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The Polyphenolic Ellagitannin Vescalagin Acts As a Preferential Catalytic Inhibitor of the α Isoform of Human DNA Topoisomerase II[S]

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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 that 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., doxorubicin) or the epipodophyllotoxin etoposide. It was recently shown that the antitumor activity of etoposide was due primarily to the inhibition of Top2α, whereas inhibition of Top2β was responsible for the development of secondary malignancies, pointing to the need for more selective Top2α inhibitors. Here, we show that the polyphenolic ellagitannin vescalagin preferentially inhibits the decatenation activity of Top2α in vitro, by a redox-independent mechanism. In CEM cells, we also show that transient small interfering RNA-mediated downregulation of Top2α but not of Top2β conferred a resistance to vescalagin, indicating that the α isoform is a preferential target. We further confirmed that Top2α inhibition was due to a catalytic inhibition of the enzyme because 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 for which cytotoxicity is due, at least in part, to the preferential inhibition of Top2α.

Introduction

DNA topoisomerases II (Top2) are nuclear enzymes that 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 monomers 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; Holm et al., 1985; Uemura et al., 1987; Downes et al., 1994), whereas Top2β is expressed at lower levels and also in quiescent cells, which suggests a link with transcription (Tsutsui et al., 2001; Lyu and Wang, 2003; Lyu et al., 2006). Both are essential for cell

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ABBREVIATIONS: Top2, DNA topoisomerase II; NHTP, nonahydroxyterphenoyl; kDNA, kinetoplast DNA; DTT, dithiothreitol; siRNA, small interfering RNA, PBS, phosphate-buffered saline; ICE, in vivo complex of enzyme; DSB, DNA double-strand break; BQ, 1,4-benzoquinone; HHDP, hexahydroxydiphenoyl; ICRF-193, 4,4′-(1,2-dimethyl-1,2-ethanediyl)bis-2,6-piperazinedione; NK314, 4-hydroxy-5-methoxy-2,3-dihydro-1*H*-[1,3]benzodioxolo[5,6-*c*]pyrrolo[1,2-*f*]-phenanthridium chloride.

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). In contrast with Top2 catalytic inhibitors that prevent Top2 binding to its substrate and/or DNA cleavage, Top2 poisons interfere with 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). Of interest, 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 to 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, 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 ellagitannins such as woodfruticosin (Kadota et al., 1990) or sanguiin H-6 (Bastow et al., 1993), as the active principal responsible for the anti-Top2 activity of these extracts. A more detailed analysis of 60 derivatives belonging to different classes of tannins, gallotannins, glucopyranosic ellagitannins, *C*-glucosidic ellagitannins, condensed tannins, and other tannins with complex structures, revealed that 36 compounds were at least more than 100-fold more potent than etoposide in inhibiting Top2 activity in vitro (Kashiwada et al., 1993). This study also showed that these compounds were catalytic inhibitors of Top2, because they could reduce the level of etoposide-induced DNA-protein cross-links in treated cells (Kashiwada et al., 1993). However, it was not reported whether they could specifically target one of the two isoforms 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 mechanism other than that of 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 from *Quercus robur* heartwood or hemisynthesized and purified as described previously (Quideau et al., 2004, 2005). Etoposide and all other chemicals were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France) unless otherwise stated. Human Top2α and Top2β were purified as reported previously (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, 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% CO_2.

Top2-Catalyzed Decatenation Assays

Top2α and Top2β catalytic activity was assessed by the decatenation assay using the catenated kinetoplast DNA from *Trypanosoma* (TopoGEN, Port Orange, FL) as a substrate. Each reaction was performed in a 20-μl final volume containing 300 ng of kDNA, 40 mM Tris/HCl, pH 7.4,10 mM $MgCl_2$, 0.5 mM EDTA, 100 mM KCl, 0.5 mM dithiothreitol, 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 before the addition of purified Top2 and kDNA and further incubated for an additional 20 min at 37°C. Reactions were stopped by the addition of 2 μl of 10× stop buffer (5% sarkosyl, 30% glycerol, and 0.125 mg/ml bromphenol blue) and directly electrophoresed on a 1% agarose gel for 45 min at 50 V. The gels were stained with ethidium bromide and visualized by UV transillumination. Positive controls of decatenation and linear DNAs (TopoGEN, Inc.) were run simultaneously. For each lane, quantitation of both open and closed circular forms of the decatenated kDNA and catenated DNA remaining in the well was performed using ImageJ software. The percentage of decatenation was then calculated for each drug concentration and normalized to that of controls in the absence of drug. Statistical analyses for each data set were performed using the unpaired *t* test. Statistically significant differences are considered for $p < 0.05$ and are indicated by asterisks in the corresponding figures.

