THE POLYPHENOLIC ELLAGITANNIN VESCALAGIN ACTS AS A PREFERENTIAL CATALYTIC INHIBITOR OF THE ALPHA ISOFORM OF HUMAN DNA TOPOISOMERASE II

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Abbreviations: DSB: DNA double-strand break; DTT: dithiothreitol; HHDP: hexahydroxydiphenoyl; ICE: In vivo Complex of Enzyme; NHTP: nonahydroxyterphenoyl; Top: DNA Topoisomerase
ABSTRACT

Polyphenolic ellagitannins are natural compounds that are often associated with the therapeutic activity of plant extracts used in traditional medicine. They display cancer-preventing activity in animal models by a mechanism which remains unclear. Potential targets have been proposed, including DNA topoisomerases II (Top2). Top2α and Top2β, the two isoforms of the human Top2, play a crucial role in the regulation of replication, transcription and chromosome segregation. They are the target of anticancer agents used in the clinic such as anthracyclines (e.g. doxorubicine) or the epipodophyllotoxin etoposide. It was recently shown that the antitumoral activity of etoposide was primarily due the inhibition of Top2α, whereas inhibition of Top2β was responsible for the development of secondary malignancies, pointing towards the need for more selective Top2α inhibitors. Here, we show that the polyphenolic ellagitanin vescalagin preferentially inhibits the decatenation activity of Top2α in vitro, by a redox-independent mechanism. In CEM cells, we also show that transient siRNA-mediated downregulation of Top2α but not Top2β conferred a resistance to vescalagin, indicating that the alpha isoform is a preferential target. We further confirmed that Top2α inhibition was due to a catalytic inhibition of the enzyme since it did not induce DNA double-strand breaks in CEM-treated cells, but prevented the formation of Top2α- rather than Top2β-DNA covalent complexes induced by etoposide. To our knowledge, vescalagin is the first example of a catalytic inhibitor which cytotoxicity is, at least in part, due to the preferential inhibition of Top2α.
INTRODUCTION

DNA Topoisomerases II (Top2) are nuclear enzymes which are essential for the suppression of topological constraints associated with DNA replication, DNA recombination and chromosome condensation and segregation during mitosis (Nitiss, 2009a; Wang, 2009). They act as dimers and cleave both strands of the DNA substrate, allowing the passage of an intact duplex molecule through the break where each of the monomer remains covalently linked to the 5'-end of the cleaved strands (Nitiss, 2009a; Wang, 2009). DNA continuity is then restored by religation of the transient break. There are two isoforms of the human Top2, Top2α (170 kDa) and Top2β (180 kDa). Top2α is expressed at high levels in proliferating cells suggesting its role in replication and chromosome segregation (DiNardo et al., 1984; Downes et al., 1994; Holm et al., 1985; Uemura et al., 1987), whereas Top2β is expressed at lower levels and also in quiescent cells which suggests a link with transcription (Lyu et al., 2006; Lyu and Wang, 2003; Tsutsui et al., 2001). Both are essential for cell division and have been the target of Top2 poisons such as etoposide used for the treatment of various malignancies (Nitiss, 2009b; Pommier et al., 2010). Conversely to Top2 catalytic inhibitors which prevent Top2 binding to its substrate and/or DNA cleavage, Top2 poisons interfere with the enzyme-DNA complexes and lead to the enhancement of Top2-DNA complexes and subsequent formation of irreversible (cytotoxic) DNA breaks (Nitiss, 2009b; Pommier et al., 2010). Interestingly, it was shown that poisoning of Top2α was responsible for the antiproliferative effect of etoposide, whereas poisoning of the β isoform led to treatment-related secondary malignancies, pointing towards the need for more selective inhibitors of the α isoform (Azarova et al., 2007).

