Original Research

Dietary factors and the risk for acute infant leukemia: evaluating the effects of cocoa-derived flavanols on DNA topoisomerase activity

Louise Lanoue[1], Kerri K Green[1], Catherine Kwik-Uribe[2] and Carl L Keen[1,3]

[1]Department of Nutrition, University of California, Davis, One Shields Ave Davis, CA 95616; [2]Analytical and Applied Sciences, Mars Incorporated, Hackettstown, NJ 07840; [3]Department of Internal Medicine, University of California, Davis, One Shields Ave Davis, CA 95616, USA

Corresponding author: Louise Lanoue. Email: llanoue@ucdavis.edu

Abstract

There is cumulative strong evidence that diets rich in flavanols can provide certain positive health benefits, particularly with respect to the cardiovascular system. Consequently, it has been suggested that increasing one's dietary intake of flavanols may be of benefit. Complicating this idea, there are reports that high intakes of certain flavonoids during pregnancy are associated with an increased risk for acute infant leukemia due to a poison effect of select polyphenolic compounds on DNA topoisomerase (topo) II activity that promotes aberrant chromosomal translocations. In the current study, we characterized the effects of select flavanols (epicatechin and catechin monomers), and select flavanol dimers and longer oligomers, on topo II activity, and on cellular toxicity *in vitro*. In contrast to the chemotherapeutic drug etoposide (VP16) and the flavonol quercetin, which strongly inhibited topo II activity and increased the formation of cleavage complexes demonstrating a poison effect, the flavanols epicatechin and catechin had little effect on topo II enzyme activity. Accordingly, several fold greater concentrations of the flavanols were required to achieve cellular toxicity similar to that of quercetin and VP16 in cultures of myeloid and lymphoid cells. Low cellular toxicity and limited topo II inhibition were also observed with a procyanidin-rich cocoa extract. Of all the flavanols tested, the dimers (B2, B5 and a mix of both) exerted the greatest inhibition of topo II and inhibited cellular proliferation rates at concentrations similar to quercetin. However, in contrast to quercetin, the dimers did not function as topo II poisons. Collectively, our *in vitro* data show that cocoa-derived flavanols have limited effects on topo II activity and cellular proliferation in cancer cell lines. We predict that these compounds are likely to have limited leukemogenic potential at physiological concentrations.

Keywords: topoisomerase II, flavanols, flavonoids, toxicity, reproduction, acute leukemia

Experimental Biology and Medicine 2010; **235**: 77–89. DOI: 10.1258/ebm.2009.009184

Introduction

Flavonoids are a large (more than 5000) group of compounds that are present in a wide variety of plant foods. The consumption of diets rich in these phytonutrients has been associated with a number of positive health effects, particularly with respect to inflammation, vascular and cognitive functions, diabetes and cancer prevention.[1–11] Given the putative health benefits of flavonoids, the growing interest in alternative medicine, the general belief that natural, herbal remedies are safe, and the numerous new foods, beverages and supplements currently being developed to contain high levels of flavonoids, it is likely that the amount of flavonoids in the typical diet will rise dramatically over the next decade. In this context, one should note that the potential positive or detrimental consequences of high flavonoid intakes during pregnancy, for the mother or child, are largely unknown, and to a great extent, with the exception of isoflavones, relatively few in-depth reproductive toxicology studies have been carried out.[12] The above is important given reports that suggest an association between pregnant mothers with the highest consumption of a limited number of select foods labeled 'flavonoid rich' and increased risk to giving birth to infants that develop acute leukemia.[13–15] It should be noted however that actual intakes of flavonoids by pregnant women have not been directly assessed in these studies.

The idea that flavonoids, which are ubiquitously distributed in diets rich in fruit, vegetables and grains, could be associated with increased risk of acute infant leukemia is

seemingly counter-intuitive. Indeed, the consumption of fresh vegetables and fruits during pregnancy seemed to be protective against these cancers.[15–17] The basic argument that has been advanced to explain a link between high flavonoid diets and leukemia is that certain flavonoids can inhibit topoisomerase activity *in vitro* at high concentrations.[15,18–23] Type II topoisomerases (topo II) are enzymes that transiently modify the DNA topology to allow for DNA replication, transcription and for chromosomal recombination and segregation.[13,24] To maintain DNA genomic integrity, topo II unwinds supercoiled DNA by cutting and religating segments of DNA, generating transient double strand breaks in the process.[25,26] When this process is interrupted by topoisomerase inhibitors called poisons, cellular accumulation of DNA strand breaks can result in cell death and this is the desired effect of anti-cancer drugs such as etoposide; the idea being that highly proliferating tumor cells have several fold greater activity of the enzyme than normal cells and are selectively killed by topo II poisons.[13,27–29] Regrettably, topo II poisons can also promote aberrant DNA recombination and illegitimate chromosomal translocations of the mixed lineage leukemia (*MLL*) gene at chromosome 11q23.[13,25,30–37] In turn these abnormal *MLL* gene rearrangements result in the formation of chimeric proteins that can disrupt hematopoieisis and cause acute lymphoid and myeloid leukemia in neonates.[31,38,39] This occurs in more than 80% of all cases of acute leukemia in infants,[13,34,36] and is associated with a poor prognosis with fewer than half of the infants that develop leukemia surviving beyond five years.[17] As the etiology of childhood leukemia is unknown, the early report of a case control study showing an association between maternal consumption of a limited number of foods containing topo II inhibitors and infant acute myeloid leukemia (AML) is of considerable interest.[40]

Because cocoa was included in the list of foods that was positively associated with an increased risk for infant AML, in the current work we determined the effects of cocoa-derived flavonoids on topoisomerase activity. Specifically, we tested the hypothesis that select flavanols in cocoa (present as monomers, dimers and higher oligomers) are not naturally occurring topoisomerase inhibitors, and that they do not induce cellular toxicity at physiologically relevant concentrations. These flavanols were studied for four reasons. First, cocoa and chocolate represent two foods that can have very high concentrations of flavanols and their oligomers, the procyanidins.[12,41] Second, the anti-topoisomerase effects of these compounds remain largely untested. Third, these products are available in relatively pure forms, and flavanol monomers and dimers have been shown to be absorbed.[42] Finally, the data from a recent prospective study suggesting that increased chocolate consumption during pregnancy reduces the risk for preeclampsia could precipitate an increased consumption of chocolate by pregnant women.[43]

Methods

Materials

Purified human topoisomerase IIα (p170 form), ATP, amsacrine, pRYG supercoiled plasmid, pRYG linear and relaxed markers, kinetoplast DNA (kDNA), a high molecular weight aggregate of interlocked (catenated) mitochondrial DNA purified from *Crithidia fasciculate*, and decatenated and linear kDNA markers were purchased from TopoGen (TopoGen, Port Orange, FL, USA). Proteinase K was purchased from Promega (Promega, Madison, WI, USA). Catechin, epicatechin, quercetin, myricetin, apigenin, genistein, etoposide (VP16), ethidium bromide, dithiothreitol (DTT), catalase, apotransferrin, bovine serum albumin (BSA), sarkosyl, bromophenol blue, glycerol and other basic chemicals were obtained from Sigma (Sigma, St Louis, MO, USA). Three types of dimers were supplied by Mars Incorporated (Mars, Hackettstown, NJ, USA): (1) procyanidin dimer B2 (97% pure; isolated from cocoa); (2) procyanidin dimer B5 (97.4% pure; isolated from cocoa); and (3) a mixture of these dimers (CD, containing B2:B5 dimers in a ratio of 6.5:1), as well as a cocoa extract (CEP), which is enriched in a mixture of flavanols and procyanidins (Table 1, Mars Incorporated). The Burkitt lymphoma (Raji) and the promyelocytic leukemia (HL-60) cell stocks were purchased from ATCC (ATCC, Manassas, VA, USA). RPMI and Iscove's modified Dubelco media, fetal bovine serum (FBS), and Hank's buffered and phosphate buffered saline solutions (HBSS, PBS) were from Gibco (InVitrogen, Carlsbad, CA, USA).

