Research Article

Delphinidin inhibits a broad spectrum of receptor tyrosine kinases of the ErbB and VEGFR family

Nicole Teller[1,2], Wilko Thiele[3,4], Ute Boettler[1,2], Jonathan Sleeman[3,4] and Doris Marko[1,2]

[1] University of Vienna, Institute of Analytical and Food Chemistry, Vienna, Austria
[2] Institute of Applied Biosciences, Section of Food Toxicology, Universität Karlsruhe (TH), Karlsruhe, Germany
[3] Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics, Karlsruhe, Germany
[4] Medical Faculty Mannheim, Centre for Biomedicine and Medical Technology, University of Mannheim, Mannheim, Germany

Delphinidin has been reported to inhibit EGFR signalling. To determine whether other receptor tyrosine kinases (RTKs) are also influenced by delphinidin, we examined its ability to inhibit the kinase activity of EGFR, ErbB2, VEGFR-2, VEGFR-3 and IGF1R in a cell-free test system. We found that delphinidin strongly inhibited the protein tyrosine kinase activity of all tested RTKs at low micromolar concentrations. In A431 and PAE cells, ligand-induced phosphorylation of the receptors was also potently suppressed, with a preference for the suppression of the activity of ErbB3 (IC_{50} ~ 100 nM) and VEGFR-3 (IC_{50} < 50 μM). Thus the inhibition of RTKs by delphinidin is not limited to cell-free assays but is also of relevance in the cellular context. The results indicate that delphinidin acts as a broad-spectrum inhibitor of RTKs. Given the crucial role of the receptors in tumour growth and metastasis, we conclude that delphinidin has the potential to act directly against tumour cells as well as to interfere with key tumour–host interactions, although the suitability of delphinidin as a drug in cancer management may be compromised by its limited stability. Nevertheless, delphinidin may represent a novel lead compound for the development of chemopreventative and chemotherapeutic intervention strategies.

Keywords: Angiogenesis / Anthocyanin / Chemoprevention / EGFR / Polyphenol

Received: November 12, 2008; revised: January 6, 2009; accepted: January 13, 2009

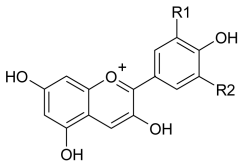
1 Introduction

Anthocyanins are naturally occurring flavonoids that are responsible for the intense colour of many fruits and vegetables [1, 2]. The average daily intake of anthocyanins is higher than that of other flavonoids with 12.5 mg/day/person in the United States [3]. In plants, only the glycosylated forms are present. Delphinidin (DEL) and malvidin (MV), so-called anthocyanidins, are the most abundant of these aglycons in food (Fig. 1). Anthocyanins as well as their aglycons have been associated with a multitude of beneficial health effects. They have been reported to be antioxidative and anti-inflammatory, to inhibit the proliferation of various tumour cell lines *in vitro* and to possess chemopreventative properties *in vivo* [4–9].

In previous studies, DEL was identified as a potent inhibitor of the epidermal growth factor receptor (EGFR) *in vitro* [5, 8, 10, 11]. EGFR belongs to the superfamily of receptor tyrosine kinases (RTKs), which are membrane spanning cell surface receptors with a related structure and tyrosine kinase activity. The ErbB-family, consisting of the EGFR and the ErbB2–4 receptors, represents an important subfamily within the RTKs because of the role of these receptors in cancer development [10–16]. In many tumour types they are overexpressed, mutated, or stimulated in an autocrine manner, resulting in crucial changes within cellular signalling pathways that regulate cell growth and differentiation. The vascular endothelial growth factor receptors 2 and 3 (VEGFR-2, and -3) as well as insulin like growth factor 1 receptor (IGF1R) are further members of the superfamily of

Correspondence: Doris Marko, Department of Analytical and Food Chemistry, University of Vienna, Währinger Straße 38, 1090 Vienna, Austria
E-mail: doris.marko@univie.ac.at
Fax: +43-1-4277-9 523

Abbreviations: A431, human vulva carcinoma cell line; **DEL,** delphinidin; **EGFR,** epidermal growth factor receptor; **FCS,** foetal calf serum; **IGF1R,** insulin like growth factor 1 receptor; **MEM,** minimum essential medium; **MV,** malvidin; **PAE,** porcine aortic endothelial; **PI3K,** phosphatidylinositol-3 kinase; **RTK,** receptor tyrosine kinases; **tyrphostin AG1478,** AG1478; **tyrphostin AG879,** AG879; **VEGFR,** vascular endothelial growth factor receptor



DEL: R1 = R2 = OH

MV: R1 = R2 = OCH_3

Figure 1. Structure of the anthocyanidins DEL and MV.

RTKs. The VEGFRs are key regulators of angiogenesis and lymphangiogenesis, and are thereby involved in tumour growth and metastasis [17, 18]. Suppression of the activity of these receptors leads to the inhibition of downstream signalling cascades such as the mitogen-activated protein kinase (MAPK) cascade and the phosphatidylinositol-3 kinase (PI3K) pathway, both regulators of cell growth and apoptosis [5, 8, 19, 20]. Their inhibition in the tumour context represents a promising therapeutic approach [21, 22].

