Research Article

Limited stability in cell culture medium and hydrogen peroxide formation affect the growth inhibitory properties of delphinidin and its degradation product gallic acid

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In the present study we investigated the stability of anthocyanidins under cell culture conditions and addressed the question whether degradation products might contribute to the cellular effects assigned to the parent compounds. Substantial degradation was found already after 30 min, measured by HPLC/DAD. However, the decrease of detectable anthocyanidins exceeded by far the formation of the respective phenolic acids. From the formed phenolic acids only gallic acid (GA) exhibited growth inhibitory properties. However, also GA was found to be degraded rapidly. Furthermore, the incubation with delphinidin (DEL) or GA resulted in a substantial formation of hydrogen peroxide. The suppression of hydrogen peroxide accumulation by catalase modified significantly the growth inhibitory effects of DEL and GA, indicating that hydrogen peroxide formation might generate experimental artefacts. In summary, the results show that the phenolic acids formed by the degradation of cyanidin (CY), pelargonidin (PG), peonidin (PN) and malvidin (MV) do not contribute to the growth inhibitory effect of the parent compound. The degradation of DEL generates a phenolic acid with substantial growth inhibitory properties (GA). However, taken into account the small proportion of generated GA and its lacking stability, the contribution of GA to the growth inhibitory properties of DEL might be limited.

Keywords: Catalase / Cyanidin / Malvidin / Peonidin / Phenolic acids

Received: January 4, 2007; revised: May 6, 2007; accepted: May 8, 2007

1 Introduction

Anthocyanins are natural food colorants widely spread in food of plant origin, intensely colored in blue, violet or red, depending on the pH and/or the presence of chelating metal ions. Anthocyanins and their aglycons, the anthocyanidins have been associated with a broad spectrum of potentially positive health effects [1–6]. Several aglycons have been reported to inhibit the growth of human tumour cells *in vitro*, with delphinidin (DEL), cyanidin (CY) and malvidin (MV) (Fig. 1) being the most effective [7–11]. The aglycons bearing vicinal hydroxy groups at the B-ring of the anthocyanidin structure (DEL and CY) are known to inhibit the protein tyrosine kinase (PTK) activity of the epidermal growth factor receptor (EGFR) [7, 8, 11, 12]. Anthocyanidins bearing methoxy substituents at the B-ring, *e.g.* MV, effectively inhibit the activity of 3′,5′-cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase subfamily 4 (3′,5′-cyclic nucleotide phosphodiesterase 4 (PDE4)) [11, 13]. Both pathways, the EGFR-regulated sig-

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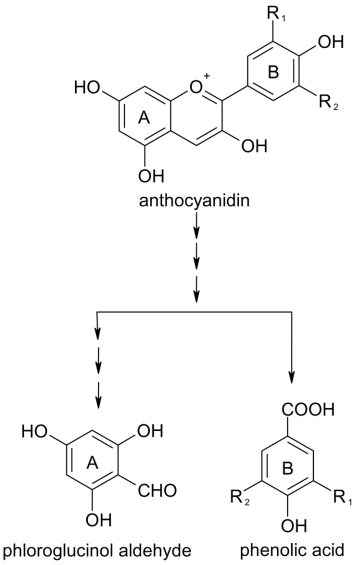
Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; **CY,** cyanidin; **DEL,** delphinidin; **EGFR,** epidermal growth factor receptor; **GA,** gallic acid; **HBA,** *p*-hydroxybenzoic acid; **MV,** malvidin; **PA,** protocatechuic acid; **PDE,** 3′,5′-cyclic nucleotide phosphodiesterase; **PG,** pelargonidin; **PN,** peonidin; **PTK,** protein tyrosine kinase; **SA,** syringic acid; **VA,** vanillic acid



Anthocyanidins	Phenolic acids	R_1	R_2
Pelargonidin (PG)	4-Hydroxybenzoic acid (HBA)	H	H
Cyanidin (CY)	Protocatechuic acid (PA)	OH	H
Delphinidin (DEL)	Gallic acid (GA)	OH	OH
Peonidin (PN)	Vanillic acid (VA)	OCH_3	OH
Malvidin (MV)	Syringic acid (SA)	OCH_3	OCH_3

Figure 1. Degradation pathway of anthocyanidins [14 – 16].

nal transduction and the cAMP pathway, are involved in the regulation of cell growth.

Under *in vitro* conditions limited chemical stability of many flavonoids, especially anthocyanidins, has been repeatedly reported [14 – 16]. But not only under *in vitro* conditions might the degradation of flavonoids occur. Anthocyanins as well as the aglycons have been reported to represent substrates for microbial degradation by human intestinal bacteria, leading to the formation of a phenolic acid arising from the B-ring and an aldehyde resulting from the A-ring (Fig. 1) [14 – 16]. After microbial fermentation as well as spontaneous chemical degradation phloroglucinol aldehyde was detected [15, 16], which might result from further degradation of the putatively generated trihydroxyphenylacetaldehyde.

However, little is known so far about the stability of anthocyanidins under cell culture conditions and the formation of the respective degradation products. Cellular effects of the anthocyanidins are subject of numerous studies, whereas the knowledge on the biological relevance of the formed degradation products and their contribution to the observed cellular effects of the parent compounds is quite limited. In addition, the formation of hydrogen peroxide under cell culture conditions, resulting from the reaction of polyphenols with yet unknown culture media constituents, is discussed to generate artefacts with respect to the cellular effectiveness of the respective polyphenols [17 – 23].