Topoisomerase II activity assay

Topo II activity was measured by the ability of topo II to decatenate a substrate (kDNA) into nicked circular (OC) and relaxed (R) products. Baseline topo II activity was determined by incubating 2 U of the enzyme with 0.5 μg kDNA for 30 min at 37°C in a 20 μL reaction mix containing 50 mmol/L Tris-HCl (pH 8.0), 120 mmol/L KCl, 10 mmol/L

Table 1 Composition of cocoa extract powder (CEP)

Compounds	Amount
Flavanol monomers (μg/mg)	
Catechin	24.2
Epicatechin	115
Flavanol oligomers (mg/g)	
Dimer	63.2
Trimer	47.2
Tetramer	38.5
Pentamer	30.6
Hexamer	25.0
Heptamer	10.5
Octamer	4.1
Nonamer	6.2
Decamer	1.3
Total oligomeric procyanidins	226.6
Polymers	6.0
Flavonols (μg/mg)	
Quercetin-3-O-glucoside	1.0
Quercetin-3-O-arabinoside	1.0
Anthocyanins (μg/mg)	
Cyanidin-3-O-galactoside	Na*
Cyanidin-3-O-arabinoside	Na
Alkaloids (%)	
Theobromine	10.3
Caffeine	1.1

*Non available

$MgCl_2$, 0.5 mmol/L DTT, 0.5 mmol/L ATP and 30 μg/mL BSA. Topo II inhibition by the flavanols (monomers, dimers and mixture) was determined by adding increasing concentrations of these compounds to the reaction mixture. Stock solutions of each of these were prepared to ensure that equal volumes of samples were added to the reaction mix. The inhibitory effects of etoposide, a known topo II poison (25 μmol/L VP16), and its vehicle control (0.2% dimethyl sulfoxide, DMSO) were also tested. The reaction was stopped with the addition of 4 μL of loading buffer (0.025% bromophenol blue and 25% glycerol) containing 5% sarkosyl and the decatenated products were separated by gel electrophoresis (1% agarose containing 0.5 μg/mL ethidium bromide) at 80 mV in tris-acetate-ethylenediaminetetraacetic acid (EDTA) or TAE buffer. kDNA product markers were included in each gel run. The amount of catenated kDNA retained in each well (W), and the formation of decatenated products were visualized by exposure of the gels to UV light using a molecular imager (BioRad, Hercules, CA, USA) and photographed. The surface area of each of these three bands was quantified using Quantity One analysis software (BioRad), where fluorescence pixel intensities were assigned arbitrary units (AU). Topo II activity (expressed in AU) was calculated as the sum of the decatenated products divided by the total amount of DNA in each lane. Additionally, the amount of nicked (OC) kDNA was quantified and analyzed independently.

Topoisomerase II poison assay

The potential topo II poison effect of flavanols was evaluated by measuring the amount of linear DNA produced after 15 min incubation at 37°C of these compounds in a reaction mix containing 0.150 μg of supercoiled pRYG, 6 U of topo II, 10 mmol/L Tris-HCl (pH 7.9), 100 mmol/L KCl, 0.1 mmol/L EDTA, 5 mmol/L $MgCl_2$, 2.5% glycerol and 1 mmol/L ATP. The reaction was terminated by adding sodium dodecyl sulfate (SDS, 1%) and samples were digested with proteinase K (1 mg/mL) for 30 min at 55°C to release the DNA from its enzymatic complex. Positive controls included known topo II poisons (VP16 and amsacrine). The accumulation of linear DNA, an indicator of cleavage complex formation was separated by gel electrophoresis and quantified as described above. The potential poison effect of select flavanols was defined as the amount of linear DNA produced above baseline conditions.

Cellular topoisomerase activity

Raji cells (3×10^6) were seeded in six-well plates in 5 mL of culture medium as described above. Cells were incubated with the test compounds for 16 h at 37°C under 5% CO_2. Cells were collected on ice in 15 mL conical tubes, rinsed and pelleted by centrifugation. For nuclei isolation, the cell pellets were washed twice in ice-cold hypotonic buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 4 mmol/L $MgCl_2$) containing 0.15% Nonidet, 0.5 mmol/L phenylmethanesulphonylfluoride (PMSF) and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The cells were lysed with a tight fitting Dounce homogenizer and the presence of nuclei was verified by phase microscopy. The nuclei extracts were transferred to microfuge tubes and pelleted by centrifugation. Proteins were extracted with the addition of a high salt buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5 mmol/L PMSF, 1 mol/L NaCl) and recovered by centrifugation (100,000 *g* for 1 h at 4°C). The protein concentration of the nuclear extracts was determined using a BioRad assay on a microplate reader with a BSA standard curve. Topoisomerase II activity of the protein extracts was determined as described above.

Cell toxicity

HL-60 and Raji cells were grown in T75 flasks in their respective media (RPMI and Iscove's modified Dubelco) supplemented with 10–20% FBS and cytotoxicity tests were carried out between passages 2 and 10 for both cell lines. To evaluate the cellular toxicity of the flavanol monomers, dimers and a procyanidin-rich cocoa extract, 5×10^5 cells were seeded in 3 mL of medium in 12-well plates and incubated with graded concentrations of the flavanols or the vehicle (50% ethanol) for 24 h at 37°C under 5% CO_2. The etoposide VP-16 (25 μmol/L) and its vehicle (DMSO) were used as positive controls. Apotransferrin (100 ng/mL) and catalase (100 U/mL) were added to the medium to exclude effects secondary to hydrogen peroxide production and other oxidation products. Prior test runs showed that these two chemicals had no influence on the proliferation rates of the cells, or their response to the test compounds (data not shown). As the cocoa extract also contains theobromine (10%) and caffeine (1%), we tested the effects of these substances in our cell model. Results obtained following the addition of caffeine and theobromine (0.1, 0.25 and 1 mg/mL) to media were similar to those obtained for control (data not shown), thus these compounds did not contribute to the effects of the cocoa extract.

The cellular toxicity associated with a 24 h exposure to the various test compounds was evaluated using trypan blue (TB) exclusion cell viability assay, and by tetrazolium (MTT) reduction in triplicate. For TB exclusion, viability was defined as the ability of cells to exclude the TB dye, indicating normal membrane integrity. For MTT reduction assay, the cells from each treatment were rinsed and pelleted at 6000 *g* for 10 min, and incubated at 37°C for 30–60 min in 300 µL of solution (25 mmol/L Hepes, 0.5 mg/mL MTT in MEM). The reaction was stopped by the addition of cold PBS. Following centrifugation, the formazan crystals were solubilized in DMSO, and read at 540 and 650 nm using a microplate reader (Wallac 1420 Multilabel counter, Perkin Elmer, Shelton, CT, USA). Only viable cells with intact mitochondrial respiratory chain enzymes will reduce the MTT compound to purple-colored formazan.

Statistical analysis

Statistical analyses were performed using SAS, version 9.0 (SAS Institute, Cary, NC, USA). Significant differences between treatments were determined by analyses of variance using generalized linear models (GLM); significant differences between groups/doses were determined by

least square means test. Statistical significance was set at $P < 0.05$. Results obtained from 3–6 different experiments are presented as means ± standard error of the means (SEM).

Results

Topoisomerase II activity

Topo II activity was assessed by measuring the decatenation of kDNA in a standard reaction. As is depicted in Figures 1–3, baseline topo II activity approximated 70–80 AU under our experimental conditions. The addition of a known topo II inhibitor (25 μmol/L VP16) to the reaction reduced topo II activity to 47–67% of baseline (a 33–53% inhibition), whereas an equivalent amount of the vehicle control (0.2% DMSO) did not significantly modulate baseline topo II activity (Figures 1b, 2b and 3b).

The addition of the flavanol monomers epicatechin and catechin to the reaction in concentrations ranging from 5 to 100 μmol/L did not significantly affect topo II activity (Figure 1). This is in contrast to the flavonol quercetin, for which 10 μmol/L was sufficient to inhibit DNA topo II activity to a greater extent than that exerted by 25 μmol/L VP16, the positive control (Figure 1 and Table 2). Similar robust inhibitory effects were observed with 25 μmol/L myricetin and genistein (data not shown). The flavanol dimers at concentrations ranging from 0 to 2.5 μmol/L did not modulate topo II activity. However, topo II activity was inhibited by the three dimers at concentrations greater than or equal to 5 μmol/L (Figure 2). At 5 μmol/L (~3 μg/mL), dimer B2 significantly reduced topo II activity by 40%, to 60% whereas dimer B5 and the cocoa dimer mixture (CD) significantly decreased topo II activity to 40% and 20% of baseline (Figure 2). At concentrations higher than 5 μmol/L (10–100 μmol/L), the dimers completely prevented kDNA decatenation (data not shown). Similar to the dimers, the procyanidin mix extracted from cocoa (CEP) significantly reduced topo II activity at concentrations higher than 2.5 μg/mL as demonstrated by the significant amount of kDNA retained in the wells at 2.5, 5 and 10 μg/mL CEP (Figure 3).

Topoisomerase poison effect

The poison effect of select flavonoids was evaluated and compared with VP16 and amsacrine, two known topo II

