RESEARCH ARTICLE

Anthocyanin-rich extracts suppress the DNA-damaging effects of topoisomerase poisons in human colon cancer cells

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Scope: The effect of two anthocyanin-rich berry extracts (A, bilberry; B, red grape) on topoisomerases was investigated in a cell-free system and in human HT29 colon carcinoma cells. In parallel, their impact on DNA integrity was determined.

Methods and results: The berry extracts suppressed the activity of topoisomerase I at concentrations $\geq 50\,\mu g/mL$. The activity of the topoisomerase II isoform was preferentially diminished ($\geq 1\,\mu g/mL$). Within HT29 cells, the extracts were found to act as catalytic inhibitors without stabilizing the cleavable complex. Although topoisomerase activity was inhibited, none of the extracts induced DNA strand breaks up to $50\,\mu g/mL$. Moreover, pre- and coincubation of HT29 cells with A ($\geq 1\,\mu g/mL$) significantly suppressed (p-value ≤ 0.001) the strand-breaking effects of camptothecin, whereas B was found to be less effective ($1\,\mu g/mL$; p-value ≤ 0.05). Both extracts were found to significantly diminish doxorubicin-mediated DNA strand breaks at concentrations $\geq 1\,\mu g/mL$ (p-value ≤ 0.001). Consistent with these results, the extracts suppressed doxorubicin-mediated enhancement of levels of topoisomerase II covalently linked to DNA in HT29 cells.

Conclusion: These results raise the possibility that high intake of berry extracts may protect DNA and thus counteract the therapeutic effectiveness of orally applied topoisomerase poisons during chemotherapy.

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1 Introduction

Anthocyanins belong to the class of flavonoids responsible for the red and blue pigmentation in fruits, berries and vegetables [1]. Dietary anthocyanins have been considered to decrease the risk of cardiovascular diseases, atherosclerosis, cancer, and diabetes associated with their anti-inflammatory and anti-oxidative properties [2]. The average daily intake of anthocyanins has been estimated to be higher than that of other flavonoids, with consumption in the United States suggested to be 12.5 mg/day/person [3]. In animal studies, anthocyanins have been shown to suppress the development of various types of cancer, *e.g.* in the esophagus, colon, and lung [4–6]. An anthocyanin-rich bilberry (*Vaccinium myrtillus*) extract, containing 15 different anthocyanins, has been found to decrease significantly the intestinal adenoma burden in the $Apc^{Min(-/-)}$ mouse model [5]. Non-glycosylated anthocyanidins and polyphenol-rich extracts potently inhibited the growth of human carcinoma cells, and this

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Abbreviations: Fpg, formamidopyrimidine-DNA glycosylase; **HT29,** human colon carcinoma cell line; **ICE bioassay,** isolating *in vivo* complexes enzyme to DNA; **kDNA,** kinetoplast DNA

inhibition is probably the corollary of their ability to impair enzymes involved in the regulation of cell proliferation and apoptosis [7–10].

In addition to their putative beneficial effects, polyphenols can exert potential adverse effects on health. An example is the ability of certain flavonoids to interfere with human topoisomerases [11–14], which might impinge on the maintenance of DNA integrity.

DNA topoisomerases are ubiquitously expressed enzymes involved in a number of nuclear processes such as replication, transcription, translation, recombination, and chromosome dynamics, by the maintenance of DNA topology [15]. Type I topoisomerase is a monomeric enzyme reversibly cleaving one DNA strand to enable, *e.g.* relaxation processes [16]. Type II topoisomerase is a dimeric and ATP-dependent enzyme that introduces a transient DNA double-strand break, permitting the passage of a second DNA double helix [17]. Topoisomerases I and II are targets of clinically used anti-cancer drugs, including derivatives of camptothecin (irinotecan and topotecan) and epipodophyllotoxin (etoposide and teniposide). These so-called topoisomerase poisons stabilize covalent topoisomerase/DNA intermediates by forming a ternary complex, thus converting the endogenous enzyme to a cellular toxin. An increase in the amount of cleavable complexes ultimately generates permanent strand breaks in the genetic material triggering cell death [18].

Certain flavonoids, *e.g.* catechin, (−)-epigallocatechin-3-gallate, genistein or quercetin, constituents of green tea, soy, or apple, respectively, have been reported to act as topoisomerase poisons [12–14, 19]. In contrast, we showed previously that anthocyanidins, anthocyanin aglycones bearing vicinal hydroxy groups in the B-ring (delphinidin, cyanidin), potently inhibit the catalytic activity of topoisomerases without stabilizing the covalent enzyme/DNA intermediate. Furthermore, the combination of the catalytic inhibitor delphinidin suppressing the formation of cleavable complexes within cells, with a topoisomerase poison targeting already formed covalent DNA–topoisomerase intermediates, decreased the DNA-damaging properties of the respective topoisomerase poisons such as camptothecin or doxorubicin [20, 21].

It is important to note that free aglycones, *e.g.* delphinidin, are generally not present in food at biologically relevant concentrations. Anthocyanidins are consumed in their glycosylated forms as anthocyanins. In the normal diet, anthocyanins occur usually as complex mixtures. Furthermore, under *in vitro* conditions, anthocyanin-rich extracts and especially anthocyanidins are known to be of limited stability [22]. Important degradation products of anthocyanidins *in vitro* and *in vivo* are protocatechuic acid and phloroglucinol aldehyde which therefore might contribute to the biological properties of anthocyanin-rich extracts [22, 23].

Present investigations centered on the potential interference of such natural anthocyanin mixtures with human DNA topoisomerases and its relevance for DNA integrity in human colon carcinoma cells. We choose two standardized anthocyanin-rich preparations (A and B) derived from bilberries and grapes, respectively, and selected degradation products. Overall, the aim of the work was to gauge the potential detrimental impact of consumption of berry extracts in the form of intact foods or food supplements on the effectiveness of topoisomerase poisons applied with therapeutic intent in cancer therapy.