During the past decades, a large number of naturally occurring compounds have been tested for their potential inhibitory activity against human Top2, including the various types
of tannins (Kashiwada et al., 1992; Kashiwada et al., 1993), which are polyphenolic compounds present in many plant extracts but were usually discarded from screening panels against cellular targets of interest despite their potential biological activities (Quideau et al., 2011). Initial studies led to the isolation and the identification of hydrolyzable dimeric ellagitannin such as woodfruticosin (Kadota et al., 1990) or sanguin H-6 (Bastow et al., 1993), as the active principle responsible for the anti-Top2 activity of these extracts. A more detailed analysis of sixty derivatives belonging to different classes of tannins, gallotannins, glucopyranosic ellagitannins, C-glucosidic ellagitannins, condensed tannins, as well as other tannins with complex structures, revealed that 36 compounds were at least more than 100-fold more potent than etoposide at inhibiting Top2 activity in vitro (Kashiwada et al., 1993). This study also showed that these compounds were catalytic inhibitors of Top2, since they could reduce the level of etoposide-induced DNA-protein crosslinks in treated cells (Kashiwada et al., 1993). However, it was not reported whether they could specifically target one of the two isoform of Top2. In this study, we investigated such selectivity for vescalagin and other polyphenolic nonahydroxyterphenoyl (NHTP)-containing C-glucosidic ellagitannins, which are found in wine aged in oak-made barrels and were shown to inhibit Top2 in vitro (Quideau et al., 2005). We show that vescalagin preferentially inhibits Top2α-mediated decatenation of kDNA in vitro in a redox-independent manner, suggesting a different mechanism than etoposide or quinone-based agents such as benzoquinone. We also demonstrate that vescalagin acts as a catalytic inhibitor of Top2 and preferentially inhibits etoposide-induced DNA-Top2α complexes in CEM cells. To our knowledge, vescalagin represents the first example of a catalytic inhibitor of Top2 preferentially targeting the α isoform of the human enzyme in cells.
MATERIALS AND METHODS

Chemicals and enzymes. The natural C-glucosidic ellagitannin vescalagin and all its analogous congeners or derivatives used in this study were extracted and purified extracted from *Quercus robur* heartwood or hemisynthesized and purified as previously described (Quideau et al., 2005; Quideau et al., 2004). Etoposide and all other chemicals were purchased from Sigma (L'Isle d'Abeau Chesnes, France) unless otherwise stated. Human purified Top2α and Top2β were purified as previously reported (Elsea et al., 1995; Kingma et al., 1997) and were a kind gift from Dr Neil Osheroff (Vanderbilt University School of Medicine, Nashville, TN).

Cell culture. The human leukemic CCRF-CEM cells were kindly provided by Dr W.T. Beck (University of Illinois at Chicago, IL). They were grown in RPMI 1640 medium (Invitrogen Life technologies SAS, Courtaboeuf, France) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Top2-catalyzed decatenation assays. Top2α and Top2β catalytic activity was assessed by the decatenation assay using the catenated kinetoplast DNA from *Trypanosoma* (Topogen, Colombus, OH) as a substrate. Each reaction was performed in 20 µL final volume containing 250 ng of kDNA, 40mM Tris/HCl pH 7.4,10 mM MgCl2, 0.5 mM EDTA, 100 mM KCl, 0.5 mM dithiotreitol, and 1 mM ATP. Reactions were initiated by the addition of kDNA and 2 units (0.15 ng) of purified human Top2 and incubated for 20 min at 37°C in the absence or in the presence of increasing concentrations of Top2 inhibitors. For DTT reactions, the drug was preincubated with DTT for 5 min prior to the addition of purified Top2 and kDNA and further incubated for additional 20 min at 37°C. Reactions were stopped by the addition of 2 µL of
10X stop buffer (5% sarkosyl, 30% glycerol and 0.125 mg/ml of bromophenol blue) and directly electrophoresed on a 1% agarose gel for 45 min at 50V. The gels were stained with ethidium bromide and visualized by UV-transillumination. Positive controls of decatenation and linear DNAs (Topogen) were run simultaneously. For each lane, quantitation of both open and closed circular forms of the decatenated kDNA and cationed DNA remaining in the well was performed using the ImageJ software. Percentage of decatenation was then calculated for each drug concentration and normalized to controls in the absence of drug. Statistical analyses for each datasets were performed using the unpaired t-test. Statistically significant differences are considered for a p<0.05 and are indicated by asterisks in the corresponding figures.

