Anthocyanidins Modulate the Activity of Human DNA Topoisomerases I and II and Affect Cellular DNA Integrity

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In the present study, we investigated the effect of anthocyanidins on human topoisomerases I and II and its relevance for DNA integrity within human cells. Anthocyanidins bearing vicinal hydroxy groups at the B-ring (delphinidin, DEL; cyanidin, CY) were found to potently inhibit the catalytic activity of human topoisomerases I and II, without discriminating between the IIα and the IIβ isoforms. However, in contrast to topoisomerase poisons, DEL and CY did not stabilize the covalent DNA-topoisomerase intermediates (cleavable complex) of topoisomerase I or II. Using recombinant topoisomerase I, the presence of CY or DEL ($\geq$1 μM) effectively prohibited the stabilization of the cleavable complex by the topoisomerase I poison camptothecin. We furthermore investigated whether the potential protective effect vs topoisomerase I poisons is reflected also on the cellular level, affecting the DNA damaging properties of camptothecin. Indeed, in HT29 cells, low micromolar concentrations of DEL (1−10 μM) significantly diminished the DNA strand breaking effect of camptothecin (100 μM). However, at concentrations $\geq$50 μM, all anthocyanidins tested (delphinidin, cyanidin, malvidin, pelargonidin, and paeonidin), including those not interfering with topoisomerases, were found to induce DNA strand breaks in the comet assay. All of these analogues were able to compete with ethidium bromide for the intercalation into calf thymus DNA and to replace the minor groove binder Hoechst 33258. These data indicate substantial affinity to double-stranded DNA, which might contribute at least to the DNA strand breaking effect of anthocyanidins at higher concentrations ($\geq$50 μM).

Introduction

Anthocyanins are naturally occurring colored plant constituents, widely spread in fruits and vegetables of the daily diet (*1*). Depending on nutrition customs, the intake of anthocyanins in Germany was estimated in 2002 to be 2.7 mg/day, with a personal variety of 0−76 mg (*2*). Anthocyanins have been associated with potentially beneficial effects on various diseases, such as diabetic retinopathy, for example (*3*), and have been suggested to possess antiinflammatory as well as chemopreventive properties (*4*). In the last years, anthocyanin preparations have gained increasing popularity on the fast expanding market of food supplements. Products are available enabling a severalfold enhancement of the daily intake above the habitual amount, raising the question whether such an enhanced intake might eventually be associated with adverse health effects.

So far, information available on the cellular effects of anthocyanins is rather limited. Like numerous other flavonoids, anthocyanins have been reported to possess antioxidative properties (*5*−*7*). We recently showed that anthocyanidins, the aglycons (Scheme 1), potently interfere with signaling cascades crucial for the regulation of cell proliferation (*8*, *9*). With respect to a risk/benefit assessment, these effects on proliferation-associated signaling cascades might be taken into account as potentially beneficial, especially in terms of chemoprevention. However, many flavonoids have been reported to interfere with human topoisomerases. This effect could be of relevance for DNA integrity (*10*−*12*). DNA topoisomerases are a group of enzymes that solve the topological problems caused by transcription, replication, chromosome condensation and segregation, and DNA repair (*13*, *14*). These enzymes change DNA topology by introducing transient breaks in the phosphodiester backbone of the DNA, enabling the release of torsion stress. In mammals, two major classes of topoisomerases are expressed, topoisomerases I and II. Topoisomerase I

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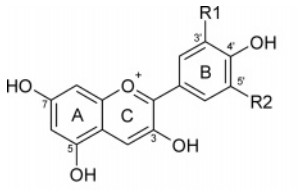
Scheme 1. Chemical Structure of Anthocyanidins



anthocyanidin

anthocyanidin	R1	R2
delphinidin (DEL)	OH	OH
cyanidin (CY)	OH	H
pelargonidin (PG)	H	H
paeonidin (PN)	OCH_3	H
malvidin (MV)	OCH_3	OCH_3

introduces a transient single strand break in the DNA double helix by the formation of a covalent DNA-topoisomerase intermediate, the so-called cleavable complex. Then, torsion stress is released by strand rotation, followed by religation of the cleaved strand (*15*). In contrast, topoisomerase II, an ATP-dependent enzyme, inserts a transient double strand break, through which a second DNA double helix is passed (*16*, *17*). Pharmacological interference with the activity of topoisomerase I or II has been reported at different steps of the catalytic cycle. The mode of interaction with the respective target enzyme is crucial with respect to potential consequences for DNA integrity. Catalytic inhibitors bind to the enzyme prior to DNA binding, thus inhibiting the formation of the cleavable complex. However, the majority of topoisomerase targeting compounds does not interfere with the cleavable complex formation but binds to and stabilizes the DNA-topoisomerase intermediate after it has formed, thus prohibiting the release and the resealing of the DNA strand. As a consequence, DNA double strand breaks are generated within cells due to collisions of the stabilized cleavable complexes with the replication forks (*18*−*20*). With respect to the potential DNA damaging properties, those compounds are considered as topoisomerase poisons, as, for example, camptothecin (CPT)[1] (topoisomerase I) or etoposide (ETO) (topoisomerase II).

In the present study, we addressed the question whether anthocyanidins target human topoisomerases I and/or II. We especially focused on the mechanism of interaction with the target enzyme(s) and the potential relevance for DNA integrity within human cells.

Materials and Methods

Materials. HT29 (human colon adenocarcinoma, ACC 299), HEK293 (human embryonal kidney cells, ACC 305), and MCF-7 (human breast adenocarcinoma, ACC 105) cells were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). Cell culture media and supplements were purchased from GIBCO Invitrogen (Karlsruhe, Germany). Delphinidin (DEL), cyanidin (CY), pelargonidin (PG), paeonidin (PN), and malvidin (MV) were obtained as chlorides from Extrasynthèse (Lyon, France), and CPT and ETO were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). For all assays, the compound solutions were freshly solved in DMSO directly before the start of the experiment, without the use of stored stock solutions, to a final DMSO content as depicted in the respective figure legends.

[1] Abbreviations: BSA, bovine serum albumin; CPT, camptothecin; CY, cyanidin; DEL, delphinidin; EGCG (−)-epigallocatechin-3-gallate; EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; ETO, etoposide; kDNA, kinetoplast DNA; MV, malvidin; PG, pelargonidin; PN, paeonidin.

Cell Culture. The cell culture was performed in humidified incubators (37 °C, 5% CO_2). HT29 and HEK293 cells were cultured in Dulbecco's modified Eagle medium, MCF-7 cells in RPMI 1640, both supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were routinely tested for the absence of mycoplasm contamination.

