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Ellagic Acid and Polyhydroxylated Urolithins Are Potent Catalytic Inhibitors of Human Topoisomerase II: An in Vitro Study

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ABSTRACT: Ellagic acid (EA), a natural polyphenol abundant in fruits and common in our diet, is under intense investigation for its chemopreventive activity resulting from multiple effects. EA inhibits topoisomerase II, but the effects on the human enzyme of urolithins, its monolactone metabolites, are not known. Therefore, the action of several synthetic urolithins toward topoisomerases II was evaluated, showing that polyhydroxylated urolithins, EA, and EA-related compounds are potent inhibitors of the α and β isoforms of human topoisomerase II at submicromolar concentrations. Competition tests demonstrate a dose-dependent relationship between ATP and the inhibition of the enzyme. Docking experiments show that the active compounds bind the ATP pocket of the human enzyme, thus supporting the hypothesis that EA and polyhydroxylated urolithins act as ATP-competitive inhibitors of human topoisomerase II.

KEYWORDS: *ellagic acid, urolithins, topoisomerase II, gyrase, ATP*

INTRODUCTION

Ellagic acid (EA) is a natural compound with a promising activity as a chemopreventive agent.[1,2] Its biological effect results from multiple actions, not completely understood, involving antioxidant activity and inhibition of several enzymes implicated in the cell cycle and in cancer proliferation.[2−6] Similarly to other biologically active polyphenols,[7−9] EA has been reported to inhibit the activity of topoisomerases II, essential enzymes that catalyze important topological events in cells.[10−12] Human topoisomerase (topo) II is an ATP-dependent enzyme present in two isoforms, types IIα and IIβ, sharing extensive amino acid sequence identity;[13] both isoforms relax, unknot, and decatenate DNA through a reaction of cleavage and religation of DNA coupled to ATP hydrolysis.[10,14] Being regulated very differently in tumor versus "normal cells", human topoisomerase II represents a good candidate for drug discovery, and drugs targeted to topoisomerase II are in clinical use.[13,14] Topoisomerase II targeted drugs have been divided in two classes: poisons and inhibitors.[12] The poison class stabilizes the cleaved DNA intermediate in the topoisomerase II reaction via formation of a drug−enzyme−DNA ternary complex (cleavable complex).[14] Conversely, catalytic inhibitors do not induce cleavable complex formation and are quite heterogeneous in their molecular structures and mechanisms of action.[15,16]

Ellagic acid (**1a**, Figure 1) and its related derivative flavellagic acid (FEA, **1c**, Figure 1) have been reported to inhibit topoisomerase II without induction of the cleavable complex.[17] EA derives from the metabolism of ellagitannins (ETs), natural polyphenols with antiproliferative and pro-apoptotic effects.[2,4,5,17−21] The biodegradation of ellagitannins in mammals is due to the intestinal microflora: their hydrolysis releases hexahydroxydiphenic acid (HHDP) residues, which are dehydrated to the bilactone ellagic acid as the main metabolite.[20] Pomegranate juice is of high interest for its effects in several types of cancer:[2,4,21] interestingly, pomegranate (*Punica granatum*) is particularly rich in EA.[20] The concentration of EA in pomegranate juice, 0.61 g/L,[22] is compatible with effects of EA on the gastrointestinal (GI) tract, and pomegranate polyphenols are metabolized and absorbed in the colon.[22] Plasma levels of EA after pomegranate juice consumption are reported to be 60−100 nM.[23,24] In mammals, dietary EA is further metabolized by the intestinal microbial flora to urolithins, monolactone compounds with a dibenzopyran-6-one scaffold.[22] Urolithins have been found in human plasma at 18.6 μM after juice consumption, although their type and quantity are highly variable depending on the gut microbiota,[22] with urolithin A (UA, **2h**, Figure 1) reported to be the most important metabolite of ETs.[22,24,25] Therefore, it has been suggested that the biological activity attributed to ellagitannins could be related to their colonic metabolites rather than to the original compounds.[25] UA is a CK2 inhibitor through recognition of the ATP binding site of the enzyme, similarly to EA,[26,27] but the topoisomerase II inhibition properties of urolithins are at present not known.

EA growth inhibition was observed in HT29[28,29] and in other colon cancer cell lines,[30] indicating possible effects of EA and its metabolites in cancer colon chemoprevention. Interestingly, in Caco-2 cells EA and urolithins arrested cell cycle progression in G2/M phase,[30] a hallmark of topoisomerase II inhibitors[31] and of the flavonoid genistein, a dual kinase and topoisomerase II inhibitor.[32] To test the hypothesis that urolithins target human topoisomerase II and may contribute to the growth inhibition effects of EA in cancer cell lines, we examined the effects of EA and of a series of recently synthesized urolithins reported to have kinase inhibition properties[27] on the catalytic activity of human topoisomerases IIα and IIβ. We enlarged our analysis to other compounds with an EA scaffold: FEA (**1c**, Figure 1) and coruleoellagic acid (CEA,**1d**, Figure 1). FEA has already been

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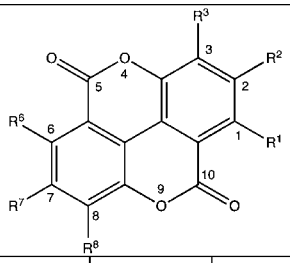
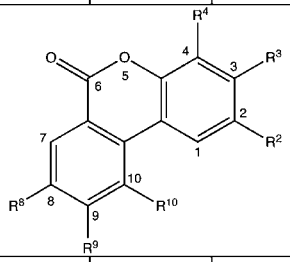