**SiRNA transfection.** ON-TARGET plus control (non-targeting) siRNA and sets of 4 siRNAs targeting Top2α (ref LU-004240-00-002) or Top2β (ref LU-004239-00-002) were purchased from Dharmacon (Lafayette, CO). Exponentially growing CEM cells were seeded in T25 flasks (500,000 cells per flask) and were transfected with 300 pmoles of siRNA using Oligofectamine transfection reagent (Invitrogen) during 96h. Then, a fraction of cells was directly used to evaluate the effect of Top2 downregulation on vescalagin cytotoxicity by cell count 72h after continuous drug treatment. Another fraction was rinsed with cold PBS, and dry cell pellets stored at -80°C for further validations of siRNA efficiencies by immunoblotting of both Top2 isoforms in total cell extracts.

**Cytotoxicity assays.**

**Cell count.** Exponentially growing cells (500,000 per T25 flask) were exposed to various concentrations of drugs for 3h or 72h and growth inhibition was evaluated by cell counting using a Coulter counter (Beckman). Results are expressed as percentage of cell growth relative to untreated cells and represent the mean ± sd of three independent experiments.
Detection of apoptotic cells was performed using the FAM-DEVD-fmk fluorochrome-labeled inhibitor of caspase (Bachem AG, Switzerland). Exponentially growing cells were seeded in 96-well plates (10,000 cells per well) and treated the following day with increasing concentrations of ellagitannins for 48h. Cells were then washed, detached by trypsin and incubated in saline solution containing 1µg/mL of the FAM-DEVD-fmk peptide according to the manufacturer’s protocol. Cells were then washed, and resuspended in fresh saline containing 2 µM propidium iodide. Following a 10 min incubation at room temperature, samples were analyzed by flow cytometry (488 nm laser) (Partec PAS, Becton Dickinson Facs- Calibur HTS, Beckman Coulter FC500). Green fluorescence was detected in FL1 and PI signal in FL3. Results of triplicate experiments are expressed as percentages of caspase 3 positive cells as a function of drug concentrations. Non-linear regressions are obtained using the Prism 4.0.1 software.

Immuno Complex of Enzyme assay. Top2-DNA covalent cleavage complexes were isolated from CEM cells using the ICE assay as previously described (Subramanian et al., 1995). Briefly, 3x10^6 cells were harvested, spun down for 5 min at 1500 g at 4°C and the pellets were directly lysed in 2 mL of 1% sarkosyl prior to Dounce homogenization. Lysates were gently layered on step gradients containing CsCl solutions (2 ml each) of the following densities: 1.82, 1.72, 1.50, and 1.45 (Shaw et al., 1975). Tubes were centrifuged at 165,000 g in a Beckman SW40 rotor for 17 h at 20ºC. DNA containing fractions were collected from the bottom of the tubes, pooled, normalized for DNA content and diluted with an equal volume of 25 mM NaPO₄ buffer (pH 6.5) prior to slot-blotting (two concentrations for each sample) onto Immobilon-P membranes with a slot-blot vacuum manifold. Then, Top2-DNA adducts were visualized by immuno-blotting with specific Top2α sc-5347 (1/2500) and Top2β sc-13059 (1/500) antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).
Measurements of DNA damage. The alkaline elution technique was used to quantify DNA double-strand breaks (DSBs) in control or treated CEM cells according to previously published procedures (Kohn, 1996). Briefly, genomic DNA of exponentially growing CEM cells was labelled with $[^{2-}{^{14}}\text{C}]$ thymidine for 18 h followed by a 2 h chase in fresh medium. Cells were then treated or not with Top2 inhibitors for 2 hours, scrapped and layered on polycarbonate filter of 2 µm pore size (Nucleopore, DMF) and directly lysed in the presence of proteinase K. The DNA was eluted with tetrapropylammonium hydroxide–EDTA buffer containing 0.1% SDS at pH 9.6. Fractions were collected over 15 h and dpm were counted using a scintillation counter (Packard). Results are expressed as % DNA retained on filters as a function of elution time and represent the mean of two independent experiments.