In the present study, we investigated the stability of anthocyanidins and the formation of the respective phenolic acids under cell culture conditions. We furthermore addressed the question whether these phenolic acids affect the growth of human colon carcinoma cells (HT29) *in vitro* and whether H_2O_2 formation might play a role in the growth inhibitory properties and the stability of both, the parent anthocyanidins and the degradation products. In addition, we investigated whether effects of the parent anthocyanidin on potential cellular targets, exemplified for the EGFR and PDE4, might be attributed to the respective degradation products.

2 Materials and methods

2.1 Chemicals

DEL, CY, pelargonidin (PG), peonidin (PN) and MV were purchased from Extrasynthèse (Genay, France). Protocatechuic acid (PA), *p*-hydroxybenzoic acid (HBA), gallic acid (GA), syringic acid (SA), vanillic acid (VA) and catalase were obtained from Sigma (Taufkirchen, Germany). For all assays the compound solutions were freshly prepared prior to the start of the experiment, without the use of stored stock solutions. All compounds and mixtures were dissolved in DMSO with a final concentration of a maximum of 1% in the respective test systems.

2.2 Cell culture

The human colon carcinoma cell line HT29 was purchased from the German Collection of Microorganism and Cell Culture in Braunschweig, Germany. HT29 cells were cultivated in DMEM (DMEM with 4500 mg/L glucose, without sodium pyruvate), supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. DMEM and the supplements were obtained from InvitrogenTM Life Technologies (Karlsruhe, Germany).

2.3 Stability of anthocyanidins and phenolic acids in cell culture medium

The compounds (anthocyanidins/phenolic acids) were dissolved in DMSO to yield 2.5 mM stock solutions. HT29 cells were seeded into 24-well culture plates at a density of 5000 cells *per* well. Seventy-two hours after cell plating, the cell culture medium was completely removed and the cells were treated with DMSO (0.1%) and the individual anthocyanidins/phenolic acids at concentrations of 25 μM. After defined periods of time (0, 0.5, 1, 3, 6, 24, 72 h) an aliquot was taken from the incubation mixture, and mixed with 10% v/v HCl (10 M) to prevent further degradation. The samples were analysed by HPLC/photodiode array (DAD).

Anthocyanidins and phenolic acids in the cell culture medium were analysed using a Nova-Pak C18

(250 × 4.6 mm id; particle size 4 μm) RP column (Waters, Eschborn, Germany). The solvent system consisted of 1% TFA in water (A) and ACN (B) with the following linear gradient: from 0 to 15 min 100% A, changing in 10 min to 11% B, then in 7 min to 14% B followed by holding there for 8 min, changing in 10 min to 20% B, and in further 10 min to 50% B and finally in 10 min to 100% B. The flow rate was 0.8 mL/min and the eluent was recorded with a DAD at 520 nm for the anthocyanidins as well as 260 and 280 nm, respectively, for the phenolic acids. Observed peaks were scanned between 200 and 600 nm.

2.4 Sulforhodamine B assay

Effects on cell growth were determined according to the method of Skehan *et al.* [24] with slight modifications. Briefly, 4500 HT29 cells were seeded *per* well into 24-well plates and allowed to grow for 48 h before treatment. Thereafter, cells were incubated with the respective drug in the absence or presence of catalase or ascorbic acid for 72 h in serum containing medium. Incubation was stopped by addition of trichloroacetic acid (50% solution). The fixed cells were stained with a 0.4% solution of sulforhodamine B. The dye was eluted with Tris-buffer (10 mM, pH 10.0) and quantified photometrically at 570 nm. Cell growth inhibition was determined as percent survival, determined by the number of treated over control cells × 100 (% T/C).

2.5 Hydrogen peroxide formation

Briefly, 40 000 HT29 cells were seeded *per* well into 24-well plates and allowed to grow for 48 h before treatment. The incubation conditions were adjusted according to the SRB-assay (see above). The formation of hydrogen peroxide was measured after several time points (15 min, 45 min, 24 h and 72 h) using the Amplex® Red hydrogen peroxide assay kit from Sigma following the manufacturer's protocol. Final hydrogen peroxide concentrations (0, 0.5, 0.75, 1, 2 and 3 μM) were used as standard curve.

2.6 Tyrosine kinase assay

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

2.7 Inhibition of phosphodiesterase activity

1.5×10^6 HT29 cells were spread in Petri dishes (d = 9 cm) and cultured for 48 h. Before harvesting, the medium was removed and the cells were washed with 3 mL of PBS. Harvesting and lysate preparation were performed at 4°C. Cells were scraped in buffer A (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 5 mM benzamidine hydrochloride, 0.5 μM trypsin inhibitor from soya beans, 0.5 mM PMSF, 0.5 mM β-mercaptoethanol, 1 μM pepstatin, 1 μM leupeptin) and homogenised with 40 strokes in a Wheaton homogeniser (tight pestle). The 100 000 × g supernatant (50 min, 4°C) was directly subjected to the PDE assay. PDE activity was determined as reported previously [11]. Briefly, PDE-containing samples were incubated at 37°C in the presence or absence of test compounds (final solvent concentration 0.3% v/v) with a mixture of cAMP and [^{3}H]-cAMP in a buffer containing 30 mM Tris-HCl, pH 7.4, 9 mM $MgCl_2$, 3 mM 5′-AMP, 3 μM cAMP resulting in a final cAMP concentration in the assay of 1 μM. The reaction was stopped at a cAMP turnover of about 20% by adding $ZnSO_4$. [^{3}H]-5′-AMP was precipitated by addition of $Ba(OH)_2$ and separated by centrifugation at 20 000 × g for 5 min. Nonhydrolysed [^{3}H]-cAMP was determined by liquid scintillation counting of the supernatant. PDE activity of each sample was determined in triplicate.