siRNA Transfection

ON-TARGETplus control (nontargeting) siRNA and sets of four siRNAs targeting Top2α or Top2β were purchased from Dharmacon RNAi Technologies (Lafayette, CO). Exponentially growing CEM cells were seeded in T25 flasks (5×10^5 cells/flask) and were transfected with 300 pmol of siRNA using Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) during 96 h. Then, a fraction of cells was directly used to evaluate the effect of Top2 down-regulation on vescalagin cytotoxicity by cell count 72 h after continuous drug treatment. Another fraction was rinsed with ice-cold PBS, and dry cell pellets were 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 (5×10^5 cells/T25 flask) were exposed to various concentrations of drugs for 3 or 72 h, and growth inhibition was evaluated by cell counting using a Coulter counter (Beckman Coulter, Fullerton, CA). Results are expressed as percentage of cell growth relative to that of untreated cells and represent the mean ± S.D. of three independent experiments.

Detection of Apoptotic Cells. Apoptotic cells were detected using the FAM-DEVD-fmk fluorochrome-labeled inhibitor of caspase (Bachem AG, Bubendorf, Switzerland). Exponentially growing cells were seeded in 96-well plates (10^4 cells/well) and treated the following day with increasing concentrations of ellagitannins for 48 h. Cells were washed, detached by trypsin, and incubated in saline solution containing 1 μg/ml FAM-DEVD-fmk peptide according to the manufacturer's protocol. Then cells were washed again and resuspended in fresh saline containing 2 μM propidium iodide. After a 10-min incubation at room temperature, samples were analyzed by flow cytometry (488-nm laser) (Partec PAS, Becton Dickinson FACSCalibur

HTS, Beckman Coulter FC500). Green fluorescence was detected in FL1 and the propidium iodide signal in FL3. Results of triplicate experiments are expressed as percentages of caspase 3-positive cells as a function of drug concentrations. Nonlinear regressions were obtained using Prism 4.0.1 software.

Immunocomplex of Enzyme Assay. Top2-DNA covalent cleavage complexes were isolated from CEM cells using the ICE assay as described previously (Subramanian et al., 1995). In brief, 3×10^6 cells were harvested and spun down for 5 min at 1500*g* at 4°C, and the pellets were directly lysed in 2 ml of 1% sarkosyl before 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 20°C. 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 $NaPO_4$ buffer, pH 6.5, before slot-blotting (two concentrations for each sample) onto Immobilon-P membranes with a slot-blot vacuum manifold. Then, Top2-DNA adducts were visualized by immunoblotting with specific Top2α (1:2500) and Top2β (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). In brief, genomic DNA of exponentially growing CEM cells was labeled with [2-^{14}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 h, scraped and layered on a polycarbonate filter of 2-μm pore size (Nucleopore; DMF, Paris, France) 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 disintegrations per min were counted using a scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Results are expressed as percentage of 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 48 h in 96-well plates and washed with PBS. Cells were then resuspended in 20 mM HEPES containing phosphatase inhibitors and fixed by the addition of paraformaldehyde (2% final). Then, cells were washed with PBS and incubated in ice-cold ethanol 70% for 2 h at −20°C. Permeabilized cells were rinsed with PBS and incubated with a rabbit anti-phospho H2AX (Ser139) antibody (Alexa Fluor 488 conjugate, 1:100 dilution; Cell Signaling Technology, Danvers, MA) overnight at 4°C. Cells were then washed and resuspended in fresh PBS, and fluorescence was analyzed by flow cytometry (Partec PAS, Becton Dickinson FACSCalibur 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 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 was extracted and purified from oak wood according to previously published procedures (Quideau et al., 2003, 2004, 2005). 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 Fig. 1). However, at a concentration of 1 μM, the four compounds, vescalagin, β-1-*O*-methylvescalagin, acutissimin B, and epiacutissimin B, showed preferential inhibition of Top2α. Decatenation of kDNA by Top2α was inhibited by approximately 50% compared with the control, whereas inhibition did not exceed 10% for Top2β (Fig. 2). Of interest, at higher concentrations of 10 and 100 μM, the parent molecule vescalagin was the only compound to retain a marked selectivity toward Top2α (Fig. 2). For vescalagin, a difference between Top2α and Top2β inhibition of 47, 45, and 32% could be observed for 1, 10, and 100 μM, respectively. For the same concentrations of castalagin, a lower difference could be seen (18, 25, and 22%, respectively), and an even more pronounced effect toward the β isoform was observed for acutissimin B, epiacutissimin B (Fig. 2), and epiacutissimin A (Supplemental Fig. 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 (Lindsey et al., 2005; Bandele and Osheroff, 2007). In contrast, quinones such as 1,4-benzoquinone (BQ) or certain polyphenols such as epigallocatechin gallate, a major constituent of green tea, are known to inhibit Top2 by a redox-dependent mechanism, and their activity is blocked by DTT (Wang et al., 2001; Lindsey et al., 2004, 2005; Bender et al., 2006; Bandele and Osheroff, 2008). Because the ellagitannin vescalagin is a polyphenol,