DSBs were also evaluated by measuring histone H2AX phosphorylation. Exponentially growing CEM cells were treated with DMSO or vescalagin for 48h in 96-well plates and washed with PBS. Cells were then resuspended in 20 mM Hepes containing phosphatases inhibitors and fixed by the addition of paraformaldehyde (2% final). Then, cells were washed with PBS and incubated in cold ethanol 70% for 2 h at -20°C. Permeabilized cells were rinsed with PBS, incubated with a rabbit anti-phospho H2AX (Ser139) antibody (Alexa Fluor® 488 conjugate, Cell signaling) (1/100 dilution) overnight at 4°C. Cells were then washed and resuspended in fresh PBS and fluorescence was analyzed by flow cytometry (Partec PAS, Becton Dickinson Facs- Calibur HTS, Beckman Coulter FC500).
RESULTS

Inhibition of Top2-mediated decatenation of kinetoplast DNA by ellagitannin compounds. This study focuses on a series of extracted and hemisynthetic nonahydroxyterphenoyl (NHTP)-bearing C-glucosidic ellagitannins in which the usual ellagitannin glucopyranose core is replaced by an open-chain glucose moiety resulting in the establishment of a C-aryl glucosidic bond (Fig. 1) (Quideau et al., 2011). These derivatives were prepared from vescalagin or castalagin that were extracted and purified from oak wood according to previously published procedures (Quideau et al., 2005; Quideau et al., 2003; Quideau et al., 2004). We first tested the effect of these ellagitannins on the catalytic activity of purified human Top2α and Top2β as measured by the in vitro decatenation of kinetoplast DNA. Overall, increasing concentrations of the ellagitannin derivatives that were tested were associated with a higher potency to inhibit Top2-mediated decatenation of kDNA (Fig. 2 & Supplemental Figure 1). However, at a concentration of 1 µM, the four compounds vescalagin, β-1-O-methylvescalagin, acutissimin B and epiacutissimin B, showed a preferential inhibition of Top2α. Decatenation of kDNA by Top2α was inhibited by approximately 50% as compared to control, whereas inhibition did not exceed 10% for Top2β (Fig. 2). Interestingly, at higher concentrations of 10 and 100 µM, the parent molecule vescalagin was the only compound to retain a marked selectivity towards Top2α (Fig. 2). For vescalagin, a difference between Top2α and Top2β inhibition of 47%, 45% and 32% could be observed for 1 µM, 10 µM and 100 µM, respectively. For the same concentrations of castalagin a lower difference could be evidenced (18%, 25%, and 22%, respectively), and even a more pronounced effect towards the β isoform was observed for acutissimin B, epiacutissimin B (Fig. 2) and epiacutissimin A (Supplemental Figure 1). These results demonstrate that vescalagin could preferentially inhibit the human Top2α in vitro.
**Top2 inhibition by vescalagin is redox-independent.** The epipodophyllotoxin etoposide, or some flavonoids such as genistein are known to inhibit Top2 by a redox-independent mechanism and their activity is not affected by the presence of reducing agents such as DTT (Bandele and Osheroff, 2007; Lindsey et al., 2004). Conversely, quinones such as 1,4-benzoquinone (BQ) or certain polyphenols such as epigallocatechin gallate (EGCG), a major constituent of green tea, are known to inhibit Top2 by a redox-dependent mechanism and their activity is blocked by DTT (Bandele and Osheroff, 2008; Bender et al., 2006; Lindsey et al., 2005; Lindsey et al., 2004; Wang et al., 2001). Because the ellagitannin vescalagin is a polyphenol, we verified whether it inhibited Top2 activity by a redox-dependent or -independent mechanism. We tested the effects of DTT on the activity of vescalagin to inhibit Top2-mediated decatenation of kDNA (Fig. 3). Vescalagin, BQ or etoposide were incubated with 500 µM DTT for 5 min prior to the addition of Top2α or Top2β. Then kDNA was added and the reaction incubated for 20 min at 37°C. DTT had no effect on the decatenation of kDNA induced by Top2α or Top2β alone or in the presence of the redox-independent Top2 poison etoposide. On the contrary, inhibition of Top2-mediated decatenation of kDNA by the redox-dependent compound BQ was completely abolished by DTT (Fig. 3). Under the same conditions, we showed that DTT had no effect on vescalagin-induced inhibition of Top2α or Top2β-induced decatenation of kDNA (Fig. 3), providing strong evidences that vescalagin is a redox-independent Top2 inhibitor.