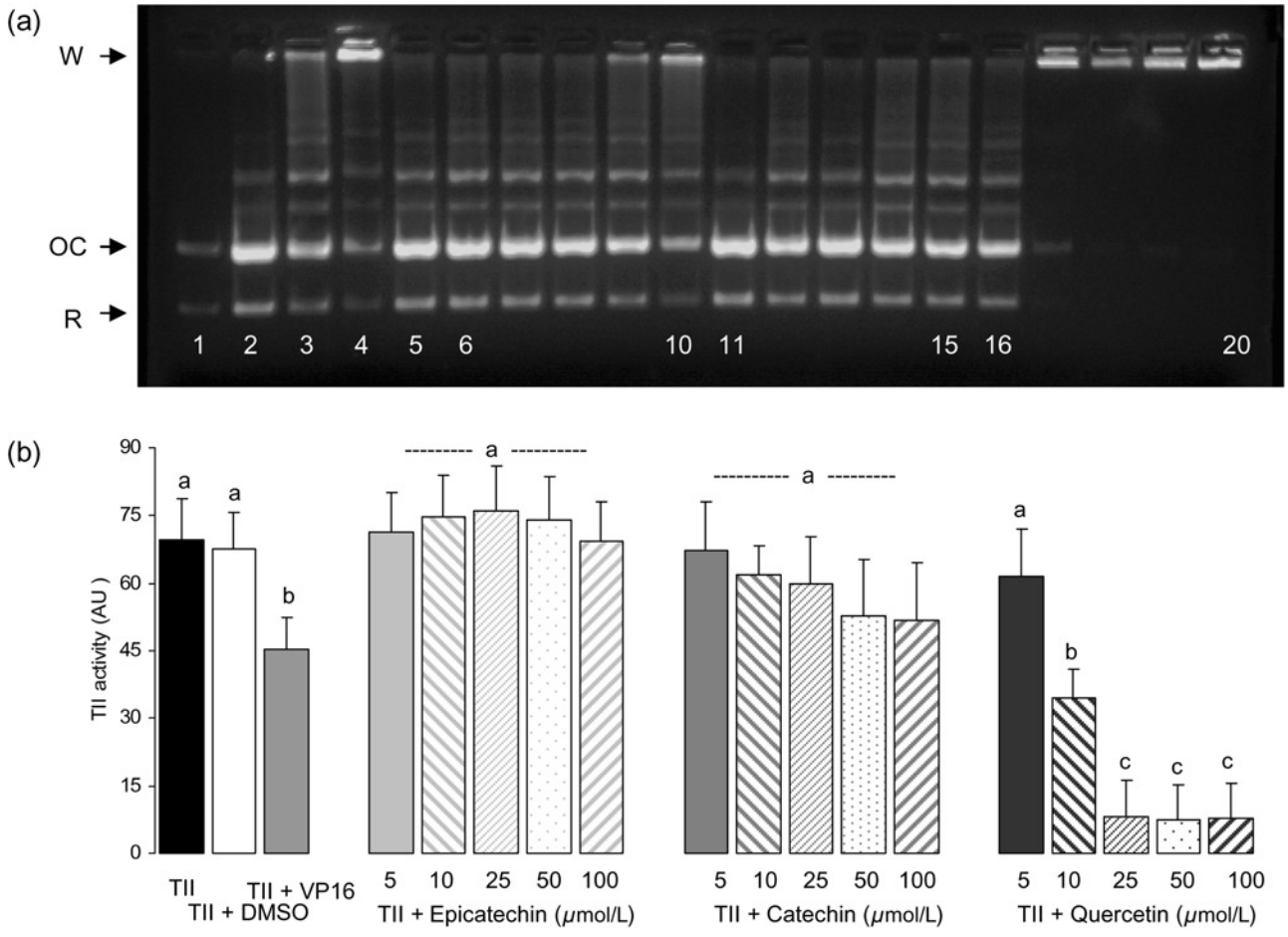

Figure 1 Effects of epicatechin, catechin and quercetin on topo II catalytic activity. (a) Representative gel electrophoresis. Lane 1: open circular (OC) and relaxed (R) DNA markers; in lanes 2 to 20: topo II was incubated with kDNA alone (lane 2); or with 25 and 50 μmol/L VP16 (lanes 3 and 4); 0.2% DMSO (lane 5); 5, 10, 25, 50 and 100 μmol/L epicatechin (lanes 6–10); 5, 10, 25, 50 and 100 μmol/L catechin (lanes 11–15); and 5, 10, 25, 50 and 100 μmol/L quercetin (lanes 16–20). The position of catenated kDNA (W) is indicated. (b) Graphic representation of topo II activity expressed in arbitrary units and calculated as the proportion of decatenated products over the total amount of kDNA. Bars are means ± SEM of 3–5 different reactions. Bars with different superscripts represent significant differences between baseline topo II activity (TII, solid black) and the various treatments. kDNA, kinetoplast DNA; SEM, standard error of the means

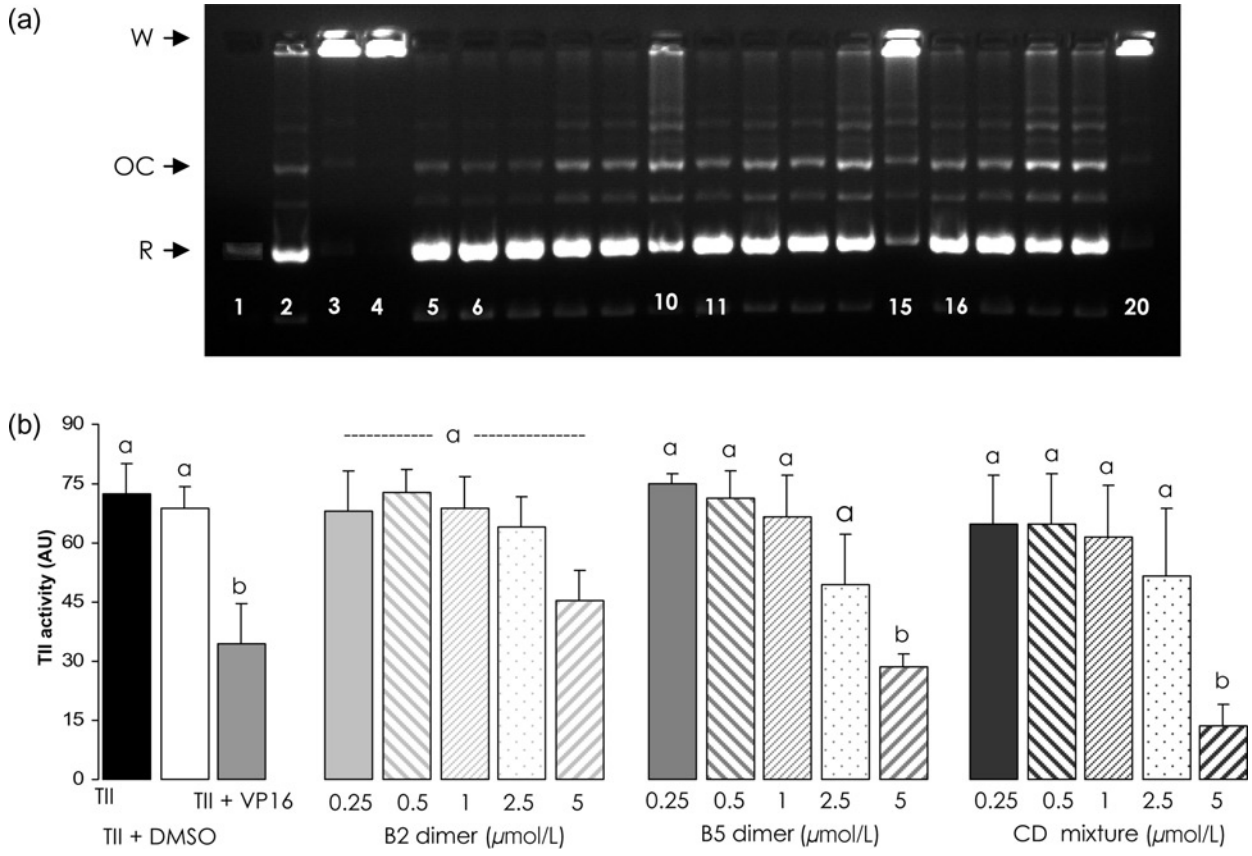

Figure 2 Effects of the flavanol dimers on topo II activity. (a) Representative gel electrophoresis. Lane 1: kDNA decatenated (OC) and relaxed (R) markers; lane 2: baseline topo II activity; lanes 3 and 4 show topo II inhibition of 25 and 50 μmol/L VP16, respectively, as kDNA is not decatenated and remains in the well; lane 5 shows no topo II inhibition of the 0.2% DMSO vehicle; lanes 6–10: B2 dimer at 0.25, 0.5, 1, 2.5 and 5 μmol/L; lanes 11–15: B5 dimer at 0.25, 0.5, 1, 2.5 and 5 μmol/L; lanes 16–20: cocoa dimer (CD) mixture at 0.25, 0.5, 1, 2.5 and 5 μmol/L. (b) Calculated topo II activity. Bars are means $\pm$ SEM of 4–5 reaction assays. Bars with different superscripts are significantly different from baseline topo II activity (TII solid bars). kDNA, kinetoplast DNA

poisons. Of all flavonoids tested, quercetin and genistein were the strongest poisons; for these compounds, 25 μmol/L resulted in significant accumulation of linear DNA, compared with 100 μmol/L for apigenin, VP-16 and amsacrine; myricetin showed no poison effect in our system (Figures 4a and b). The flavanols epicatechin and catechin were not effective poisons as 200 μmol/L concentrations of these monomers resulted in significantly less linear DNA formation than the amount produced with either the positive controls or quercetin and genistein (Figure 4c). Similar to the monomers, the three types of cocoa-derived dimers and procyanidins showed very limited poison effects; as shown in Figures 5a–d, high concentrations of these products were not associated with the formation of stable cleavage complexes, measured in our assays by linear DNA.

Cellular topoisomerase activity

Prior to measuring topo II activity in Raji cells, we established the effects of the addition of 25 μmol/L VP16 or its vehicle to the culture medium on cell viability over a 24-h period. Raji cell number and metabolic competence were largely unaffected up to 12 h, while 40% of cells were dead after a 24-h exposure to the etoposide (Figure 6). These data suggest that by 24 h, significant topo II inhibition had already occurred and residual topo II activity may not have been detectable by our assay. Thus, cellular topoisomerase activity was evaluated at 16 h, when 80% of the cells remained viable in the presence of 25 μmol/L VP16. In these conditions, nuclear topo II activity tended to be lower in cells exposed to VP16 compared with vehicle (DMSO), but the difference between the two groups was not statistically significant (Figure 7). Raji cells grown in the presence of epicatechin (100 μmol/L), quercetin (50 μmol/L), cocoa dimer mix (100 μmol/L) and two concentrations of cocoa extract procyanidins (100 and 250 ng/mL) had similar nuclear topo II activities as that measured in cells treated with the ethanol control; and all flavanoid treatments yielded higher topo II activity than VP16 (Figure 7). Thus, none of the flavanols inhibited topo II activity in Raji cells, at the doses tested.