Preparation of Nucleic Extract. Human MCF-7 cells (3×10^7) were resuspended in 7.5 mL of lysis buffer {0.3 M sucrose; 0.5 mM [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid (EGTA), pH 8.0; 60 mM KCl; 15 mM NaCl; 15 mM HEPES, pH 7.5; 150 μM spermine; and 50 μM spermidine}, and 0.5 mL of lysis buffer with Triton X-100 (13.5:1 v/v) was added. The cells were lysed on ice for 15 min. After centrifugation (150*g*, 10 min), the supernatant was removed and the pellet was resuspended in extraction buffer (100 mM NaCl; 5 mM KH_2PO_4, pH 7.5; 1 mM phenylmethanesulfonyl fluoride; and 1 μL/mL β-mercaptoethanol) to 3×10^7 nuclei per mL. A 10% volume of 5 M NaCl was carefully added to precipitate DNA on ice for 20 min. After centrifugation, the supernatant was collected, 1.5 × volume of glycerol was added, and aliquots were stored at −20 °C until use in relaxation assays.

Expression and Purification of Recombinant Human Topoisomerases I, IIα, and IIβ. Topoisomerase I. The plasmid pHT143 (*21*) was transformed into the yeast strain *Saccharomyces cerevisiae* top1 null strain RS190 (*22*). The cells were grown, and expression was induced as described (*23*). After induction, yeast cells were frozen in liquid nitrogen. To extract topoisomerase I, cells were mixed with an equal volume of 0.5 mm glass beads (BioSpec Products Inc., United States) and two volumes of extraction buffer [50 mM Tris-HCl, pH 7.5; 500 mM NaCl; 1 mM EDTA; 1 mM DTT; 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride; and 1 mM phenylmethanesulfonyl fluoride] and using a bead beater (BioSpec Products Inc.) cracked with five 1 min bursts, each followed by a 1 min break. This and all following steps were carried out at 4 °C. After sedimentation of debris (5000*g*, 30 min, 4 °C), ammonium sulfate was added to a final concentration of 2 M, and after 24 h at 4 °C, the precipitate was collected (22000*g*, 30 min, 4 °C). The ammonium sulfate precipitate was dissolved [50 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1 mM EDTA; 1 mM DTT; 1 mM phenylmethanesulfonyl fluoride; 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and 10% (v/v) glycerol] and absorbed to a heparin-sepharose (Amersham Bioscience, Freiburg, Germany) column. The column was washed with 400 mM and eluted with 800 mM NaCl in buffer A [50 mM Tris-HCl, pH 7.5; 0.5 mM phenylmethanesulfonyl fluoride; 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and 10% (v/v) glycerol]. The eluate was diluted with two volumes of buffer A, cleared by centrifugation (22000*g*, 30 min, 4 °C), and loaded onto a Ni−NTA sepharose (Qiagen, Hilden, Germany) column. The Ni−NTA column was washed with 20 mM and eluted with 200 mM imidazol [15 mM HEPES KOH, pH 7.5; 150 mM NaCl; 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride; and 10% (v/v) glycerol]. The eluate was diluted [1 volume of 15 mM HEPES KOH, pH 7.5; 0.5 mM EDTA; 1 mM DTT; 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and 10% (v/v) glycerol], cleared by centrifugation (22000*g*, 30 min, 4 °C), and loaded onto a Source 15S (Amersham Bioscience) column. After sample application, the column was washed [15 mM HEPES KOH, pH 7.5; 80 mM NaCl; 1 mM DTT; and 10% (v/v) glycerol] and finally eluted [15 mM HEPES KOH, pH 7.5; 400 mM NaCl; 0.5 mM EDTA; 1 mM DTT; 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; and 60% (v/v) glycerol]. Using this procedure, topoisomerase I was purified to apparent homogeneity as determined by SDS gel electrophoresis followed by protein staining with silver nitrate. The specific activity of purified topoisomerase I was determined in a relaxation assay with serial dilution of the enzyme and calculated to be approximately 3×10^6 units/mg topoisomerase I.

Topoisomerase II. The plasmid YEpWOB6 in which the first five residues of yeast topoisomerase II is fused to the 29th

residue of human topoisomerase IIα has been described previously (*24*). A similar system enabling overexpression of human topoisomerase II*β* residue 46-1621 fused to the first five residues of yeast topoisomerase II has previously been described (*25*). Using linker PCR cloning, we replaced the topoisomerase IIα sequence in YePWOB6 with the topoisomerase II*β* sequence (residue 46-1621), giving rise to YePWOB6TopoII*β*. Basically, this plasmid has the same characteristics as the YEphTOP*β* previously described by Austin et al. (*25*). The yeast strain *S. cerevisiae* JEL1 transformed with YEpWOB6 or YePWOB6-TopoII*β* was grown, and expression was induced as described (*25*). Crude extract was generated using a bead beater as described for topoisomerase I (extraction buffer included 1 M NaCl). Proteins in the extract were fractioned using a two-step ammonium sulfate precipitation. First, 1/8 volume of saturated ammonium sulfate was added, and precipitated proteins were discarded (15000*g*, 30 min, 4 °C). Next, one volume of saturated ammonium sulfate was added, and after incubation (24 h at 4 °C), proteins were sedimented (15000*g*, 30 min, 4 °C) and renaturated as described for topoisomerase I. Dissolved proteins were absorbed to a heparin-sepharose column. After the column was washed (50 mM Tris-HCl, pH 7.5; 350 mM NaCl; 0.5 mM EDTA; 1 mM DTT; 0.5 mM phenylmethanesulfonyl fluoride; and 10% (v/v) glycerol], topoisomerase IIα or II*β* was eluted with a linear salt gradient beginning with 400 mM and ending with 1300 mM NaCl in buffer A. Peak fractions were collected, diluted to a final NaCl concentration of 250 mM with buffer A, loaded on a HiTrap Heparin HP (Amersham Bioscience) column, washed with 350 mM NaCl in buffer A, and eluted as above. Human topoisomerase IIα and II*β* were thus purified to apparent homogeneity as determined by SDS gel electrophoresis followed by protein staining with silver nitrate. The specific activities of purified topoisomerase IIα or II*β* were determined in a relaxation assay with serial dilution of the enzyme, and in both cases, the activities were found to be approximately 1.2×10^5 units/mg enzyme.

Topoisomerase Activity. Topoisomerase I. For the detection of catalytic topoisomerase I activity, a plasmid relaxation assay was carried out. Plasmid DNA (250 ng of pUC18) was incubated in a final volume of 30 μL [containing 0.3 μL of nucleic extract; 10 mM Tris, pH 7.9; 100 mM KCl; 10 mM $MgCl_2$; 0.5 mM DTT; 0.5 mM EDTA; and 0.03 mg/mL bovine serum albumin (BSA)] for 30 min at 37 °C. The reaction was stopped by the addition of 1/10 volume of 5% (w/v) SDS. 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; 1 mM EDTA, pH 8.5; and 20 mM acetic acid). Subsequently, the gel was stained in 10 μg/mL ethidium bromide for 20 min. The fluorescence of ethidium bromide was documented with a Lumi Imager (Roche, Mannheim, Germany).