2 Materials and methods

2.1 Chemicals

Camptothecin, doxorubicin, phloroglucinol aldehyde, protocatechuic acid and menadione were purchased from Sigma-Aldrich (Taufkirchen, Germany). Extract A, a standardized extract of bilberries, and extract B, an extract of red grape pomace and waste product of red wine and grape juice production, were kindly provided by Indena SpA (Milan, Italy). A is generated by Indena and B by Bagnarese SpA (Bagnara di Romagna, Italy). A is prepared by an industrial proprietary process ensuring constant and reproducible anthocyanin composition (36% w/w). Anthocyanin constituents of A and their approximate relative abundance (% in brackets, by HPLC analysis) compared with total anthocyanins (= 100%) are delphinidin-3-galactoside (16%), delphinidin-3-glucoside (14%), and delphinidin-3-arabinoside (12%); cyanidin-3-galactoside (10%), cyanidin-3-glucoside (11%), and cyanidin-3-arabinoside (8%); petunidin-3-galactoside (3%), petunidin-3-glucoside (8%), and petunidin-3-arabinoside (2%); peonidin-3-galactoside (1%), peonidin-3-glucoside (4%), and peonidin-3-arabinoside (1%); malvidin-3-galactoside (3%), malvidin-3-glucoside (5%), and malvidin-3-arabinoside (2%) (Indena, datasheet). The other constituents of A are polyphenols other than anthocyanins (phenolic acids, flavonols, proanthocyanidins ∼18%), carbohydrates (∼20%), aliphatic alcohols (∼9%), fats (∼0.04%), nitrogen compounds (∼1%), and ash (∼0.7%) with the remaining ∼15% undefined. The anthocyanin content of B is 22% w/w. It contains the following five anthocyanins with their approximate relative abundance in percentage of total anthocyanins (= 100%) in parentheses: delphinidin-3-glucoside (7%), cyanidin-3-glucoside (16%), petunidin-3-glucoside (12%), peonidin-3-glucoside (20%), and malvidin-3-glucoside (40%) (Bagnarese, datasheet). Minor anthocyanin constituents, which altogether amount to 5% of total anthocyanins, are delphinidin-3-glucoside acetate, cyanidin-3-glucoside acetate, petunidin-3-glucoside acetate, peonidin-3-glucoside acetate, malvidin-3-glucoside acetate, delphinidin-glucoside *p*-cumarate, cyanidin-3-glucoside *p*-cumarate, petunidin-3-glucoside *p*-cumarate, peonidin-3-glucoside *p*-cumarate, and malvidin-3-glucoside *p*-cumarate. The other constituents of B are polyphenols other than anthocyanins (flavan-3-ols, *i.e.* catechins and procyanidins ∼12%), carbohydrates (∼25%), organic acids (∼14%), aliphatic alcohols (∼9%), nitrogen compounds (∼1%), and

ash (~1%) with the remaining ~16% undefined. The identity of the major anthocyanins in either mixture was confirmed by HPLC mass spectrometric analysis [5].

2.2 Cell culture

HT29 (human colon adenocarcinoma, ACC 299) cells were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). The cell culture was performed in humidified incubators (37°C, 5% CO_2). HT29 cells were cultured in DMEM as previously reported [21].

2.3 Suppression of hydrogen peroxide formation

Single polyphenols and polyphenol-rich extracts are known to generate substantial amounts of hydrogen peroxide (H_2O_2) within the cell culture medium, possibly influencing biological endpoints *in vitro* [22, 24, 25]. Therefore, the formation of H_2O_2 under the applied cell culture conditions was monitored as described previously [22]. Briefly, HT29 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% fetal calf serum) conditions for 24 h and then incubated with the test 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. Due to the observed H_2O_2-accumulating effects of the tested anthocyanin rich extracts, all experiments in cell culture systems were performed in the presence of catalase (100 U/mL), suppressing the level of H_2O_2 to the range of the solvent control (data not shown).

2.4 Sulforhodamine B assay

The effects on cell growth were determined according to the method of Skehan *et al.* [26] with slight modifications [22]. 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 for 72 h in serum-containing medium. For the pre- and coincubation experiments, cells were pretreated for 60 min with extract A, B, followed by coincubation of doxorubicin, campthothecin and A, B for 23 h in serum-containing medium. Incubation was stopped by the 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 Preparation of nucleic extract

Nucleic extract was prepared from Human MCF-7 cells published in [20].

2.6 Topoisomerase activity

2.6.1 Topoisomerase I

The interference with the catalytic activity of topoisomerase I was determined using a plasmid relaxation assay. Plasmid DNA (250 ng of pUC 18) was incubated in a final volume of 30 μL containing 0.3 μL of nucleic extract; 10 mM Tris-HCl; pH 7.9; 100 mM KCl; 10 mM $MgCl_2$; 0.5 mM DTT; 0.5 mM EDTA; and 0.03 mg/mL BSA for 30 min at 37°C. The reaction was stopped by the addition of one-tenth volume of 5% w/v SDS. Afterwards, the samples were treated with 1 mg/mL proteinase K at 37°C for 30 min. Gel electrophoresis was performed at 4.5 V/cm in 1% w/v agarose gels with Tris-acetate/EDTA buffer (40 mM Tris-HCl; 1 mM EDTA, pH 8.5; and 20 mM acetic acid). Subsequently, the gel was stained in 10 μg/mL ethidium bromide solution for 20 min. The fluorescence of ethidium bromide was detected with the LAS-3000 system (Fujifilm, Raytest, Germany).

2.6.2 Topoisomerase II

The catalytic activity of topoisomerase II was detected using catenated kinetoplast DNA (kDNA) in a cell-free decatenation assay. The kDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb), which can be released by topoisomerase II. kDNA (200 ng) (TopoGen, Columbus, OH, USA) was incubated in a final volume of 30 μL containing 40 ng of topoisomerase IIα (50 mM Tris, pH 7.9; 120 mM KCl; 10 mM $MgCl_2$; 1 mM ATP; 0.5 mM DTT; 0.5 mM EDTA; and 0.03 mg/mL BSA) at 37°C for 1 h. The reaction was stopped by the addition of one-tenth volume of 1 mg/mL proteinase K in 10% w/v SDS followed by incubation at 37°C for 30 min. Gel electrophoresis was performed in the absence of ethidium bromide at 60 V for 3 h in a 1% w/v agarose gels with Tris-acetate/EDTA buffer (40 mM Tris; 1 mM EDTA, pH 8.5; and 20 mM acetic acid). Subsequently, the gel was stained in 10 μg/mL ethidium bromide solution for 20 min. The fluorescence of ethidium bromide was detected with the LAS-3000 system (Fujifilm, Raytest, Germany) with the Image Analyzer software (AIDA 3.52) for quantification. Arbitrary Light Units were plotted as test over control (%).

