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Impact of Delphinidin on the Maintenance of DNA Integrity in Human Colon Carcinoma Cells

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Delphinidin has been found to possess DNA strand-breaking properties in cell culture. In the present study, we demonstrated that the extent of DNA damage by delphinidin is not affected by the expression of tyrosyl-DNA-phosphodiesterase 1, indicating that the induction of DNA strand breaks is not predominantly topoisomerase-mediated. However, the DNA-damaging properties of delphinidin were decreased by the addition of catalase to the cell culture medium, counteracting delphinidin-mediated hydrogen peroxide formation. Under these conditions, delphinidin showed clearly antioxidative properties in HT29 cells, preventing menadione-induced oxidative DNA damage. In contrast, in the absence of catalase, delphinidin lacked antioxidative properties. In conclusion, delphinidin acted as an effective antioxidant within intact cells if the formation of hydrogen peroxide was prevented. In the absence of catalase, the accumulated hydrogen peroxide appears to play a substantial role for the observed DNA-damaging properties of delphinidin and the apparent lack of antioxidative properties of this anthocyanidin.

KEYWORDS: Oxidative stress; comet assay; topoisomerase; TDP-1; hydrogen peroxide

INTRODUCTION

Anthocyanins are natural food colorants widely distributed in foods of plant origin. Anthocyanins represent water-soluble anthocyanidin glycosides. The flavylium cation, the anthocyanidin, is bound predominantly at the C3- or the C5-position to a sugar moiety. More than 6000 anthocyanins occur naturally in plants. Delphinidin (**Figure 1**) glycosides are among the most abundant anthocyanins, for example, in bilberries, blueberries, or crowberries (*1, 2*). The consumption of anthocyanin-rich fruits has been associated with a spectrum of positive effects on human health (*3*−*5*), leading to an increasing popularity of respective fortified preparations on the expanding market of food supplements. However, the cellular mechanisms of action of these flavonoids have not yet been fully elucidated. As with many other flavonoids, anthocyanidins have been suggested to act as antioxidants (*6, 7*), but studies were predominantly performed in cell-free test systems. Under the chosen experimental conditions, delphinidin exhibited substantial antioxidative capacity (*8, 9*). In contrast, under cell culture conditions, these findings could not be clearly confirmed. Anthocyanidins as well as their glycosides failed to reduce oxidative DNA damage in HT29 cells (*8*) or smooth muscle cells (*10*). Recent findings on the formation of hydrogen peroxide in cell culture medium in the presence of red wine anthocyanins (*11*) or the free aglycones as well as respective degradation products of anthocyanidins (*12*) raised the question as to whether experimental artifacts might play a role for the lack of antioxidative efficacy of anthocyanidins under cell culture conditions.

Thus, with respect to the maintenance of DNA integrity within cells, delphinidin apparently fails to protect against oxidative damage. Moreover, delphinidin was even found to mediate DNA strand-breaking effects in cell culture (*13*). The underlying mechanism of action has not been completely elucidated so far. We recently showed that anthocyanidins bearing vicinal hydroxy groups at the B ring, such as delphinidin or cyanidin, represent potent inhibitors of human topoisomerases. Topoisomerases are enzymes, which regulate the DNA topology and are thereby essential for catalytic DNA processes like replication, transcription, and chromatin condensation. The enzymes generate transient DNA strand breaks in the sugar phosphate backbone of the DNA, forming the so-called cleavable complex as a covalent topoisomerase-DNA intermediate (*14, 15*). Because of their mode of interference with the catalytic cycle, topoisomerase inhibitors can be separated into two classes: catalytic inhibitors, inactivating the enzyme prior to its binding to DNA, and topoisomerase poisons, stabilizing the cleavable complex (*16, 17*). Because of the collision of the stabilized cleavable complex with the replication fork, DNA damage can occur. Via this mechanism, topoisomerase poisons enhance the amount of DNA strand breaks in cells (*17, 18*). Recently, tyrosyl-DNA-phosphodiesterase-1 (TDP-1) has been identified as a repair enzyme releasing the trapped topoisomerase from the DNA (*19, 20*). Overexpression of TDP-1 was found to decrease the extent of