Cellular toxicity

Overall, assessment of cell toxicity using the MTT reduction assay yielded results similar to those of cell viability as measured by TB exclusion for both cell lines; hence only the cell viability data in HL-60 are presented here (Figure 8). Exposure (24 h) to high concentrations of epicatechin and catechin was largely non-toxic to the cells in contrast to quercetin, which was associated with

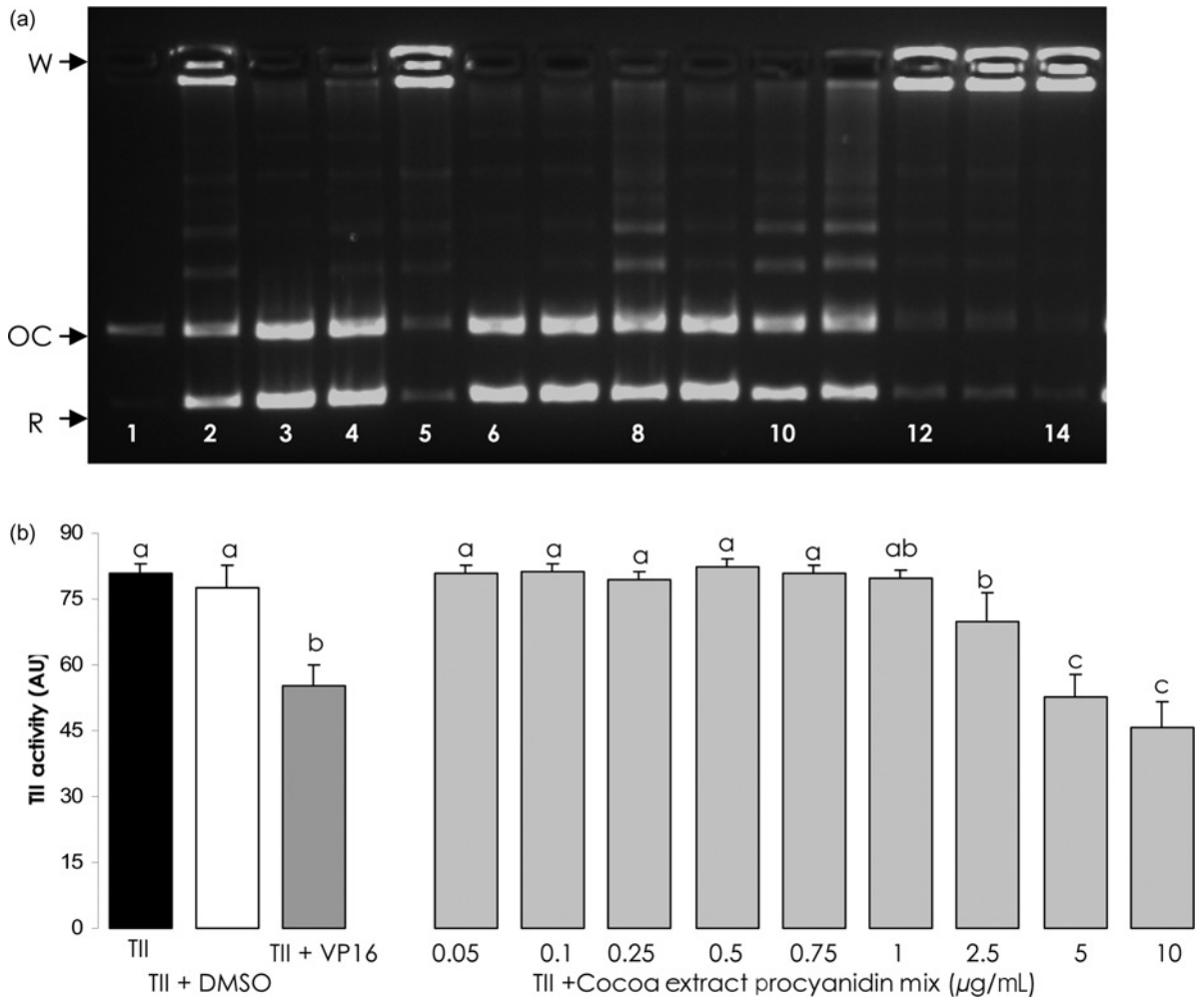

Figure 3 Effects of procyanidin-rich cocoa extract and VP16 on topo II activity. (a) Representative gel electrophoresis. Lane 1 shows the decatenated markers; in lanes 2–14 topo II was incubated with kDNA alone (lane 3); or with 25 and 50 μmol/L VP16 (lanes 2 and 5); 0.2% DMSO (lane 4); or with 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5 and 10 μg/mL, cocoa extract (CE, lanes 6–14). (b) Calculated topo II activity. Bars are means $\pm$ SEM of 8–12 independent assays. Bars with different superscripts indicate a significant difference between baseline topo II activity (TII solid black) and a given concentration of the cocoa extract. kDNA, kinetoplast DNA; SEM, standard error of the means

a significant reduction in cell number at lower concentrations. Specifically, a media concentration of 10 mmol/L of flavanol monomers reduced cell numbers to amounts similar to that of 25 μmol/L VP16 compared with 300 μmol/L for quercetin (Figure 8a). The inclusion of VP16 to culture medium not only prevented the normal rate of cell proliferation over 24 h, but also was associated with a 44% reduction in cell viability (Figure 8a – c). These effects may be the consequence of VP16-mediated topo II inhibition, which strongly impairs cellular replication and induces cell death.

HL-60 exposure to the B2, B5 and CD dimers at concentrations ranging from 10 to 300 μmol/L did not result in significant loss of cell viability (Figure 8b). For the dimer B2, supplementation of the culture medium at concentrations of 300 μmol/L was associated with a significant reduction in cell number compared with the alcohol vehicle control; although this reduction in cell viability

Table 2 Concentrations of monomers, dimers and cocoa extract resulting in topo II inhibition similar to that of 25 μmol/L VP16

	Concentration of flavonoids that resulted in topo II inhibition similar to VP16	Concentration of flavonoids that resulted in topo II poison similar to VP16	Concentration of flavonoids that resulted in cellular toxicity similar to VP16
Epicatechin	>100 μmol/L or 29 μg/mL	200 μmol/L or 58 μg/mL	10 mmol/L or ~3 mg/mL
Catechin	>100 μmol/L or 29 μg/mL	100 μmol/L or 29 μg/mL	10 mmol/L or 3 mg/mL
Quercetin	10 μmol/L or ~3.5 μg/mL	25 μmol/L or 9 μg/mL	300 μmol/L or 100 μg/mL
Dimer B2	>5 μmol/L or ~3 μg/mL	>100 μmol/L or 60 μg/mL	>300 μmol/L or 90 μg/mL
Dimer B5	5 μmol/L or ~3 μg/mL	>100 μmol/L or 60 μg/mL	>300 μmol/L or 90 μg/mL
Cocoa Dimer Mix (CD)	5 μmol/L or ~3 μg/mL	>100 μmol/L or 60 μg/mL	>300 μmol/L or 90 μg/mL
Procyanidin-rich cocoa extract (CEP)	2.5 μg/mL	10 μg/mL	>1.5 mg/mL