Topoisomerase II. Effects on the catalytic activity of topoisomerase II were determined using a decatenation assay. Catenated kinetoplast DNA (kDNA) (200 ng, TopoGEN, Ohio) was used as a substrate. kDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb), which can be released by topoisomerase II. The kDNA was incubated in a final volume of 30 μL (containing 40 ng of topoisomerase II; 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 60 min. The reaction was stopped by the addition of 1/10 volume of 1 mg/mL proteinase K in 10% (w/v) SDS and incubation at 37 °C for further 30 min. Gel electrophoresis and detection were performed as described above.

Stabilization of the Cleavable Complex. Plasmid DNA (250 ng of pUC18) was incubated in a final volume of 30 μL (10 mM Tris, pH 7.9; 100 mM KCl; 10 mM $MgCl_2$; 0.5 mM DTT; 0.5 mM EDTA; and 0.03 mg/mL BSA) with 50 ng of topoisomerase I for 30 min at 37 °C. The reaction was stopped by the addition of 1/10 volume of 5% (w/v) SDS. The samples were subsequently treated with 1 mg/mL proteinase K at 37 °C for 30 min. Gel electrophoresis was performed at 0.4 V/cm in 1% (w/v) agarose gels with 0.5 μg/mL ethidium bromide in Tris acetate/EDTA buffer (40 mM Tris; 1 mM EDTA, pH 8.5; and 20 mM acetic acid) overnight. Gel detection was performed as described above.

Topoisomerase II Immunoband Depletion. For immunoband depletion, 10^6 HEK293 cells were incubated for 30 min at 37 °C in 100 μL of drug-containing medium. The samples were transferred on ice, and 2-fold lysis buffer [250 mM Tris, pH 6.8; 4% (w/v) SDS; 0.04% (w/v) bromophenol blue; 4% (v/v) glycerol; 20 mM DTT; 20 mM EDTA; 2 mM phenylmethanesulfonyl fluoride; and 2.5 M urea], containing the respective drug concentration, was added. The cell suspension was sonicated and heated to 95 °C for 10 min. DTT was added (2 μL of 1 M stock solution), and 20−30 μL was loaded onto a 7% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane. The membranes were subsequently blocked for 1 h with a solution of 5% (w/v) milk powder [20 mM Tris, pH 7.6; 130 mM NaCl; and 0.1% (v/v) Tween-20]. Incubation with rabbit polyclonal antibodies [diluted 1:500 (v/v) in blocking solution] against topoisomerase IIα or II*β* (Santa Cruz, Heidelberg, Germany) was performed at 4 °C overnight. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody [diluted 1:2000 (v/v) in blocking solution, Cell Signaling Technology, Beverly, United States]. Protein bands were finally visualized with the LumiGLO system (Cell Signaling Technology), and chemiluminescence was detected with a Lumi Imager (Roche) using the Lumi Analyst 3.1 software for quantification.

DNA Binding. The assay was performed according to the method of Morgan et al. (*26*) with slight modifications adapted to 96-well plates. A decrease in the fluorescence intensity as a result of the replacement of the intercalator ethidium bromide (1 μM) in double-stranded DNA (calf thymus DNA) was measured at λ_{ex} of 544 nm and λ_{em} of 590 nm. A decrease in the fluorescence intensity resulting from the replacement of the minor groove binder Hoechst 33258 (1 μM) was measured at λ_{ex} of 355 nm and λ_{em} of 460 nm.

Single Cell Gel Electrophoresis (Comet Assay). Single-cell gel electrophoresis was performed according to the method of Gedik et al. (*27*). HT29 cells (3×10^5 in 5 mL of serum containing medium) were spread into Petri dishes (5.5 cm diameter) 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 DMSO (1% v/v), 100 μM CPT, or anthocyanidins in serum free medium. For the coincubation experiments, HT29 cells were preincubated for 30 min with the solvent control (0.2% v/v DMSO) or the respective anthocyanidin (DEL or MV), followed by 1 h of coincubation of the respective compounds with 100 μM CPT. Thereafter, aliquots corresponding to 70000 cells were centrifuged (5 min, 200*g*). 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, 10 mM Tris, 1% (w/v) *N*-laurylsarcosylsodium salt, 1 mL of Triton-X-100, and 10 mL of DMSO]. 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 ($\lambda_{ex} = 546 \pm 12$ 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 parametrized with respect to tail intensity (intensity of

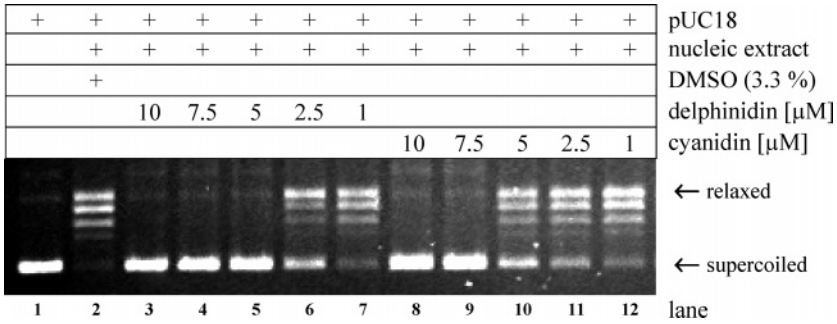


Figure 1. Catalytic activity of topoisomerase I measured by DNA relaxation. Supercoiled pUC18 plasmid DNA (lane 1) was incubated for 30 min at 37 °C with nucleic extract from MCF-7 cells (lanes 2−12) in the absence (lane 2) or presence of decreasing amounts of DEL (lanes 3−7) or CY (lanes 8−12). The reaction was stopped with 1% (w/v) SDS, and after digestion with proteinase K, samples were subjected to submarine 1% agarose gel electrophoresis. UV-transilluminated gels were documented 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 identical experiments with similar outcome.

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

Inhibition of the Catalytic Activity of Topoisomerase I. Topoisomerase I activity was determined as relaxation of supercoiled pUC18 plasmid DNA by nucleic extract from MCF-7 cells. To eliminate topoisomerase II activity contributing to plasmid relaxation, experiments were carried out in the absence of ATP. DEL and CY were found to mediate substantial inhibition of topoisomerase I activity at a concentration of 2.5 μM (Figure 1, compare lanes 6 and 11 with 2). The catalytic activity of topoisomerase I was completely blocked at a concentration of 5 μM DEL or 7.5 μM CY, respectively (Figure 1, lanes 5 and 9). The anthocyanidins PN, PG, and MV did not affect the catalytic activity of topoisomerase I up to 100 μM (data not shown).

Lack of Stabilization of the Cleavable Complex of Recombinant Topoisomerase I. Knowing that CY and DEL inhibit topoisomerase I activity, we next addressed the question whether the anthocyanidins act as pure catalytic inhibitors or as topoisomerase poisons, stabilizing the cleavable complex. The electrophoretic conditions were modified (see Materials and Methods section) to allow the detection of circular DNA bearing a single strand break (open circular plasmid), resulting from the stabilization of the cleavable complex, as shown for the positive control CPT (Figure 2A, lanes 6 and 13). In contrast to CPT, induction of open circular DNA was not detected with CY or DEL up to 10 μM (Figure 2A). Even up to 100 μM, CY or DEL did not induce the production of open circular DNA by topoisomerase I (data not shown). Similarly, formation of open circular DNA was not found by incubation with MV up to 100 μM (data not shown). Thus, the inhibition of topoisomerase I-catalyzed DNA relaxation shown in Figure 1 is not due to stabilization of the topoisomerase I-DNA complex, excluding that CY and DEL could be acting as topoisomerase I poisons.