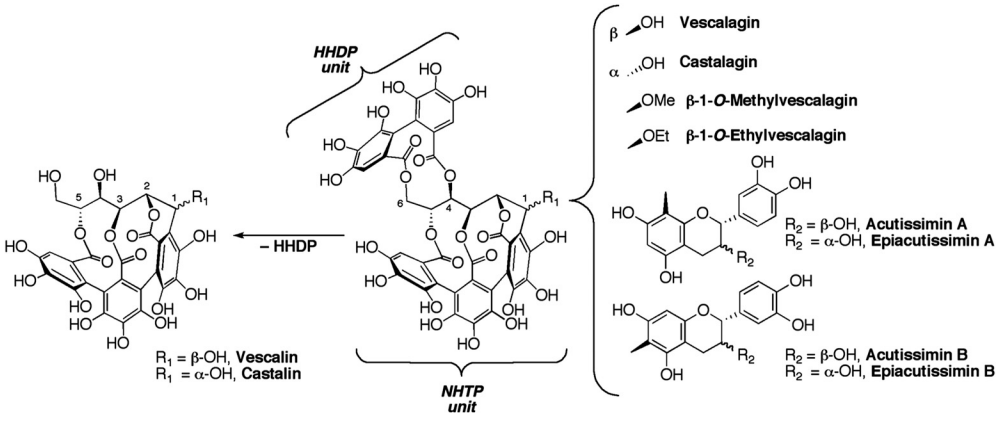

Fig. 1. Structures of the polyphenolic ellagitannins tested in this study.