In the present study, we investigated the inhibitory properties of DEL towards a spectrum of RTKs, with special emphasis on the question of specificity and whether the inhibitory effects are limited to cell-free conditions or are also of relevance within intact cells. For comparison, the methoxylated analogue MV was included in the assays. Furthermore, recent studies have shown that the incubation of cells with polyphenols might lead to the formation of hydrogen peroxide in the cell culture medium, which could trigger a cellular response and generate experimental artefacts [23 – 27]. Thus, in the present study, we also determined the accumulation of hydrogen peroxide within the cell culture medium and its influence on EGFR phosphorylation.

2 Materials and methods

2.1 Chemicals

DEL and MV were purchased from Extrasynthèse (Genay, France). The EGFR-specific inhibitor tyrphostin AG1478 (AG1478) and the ErbB2-specific inhibitor tyrphostin AG879 (AG879) were purchased from Sigma – Aldrich (Taufkirchen, Germany). For all assays the compound solutions were freshly prepared directly prior to the experiment, 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 maximally 1% v/v.

2.2 Cell culture

The human vulva carcinoma cell line (A431) was cultivated in minimum essential medium (MEM) (Sigma, Taufkirchen, Germany). The medium was supplemented with 10% foetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin. MEM and all supplements were obtained from Invitrogen™ Life Technologies (Karlsruhe, Germany). The production, characterization and culturing of porcine aortic endothelial (PAE) cells and their VEGFR-2 and VEGFR-3 transfectants have been described previously [28, 29]. Where indicated, cells were treated with 100 Units/mL catalase.

2.3 Hydrogen peroxide formation

Briefly, A431 cells were seeded into 24-well plates at 1×10^5 cells *per* well and allowed to grow for 48 h before treatment. Cells were further cultivated under serum-reduced (1% FCS) conditions for 24 h, and then incubated with the appropriate compound for 45 min in serum-free medium. The formation of hydrogen peroxide was subsequently measured using the Amplex® Red hydrogen peroxide assay kit from Sigma according to the manufacturer's instructions. Hydrogen peroxide concentrations of 0, 0.5, 0.75, 1.0, 2.0, 3.0 and 4.0 μM were used to generate a standard curve.

2.4 *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 microtitre 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 glutathione S-transferase (GST)-kinase, and 25 μL 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 anti-phosphotyrosine 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 three 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.

2.5 Western blot analysis

Aliquots of 2×10^6 A431 cells were seeded into Petri dishes 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 desired compound 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. Cells were lysed at 4°C in 0.2 mL 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 (20 000 × *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 Signalling Technology, Beverly, MA, USA). α-tubulin antibodies were employed as a loading control (Santa Cruz, Heidelberg, Germany). Anti-mouse or anti-rabbit IgG peroxidase conjugates (Santa Cruz, Heidelberg, Germany) were used as appropriate secondary antibodies. A chemoluminescent signal was then obtained with Lumi-GLO (Cell Signalling 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 (T/C) [%].

2.6 Ligand-induced VEGFR phosphorylation assays

Ligand-induced VEGFR phosphorylation assays were performed as previously described [28, 29]. 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 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 the following 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 phenylmethanesulphonyl fluoride, 0.1 Units/mL aprotinin and 5 mM Na_3VO_4. The lysates were immunoprecipitated with 8 µg anti-VEGFR-2 (C-1158, Santa Cruz, Heidelberg, Germany) or anti-VEGFR-3 antibody (AF743, R & D Systems) and 80 µL protein G-Sepharose (Amersham). Half the volume of each sample was loaded onto two SDS-Gels and Western blotted. The blots were probed with anti-phosphotyrosine Ig (PY20-hrpo, Becton Dickinson) or with specific anti-receptor Ig (VEGFR-3: AFL4, eBioscience; VEGFR-2: AF357, R&D Systems), respectively to control the loading of the samples.

3 Results

3.1 DEL induces hydrogen peroxide formation in cell cultures

The formation of hydrogen peroxide within the cell culture medium in response to polyphenols may account for some of the reported cellular effects of these compounds [23 – 27]. We therefore first set out to determine whether this is also the case for DEL in cultures of A431 cells, and to establish conditions in which specific effects of DEL on RTK activity in these cells could be studied. Hydrogen peroxide production was measured using the amplex red hydrogen peroxide assay kit after 45 min of incubation of the cells with DEL. A concentration-dependent formation of hydrogen peroxide was observed, with a maximum of 3.6 ± 0.2 µM H_2O_2 being obtained with the highest DEL concentration applied (100 µM) (Fig. 2). In the presence of catalase (100 Units/mL), the hydrogen peroxide formation was effectively suppressed to the level of the solvent control. Thus, in the presence of catalase only 0.7 ± 0.02 µM H_2O_2 were detected after 45 min of incubation with 100 µM DEL. In further experiments, catalase was therefore always added to cultures. Thus, nonspecific artefacts due to H_2O_2 production could be distinguished from specific effects of DEL on RTK activity.