Protective Effect of CY or DEL against Topoisomerase I Poison. These findings prompted us to consider CY and DEL to exert their effect on topoisomerase I DNA catalysis before DNA cleavage. To investigate this, we determined the impact of these compounds on cleavable complex formation induced by the established topoisomerase I poison CPT. In coincubation experiments, pUC18 and topoisomerase I were preincubated with CY or DEL for 5 min prior to the addition of CPT. Already in the presence of 1 μM CY or DEL, a decrease in the open circular plasmid DNA form induced by CPT (100 μM) was observed (Figure 2B, lanes 5 and 8). Concentrations of CY or DEL $\geq$ 5 μM abolished completely the induction of open circular plasmid DNA by CPT (Figure 2B, lanes 4 and 7). In contrast, the formation of open circular DNA as a result of CPT treatment remained unaffected by preincubation with up to 100 μM MV (data not shown).

Inhibition of the Catalytic Activity of Recombinant Topoisomerases IIα and II*β*. To examine whether anthocyanidins have similar effects on human topoisomerase II, we determined the catalytic activity of recombinant human topoisomerase II in a decatenation assay. In the catenated form, kDNA cannot enter an agarose gel, whereas single circles released by catalytic active topoisomerase II from the catenated network will migrate into the gel (Figure 3, compare lane 1 with 2). MV, PG, and PN showed no effect on the catalytic activity of topoisomerase IIα and IIβ up to 100 μM. Exemplarily, the results for topoisomerase IIβ are shown in Figure 3 (lanes 3−8). CY and DEL were found to inhibit the catalytic activity of topoisomerase IIβ at concentrations exceeding 5 μM. At 10 μM CY or DEL, the catalytic activity was found to be completely blocked (Figure 3, lanes 9 and 14). Similar results were obtained with recombinant topoisomerase IIα (data not shown).

No Stabilization of the Topoisomerase II Cleavable Complex by Anthocyanidins in HEK293 Cells. To corroborate the results obtained with recombinant topoisomerase II in vitro, we investigated the effect of anthocyanidins on the topoisomerase II cleavable complex within human cells. By Western blot analysis, we investigated changes in the amount of topoisomerase protein covalently linked to the DNA. Under the chosen experimental conditions, topoisomerase protein, trapped covalently bound to the DNA in a stabilized cleavable complex, cannot migrate into a SDS-polyacrylamide gel, resulting in a decline of the immunoband signal in the subsequent Western blot analysis. The incubation of HEK293 cells with the topoisomerase II poison ETO resulted in a highly significant reduction of the topoisomerase IIα and IIβ signals, as compared to the solvent

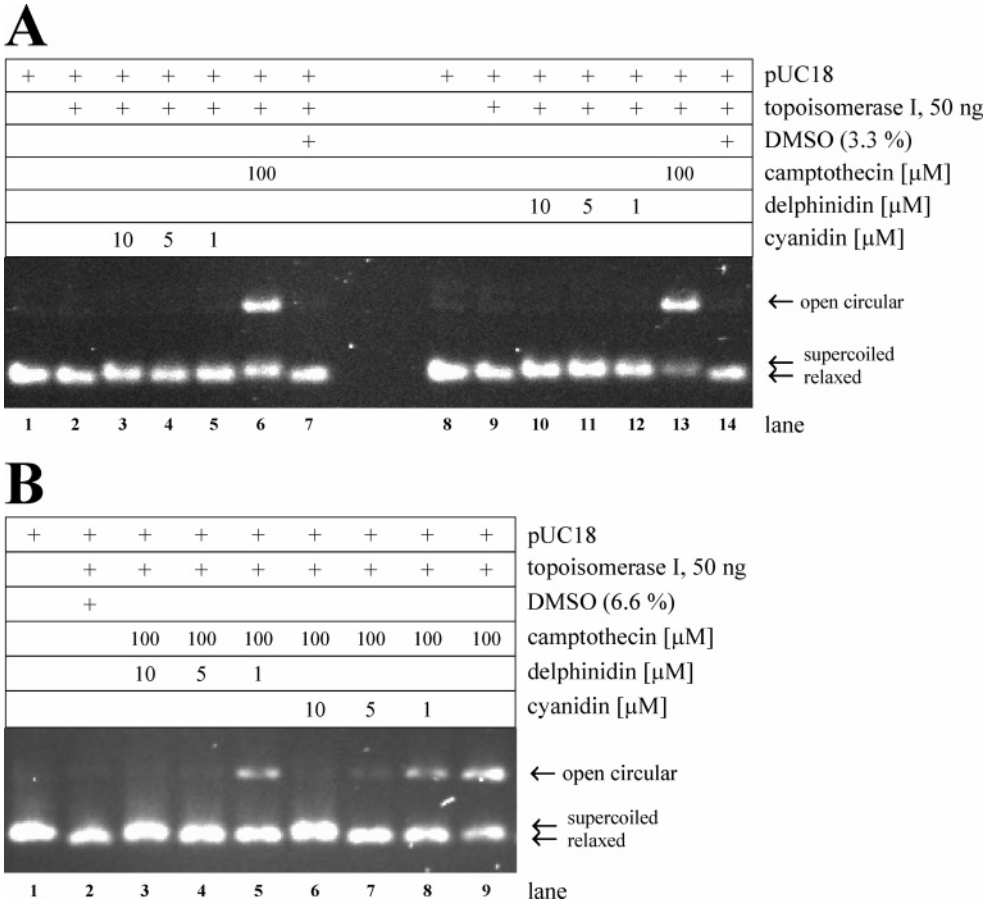


Figure 2. Topoisomerase I DNA cleavage assays. (A) Topoisomerase I cleavable complex assay for CY and DEL. Supercoiled pUC18 plasmid DNA was incubated for 30 min at 37 °C with 50 ng of recombinant human topoisomerase I. The reaction was stopped with 1% (w/v) SDS, and after digestion with proteinase K, samples were subjected to submarine 1% agarose gel electrophoresis in the presence of ethidiumbromide. UV-transilluminated gels were documented by digital photography. Incubation was performed in the absence (lanes 2, 7, 9, and 14) or presence of CY (lanes 3−5), DEL (lanes 10−12), or CPT (lanes 6 and 13). (B) Coincubation experiments with CY or DEL and CPT (100 μM). pUC18 plasmid DNA was incubated with 50 ng of recombinant topoisomerase I and DEL (lane 3−5) or CY (lane 6−8) for 5 min prior to the addition of 100 μM CPT and further incubation for 30 min. Incubation of pUC18 and topoisomerase I with 100 μM CPT is shown in lane 9. Open circular DNA is formed when the covalent enzyme-DNA-intermediate is stabilized, indicative for topoisomerase poisons (see panel A, lanes 6 and 13, or panel B, lane 9). Representative gels from two to four independent experiments with similar outcomes.