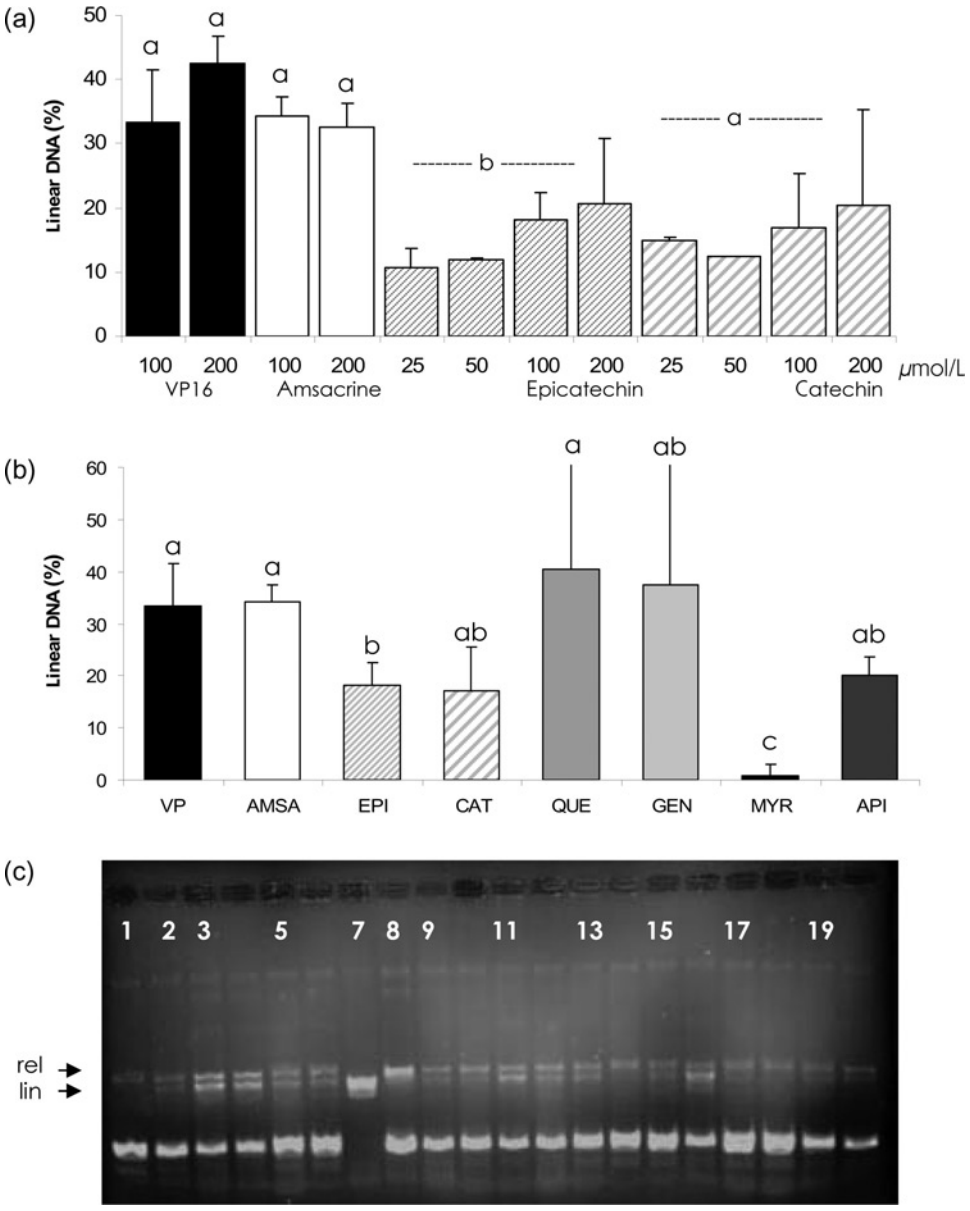

Figure 4 Poison effect of flavonoids. (a,c) Graphic representations of cleavable complex (linear DNA) formation following 15 min incubation with different flavonoids. (b) Representative gel electrophoresis of a reaction containing pRYG alone (lane 1); with topo II (lane 1; with topo II and VP16 and amsacrine (100–200 μmol/L, lanes 3–6); lanes 7 and 8 show the linear (lin) and relaxed (rel) markers; lanes 9 to 20 reaction mix containing pRYG, topo II and 100 and 200 μmol/L of epicatechin (9–10), catechin (11–12), quercetin (13–14), genistein (15–16), myricetin (17–18) and apigenin (19–20). (a) Solid bars are VP16 effects; open bars are amsacrine and stippled bars are epicatechin and catechin. (c) Topo II poison effect of 100 μmol/L VP16, amsacrine (AMSA), epicatechin (EPI), catechin (CAT), quercetin (QUE), genistein (GEN), myricetin (MYR), and apigenin (API). Bars are means $\pm$ SEM of 3–6 assays. Bars with different superscripts indicate a significant difference ($P < 0.05$). SEM, standard error of the means

(−25%) was not as severe as that in response to 25 μmol/L VP16 (−50%).

With respect to the cocoa extract, HL-60 cells were not affected by CEP at the doses tested (Figure 8c), as cell counts were similar to the vehicle for CEP concentrations ranging between 0.1 and 1.5 mg/mL. Moreover, at these doses, HL-60 cell numbers were significantly higher than the counts measured after 24-h incubation with VP16, suggesting a low toxicity of these compounds. Consistent with the other series of experiments, VP16 was toxic to both cell lines, and it inhibited cell proliferation.

Discussion

The experiments described in this report were designed to test the DNA topo II inhibitory effects of flavanol monomers, and select flavanol dimers and oligomers using standard *in vitro* assays. Our results show that, compared with known anti-topo II agents, cocoa-derived flavanols, particularly the monomers were not effective inhibitors of topo II activity and did not exert strong poison effect. This is in contrast to several other types of flavonoids, for which we and others show significant topo II inhibition using similar

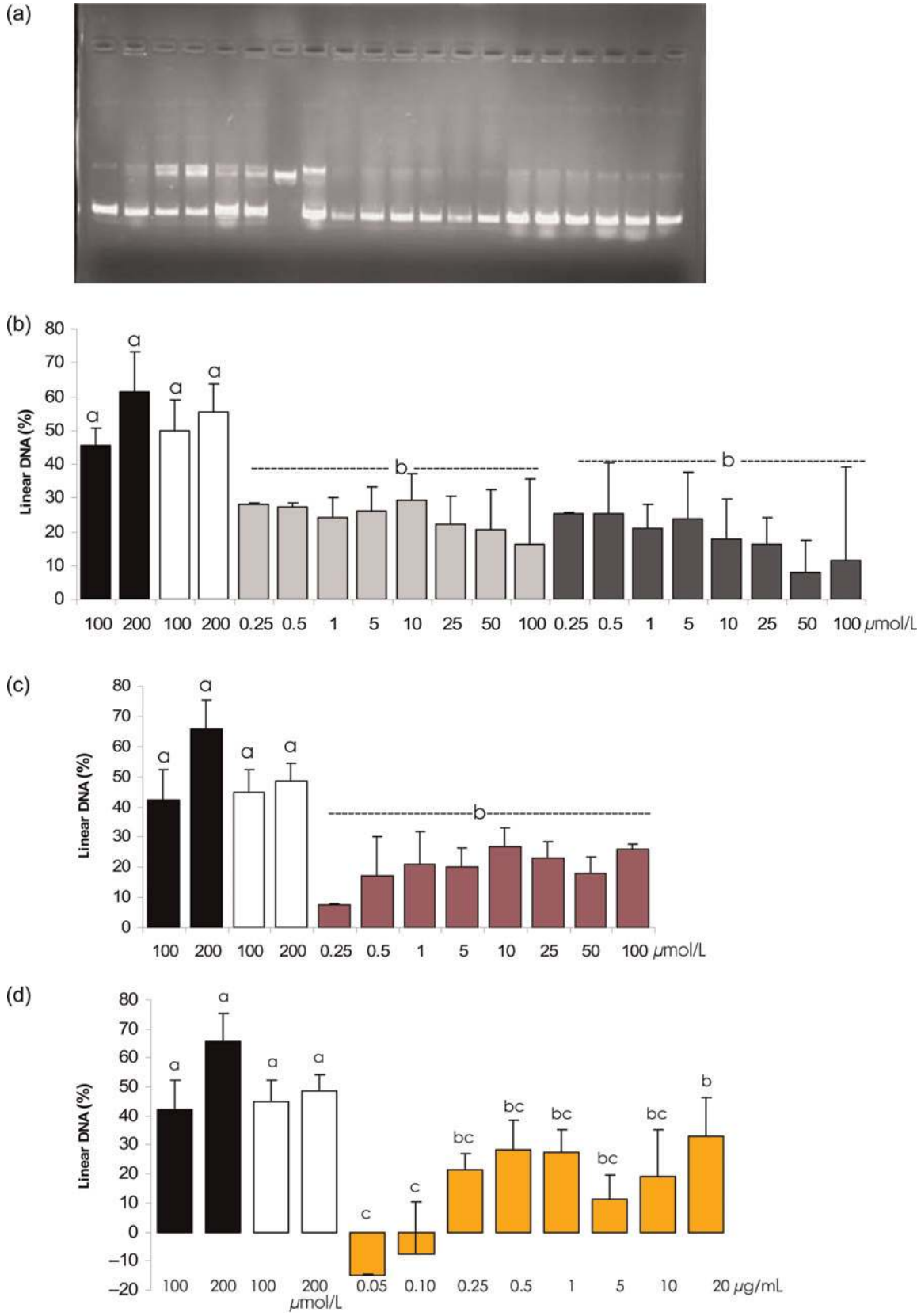

Figure 5 Poison effect of flavanol dimers and oligomers. (a) Representative gel electrophoresis: pRYG (lane 1); pRYG and topo II (lane 2); with VP16 and amsacrine (100–200 μmol/L, lanes 3–6); lanes 7 and 8 show the linear (lin) and relaxed (rel) markers; with B2 (lanes 9–14) and B5 (lanes 15–20) at 1, 5, 10, 25, 50 and 100 μmol/L. (b–d) Graphic representations of cleavage complex formation following 15 min incubation with flavanol dimers. Solid bars are VP16 effects; open bars are amsacrine, gray bars are dimers B2 and B5 (b); magenta bars are cocoa dimer mix (CD) (c); yellow bars are cocoa extract procyanidin oligomers (d). Bars are means $\pm$ SEM of 4–6 assays. Bars with different superscripts indicate a significant difference between VP16 and the flavanols