3.2 DEL inhibits the protein tyrosine kinase activity of a panel of RTKs

The effect of DEL and its methoxylated analogue MV on the protein tyrosine kinase activity of five different RTKs (VEGFR-3, VEGFR-2, EGFR, ErbB2, IGF1R) was determined in cell-free *in vitro* tyrosine kinase assays. Significant inhibition of the protein tyrosine kinase activity of the EGFR, VEGFR-2 and VEGFR-3 kinase domains was only observed at concentrations ≥10 µM of MV. The receptors ErbB2 and IGF1R were significantly decreased in their pro-

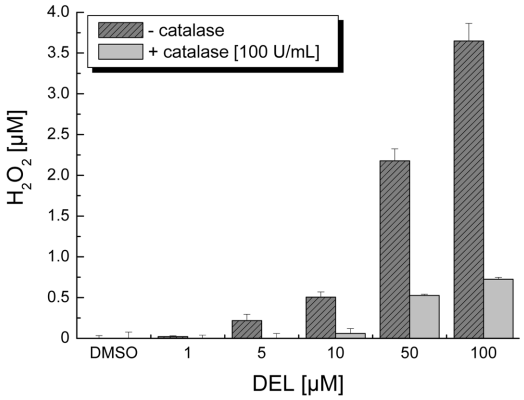


Figure 2. Hydrogen peroxide formation in cell culture medium (MEM) by DEL in the presence or in the absence of catalase (100 Units/mL) measured after 45 min of incubation. The data presented are the mean ± range of two independent experiments, each performed in duplicate.

tein tyrosine kinase activity only at concentrations ≥50 µM of MV. At this concentration however, no significant differences in the degree of inhibition of the protein tyrosine kinase activity of the tested RTKs were observed, with all tested RTKs being suppressed by about 50% (Fig. 3A). In contrast, a significant inhibitory effect of DEL on protein tyrosine kinase activity was observed at more than ten fold lower concentrations of DEL compared to MV. An initial decrease in the protein tyrosine kinase activity was found at concentrations ≥1 µM of DEL for the VEGFR-2/3 and EGFR. At concentrations ≥5 µM DEL a significant inhibition of the protein tyrosine kinase activity was observed for all receptors. An apparent preference for the inhibition of the protein tyrosine kinase activity of VEGFR-2 (IC_{50} = 2.8 ± 1.3 µM) and EGFR (IC_{50} = 1.0 ± 0.2 µM) was seen, mirrored in their IC_{50} values (Fig. 3B). However, with an increased substance concentration (10 µM DEL), the protein tyrosine kinase activity of both IGF1R and VEGFR-2 was not further reduced and apparently reached a steady state.

3.3 DEL inhibits ligand-induced ErbB receptor autophosphorylation

We next addressed the question whether the kinase inhibitory effects of DEL are limited to the cell-free test system, or are also of relevance in the cellular context. To this end, the activity of EGFR, ErbB2 and ErbB3 after treatment with DEL was measured as a change in the phosphorylation status of these RTKs. The A431 that overexpresses all three ErbB receptors was used for these experiments. Two-fold higher concentrations of DEL were necessary to achieve significant reduction in ligand-induced phosphorylation of EGFR in A431 cells in the absence of catalase (IC_{50} = 72 ± 32 µM) [10] compared to experiments in which catalase was added (IC_{50} = 33 ± 14 µM) (Fig. 4A). Further-

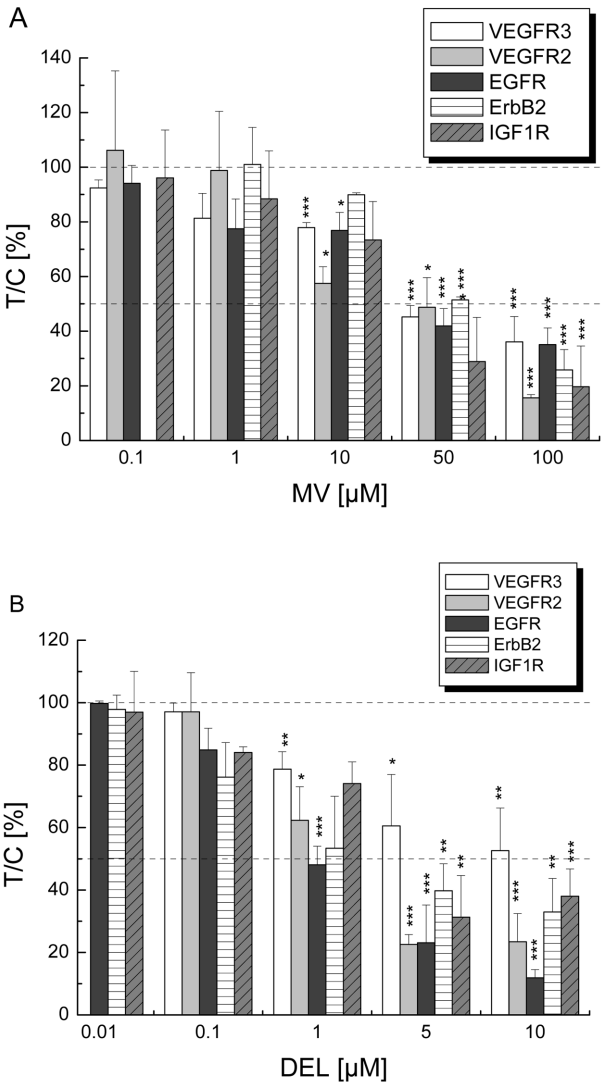


Figure 3. Inhibition by MV (A) and DEL (B) of the tyrosine kinase activity of recombinant kinase domains derived from VEGFR-2, VEGFR-3, EGFR, ErbB2 and IGF1R. The phosphorylation of tyrosine residues on a poly(Glu/Tyr)$^{4:1}$ peptide substrate by the recombinant kinase domains in the presence or absence of DEL or MV was determined by ELISA. The data presented are the mean ± SD of at least three independent experiments, each performed in triplicate. Statistical significance was calculated by Student's *t*-test (* = $p < 0.05$; ** $p < 0.01$; *** = $p < 0.005$).

