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Anthocyanin-Rich Blackberry Extract Suppresses the DNA-Damaging Properties of Topoisomerase I and II Poisons in Colon Carcinoma Cells

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ABSTRACT: In the present study, we addressed the question whether cyanidin-3-glucoside (C3G) or complex C3G-rich blackberry extracts affect human topoisomerases with special emphasis on the contribution of the potential degradation products phloroglucinol aldehyde (PGA) and protocatechuic acid (PCA). In HT29 colon carcinoma cells a C3G-rich blackberry extract suppressed camptothecin- (CPT-) or doxorubicin- (DOX-) induced stabilization of the covalent DNA−topoisomerase intermediate, thus antagonizing the effects of these classical topoisomerase poisons on DNA integrity. As a single compound, C3G (100 μM) decreased the DNA-damaging effects of CPT as well, but did not significantly affect those induced by DOX. At the highest applied concentration (100 μM), cyanidin protected DNA from CPT- and DOX-induced damage. Earlier reports on DNA-damaging properties of cyanidin were found to result most likely from the formation of hydrogen peroxide as an artifact in the cell culture medium when the incubation was performed in the absence of catalase. The suppression of hydrogen peroxide accumulation, achieved by the addition of catalase, demonstrated that cyanidin does not exhibit DNA-damaging properties in HT29 cells (up to 100 μM). The observed effects on topoisomerase interference and DNA protection against CPT or DOX were clearly limited to the parent compound and were not observed for the potential cyanidin degradation products PGA and PCA.

KEYWORDS: DNA damage, topoisomerase, cyanidin, camptothecin, doxorubicin

INTRODUCTION

Epidemiological studies emphasize that a diet rich in fruits and vegetables is associated with a lower incidence of cancer.[1,2] Anthocyanins represent a class of colored plant constituents comprising a color spectrum from red to blue that occur in many fruits and vegetables of the daily diet. In the United States, the consumption of anthocyanins has been estimated to be higher than that of other flavonoids such as quercetin.[3] In addition, based on their proposed health benefits, anthocyanins are widely consumed with functional foods and food supplements.[4]

Berry anthocyanins have been reported to exhibit anticancer properties in vivo.[5−8] In vitro studies have demonstrated that several overlapping mechanisms of action might contribute to their biological effects, including the inhibition of receptor tyrosine kinases,[9−11] the induction of apoptosis,[12−14] and the interference with human topoisomerases.[15−17] We previously reported that the free aglycone delphinidin, a so-called anthocyanidin, acts as a catalytic topoisomerase inhibitor in human colon carcinoma cells and diminishes the DNA-damaging properties of classical topoisomerase poisons.[17]

The bioavailability of anthocyanins is reported to be quite limited depending on the structure of the aglycone and the sugar moieties.[18,19] Under in vitro conditions, limited chemical stability of the nonglycosylated anthocyanidins has been repeatedly reported.[6,20−22] Several authors have confirmed that protocatechuic acid (PCA) is the dominant degradation product of cyanidin in cell culture.[20−22] In addition, anthocyanins as well as their respective aglycones, have been discussed to represent substrates for microbial degradation by human intestinal bacteria, also resulting in the formation of the respective benzoic acids and phloroglucinol aldehyde (PGA[23−25]).

So far, the cellular effects of the free aglycons, the anthocyanidins, have been part of numerous studies, whereas knowledge on the biological relevance of the glycosylated anthocyanins, as well as the formed degradation products, which have a higher chemical and microbial stabilities, is quite limited. Furthermore, several reports have discussed the question of whether the formation of hydrogen peroxide under cell culture conditions, resulting from the reaction of polyphenols with yet-unknown culture medium constituents, leads to in vitro artifacts, thereby interfering with the cellular effectiveness of the respective polyphenols.[26−30]

In the present study, we addressed the question whether interference with topoisomerases is limited to the free anthocyanidins or whether the respective glycosides, exemplified for

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cyanidin-3-glucoside (C3G), or even complex C3G-rich blackberry extracts also affect human topoisomerases. The aim of the study was to elucidate whether C3G-rich blackberry extracts, already available as food supplements, are likely to suppress the DNA-damaging properties of topoisomerase poisons used clinically for cancer treatment with special emphasis on the potential contribution of C3G and its corresponding degradation products. For comparison, the aglycone cyanidin, which is not present in native blackberry extracts but might be liberated after bacterial deglycosylation, was included in the testing.

MATERIALS AND METHODS

Chemicals. Cyanidin was purchased from Extrasynthèse (Genay, France), whereas camptothecin (CPT), doxorubicine (DOX), protocatechuic acid (PCA), phloroglucinol aldehyde (PGA), and catalase were obtained from Sigma-Aldrich Gmbh (Munich, Germany). Cyanidin-3-glucoside (C3G) was obtained from Carl Roth GmbH (Karlsruhe, Germany). For all assays, the compound solutions were prepared in dimethyl sulfoxide (DMSO) before the beginning of the experiment, without use of stored stock solutions.