2.7 Isolating *in vivo* complexes of enzyme to DNA (ICE bioassay)

About 1.2 million HT29 cells were spread into Petri dishes (two Petri dishes for one concentration) and allowed to grow

for 48 h. Thereafter, the cells were incubated with the solvent control (1% v/v), camptothecin (100 μM), doxorubicin (10 μM), A or B for 1 h under serum-free conditions in the presence of catalase (100 U/mL). For the coincubation experiments, HT29 cells were preincubated for 30 min with the solvent control (0.2% v/v DMSO), the respective berry extract (A or B), or the degradation product protocatechuic acid, followed by 1 h coincubation of the respective compounds with 100 μM camptothecin or 10 μM doxorubicin. Thereafter, the ICE bioassay was performed with slight modifications as described previously by [24]. The medium was removed, and the cells were abraded at room temperature in 3 mL TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1% w/v, *N*-laurylsarcosyl sodium salt). Cell lysates were layered onto a cesium chloride gradient in polyallomer tubes (14 mL; SW40, Beckman Coulter GmbH, Krefeld, Germany). One gradient consisted of four layers (2 mL/layer) of cesium chloride with a decreasing density from the bottom to the top. The tubes were centrifuged at 100 000 × *g* for 24 h at 20°C. The gradients were fractionated (300 μL/fraction) from the bottom of the tubes. The fractions were blotted onto a nitrocellulose membrane using a slot blot apparatus (Minifold II, Whatman®/Schleicher & Schuell, Dassel, Germany). Topoisomerase protein was detected using rabbit polyclonal antibodies specific for either topoisomerase I (100 kDa), topoisomerase IIα (170 kDa), or topoisomerase IIβ (180 kDa) at a 1:250 or 1:500 dilution, respectively. An anti-rabbit IgG peroxidase conjugate (1:2000) was used as the secondary antibody. All antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The respective chemoluminescent signals (Santa Cruz Biotechnology) were analyzed using the LAS 3000 imager with the AIDA Image Analyzer 3.52 software for detection (Fujifilm, Raytest).

2.8 Single-cell gel electrophoresis (comet assay)

HT29 cells (3×10^5 in 5 mL medium containing 10% fetal calf serum v/v) were spread into Petri dishes (5 cm) and allowed to grow for 48 h prior to treatment. In the experiments with single compounds, HT29 cells were treated for 1 h with the solvent control (1% v/v DMSO), 20 μM menadione, A or B in serum-free medium in the presence of catalase (100 U/mL). For the coincubation experiments, HT29 cells were treated like described above. Thereafter, aliquots containing 70 000 cells were centrifuged (5 min, 200 × *g*). Single-cell gel electrophoresis was performed encoded according to the method of Gedik *et al.* [27]. The resulting cell pellet was resuspended in 65 μL 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 removing the cover glass, slides were immersed for 1 h at 4°C in lysis solution (89 mL lysis stock solution, 2.5 mM sodium chloride, 100 mM EDTA, 10 mM Tris, 1% w/v *N*-laurylsarcosyl sodium salt; 1 mL Triton-X-100, 10 mL DMSO). For the additional detection of oxidative DNA damage, slides were washed three times in enzyme buffer (40 mM HEPES, pH 8.0, 0.1 M potassium chloride, 0.5 mM EDTA, 0.2 mg/mL BSA), covered with 50 μL of either enzyme buffer or formamidopyrimidine-DNA glycosylase (Fpg-enzyme) and incubated for 30 min at 37°C. Subsequently, DNA was allowed to unwind (300 mM NaOH, 1 mM EDTA, pH 13.5, 20 min, 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 ($\lambda_{ex} = 546 \pm 12$ nm; $\lambda_{em} \geq 590$ nm). Slides were subjected to computer-aided image analysis (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 parameterized with respect to tail intensity (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.

3 Results

3.1 Effect of anthocyanin-rich berry extracts on cell growth *in vitro*

The effect of A and B on the growth of the cell line HT29 was determined using the sulforhodamine B assay. As previously reported, polyphenols generates hydrogen peroxide with so far unknown cell media constituents [22, 24, 25]. Therefore, all cell culture experiments were performed in the presence of catalase (100 U/mL) to exclude artificial hydrogen peroxide mediated effects. Incubation of HT29 cells with A or B up to 500 μg/mL for 72 h led to an inhibition of cell growth but without reaching an IC_{50} value (Fig. 1).

3.2 Effects of anthocyanin-rich berry extracts on the catalytic activity of topoisomerases I and II

Topoisomerase I activity was determined as the relaxation of supercoiled pUC18 plasmid DNA by nucleic extract from MCF-7 cells. The assay was performed in the absence of ATP to exclude topoisomerase II activity. Both extracts potently inhibited the catalytic activity of topoisomerase I at a concentration of 25 μg/mL (Fig. 2A, lanes 4 and 8). The

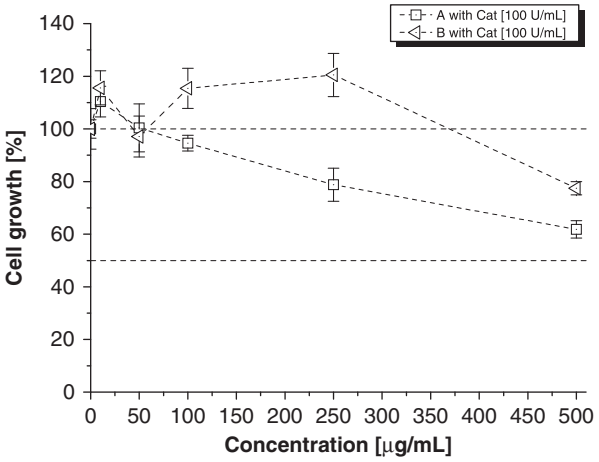

Figure 1. Inhibition of cell growth in HT29 cells by A and B in the presence of catalase (CAT, 100 U/mL) determined in the sulforhodamine B assay (72 h). Growth inhibition was calculated as percent survival of treated cells over control cells (treated with the solvent 1% DMSO) × 100 (T/C, %). The values given are the mean ± SD of four independent experiments, each performed in quadruplicates.

catalytic activity of topoisomerase I was completely suppressed by either extract at concentrations ≥ 50 μg/mL (Fig. 2A, lanes 5–6 and 9–10). The topoisomerase I poison camptothecin was used as the positive control (Fig. 2A, lane 11).