more, DEL was found to decrease the ligand-induced autophosphorylation of the ErbB2 receptor in A431 cells (IC_{50} = 51 ± 23 µM), albeit slightly less effectively than was the case for EGFR (Fig. 5). Importantly, DEL was able to inhibit heregulin-induced phosphorylation of ErbB3 much more potently than it could suppress the EGF-stimulated phosphorylation of EGFR and ErbB2. Inhibition of the heregulin-stimulated phosphorylation of ErbB3 was already observed at a concentration of 0.01 µM DEL. The phosphorylation of the receptor was reduced by more than

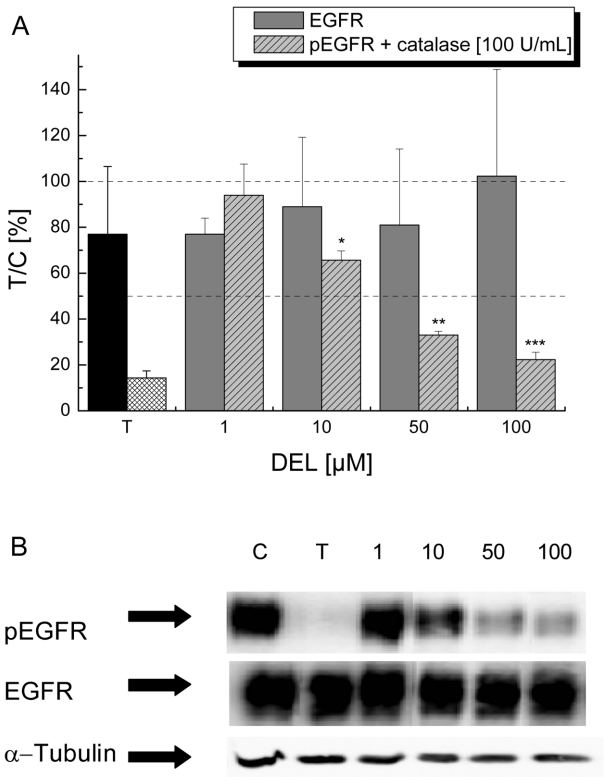


Figure 4. Western blot analysis of the effect on phosphorylation and expression of EGFR protein in A431 cells after 45 min treatment with DEL (catalase 100 Units/mL) (A). The data are presented as a percentage of the solvent control stimulated by 100 ng/mL EGF. The presented data are the mean ± SD of at least three independent experiments with similar outcomes. Statistical significance was calculated by Student's *t*-test (* = $p < 0.05$; ** $p < 0.01$; *** = $p < 0.005$). (B) Representative Western blot experiment. (C, solvent control (1% DMSO as final concentration); T, AG1478 + AG879 EGFR and ErbB2 specific inhibitors (5 μM)).

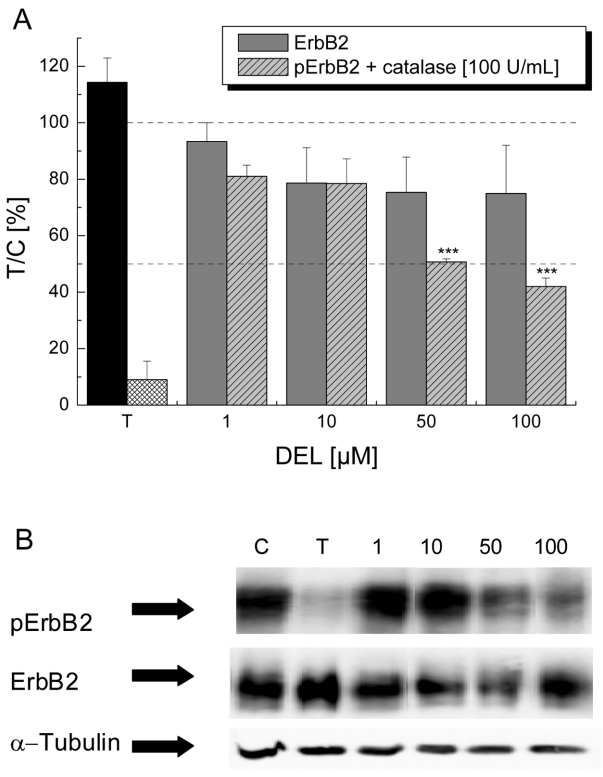


Figure 5. (A) Western blot analysis of the effect on phosphorylation and expression of ErbB2 receptor protein in A431 cells after 45 min treatment with DEL. The data are presented as% of the solvent control stimulated by 100 ng/mL EGF. The presented data are the mean ± SD of at least three independent experiments with similar outcome. Statistical significance was calculated by Student's *t*-test (* = $p < 0.05$; ** $p < 0.01$; *** = $p < 0.005$). (B) Representative Western blot experiment. (C, solvent control (1% DMSO as final concentration); T, AG1478 + AG879 EGFR and ErbB2 specific inhibitors (5 μM)).

50% at 0.1 μM DEL (Fig. 6A). Whereas the protein levels of the EGFR and ErbB2 receptor were not modulated by DEL, ErbB3 protein levels were diminished by DEL in a concentration-dependent manner at concentrations above 0.1 μM (Fig. 6A and B). The amount of ErbB3 protein was reduced to 50% after incubation with 20 μM DEL.