Blackberry Extract. The blackberry extract was an XAD-7-purified extract and was obtained from Prof. P. Winterhalter, University of Braunschweig (Braunschweig, Germany). Anthocyanin measurements were carried out by high-performance liquid chromatography (HPLC)/diode array detector (DAD)/tandem mass spectrometry (MS/MS). Analysis was performed on an HP 1200 series instrument (Agilent Technologies, Waldbronn, Germany) equipped with a degaser, a binary HPLC pump, a DAD, an autoinjector, and a column oven. For HPLC conditions, please see ref 31. The eluent was recorded with diode array detection at 520 nm for quantification of the anthocyanins. For identification, the HPLC system was directly coupled to a hybrid triple-quadrupole linear ion-trap mass spectrometer (3200 QTrap; AB Sciex, Darmstadt, Germany), equipped with a TurboIonSpray source. The analytes were detected in positive-ion mode at a vaporizer temperature of 650 °C and an ion-spray voltage of 5.5 kV. Nitrogen was used for both curtain gas and collision gas, at 10 psi and medium, respectively. The collision energy and declustering potential were set to 10 V. Data acquisition was performed in full scan mode (enhanced mass spectrometry mode). Identification and quantification of further polyphenols (flavonoids, phenolic acids) was performed as described previously.[32,33] Quantification of all compounds (anthocyanins, flavonoids, phenolic acids) was performed by external calibration using commercially available reference compounds. Calibration curves for the different polyphenols were constructed in the range of 0.05−100 μM in which the response was linear. The quantification of those polyphenols that are not commercial available was based on a representative standard of the same polyphenol class. The limits of detection ranged between 200 fmol and 6 pmol.

The anthocyanin content in the blackberry extract was 100 mg/(g of extract). C3G, cyanidin-3-dioxalyl glucoside, cyanidin-3-rutinoside, cyanidin pentoside, and two further cyanidin derivatives were identified, with C3G [76 mg/(g of extract)] as the major compound (Table 1).

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). Cell culture medium was supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS). DMEM and the supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

Table 1. Composition of the Blackberry Extract

compound	concentration [μg/(g of extract)]
cyanidin-3-glucoside	76000
cyanidin pentoside	11600
cyanidin-3-dioxalylglucoside	2000
cyanidin-3-rutinoside	800
two unidentified cyanidin derivatives	9600
gallic acid	19.7
protocatechuic acid	46.0
coumaric acid	1.1
caffeic acid	3.5
epicatechin	31.9
quercetin	0.7
quercetin-3-glucoside	8.3
quercetin glucuronide	8.7
kaempferol glucuronide	35.7
procyanidin B2	9.8

Cell Growth Inhibition (SRB Assay). Briefly, 5000 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 (100 U/mL) for 72 h in serum-containing medium. Effects on cell growth were determined according to the method of Skehan et al.[34] previously described by Kern et al.[20]

Hydrogen Peroxide Formation. Briefly, 40000 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, with incubation in the presence of catalase (100 U/mL) in serum-containing medium. 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 (Taufkirchen, Germany) following the manufacturer's protocol.

Single-Cell Gel Electrophoresis (Comet Assay). HT29 cells (3×10^5 in 5 mL of serum-containing medium) were spread into Petri dishes (d = 5 cm) 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% (v/v) DMSO] or the test compounds in serum-free medium 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] or the respective test compound and were then coincubated for 1 h with 100 μM CPT or 10 μM doxorubicin. The comet assay was performed according to the method of Gedik et al.[35] previously reported by Esselen et al.[17]

Isolating in Vivo Complexes of Enzyme to DNA (ICE Bioassay). The ICE bioassay was performed with slight modifications as described previously by Esselen et al.[17] According to this method, 1.2×10^6 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) DMSO], CPT, DOX, or blackberry extract under serum-free conditions. For the coincubation experiments, HT29 cells were treated for 30 min with the solvent control [0.2% (v/v) DMSO; 1% (v/v) DMSO in the case of C3G] or blackberry

Table 2. Effects of the Test Compounds on the Growth of Human Colon Carcinoma Cells Using the Sulforhodamine B Assay[a])

	growth inhibition IC_{50}	
substance	none	catalase (100 U/mL)
blackberry extract	120 ± 30 μg/mL	>200 μg/mL
cyanidin	57 ± 3 μM[b]	–[c]
cyanidin-3-glucoside	>300 μM	–[c]
phloroglucinol aldehyde	175 ± 67 μM	160 ± 17 μM
protocatechuic acid	>300 μM[d]	–[c]