To examine whether anthocyanin-rich extracts can inhibit topoisomerase II, we determined the catalytic activity of recombinant human topoisomerase II in a decatenation assay. In its catenated form, kDNA cannot enter the agarose gel, whereas minicircles, released by catalytically active topoisomerase II, migrate into the gel (Fig. 2B, compare lane 1 with 2). A and B were found to inhibit the catalytic activity of topoisomerase II at concentrations exceeding 1 μg/mL. The catalytic activity of topoisomerase IIα was fully blocked at 5 μg/mL A or B, respectively (Fig. 2B, lanes 5–6 and 9–11). Doxorubicin, a topoisomerase II poison, served as the positive control (Fig. 2B, lane 12). Comparable results were obtained with recombinant topoisomerase IIβ (Fig. 2C).

In conclusion, the results indicate that anthocyanins exhibit inhibitory preference for the topoisomerase II isoenzyme family without discriminating between the two topoisomerase II isoforms in the cell-free test systems.

3.3 Effects of anthocyanin-rich berry extracts on topoisomerase binding to DNA in HT29 cells

The ICE bioassay was used to determine the amount of topoisomerase covalently linked to the DNA. Treatment of HT29 cells for 1 h with camptothecin (100 μM) increased the amount of topoisomerase I covalently linked to DNA (Fig. 3A, fractions 6–9), whereas, as expected, doxorubicin

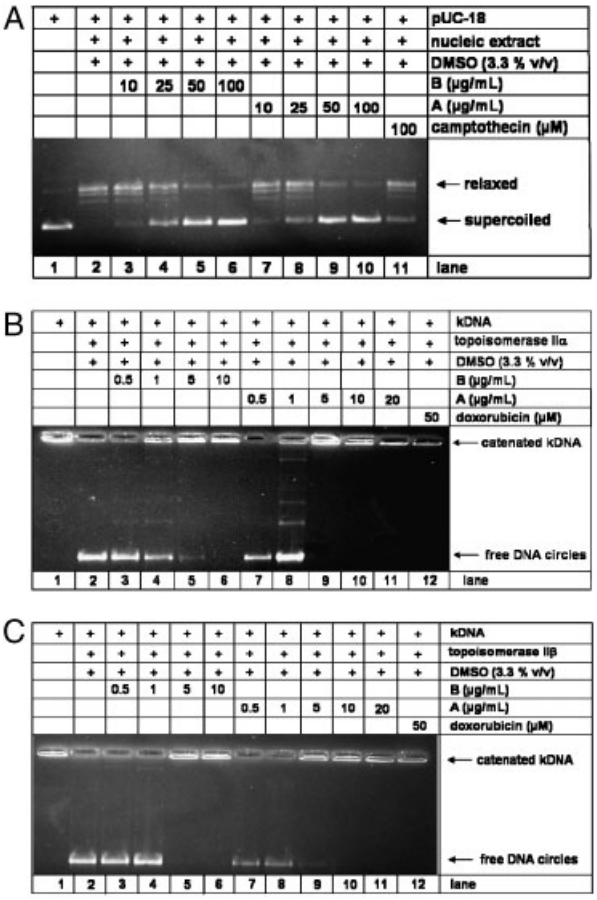

Figure 2. (A) Representative gel of relaxation assay for topoisomerase I. Supercoiled pUC18 plasmid DNA (250 ng, lane 1) was incubated for 30 min at 37°C with nucleic extract from MCF-7 cells (lanes 2–11) in the presence of 3.3% DMSO (lane 2) or of increasing concentrations of B (lanes 3–6) or A (lanes 7–10), respectively, or with camptothecin as a positive control (lane 11). The reaction was stopped with 1% w/v SDS, and after digestion with proteinase K, samples were separated in 1% agarose gels in the absence of ethidium bromide. The ethidium bromide-labeled DNA was documented under UV light by digital photography. Active topoisomerase I converts the supercoiled pUC18 plasmid DNA into the relaxed form (see arrows). Shown is a representative gel out of three independent experiments. (B) Representative gel of a decatenation assay with topoisomerase IIα. Lane 1 shows catenated kDNA not exposed to topoisomerase IIα enzyme. Active topoisomerase II releases single free DNA circles from the catenated DNA network treated with the solvent control DMSO (lane 2). Increasing concentrations of B (lanes 3–6) or A (lanes 7–11) show an inhibitory effect of the catalytic activity ≥ 1 μg/mL as well as the positive control doxorubicin (lane 12). (C) Representative gel of a decatenation assay with topoisomerase IIβ. Increase in concentrations of A or B shows an inhibitory effect of the catalytic activity ≥ 5 μg/mL (lanes 3–11) as well as the positive control doxorubicin (lane 12).

(10 μM) enhanced the level of topoisomerases IIα and IIβ in the DNA peak fractions (Fig. 3A, fractions 6–10). In contrast, neither A nor B alone up to 100 μg/mL affected the extent of DNA/topoisomerase binding (Fig. 3A). These

findings are in accordance with the hypothesis that anthocyanins act as catalytic inhibitors targeting topoisomerases prior to their binding to the DNA within intact colon carcinoma cells. If this hypothesis is indeed correct, one may expect that anthocyanins can diminish the formation of the transient DNA–topoisomerase complex, thus undermining the effect of topoisomerase poisons which target the cleavable complex.

To test this hypothesis, the ICE assay was used for competition studies in HT29 cells (Fig. 3B–D). HT29 cells were pretreated with A or B for 30 min and thereafter coincubated for 1 h with anthocyanin-rich extract and camptothecin (100 μM) or doxorubicin (10 μM) in combination. At a concentration $\geq$10 μg/mL, A decreased the level of doxorubicin-stabilized topoisomerase IIα/DNA intermediates (Fig. 3C). However, A at concentrations of up to 30 μg/mL did not alter the levels of camptothecin-induced topoisomerase I/DNA intermediates (Fig. 3B) and doxorubicin-stabilized topoisomerase IIβ/DNA complexes (Fig. 3D). The amount of topoisomerase IIα/DNA complexes induced by doxorubicin (10 μM) was effectively diminished by coincubation with B $\geq$10 μg/mL (Fig. 3C), whereas only a marginal decrease of topoisomerase IIβ/DNA intermediates was detected (Fig. 3D). In accordance to A, amounts of cleavable complexes stabilized by camptothecin were not affected by coincubation with B up to 30 μg/mL (Fig. 3B).

