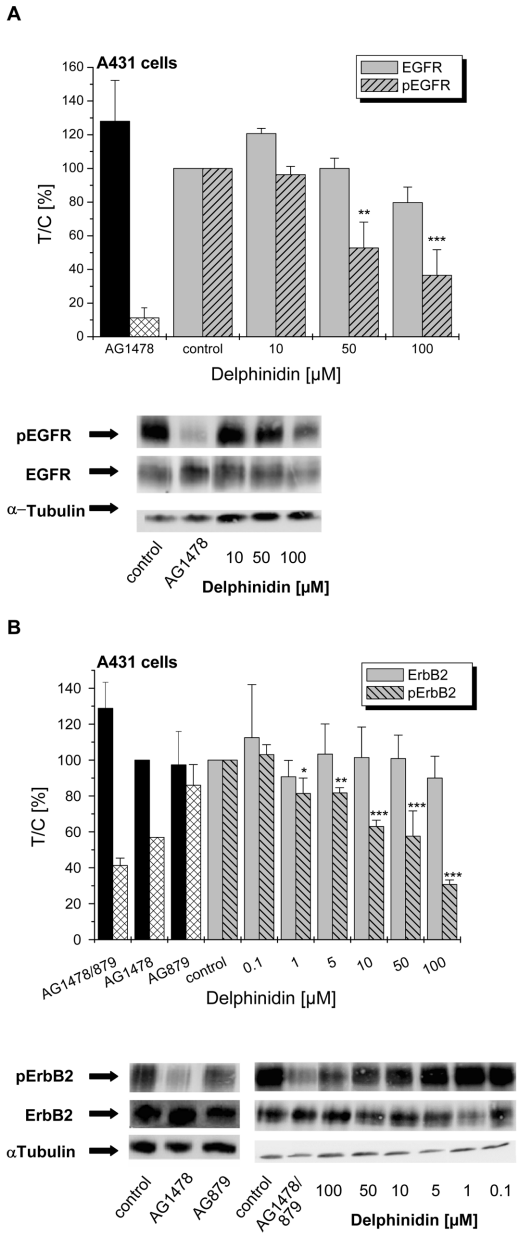


Figure 2. Western blot analysis of the (A) EGFR and the (B) ErbB2 receptor protein in A431 cells after 45 min treatment with delphinidin. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of three independent experiments with similar outcome, as shown for one representative experiment. The significances indicated were calculated in relation to the lowest applied concentration (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.001$); AG1478 EGFR specific inhibitor (10 μM); AG879 ErbB2 specific inhibitor (10 μM); AG1478/879 EGFR/ErbB2 specific inhibitor (5 μM).

decrease of ErbB2 receptor phosphorylation was detected in A431 cells. At a concentration of 1 μM delphinidin, a slight but significant reduction of ErbB2 receptor phosphorylation was observed (IC_{50} = 60 ± 21 μM, Fig. 2B). At

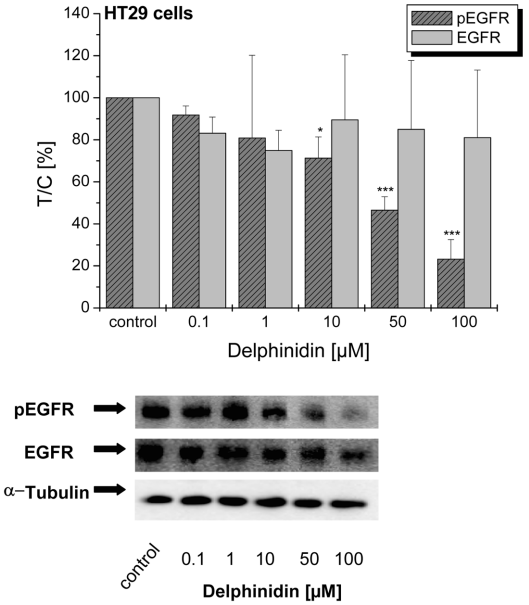


Figure 3. Western blot analysis of the EGFR protein in HT29 cells after 45 min treatment with delphinidin. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of four independent experiments with similar outcome, as shown for one representative experiment. The significances indicated were calculated in relation to the lowest applied concentration (* = $p < 0.05$; *** = $p < 0.001$); AG1478/879 EGFR/ErbB2 specific inhibitor (5 µM).