	R1	R2	R3	R6	R7	R8
1a (EA)	-H	-OH	-OH	-H	-OH	-OH
1b	-H	$-OCH_3$	$-OCH_3$	-H	$-OCH_3$	$-OCH_3$
1c (FEA)	-OH	-OH	-OH	-H	-OH	-OH
1d (CEA)	-OH	-OH	-OH	-OH	-OH	-OH



	R2	R3	R4	R8	R9	R10
2a	-H	-OH	-OH	-OH	-OH	-OH
2b	-OH	-OH	-OH	-OH	-OH	-OH
2c	-H	-OH	-H	$-OCH_3$	-H	-H
2d (UB)	-H	-OH	-H	-H	-H	-H
2e	-H	-OH	-Br	$-OCH_3$	-H	-H
2f	-H	-OH	$-CH_3$	-OH	-H	-H
2g	-H	-OH	-Br	-OH	-H	-H
2h (UA)	-H	-OH	-H	-OH	-H	-H
2i	-H	-OH	$-NO_2$	$-OCH_3$	-H	-H

[a] numbering according to Chemical Abstract (Registry Number: 476-66-4)

[b] numbering according to Chemical Abstract (Registry Number: 131086-94-7)

Figure 1. Molecular structures of the tested ellagic acid and urolithin derivatives. All compounds referring to ellagic acid (EA) molecular structure are designated **1** plus a letter, which is "**a**" for EA. "**1**" therefore designates the bilactone compounds. Urolithins are designated by "**2**", again with a letter specifying univocally the identity and position of substituents. Numbering of the structure of EA is according to Chemical Abstracts Service (CAS Registry No. 476-66-4). Numbering of urolithins is also according to Chemical Abstracts Service (CAS Registry No. 131086-94-7).

reported to inhibit topoisomerase II,[17] whereas CEA showed distinct properties as an antiplasmodial agent,[33] but no data are available on its human topoisomerase II inhibition properties.

In the bilactone series, we demonstrate that EA, FEA, and CEA are potent inhibitors of the catalytic activity of human topoisomerase II and that hydroxyl groups must be free: when all hydroxyls of EA are methylated, as in compound **1b**, enzyme inhibition is lost. In the urolithin series we show that urolithins **2a** and **2b** are also potent inhibitors of the human enzymes. Whereas **2b** was never isolated as a metabolite of EA, **2a** corresponds to urolithin M5, a pentahydroxy urolithin, previously identified as an ellagic acid metabolite in mammals.[34] Polyhydroxylation is necessary for enzyme inhibition: when hydroxyls are removed or substituted as in urolithins **2c**−**2i**, human topoisomerase II inhibition is lost or becomes irrelevant in physiological conditions. Furthermore, we show that the loss of the condensed cyclic system of ellagic acid and urolithins leads to inactivity toward the enzyme.

Because EA has been reported to inhibit the bacterial topoisomerase II, DNA gyrase,[35] we also tested all derivatives for the inhibition of the catalytic activity of the bacterial enzyme. However, none of the compounds inhibited gyrase at submicromolar concentrations, although they could interfere with novobiocin, a potent gyrase inhibitor recognizing the ATP binding site of the enzyme. To clarify the mechanism of action of EA and polyhydroxylated urolithins, we performed ATP competition assays, demonstrating that potent inhibition of human topoisomerase II is antagonized by ATP. Our data are strengthened by molecular docking experiments that confirmed the formation of energetically stable complexes between the ATP-binding domain of human topoisomerase II and the potent hexahydroxylated urolithin derivative **2b**.

Our results, besides adding new evidence of the molecular mechanism of action of EA on human topoisomerase II, indicates that EA, but not its metabolites UA and UB, is a potent human topo II inhibitor at concentrations achievable by

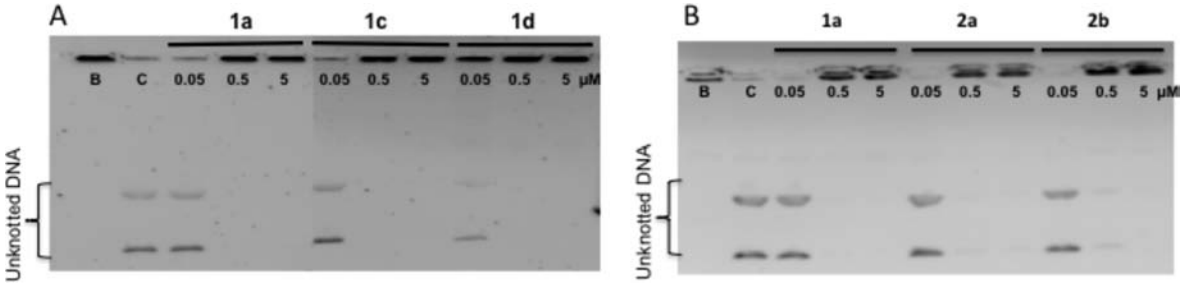


Figure 2. Ellagic acid and urolithin derivatives inhibit human topoisomerase IIα activity at submicromolar concentrations: (A) ellagic acid derivatives (bilactone series); (B) urolithin derivatives (monolactone series). Two hundred nanograms of kDNA was incubated with increasing concentrations of the test compounds and human topoisomerase IIα as indicated and finally loaded in 1% agarose gel in 1× TBE (90 mM Tris, 90 mM boric acid, 20 mM EDTA) containing EtBr at 0.5 μg/mL; running buffer 1× TBE + 0.5 μg/mL EtBr; gel run was 1.5 h at 4 V/cm. B, blank; C, control (+ enzyme). Ellagic acid (**1a**) was included in both panels as control. Only active compounds are shown.

ingestion of food rich in ellagitannins and may contribute to protective effects in the GI tract.