[a] HT29 cells, 72 h incubation, ±catalase. [b] Previously reported in ref 33. [c] No inhibition up to 300 μM. [d] Previously reported in ref 20

extract and then coincubated for 1 h with 10 μM CPT or 10 μM DOX. The medium was removed, and the cells were abraded at room temperature in 3 mL of TE buffer [10 mM tris-(hydroxymethyl)aminomethane (Tris), pH 8.0, 1 mM ethylenediaminetetraacetic acid (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 DNA content in the single fractions was determined by measuring the absorbency at 260 nm using a NanoDrop spectrophotometer (PeqLab Biotechnologie GmbH, Erlangen, Germany), and all fractions were blotted onto a nitrocellulose membrane using a slot blot apparatus (Minifold II, Whatman/Schleicher & Schuell, Dassel, Germany). Topoisomerase was detected using a rabbit polyclonal antibody against topoisomerase I (1:250 dilution), topoisomerase IIα (1:500 dilution), or topoisomerase IIβ (1:500 dilution). An antimouse IgG peroxidase conjugate (1:2000) or an antirabbit IgG peroxidase conjugate (1:2000) was used as secondary antibodies. All antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The respective chemoluminescent signals (LumiGLO, Cell Signaling Technology, Danvers, MA) were analyzed using the LAS 3000 with the AIDA Image Analyzer 3.52 software for quantification (Raytest, Straubenhardt, Germany). Arbitrary light units were plotted as the ratio of test to control (%).

RESULTS

Inhibition of Tumor Cell Growth in Vitro. The effects of blackberry extract, the major anthocyanin C3G, and the potential degradation products PCA and PGA on the growth of HT29 cells were determined using the SRB assay. The blackberry extract potently inhibited the growth of HT29 cells with a half-maximal inhibitory concentration (IC_{50}) of 120 ± 30 μg/mL (Table 2). The presence of catalase significantly diminished its growth inhibitory properties (IC_{50} ≥ 200 μg/mL). In the absence of catalase, C3G exhibited marginal growth inhibitory properties without reaching an IC_{50} value of up to 300 μM in the cell culture medium. In the presence of catalase, no effect on tumor cell growth was observed (Table 2). We reported previously that the aglycone cyanidin suppresses the growth of HT29 cells in micromolar concentrations,[36] yet the present study revealed that

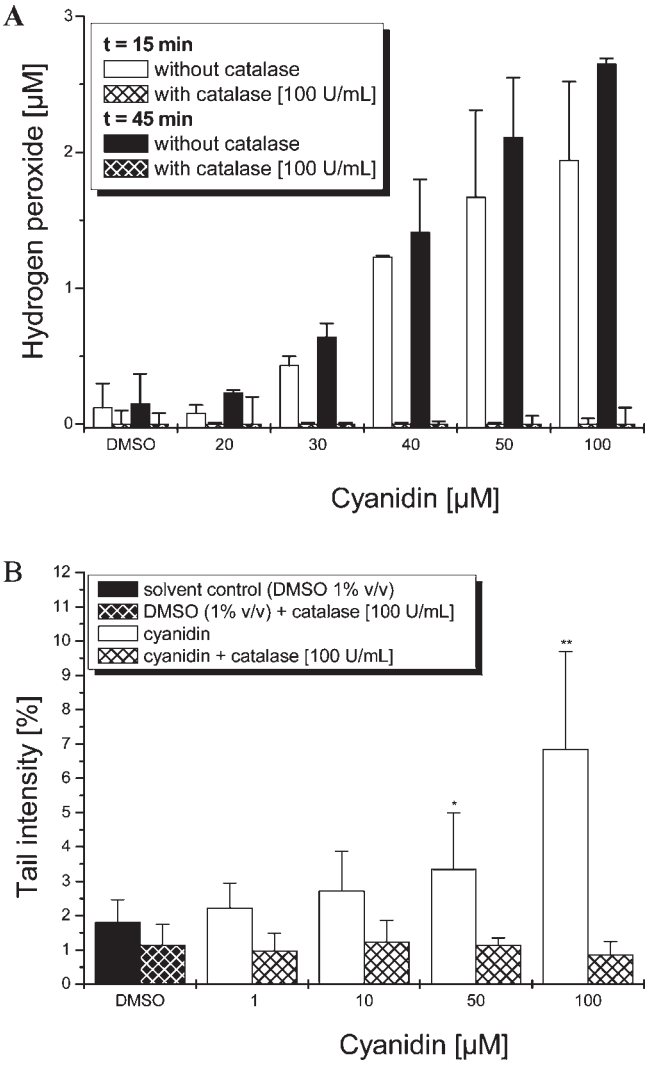

Figure 1. (A) Hydrogen peroxide levels in serum-containing cell culture medium of HT29 cells after incubation with cyanidin in the presence and absence of catalase (100 U/mL) for 15 and 45 min. The data presented are the means ± standard deviations of at least two independent experiments, each performed in duplicate. (B) Single-cell gel electrophoresis (comet assay) with HT29 cells. Cells were treated under serum-free conditions with cyanidin in the presence or absence of catalase (100 U/mL). The data presented are the means ± standard deviations of at least three independent experiments, each performed in duplicate. Significances indicated refer to the significance level as compared to the respective control (Student's *t*-test * = $p < 0.05$, ** = $p < 0.01$).

the addition of catalase to the cell culture medium is associated with a complete loss of growth inhibitory properties in concentrations up to 300 μM (Table 2). Among the degradation products, PGA potently diminished the growth of HT29 cells (IC_{50} = 175 ± 67 μM). Of note, the presence of catalase did not affect its growth inhibitory properties (Table 2). In contrast, growth inhibition mediated by PCA as previously described in ref 20 was slightly diminished in the presence of catalase (Table 2).