In intact HT29 cells, the anthocyanin-rich extracts exhibited inhibitory preference for the topoisomerase IIα isoform. In this competition study, the degradation product protocatechuic acid was included in the tests to discriminate between the effects of intact and degraded anthocyanins. In contrast to the extracts, the degradation product protocatechuic acid did not alter the amount of the camptothecin- and doxorubicin-induced topoisomerases I and II/DNA complexes up to 250 μM protocatechuic acid (data not shown).

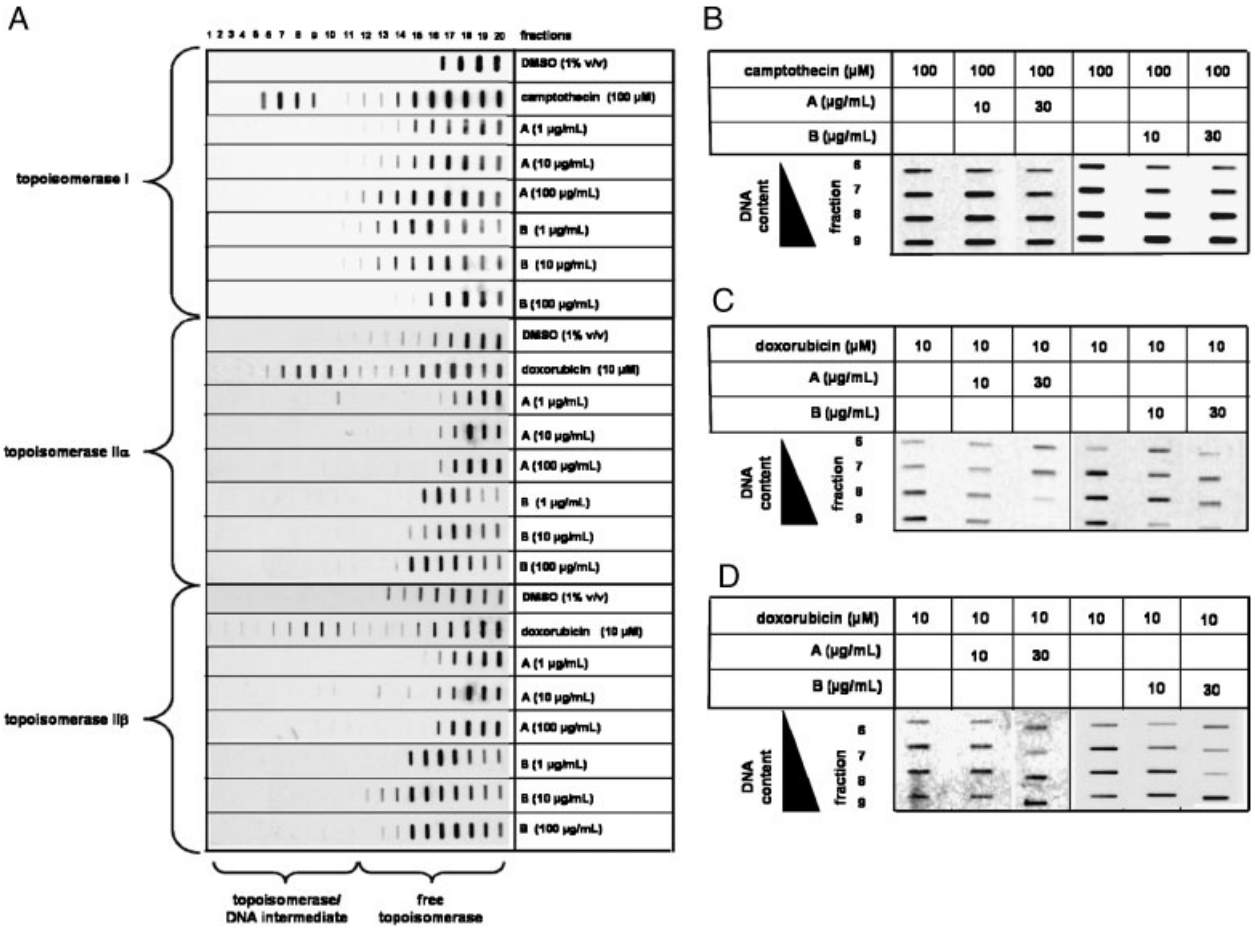

Figure 3. (A) Detection of the level of cleavable complexes in HT29 cells using the ICE assay. Cells were treated with the respective test compound for 1 h. Representative gels of three independent experiments were shown. Impact of A and B in the presence of catalase (100 U/mL) on the (B) camptothecin-stabilized topoisomerase I/DNA intermediates and (C+D) on the doxorubicin-stabilized topoisomerase IIα/IIβ/DNA intermediates in the ICE assay. HT29 cells were pretreated with the extracts for 30 min followed by coincubation with the respective extract and camptothecin (100 μM) or doxorubicin (10 μM) for 1 h. Representative gels of four independent experiments were shown.

3.4 Effects of anthocyanin-rich berry extracts and degradation products on DNA integrity

The effect of anthocyanin-rich extracts on DNA integrity was reflected by DNA strand breaks measured by single-cell gel electrophoresis (comet assay) in HT29 cells incubated with berry extracts. In addition to the basic comet assay, treatment with the base excision DNA repair enzyme Fpg was performed to indicate potential oxidative DNA damage. Extract A increased the rate of DNA strand breaks in HT29 cells in comparison to the respective solvent control (1% DMSO v/v), whereas no enhancement of Fpg-sensitive sites was observed (Fig. 4). The level of DNA strand breaks as well as amount of Fpg-sensitive sites was not enhanced by the treatment of HT29 cells with B in comparison to the respective solvent control. Only at the highest concentration of B (50 μg/mL), the rate of Fpg-sensitive sites was increased compared with the respective basic DNA strand breaks (without Fpg) (Fig. 4). The degradation products protocatechuic acid and phloroglucinol aldehyde do not exhibit any DNA-damaging properties in the basic comet assay up to 100 μM (data not shown).