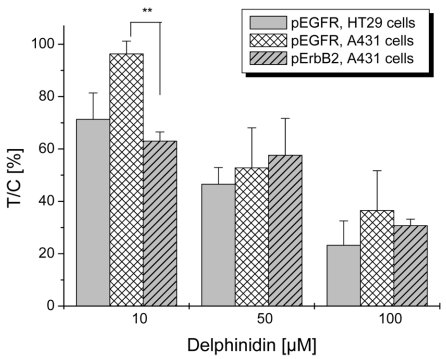


Figure 4. Comparison of the effects of delphinidin on phosphorylated EGFR and ErbB2 receptor protein in HT29 and A431 cells. Arbitrary light units were plotted as T/C (%). The presented data are the mean ± SD of three independent experiments with similar outcome. The indicated significance (** = $p < 0.005$) was calculated in relation to the pEGFR in the same concentration (10 µM).

a concentration of 10 µM, delphinidin exhibited significantly higher inhibitory effects against the ErbB2 receptor (Fig. 4). However, at concentrations ≥50 µM delphinidin affected the phosphorylation of both receptors to an almost similar extent. In contrast to delphinidin, the methoxylated

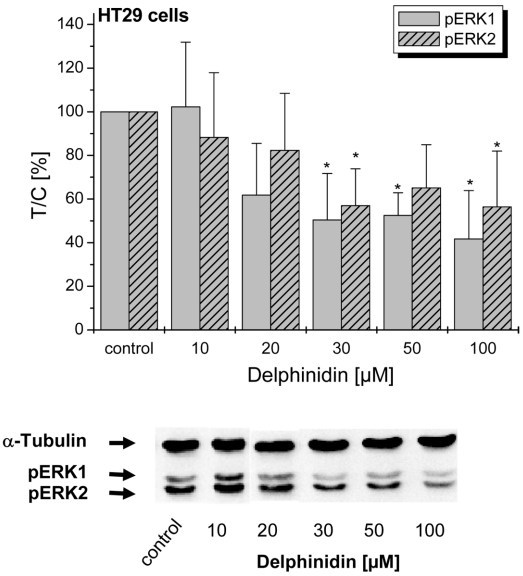


Figure 5. Western blot analysis of the phosphorylated ERK1/ERK2 proteins in HT29 cells after 45 min treatment with delphinidin. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of four independent experiments with similar outcome, as shown for one representative experiment. The significances indicated were calculated in relation to the lowest applied concentration (* = $p < 0.05$).

anthocyanidin malvidin did not affect the autophosphorylation of both RTKs EGFR and ErbB2 receptor up to 100 µM (data not shown).

Downstream in the signalling cascade, delphinidin was found to significantly diminish phosphorylated ERK1 and ERK2 in HT29 cells at a concentration of 30 µM. However, enhanced substance concentration failed to intensify the effect, apparently reaching a steady state (Fig. 5).

3.2 Quercetin

Treatment of HT29 cells with the flavonol quercetin potently suppressed the autophosphorylation of the EGFR with an IC_{50}-value of 0.6 ± 0.1 µM (Fig. 6, Table 1). Subsequently, a significant decrease of phosphorylated ERK1 and ERK2 was observed, with comparable IC_{50}-values for both isoforms (ERK1: 7.7 ± 2.1 µM; ERK2: 6.6 ± 3.5 µM) (Fig. 7).

In contrast to the potent inhibition of the EGFR phosphorylation, quercetin only marginally affected the phosphorylation of the ErbB2 receptor in A431 cells (Fig. 8).

3.3 EGCG

In several studies the green tea catechin EGCG has been reported to possess inhibitory properties on the EGFR activity [4, 24]. In the present study, we investigated the

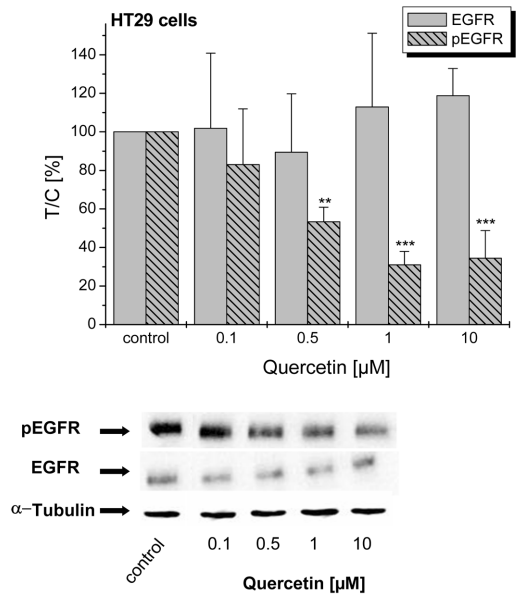


Figure 6. Western blot analysis of the EGFR protein in HT29 cells after 45 min treatment with quercetin. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of four independent experiments with similar outcome, as shown for one representative experiment. The significances indicated were calculated in relation to the lowest applied concentration (** = $p < 0.005$; *** = $p < 0.001$).