MATERIALS AND METHODS

Chemicals. EA was from Sigma, whereas all other derivatives except **1d** were synthesized as previously reported.[27] CEA **1d** was synthesized as reported in ref 33. The compounds' stock solutions were made in DMSO and maintained at −20 °C.

Inhibition of Human Topoisomerase II. Human topoisomerase II assays are performed according to the protocol reported by several authors[36,37] with some minor modifications. A standard assay is made in a final volume of 20 μL containing 200 ng of kinetoplast DNA (kDNA), 40 mM Tris-HCl, pH 7.9, 80 mM KCl, 5 mM DTT, 15 μg/mL BSA, 10 mM $MgCl_2$, 1 mM ATP, 0.066 U topoisomerase II α or 2.5 U topoisomerase II β, and Milli-QH_2O up to a final volume of 18 μL. Human topoisomerase II α was from Inspiralis Ltd. (UK), whereas human topo IIβ was kindly provided by Dr. Giovanni Capranico, University of Bologna. The reactions are started by adding 2 μL of compound solution, to have the desired final concentration, and incubated for 60 min at 37 °C. All of the reactions are stopped with 3 μL of gel loading buffer made with 49% TE (10 mM Tris, 1 mM EDTA, 20 mM NaCl, pH 7.4), 49% glycerol, 2% SDS, 0.025% bromophenol blue, and 0.025% xylene cyanol. Samples are resolved by electrophoresis on a 1% agarose gel running in 1× TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA), both containing 0.5 μg/mL ethidium bromide. DNA bands are visualized by UV and photographed using the Geliance 600 Imaging System (Perkin-Elmer). The arrays for the electrophoresis are the Sub-Cell GT type provided by Bio-Rad. Densitometric analysis was peformed using the software Gene tools from Perkin-Elmer. The control containing only kDNA corresponds to 0% activity, and 100% activity corresponds to the sample containing kDNA and enzyme. The concentration of the inhibitor preventing 50% of the substrate (knotted DNA) from being converted into the reaction product (unknotted DNA) can be determined from a standard sigmoid curve. By averaging at least three experiments, the IC_{50} values ± SD were determined. The competition assays were made under the same conditions as the standard decatenation assays, but with the ATP concentration varied from 1 mM (standard) to 2.5 mM.

Inhibition of *Escherichia coli* DNA Gyrase. This assay is performed according to the method of Sayer[38] with minor modifications. The standard assay is performed for a final volume of 20 μL containing 0.5 μg of relaxed pBR322 in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% w/v glycerol, and 0.1 mg/mL albumin and adding 1 U of *E. coli* DNA gyrase (Inspiralis Ltd.) and 2 μL of compound to have the desired final concentration. After 30 min at 37 °C, the reactions are stopped with 5 μL of the same dye solution used for the unknotting assays. Samples are resolved by electrophoresis on a 1% agarose gel in 1× TBE buffer and stained post run with a 0.5 μg/mL ethidium bromide solution for 15 min. DNA bands are visualized by UV and photographed.

Molecular Docking Simulations. *Target Structures.* The ATPase domain of a human type IIA DNA topoisomerase was retrieved from the PDB (PDB code 1ZXM).[39] Hydrogen atoms were added using standard geometries to the protein structure with the Molecular Operation Environment (MOE, version 2010.10) program.[40] All water molecules within 4.5 Å from the adenylyl imidodiphosphate cofactor and with an oxygen B-factor <25 Å^2 have been retained as integral parts of the putative ATP binding region. To appropriately assign ionization states and hydrogen positions, the Protonate-3D tool implemented by MOE was used.[40] To minimize contacts among hydrogens, the structures were subjected to Amber94[41] force field minimization until the rms of conjugate gradient was <0.1 kcal/mol/Å, keeping the heavy atoms fixed at their crystallographic positions.

Molecular Docking Protocol. All docked ligands were built using the "Builder" module of MOE.[40] Ligands were docked into the putative ATP-binding sites using flexible MOE-Dock methodology.[40] The purpose of MOE-Dock is to search for favorable binding configurations between a small, flexible ligand and a rigid macromolecular target. Searching is conducted within a user-specified 3D docking box, using "tabù search" protocol[42] and MMFF94 force field.[43] Charges for ligands were imported from the MOPAC program[44] output files. MOE-Dock performs a user-specified number of independent docking runs (55 in the present case) and writes the resulting conformations and their energies to a molecular database file. The resulting ligand−top2 complexes were subjected to MMFF94 all-atom energy minimization until the rms of the conjugate gradient was <0.1 kcal/mol/Å. GB/SA approximation[45] has been used to model the electrostatic contribution to the free energy of solvation in a continuum solvent model. The interaction energy values were calculated as the energy of the complex minus the energy of the ligand, minus the energy of top2: $\Delta E_{inter} = E_{(complex)} - (E_{(L)} + E_{(top2)})$.

DNA Binding. DNA binding was determined by thermal denaturation assay (fluorescence quenching assay, FQA) essentially as described.[46] Briefly, oligonucleotides were labeled at the 5′ end of the forward strand with FAM (6′-carboxyfluorescein) and with DAB (dabcyl, *N*-4′-carboxy-4-(dimethylamino)azobenzene) at the 3′-end of the reverse strand. FAM maximum emission wavelength is at 517 nm when excited at 494 nm (ε_{494} = 83000 cm^{-1} M^{-1}) and is efficiently quenched by DAB. The double-stranded oligo sequences were obtained by annealing FAM-ACTATTCCCGGGTAATGA with TCATTACCCGGGTAAAGT-DAB and FAM-GTCCCTGTTCGGGCGCCA with TGGCGCCCGAACAGGGAC-DAB. Melting reactions took place in glass capillaries containing 20 μL of the incubation mixture: 2 μL of compound at the final concentration of 1, 10, and 100 μM; 4 μL of HB buffer (HB 20: 10 mM Tris, 1 mM EDTA, 20 mM NaCl, pH 7.5). Before the assay took place, forward and reverse strand oligo solutions were mixed at equimolar concentrations, denatured at 95 °C for 5 min, and allowed to anneal at room temperature for 1 h. Thermal protocol of melting

was a ramp 30−95 °C modified by 1 °C/min by the Light Cycler (Roche) apparatus. Fluorescence in each capillary was read at 520 nm by the same apparatus.