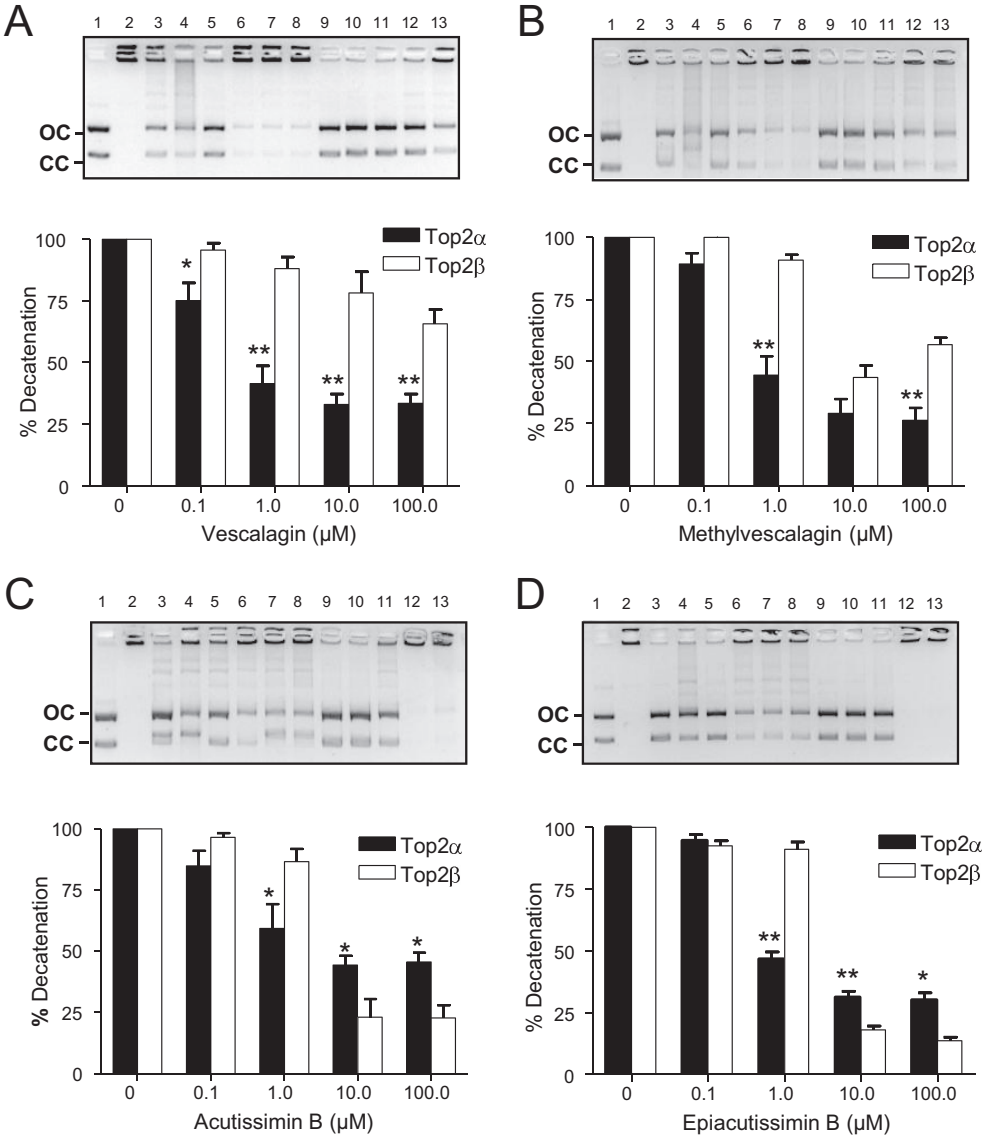

Fig. 2. Inhibition of Top2-mediated decatenation of kDNA by ellagitannins. For each reaction, 300 ng of kDNA was incubated for 20 min at 37°C with 0.15 ng of purified recombinant Top2α or Top2β in the absence or in the presence of 0.1, 1, 10, or 100 μM concentration of each ellagitannin compound. Reactions 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 the remaining kDNA were quantitated using ImageJ software, and Top2 inhibition was expressed as a percentage of decatenation compared with that for controls (enzyme alone). Results are the mean ± S.D. ($n = 4$). 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 to 8, same as lanes 3 + 0.1, 1, 10, or 100 μM ellagitannin compound, respectively; lanes 9 to 13, same as lanes 3 and 5 to 8 with Top2β; lanes 4, etoposide (100 μM). Statistical significance as evaluated by the unpaired *t* test: *, $p < 0.05$; **, $p < 0.01$.

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 was incubated with 500 μM DTT for 5 min before the addition of Top2α or Top2β. Then kDNA was added, and the reaction was 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 evidence that vescalagin is a redox-independent Top2 inhibitor.

Top2α Is a Preferential Target of Vescalagin in CEM Cells. Because 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 down-regulation of Top2α or Top2β on the sensitivity of CEM cells to vescalagin. CEM cells were transfected with either siRNA specifically targeting Top2α or Top2β or with nontargeting (control) siRNA and were treated with increasing concentrations of vescalagin (Fig. 4A). The results show that transient down-regulation of Top2α conferred a 2- to 3-fold higher level of resistance to vescalagin than Top2β silencing, even though better silencing of Top2β could be achieved (Fig. 4B). These results demonstrate 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 for the α isoform in CEM-treated cells (Fig. 5). Using the ICE assay, which can