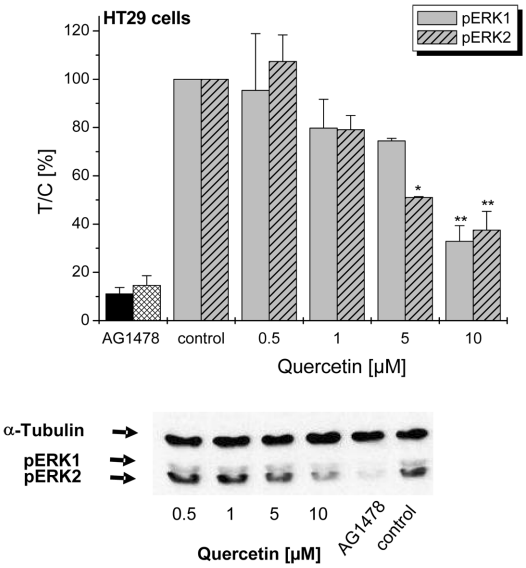


Figure 7. Western blot analysis of the phosphorylated ERK1/ERK2 proteins in HT29 cells after 45 min treatment with quercetin. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of four independent experiments with similar outcome, as shown for one representative experiment. The significances indicated were calculated in relation to the lowest applied concentration (* = $p < 0.05$; ** = $p < 0.005$); AG1478 EGFR specific inhibitor (1 µM).

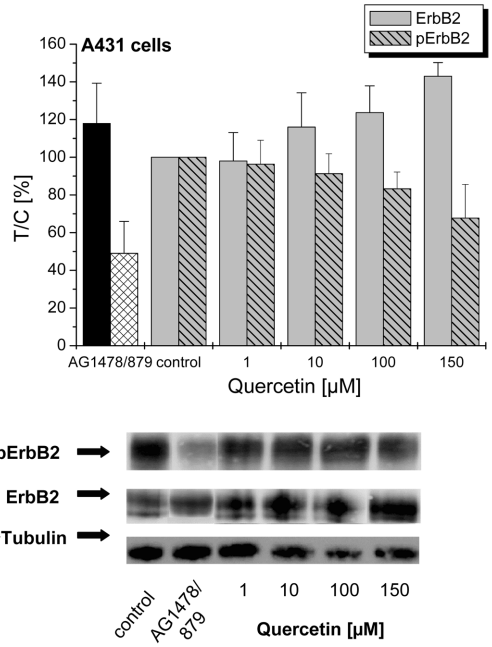


Figure 8. Western blot analysis of the ErbB2 receptor protein in A431 cells after 45 min treatment with quercetin. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of three independent experiments with similar outcome, as shown for one representative experiment. AG1478/879 EGFR/ErbB2 specific inhibitor (5 µM).

impact of EGCG on the ErbB2 receptor phosphorylation in A431 cells. A significant inhibition of the ErbB2 receptor phosphorylation was observed at EGCG concentrations ≥ 100 µM. However, up to 150 µM an IC_{50}-value was not achieved (Fig. 9).

4 Discussion

Polyphenols of different structural classes have been reported to target the EGFR. However, several of these studies were performed in cell-free systems [5 – 7]. In the present study, we addressed the question whether these inhibitory effects are of relevance within intact cells. Furthermore, we investigated whether the effect is target specific or whether related RTKs might also be affected as exemplified for the ErbB2 receptor. Effective inhibition of receptor phosphorylation results in the suppression of the subsequent MAPK cascade. Therefore, we studied whether the impact on the upstream located receptor is mirrored by a decrease in the activity of the downstream localised kinases ERK1/2.