Hydrogen Peroxide Accumulation and Influence of DNA Integrity. To assess whether cyanidin leads to increased extracellular hydrogen peroxide formation in DMEM media, HT29 cells were incubated with cyanidin for 72 h. The hydrogen peroxide

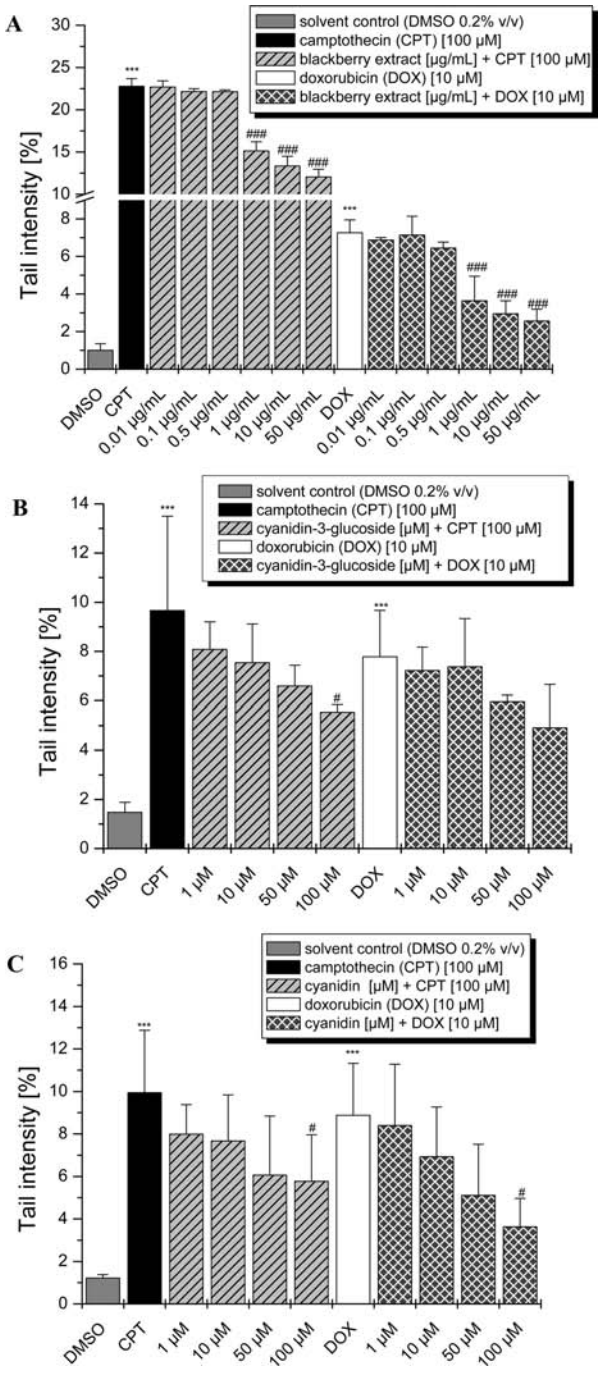

Figure 2. Impact of the test compounds (A) blackberry extract, (B) cyanidin-3-glucoside, and (C) its aglycone cyanidin on the DNA-strand-breaking properties of camptothecin (CPT) or doxorubicin (DOX) in HT29 cells in the presence of catalase (100 U/mL) tested in the comet assay. For the coincubation experiments, HT29 cells were preincubated for 30 min with the solvent control (0.2% DMSO) or the respective test compound and then coincubated for 1 h with 100 μM CPT or 10 μM DOX. DMSO [0.2% (v/v)] was included in the testing as the solvent control. All incubations were performed in the presence of catalase (100 U/mL). The data presented are the means ± standard deviations of at least three independent experiments, each performed in duplicate. Significances indicated refer the significance level compared to the respective control (Student's *t*-test *** = $p < 0.001$) or to the respective topoisomerase poison (CPT or DOX; Student's *t*-test # = $p < 0.05$, ### = $p < 0.001$).

concentration in the cell culture medium was measured after 15 min, 45 min, 24 h, and 72 h. The short-time incubation of cyanidin (15 min) determined the concentration-dependent formation of hydrogen peroxide in the micromolar range (Figure 1A), which was slightly enhanced after 45 min (Figure 1A). Coincubation with catalase reduced the hydrogen peroxide concentration in the cell culture medium to the level of the solvent control (Figure 1A). After both 24 and 72 h, no hydrogen peroxide accumulation in the medium of HT29 cells was detected (data not shown).