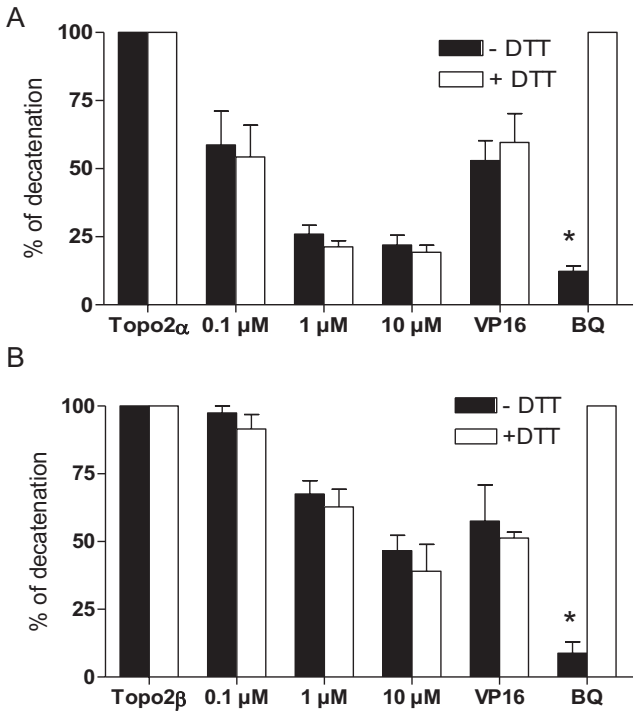

Fig. 3. Effects of DTT on the ability of vescalagin to inhibit Top2-mediated decatenation of kinetoplast DNA. Vescalagin was incubated without or with 500 μM DTT for 5 min at room temperature before its addition to the reaction mix containing 2 units of purified Top2α (A) or Top2β (B). Then, 300 ng of 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 the legend to Fig. 2. Control reactions were performed in the absence of compounds or in the presence of 100 μM etoposide (VP16) or 25 μM BQ. Results are expressed as percentages of decatenation relative to that of controls and represent the mean ± S.D. (n = 3). *, $p < 0.01$.

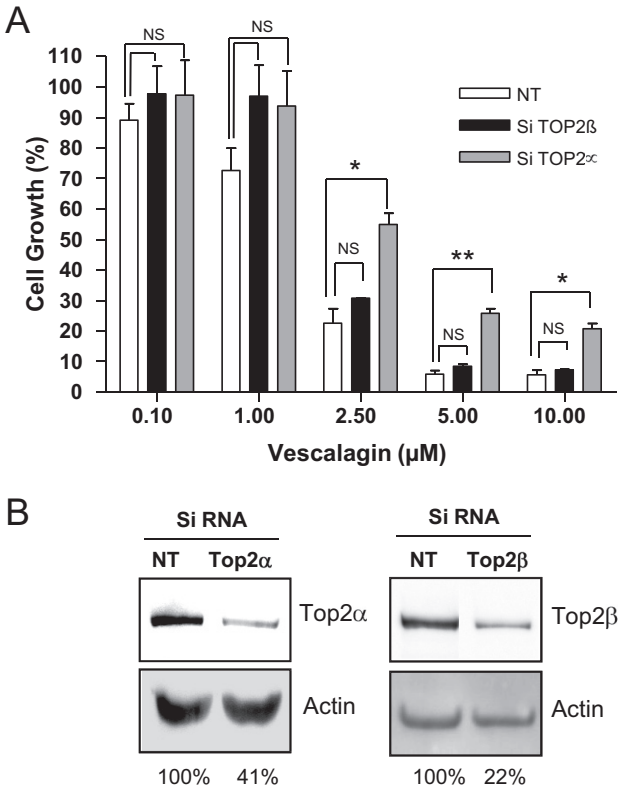

Fig. 4. Effects of siRNA-mediated down-regulation of Top2α or Top2β on the cell sensitivity of CEM cells to vescalagin. A, CEM cells were transiently transfected with nontargeting siRNA (NT) or siRNA directed against Top2α (Si Top2α) or Top2β (Si Top2α) for 96 h and further treated with DMSO or indicated concentrations of vescalagin for 72 h. Cell survival was determined by cell counting as described under *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.

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 pretreated with vescalagin, confirming that vescalagin could act as a catalytic inhibitor of Top2 (Fig. 5B). Of interest, this inhibition was more pronounced in the case of the α 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). In 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% compared with control) in the presence of a 250 μM concentration of the drug. In contrast with 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.