3.5 Effects of anthocyanin-rich berry extracts on DNA strand breaking properties of topoisomerase poisons and outcome for cytotoxicity

Considering the activity of the berry extracts as catalytic topoisomerase inhibitors, we addressed the question whether A or B modulates the DNA-damaging properties of camptothecin or doxorubicin. HT29 cells were pretreated for 30 min with the respective anthocyanin-rich extract, followed by incubation for 1 h with camptothecin (100 μM, Fig. 5A) or doxorubicin (10 μM, Fig. 5B) in combination with the respective extract. Treatment of HT29 cells with camptothecin (Fig. 5A) or doxorubicin (Fig. 5B) alone resulted in a significant increase in DNA strand breaks.

Either extract diminished the DNA-damaging effects of topoisomerases I poison camptothecin in a concentration-dependent manner (Fig. 5A). Pre- and coincubation with

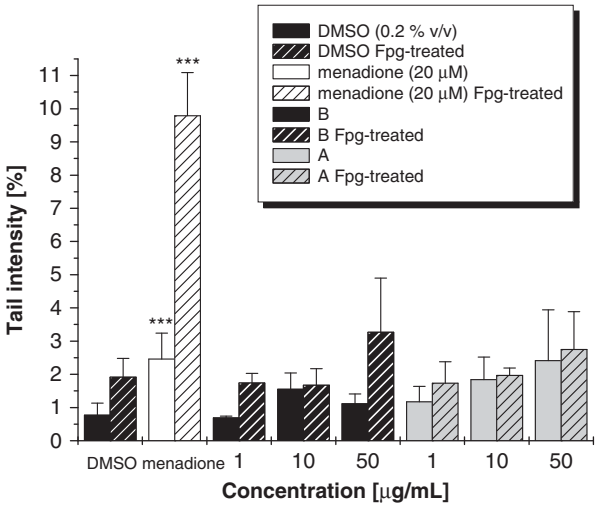

Figure 4. Single-cell gel electrophoresis (comet assay) with (A) and (B) treated HT29 cells in the presence of catalase (100 U/mL). The DNA repair enzyme formamidopyrimidine-DNA glycosylase (Fpg) was used to detect the potential oxidative DNA damage. The cells were treated 1 h with A or B. The redoxcycler menadione (20 μM) was included in the testing as a positive control for oxidative DNA damage (without catalase).

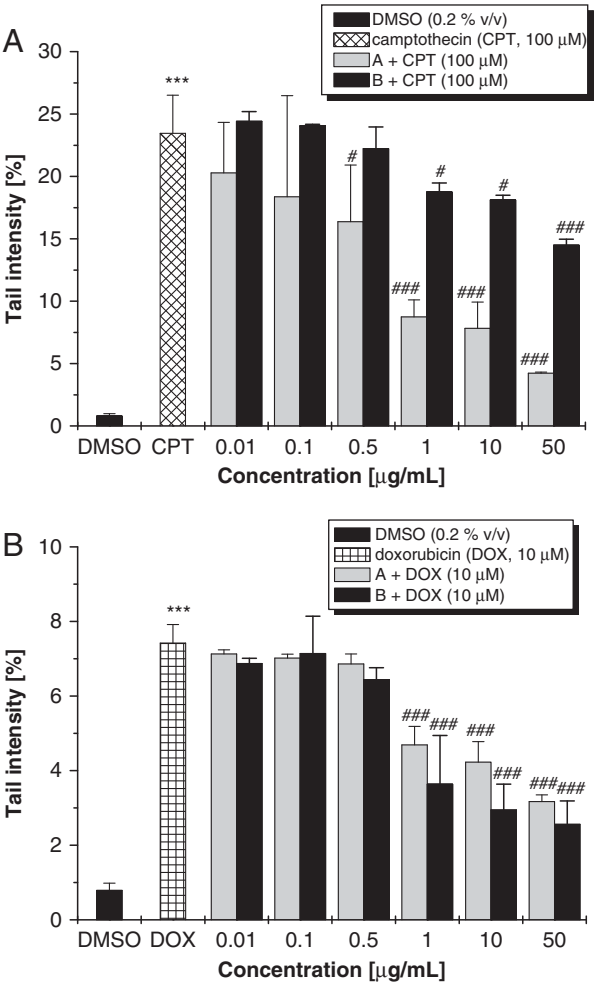

Figure 5. Impact of A and B in the presence of catalase (100 U/mL) on (A) camptothecin – or (B) doxorubicin – induced DNA damage. For the coincubation experiments, HT29 cells were preincubated for 30 min with the solvent control (0.2% DMSO) or the anthocyanin-rich extract, followed by 1 h coincubation of A or B with (A) 100 μM camptothecin or (B) 10 μM doxorubicin. The data presented are the mean ± SD of at least three independent experiments, each performed in duplicate. Significances indicated refer the significance level compared with the respective control (Mann–Whitney *U*-test, $^{***}p<0.001$) or to the respective topoisomerase poison (Mann–Whitney *U*-test, camptothecin or doxorubicin; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$).

A at concentrations of $\geq 0.5\,\mu g/mL$ resulted in a significant decrease of camptothecin-mediated DNA strand breaks. A showed a more pronounced effect compared with B as it decreased the DNA damage at concentrations of $\geq 1\,\mu g/mL$. With respect to the topoisomerases II poison doxorubicin, both berry extracts suppressed its strand breaking properties to a similar extent (Fig. 5B).

Furthermore, the effects of A and B on doxorubicin and camptothecin-induced DNA damage were investigated with respect to cytotoxicity. HT29 cells were pretreated with A or B (50 μg/mL) for 60 min and thereafter coincubated for 23 h with A or B (50 μg/mL) and doxorubicin (0.01–100 μM) in combination (Fig. 6A). In contrast to the incubation with doxorubicin alone, pre- and coincubation of doxorubicin with A reduced the cytotoxic properties of doxorubicin. In the highest concentration, doxorubicin (100 μM) suppressed cell growth to $32.1 \pm 16.3\%$ in relation to the solvent control, whereas coincubation with A potently decreased cytotoxicity ($51.5 \pm 9.0\%$; Fig. 6A). Furthermore, B suppressed the cytotoxicity of doxorubicin in a more pronounced way than A (87.3 ± 5.9; Fig. 6A). According to the above-described coincubation protocol, the effects of A and B on camptothecin-induced cytotoxicity were investigated. In HT29 cells, 100 μM camptothecin suppressed cell proliferation to $59.8 \pm 7.9\%$. In contrast pre- and coincubation with A or B resulted in reduced cytotoxicity in the highest concentration of camptothecin (100 μM) with $79.4 \pm 9.5\%$ cell growth, respectively, 78.4 ± 26.4 for pre- and coincubation with compound B (Fig. 6B).