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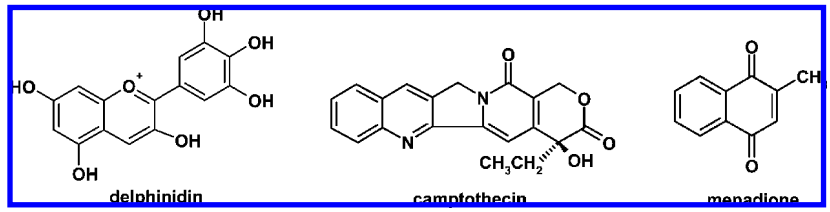

Figure 1. Structures of the anthocyanidin delphinidin, the topoisomerase I poison camptothecin, and the redox cycler menadione.

DNA damage by the topoisomerase poisons camptothecin and etoposide (*21*). In cell-free systems, delphinidin has been shown to act as a catalytic inhibitor of human topoisomerases (*13*); however, within intact cells, topoisomerase-mediated DNA damage cannot yet be excluded. In the present study, we addressed the question as to whether the overexpression of TDP-1 affects the DNA-damaging properties of delphinidin in cell culture, indicating the occurrence of topoisomerase poisoning. Furthermore, the contribution of hydrogen peroxide formation to the observed DNA strand-breaking effects and the reported lack of antioxidative properties (*8, 10*) under cell culture conditions of delphinidin have been investigated.

MATERIALS AND METHODS

Chemicals. Delphinidin was purchased from Extrasynthèse (France), and the topoisomerase poison camptothecin and the redox cycler menadione were from Sigma Aldrich (Germany). For all assays, the compound solutions were prepared in dimethyl sulfoxide (DMSO) before the beginning of the experiment, without using stored stock solutions.

Cell Culture. The human colon carcinoma cell line HT29 was cultivated in Dulbecco's modified Eagle's medium (DMEM with 4500 mg/L glucose, without sodium pyruvate). The cell culture medium was supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The transfected human embryonic kidney cells (HEK 293-GFP, HEK 293-GFP-TDP-1, and HEK 293-GFP-TDP-1-H263A) (*21*) were cultivated in the same medium containing 0.4 mg/mL puromycin. DMEM and the supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

Cell Incubation. HEK 293 cells were divided into aliquots containing 1×10^6 cells each and incubated for 1 h with DMSO (1% v/v), 100 μM camptothecin, or delphinidin in 1 mL of serum-free medium in round-bottom vials. Thereafter, aliquots corresponding to 70000 cells were centrifuged (5 min, 300*g*), and the pellet was used for the comet assay.

HT29 cells [3×10^5 in 5 mL of medium containing 10% FCS (v/v)] were spread into Petri dishes (5.5 cm^2) and allowed to grow for 48 h prior to treatment with drugs. In the experiments with single compounds, HT29 cells were treated for 1 h with the solvent control 1% DMSO v/v, 20 μM menadione, or delphinidin in serum-free medium. For the coincubation experiments, HT29 cells were preincubated for 1 h with the solvent control (1% DMSO v/v) or different concentrations of delphinidin, followed by 1 h of incubation of 20 μM menadione. Thereafter, aliquots containing 70000 cells were centrifuged (5 min, 300*g*).