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

Vescalagin Exhibits Antiproliferative Activity in a Variety of Cancer Cell Lines. 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 cell lines, HeLa, DU145, and A375, using a multiplexed approach (Schembri et al., 2009), which allows one to distinguish between cytostatic and proapoptotic effects (Supplemental Fig. 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 cell line A375, whereas CEM and the prostate cancer cells DU145 seem more resistant to apoptosis induction. In contrast, in HeLa cells and to a lesser extent in DU145 and A375 cells, vescalagin induced an inhibition of proliferation already at 30 μM, whereas CEM cells were more resistant to this cytostatic effect. Although it is reasonable to think that Top2α inhibition is involved in the anticancer effects of vescalagin in these cell types, triggering of multiple signaling 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).

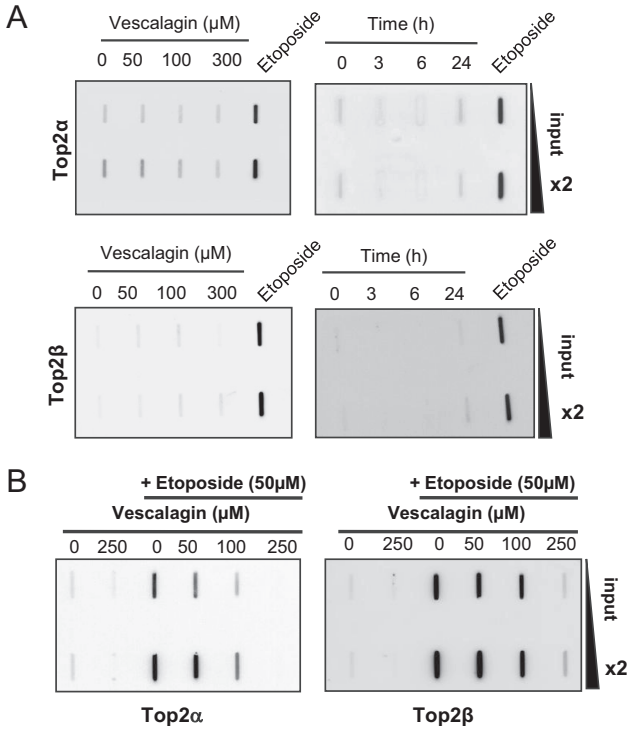

Fig. 5. Vescalagin is a selective catalytic inhibitor of Top2α. A, measurements of Top2α- or Top2β-DNA covalent complexes in DNA-containing fractions of CEM cells (two concentrations used) after treatment with vescalagin using the ICE assay as described under *Materials and Methods*. Cells were treated for 3 h with the indicated concentrations or with 50 μM vescalagin for the 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 3 h before the addition of 50 μM etoposide and incubated for an additional h. Top2-DNA complexes were quantitated using ImageJ software.

Discussion

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 four main classes of tannins, gallotannins, ellagitannins, complex tannins, and condensed tannins, to inhibit human topoisomerase II in vitro and identified 36 derivatives that 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 toward Top2α or Top2β was never addressed. This is of importance because inhibition of each of these isoforms seems to have distinct biological effects. 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 (Errington et al., 1999; Azarova et al., 2007). In contrast, the β isoform seems to play a prominent role in the occurrence of secondary malignancies because 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 nonreplicating cells could uncover DNA double-strand breaks (Azarova et al., 2007) responsible for translocations leading to specific leukemias induced by Top2 poisons [for reviews, see Felix (1998) and Mistry et al. (2005)], pointing to the need for more selective Top2α inhibitors.

In this study, we tested the inhibitory activity of several ellagitannins toward 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 had already been shown that the potency of these derivatives toward Top2 was not linked to the number of phenolic hydroxyl groups present on the molecule, because ellagitannin dimers and tetramers showed the same activity as the corresponding monomers (Kashiwada et al., 1993). Likewise, 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 epigallocatechin gallate, which is known to poison