Delphinidin was found to effectively suppress the phosphorylation of the EGFR in HT29 and A431 cells, showing that the potent inhibitory properties are not limited to the

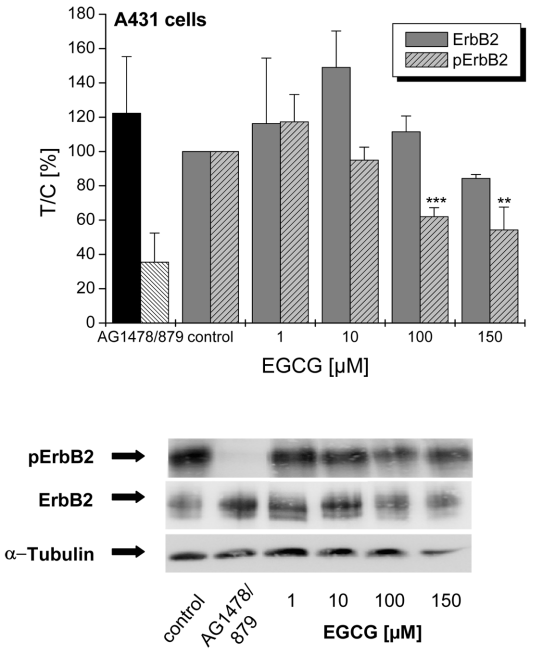


Figure 9. Western blot analysis of the ErbB2 receptor protein in A431 cells after 45 min treatment with EGCG. Arbitrary light units were plotted as T/C (%), with the respective solvent control (1% DMSO) stimulated by 100 ng/mL EGF set as 100%. The presented data are the mean ± SD of three independent experiments with similar outcome, as shown for one representative experiment. The significances indicated were calculated to the lowest applied concentration (** = $p < 0.005$; *** = $p < 0.001$). AG1478/879 EGFR/ErbB2 specific inhibitor (5 µM).

isolated receptor preparation (Figs. 2A, 3, 4; Table 1). Furthermore delphinidin was identified as a potent inhibitor of the ErbB2 receptor, diminishing the ErbB2 receptor phosphorylation in A431 cells in the same concentration range as observed for the EGFR (Fig. 2B and 4). The ErbB2 receptor acts predominantly as a partner of the EGF-stimulated EGFR resulting in the formation of an active EGFR-ErbB2 heterodimer [15]. With respect to the phosphorylation status, delphinidin did not differentiate between the EGFR and the ErbB2 receptor, indicating a lack of specificity (Fig. 4, Table 1). However, to affect the phosphorylation status of both receptors delphinidin might either act as a dual inhibitor, targeting each of the receptors, or affect primarily the EGFR, leading to the suppression of the ErbB2 receptor phosphorylation by inhibiting the crossphosphorylation sequence [25]. Our studies on the ErbB2 receptor activity with the specific EGFR inhibitor AG1478 and the ErbB2 inhibitor AG879 separately and in combination indicated that in the applied cell lines an EGFR-ErbB2 heterodimer is indeed present.

Downstream in the signalling cascade delphinidin significantly inhibited the activity of ERK1/2 without preference for one ERK isoform (Fig. 5). In comparison to the effects on the upstream located EGFR, delphinidin was less potent

Table 1. Suppression of the phosphorylation status of the EGFR and the ErbB2 receptor in HT29 cells and A431 cells, respectively

Compound	EGFR phosphorylation IC_{50} (µM)		ErbB2 phosphorylation IC_{50} (µM)
	HT29 cells	A431 cells	A431 cells
Delphinidin	54 ± 11	71 ± 32	60 ± 21
Quercetin	0.6 ± 0.1	nt	>150
EGCG	nt	nt	>150

nt, not tested.

with respect to the suppression of ERK1/2 activity. Thus, in addition to the effects on the EGFR further potentially compensating cellular effects of delphinidin have to be considered. The concentration range leading to a significant reduction of ERK1/2 phosphorylation (Fig. 5) is in accordance with earlier findings on the suppression of the phosphorylation of the subsequent transcription factor Ets like kinase 1 (ELK-1) by delphinidin, measured in a reporter gene assay [5].

In contrast to delphinidin, its methoxylated analog malvidin showed no inhibitory effect on the autophosphorylation of the EGFR and/or the ErbB2 receptor in intact cells. These results are in accordance with earlier studies on the effect of malvidin on the PTK activity of the EGFR in a cell-free test system, showing that within the class of anthocyanidins vicinal hydroxyl groups are prerequisite for potent EGFR inhibition [5, 6]. However, although malvidin did not affect the upstream located RTKs, a potent suppression of the activity of the downstream localised kinases ERK1/2 was shown previously [5, 26]. Thus, it is tempting to speculate that the effective suppression of ERK1/2 phosphorylation by malvidin results from the interference with crosstalking signalling pathways such as *e.g.* the 3′,5′-cyclic adenosine monophosphate (cAMP) pathway [6].