Single Cell Gel Electrophoresis (Comet Assay). Single cell gel electrophoresis was performed according to the method of Gedik et al. (*22*). The resulting cell pellet was resuspended in 65 μL of low-melting agarose and distributed onto a frosted glass microscope slide, precoated with a layer of normal-melting agarose. The slides were coverslipped and kept at 4 °C for 10 min to allow solidification of the agarose. After the cover glass was removed, slides were immersed for 1 h at 4 °C in lysis solution [89 mL of lysis stock solution, 2.5 mM NaCl, 100 mM EDTA, 100 mM Tris, 1% (w/v) *N*-laurylsarcosyl sodium salt, 1 mL of Triton-X-100, and 10 mL of DMSO]. Afterward, the slides were washed briefly three times with fpg buffer [40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0, with KOH], and the treatment with the DNA repair enzyme formamidopyrimidin-DNA-glycosylase (fpg) followed for 30 min at 37 °C. This step was only necessary if oxidative DNA damage should be detected. Subsequently, DNA was allowed to unwind (300 mM NaOH; 1 mM EDTA, pH 13.5; 20 min; and 4 °C) followed by horizontal gel electrophoresis at 4 °C for 20 min (25 V, 300 mA). Thereafter, the slides were washed three times with 0.4 M Tris HCl, pH 7.5, and stained with ethidium bromide (40 μL per coverslip, 20 μg/mL). Fluorescence microscopy was performed with a Zeiss Axioskop 20 ($\Delta_{ex} = 546 \pm 12$ nm; $\Delta_{em} > 590$ nm). Slides were analyzed with a Comet Assay III System (Perceptive Instruments, Suffolk, Great Britain), scoring 50 images per slide randomly picked from each electrophoresis. For each concentration of drug, two slides were independently processed and analyzed. The results were parametrized with respect to tail intensity (TI; intensity of the DNA in the comet tail calculated as percentage of overall DNA intensity in the respective cell). Such quantitative data were always derived from at least three independent sets of experiments and from the evaluation of 100 individual cells per concentration (50/slide) in each experiment. In parallel to the comet assay, viability of the cells was determined by trypan blue exclusion.

RESULTS AND DISCUSSION

Impact of TDP-1 Expression. To test the hypothesis of whether DNA strand breaks induced by treatment with delphinidin are topoisomerase-mediated, the impact of delphinidin on the DNA integrity of cells overexpressing human tyrosyl-DNA-phosphodiesterase 1 (TDP-1) was investigated by single cell gel electrophoresis (comet assay). HEK293-GFP-TDP-1 cells express the active form of this repair enzyme as a fusion protein with GFP. HEK293-GFP-TDP-1-H263A cells used as a negative control express a fusion protein of GFP and an inactive form of the TDP-1, caused by a point mutation at histidine 263 being converted to alanine. Cells overexpressing unfused GFP were included in the testing as an additional negative control (*21*).

After 1 h of treatment with camptothecin (100 μM), the TI measured for the HEK293-GFP-TDP-1 cells was significantly lower than those measured for the HEK293-GFP and HEK293-GFP-TDP-1-H263A cells (**Figure 2**) ($TI_{GFP} = 13.0 \pm 2.7\%$, $TI_{TDP-1} = 7.8 \pm 2.1\%$, and $TI_{H263A} = 13.0 \pm 2.6\%$). In contrast, no significant difference in the intensity of DNA damage between the different clones was observed after incubation with delphinidin (**Figure 2**) ($TI_{GFP} = 7.0 \pm 3.6\%$, $TI_{TDP-1} = 7.0 \pm 3.3\%$, and $TI_{H263A} = 6.4 \pm 2.6\%$).

Hydrogen Peroxide Accumulation and DNA Damage. The impact of hydrogen peroxide on the DNA-damaging properties of delphinidin was determined by the addition of catalase to the cell culture medium (100 U/mL). In the absence of catalase, delphinidin increased concentration dependently the rate of DNA strand breaks in HT29 cells as compared to the respective solvent control (1% DMSO v/v). In concentrations ≥ 50 μM, the DNA strand-breaking properties of delphinidin were significantly enhanced (white bars, **Figure 3**). Also, in the presence of catalase (gray striped bars, **Figure 3**), an increase of DNA damage was observed at 50 μM delphinidin. At that concentration, the addition of catalase to the cell culture medium did not affect the DNA-damaging effect of delphinidin. However, the presence of catalase prevented a further increase of DNA strand breaks at 100 μM delphinidin but could not totally eliminate the strand-breaking properties of delphinidin.