3 Results

3.1 Stability of anthocyanidins

The degradation of anthocyanidins and the formation of the respective phenolic acids during 72 h incubation of HT29 cells were determined by HPLC/DAD. The anthocyanidins were applied in a concentration below the IC_{50} values in the sulforhodamine B assay (25 μM) [11]. DEL was no longer detectable after 30 min of incubation (Fig. 2A). Substantial decrease was also observed for the other anthocyanidins CY (Fig. 2B), PG (Fig. 2C), PN (Fig. 2D) and MV (Fig. 2E). After 3 h of incubation less than 10% of the applied concentration of PG, PN and MV was still detectable, whereas CY has completely vanished. The disappearance of detectable anthocyanidins under the respective cell culture conditions

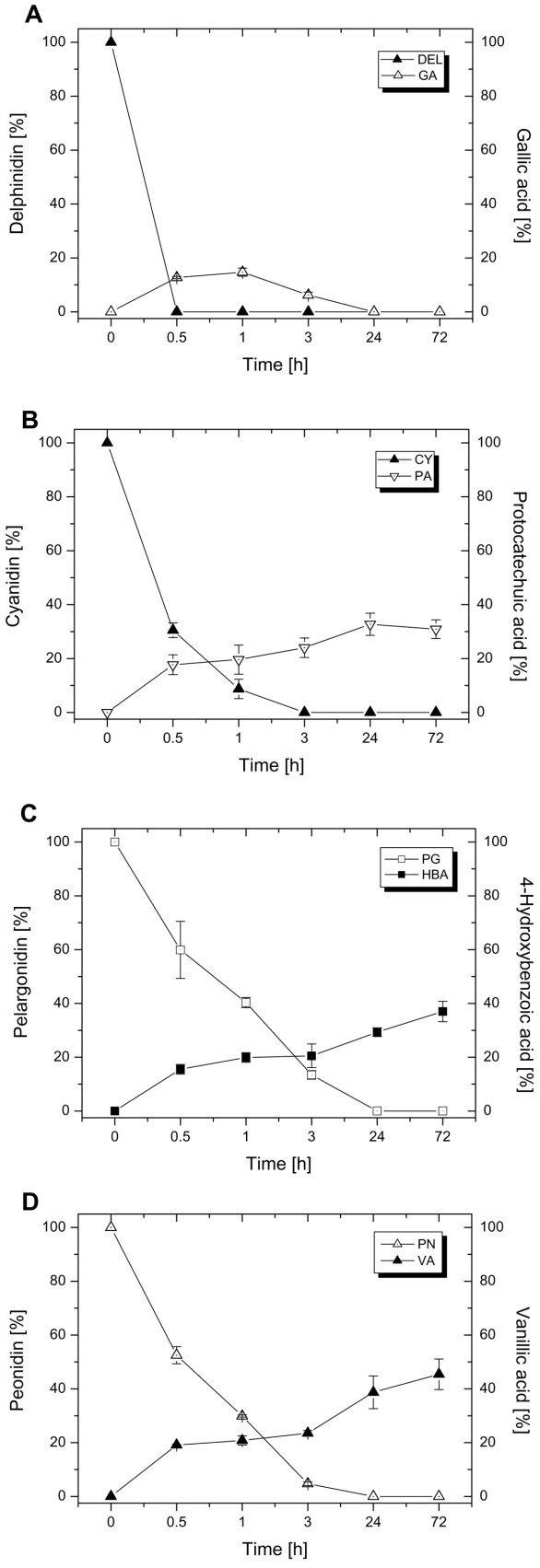


Figure 2. Analysis of (A) DEL, (B) CY, (C) PG, (D) PN and (E) MV and the formation of the respective phenolic acids in the culture medium (serum containing DMEM, 10% FCS) of HT29 cells, incubated with 25 μM of the anthocyanidins by HPLC/DAD. Values are expressed as relative peak area (in%) as mean ± SD of at least two independent experiments using different cell passages. The concentration of the formed phenolic acids was calculated with respect to the applied concentration of the respective anthocyanidin.

could be summarised as DEL > CY ≈ MV >PN ≈ PG (Fig. 3A). The disappearance of anthocyanidins from the cell culture medium was associated by the formation of the respective phenolic acids (Figs. 2A – E, 3A). However, in all cases the amount of the formed phenolic acid did not correspond to the disappearance of the parent anthocyanidin. Furthermore, the amount of the respective degradation products, with the exception of GA, continued to increase up to 72 h even though the anthocyanidins appeared to be almost totally degraded after 3 h (Fig. 3A). The formation of GA reached an apparent maximum after 1 h, corresponding to 20% of the applied DEL concentration, and was not longer detectable after 24 h (Figs. 2A, 3B).

Also in the presence of catalase DEL completely disappeared within 30 min of incubation (Fig. 3B). However, under these conditions the extent of GA formation was diminished after 30 min and 1 h incubation. But the generated GA was detectable up to 6 h, indicating slightly enhanced stability of the phenolic acid. All over, the total amount of generated GA was lower in the presence of catalase (Fig. 3B). Considering the apparent rapid disappearance of GA, the stability of this phenolic acid under cell culture conditions was investigated. When GA was directly applied to the cell culture medium of HT29 cells a time dependent decrease of detectable compound was observed with about 50% loss already after 1 h of incubation (Fig. 3B). After 6 h incubation GA was no more detectable in the cell culture medium. In the presence of catalase, the loss of GA was slightly retarded. However, after 6 h of incubation only 6.5 ± 0.8% of the applied GA concentration was still detectable in the cell culture medium indicating substantial decay (Fig. 3B).