**Top2α is a preferential target of vescalagin in CEM cells.** Since vescalagin is a polyphenolic compound that could potentially alter the function of multiple proteins, we then investigated whether the preferential inhibition of Top2α could also be observed in a cellular context. For this purpose, we measured the effect of the specific downregulation of Top2α or
Top2β on the sensitivity of CEM cells to vescalagin. CEM cells were either transfected with siRNA specifically targeting Top2α or Top2β, or with non-targeting (control) siRNA, and were treated with increasing concentrations of vescalagin (Fig. 4A). The results show that transient downregulation of Top2α conferred a 2- to 3-fold higher level of resistance to vescalagin than Top2β silencing, even though a better silencing of Top2β could be achieved (Fig. 4B). These results demonstrate a preferential targeting of the α isoform of Top2 in CEM cells and are consistent with its preferential inhibition that was observed in vitro, especially for low concentrations of the drug. They also demonstrate that sensitivity of CEM cells to vescalagin is, at least in part, inversely correlated with Top2 levels.

**Vescalagin is a preferential catalytic inhibitor of Top2α in CEM cells.** Vescalagin was previously shown to inhibit the formation of etoposide-induced protein-linked DNA breaks in KB cells, suggesting that this derivative was a catalytic inhibitor of Top2 (Kashiwada et al., 1993). We then investigated whether catalytic inhibition of Top2 by vescalagin was also selective of the α isoform in CEM-treated cells (Fig. 5). Using the ICE assay, which can directly assess the amount of DNA-Top2α or -Top2β cleavage complexes in cells, we confirmed that vescalagin could not induce Top2 trapping as opposed to the Top2 poison etoposide (Fig. 5A), even when high concentrations of vescalagin were used or when duration of treatment was prolonged.

We also show that DNA-Top2 cleavage complexes induced by etoposide are inhibited when cells are pre-treated with vescalagin, confirming that vescalagin could act as a catalytic inhibitor of Top2 (Fig. 5B). Interestingly, this inhibition was more pronounced in the case of the alpha isoform of Top2. The amount of Top2α-DNA complexes induced by etoposide was reduced by ~70% in the presence of 100 µM vescalagin, and cleavage complexes were completely suppressed for a concentration of 250 µM (Fig. 5B). By contrast, the amount of
Top2β-DNA cleavage complexes was only reduced by 50% in the presence of 100 µM vescalagin and complexes could still be detectable (~8% as compared to control) in the presence of 250 µM of the drug. Conversely to etoposide, we also found that vescalagin did not induce significant DNA double-strand breaks as measured by alkaline elution (Fig. 6A) or by the phosphorylation of H2AX (Fig. 6B), which is consistent with our previous observations.

Together, these results demonstrate that the alpha isoform of Top2 is a preferential target of vescalagin in cells and that vescalagin is a catalytic inhibitor of Top2.

**Vescalagin exhibit antiproliferative activity in a variety of cancer cell lines.** In order to address whether vescalagin could exert a cytotoxic effect, we evaluated the consequences of vescalagin treatment on cell proliferation and apoptosis in three additional cancer cells lines, HeLa, DU145 and A375 using a multiplexed approach (Schembri et al., 2009), which allows to distinguish between cytostatic and pro-apoptotic effects (Supplemental Figure 2). The results show that vescalagin can induce apoptosis or proliferation arrest depending on the cell type: 100 µM vescalagin induces apoptosis in the epithelial carcinoma cell line HeLa and the melanoma cells A375, while CEM and the prostate cancer cells DU145 seem more resistant to apoptosis induction. Conversely, in Hela cells, and to a lesser extent in DU145 and A375 cells, vescalagin induced an inhibition of proliferation already at 30 µM, while CEM cells were more resistant to this cytostatic effect. While it is reasonable to think that Top2α inhibition is involved in the anticancer effects of vescalagin in these cell types, triggering of multiple signalling pathways for longer incubation times may also contribute to these effects. It is also interesting to note that the sensitivity of A375 cells to vescalagin confirmed previous observations in another melanoma cell line (Kashiwada et al., 1992).
Because of its essential role in cell proliferation and cell division, the human topoisomerase II is the nuclear target of various anticancer agents such as doxorubicin or etoposide, which have been used in the clinic for more than 30 years. In the search for new topoisomerase II inhibitors originating from plants, a previous study investigated the ability of 60 compounds from the 4 main classes of tannins, gallotannins, ellagitannins, complex tannins and condensed tannins, to inhibit human topoisomerase II in vitro and identified 36 derivatives which could inhibit Top2 with a higher potency than that of etoposide (Kashiwada et al., 1993). More recently, we also found that new polyphenolic C-glucosidic ellagitannins isolated from wine aged in oak barrels could also inhibit Top2 with high potency (Quideau et al., 2005). However, the specificity of these ellagitannins towards Top2α or Top2β was never addressed. This is of importance because inhibition of each of these isoforms seems to have distinct biological impacts. Poisoning of Top2α is thought to play a major role in the antiproliferative effect of Top2 poisons because it is expressed in replicating cancer cells (Azarova et al., 2007; Errington et al., 1999). By contrast, the β isoform seems to play a prominent role in the occurrence of secondary malignancies since its suppression in the skin of mice showed a diminution of etoposide-induced melanomas (Azarova et al., 2007). It was proposed that specific degradation of Top2β-DNA complexes in non-replicating cells could uncover DNA double-strand breaks (Azarova et al., 2007) responsible for translocations leading to specific leukemias induced by Top2 poisons [see (Felix, 1998; Mistry et al., 2005) for reviews], pointing towards the need for more selective Top2α inhibitors.