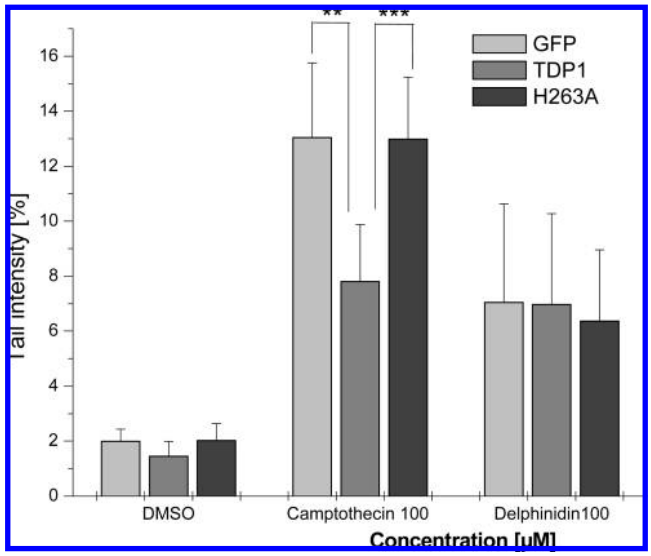

Figure 2. Single cell gel electrophoresis (comet assay) with different HEK cell lines HEK293-GFP (GFP), HEK293-GFP-TDP-1 (TDP-1), and HEK293-GFP-TDP-1-H263A (H263A). The tail intensities presented are the means ± SD of at least three independent experiments, each performed in duplicate. Significances indicated refer to the significance level as compared to the indicated concentration (Mann−Whitney U test, ** = $p < 0.01$; *** = $p < 0.001$).

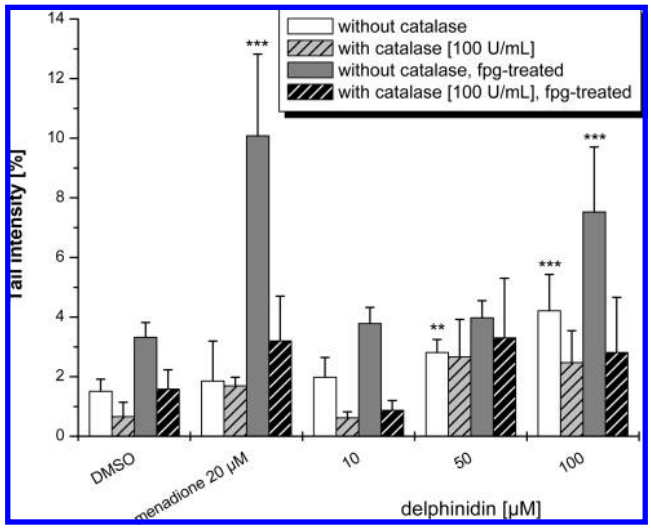

Figure 3. Single cell gel electrophoresis (comet assay) with delphinidin-treated HT29 cells in the presence (gray and black bars) or absence (white and dark gray bars) of catalase. Fpg was used to detect oxidative DNA damage (dark gray and black bars). The tail intensities presented are the means ± SD of at least three independent experiments, each performed in duplicate. Significances indicated refer to the significance level of the respective concentration as compared to the respective control (Mann−Whitney U test, ** = $p < 0.01$; *** = $p < 0.001$).

The treatment of HT29 cells with the redox cycler menadione at a concentration of 20 μM in the presence (gray striped bars) or absence (white bars) of catalase only marginally enhanced the rate of DNA strand breaks as compared to the respective solvent control (**Figure 3**). Postincubation treatment of the test samples with fpg was used as an indication for oxidative DNA damage. In the absence of catalase, a highly significant increase of fpg-sensitive sites was observed at 100 μM delphinidin (dark gray bars), which was completely prevented by the addition of catalase to the cell culture medium (black striped bars, **Figure 3**). Thus, the suppression of hydrogen peroxide accumulation in the cell culture medium avoided putative oxidative DNA damage at high delphinidin concentrations.

In the absence of catalase (dark gray bars), the level of fpg-sensitive sites was significantly enhanced in menadione-treated cells. However, the presence of catalase (black striped bars) totally diminished the effect of the redox cycler menadione (**Figure 3**).