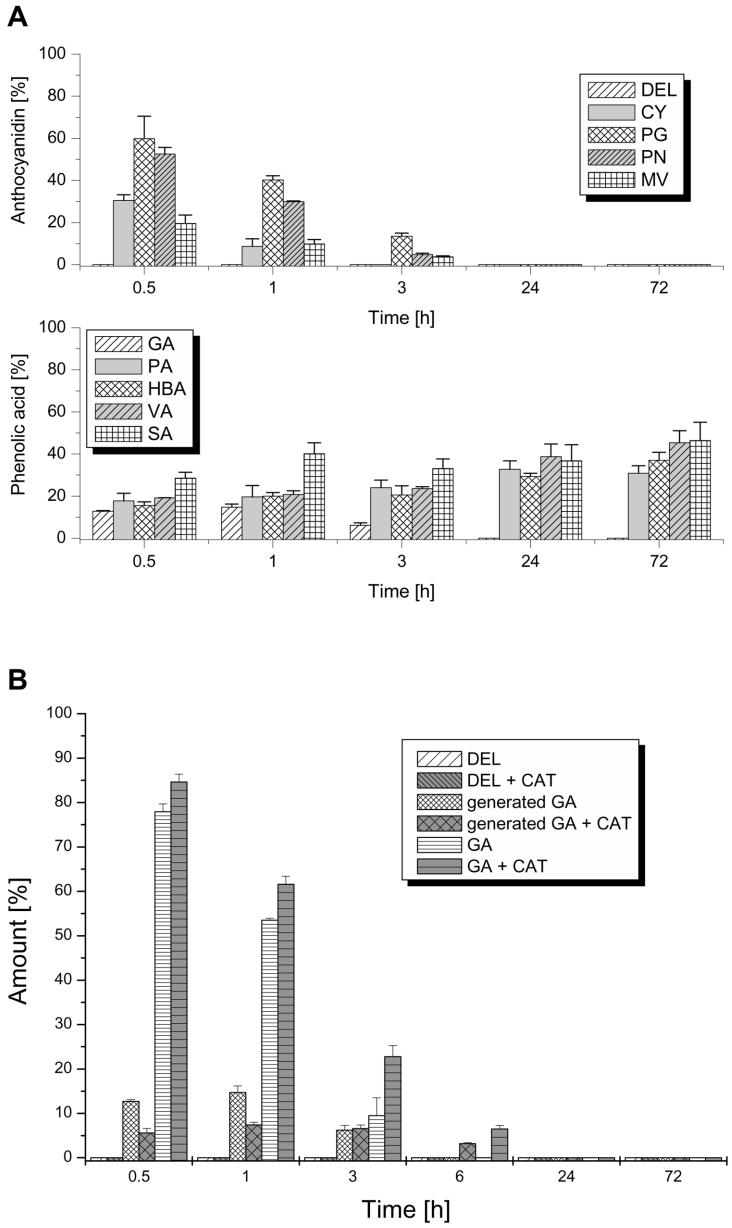


Figure 3. (A) Comparison of the disappearance of the parent anthocyanidins and the formation of phenolic acids in the cell culture medium of HT29 cells. (B) Effect of the presence of catalase in the cell culture medium on the stability of DEL and GA. Values are expressed as relative peak area (in%) as mean ± SD of at least two independent experiments using different cell passages.

3.2 Inhibition of tumour cell growth in vitro

The effect of degradation products of anthocyanidins on the growth of HT29 cells was determined using the sulforhodamine B assay. Among the phenolic acids resulting from anthocyanidin degradation, only GA exhibited substantial growth inhibitory properties (Fig. 4A, Table 1). The presence of catalase or ascorbic acid did not significantly affect the growth inhibitory effectiveness of GA up to a concentration of 40 μM (Fig. 4B). At a concentration of 50 μM GA, the addition of catalase resulted in a slight but significant decrease of the growth inhibitory effect. However, at GA concentrations ≥60 μM the presence of catalase or ascorbic acid led to a clear loss of growth inhibitory effectiveness.

Considering the substantial effect of catalase or ascorbic acid on the growth inhibitory effect of GA, we investigated whether the growth inhibition mediated by the parent compound DEL might also be affected. The presence of ascorbic acid did not significantly influence the inhibition of cell growth by DEL up to a concentration of 35 μM (Fig. 4C). However, at 40 μM, DEL, ascorbic acid significantly diminished the growth inhibitory effect of DEL by about 15%.