RESULTS AND DISCUSSION

EA, FEA, CEA, and Polyhydroxylated Urolithins Are Potent Inhibitors of Human Topoisomerases IIα and IIβ. To evaluate the activity of the test compounds on human topoisomerases II (isoform α and isoform β), we have employed the unknotting assay,[37] based on the conversion of knotted kDNA, unable to migrate in the gel, to its unknotted forms that could be visualized by agarose gel electrophoresis as lower mobility bands (Figure 2).

It is evident from Figure 2A that EA (**1a**) inhibits human topoisomerase II at submicromolar concentrations. Experiments performed at least in triplicate allowed us to determine the IC_{50} reported with their standard deviation (SD) in Table 1.

Table 1. Inhibition of Human Topoisomerases II Unknotting Activity by Ellagic Acid and Urolithin Derivatives

	IC_{50}[a] (μM)	
	topo IIα	topo IIβ
1a (EA)	0.27 ± 0.05	0.25 ± 0.15
1b	na[b]	na
1c (FEA)	0.28 ± 0.16	0.21 ± 0.14
1d (CEA)	0.16 ± 0.07	0.12 ± 0.03
2a	0.17 ± 0.03	0.10 ± 0.01
2b	0.08 ± 0.02	0.12 ± 0.03
2c	na	na
2d (UB)	na	na
2e	na	na
2f	na	na
2g	na	na
2h (UA)	na	na
2i	na	na

[a]Data represent the mean of three independent experiments ± SD. [b]na, not active up to 5 μM.

Doxorubicin, a well-known topoisomerase II poison, was tested at the same experimental conditions, reporting an IC_{50} = 2.69 ± 0.43 μM, strengthening the finding that ellagic acid and flavellagic acid are potent inhibitors of human topoisomerase IIα. Coruleoellagic acid (**1d**), a hexahydroxy-substituted compound, is the most active of the bilactone series (Figure 2A), with an IC_{50} value of 0.16 ± 0.07 μM. This is the first report of potent topoisomerase II inhibition by CEA. Compound **1b** was inactive at the concentrations of our tests and up to 10 μM, thus verifying the importance of free hydroxyls for obtaining potent inhibitors of human topoisomerase IIα (Table 1).

In the case of urolithins, the main metabolites of EA, **2h** and **2d**, respectively urolithins A and B,[22] are inactive up to 5 μM, a concentration that we chose as a limit in our series of potent derivatives, and therefore we considered them "inactive" in our experimental conditions (Table 1). Such lack of biological relevance toward human topoisomerase II is shared by all monolactone derivatives **2c−2i**, where free hydroxyls are methylated or substituted by other functional groups (Table 1). Conversely, the two urolithin derivatives **2a** and **2b** exhibit activity comparable or superior to the those of the bilactone compounds (Figure 2B and Table 1). Interestingly, **2a**, a pentahydroxyurolithin identified as a urinary metabolite of EA,[34] bears additional hydroxyl groups introduced at positions 4 and 9 of urolithin A, whereas in the case of **2b**, OH groups are present at positions 2, 4, and 9 relative to **2h** (UA). The hexahydroxyurolithin **2b**, a synthetic compound that was never isolated as a metabolite in vivo, is the most active compound of the whole series (IC_{50} = 0.08 ± 0.02 μM on human topoisomerase IIα). To the best of our knowledge, this is the first report of such potent inhibition of human topoisomerase II by synthetic urolithins, strengthening the increasing interest of pharmaceutical research for this class of compounds. To further analyze the novel compounds, we performed unknotting assays with human topoisomerase IIβ, demonstrating comparable activity to the α isoform, as reported in Table 1.

Besides urolithins, another product of ellagitannin metabolism is 3,4,5-trihydroxybenzoic acid, or gallic acid (GA, Figure 3A).[20] GA, which has been reported to be cytotoxic in vitro,[21] may contribute to the observed activities of ETs by inhibition of human topoisomerase IIα. Using EA as positive control and 0.05, 0.5, and 5 μM as compound concentrations, we performed the unknotting assay with human topoisomerase IIα (Figure 3B), showing that GA does not inhibit human topoisomerase IIα up to our cutoff concentration of 5 μM. We therefore considered it to be "inactive", thus demonstrating that a condensed ring system bearing at least one lactone group must be retained for the potent enzyme inhibition exhibited by EA and polyhydroxylated urolithins.