3.4 DEL inhibits ligand-induced VEGFR autophosphorylation

To evaluate whether the kinase activities of other related RTKs such as VEGFR-2 and VEGFR-3 are also inhibited by DEL at the cellular level, PAE cells transfected with either VEGFR-2 or VEGFR-3 were incubated with DEL in the presence of catalase (100 Units/mL). The activation of these receptors by their ligands ($VEGF_{165}$ and VEGF-C-Cys, respectively) is indicated by phosphorylation of the receptors. The effect of DEL on ligand-induced autophosphorylation was determined by immunoprecipitation of the receptors followed by Western blot analysis.

Under these experimental conditions, we found that DEL noticeably reduced the phosphorylation status of VEGFR-2 at a concentration of 5 μM. An approximately 50% inhibition was achieved with 50 μM DEL. An even more pronounced effect of DEL on ligand-induced VEGFR-3 autophosphorylation was observed with a complete inhibition of phosphorylation at 50 μM DEL. DEL had no effect on the protein levels of either VEGFR-2 or VEGFR-3 (Fig. 7).

4 Discussion

There is increasing interest in naturally occurring polyphenols that are found in foodstuffs as potential regulators of molecular processes involved in cancer, metastasis and cardiovascular disease. Here we demonstrate that DEL, an anthocyanidin found in a variety of fruits and vegetables, is

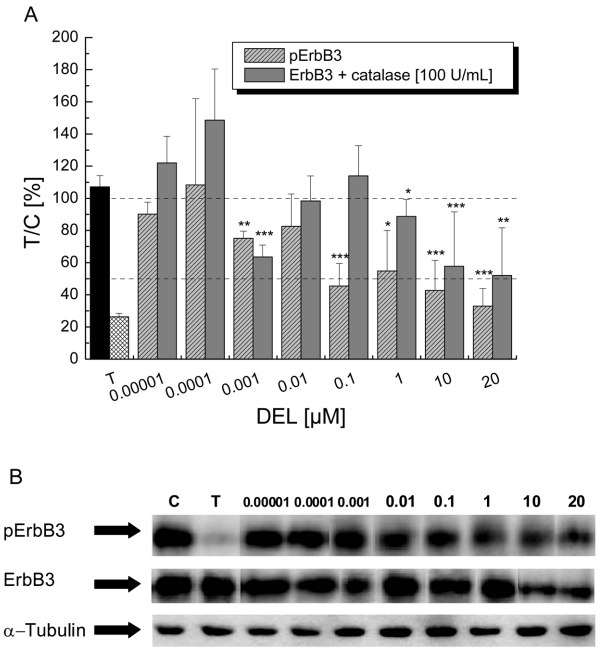


Figure 6. (A) Western blot analysis of the effect on phosphorylation and expression of ErbB3 receptor protein in A431 cells after 45 min treatment with DEL. The data are presented as a percentage of the solvent control stimulated by 20 ng/mL heregulin. The presented data are the mean ± SD of at least three independent experiments with similar outcomes. Statistical significance was calculated by Student's *t*-test (* = $p < 0.05$; ** $p < 0.01$; *** = $p < 0.005$). (B) Representative Western blot experiment. (C, solvent control (1% DMSO as final concentration); T, AG1478 + AG879 EGFR and ErbB2 specific inhibitors (5 µM)).

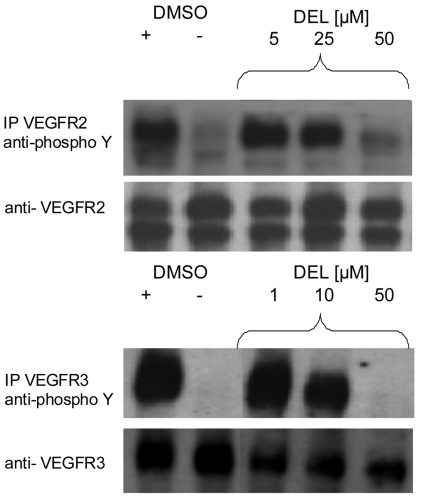


Figure 7. Effect on phosphorylation and expression of VEGFR-2 and VEGFR-3 protein in PAE cells after 60 or 45 min treatment respectively, with DEL. The cells were stimulated for 5 min with 400 ng/mL VEGF-C-Cys (VEGFR-3) or 8 min with 30 ng/mL $VEGF_{165}$ (VEGFR-2). VEGFR proteins were immunoprecipitated and Western blotted. Figures are representatives of Western blot experiments; C, solvent control (1% DMSO as final concentration), (+) stimulated with ligand, (–) not stimulated cells.

a broad-spectrum inhibitor of RTK activity, but is a more potent inhibitor of some RTKs compared to others. Our data also indicate that hydrogen peroxide formation in response to DEL may account for some of the previously reported cellular effects of this molecule.