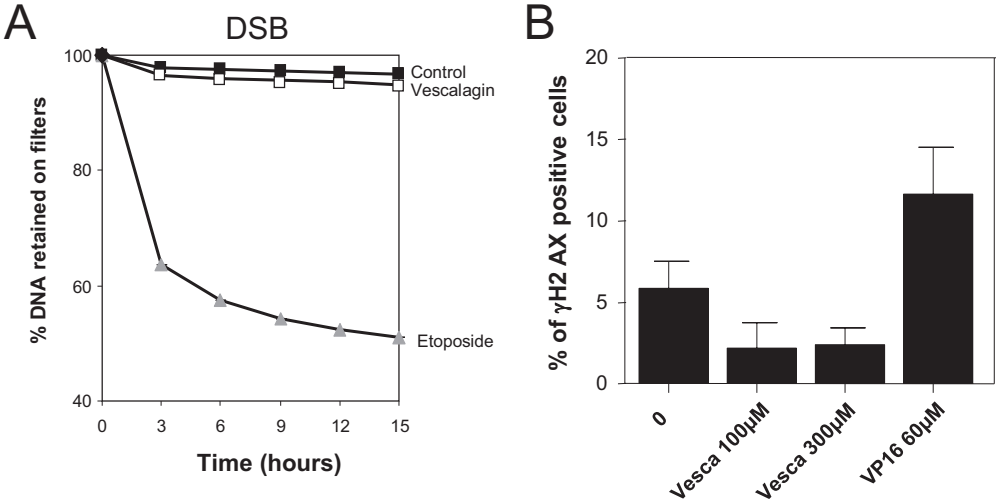

Fig. 6. DNA damage induced by vescalagin in CEM cells. A, measurements of DSBs using the alkaline elution technique after a 2-h treatment with 50 μM vescalagin (Vesca) or etoposide (VP16). B, measurements of DSBs by the detection of histone H2AX phosphorylation by flow cytometry as indicated under *Materials and Methods*.

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 NHTP-bearing analogs, vescalin and castalin, in which such a medium ring-forming biarylic unit is absent, exhibited only weak activity against Top2.

Of interest, 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 lesser 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 than with 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 toward Top2α. We also noticed that methylation of the C-1 hydroxyl group of vescalagin did not significantly affect preferential inhibition of Top2α, whereas substitutions by an ethyl group (Supplemental Fig. 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 of 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 investigation. Taken 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 have an impact on the selectivity toward Top2α and that the best selectivity is observed with a β-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, because transient down-regulation of Top2α conferred higher resistance of CEM cells to this ellagitannin compared with the transient repression of Top2β. This finding 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 the fact that preferential inhibition of cellular Top2α results from catalytic inhibition of the enzyme (Kashiwada et al., 1993). When this effect was compared with that of classic catalytic inhibitors such as the bisdioxopiperazine 4,4′-(1,2-dimethyl-1,2-ethanediyl)bis-2,6-piperazinedione (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; Oestergaard et al., 2004; Nitiss, 2009b), reduction of Top2α induced a minor increase in sensitivity to ICRF-193 and reduction of Top2β levels had no effect (Supplemental Fig. 3). This result suggests that inhibition of Top2α by vescalagin is probably occurring by a mechanism other than ICRF-193, which remains to be further investigated.

In the search for a 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 4-hydroxy-5-methoxy-2,3-dihydro-1*H*-[1,3]benzodioxolo[5,6-*c*]pyrrolo[1,2-*f*]-phenanthridium chloride (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). As an alternative, 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 quinoline aminopurine compound 1 (Chène et al., 2009), thiosemicarbazones (Huang et al., 2010), *N*-fused imidazoles (Baviskar et al., 2011), or xanthone analogs (Jun et al., 2011), which inhibit the catalytic activity of Top2α by an ATP-competitive mechanism. Apart from quinoline aminopurine compound 1, which inhibits both isoforms (Chène et al., 2009), it is not known whether these derivatives also inhibit the catalytic activity of Top2β, which is expressed in postmitotic cells (Watanabe et al., 1994; Lyu and Wang, 2003) and nonproliferating tissues such as the adult heart (Capranico et al., 1992). Development of a selective Top2α catalytic inhibitor would therefore be useful because 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-E-Kamal et al., 2007). Likewise, vescalagin, which is readily available from fagaceous woody plant sources (Quideau et al., 2003, 2005), 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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Contributed new reagents or analytic tools: Jacquet, Pouységu, and Deffieux.

Performed data analysis: Auzanneau, Montaudon, Puyo, Deffieux, Pouységu, Elkaoukabi-Chaibi, De Giorgi, Ichas, and Pourquier.

Wrote or contributed to the writing of the manuscript: Auzanneau, Montaudon, Quideau, and Pourquier.

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