Ellagic Acid and Urolithin Derivatives Are Very Weak Inhibitors of DNA Gyrase. The good preliminary results

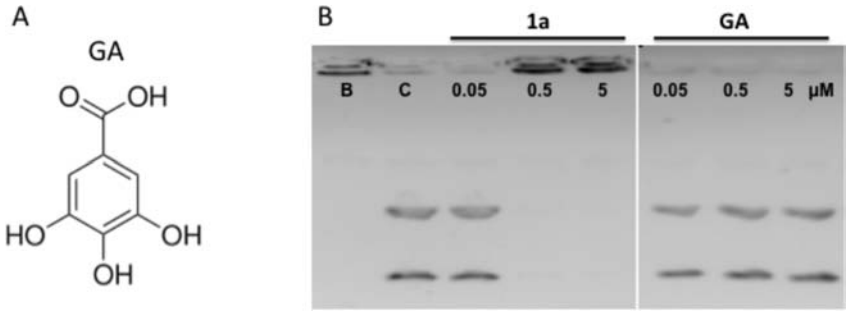


Figure 3. Gallic acid does not inhibit human topoisomerase IIα: (A) chemical structure of gallic acid (GA); (B) unknotting activity by GA. Two hundred nanograms of kDNA was incubated with increasing concentrations of the test compounds and human topoisomerase IIα as indicated under Materials and Methods and finally loaded in 1% agarose gel in 1× TBE (90 mM Tris, 90 mM boric acid, 20 mM EDTA) containing EtBr at 0.5 μg/mL; running buffer 1× TBE + 0.5 μg/mL EtBr; gel run was 1.5 h at 4 V/cm. B, blank; C, control (+ enzyme).

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obtained on the two isoforms of human topoisomerase II prompted us to investigate the activity of the test compound on DNA gyrase. In fact, earlier literature reported that ellagic acid could inhibit DNA gyrase with a medium potency, whereas monolactones derived from ellagic acid were reported to be even less potent.[35] Gyrase activity is conveniently assayed by following the supercoiling of relaxed plasmid DNA, using as positive control the antibiotic novobiocin, a well-known catalytic inhibitor of bacterial topoisomerases that acts on inhibiting ATP binding to its site of the gyrase B subunit.[47,48] We therefore tested ellagic acid and all other derivatives on *E. coli* DNA gyrase: none of the compounds shown in Figure 1 inhibit the bacterial enzyme at the concentrations active on human topoisomerases II, that is, below 5 μM, indicating a selective targeting of the mammalian enzyme at submicromolar concentrations. In accord with earlier literature data,[35] inhibition of the catalytic activity of *E. coli* gyrase by **1a** (ellagic acid) as well as by the representative urolithin **2a** becomes evident only at higher concentrations, as seen from the decrease in the formation of the negatively supercoiled plasmid band in Figure 4 starting from 50 μM, whereas the positive control novobiocin exhibits potent and full inhibition of DNA gyrase at 10 μM (Figure 4).

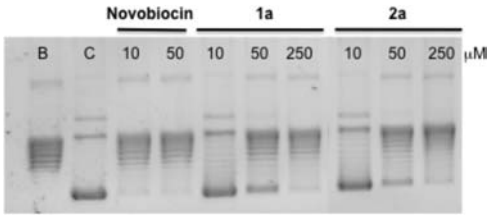

Figure 4. Ellagic acid and urolithins are weak inhibitors of *E. coli* DNA gyrase. Each sample containing 0.5 μg of relaxed pBR322, 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% w/v glycerol, 0.1 mg/mL albumin, 1 U gyrase, compound and Milli-Q H_2O up to 20 μL was incubated for 30 min at 37 °C, stopped, and resolved by 1% agarose gel in 1× TBE; running buffer 1× TBE; run ~1.5 h at 4 V/cm. Staining was done with 0.5 μg/mL ethidium bromide. B, blank relaxed plasmid (no enzyme and no compound); C, positive control (plus DNA gyrase).

However, when novobiocin was co-incubated with excess but noninhibitory concentrations of ellagic acid (10 and 50 μM), a clear reduction of the inhibitory effect of the antibiotic on the inhibition of *E. coli* DNA gyrase was observed, indicating antagonism for the active site on the enzyme (Figure 5).

From these results we can conclude that ellagic acid and urolithin derivatives inhibit bacterial DNA gyrase only at much higher concentrations than those achieved for the inhibition of mammalian topoisomerases II. However, ellagic acid can antagonize novobiocin's potent effect on the DNA gyrase, suggesting a shared binding site on the bacterial enzyme.

Ellagic Acid and Its Derivatives Compete with ATP. We have clearly established that ellagic acid and polyhydroxylated urolithin derivatives are potent inhibitors of the catalytic activity of human topoisomerases II and weak inhibitors of bacterial gyrase. However, the interference with novobiocin activity suggests a partial overlap on the ATP binding domain, as reported for other compounds.[49] Mammalian topoisomerases II are ATP-dependent enzymes, and, recently, catalytic inhibitors of topoisomerase II were rationally designed to fit into the ATP binding site of the enzyme.[50,51]

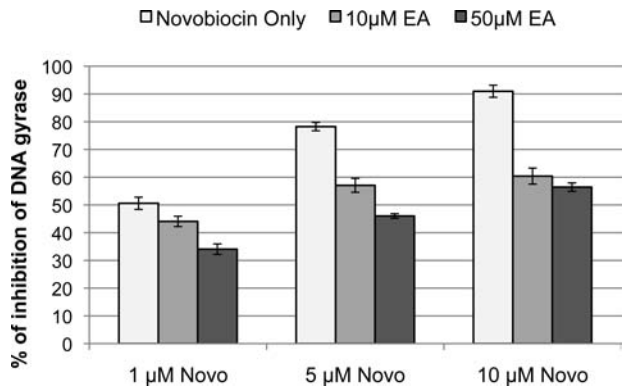

Figure 5. Effect of ellagic acid (EA) on the inhibition by novobiocin of *E. coli* DNA gyrase. Relaxed pBR322 (0.5 μg) was incubated with *E. coli* DNA gyrase and increasing concentrations of novobiocin (1, 5, and 10 μM) alone and in the presence of fixed concentrations of ellagic acid (10 and 50 μM). The bar graph indicates the observed percentage of inhibition of DNA supercoiling by novobiocin in the absence and presence of noninhibitory concentrations of ellagic acid.