4.1 Hydrogen peroxide formation by polyphenols, a potential source of artefacts

Recent studies have demonstrated that polyphenols can induce the formation of hydrogen peroxide under cell culture conditions [23–27]. The accumulation of hydrogen peroxide in the cell culture medium could itself trigger cellular responses, but has also been shown to affect the stability of the test compounds [7, 27]. Both of these factors might result in the generation of experimental artefacts, leading to misinterpretation of the bioactive properties of the tested polyphenols. In the present study, we showed that the anthocyanidin DEL at a concentration of ≥10 µM rapidly generated substantial amounts of hydrogen peroxide in the cell culture medium of A431 cells. The formation of hydrogen peroxide could be effectively suppressed by the addition of catalase (100 Units/mL) to the cell culture medium (Fig. 2). In the presence of catalase, the effects of DEL on the phosphorylation status of EGFR were clearly enhanced (IC_{50} 33 ± 14 µM) (Fig. 4A). This is in line with the earlier findings, in which intensified suppression of EGFR phosphorylation by apple polyphenols was observed in the presence of catalase [7]. The mechanism by which hydrogen peroxide is formed as a consequence of the incubation cultured cells with DEL remains to be determined, but could be dependent on a number of factors, including medium composition or the cell type. For example, we recently showed that the incubation of human colon carcinoma cell lines (HT29) with DEL in DMEM leads to hydrogen peroxide concentrations in the cell culture medium of up to 11 µM [27]. In contrast, the generation of hydrogen peroxide within the MEM medium used here for cultivation of A431 cells was three-fold lower, and could be effectively suppressed by the addition of catalase at the level of the solvent control. Together these findings indicate that care needs to be taken to control for nonspecific effects of polyphenols due to the production of hydrogen peroxide in culture medium.

4.2 DEL inhibits a broad spectrum of RTKs, but most potently ErbB3 and VEGFR-3

Cyanidin and DEL bear vicinal hydroxyl groups at the phenyl ring at the 2-position (B-ring) and have been previously characterized as potent inhibitors of the kinase activity of EGFR. Conversely, the methoxylated analogue MV was found to be completely inactive, even up to a concentration

of 100 μM [5]. In line with these earlier findings, we found that DEL inhibited the kinase activity of EGFR more potently than did MV. Importantly, a comparable difference in inhibitory potency between DEL and MV was observed for all tested receptors (EGFR, ErbB2, VEGFR-2, VEGFR-3 and IGF1R), indicating that vicinal hydroxyl groups at the B-ring (DEL) rather than methoxy groups (MV) are required for potent inhibition of protein tyrosine kinase activity, not only for inhibition of EGFR but also for a broader panel of RTKs.

In A431 cells, the EGF-stimulated phosphorylation of both EGFR and ErbB2 was comparably suppressed by DEL (Fig. 4 and 5), whereas the heregulin-stimulated activation of the ErbB3 receptor was inhibited much more effectively (Fig. 6A). The IC_{50} value for the reduction of ligand-induced ErbB3 phosphorylation was in the nanomolar range, indicating a preference of DEL towards the inhibition of the autophosphorylation of this receptor. Taken together, the inhibitory properties of DEL towards ErbB family members can be summarized as ErbB3 ⩾ EGFR - ≈ ErbB2 receptor.

In addition to suppressing ErbB3 phosphorylation, DEL also diminished the protein level of ErbB3 in a concentration-dependent manner. In contrast, the protein content of EGFR and ErbB2 remained unchanged (Fig. 5 – 7). This suggests that DEL does not only affect the ligand-induced autophosphorylation status of the ErbB3 receptor but might also enhance the internalization and endocytosis of this receptor. Of note, impaired endocytotic breakdown of RTKs is often associated with increased and uncontrolled signalling, associated with carcinogenesis [30]. In addition, the endocytosis of the ErbB3 receptor has been reported to be less efficient than EGFR endocytosis [30, 31]. A recent publication reports the inhibition of the activity of the EGFR by DEL [11]; however, they did not use a hydrogen peroxide scavenging system and incubated the cells for 3 h. This latter point is important because DEL has only limited stability under tissue culture conditions, and is no longer detectable by HPLC/DAD after 30 min of incubation [27]. We took these aspects into account in our study, and used a short incubation time and included catalase in the cultures to decrease DEL degradation and prevent experimental artefacts by the formation of hydrogen peroxide, as reported previously [7]. Our findings therefore now allow firm conclusions regarding the inhibitory effect of DEL on the EGFR to be made.

Within the superfamily of RTKs, the receptors of the VEGFR family are key regulators of angiogenesis and lymphangiogenesis [17, 18]. An inhibition of VEGFR-2 phosphorylation by DEL has been previously reported [32]. However, high concentrations of VEGF (100 ng/mL) were used in this study, a concentration which is not permissive for effective VEGFR-2 activation [33]. Furthermore, this study did not use a hydrogen peroxide scavenging system and employed a long incubation time of 18 h. In the present study we used therefore a short incubation time, included catalase and employed a physiologically relevant concentration of VEGF. At a concentration of 50 μM, DEL suppressed the phosphorylation of the VEGFR-2 to about 50%, whereas at this concentration the phosphorylation of VEGFR-3 was completely abrogated. Thus, a clear preference for the suppression of the phosphorylation of VEGFR-3 in cells was observed (Fig. 7).

While DEL not only inhibited RTKs in cell-free *in vitro* kinase assays but also within intact cells, it is important to note that suppression of the phosphorylation of EGFR, ErbB2 and VEGFR-2 in intact cells occurred within a 10- to 30-fold higher concentration range in comparison to the inhibition of the protein tyrosine kinase activity in the cell-free test system. This might conceivably be due in part to limited cellular uptake and/or low stability of the test compound under cell culture conditions. However, DEL had a far more potent inhibitory effect on ErbB3 and VEGFR-3 than the other tested RTKs, with inhibitory properties in cell culture in a concentration range comparable to those obtained using the *in vitro* kinase assays. These data therefore underscore a preference for DEL towards these two receptors in the cellular context.