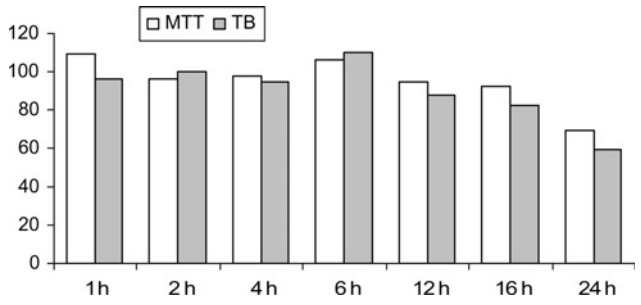

Figure 6 Time course response to 25 μmol/L VP16. Raji cells (0.6×10^5/mL) were cultured in medium containing 25 μmol/L VP16, or its vehicle (DMSO), and collected at 1, 2, 4, 6, 12, 16, 24 h of treatment. Cell viability was determined by trypan blue (TB) assay and metabolic competence by MTT reduction. Data are expressed as % of control for each time point

in vitro conditions.[18–22] For example, Strick *et al.*[22] reported that quercetin, fisetin, luteolin, apigenin and genistein inhibited topo II activity at concentrations similar to that of the anti-cancer drug etoposide (VP16). Thus, the type and degree of topo II interference by flavonoids can be predicted from the structural differences between classes of flavonoids.[20,21,44,45] Flavanoids with a planar conformation (due to C2–C3 unsaturation and a C4 keto group in the C ring), and with multiple hydroxyl groups on their B ring are effective topo II poisons *in vitro*.[19,20,27] Flavanols, such as epicatechin, have a fully saturated C ring and are not fully hydroxylated, have seemingly significantly low ability to interact or inhibit the enzyme. This is important, given that flavanols (from tea, wine and cocoa) account for more than 80% of the total dietary flavonoid intake of US adults[46]; and relevant, given the numerous health benefits that have been recently associated with the intake of diets rich in these nutrients.[41,47]

It should also be noted that in our system, the flavanol dimers and oligomers exerted some anti-topo II activity at relatively low concentrations. Although the cocoa extract contained 31% epicatechin (37% monomer), it provided 10–20-fold greater topo II inhibition than the flavanols, suggesting a potential synergistic effect among the various fractions of this complex mix of polyphenols. A limited number of investigations have studied the topo II inhibitory effects of more complex flavonoids by testing either purified oligomers or crude plant extract samples containing a mixture of compounds including flavonoids. These investigations have reported significant topo II inhibition using a similar *in vitro* enzymatic assay, or other indirect markers of topoisomerase activity.[48–50] Agents that inhibit topoisomerase activity are categorized as poisons or as catalytic inhibitors. Topoisomerase poisons act by stabilizing the cleavage complex intermediates formed during the enzymatic reaction and have been associated with illegitimate rearrangements of the *MLL* gene and incidence of acute leukemia. Our data indicate that the flavanol oligomers are not topo II poisons. Although catechins are considered topo II poisons by some,[13,17,23] the published data show that flavanols are effective topo II inhibitors only as phenolic extracts,[51,52] tannins[53] or gallic derivatives,[18,54,55] or at very high levels of catechin.[21] The distinction cannot be overemphasized, given recent reports that physiological concentrations of flavanol monomers and dimers are associated with positive cardiovascular effects.[1,11,56,57]

We also evaluated the toxicity of flavanols in myeloid and lymphoid cancer cells and correlated these results with that from the topo II activity assays. There was a strong concordance with both sets of data (Table 2). To illustrate, the flavanol monomers showed both weak topo II inhibitory activity (30 μg/mL were needed to match VP16), and limited functional or downstream effects (3 mg/mL were needed to induce cell toxicity). In contrast, dimers and oligomers inhibited topo II activity at lower concentrations than the monomers (3 μg/mL) and also exerted cellular toxicity at lower concentrations than the monomers (90 μg/mL, Table 2). In our cell culture experiments, the effects of the

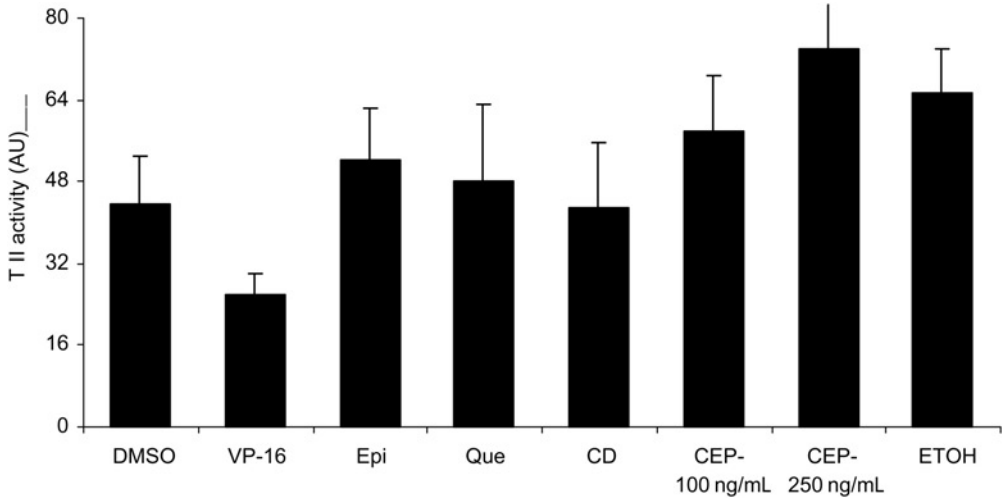

Figure 7 Nuclear topo II activity in Raji cells following a 16-h exposure to VP16, quercetin or flavanol monomers, dimer and oligomers. Raji cells (3×10^6) were seeded in six-well plates containing 5 mL medium supplemented with catalase (100 U) and apotransferrin (100 μg/mL). The etoposide (25 μmol/L), quercetin (Que, 50 μmol/L), epicatechin (Epi, 100 μmol/L), dimer mixture (CD, 100 μmol/L) and cocoa extract (CEP, 0.1–0.25 ng/mL), and their respective vehicle (DMSO, 50% ethanol) were added to each well from frozen stocks, and cells were incubated for 16 h. Nuclear proteins were extracted as described and topo II activity was measured on equal amounts of proteins. Values are means $\pm$ SEM of 5–8 experiments, expressed in arbitrary units (AU). ANOVA: effect of treatment: $P < 0.08$. ANOVA, analysis of variance; SEM, standard error of the means

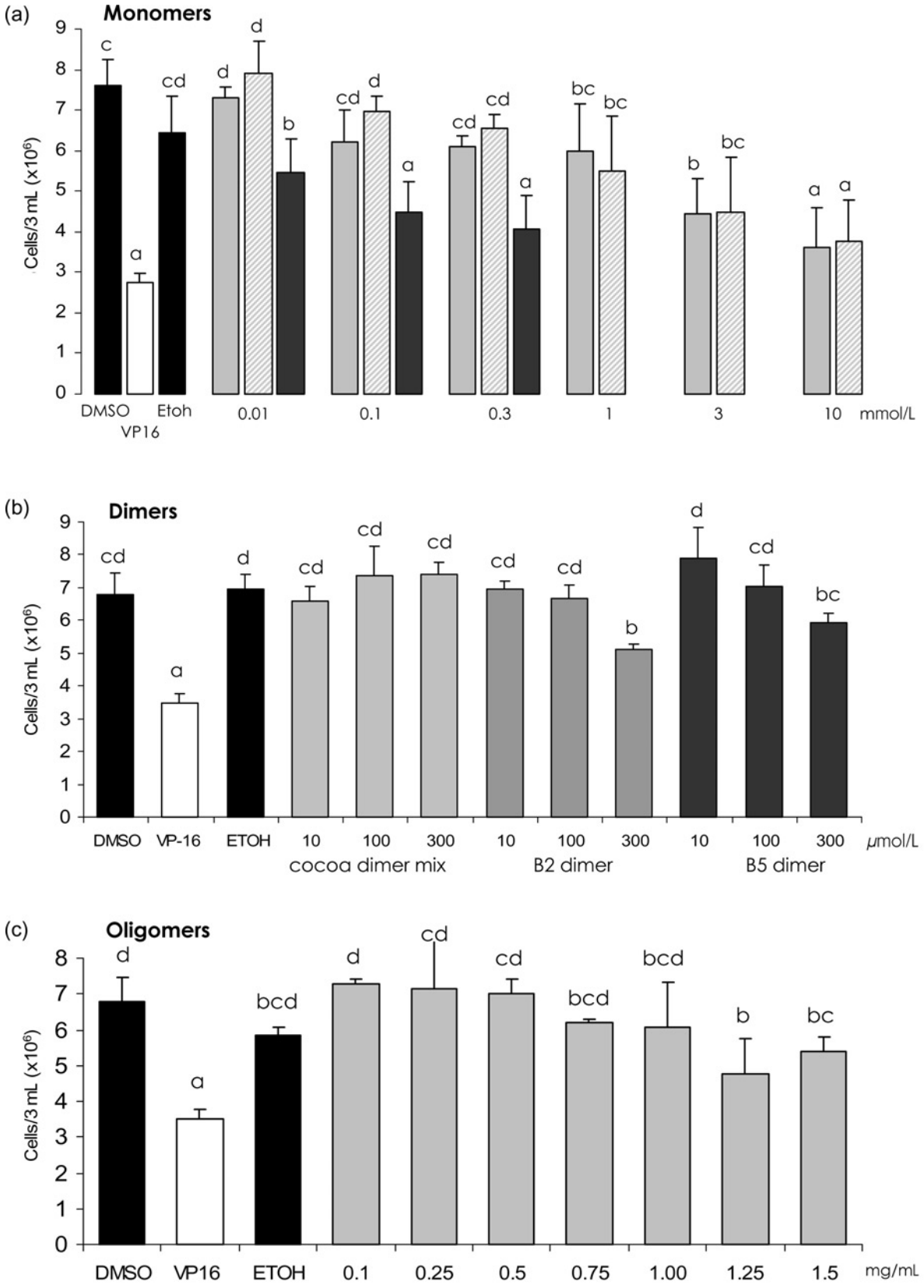

Figure 8 Flavanols show limited toxicity *in vitro*. HL-60 cells (1.5×10^5/mL) were grown for 24 h in 3 mL of media containing 25 μmol/L VP16 (white bars), or the vehicles (50% ethanol [ETOH] and 10% DMSO, black bars) and (a) epicatechin and catechin (gray bars) or quercetin (solid bars); (b) flavanol dimers; and (c) cocoa extract procyanidins. Bars represent cell viability measured by trypan blue exclusion assay and are means $\pm$ SEM, $n = 3$–5 replicates. Bars with different superscripts are significantly different from one another ($P < 0.05$)