In the attempt to clarify the molecular mechanism of action of EA and its related polyhydroxylated derivatives toward human topoisomerase II, we have performed an ATP competition assay with human topoisomerase II. Results of the unknotting assay with human topoisomerase II and ellagic acid (**1a**) at different ATP concentrations are reported in Figure 6.

Increasing ATP concentrations lead to a reduction in the potency of EA (**1a**) toward the inhibition of human topo II,

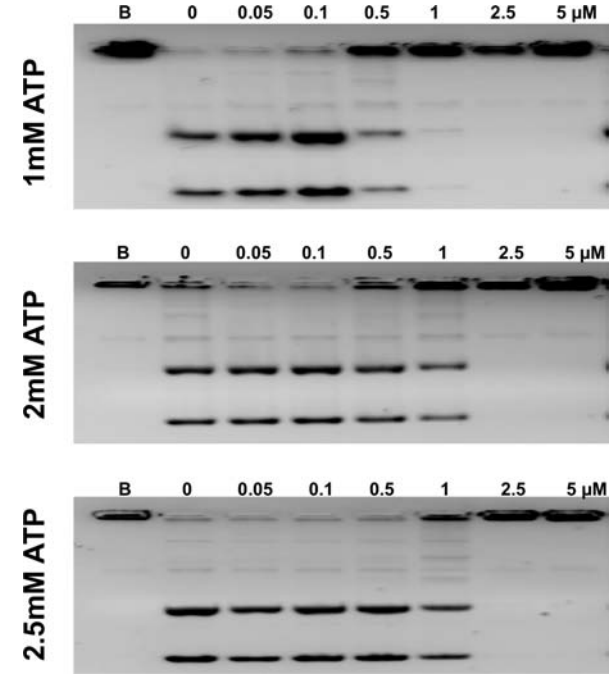

Figure 6. ATP antagonizes the topoisomerase II inhibition by ellagic acid. Two hundred nanograms of kDNA was incubated with increasing concentrations of the test compounds and human topoisomerase IIα. In the competition assay the concentration of ATP was increased from 1 mM (standard assay) to 2.5 mM as indicated in each panel. The samples were finally loaded in 1% agarose gel in 1× TBE with 0.5 μg/mL EtBr. Running buffer 1× TBE + 0.5 μg/mL EtBr; run ~1.5 h at 4 V/cm. B, blank; 0, control (+ enzyme).

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evidenced by its increase in IC_{50} value, reported in Table 2. This observation is not limited to EA, but it is observed also for compound **1d** (coruleoellagic acid) and for the two urolithins **2a** and **2b**, which also compete with ATP (Table 2), indicating that polyhydroxylated EA and urolithins act as catalytic inhibitors of topoisomerase II through the recognition of the ATP binding pocket of the human enzyme.

Table 2. Effect of ATP on the Inhibition of Human Topoisomerase IIα by Ellagic Acid and Urolithin Derivatives

	IC_{50}[a] (μM)		
	1 mM ATP	2 mM ATP	2.5 mM ATP
1a (EA)	0.27 ± 0.05	0.51 ± 0.05	0.99 ± 0.06
1d (CEA)	0.16 ± 0.07	0.23 ± 0.04	0.35 ± 0.02
2a	0.17 ± 0.03	0.24 ± 0.03	0.68 ± 0.03
2b	0.08 ± 0.02	0.19 ± 0.07	0.25 ± 0.08

[a]Data represent the mean of three independent experiments ± SD.

Molecular Modeling Studies. The hypothesis of the competition with the ATP molecule is supported by modeling data that underscore the importance of specific amino acid residues and water molecules in the binding with both the ATP molecule and the test derivatives. The ATP-binding pocket is in fact small and rich in polar residues, promoting the formation of hydrogen bonds between the amino acids and molecules rich in hydroxyls. In Figure 7 we report the crystallographic structure of AMP-PNP (a nonhydrolyzable ATP analogue, colored in cyan) [39] superimposed with the hypothetical binding mode of urolithin **2b** (colored in pink) within the same ATP-binding pocket of the human topoisomerase IIα.

Compound **2b** is held in the same place of the AMPPNP through a complex network of hydrogen bonds, similarly to what was described for the adenine moiety of the AMPPNP. In urolithin **2b**, the two phenolic O−H groups at positions 8 and 9 interact through direct hydrogen bonding to the Asn-120 side-chain carbonyl plus water-mediated contacts to the side-chain groups of Asn-91, Asn-120, and Thr-215. On the other hand, the phenolic O−H group at position 4 is within hydrogen-bonding distance of the backbone C═O group of Ile-141 and can also interact through an additional dipole-charge interaction with the side chain group of Lys-168. The two phenolic O−H groups at positions 3 and 2 exhibit an extended network of hydrogen bonding with three consecutive residues (147−149) of the ATP binding site of the human enzyme. Indeed, all crucial interactions mediated by the phenolic O−H groups compound **2b** are almost conserved into the hypothetical binding motif of EA. In particular, the phenolic groups at positions 3 and 8 of EA (respectively 4 and 9 in the urolithin scaffold) play a crucial role in determining the stability of both EA and urolithin scaffolds into the ATP-binding site of the human topoisomerase IIα. On the basis of such structure−activity information, it is possible to propose a hypothetical binding mode for polyhydroxylated EA derivatives summarized in Figure 8.