In contrast, in the presence of catalase, the growth inhibitory effect of 25 μM DEL was significantly enhanced (Fig. 4C, Table 1). Unexpectedly, higher DEL concentrations resulted in the recurrence of cell proliferation. As a consequence, the growth modulating effect of DEL in the presence of catalase was monitored over a broader concentra-

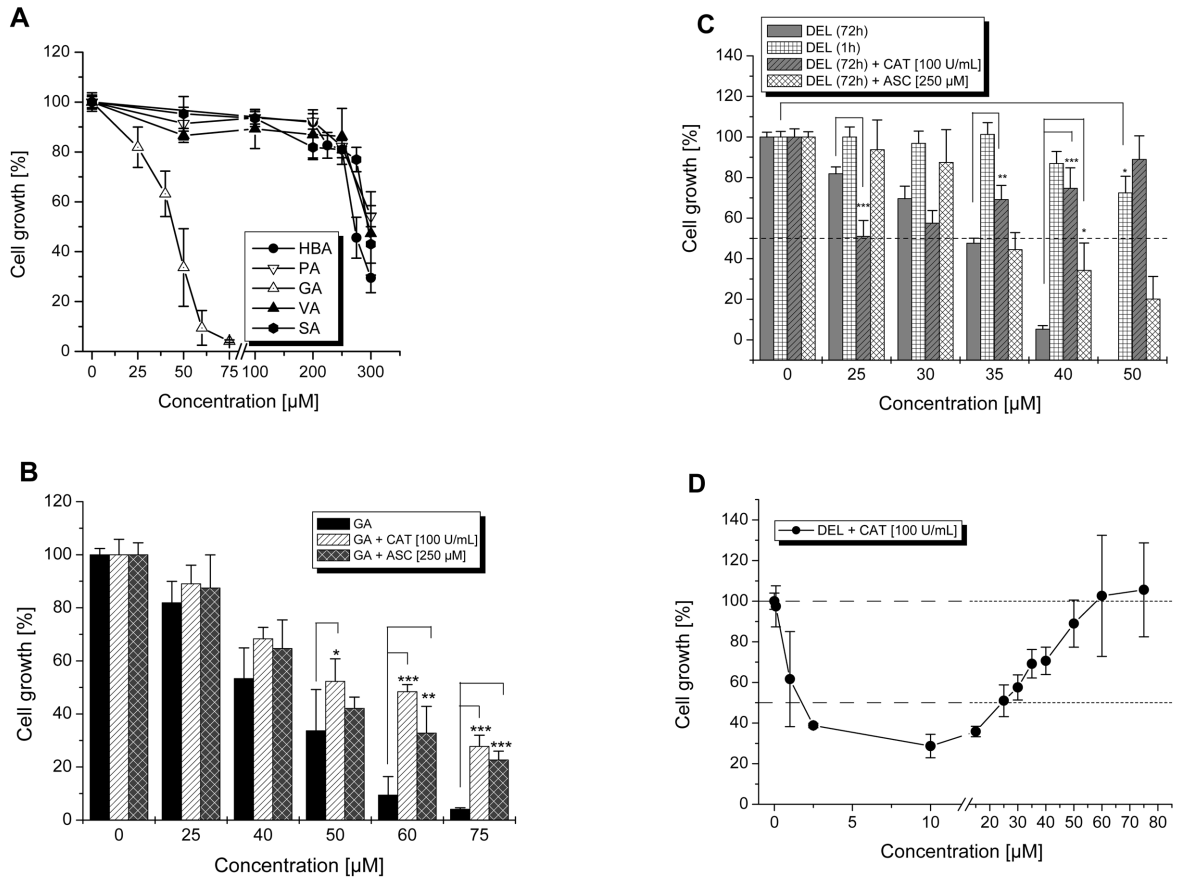


Figure 4. Inhibition of tumour cell growth in the sulforhodamine B assay [24] by (A) phenolic acids, (B) GA in the presence or absence of catalase (CAT) or ascorbic acid (ASC), (C) DEL in the presence or absence of catalase or ascorbic acid and (D) detailed study on the modulation of the growth inhibitory effect of DEL by catalase. HT29 cells were incubated with the respective compound in serum containing medium for 72 or 1 h (DEL) followed by 71 h postincubation with cell culture medium. Growth inhibition was calculated as percent survival of treated cells over control cells (treated with the solvent 1% DMSO) × 100 [T/C%]. The values given are the mean ± SD of at least three independent experiments, each performed in quadruplicate.

tion range (Fig. 4D). In the presence of catalase, substantial growth inhibition of DEL was already observed at a concentration of 1 µM, suppressing the growth of HT29 cells by about 40%. Concentration-dependent inhibition of cell growth was observed up to an apparent inhibitory maximum at 10 µM DEL, followed by a successive increase in cell growth at concentrations ≥20 µM. At a concentration of 60 µM DEL the growth inhibitory effect was completely abolished with a rate of cell growth corresponding to the untreated control (Fig. 4D).

Considering the apparent limited stability of DEL under cell culture conditions we investigated whether 1 h incubation with DEL followed by 71 h postincubation with serum containing medium without DEL was sufficient for effective growth inhibition. In the highest test concentration (50 µM) DEL showed a slight but significant growth inhibitory effect, however the effect was only marginal in comparison to 72 h incubation with DEL (Fig. 4C).

3.3 Hydrogen peroxide formation

The incubation of HT29 cells with the anthocyanidin DEL led to a concentration dependent formation of hydrogen peroxide (Fig. 5). However, hydrogen peroxide concentrations >10 µM were only detected in the short time experiments (15 and 45 min; Figs. 5A and B). After 24 h (Fig. 5C) and 72 h (data not shown) the hydrogen peroxide levels were in the concentration range of the solvent control. GA in the highest test concentration (75 µM) generated hydrogen peroxide levels up to 12 µM after 15 min (Fig. 6A). This concentration increased further up to 19 µM after 45 min (Fig. 6B). No enhanced hydrogen peroxide concentrations were measured after longer incubation periods (24 h, Fig. 6C; 72 h, data not shown). The presence of 100 U/mL of catalase or 250 µM of sodium ascorbate were found to be sufficient to diminish the hydrogen peroxide concentration to the level of the solvent control (DMSO, 1%) (Figs. 5 and 6).