The impact of hydrogen peroxide formation on the DNA-damaging properties of cyanidin was investigated in the comet assay. To determine a possible contribution of hydrogen peroxide to the DNA-damaging properties of cyanidin, HT29 cells were incubated with cyanidin in either the absence or the presence of catalase (100 U/mL). In line with earlier results,[16] in the absence of catalase, cyanidin increased the amount of DNA strand breaks in HT29 cells in a concentration-dependent fashion (Figure 1B). At concentrations of ≥ 50 μM cyanidin, a significant increase of DNA strand breakage was observed. In contrast, in the presence of catalase (Figure 1B), cyanidin did not induce DNA damage in HT29 cells up to 100 μM.

Furthermore, the DNA-strand-breaking properties of the anthocyanin C3G (up to 100 μM) and the degradation products PGA and PCA (up to 100 μM) were investigated in the comet assay. In the presence of catalase, none of the test compounds induced DNA strand breakage in HT29 cells after 1 h of incubation (data not shown).

Modulation of DNA-Strand-Breaking Properties of Classical Topoisomerase Poisons. We reported previously that hydroxylated anthocyanidins such as delphinidin act as a catalytic topoisomerase inhibitor, suppressing the stabilizing effect of the topoisomerase poisons CPT,[16] etoposide, and DOX[17] on the covalent topoisomerase−DNA intermediate, thus decreasing the DNA-damaging potential of these antineoplastic agents in human colon carcinoma cells. In the present study, we addressed the question whether these effects are limited to the free aglycon (cyanidin) or whether a respective glycoside (C3G) or even a C3G-rich complex extract (blackberry) affects the rate of topoisomerase poison-induced DNA strand breaks as well. Furthermore, we also addressed the question whether putative anthocyanin degradation products (PGA, PCA) contribute to the potential DNA-protective effect of the extract.

HT29 cells were pretreated for 30 min with the respective test compound and then coincubated with CPT (100 μM) or DOX (10 μM) for 1 h. As expected, treatment of HT29 cells with CPT or DOX as single compounds resulted in a significant increase of DNA strand breaks (Figure 2A-C).

In the presence of blackberry extract (≥ 1 μg/mL), the DNA-damaging effects of CPT and DOX were diminished to a significant extent and in a concentration-dependent fashion (Figure 2A). The major anthocyanin of the blackberry extract, C3G, significantly reduced the rate of DNA strand breaks induced by CPT at a concentration of 100 μM. In the case of DOX (in combination with C3G), a slight but not significant decrease of DNA damage was observed (Figure 2B). The treatment with the aglycone cyanidin resulted in a concentration-dependent decrease of the amount of CPT- and DOX-induced DNA strand breaks, with a significant effectiveness at 100 μM cyanidin (Figure 2C). In contrast, the potential degradation products PCA and PGA did not affect the DNA-strand-breaking properties of the topoisomerase poisons CPT and DOX (data not shown).