Finally, comparing the ATP-binding site of the topoisomerase IIα with the corresponding binding site of the bacterial DNA gyrase, it is worth emphasizing that the cavity volume of human topoisomerase IIα is larger (around 700 Å^3) than in bacterial DNA gyrase (around 500 Å^3) as shown in Figure 9. Consequently, ellagic acid and urolithin derivatives are sterically better accommodated within the ATP-binding site of the topoisomerase IIα, supporting the experimental evidence that ellagic acid and urolithin derivatives inhibit bacterial DNA gyrase only at much higher concentrations than those achieved for the inhibition of mammalian topoisomerases II.

Ellagic Acid Derivatives Do Not Intercalate into DNA. Our data indicate that EA and polyhydroxylated derivatives belonging to the bi- and monolactone series are potent inhibitors of the unknotting activity of the human top-

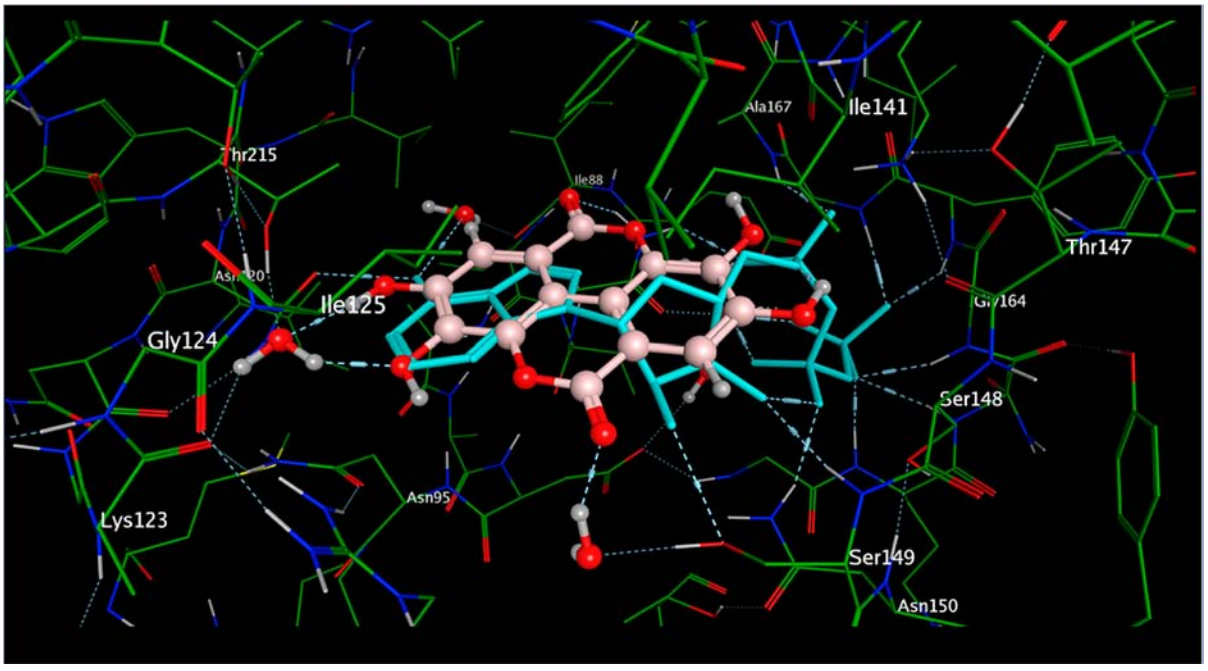

Figure 7. Urolithin **2b** recognizes the ATP-binding site of human topoisomerase IIα. The crystallographic structure of the AMP−PNP (cyan) complexed to the ATP-binding site of the topoisomerase IIα (PDB code 1ZXM) is shown superimposed with the hypothetical binding mode of urolithin **2b** (pink) modeled in the ATP-binding pocket of human topoisomerase II.

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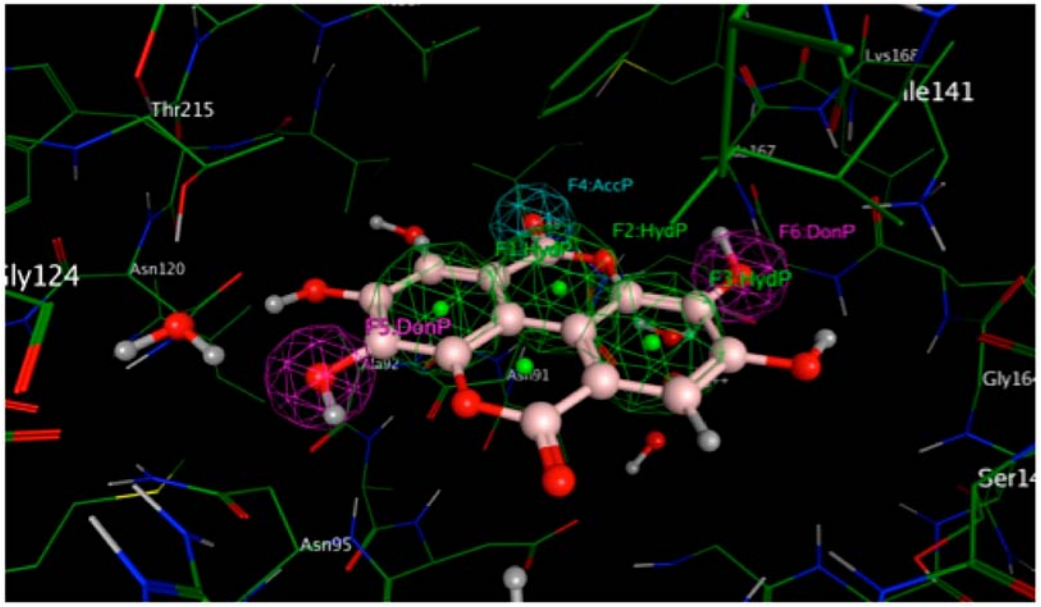

Figure 8. Hypothetical binding mode of EA derivatives to human topoisomerase II.