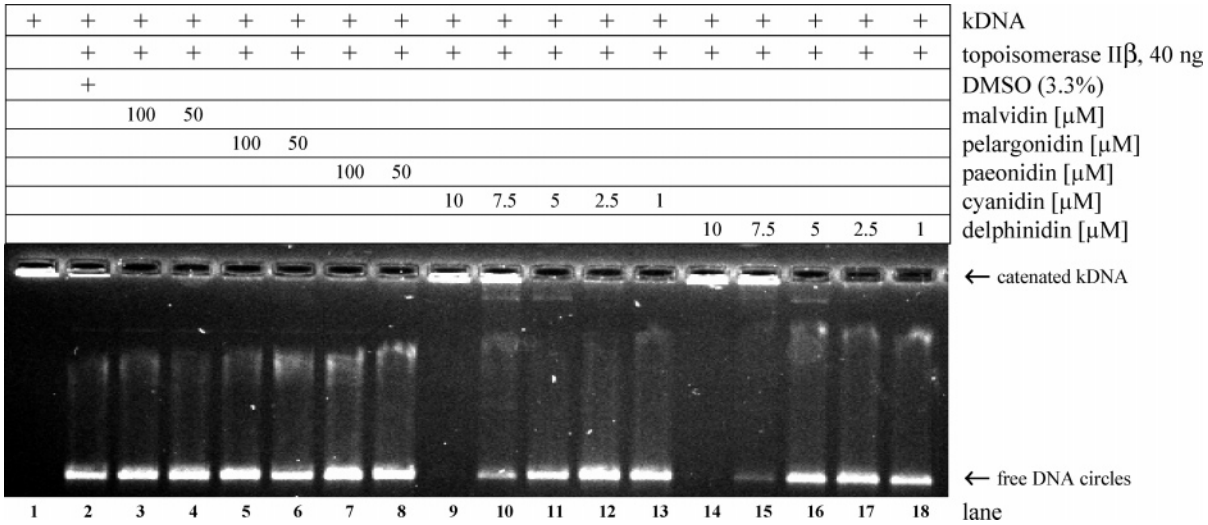


Figure 3. Topoisomerase II catalytic activity (recombinant enzyme) was determined as the decatenation of kDNA from *Crithidia fasciculata*. Recombinant topoisomerase II (40 ng) was added, and reactions were carried out for 60 min at 37 °C in the absence (lane 2) or presence of decreasing amounts of MV (lanes 3 and 4), PG (lanes 5 and 6), PN (lanes 7 and 8), CY (lanes 9−13), or DEL (lanes 14−18). The reaction was stopped with 1% (w/v) SDS, and after digestion with proteinase K, samples were subjected to submarine 1% agarose gel electrophoresis. UV-transilluminated gels were documented by digital photography. Lane 1 shows substrate not reacted with enzyme (catenated kDNA, see arrow). Only active topoisomerase II can release single DNA circles from the catenated DNA network (free DNA circles, see arrow). Shown is a representative gel of three independent experiments with similar outcome.

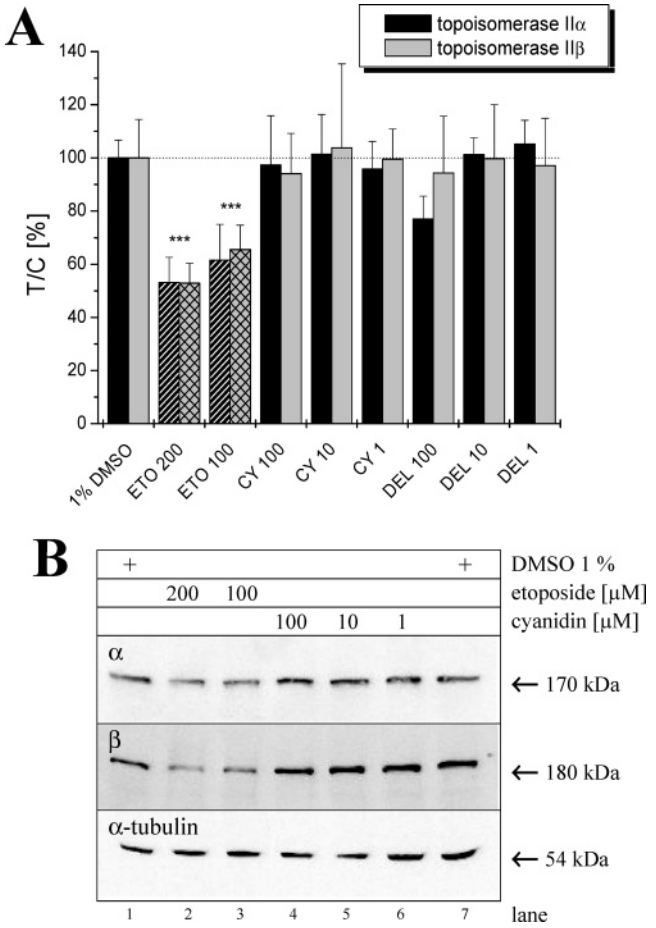


Figure 4. Immunoband depletion assay with HEK293 cells for topoisomerase IIα + β. HEK293 cells (10^6) were incubated for 30 min at 37 °C in 100 μL of drug containing medium. Lysis buffer, containing the appropriate drug concentration, was added, and the cell suspension was sonicated and heated to 95 °C for 10 min. Cell lysates were applied to SDS gel electrophoresis on 7% polyacrylamide gels, followed by Western blotting and immunostaining with rabbit peptide antibodies specific for topoisomerase IIα, IIβ, or α-tubulin. (A) Arbitrary light units were plotted as test over control (T/C, %) for the incubation with DEL or CY (full bars) in comparison to the positive control ETO (hatched bars). Black bars indicate the level of detectable topoisomerase IIα, and gray bars are for topoisomerase IIβ. Data show the means ± SD from four to six independent experiments. The significances indicated are calculated as compared to the control using Students t-test (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$). (B) Representative Western blots for immunoband depletion of topoisomerase IIα (top) or topoisomerase IIβ (middle) by CY and ETO. The content of α-tubulin was determined as a loading control (bottom).

control (Figure 4A; Figure 4B, lanes 2 and 3). A slight but not statistically significant immunoband depletion was observed only in the highest concentration of DEL (100 μM) and was limited to the IIα isoform (Figure 4A). By treatment with CY, no immunoband depletion of both topoisomerase II isoforms was detected (Figure 4A,B, top and middle). Representative Western blots for the incubation with CY are shown in Figure 4B. The content of α-tubulin, which was determined as a loading control, remained unaffected (Figure 4B, bottom). Thus, CY and DEL appear to inhibit the catalytic activity of topoisomerase II without stabilizing the topoisomerase II cleavage complex.