In this study, we tested the inhibitory activity of several ellagitannins towards Top2α and Top2β catalytic activity. All of the ellagitannins in which a 2,3,5-NHTP unit and a 4,6-hexahydroxydiphenoyl (HHDP) unit are connected to the open-chain glucose core, strongly
inhibited Top2-mediated decatenation of kDNA in a concentration-dependent manner. It was already shown that the potency of these derivatives towards Top2 was not linked to the number of phenolic hydroxyl groups present on the molecule, since ellagitannin dimers and tetramers showed the same activity than the corresponding monomers (Kashiwada et al., 1993). In this line, we directly assessed the role of hydroxyl groups by evaluating the effect of DTT on vescalagin-induced Top2 inhibition and found that DTT had no effect, further strengthening the fact that this ellagitannin inhibits Top2 by a redox-independent mechanism. In that respect, vescalagin drastically differs from other active polyphenolic compounds such as the flavanoid EGCG that is known to poison both isoforms of Top2 by a redox-dependent mechanism similar to that of quinone-based compounds (Bandele and Osheroff, 2008). Our results suggest that the biarylic HHDP unit esterified at the O4- and O6-positions of the glucose core constitutes by itself a key structural determinant for Top2 inhibition by these ellagitannins. Indeed, the two NHTTP-bearing analogues, vescalin and castalin, in which such a medium-ring-forming biarylic unit is absent, exhibited only a weak activity against Top2.

Interestingly, we also found that vescalagin, as well as its β-1-O-methylated derivative exerted a significant preferential inhibition of the α isoform of Top2 at 1 µM concentration. A similar preference was also observed for the two flavanoellagitannins acutissimin B and epiacutissimin B. Vescalagin, and β-1-O-methylvescalagin to a lower extent, were the only compounds for which this selectivity was retained for higher concentrations of 10 and 100 µM. It is presently difficult to address the mechanistic basis for this Top2α selectivity on a structural point of view. Because preferential inhibition of Top2α was more pronounced in the case of vescalagin as compared to its C-1 α-epimer castalagin, one could hence suggest that the β-orientation of the C-1 hydroxyl group of vescalagin might play a role in favoring selectivity towards Top2α. We also noticed that methylation of the C-1 hydroxyl group of
vescalagin did not significantly affect the preferential inhibition of Top2α, whereas substitutions by an ethyl group (Supplemental Figure 1) or longer aliphatic chains (not shown) led to equal inhibition of both Top2 isoforms. When bulkier and phenolic substituents are present at the same position regardless to their orientations, such as in the case of the flavanoellagitannins tested, a preferential inhibition of Top2β could even be observed for high concentrations of the drugs. The mechanism of this concentration-dependent switch in inhibitory selectivity is presently unknown and awaits further investigations. Together, these results suggest that both the nature (aliphatic or phenolic) and the steric demand (length and/or bulk) of the substituents at C-1 may impact the selectivity towards Top2α, and that the best selectivity is observed with a beta-oriented hydroxyl or methoxy group at C-1 of 4,6-HHDP-bearing C-glucosidic ellagitannins.