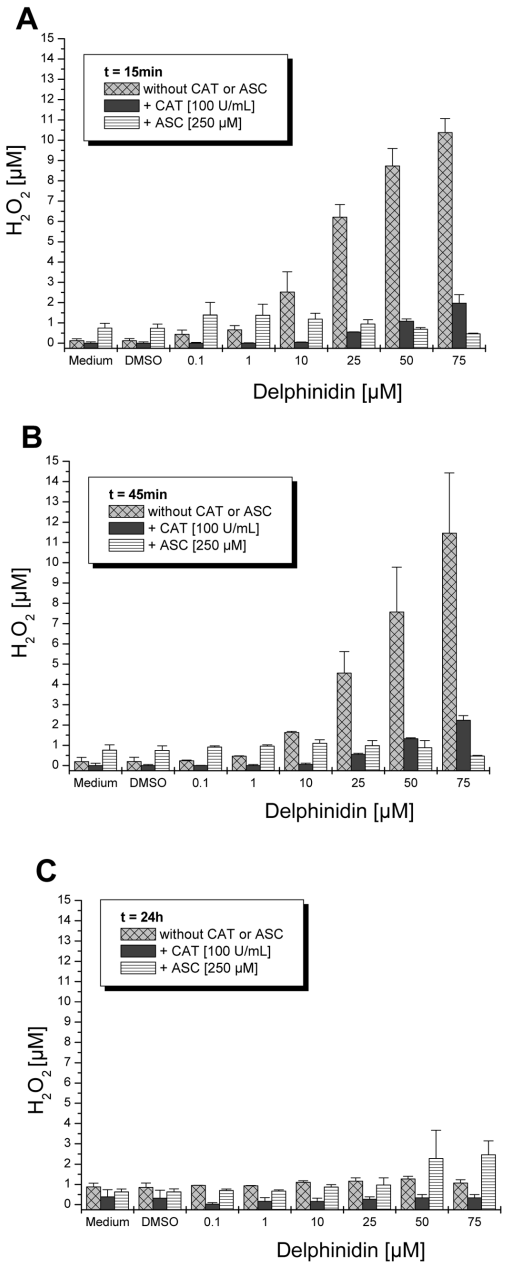


Figure 5. Hydrogen peroxide levels in serum containing cell culture medium of HT29 cells after incubation with DEL in the presence and absence of catalase (CAT, 100 U/mL) or sodium ascorbate (ASC, 250 µM) for (A) 15 min, (B) 45 min and (C) 24 h. The data presented are the mean ± SD of at least two independent experiments, each performed in duplicate.

3.4 Interference with potential cellular targets

We furthermore investigated whether degradation products affect similar cellular targets like the parent anthocyanidins. Anthocyanidins bearing vicinal hydroxy groups at the B-ring represent potent inhibitors of the PTK activity of the EGFR. Modulation of the EGFR activity by anthocyanidin degradation products was determined using an enzyme-linked immunosorbent assay (ELISA). GA was identified as a weak inhibitor of the PTK activity of the EGFR, whereas the other phenolic acids, if at all, mediated only marginal effects (Fig. 7, Table 1).

Table 1. Effect of DEL and phenolic acids on the growth of human colon carcinoma cells using the sulforhodamine B assay (HT29 cells, 72 h incubation, ± catalase or ascorbic acid) and the PTK activity of the EGFR (ELISA)

Substance	Growth inhibition IC_{50} (µM)			EGFR ELISA IC_{50} (µM)
	None	Catalase (100 U/mL)	Ascorbic acid (250 µM)	
HBA	269 ± 26	n.t.	n.t.	–[a]
PA	>300	n.t.	n.t.	–[a]
GA	42 ± 8	49 ± 1	48 ± 7	189 ± 15
VA	297 ± 88	n.t.	n.t.	–[a]
SA	295 ± 75	n.t.	n.t.	>300
DEL	35 ± 5[b]	–[c]	36 ± 11	1.1 ± 0.2[b]

a) No inhibition up to 300 µM.
b) Previously reported in ref. [11]. n.t.: not tested.
c) Not calculated (u-shaped curve, Fig. 4D).

Anthocyanidins bearing methoxy substituents at the B-ring are known to inhibit the hydrolysis of cAMP by cAMP-specific phosphodiesterases (PDE4). PDE4 activity was measured according to the method of Poech [25]. VA and SA, the phenolic acids resulting from the degradation of the PDE-inhibitory anthocyanidins PN and MV, did not affect cAMP-hydrolysis up to 300 µM (data not shown).

4 Discussion

Several anthocyanidins have been reported to inhibit the growth of human tumour cells *in vitro*, with DEL, CY and MV being the most potent [7 – 11]. In accordance with studies on the stability of anthocyanidins in aqueous solutions [14, 15], a substantial loss of detectable aglycons in the cell culture supernatant of HT29 cells was observed already after 30 min of incubation (Figs. 2A – E). Concomitantly, the formation of the respective phenolic acids was observed. The velocity of disappearance of the aglycons from the cell culture medium could be summarised as DEL > CY ≈ MV > PN ≈ PG (Fig. 3A). These data are in line with studies showing that methoxylated anthocyanidins are rather more stable than catecholic aglycons [14]. Noteworthy, for all five anthocyanidins the amount of the generated respective phenolic acid accounted only for a minor part of the loss of aglycon, indicating additional factors to play a role.