Anthocyanidins Compete with Ethidium Bromide and Hoechst 33258. It could be speculated that CY and DEL inhibit topoisomerases by intercalating into

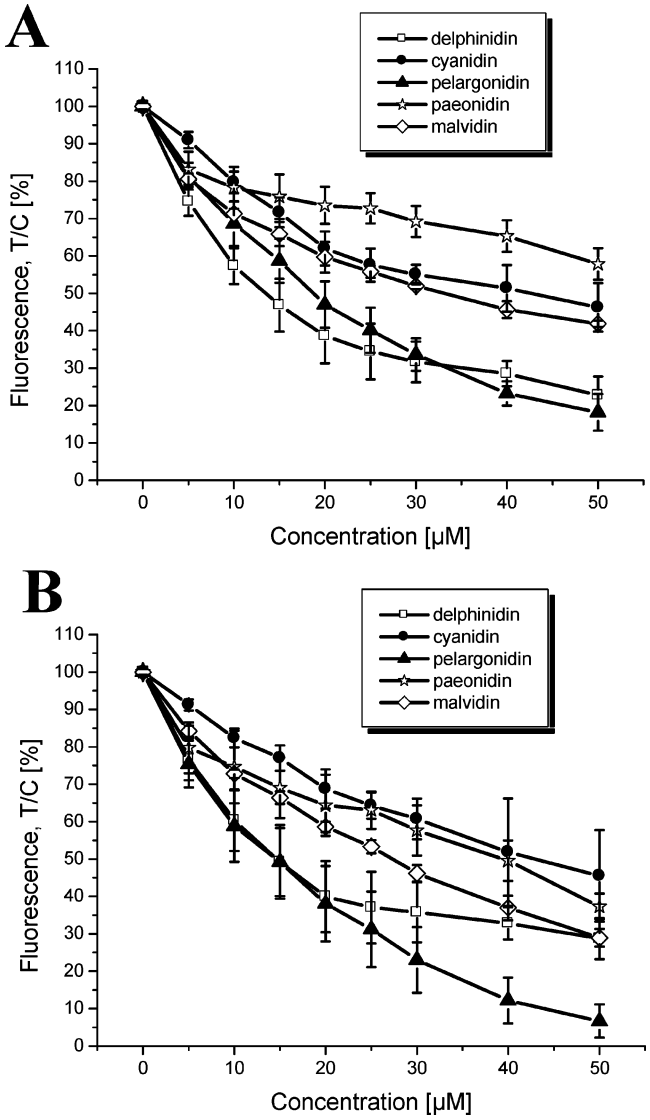


Figure 5. Fluorescence competition assays with calf thymus DNA. The ordinate shows the fluorescence of ethidiumbromide or Hoechst 33258 calculated as percent of control (T/C, %). Competition with ethidiumbromide or Hoechst 33258 is detected as a loss of fluorescence. (A) Competition with the intercalator ethidium bromide. (B) Competition with Hoechst 33258, a minor groove binder. The data presented are the means ± SD of at least three independent experiments, each performed in quintuplicate.

DNA double strands, rendering DNA a less fitting substrate for the enzymes. Whether anthocyanidins intercalate into DNA was investigated using an ethidium bromide fluorescence competition assay. The replacement of ethidium bromide in the DNA double helix (calf thymus DNA) results in a detectable loss of fluorescence. All anthocyanidins tested were found to compete with ethidium bromide for DNA intercalation (Figure 5A). DEL and PG were the most effective compounds with EC_{50} values of 13.7 ± 1.5 μM for DEL and 19.8 ± 2.1 μM for PG. MV and CY were slightly less effective with EC_{50} values of 33.4 ± 1.8 and 42.0 ± 4.5 μM, respectively.

The affinity of anthocyanidins to the minor groove of DNA was studied in a fluorescence competition assay with the minor groove binder Hoechst 33258. All anthocyanidins tested effectively competed with Hoechst 33258 for binding to the minor groove of the DNA. DEL and PG were found to be equipotent with EC_{50} values of 15.2 ± 1.9 and 15.3 ± 2.0 μM, respectively. The EC_{50} values