We further demonstrated that preferential inhibition of Top2α by vescalagin also occurred in cells, as transient downregulation of Top2α conferred a higher resistance of CEM cells to this ellagitannin as compared to the transient repression of Top2β. This is actually consistent with in vitro data showing a higher potency of vescalagin to inhibit the catalytic activity of the α isoform and to reduce the formation of etoposide-induced Top2α-DNA complexes, especially for low concentrations of the drug. This is also in accordance with the fact that inhibition of etoposide-induced Top2 trapping by vescalagin was more pronounced for Top2α than for Top2β, further confirming that preferential inhibition of cellular Top2α results from a catalytic inhibition of the enzyme (Kashiwada et al., 1993). When this effect was compared to classical catalytic inhibitors such as the bisdioxopiperazine ICRF-193, which stabilizes the closed clamp form of the enzyme (Roca et al., 1994) but was also shown to induce Top2 poisoning (Huang et al., 2001; Nitiss, 2009b; Oestergaard et al., 2004), reduction of Top2α induced a minor increase in sensitivity to ICRF-193 and reduction of
Top2β levels had no effect (Supplemental Figure 3). This suggests that inhibition of Top2α by vescalagin is probably occurring by a different mechanism than ICRF-193 that remains to be further investigated.

In the search for new selective Top2α derivative that could reduce the occurrence of secondary malignancies that are attributed to the processing of stabilized DNA-Top2β cleavage complexes, the benzo[c]phenanthridine alkaloid NK314 was the first derivative to be identified (Toyoda et al., 2008). NK314 selectively targets the α isoform in vitro and in Nalm-6 pre-B cells by inducing Top2α-DNA complexes and DNA double-strand breaks (Onda et al., 2008; Toyoda et al., 2008). Alternatively, the use of catalytic inhibitors of Top2 could also prevent DNA cleavage and reduce drug-induced chromosomal rearrangements. Recent studies reported the synthesis of new derivatives such as the purine analog QAP1 (quinoline aminopurine compound 1) (Chene et al., 2009), thiosemicarbazones (Huang et al., 2010), N-fused imidazoles (Baviskar et al., 2011), or xanthone analogues (Jun et al., 2011) which inhibit the catalytic activity of Top2α by an ATP-competitive mechanism. Apart from QAP1 which inhibits both isoforms (Chene et al., 2009), it is not known whether these derivatives also inhibit the catalytic activity of Top2β which is expressed in post-mitotic cells (Lyu and Wang, 2003; Watanabe et al., 1994) and non-proliferating tissues such as the adult heart (Capranico et al., 1992). Developing selective Top2α catalytic inhibitor would therefore be useful since Top2β was shown to be involved in anthracyclin-induced cardiotoxicity (Lyu et al., 2007) and was also required for neuronal differentiation and the expression of a number of neuronal genes (Lyu and Wang, 2003; Nur et al., 2007). In this line, vescalagin that is readily available from fagaceous woody plants sources (Quideau et al., 2005; Quideau et al., 2003), could serve as a basis for the development of catalytic inhibitors of Top2α with reduced toxicity that could be used in cancer chemotherapy.
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REFERENCES


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FIGURES LEGEND

**Figure 1**: Structures of the polyphenolic ellagitannins tested in this study.

**Figure 2**: Inhibition of Top2-mediated decatenation of kinetoplast DNA (kDNA) by ellagitannins. For each reaction, 300 ng of kDNA was incubated for 20 min at 37°C with 0.15 ng of purified recombinant Top2α or Top2β in the absence or in the presence of 0.1, 1, 10 or 100 µM of each ellagitannin compound. Reactions were stopped by the addition of 0.5% sarkosyl. Then, reaction products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Decatenated DNAs, i.e. open circular (OC) and closed circular (CC) forms as well as remaining kDNA were quantitated using ImageJ software and Top2 inhibition expressed as percentage of decatenation as compared to controls (enzyme alone). Results are the mean ± SD (n=4). Bar graphs correspond to the quantitations of the gels shown above for vescalagin (A), β-1-O-methylvescalagin (B), acutissimin B (C), and epiacutissimin B (D). Lanes 1: control decatenated kDNA; lanes 2: control kDNA; lanes 3: kDNA + Top2α; lanes 5-8: same as lane 3 + 0.1, 1, 10, 100 µM of ellagitannin compound, respectively; lanes 9-13: same as lanes 3, 5-8 with Top2β. Lane 4: etoposide (100 µM). Asterisks indicate statistical significance as evaluated by the unpaired t-test: (*) p<0.05; (**) p<0.01