flavanols on cell proliferation and metabolic activity were tested at μmol/L and mmol/L concentration ranges, amounts 50–2000-fold greater than plasma epicatechin levels (1–5 μmol/L) measured following the ingestion of a cocoa-rich product.[11,42,58] Although these experimental conditions (*in vitro* assays of supraphysiological concentrations) may limit our conclusions regarding potential clastogenic effects of flavanols, a recent *in vitro* study also showed that epicatechin, catechin and dimers B1 and B2 exerted no cytotoxicity against various cancer cell lines unless they contained galloyl groups (gallic acid).[59] Thus, it is likely that these flavanols have limited clastogenic or genotoxic potential *in vivo* when consumed as part of a normal diet.

We think it is important to iterate the fact that our data were generated using *in vitro* systems similar to those used by others to evaluate effects on topo II activity and that inference to any effects with regard to human biology and reproduction is restricted. Moreover, we tested for effects using high concentrations of unmetabolized flavanols, which are likely to be different from the effects of circulating flavanols metabolites. Current data suggest that primarily monomers, and to a lesser extent dimers B2 and B5, are transported intact through the gut[42,60,61]; the longer oligomers are thought to be poorly absorbed.[47,62] With respect to dimer B2, the concentration that inhibited topo II activity (5 μmol/L) in our study was 100-fold higher than the average B2 plasma concentration (50 nmol/L) that occurs following the consumption of a flavanol-rich beverage.[42] The maximal blood concentration of monomers rarely reaches 5 μmol/L, and they are usually present as a combination of methylated, sulfated and glucuronidated metabolites.[47,63,64] With this in mind, and knowing that flavanols are rapidly eliminated from plasma,[11,47] the likelihood that significant concentrations of procyanidins reach the nuclei of hematopoeitic progenitor cells in the fetus following maternal consumption of chocolate is very small. Even though the bioavailability and biotransformation of flavonoids are not fully understood, it is unlikely that either the concentrations (amount) or the forms of substrates that generated leukemogenic effects *in vitro* are achieved in the fetal tissues. For example, Chu *et al.*[65,66] measured picomolar amounts of epicatechin in plasma and in certain tissues of GD 15.5 rat fetuses following the oral dosage of dams with large amounts of green tea extract (epigallocatechin gallate [EGCG] appeared more readily transported to the fetal compartment). Therefore, the risk for acute leukemia from intakes of flavanol-rich foods would seem to be limited.

With the exception of a few specific nutrients, the contribution of different nutritional factors to pregnancy outcome is poorly understood.[67] Similarly, the potential positive or detrimental consequences of high flavonoid intakes during pregnancy for the mother or child are largely unknown, and to a great extent, with the exception of isoflavones, relatively few in-depth reproductive toxicology studies have been carried out. Flavonoids, as common components of a plant food-rich diet have been reported to improve certain aspects of health and to decrease mortality in recent epidemiological and intervention trials.[12] However, it has been speculated that large intakes of flavonoid-rich foods during pregnancy can increase the risk for acute infant leukemia due to chromosomal translocations secondary to flavonoid-induced reductions in topoisomerase activity.[23] The concerns that various flavonoids might contribute to infant leukemia are legitimate, but this area of research clearly warrants further investigation. The list of agents capable of inhibiting topo II *in vitro* is extensive but evidence of them being a risk factor for leukemia during pregnancy is limited and contradictory; this is partly driven by the rare occurrence of the disorder, approximately 7.2 per 100,000 births,[68] which calls for case-control studies, leading to recall bias[15,32] and the fact that the etiology of acute childhood leukemia remains to be fully understood. The suggestion that the consumption of certain high flavanol foods during a vulnerable period such as pregnancy may lower the risk of preeclampsia, but increase the risk of giving birth to a child that will develop acute leukemia sends a confusing message to expecting mothers. However, while additional studies aimed at the evaluation of the benefits and risks of high dietary intakes of isolated flavonoids on pregnancy outcome in whole animal models would be of value, the results of the present study strongly argue against the hypothesis that physiological concentrations of flavanols, or oligomers of flavanols, pose a reproductive risk.

ACKNOWLEDGMENT

Supported by a grant from the NIEHS Center for Environmental Health Sciences and by the Vitamin Cases Consumer Settlement Fund. Mars Incorporated has, in part, financially supported this work.

REFERENCES

1 Balzer J, Rassaf T, Heiss C, Kleinbongard P, Lauer T, Merx M, Heussen N, Gross HB, Keen CL, Schroeter H, Kelm M. Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial. *J Am Coll Cardiol* 2008;**51**:2141–9

2 Corder R, Mullen W, Khan NQ, Marks SC, Wood EG, Carrier MJ, Crozier A. Oenology: red wine procyanidins and vascular health. *Nature* 2006;**444**:566

3 Corder R. Red wine, chocolate and vascular health: developing the evidence base. *Heart* 2008;**94**:821–3

4 Grassi D, Aggio A, Onori L, Croce G, Tiberti S, Ferri C, Ferri L, Desideri G. Tea, flavonoids, and nitric oxide-mediated vascular reactivity. *J Nutr* 2008;**138**:1554S–60S

5 Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, Jacobs DR Jr. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am J Clin Nutr* 2007;**85**:895–909

6 Moon YJ, Wang X, Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol In Vitro* 2006;**20**:187–210

7 Mursu J, Nurmi T, Tuomainen TP, Salonen JT, Pukkala E, Voutilainen S. Intake of flavonoids and risk of cancer in Finnish men: The Kuopio Ischaemic Heart Disease Risk Factor Study. *Int J Cancer* 2008;**123**:660–3

8 Nichenametla SN, Taruscio TG, Barney DL, Exon JH. A review of the effects and mechanisms of polyphenolics in cancer. *Crit Rev Food Sci Nutr* 2006;**46**:161–83

9 Nurk E, Refsum H, Drevon CA, Tell GS, Nygaard HA, Engedal K, Smith AD. Intake of flavonoid-rich wine, tea, and chocolate by elderly men and women is associated with better cognitive test performance. *J Nutr* 2009;**139**:120–7

10 Perez-Vizcaino F, Duarte J, Andriantsitohaina R. Endothelial function and cardiovascular disease: effects of quercetin and wine polyphenols. *Free Radic Res* 2006;**40**:1054–65