From the phenolic acids formed by the degradation of anthocyanidins, only GA was found to possess substantial growth inhibitory properties (Fig. 4A, Table 1). These data are in line with the earlier reports on growth inhibitory effects of GA in human colon carcinoma cells [26 – 29]. The growth inhibitory effect of GA on HT29 cells was com-

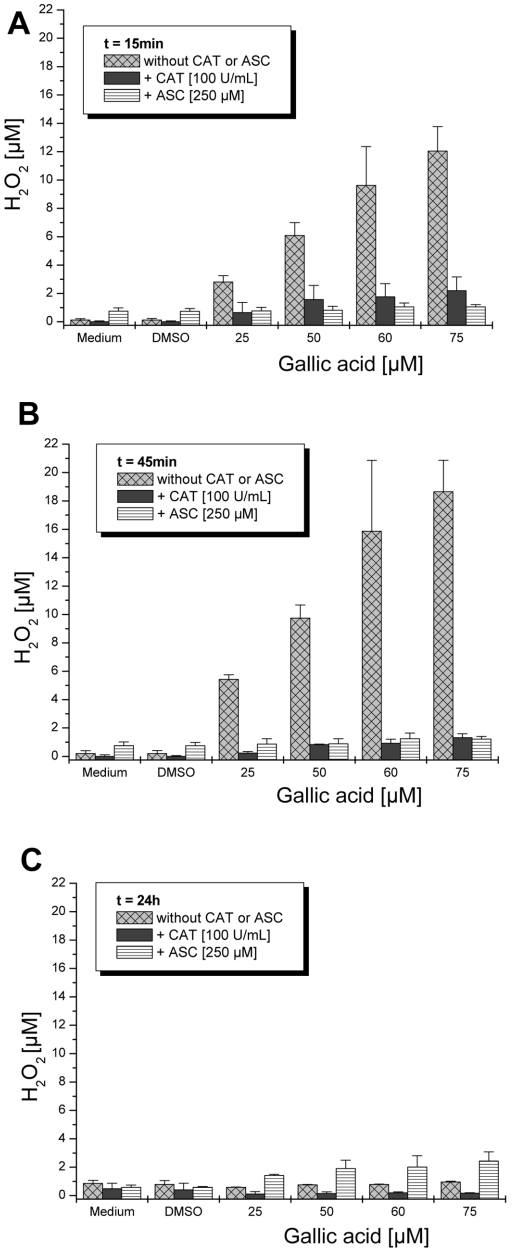


Figure 6. Hydrogen peroxide levels in serum containing cell culture medium of HT29 cells after incubation with GA in the presence and absence of catalase (CAT, 100 U/mL) or sodium ascorbate (ASC, 250 µM) for (A) 15 min, (B) 45 min and (C) 24 h. The data presented are the mean ± SD of at least two independent experiments, each performed in duplicate.

parable to the effect observed by incubation with the parent anthocyanidin DEL (Table 1). Therefore, it cannot be excluded, that the formation of GA might at least contribute to the growth inhibitory effects of DEL. However, not only DEL but also CY, MV and PN mediate growth inhibitory effects in cell culture [7 – 11]. In contrast to GA, the phenolic acids resulting from the degradation of CY, MV and PN (PA, SA, VA) were found to possess only marginal

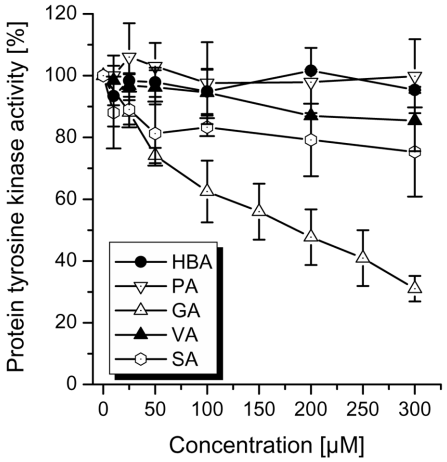


Figure 7. Inhibition of the tyrosine kinase activity of the EGF-receptor by phenolic acids. The phosphorylation of tyrosine residues of a poly (Glu/Tyr) peptide was determined by ELISA using an antiphosphotyrosine antibody linked to peroxidase. The data presented are the mean ± SD of three independent experiments, each performed in triplicate.

growth inhibitory properties. Thus, it is rather unlikely that these phenolic acids are responsible for the growth inhibitory effects observed by incubation with CY, MV or PN.

Due to the reactivity of the putatively generated aldehyde, which is postulated as a common degradation product of all anthocyanidins [15, 16], the probable contribution of this compound to the growth inhibitory properties of the parent anthocyanidins could not be determined. Irrespective of the B-ring substitution pattern, anthocyanidins are expected to form the same aldehyde. Taking into account that the majority of the generated phenolic acids were found to be inactive (Fig. 4A) and speculating that the aldehyde might have substantial impact on cell growth, the anthocyanidins CY, PG, PN and MV should mediate similar growth inhibitory effects, which is not the case [11]. However, the data on the stability of the anthocyanidins indicate that the substitution pattern of the B-ring affect the stability of the anthocyanidins under the applied cell culture conditions and thus presumably the release of the reactive aldehyde. Under the hypothesis that the aldehyde might be responsible for the growth inhibitory properties of the anthocyanidins, DEL has to be postulated to be the most potent because of its fast disappearance, indicating a quick degradation. Indeed, the growth inhibitory properties of the aglycons (DEL > CY ≈ MV > PN > PG), reported earlier [11], are almost in accordance with the hypothetic degradation rate, thus indicating that the formation of a reactive aldehyde might indeed contribute to the growth inhibitory effect. However, the rate of the disappearance of the parent anthocyanidin is not necessarily identical with its degradation. For all anthocyanidins the disappearance of the parent compound exceeded by far the formation of the respective phenolic acid, indicating that additional factors might con-

tribute, for example, like protein binding. This hypothesis is supported by the finding that at least for MV, PN and PG the level of the respective phenolic acids was still increasing although no intact anthocyanidin was anymore detectable (Figs. 2A – E).