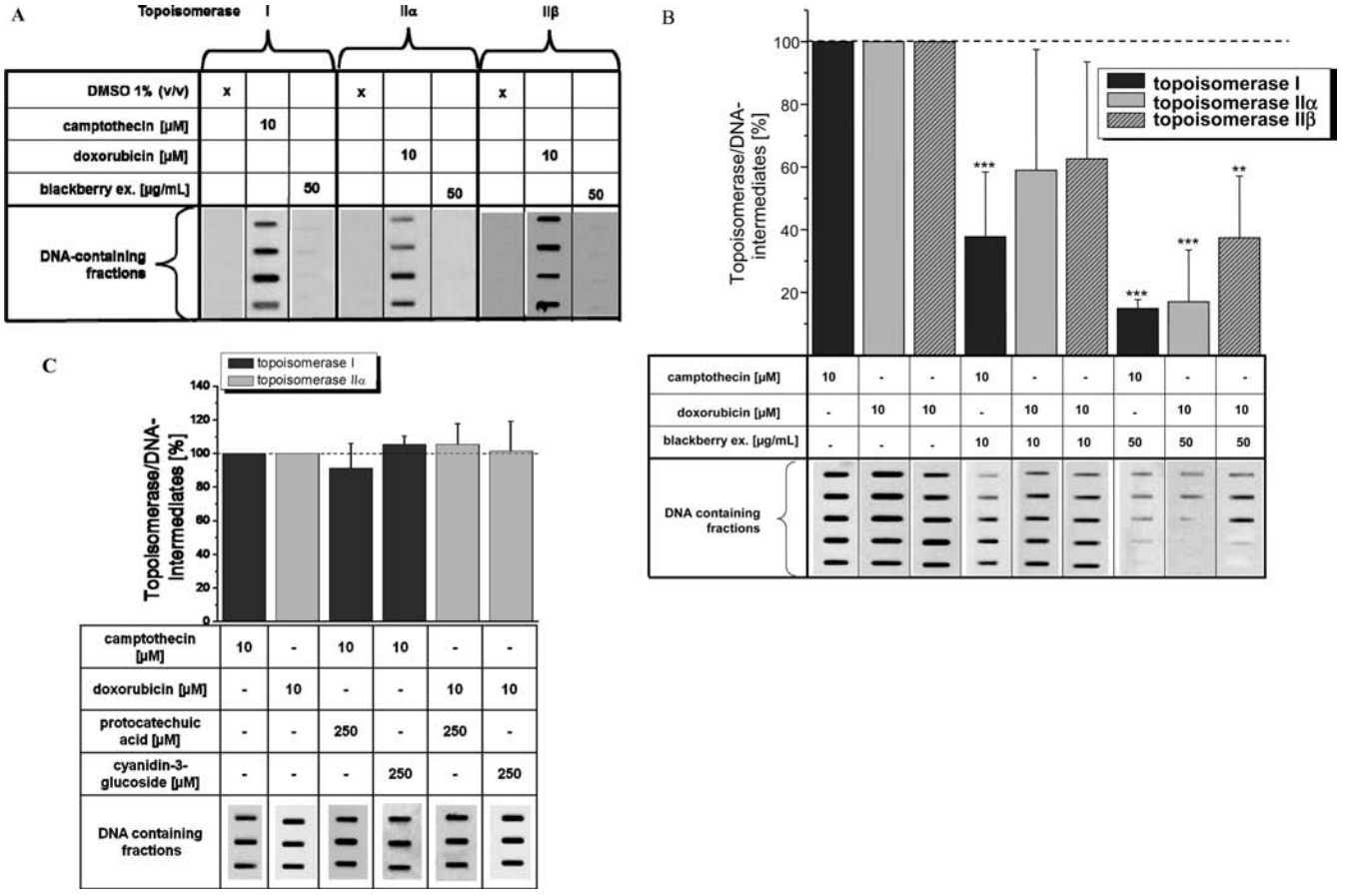

Figure 3. (A) Detection of the covalent topoisomerase I and topoisomerase IIα/DNA intermediate in HT29 cells in the ICE assay. Cells were treated with the respective test compound for 1 h in the presence of catalase (100 U/mL). A representative immunoblot of three independent experiments is shown. Impacts of (B) blackberry extract and (C) C3G and protocatechuic acid on the camptothecin- or doxorubicin-stabilized enzyme/DNA complexes in the ICE assay. HT29 cells were pretreated with blackberry extract, C3G, or protocatechuic acid for 30 min and then coincubated with blackberry extract, C3G, or protocatechuic acid and CPT (10 μM) or DOX (10 μM) for 1 h. The treatment was performed in the presence of catalase (100 U/mL). A representative immunoblot is shown. The level of topoisomerase/DNA intermediate was calculated as the ratio of coincubated cells to control cells (treated with the respective topoisomerase poison) with respect to DNA content × 100% (T/C, %). The data presented are the means ± standard deviations of three independent experiments. Significances indicated refer the significance level compared to the respective topoisomerase poison CPT or DOX (Student's *t*-test ** = $p < 0.01$, *** = $p < 0.001$).

Blackberry Extract Diminishes Cleavable Complex Stabilization by Topoisomerase Poisons. Delphinidin was characterized as a pure catalytic topoisomerase inhibitor within intact human colon carcinoma cells.[17] However, so far, little is known about the impact of glycosylated anthocyanins and their degradation products on topoisomerase inhibition within intact tumor cells. In this study, we used the ICE bioassay[17] to directly determine the amount of topoisomerase covalently linked to the DNA (see Materials and Methods). After 1 h of incubation, CPT (10 μM) potently enhanced the amount of topoisomerase I in the DNA-containing fractions, indicative of a cleavable complex (covalent DNA−topoisomerase intermediate) stabilization (Figure 3A). The topoisomerase II poison DOX (10 μM) highly elevated the level of topoisomerase IIα (Figure 3A) and IIβ (data not shown) bound to DNA. In contrast, treatment of HT29 cells with the anthocyanin-rich blackberry extract (50 μg/mL) did not result in a stabilization of the topoisomerase/DNA intermediate, presented for topoisomerase I and IIα as examples in Figure 3A.

The above-mentioned results indicate that blackberry anthocyanins target topoisomerases prior to their binding to the DNA. To extend this hypothesis, the ICE assay was used for competition studies in cultured HT29 cells (Figure 3B). HT29 cells were pretreated with the blackberry extract, C3G, or the degradation product PCA for 30 min and then coincubated with the extract, C3G or PCA, and CPT or DOX in combination for 1 h. Blackberry extract at a concentration of 10 μg/mL significantly reduced the amount of topoisomerase I covalently bound to DNA stabilized by 10 μM CPT (Figure 3B). At a concentration of 50 μg/mL blackberry extract, the DNA−topoisomerase I binding was diminished by up to 20% in comparison to the respective CPT-treated control cells. The levels of topoisomerase IIα and IIβ in the DNA peak fractions stabilized by DOX (10 μM) were significantly decreased after pre- and coincubation with 50 μg/mL blackberry extract (Figure 3B), with a slightly more pronounced effect on the topoisomerase IIα isoform. In contrast, C3G in concentrations up to 250 μM and the tested degradation product PCA at a concentration of 250 μM did not modulate the amount of topoisomerase I/IIα covalently bound to DNA (Figure 3C).