11 Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M. (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* 2006;**103**:1024–9
12 Erdman JW Jr, Balentine D, Arab L, Beecher G, Dwyer JT, Folts J, Harnly J, Hollman P, Keen CL, Mazza G, Messina M, Scalbert A, Vita J, Williamson G, Burrowes J. Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31–June 1, 2005, Washington, DC. *J Nutr* 2007;**137**:718S–37S
13 Felix CA, Kolaris CP, Osheroff N. Topoisomerase II and the etiology of chromosomal translocations. *DNA Repair (Amst)* 2006;**5**:1093–108
14 Ross JA, Potter JD, Robison LL. Infant leukemia, topoisomerase II inhibitors, and the MLL gene. *J Natl Cancer Inst* 1994;**86**:1678–80
15 Tower RL, Spector LG. The epidemiology of childhood leukemia with a focus on birth weight and diet. *Crit Rev Clin Lab Sci* 2007;**44**:203–42
16 Jensen CD, Block G, Buffler P, Ma X, Selvin S, Month S. Maternal dietary risk factors in childhood acute lymphoblastic leukemia (United States). *Cancer Causes Control* 2004;**15**:559–70
17 Spector LG, Xie Y, Robison LL, Heerema NA, Hilden JM, Lange B, Felix CA, Davies SM, Slavin J, Potter JD, Blair CK, Reaman GH, Ross JA. Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group. *Cancer Epidemiol Biomarkers Prev* 2005;**14**:651–5
18 Austin CA, Patel S, Ono K, Nakane H, Fisher LM. Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives. *Biochem J* 1992;**282**(Part 3):883–9
19 Bandele OJ, Osheroff N. Bioflavonoids as poisons of human topoisomerase II alpha and II beta. *Biochemistry* 2007;**46**:6097–108
20 Bandele OJ, Clawson SJ, Osheroff N. Dietary polyphenols as topoisomerase II poisons: B ring and C ring substituents determine the mechanism of enzyme-mediated DNA cleavage enhancement. *Chem Res Toxicol* 2008;**21**:1253–60
21 Constantinou A, Mehta R, Runyan C, Rao K, Vaughan A, Moon R. Flavonoids as DNA topoisomerase antagonists and poisons: structure-activity relationships. *J Nat Prod* 1995;**58**:217–25
22 Strick R, Strissel PL, Borgers S, Smith SL, Rowley JD. Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia [see comments]. *Proc Natl Acad Sci USA* 2000;**97**:4790–5
23 Ross JA. Dietary flavonoids and the MLL gene: a pathway to infant leukemia? [comment]. *Proc Natl Acad Sci USA* 2000;**97**:4411–3
24 Mueller-Planitz F, Herschlag D. Coupling between ATP binding and DNA cleavage by DNA topoisomerase II: A unifying kinetic and structural mechanism. *J Biol Chem* 2008;**283**:17463–76
25 McClendon AK, Osheroff N. DNA topoisomerase II, genotoxicity, and cancer. *Mutat Res* 2007;**623**:83–97
26 Schoeffler AJ, Berger JM. Recent advances in understanding structure-function relationships in the type II topoisomerase mechanism. *Biochem Soc Trans* 2005;**33**:1465–70
27 Kellner U, Sehested M, Jensen PB, Gieseler F, Rudolph P. Culprit and victim – DNA topoisomerase II. *Lancet Oncol* 2002;**3**:235–43
28 Robert J, Larsen AK. Drug resistance to topoisomerase II inhibitors. *Biochimie* 1998;**80**:247–54
29 Wilstermann AM, Bender RP, Godfrey M, Choi S, Anklin C, Berkowitz DB, Osheroff N, Graves DE. Topoisomerase II – drug interaction domains: identification of substituents on etoposide that interact with the enzyme. *Biochemistry* 2007;**46**:8217–25
30 Emerenciano M, Koifman S, Pombo-de-Oliveira MS. Acute leukemia in early childhood. *Braz J Med Biol Res* 2007;**40**:749–60
31 Greaves M. Pre-natal origins of childhood leukemia. *Rev Clin Exp Hematol* 2003;**7**:233–45
32 Lightfoot TJ, Roman E. Causes of childhood leukaemia and lymphoma. *Toxicol Appl Pharmacol* 2004;**199**:104–17
33 Aplan PD. Causes of oncogenic chromosomal translocation. *Trends Genet* 2006;**22**:46–55
34 Chowdhury T, Brady HJ. Insights from clinical studies into the role of the MLL gene in infant and childhood leukemia. *Blood Cells Mol Dis* 2008;**40**:192–9
35 Greaves M. In utero origins of childhood leukaemia. *Early Hum Dev* 2005;**81**:123–9
36 Ross JA. Environmental and genetic susceptibility to MLL-defined infant leukemia. *J Natl Cancer Inst Monogr* 2008;**39**:83–6
37 Sung PA, Libura J, Richardson C. Etoposide and illegitimate DNA double-strand break repair in the generation of MLL translocations: new insights and new questions. *DNA Repair (Amst)* 2006;**5**:1109–18
38 Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. *Blood* 2003;**102**:2321–33
39 Rowley JD. Chromosome translocations: dangerous liaisons revisited. *Nat Rev Cancer* 2001;**1**:245–50
40 Ross JA, Potter JD, Reaman GH, Pendergrass TW, Robison LL. Maternal exposure to potential inhibitors of DNA topoisomerase II and infant leukemia (United States): a report from the Children's Cancer Group. *Cancer Causes Control* 1996;**7**:581–90
41 Aron PM, Kennedy JA. Flavan-3-ols: nature, occurrence and biological activity. *Mol Nutr Food Res* 2008;**52**:79–104
42 Holt RR, Lazarus SA, Sullards MC, Zhu QY, Schramm DD, Hammerstone JF, Fraga CG, Schmitz HH, Keen CL. Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am J Clin Nutr* 2002;**76**:798–804
43 Triche EW, Grosso LM, Belanger K, Darefsky AS, Benowitz NL, Bracken MB. Chocolate consumption in pregnancy and reduced likelihood of preeclampsia. *Epidemiology* 2008;**19**:459–64
44 Snyder RD, Arnone MR. Putative identification of functional interactions between DNA intercalating agents and topoisomerase II using the V79 *in vitro* micronucleus assay. *Mutat Res* 2002;**503**:21–35
45 Ferguson LR. Role of plant polyphenols in genomic stability. *Mutat Res* 2001;**475**:89–111
46 Chun OK, Chung SJ, Song WO. Estimated dietary flavonoid intake and major food sources of U.S. adults. *J Nutr* 2007;**137**:1244–52
47 Cooper KA, Donovan JL, Waterhouse AL, Williamson G. Cocoa and health: a decade of research. *Br J Nutr* 2008;**99**:1–11
48 Jo JY, Gonzalez de Mejia E, Lila MA. Effects of grape cell culture extracts on human topoisomerase II catalytic activity and characterization of active fractions. *J Agric Food Chem* 2005;**53**:2489–98
49 Ramirez-Mares MV, Chandra S, de Mejia EG. *In vitro* chemopreventive activity of *Camellia sinensis, Ilex paraguariensis* and *Ardisia compressa* tea extracts and selected polyphenols. *Mutat Res* 2004;554:53–65
50 Gonzalez de Mejia E, Song YS, Ramirez-Mares MV, Kobayashi H. Effect of *Yerba mate* (*Ilex paraguariensis*) tea on topoisomerase inhibition and oral carcinoma cell proliferation. *J Agric Food Chem* 2005;**53**:1966–73
51 Jo JY, de Mejia EG, Lila MA. Cytotoxicity of bioactive polymeric fractions from grape cell culture on human hepatocellular carcinoma, murine leukemia and non-cancerous PK15 kidney cells. *Food Chem Toxicol* 2006;**44**:1758–67
52 Jo JY, Gonzalez de Mejia E, Lila MA. Catalytic inhibition of human DNA topoisomerase II by interactions of grape cell culture polyphenols. *J Agric Food Chem* 2006;**54**:2083–7
53 Quideau S, Jourdes M, Lefeuvre D, Montaudon D, Saucier C, Glories Y, Pardon P, Pourquier P. The chemistry of wine polyphenolic C-glycosidic ellagitannins targeting human topoisomerase II. *Chemistry* 2005;**11**:6503–13
54 Bandele OJ, Osheroff N. (−)-Epigallocatechin gallate, a major constituent of green tea, poisons human type II topoisomerases. *Chem Res Toxicol* 2008;**21**:936–43
55 Suzuki K, Yahara S, Hashimoto F, Uyeda M. Inhibitory activities of (−)-epigallocatechin-3-O-gallate against topoisomerases I and II. *Biol Pharm Bull* 2001;**24**:1088–90
56 Heiss C, Finis D, Kleinbongard P, Hoffmann A, Rassaf T, Kelm M, Sies H. Sustained increase in flow-mediated dilation after daily intake of high-flavanol cocoa drink over 1 week. *J Cardiovasc Pharmacol* 2007;**49**:74–80
57 Schewe T, Steffen Y, Sies H. How do dietary flavanols improve vascular function? A position paper. *Arch Biochem Biophys* 2008;**476**:102–6
58 Wang JF, Schramm DD, Holt RR, Ensunsa JL, Fraga CG, Schmitz HH, Keen CL. A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. *J Nutr* 2000;**130**:2115S–9S
59 Actis-Goretta L, Romanczyk LJ, Rodriguez CA, Kwik-Uribe C, Keen CL. Cytotoxic effects of digalloyl dimer procyanidins in human cancer cell lines. *J Nutr Biochem* 2008;**19**:797–808
60 Spencer JP, Schroeter H, Shenoy B, Srai SK, Debnam ES, Rice-Evans C. Epicatechin is the primary bioavailable form of the procyanidin

dimers B2 and B5 after transfer across the small intestine. *Biochem Biophys Res Commun* 2001;**285**:588–93
61 Spencer JP. Metabolism of tea flavonoids in the gastrointestinal tract. *J Nutr* 2003;**133**:3255S–61S
62 Yang CS, Sang S, Lambert JD, Lee MJ. Bioavailability issues in studying the health effects of plant polyphenolic compounds. *Mol Nutr Food Res* 2008;**52**(Suppl. 1):S139–51
63 Schroeter H. Flavonoids: neuroprotective agents? Modulation of oxidative stress-induced MAP Kinase signal transduction. In: Packer CR-EaL, ed. *Flavonoids in Health and Disease*. New York: Marcel Dekker, 2004:233–72
64 Spencer JP, Abd-el-Mohsen MM, Rice-Evans C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch Biochem Biophys* 2004;**423**:148–61
65 Chu KO, Wang CC, Chu CY, Chan KP, Rogers MS, Choy KW, Pang CP. Pharmacokinetic studies of green tea catechins in maternal plasma and fetuses in rats. *J Pharm Sci* 2006;**95**:1372–81
66 Chu KO, Wang CC, Chu CY, Choy KW, Pang CP, Rogers MS. Uptake and distribution of catechins in fetal organs following *in utero* exposure in rats. *Hum Reprod* 2007;**22**:280–7
67 Keen CL, Hanna LA, Lanoue L, Uriu-Adams J, Rucker RB, Clegg MS. Developmental consequences of trace mineral deficiencies in rodents: acute and long-term effects. *J Nutr* 2003;**133**:1477S–80S
68 Smith MA, Gloeckler-Ries LA, Gurney JG, Ross JA. Leukemia. SEER Pediatric Monograph, NIH Pub 99-4649, 1999

(Received June 11, 2009, Accepted October 5, 2009)