4.3 Potential anti-cancer activities of DEL

The RTK inhibitory properties of DEL support the notion that this molecule could have chemopreventative or chemotherapeutic properties. In previous studies, we have shown that the suppression of EGFR in A431 cells is associated with growth inhibition of the tumour cells [7, 19, 27]. In the presence of catalase in the cell culture medium, we have also reported potent growth inhibition by DEL at low micromolar concentrations of HT29, although at higher concentrations this effect was abrogated [27]. Taking into account that the ErbB3 receptor represents a crucial upstream activator of the PI3K pathway, the so-called survival pathway, effective inhibition of this protein represents a possible means for preventing tumour cell survival and carcinogenesis [15, 16]. The flavonoid isoliquiritigenin has already been identified as an inhibitor of both ErbB3 activity as well as the downstream PI3K cascade [19].

VEGFR-2 and VEGFR-3 both play roles in the regulation of angiogenesis, an important driving force behind tumour growth. Furthermore, VEGFR-3 also plays a crucial role in tumour-induced lymphangiogenesis, a process that promotes tumour dissemination [29]. DEL or derivatives thereof might therefore be of relevance as anti-angiogenesis agents, or for the prevention of lymphogenic metastasis. In this regard it is interesting that DEL has recently been reported to inhibit the activity of another RTK involved in angiogenesis, the platelet derived growth factor receptor [34]. However, it is important to note that these experiments were performed in the absence of a hydrogen peroxide scavenging system.

4.4 Potential physiological effects of DEL as a dietary constituent

The average daily intake of the glycosides, the anthocyanins, is estimated as 12.5 mg/day/person in the Unites States [3]. In the plasma, low concentrations of DEL glycosides are present as unmetabolized forms with only small amounts of metabolites and no DEL aglycons [35]. The anthocyanins may be deglycosylated and/or degraded by microbes in the colon [36]. Thus, with the exception of ErbB3 that is already inhibited by DEL in the nanomolar range, it appears unlikely that systemic concentrations of DEL would be reached under normal dietary intake that would be sufficient to achieve substantial inhibition of many RTKs. However, local concentrations of DEL within the gastrointestinal tract might occur in the RTK inhibitory range, and thus ErbB3 and VEGFR-3 in the intestine, for example, could be affected by the consumption of dietary sources of anthocyanins such as enriched functional food or food supplements.

In summary, we have identified the anthocyanidin DEL as an inhibitor of the activity of a spectrum of RTKs associated with carcinogenesis and tumour progression. DEL was found to decrease the activity of the tested receptors within living cells. Moreover, a preferred inhibition of the activity of ErbB3 and VEGFR-3 was observed. These effects might contribute to the chemopreventative properties of DEL, probably interfering with the regulation of cell survival, and may have applications in the management of cancer.

The study was funded by grants from the Deutsche Forschungsgemeinschaft to D. Marko as part of the FlavoNet (MA 1659/6-1/2), and also to J. Sleeman under the auspices of the Schwerpunkt Program SPP1190 'Tumour-Vessel interface'.

The authors have declared no conflict of interest.

5 References

[1] Mazza, G., Anthocyanins in grapes and grape products, *Crit. Rev. Food Sci. Nut.* 1995, *35*, 1 – 20.

[2] Mazza, G., Miniati, E., *Anthocyanins in Fruits, Vegetables, and Grains*, CRC Press, Boca Raton 1993, 1 – 28.

[3] Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., *et al*., Concentrations of anthocyanins in common foods in the united states and estimation of normal consumption, *J. Agric. Food Chem.* 2006, *54*, 4069 – 4075.

[4] Wang, H., Nair, M. G., Strasburg, G. M., Chang, Y. C., *et al*., Antioxidant and antiinflamatory activities of anthocyanins and their aglycons, cyanidine, from tart cherries, *J. Nat. Prod.* 1999, *62*, 294 – 296.

[5] Meiers, S., Kemény, M., Weyand, U., Gastpar, R., *et al*., The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor, *J. Agric. Food Chem.* 2001, *49*, 958 – 962.

[6] Zhao, C., Giusti, M. M., Malik, M., Moyer, M. P., *et al*., Effects of commercial anthocyanin rich extracts on colonic cancer and nontumorigenic colon cell growth, *J. Agric. Food Chem.* 2004, *52*, 6122 – 6128.

[7] Fridrich, D., Kern, M., Pahlke, G., Volz, N., *et al*., Apple polyphenols diminish the phosphorylation of the epidermal growth factor receptor in HT29 colon carcinoma cells, *Mol. Nutr. Food Res.* 2007, *51*, 594 – 601.

[8] Marko, D., Puppel, N., Tjaden, Z., Jakobs, S., *et al*., The substitution pattern of anthocyanidins affects different cellular signaling cascades regulating cell proliferation, *Mol. Nutr. Food Res.* 2004, *48*, 318 – 325.

[9] Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., *et al*., Berry anthocyanins as novel antioxidants in human health and disease prevention, *Mol. Nutr. Food Res.* 2007, *51*, 675 – 683.

[10] Fridrich, D., Teller, N., Esselen, M., Pahlke, G., *et al*., Comparison of delphinidin, quercetin and (–)-epigallocatechin-3-gallate as inhibitors of the EGFR and the ErbB2 receptor phosphorylation, *Mol. Nutr. Food Res.* 2008, *52*, 815 – 822.

[11] Afaq, F., Zaman, N., Khan, N., Syed, D. N., *et al*., Inhibition of epidermal growth factor receptor signaling pathway by delphinidin, an anthocyanidin in pigmented fruits and vegetables, *Int. J. Cancer* 2008, *123*, 1508 – 1515.