Antioxidative Properties in Cell Culture. To study potential antioxidative effects of delphinidin in cell culture, HT29 cells were pretreated for 1 h with delphinidin prior to a challenge of the cells for 1 h with the redox cycler menadione. The pretreatment with delphinidin and the incubation with the solvent control were performed in the presence or the absence of catalase. We showed in **Figure 3** that the coincubation of menadione and catalase (100 U/mL) lead to a complete loss of menadione's redox activity. Therefore, the postincubation of HT29 cells with the redox cycler has always been carried out in the absence of catalase. Treatment with menadione alone at a concentration of 20 μM resulted in a significant increase of fpg-sensitive sites in the comet assay (striped black bar) as compared to the incubation without fpg (black bar, **Figure 4**) indicative for oxidative DNA damage ($TI_{basic} = 2.5 \pm 0.8\%$ and $TI_{fpg} = 9.8 \pm 1.3\%$). In the absence of catalase, the preincubation of HT29 cells with delphinidin failed to suppress the induction of fpg-sensitive sites caused by menadione (striped gray bars, **Figure 4**). Moreover, the extent of basic (gray bars, **Figure 4**) and oxidative DNA damage (striped gray bars, **Figure 4**) appeared to be enhanced by preincubation with delphinidin at concentrations in which delphinidin alone mediated strand-breaking properties (≥50 μM, **Figure 3**). In contrast, in the presence of catalase at the preincubation with delphinidin, the menadione-caused induction of fpg-sensitive sites was effectively diminished in a concentration-dependent manner with an apparent maximum at 10 μM delphinidin ($TI_{fpg} = 3.0 \pm 1.1\%$, hatched black bars) as compared to the rate of DNA damage observed by the redox cycler menadione after fpg posttreatment (striped black bar, **Figure 4**). Moreover, the highly enhanced level of DNA strand breaks, resulting from the preincubation with 50 μM delphinidin and a subsequent treatment with menadione in the absence of catalase (gray bars) were in the presence of catalase (black bars) nearly diminished to the level of the respective solvent control (white bars, Figure **4**).

Anthocyanins and their aglycones, the anthocyanidins, have been associated with a broad spectrum of beneficial health effects. However in vivo, anthocyanins and the respective aglycones possess only limited bioavailability, but irrespective of the apparent low systemic bioavailability, enhanced local concentrations in the gastrointestinal tract might have to be considered, especially under respective intake habits. Kahle et al. (*23*) showed that up to 70% of blueberry anthocyanins can reach the colon under physiological circumstances, depending on the sugar moiety. In the present study, the question was addressed as to whether anthocyanidins are of relevance for the maintenance of DNA integrity in human colon carcinoma cells. We previously showed that delphinidin exhibits DNA-damaging properties in HT29 cells at concentrations ≥50 μM (*13*). However, the underlying mechanism of action is not completely elucidated so far. Several flavonoids have been reported to interfere with human topoisomerases (*24*−*28*). Many of these flavonoids, such as the soy isoflavone genistein (*26*) or the green tea catechin (−)-epigallocatechin-3-gallate (*25*), were found to act as topoisomerase poisons, stabilizing the covalent DNA-topoisomerase intermediate. The collision of this stabilized