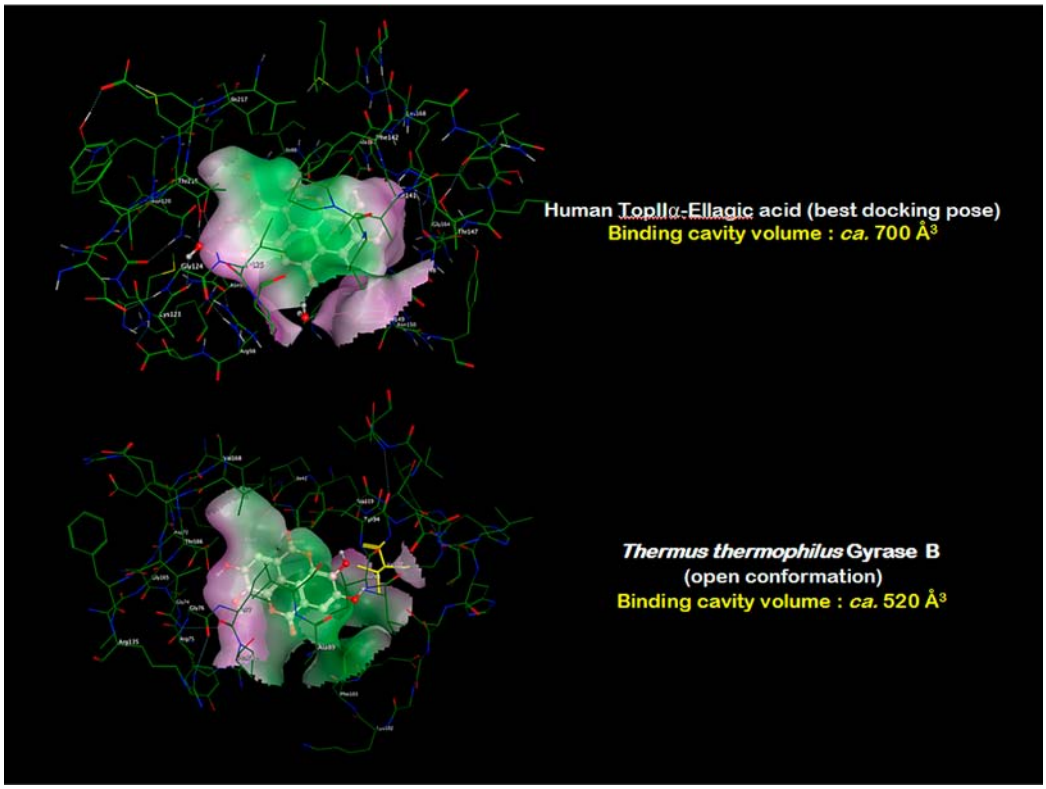

Figure 9. Ellagic acid is better accommodated in the ATP-binding site of human topoisomerase II: comparison between the ATP-binding site of the human topoisomerase IIα (PDB code 1ZXM, upper panel) and the corresponding binding site of the *Thermus thermophilus* DNA gyrase (PDB code 1KIJ, bottom panel). Ellagic acid was docked in both ATP-binding sites.

oisomerases II through recognition of the ATP-binding pocket of the mammalian enzyme, a behavior resembling that of synthetic biphenols recently described as potent catalytic inhibitors of human topoisomerase II unable to bind DNA.[52] To exclude the contribution of DNA binding to the topoisomerase II inhibition, we measured by thermal denaturation the ability of the tested derivatives to increase the thermal stability of double-stranded DNA, using as a positive control doxorubicin, a DNA intercalator and a potent topoisomerase II poison. None of the compounds tested affected the ΔT_m of dsDNA up to 100 μM, whereas doxorubicin at 5 μM exhibited a clear stabilizing effect (ΔT_m = 16 °C) on two different oligonucleotide pairs, similarly to other known intercalators.[46] Therefore, we can safely conclude that our tested derivatives inhibit topoisomerase II catalytic activity through a molecular mechanism not related to DNA

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intercalation, differently from doxorubicin and other topoisomerase II poisons.

Ellagic acid and polyhydroxylated urolithins therefore are potent inhibitors of the human enzyme with a mechanism of action distinct from that exhibited by the topo II drugs used in cancer chemotherapy. In the gastrointestinal tract EA's inhibitory activity toward human topo II may contribute to the growth inhibition effects observed on cancer cell lines. Among its metabolites, UA cannot be related to topo II effects, but other metabolites such as the pentahydroxyurolithin **2a**, also referred to as M5 by other authors,[34] may additionally contribute to EA's activity through their action on human topo II. Indeed, although the polyhydroxy urolithins identified as potent topo II inhibitors are minor or absent in food, they can be exploited in the development of anticancer drugs. This is particularly relevant from a pharmaceutical point of view because urolithins are more bioavailable than EA. Our results on the effects of these compounds on human topoisomerase II add new insights for understanding the complex mechanism of action of these important polyphenols in the difficult task of translating natural compounds and their metabolites into drugs with chemopreventive effects.[53] In this respect, we acknowledge that, once again, an original chemical and pharmacological idea comes from natural sources.

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ABBREVIATIONS USED

EA, ellagic acid; FEA, flavellagic acid; CEA, coruleoellagic acid; UA, urolithin A; UB, urolithin B; ETs, ellagitannins; GA, gallic acid; kDNA, kinetoplast DNA.

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