The flavonol quercetin has been reported earlier as an inhibitor of the PTK activity of the EGFR [7, 23, 24]. From the flavonoids included in this study, quercetin exhibited by far the most potent inhibitory properties on the phosphorylation status of the EGFR in HT29 cells (Fig. 6; Table 1). The potent inhibition of the EGFR phosphorylation by quercetin was found to be associated with an effective suppression of the activity of the subsequent MAP kinases ERK1/2 (Fig. 7), however only at higher concentrations compared to the effects at the level of the upstream localised EGFR. These data indicate that in HT29 cells quercetin succeeds to downregulate the activity of the EGFR-MAPK-signalling cascade, but apparently with a loss of its potency. In contrast, Bhatia *et al.* (2001) observed only a marginal effect on the EGFR activity in A431 cells up to 200 µM [24], indicating cell-type specificity. However, in a reporter gene approach in A431 cells a decrease of the phosphorylation of the transcription factor ELK-1 by quercetin treat-

ment was reported [7]. Interestingly, in A431 cells, quercetin has also only marginal inhibitory properties on the activity of the ErbB2 receptor (Fig. 8). The results in A431 cells indicate that alternative upstream signalling elements are affected by quercetin responsible for the suppression of ERK1/2 activity measured as ELK-1 phosphorylation.

The green tea catechin EGCG has been repeatedly described as a potent inhibitor of the activity of the EGFR in isolated test systems as well as in intact cells [4, 5, 7, 24, 27]. Treatment of A431 cells with EGCG diminishes concentration-dependent the autophosphorylation of the EGFR (70 – 80% inhibition at 200 μM, 24). In A431 cells also the phosphorylation of the ErbB2 receptor was significantly suppressed by EGCG (Fig. 9), albeit less effective compared to delphinidin (Fig. 2B; Table 1).

Polyphenols represent a wide variety of compounds in our diet and occur mainly as glycosides. The bioavailability appears to differ strongly between the various polyphenols, and the most abundant polyphenols in our diet are not necessarily those that have the best bioavailability profile. Delphinidin glycosides are detected in low concentrations as unmetabolised forms in the plasma, whereas only small amounts of metabolites and no delphinidin aglycone could be determined [28]. Due to unsatisfying overall recovery rates, still characteristic for bioavailability studies with anthocyanins, it cannot be excluded that the systemic bioavailability might be underestimated [29 – 31]. But, even if some underestimation of the plasma levels are taken into account, it appears unlikely that systemic concentrations of delphinidin are reached which are sufficient for effective inhibition of the EGFR and the ErbB2 receptor. However, locally in the gastrointestinal tract respective concentrations might be reached especially by the consumption of respectively enriched functional food or food supplements. Studies with ileostoma patients showed that the majority of anthocyanins are detected in the ileostoma fluid demonstrating that substantial amounts of these compounds indeed reach the colon [32].

The bioavailability of quercetin appears to exceed that of anthocyanins, however, different studies reported that quercetin is present in the plasma predominantly in conjugated forms [29]. Thus, the relevance of EGFR inhibition *in vivo* is still unclear. In contrast to quercetin, the catechin EGCG has been reported to be present in plasma in a large proportion (1 μmol/L) in a free form, which might indeed affect receptor activity of respective target tissue [33].

In summary, we found that the inhibitory properties of delphinidin are not limited to the isolated EGFR, but are also of relevance within intact cells. Among the tested flavonoids, quercetin was identified as the most potent inhibitor of the phosphorylation of the EGFR in HT29 cells, effectively suppressing the subsequent MAPK cascade. Concomitantly, delphinidin, quercetin and EGCG were found to suppress the phosphorylation of the ErbB2 receptor, with delphinidin exhibiting the strongest inhibitory properties. Their potency on ErbB2 receptor phosphorylation can be summarised as delphinidin > EGCG > quercetin. The effectiveness of delphinidin towards the EGFR and ErbB2 receptor was comparable, indicating a broader activity against RTKs. In low micromolar concentrations delphinidin even showed some preference towards the ErbB2 receptor. Thus, quercetin and delphinidin appear to differ in their activity profile towards the ErbB receptor family members. Whereas quercetin was most effective against the EGFR, delphinidin exhibited some preferences toward the ErbB2 receptor.

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The authors have declared no conflict of interest.

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