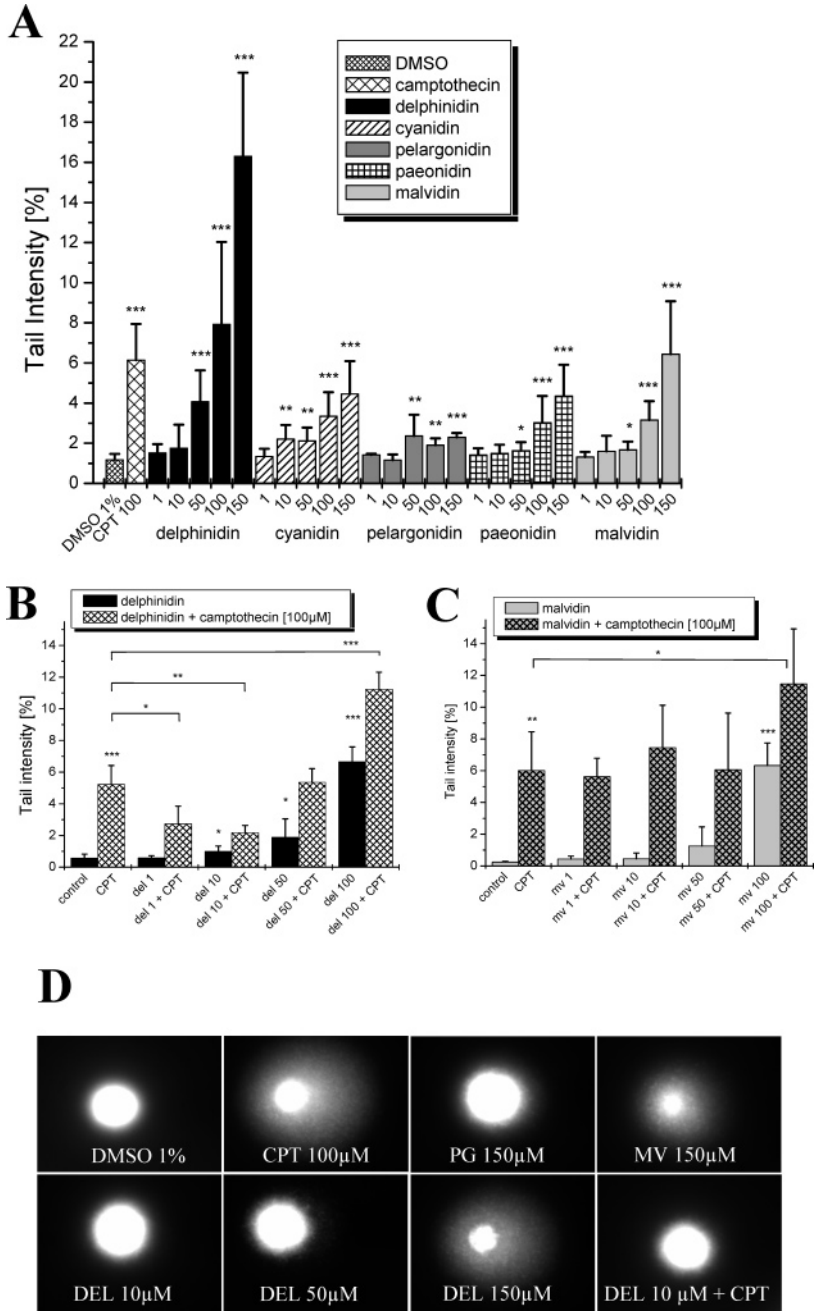


Figure 6. Single-cell gel electrophoresis (comet assay) with HT29 cells. Cells were treated adherent in serum free medium with compounds for either 1 (A) or 1.5 h (B and C) when coincubated with CPT as described in the Materials and Methods. The data presented are the means ± SD of at least three independent experiments, each performed in duplicate. Significances indicated refer to the significance level as compared to the respective control (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$). (A) Induction of DNA strand breaks by incubation of HT29 cells with the respective compounds. The cells were treated with the compounds for 1 h. (B) Effect of DEL on the DNA strand breaking properties of CPT (100 μM). Incubations with DEL are shown in black bars, and coincubations with 100 μM CPT are shown in hatched bars. (C) Effect of MV on the DNA strand breaking properties of CPT (100 μM). Incubations with MV are shown in gray bars, and coincubations with 100 μM CPT are shown in hatched bars. (D) Representative comet assay images of HT29 cells incubated with DMSO (1%), CPT (100 μM), PG (150 μM), MV (150 μM), DEL (10 μM), DEL (50 μM), DEL (150 μM), and DEL (10 μM) coincubated with CPT (100 μM). For the coincubation experiments, HT29 cells were preincubated for 30 min with the solvent control (0.2% DMSO) or the respective anthocyanidin (DEL), followed by 1 h of coincubation of the DEL with 100 μM CPT.

in decreasing order for MV, PN, and CY were 28.0 ± 2.3, 38.1 ± 3.4, and 43.5 ± 2.0 μM. Clearly, all anthocyanidins interact with DNA, whereas only CY and DEL inhibit topoisomerases and the others do not. Thus, a simple correlation between the property of interacting with DNA and their ability of inhibiting topoisomerases can be excluded. Consequently, the effects of CY and DEL on topoisomerases are unlikely to be related to the compounds direct interactions with DNA.

Anthocyanidins Induce DNA Strand Breaks in the Comet Assay. We furthermore investigated the effect of anthocyanidins on DNA integrity, measured as DNA strand breaks by single cell gel electrophoresis (comet assay). The induction of DNA strand breaks was studied in HT29 cells (human colon carcinoma) using the topoisomerase I poison CPT as a positive control (Figure 6A). At low micromolar concentrations (1 μM), all anthocyanidins tested showed no effect on DNA integrity

(Figure 6A). At a concentration of 10 μM, CY already induced a slight but significant increase in DNA strand breaks, whereas the other anthocyanidins showed no significant effect. At 50 μM, all anthocyanidins tested were found to induce at least a slight but significant increase of DNA strand breaks, with DEL being the most potent analogue (Figure 6A,D). At the highest concentration tested (150 μM), the DNA strand breaking potential of anthocyanidins can be summarized as DEL $\gg$ MV $>$ CY $\cong$ PN $>$ PG.

DEL but not MV Modulate the DNA Strand Breaking Properties of CPT. The data presented in Figure 2 suggested that DEL might prevent the stabilization of the cleavable complex by the topoisomerase I poison CPT. To corroborate this hypothesis, we investigated if the presence of DEL is of relevance for the properties of CPT to damage DNA within human cells. HT29 cells were pretreated for 30 min with DEL, followed by a coincubation of DEL and CPT (100 μM) for 1 h. The incubation of CPT alone (1 h) resulted in a significant induction of DNA damage (Figure 6B,D). In the presence of DEL at low micromolar concentrations (1 and 10 μM), the CPT-mediated DNA damage was found to be significantly reduced (Figure 6B,D). The pre-/coincubation of 10 μM DEL with 100 μM CPT results in an almost complete suppression of DNA stand breakage [compare Figure 6D CPT (100 μM) with DEL (10 μM) + CPT (100 μM)]. In the presence of 50 μM DEL, a recurrence of DNA strand breaks was observed, reaching a level mediated by CPT alone. The combination of 100 μM DEL with CPT resulted in a substantial increase in DNA damage, corresponding approximately to the sum of the strand breaking effects of the single compounds.

To test the hypothesis whether topoisomerase I catalytic inhibition, as to be expected from DEL, or mere intercalative/minor groove binding properties are likely to be responsible for the effects shown in Figure 6B, we performed analogous experiments with the combination of MV and CPT (Figure 6C). In the presence of MV (up to 50 μM), the DNA damaging effect of CPT remained unaffected. The combination of 100 μM MV with CPT even enhanced the level of DNA strand breaks, an effect that was comparable to the combination of 100 μM DEL with CPT.

Discussion

In the present study, we show that the anthocyanidins CY and DEL potently inhibit the catalytic activity of topoisomerase I and II, without discriminating between the isoforms IIα and IIβ (Figures 1 and 3). The data clearly show that inhibitory properties are strictly limited to analogues bearing vicinal hydroxy groups at the B-ring (CY, DEL). Many flavonoids of different structural classes have been reported to target human topoisomerases (*11*, *28*−*30*). The majority of these compounds have been described to affect both topoisomerase I and topoisomerase II. Among the flavonoids targeting topoisomerases, the green tea catechin (−)-epigallocatechin-3-gallate (EGCG) and the flavonol quercetin are some of the most potent compounds, exhibiting IC_{50} values in the low micromolar range (*29*−*31*). In the present study, we show that the anthocyanidins CY and DEL are comparable to potent flavonoids such as EGCG or quercetin with respect to their potency to inhibit the catalytic activity of topoisomerases I and II. Within the class of flavons/flavonols, vicinal hydroxy groups at the B-ring have been reported as an important structural element for efficient topoisomerase II targeting (*11*). This is in accordance with our findings that within the group of anthocyanidins topoisomerase inhibitory properties are limited to the analogues bearing vicinal hydroxy groups at the B-ring.

The mode of interaction with the target enzyme is crucial for potential consequences on DNA integrity. The majority of topoisomerase targeting drugs belongs to the so-called class of topoisomerase poisons, stabilizing the covalent DNA-topoisomerase intermediate (*32*, *33*). Most of the flavonoids targeting topoisomerases have been reported to act as topoisomerase poisons (*11*, *29*, *31*, *34*−*37*). In contrast, our results show that CY and DEL do not affect the stability of the cleavable complex of topoisomerase I or II (Figures 2A and 4), indicating that these compounds might represent catalytic inhibitors.