DISCUSSION

We reported previously that the anthocyanidin delphinidin acts as a catalytic topoisomerase inhibitor in human colon carcinoma cells and diminishes the DNA-damaging properties of classical topoisomerase poisons,[17] indicating an additional molecular mechanism to be considered in the spectrum of bioactive features of this food constituent. These results raised the question whether high consumption of anthocyanidins in humans might compromise the outcome of respective cancer therapy. However, with respect to the potential relevance of these findings, it has to be taken into account that free anthocyanidins are rare in natural food items and rather are consumed predominantly in their glycosylated form within complex polyphenol mixtures. Further, it must be taken into account that intensive degradation might occur within the gastrointestinal tract, probably contributing to the bioactivity of these products. Thus, in the present study, we compared the effects of the free aglycon (cyanidin) with those of a respective glycoside (C3G), a complex blackberry extract containing C3G as the major anthocyanidin (Table 1), and the potential cyanidin degradation products PCA and PGA.

The effects on tumor cell growth were determined. The blackberry extract was found to diminish cell growth of HT29 cells, although the presence of catalase significantly attenuated this effect (Table 2). In accordance with earlier reports,[36] the free aglycone cyanidin was found to exhibit potent growth inhibitory properties. The addition of catalase resulted in a loss of any growth inhibitory properties of cyanidin as well as the respective glycoside (C3G) (Table 2), suggesting that the formation of hydrogen peroxide acts as a major contributor to the observed growth inhibitory effects, thus generating experimental artifacts that are presumably limited to the conditions of the cell culture medium. Different authors have reported substantial hydrogen peroxide formation under cell culture conditions.[26−30] Short-time incubation of HT29 cells with cyanidin (15 min) indeed demonstrated the concentration-dependent formation of hydrogen peroxide in the cell culture medium in the micromolar range, even being slightly enhanced after 45 min (Figure 1). Addition of catalase effectively diminished the hydrogen peroxide concentration in the cell culture medium down to the level of the solvent control. Yet, after long-time incubation (24 and 72 h), no enhanced hydrogen peroxide level was detected (data not shown). The observation that hydrogen peroxide accumulation seems to be an early event, yet changing cell signaling constantly, is in line with earlier findings.[20] Furthermore, the contribution of hydrogen peroxide to the detected growth inhibition of the test compounds in the absence of catalase can be assumed. Taken together, the data enforce the necessity of adding a H_2O_2-scavenging system to in vitro cell culture assays when effects of polyphenol structures are applied for testing.

Several publications have discussed the possibility that biological effects might be mediated not by anthocyanins themselves[7,37,38] but rather by the released aglycons or even respective degradation products such as PGA and PCA.[20,21] Of note, only PGA displayed growth inhibition properties (Table 2). In 2009, Woodward et al. investigated the degradation of C3G to PGA and PCA.[39] In accordance with our results, concentrations where growth inhibitory effects of PGA were achieved were nearly 100-fold higher than the maximum concentrations that might arise from the degradation of C3G in the blackberry extract concentrations applied in the present study. Thus, the detected effects of PGA on cell growth appear not to be of relevance for the influence of the overall extract.

To investigate whether generated hydrogen peroxide might also contribute to the DNA-damaging properties of cyanidin, a comet assay was performed in both the presence and the absence of catalase. In line with earlier results,[16] in the absence of catalase, cyanidin did increase the amount of DNA strand breaks in HT29 in a concentration-dependent fashion (Figure 1B). Yet, the addition of catalase reduced this effect to the level of the solvent control. Furthermore, neither the blackberry extract nor the isolated constituent C3G nor the degradation products PGA and PCA induced DNA strand breaks in HT29 cells in the presence of catalase (data not shown). In contrast, the blackberry extract (Figure 2A), C3G (Figure 2B), and cyanidin (in the presence of catalase, Figure 2C) were identified as protectors against DNA-damaging topoisomerase poisons. Because 76% of the blackberry anthocyanins within the tested extract represents C3G, the contribution of C3G to the detected DNA-protective properties of the blackberry extract from topoisomerase I poisons (CPT) can be assumed. Hence, the more potent protection from topoisomerase I poison-caused damage by C3G might also explain the stronger protective effects of the C3G-rich extract from DNA damage caused by CPT (Figure 2A).

The high DNA-protective properties of cyanidin are in line with previously reported data, where we demonstrated that the anthocyanidin delphinidin acts as a catalytic topoisomerase inhibitor suppressing the DNA-strand-breaking effects of different topoisomerase poisons (CPT,[16] etoposide, and DOX[17]). Of note, the cyanidin degradation products PCA and PGA did not protect HT29 cells from the DNA-strand-breaking properties of the two topoisomerase poisons (data not shown). Therefore, the potent DNA-protecting properties can be attributed to the parent compound, implying that degradation is expected to result in a loss of DNA-protective properties.