**Figure 3**: Effects of DTT on the ability of vescalagin to inhibit Top2-mediated decatenation of kinetoplast DNA. Vescalagin was incubated without or with 500 µM DTT for 5 min at room temperature prior to its addition to the reaction mix containing 2 units of purified Top2α or Top2β. Then, 300 ng kDNA was added and incubated for additional 20 min at 37°C and reactions were stopped by the addition of 0.5% sarkosyl. Reaction products were processed as described in Figure 2. Control reactions were performed in the absence of compounds or in
MOL #77537

the presence of 100 µM etoposide or 25 µM 1,4-benzoquinone (BQ). Results are expressed as percentages of decatenation relative to controls and represent the mean ± SD (n=3). (*) p<0.01

**Figure 4:** Effects of siRNA-mediated downregulation of Top2α or Top2β on the cell sensitivity of CEM cells to vescalagin. (A) CEM cells were transiently transfected with nontargeting siRNA or siRNA directed against Top2α or Top2β for 96 h and further treated with DMSO or indicated concentrations of vescalagin for 72 h. Cell survival was determined by cell counting as described in Materials and Methods. (B) Measurements of Top2α and Top2β protein levels by western blotting using total cell extracts (50 µg) from CEM cells at the time of drug treatment. Actin was used as loading control. Results are the mean of two independent experiments performed in duplicate. (*) p<0.05, (**) p<0.01; (NS) not significant

**Figure 5:** Vescalagin is a selective catalytic inhibitor of Top2α. (A) Measurements of Top2α- or Top2β-DNA covalent complexes in DNA-containing fractions of CEM cells (two concentrations used) following treatment with vescalagin using the ICE assay as described in Materials and Methods. Cells were treated for 3h with indicated concentrations or with 50 µM vescalagin for indicated times. Treatment with etoposide (50 µM, 1 h) was used as a positive control. (B) Inhibition of etoposide-induced Top2α- or Top2β-DNA cleavage complex formation by vescalagin in CEM cells using the ICE assay. Cells were treated with 0, 50, 100, or 250 µM vescalagin for 3h prior to the addition of 50 µM etoposide and incubated for an additional hour. Top2-DNA complexes were quantitated using the ImageJ software.

**Figure 6:** DNA-damage induced by vescalagin in CEM cells. (A) Measurements of DNA double-strand breaks (DSB) using the alkaline elution technique following a 2h treatment
MOL #77537

with 50 µM vescalagin or etoposide. (B) Measurements of DSBs by the detection of histone H2AX phosphorylation by flow cytometry as indicated in Materials and Methods.
Figure 1

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Figure 2
Figure 3

A

B
Figure 4

A

Cell Growth (%) vs. Vescalagin (µM)

B

Si RNA

NT Top2α

0.10 1.00 2.50 5.00 10.00

Vescalagin (µM)

0 10 20 30 40 50 60 70 80 90 100 110

NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS

Actin

Top2α

Top2β

Actin

100% 41% 100% 22%

Figure 4
Figure 5

A

Vescalagin (µM) | Time (h)  
---|---
0 | 0 | 3 | 6 | 24 | 0 | 50 | 100 | 300 | 0 | 50 | 100 | 300

Top2α | Top2β  
Vescalagin (µM) | Etoposide  
0 | 50 | 100 | 300 | 0 | 50 | 100 | 300 | 0 | 50 | 100 | 300

B

+ Etoposide (50µM)  
Vescalagin (µM) | Vescalagin (µM)  
0 | 250 | 0 | 50 | 100 | 250 | 0 | 250 | 0 | 50 | 100 | 250

Top2α | Top2β  
Vescalagin (µM) | Etoposide  
0 | 250 | 0 | 50 | 100 | 250 | 0 | 250 | 0 | 50 | 100 | 250
Figure 6

A

% DNA retained on filters

Time (hours)

0 3 6 9 12 15

0 40 60 80 100

Control

Etoposide

B

% of γH2AX positive cells

0 5 10 15 20

Vesca 100μM

Vesca 200μM

VH1 50μM

Figure 6