Furthermore, it has to be considered that incubation with DEL, putatively releasing both, GA and the aldehyde, did not exceed the growth inhibitory effect of GA alone (Table 1). Thus, the generation of a reactive aldehyde might contribute, but is unlikely to solely cause the growth inhibitory effects of anthocyanidins. In the case of DEL, the formation of the growth inhibitory phenolic acid GA might indeed play a role for its effective growth inhibitory properties. However, the limited chemical stability of GA has to be taken into account. PA, VA and SA are unlikely to play a role for the growth inhibitory effect of the respective anthocyanidin.

Currently, the formation of hydrogen peroxide by the reaction of phenolic compounds with the constituents of the cell culture medium is discussed to affect the cells, generating experimental artefacts limited to these cell culture conditions and without relevance for the *in vivo* situation. The yet unknown mechanisms of the formation of hydrogen peroxide in cell culture has been reported so far already for a series of phenolic compounds including GA [17 – 23]. Therefore, we addressed the question whether the observed cellular effects in our cell culture experiments were modulated by the presence of catalase or sodium ascorbic acid, suppressing hydrogen peroxide accumulation. After short time incubation of HT29 cells with DEL, the formation of hydrogen peroxide was observed at concentrations $\geq$10 μM (Figs. 5A and B). GA was found to even exceed the hydrogen peroxide generating properties of DEL (Figs. 6A and B). In the presence of 100 U/mL catalase [30] or 250 μM sodium ascorbate the accumulation of hydrogen peroxide was effectively suppressed to the level of the solvent control (Figs. 5 and 6). After 24 or 72 h of incubation with either test compound hydrogen peroxide was in the range of the solvent control independent of the presence of catalase or sodium ascorbate.

In the sulforhodamine B assay, the presence of catalase or ascorbic acid substantially affected the growth inhibitory properties of GA and DEL (Figs. 4B – D). At a concentration $\geq$50 μM GA, the presence of catalase or ascorbic acid diminished the growth inhibitory effectiveness to an almost similar extent. Nevertheless, also under these experimental conditions, GA exhibited substantial growth inhibitory properties (Fig. 4B, Table 1).

In the presence of ascorbic acid the formation of hydrogen peroxide might contribute to the growth inhibitory effect of DEL at concentrations $\geq$40 μM (Fig. 4C). Unexpectedly, incubations in the presence of catalase revealed a u-shaped curve of cell growth (Fig. 4D). The presence of catalase substantially decreased the concentration of DEL mediating effective growth inhibition. However, at concentrations $\geq$20 μM a recurrence of cell growth was observed, suggesting an overlay of the initial growth inhibitory effect by a second, so far unknown growth stimulating mechanism.

The EGFR and its subsequent signalling cascade are known to play a role in the regulation of cell proliferation. Several flavonoids of different classes have been shown to inhibit the PTK activity of the EGFR [7, 11, 12, 31 – 34]. We previously reported that the anthocyanidins DEL and CY represent potent inhibitors of this receptor tyrosine kinase [7, 11]. Vicinal hydroxy groups at the B-ring were identified as crucial structural features for effective EGFR inhibition [11, 34]. In contrast to the native anthocyanidins DEL and CY, the resulting phenolic acids were either weak inhibitors (GA) or even inactive (PA) (Fig. 7). These data let assume that in addition to the substituted B-ring the respective flavonoid structure plays a crucial role for effective inhibition of EGFR activity. The effective growth inhibition mediated by GA indicates a different, so far unknown, mechanism of action.

A comparable deactivating effect of the degradation was observed for MV. As many other flavonoids, MV has been reported to inhibit the activity of cAMP-specific phosphodiesterases (PDE4) [11, 13, 35, 36]. We have previously shown that within the class of anthocyanidins methoxy residues at the B-ring are prerequisite for the effective inhibition of PDE4 [11]. In contrast, the respective phenolic acids SA and VA were found to be inactive up to 300 μM (data not shown).

Taken our data together, it is unlikely that exclusively the degradation products are responsible for the growth inhibitory effects of anthocyanidins in cell culture systems. However, the velocity of the formation and the amount of the reactive aldehyde might contribute to growth inhibition. In the case of DEL an additional growth inhibitory impact by the release of GA has to be taken into account. The generated phenolic acids are, if at all, weak inhibitors of the EGFR and inactive towards cAMP-hydrolysing PDE. Thus, inhibitory effects on these potential target enzymes, measured by application of the parent anthocyanidins, can be attributed to the native compounds and are not merely the effect of degradation products.

GA and DEL were found to generate substantial amounts of hydrogen peroxide under cell culture conditions, which has to be considered a probable source for experimental artefacts.

The study was performed as a part of the FlavoNet, supported by the grant MA1659/4-1/2 of the Deutsche Forschungsgemeinschaft (DFG).

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