Interestingly, the complex blackberry extract turned out to be more potent than expected based on the anthocyanin content, indicating the presence of further constituents with DNA-protective qualities.

To gain further information on the mode of action, the amount of topoisomerase covalently linked to the DNA in HT29 cells was determined by the use of the ICE bioassay.[17] Different authors have reported that flavonoids suppress the activity of human topoisomerases and thereby alter DNA integrity.[16,40,41] Anthocyanidins bearing vicinal hydroxyl groups at the B-ring, such as cyanidin or delphinidin, could be identified as pure catalytic topoisomerase inhibitors, lacking stabilizing effects on the covalent enzyme/DNA intermediate, whereas polyphenols such as genistein or (−)-epigallocatechin-3-gallate seem to act as topoisomerase poisons thereby forming a covalent complex with the DNA−topoisomerase intermediate.[15,17,40,41] In a recent study berry extracts (bilberry, grape), rich in glycosylated anthocyanins, potently diminish topoisomerase I and topoisomerase II activity with a preference to topoisomerase II.[42]

In contrast to CPT, which induced cleavable complex stabilization of topoisomerase I (Figure 3A), or DOX, which strongly elevated the levels of topoisomerase IIα (Figure 3A) and IIβ bound to DNA, the anthocyanin-rich blackberry extract (50 μg/mL) did not result in a stabilization of the topoisomerase−DNA intermediate (up to 50 μg/mL; Figure 3A) and thus appears not to act as a topoisomerase poison.

To evaluate whether the blackberry anthocyanins, C3G, or the cyanidin degradation product PCA might target topoisomerases prior to their binding to DNA, as reported previously for the free aglycone delphinidin, the ICE assay was used for competition studies. Pretreatment of HT29 cells with the blackberry extract (50 μg/mL) for 30 min before coincubation with CPT resulted in a significantly reduced amount of topoisomerase I covalently bound to DNA, already at low extract concentrations (Figure 3B). Furthermore, the level of DOX- (10 μM) stabilized topoisomerase IIα and IIβ in the DNA peak fractions was significantly decreased by coincubation with blackberry extract, with a slightly more pronounced effect on the topoisomerase IIα isoform (Figure 3B). Thus, the blackberry extract again exhibited a slightly more potent capacity to prevent from topoisomerase I poison- (CPT-) induced DNA damage, supporting the results detected in the comet assay. In contrast to the blackberry extract, C3G (250 μM) and the degradation product PCA (250 μM) did not modulate the stability of the DNA/topoisomerase complex I and IIα (Figure 3C). Thus, a contribution of C3G or its degradation product PCA to the effects of the blackberry extract seems to be unlikely.

Taken together, our data show that pretreatment of cells with blackberry extract diminishes the cleavable complex stabilizing effect of topoisomerase I and II poisons. In cell-free test systems, cyanidin was already identified as a potent inhibitor of topoisomerase activity.[16] Taking into account the DNA-protective effects of C3G seen in the comet assay, a contribution of C3G to the DNA-protective properties of the blackberry extract seems to be conceivable, also presumably not mediated by decreasing the levels of covalent DNA−topoisomerase intermediates. Yet, earlier studies also identified complex polyphenolic extracts as even more potent modulators of topoisomerase activity than isolated constituents. Hence, synergistic effects and/or a contribution of yet-unknown constituents might have to be considered. Oligomeric and polymeric procyanidins, also present in berries and grapes, have already been identified as topoisomerase inhibitors;[15,43,44] thus, a contribution of such structures to the effects seen here might also be of relevance.

In summary, our data identified a C3G-rich blackberry extract as a potent suppressor of topoisomerase activity in vitro, interfering with classical topoisomerase poisons thus leading to the protection of DNA. Furthermore, the blackberry extract suppresses DNA complex formation by topoisomerase poisons in HT29 cells. To estimate a possible relevance of the detected DNA-protecting properties of the C3G-rich blackberry under in vivo conditions, their bioavailability must be taken into account. Studies in 2006 by Kahle et al. on the bioavailability of a blueberry extract for ileostomy patients indicated that, depending on the sugar moiety of the anthocyanins, up to 85% could be determined in the ileostomy bags and, therefore, could reach the colon.[45] Assuming a consumption of 200 g of blackberries with an anthocyanin content of ~50 mg of total anthocyanins in 100 g (fresh weight),[46] a local concentration in the gastrointestinal tract comparable to the bioactive concentrations applied here is achievable. Thus, a relevance of the biological activity of the blackberry extract detected here under in vivo conditions seems to be reasonable. In the case of coapplication with therapeutically applied topoisomerase poisons, as prescribed in several chemotherapeutic schemes, this could lead to a reduced effectiveness of the chemotherapeutics, at least locally in the gastrointestinal tract.

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ABBREVIATIONS USED

C3G, cyanidin-3-glucoside; CPT, camptothecin; DOX, doxorubicin; ICE bioassay, isolating in vivo complexes of enzyme to DNA; PCA, protocatechuic acid; PGA, phloroglucinol aldehyde

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