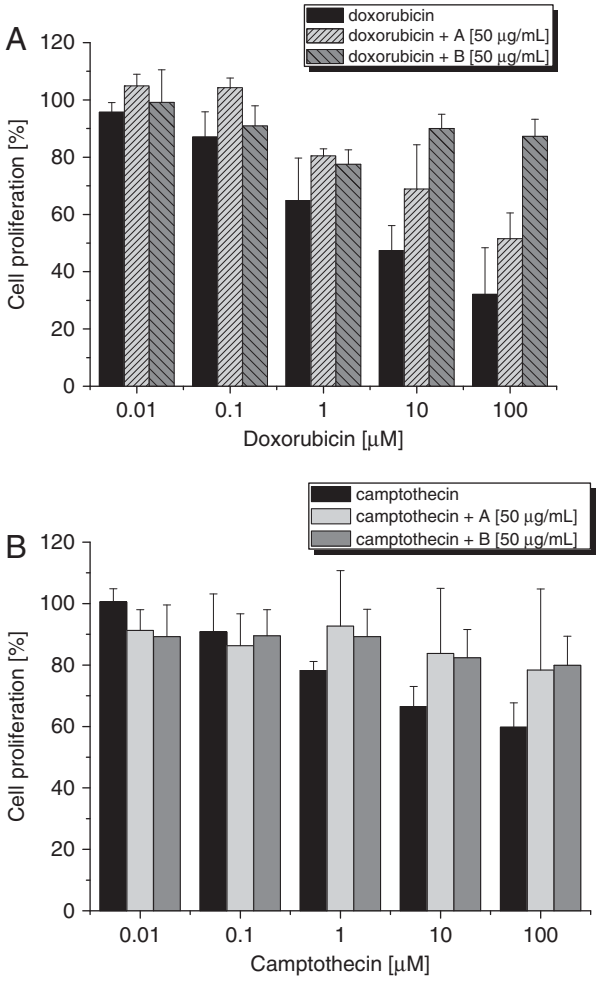

Figure 6. Inhibition of cell growth in HT29 cells by doxorubicin (24 h) and pre- (1 h) and coincubation (23 h) of doxorubicin and A or B (50 μg/mL) in the presence of catalase (CAT, 100 U/mL) determined in the sulforhodamine B assay (A). Inhibition of cell growth in HT29 cells by camptothecin (24 h) and pre- (1 h) and coincubation (23 h) of camptothecin and A or B (50 μg/mL) in the presence of catalase (100 U/mL) determined in the sulforhodamine B assay (B). 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 four independent experiments, each performed in quadruplicates.

4 Discussion

Anthocyanidins have been shown to potently inhibit the growth of human-derived carcinoma cells, and this inhibition is probably the corollary of their ability to impair enzymes involved in the regulation of cell proliferation and apoptosis [9, 28, 29]. We showed in the present study that anthocyanin-rich berry extracts inhibit the growth of HT29 colon carcinoma cells (Fig. 1), consistent with the results of Jing *et al.* [8]. Anthocyanidins probably released from anthocyanins undergo rapid degradation under cell culture conditions generating respective phenolic acids and phloroglucinol aldehyde [23]. Phenolic acids, like protocatechuic acid, were shown to have marginal effects on cell growth [22]. Therefore, a substantial contribution of the degradation products to the growth inhibitory properties of the anthocyanin-rich extracts seems unlikely. Many flavonoids suppress the activity of human topoisomerases and therefore affect DNA integrity [13, 19, 20]. Topoisomerase inhibitors can be categorized into two classes. Members of the first group act as pure catalytic inhibitors without stabilizing the covalent enzyme/DNA intermediate. The second group consists of topoisomerase poisons, which form a covalent ternary complex with the DNA–topoisomerase intermediate [16, 17].

Anthocyanidins bearing vicinal hydroxy groups in the B-ring, exemplified by cyanidin and delphinidin, have been shown to act as pure catalytic topoisomerase inhibitors, whereas other polyphenols like genistein or (−)-epigallocatechin-3-gallate exhibit topoisomerase poisoning properties [12, 13, 19, 21]. In the study presented here, berry extracts rich in anthocyanins were found to potently diminish topoisomerase I and II activity (Fig. 2A–C). In cell-free test systems, both extracts completely inhibited topoisomerase I activity at the same concentration range ($\geq 50\,\mu g/mL$). In comparison, the aglycones cyanidin and delphinidin

affect topoisomerase I and II activity in concentrations $\geq$7.5 μM [20]. Of note, both anthocyanin-rich extracts, irrespective of their differing polyphenol composition, inhibited topoisomerase II more potently than topoisomerase I.

Interestingly, the isolated glycosides cyanidin-3-glucoside and delphinidin-3-rutinoside failed to affect topoisomerase activity at concentrations up to 100 μM (unpublished data). These findings are consistent with reports according to which glycosides are less potent effectors of potential cellular targets compared with the free aglycones [30–32]. In contrast, extracts containing complex polyphenolic mixtures were found to potently affect the topoisomerase activity implicating a contribution of yet unknown constituents, or synergistic or additive effects. One may speculate that oligomeric and/or polymeric procyanidins contained in berries and grapes might have contributed to the modulation of topoisomerase activity by the extracts observed here, because some procyanidins have been previously characterized as topoisomerase inhibitors [12, 33, 34].