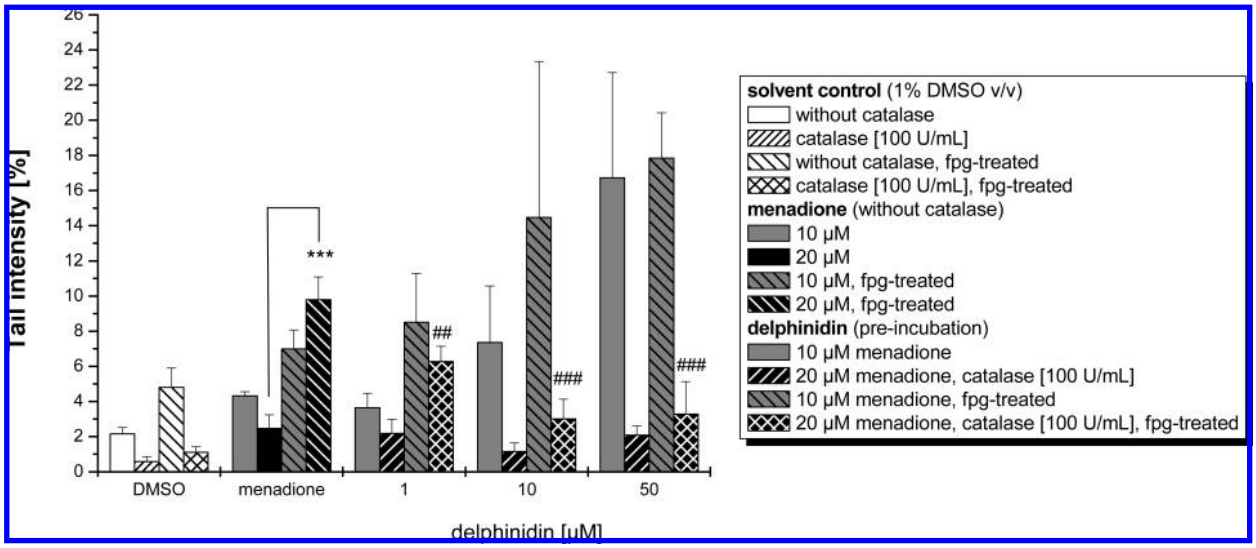

Figure 4. Single cell gel electrophoresis (comet assay) with HT29 cells. The cells were treated with delphinidin or DMSO in the absence (white and dark gray bars) or presence (gray and black bars) of catalase for 1 h and afterward incubated with 10 μM, respectively, 20 μM menadione for 1 h. Fpg was used to detect oxidative DNA damage (dark gray and black bars). The tail intensities presented are the means ± SD of at least three independent experiments, each performed in duplicate. Significances indicated refer to the significance level as compared to the respective untreated control (Mann−Whitney U test, *** = $p < 0.001$) or the respective positive control (menadione 20 μM, fpg-treated; Mann−Whitney U test, ## = $p < 0.01$, ### = $p < 0.001$).

complex with an approaching replication fork might lead to DNA strand breakage. As reported previously, anthocyanidins bearing vicinal hydroxy groups at the B ring interfere with the catalytic activity of human topoisomerases I and II (*13*). Thus, topoisomerase-mediated DNA damage within cells could not be excluded. The contribution of topoisomerase poisoning to the DNA-damaging effects of delphinidin was studied in cells overexpressing TDP-1, a repair enzyme that removes adducts, for example, of topoisomerase I from the 3′-phosphate of DNA breaks (*19*−*21*). We previously reported that the overexpression of TDP-1 in HEK293 cells diminishes the extent of DNA damage by the topoisomerase I poison camptothecin as well as by the topoisomerase II poison etoposide (*21*). In contrast, TDP-1 expression did not affect the DNA-damaging properties of delphinidin (**Figure 2**). These results are in line with earlier reports demonstrating that delphinidin acts as a catalytic topoisomerase inhibitor in cell-free test systems without affecting the stability of the covalent DNA-topoisomerase intermediate (*13*). Taken together, it can be excluded that the DNA-damaging properties of delphinidin are due to "classical" topoisomerase poisoning, the stabilization of the DNA-topoisomerase intermediate. Nevertheless, considering the potent inhibition of the catalytic activity of topoisomerases I and II in cell-free test systems, it cannot be excluded that in intact cells topoisomerase activity might also be affected. Even if the stability of the DNA-topoisomerase intermediate remains unaffected, the suppression of topoisomerase activity, for example, by inhibiting the binding of the enzyme to the DNA, might lead to an accumulation of torsion stress and, as a result, to DNA breakage (*21, 24*).