If CY and DEL indeed act as catalytic inhibitors, binding of the enzyme to the DNA, and therefore the formation of the cleavable complex, should be prevented. As a consequence, the cleavable complex stabilization by a topoisomerase I poison, such as CPT, should be counteracted. In respective coincubation experiments with recombinant topoisomerase I, we showed that in topoisomerase inhibitory concentrations ($\geq$1 μM) CY and DEL effectively block the cleavable complex stabilizing effect of CPT (100 μM) (Figure 2B). Anthocyanidins lacking topoisomerase inhibitory properties, such as MV, did not affect the stabilization of the cleavable complex by CPT, indicating that indeed the catalytic inhibition of topoisomerase I is crucial for the protective effect of CY or DEL. We further showed that inside intact cells, the protective effect of DEL is reflected as a suppression of the DNA damaging properties of CPT in the comet assay (Figure 6B). In accordance with the results using recombinant topoisomerase I, also in the comet assay, only the topoisomerase inhibitory analogue DEL, but not MV, was found to affect the strand breaking effects of CPT (compare Figure 6B with 6C). Interestingly, the protective effect of CY and DEL appears to be limited to topoisomerase I. In intact cells (immunoband depletion, Figure 4), no indication was found that anthocyanidins might stabilize the topoisomerase II cleavable complex, in contrast to the observed effects of the known topoisomerase II poison ETO.

Considering the potential catalytic inhibitory activity of CY and DEL, the question has to be raised whether the inhibition of the catalytic activity results from a specific interaction directly with the target protein or whether more unspecific effects such as DNA intercalation or binding to the minor groove of the DNA might be responsible. We found that all anthocyanidins tested effectively compete with ethidium bromide for the intercalation into double-stranded DNA and are also able to replace Hoechst 33258, a minor groove binder (Figure 5A,B). These data indicate that all anthocyanidins tested exhibit substantial affinity to the DNA, as reported previously (*38*, *39*). However, DEL and PG were found to be equipotent in both test systems (Figure 5A,B), but only DEL induced potent topoisomerase inhibition, whereas PG was completely inactive (Figure 3). These results indicate that in the case of the anthocyanidins CY and DEL, the affinity to the DNA is of minor importance for the topoisomerase inhibitory properties, as well as for the protective effects of these compounds against the cleavable complex stabilization by CPT.

Considering the potent inhibition of the catalytic activity of topoisomerases by CY and DEL and the apparent affinity of anthocyanidins to double-stranded DNA, we investigated the effect of these compounds on DNA integrity within human cells. HT29 cells were chosen for testing as a human cell line originating from the gastrointestinal tract. After only 1 h of incubation, all anthocyanidins tested were found to induce DNA strand breaks in the comet assay at concentrations ≥50 μM (Figure 6A). Several flavonoids, especially those acting as topoisomerase poisons, have been reported to possess DNA damaging properties (*12*, *40*−*42*).

Our finding of an induction of DNA strand breaks by anthocyanidins (Figure 6A) raises the question whether these results are of relevance with respect to food safety. In vivo anthocyanins and the respective aglycons have been reported to possess only limited bioavailability (*43*−*49*). However, the available studies on the bioavailability of anthocyanins suffer from low recovery rates, leaving open the question upon the fate of the majority of the ingested compounds. The highest plasma concentration reported so far after the ingestion of 20 g of chokeberry extract (1.3 g of CY-3-glycosides) was about 1 μM (*50*). After oral intake of 400 mL of red grape juice (283.5 mg of total anthocyanins), a maximum plasma concentration of 337 ng anthocyanins/mL (0.7 μM when calculated as CY-3-GLC) was observed after 30 min (*46*). On the basis of the available studies, disregarding the unsatisfying recovery rate, the oral intake of anthocyanins with the diet as well as the intake of food supplements according to the manufactures recommendations should not result in plasma concentrations of anthocyanidins in the range where DNA strand breaks were here shown to be induced in vitro. However, the question is still open whether there are organs or cell types within the body that exhibit enhanced uptake of anthocyanins or the respective aglycons, resulting eventually in different biological responses. On the other hand, irrespective of the apparent low systemic bioavailability, enhanced local concentrations in the gastrointestinal tract might have to be considered, especially under enhanced intake habits. In the present study, we showed that already at concentrations ≥1 μM CY or DEL affect the cleavable complex stabilization by CPT (Figure 2B). In intact cells, 1 μM DEL is sufficient to significantly decrease the DNA strand breaking effect of 100 μM CPT (Figure 6B). Considering the studies on bioavailability, our data indicate that at least locally in the gastrointestinal tract concentrations might be reached where the reported cellular effects of anthocyanidins on the interference with topoisomerases might be of relevance.

In summary, the anthocyanidins CY and DEL represent potent inhibitors of the catalytic activity of topoisomerase I and II, without affecting the stability of the respective cleavable complex. The effect of anthocyanidins on DNA integrity appears to depend on the concentration range. In low micromolar concentrations, an apparent protective effect against the DNA damaging properties of topoisomerase I poisons might be considered. At higher concentrations (≥50 μM), DNA strand breaks are induced by all anthocyanidins tested, an effect that might result from the observed affinity to double-stranded DNA.

The present study shows that with respect to the inhibition of catalytic topoisomerase activity, anthocyanidins are among the most potent flavonoids, comparable, e.g., to the green tea catechin EGCG (IC_{50} values of 3−5 μM) (30) or the flavonol quercetin (IC_{50} values about 4−23 μM) (*11*, *29*). In contrast to most of the flavonoids targeting topoisomerases (*11*, *29*, *31*, *35*, *51*), CY and DEL apparently do not represent topoisomerase poisons. However, with respect to food safety, it cannot be excluded that the effective inhibition of the catalytic activity might imply undesired effects on DNA integrity as well, e.g., by hindering essential topological changes in the DNA.

We further showed that CY and DEL prevent the cleavable complex stabilization by the topoisomerase I poison CPT (Figure 2B). This effect is reflected in intact cells as a suppression of the DNA damaging properties of CPT in the presence of DEL (Figure 6B). The potential protection vs topoisomerase I poisons by CY or DEL might be considered as desirable, regarding the intake of topoisomerase I poisons as constituents of the daily diet such as quercetin or many other flavonoids (*31*). However, enhanced intake of anthocyanins or anthocyanidins, respectively, might be taken into account to be counterproductive when topoisomerase I poisons are used as a part of anticancer therapy, e.g., in the treatment of colon carcinomas (*52*, *53*).

So far, several potential cellular activities of anthocyanidins have been reported, including interference with cellular signaling cascades, regulating cell proliferation (*8*, *9*), antioxidative properties (*6*, *7*, *10*), and, as reported here, inhibition of topoisomerase activity. Taken together, anthocyanidins have to be considered as potent bioactive compounds, interfering with biological processes in a pleiotropic and contradictory way. The data presented here indicate that an uncontrolled increase in consumption of these compounds might be accompanied by adverse effects. Therefore, the ingestion of concentrated preparations of anthocyanins/anthocyanidins, coming into lifestyle fashion lately, must be considered with utmost care.

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