As anthocyanin-rich extracts were shown to act as catalytic topoisomerase inhibitors without entailing enzyme/DNA association, we surmised that they might prevent the formation of the cleavable complex. We explored whether the topoisomerase cleavable complex stabilized by an enzyme poison is affected by anthocyanin-rich extracts in intact human colon carcinoma cells. Under cell-free conditions, complete inhibition of the activity of topoisomerase I was detected (Fig. 2A). In intact HT29 cells, A or B (Fig. 3B) were insufficient to counteract topoisomerase poisoning by camptothecin. Consistent with the decatenation assay, A and B at concentrations of $\geq$10 μg/mL potently suppressed the topoisomerase IIα/DNA intermediate stabilized by doxorubicin, whereas suppression of the ternary doxorubicin/topoisomerase IIβ/DNA complex required at least 30 μg/mL B. In contrast to the studies using recombinant topoisomerases IIα and β, where A or B exhibited no preference for one isoform, a more pronounced effect on topoisomerase IIα was observed in HT29 cells. Moreover, the tested degradation product protocatechuic acid was not able to prevent topoisomerase poisoning by camptothecin or doxorubicin. All these results suggest that anthocyanin-rich extracts and not their respective degradation products interfere with human topoisomerase prior to binding of the enzyme to DNA, as has been reported previously for the anthocyanidin delphinidin [21]. Delphinidin as a free aglycone has not been detected in berry extracts [5, 9]. Nevertheless, generation of delphinidin from precursor anthocyanins *in vivo* (*e.g.* in the gastrointestinal tract) cannot be excluded, so that it could contribute to the topoisomerase-inhibitory properties of the berry extracts. A comparison of the suppression of the doxorubicin-induced cleavable complex between A or B and delphinidin intimates that other topoisomerase II-active compounds were probably present in the extract. The impact of isolated anthocyanins on topoisomerases in human cancer cells is poorly understood. In the light of the reported effectiveness of glycosylated flavonoids on potential cellular targets in comparison to their respective aglycones [30–32], a contribution of yet unknown berry extract constituents to anti-topoisomerase activity is possible.

With respect to the potent inhibition of the catalytic activity of topoisomerases by A and B, we investigated the effect of these extracts on DNA integrity in the human colon carcinoma cell line HT29. Anthocyanidins have shown before to cause DNA strand breaks in HT29 cells after only 1 h of incubation [20]. In contrast, the anthocyanin-rich extracts as well as the tested degradation products did not induce DNA strand breaks in the comet assay. Consistent with the results for delphinidin [35], neither A nor B increased the amount of Fpg-sensitive sites indicative of oxidative stress DNA damage. Several flavonoids, especially topoisomerase poisonous ones, possess DNA-damaging properties [36, 37]. It is conceivable that under *in vivo* conditions in the gastrointestinal tract, further products of berry extract degradation with putative DNA strand breaking effects are generated.

Under *in vivo* conditions anthocyanins and their respective aglycones have been reported to possess only limited systemical bioavailability [38]. Of special interest for the presented study here was the local availability of the anthocyanins in the colon. *In vivo* studies with ileostoma probands have shown that depending on the sugar moiety and the degree of methoxylation, blueberry anthocyanins up to 80% could reach the colon unmetabolized [39]. Thus, although the systemic bioavailability appears to be limited in the gastrointestinal tract, high local anthocyanin concentrations might be achieved, especially by consumption of biofactor-enriched functional food. Furthermore, concentrations used in the present *in vitro* study are in a consumer relevant range for local effectiveness of anthocyanins. Consumption of $\geq$100 mg of anthocyanins could be achieved easily with daily intake of berries and other fruits [3].

In the present study, we found that A and B at concentrations of $\geq$1 μg/mL significantly suppressed the DNA strand breaking effect of camptothecin. A was a more potent suppressor of camptothecin-induced DNA damage than B but both extracts diminished the DNA strand breaking properties of doxorubicin to the same extent (Fig. 5B). In contrast, the degradation products protocatechuic acid and phloroglucinol aldehyde had no effect on the DNA strand breaking properties of camptothecin and doxorubicin (data not shown). Concomitantly, coincubation of doxorubicin with extract A and in a more pronounced way for extract B led to decreased cytotoxic effects on HT29 cells in comparison to doxorubicin incubation (Fig. 6A). Furthermore, coincubation of camptothecin with the extracts A and B revealed a slight reduction in the cytotoxic effects of camptothecin (Fig. 6B). These properties of A and B were observed in concentrations of a potent suppression of DNA strand breaks indicating a role of this mode of action for the attenuation of the cytotoxicity of doxorubicin and camptothecin.

The lack of effectiveness of A against cleavable complex stabilization by camptothecin suggests that as yet unknown mechanisms of actions contribute to its potent DNA protective effects. One may proffer modulation of DNA repair as the putative candidate mechanism. Consistent with this notion, we have previously observed that the treatment of HT29 cells with delphinidin for 1.5 h increased the relative transcript level of genes involved in DNA double strand break repair [21].

In the context of the increased use and enrichment of anthocyanins in food and food supplements, the effects observed here must be taken into account. In the literature, a wide range of putative beneficial health effects have been attributed to anthocyanins including chemopreventive properties in animal and human intervention studies ([4–6, 40], reviewed in [41]). The proposed mechanisms of action comprise a wide range of cellular effects, *e.g.* anti-oxidative properties and the modulation of signaling elements associated with cell growth and apoptosis [9, 10, 28, 29]. However, single studies revealed potentially undesired effects of anthocyanins like prooxidative properties and the inhibition of human topoisomerases [11–14, 42, 43]. Here, the observed suppression of DNA-damaging properties of topoisomerase poisons and the subsequent attenuation of cytotoxicity by anthocyanin-rich extracts might be a desirable effect for healthy individuals being exposed to dietary topoisomerase poisons like (−)-epigallocatechin-3-gallate [12, 14]. In contrast, the same mechanism of action might be of relevance during chemotherapy. A coapplication with chemotherapeutical topoisomerase poisons may lead to a reduced effectiveness of the therapy. Therefore, further studies *in vivo* on the role of anthocyanins during chemotherapy are indispensable and demand further investigations.

In summary, the present study shows that anthocyanin-rich extracts potently diminished the catalytic activity of topoisomerases with a preference for topoisomerase II in cell-free systems. In intact colon carcinoma cells, stabilization of the cleavable complex after extract treatment was not observed, indicative of pure catalytic topoisomerase inhibition. We further demonstrated that the interference of the extracts with the regulation of DNA topology did not result in DNA strand breaks. However, the anthocyanin-rich extracts potently suppressed the DNA-damaging properties of the topoisomerase poisons camptothecin and doxorubicin, but the reduction of topoisomerase poison-induced DNA strand breaks could not be exclusively linked to effects on cleavable complex formation. These results raise the possibility that high intake of berry extracts may protect DNA and thus counteract the therapeutic effectiveness of orally applied topoisomerase poisons.

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