We furthermore addressed the question as to whether oxidative stress might contribute to the DNA-damaging effects of delphinidin in HT29 cells previously reported in ref *13*. Reactive oxygen species (ROS) like the hydroxyl radical and hydrogen peroxide represent important factors for the induction of DNA damage. ROS are generated intra- and extracellularly. Under cell culture conditions, many flavonoids have been associated with the formation of hydrogen peroxide, which affects the stability of the compounds and might lead to experimental artifacts (*11, 12, 29, 30*). The extent of hydrogen peroxide formation depends on various parameters such as exposure time, the structure of the polyphenol, the medium composition, and the presence of serum and of cells (*29, 30*). We previously reported that the incubation of HT29 cells with delphinidin leads to the formation of substantial amounts of hydrogen peroxide within 15 min of incubation, whereas the addition of catalase (100 U/mL) to the cell culture medium effectively suppresses the accumulation of hydrogen peroxide (*12*). In the present study, we showed that the addition of catalase to the cell culture medium diminished the extent of DNA damage by delphinidin. Especially, an intensive increase of fpg-sensitive sites, as seen in the absence of catalase, indicative for oxidative DNA damage, was effectively suppressed (**Figure 3**). Thus, in contrast to results obtained without prevention of hydrogen peroxide accumulation, delphinidin did not exhibit prooxidative activity in HT29 cells when catalase was added to the cell culture medium. Noteworthy, the addition of catalase did not completely abolish the DNA-damaging effects of delphinidin, which might be due to the potential contribution of the interference of delphinidin with topoisomerase activity to the DNA breakage. In the literature, a fast degradation of delphinidin to phloroglucinol aldehyde and the respective phenolic acid gallic acid under in vitro conditions has been reported (*12, 31*). In comparison to the parent compound also, its degradation product gallic acid was found to accumulate substantial amounts of hydrogen peroxide under cell culture conditions (*12*). Moreover, gallic acid itself possesses slight DNA-damaging properties (*32, 33*). Therefore, a contribution of the degradation product to the DNA strand-breaking effects of delphinidin could not be excluded.

Like many flavonoids, anthocyanidins have been reported to represent potent antioxidants (*5, 6*). However, most of these studies were performed in cell-free test systems. Under cell culture conditions, anthocyanidins have been repeatedly reported to lack substantial antioxidative activity (*8, 10*). In vivo, however, anthocyanidins appear to act as antioxidants, protecting blood cells against oxidative stress (*34*). In the present study, we addressed the question as to whether the apparent lack of antioxidative properties of anthocyanidins (*8, 10*) in vitro might

be an artifact of hydrogen peroxide formation in cell culture. In accordance with the above-mentioned in vitro studies, delphinidin failed to suppress the DNA-damaging effects of the redox cycler menadione, when the incubation with delphinidin was performed in the absence of catalase (**Figure 4**). Under these experimental conditions, delphinidin even enhanced the extent of DNA damage. In contrast, delphinidin was found to clearly possess antioxidative properties, effectively suppressing the effects of menadione, when the accumulation of hydrogen peroxide in the cell culture medium was suppressed by the addition of catalase (**Figure 4**). Noteworthy, an apparent maximum of antioxidative effects was already reached at 10 μM delphinidin. The data indicate that the observed pro-oxidative effects of anthocyanidins are cell culture artifacts, thus tempting to speculate that not the respective flavonoids (*35, 36*) but under cell culture conditions formed hydrogen peroxide induces oxidative DNA damage.

The delphinidin degradation product gallic acid has been reported to act as a potent antioxidant in vitro (*37, 38*). Therefore, it cannot be excluded that the formation of gallic acid might at least contribute to the antioxidative properties of delphinidin. However, the limited chemical stability of gallic acid makes it rather unlikely to solely cause the antioxidative effects observed after incubation with delphinidin.

In summary, delphinidin was found to act as a potent antioxidant within intact cells if experimental artifacts resulting from the formation of hydrogen peroxide were prevented by the addition of catalase. In the absence of catalase, the accumulated hydrogen peroxide appears to play a substantial role for the observed DNA-damaging properties of delphinidin and the apparent lack of antioxidative properties of this anthocyanidin under these cell culture conditions. However, hydrogen peroxide formation was not solely responsible for the DNA-damaging properties of delphinidin. Although not acting as a topoisomerase poison, the interference with topoisomerase activity, leading to enhanced torsion stress, might contribute to the extend of DNA strand breaks.

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