[12] Yarden, Y., Sliwkowski, M. X., Untangling the ErbB signalling network, *Nat. Rev. Mol. Cell Biol.* 2001, *2*, 127 – 137.

[13] Olayioye, M. A., Neve, R. M., Lane, H. A., Hynes, N. E., The ErbB signalling network: Receptor heterodimerization in development and cancer, *EMBO J.* 2000, *19*, 3159 – 3167.

[14] Hynes, N. E., Lane, H. A., ErbB receptors and cancer: The complexity of target inhibitors, *Nat. Rev. Cancer* 2005, *5*, 341 – 354.

[15] Karamouzis, M. V., Badra, F. A., Papavassiliou, A. G., Breast cancer: The upgraded role of Her-3 and Her-4, *IJBCB* 2007, *39*, 851 – 856.

[16] Holbro, T., Civenni, C., Hynes, N. E., The ErbB receptors and their role in cancer progression, *Exp. Cell Res.* 2003, *284*, 99 – 110.

[17] Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., *et al*., Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation, *J. Biol. Chem.* 1998, *273*, 30336 – 30343.

[18] Korpelainen, E. I., Alitalo, K., Signaling angiogenesis and lymphangiogenesis, *Curr. Opin. Cell Biol.* 1998, *10*, 159 – 164.

[19] Jung, J. I., Chung, E., Seon, M. R., Shin, H. K., *et al*., Isoliquiritigenin (ISL) inhibits ErbB3 signaling in prostate cancer cells, *BioFactors* 2006, *28*, 159 – 168.

[20] Kern, M., Tjaden, Z., Ngiewih, Y., Puppel, N., *et al*., Inhibitors of the epidermal growth factor receptor in apple juice extract, *Mol. Nutr. Food Res.* 2005, *49*, 317 – 328.

[21] Thiele, W., Sleeman, J. P., Tumor-induced lymphangiogenesis: A target for cancer therapy? *J. Biotechnol.* 2006, *25*, 224 – 241.

[22] Schenone, S., Bondavalli, F., Botta, M., Antiangiogenic agents: An update on small molecule VEGFR inhibitors, *Curr. Med. Chem.* 2007, *14*, 2495 – 2516.

[23] Long, L. H., Clement, M. V., Halliwell, B., Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin and quercetin to commonly used cell culture media, *Biochem. Biophys. Res. Commun.* 2002, *273*, 50 – 53.

[24] Lapidot, T., Walker, M. D., Kanner, J., Can apple antioxidants inhibit tumor cell proliferation? Generation of H(2)O(2) during interaction of phenolic compounds with cell culture media, *J. Agric. Food Chem.* 2002, *50*, 3156 – 3160.

[25] Roques, S. C., Landrault, N., Teissèdre, P. L., Laurent, C., *et al*., Hydrogen peroxide generation in cacao-2 cell culture medium by addition of phenolic compounds: Effect of ascorbic acid, *Free Radic. Res.* 2002, *36*, 593 – 599.

[26] Lee, K. W., Hur, H. J., Lee, H. J., Lee, C. Y., Antiproliferative effects of dietary phenolic substances and hydrogen peroxide, *J. Agric. Food Chem.* 2005, *53*, 1990 – 1995.

[27] Kern, M., Fridrich, D., Reichert, J., Skrbek, S., *et al*., Limited stability in cell culture medium and hydrogen peroxide formation affect the growth inhibitory properties of delphinidin and its degradation product gallic acid, *Mol. Nutr. Food Res.* 2007, *51*, 1163 – 1172.

[28] Kroll, J., Waltenberger, J., The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways porcine aortic endothelial cells, *J. Biol. Chem.* 1997, *272*, 32521 – 32527.

[29] Kirkin, V., Mazitschek, R., Krishnan, J., Steffen, A., *et al*., Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2, *Eur. J. Biochem.* 2001, *268*, 5530 – 5540.

[30] Roepstorff, K., Grøvdal, L., Grandal, M., Lerdrup, M., *et al*., Endocytic downregulation of ErbB receptors: Mechanisms and relevance in cancer, *Histochem. Cell Biol.* 2008, *129*, 563 – 578.

[31] Baulida, J., Kraus, M. H., Alimandi, M., Di Fiore, P. P., *et al*., All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired, *J. Biol. Chem.* 1996, *271*, 5251 – 5257.

[32] Lamy, S., Blanchette, M., Michaud-Levesque, J., Lafleur, R. *et al*., Delphinidin, a dietary anthocyanidin, inhibits vascular endothelial growth factor receptor-2 phosphorylation, *Carcinogenesis* 2006, *27*, 989 – 996.

[33] Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., *et al*., Proteolytic processing regulates receptor specificity and activity of VEGF-C, *EMBO J.* 1997, *16*, 3898 – 3911.

[34] Lamy, S., Beaulieu, E., Labbé, D., Bédard, V., *et al*., Delphinidin, a dietary anthocyanidin, inhibits platelet derived growth factor ligand/receptor (PDGF/PDGFR), *Carcinogenesis* 2008, *29*, 1033 – 1041.

[35] Matsumoto, H., Ichiyanagi, T., Iida, H., Ito, K., *et al*., Ingested delphinidin-3-rutinoside is primarily excreted to urine as the intact form and to bile as the methylated form in rats, *J. Agric. Food Chem.* 2006, *54*, 578 – 582.

[36] Keppler, K., Humpf, H. U., Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora, *Bioorg. Med. Chem.* 2005, *13*, 5195 – 5205.

