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Effects of Olive Metabolites on DNA Cleavage Mediated by Human Type II Topoisomerases

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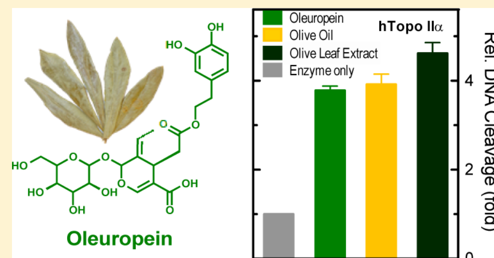
ABSTRACT: Several naturally occurring dietary polyphenols with chemopreventive or anticancer properties are topoisomerase II poisons. To identify additional phytochemicals that enhance topoisomerase II-mediated DNA cleavage, a library of 341 Mediterranean plant extracts was screened for activity against human topoisomerase IIα. An extract from *Phillyrea latifolia* L., a member of the olive tree family, displayed high activity against the human enzyme. On the basis of previous metabolomics studies, we identified several polyphenols (hydroxytyrosol, oleuropein, verbascoside, tyrosol, and caffeic acid) as potential candidates for topoisomerase II poisons. Of these, hydroxytyrosol, oleuropein, and verbascoside enhanced topoisomerase II-mediated DNA cleavage. The potency of these olive metabolites increased 10−100-fold in the presence of an oxidant. Hydroxytyrosol, oleuropein, and verbascoside displayed hallmark characteristics of covalent topoisomerase II poisons. (1) The activity of the metabolites was abrogated by a reducing agent. (2) Compounds inhibited topoisomerase II activity when they were incubated with the enzyme prior to the addition of DNA. (3) Compounds were unable to poison a topoisomerase IIα construct that lacked the N-terminal domain. Because hydroxytyrosol, oleuropein, and verbascoside are broadly distributed across the olive family, extracts from the leaves, bark, and fruit of 11 olive tree species were tested for activity against human topoisomerase IIα. Several of the extracts enhanced enzyme-mediated DNA cleavage. Finally, a commercial olive leaf supplement and extra virgin olive oils pressed from a variety of *Olea europea* subspecies enhanced DNA cleavage mediated by topoisomerase IIα. Thus, olive metabolites appear to act as topoisomerase II poisons in complex formulations intended for human dietary consumption.



A broad spectrum of anticancer drugs comes directly from natural sources or is derived from natural products. Many of these compounds are botanical in nature. Plants have been used for culinary and medicinal purposes for millennia. The Mediterranean basin is home to a biologically diverse plant biome and is a particularly rich source of botanicals with medicinal properties.[1−3] Many of these have been used in traditional Bedouin and Israeli medicine since antiquity. Phytochemicals derived from Mediterranean plant species have been shown to provide a variety of health benefits and display anti-inflammatory, anticancer, cardioprotective, and chemopreventative properties.[1−3]

Type I and type II topoisomerases, which are essential enzymes, are important targets for plant-derived anticancer drugs. For example, topotecan, a topoisomerase I-targeted drug, is derived from camptothecin, which is found in the yew tree (*Camptotheca acuminata*).[4,5] Etoposide, a topoisomerase II-targeted drug, is derived from podophyllotoxin, which is found in Mayapples (*Podophyllum peltatum*).[6−9] Type II topoisomerases also are targeted by a variety of dietary phytochemicals with chemopreventative properties,[10] including bioflavonoids (from soy, fruits, and vegetables),[11−13] catechins (from green tea),[14,15] curcumin (from turmeric),[16,17] thymoquinone (from black seed),[18] and isothiocyanates (from cruciferous vegetables).[19] Although diverse in structure, these compounds act by increasing levels of covalent topoisomerase II-cleaved DNA complexes (cleavage complexes), which are formed as requisite intermediates during the critical DNA strand passage activity of the enzyme.[10,20−24] They are termed "topoisomerase II poisons" to distinguish them from compounds that inhibit the catalytic activity of the enzyme without increasing levels of topoisomerase II-mediated DNA cleavage.[10,20−24] Topoisomerase II−DNA cleavage complexes are intrinsically dangerous to cells. At high

Received: February 17, 2015
Revised: June 29, 2015
Published: July 1, 2015

ACS Publications © 2015 American Chemical Society

DOI: 10.1021/acs.biochem.5b00162
Biochemistry 2015, 54, 4531−4541

levels, these complexes can induce cell death pathways or trigger chromosomal translocations.[10,20−24]

Humans encode two isoforms of topoisomerase II, α and β.[20,25−28] Topoisomerase IIα is an essential enzyme that is expressed at high concentrations in proliferating cells. It is the enzyme responsible for decatenating daughter chromosomes prior to mitosis. Topoisomerase IIβ is expressed in all cell types and appears to play important roles in transcription. Both enzyme isoforms are cellular targets for anticancer drugs and other topoisomerase II poisons.[10,20−24,29]

Topoisomerase II poisons function by two distinct mechanisms. Drugs such as etoposide interact noncovalently at the interface between the enzyme active site and DNA.[10,20,30] They bind to the protein and intercalate into the cleaved scissile bond, thus inhibiting the ability of topoisomerase II to religate the cut DNA. Compounds that utilize this mechanism are termed interfacial topoisomerase II poisons.[30] Genistein and several other bioflavonoids (flavones, isoflavones, and flavonols) utilize this mechanism.[12,13] In contrast, compounds containing reactive protein modification groups, such as quinones and isothiocyanates, form covalent adducts with cysteine (and potentially other) amino acid residues that are distal to the active site of the type II enzyme.[10,19,20,31−34] Compounds that utilize this mechanism are termed covalent topoisomerase II poisons.[10,20] Although the details underlying the basis for the DNA cleavage enhancement by covalent poisons have yet to be fully delineated, these reactive compounds are believed to act by affecting the closure of the N-terminal protein gate.[33,35,36] As examples, epigallocatechin gallate (EGCG), curcumin oxidation products, thymoquinone, and sulforaphane appear to function utilizing this latter mechanism.[14,17−19]

Covalent poisons can be distinguished from interfacial poisons by two hallmark characteristics.[17,20,31,32] Because the oxidation state of covalent poisons is critical for their adduction chemistry, reducing agents, such as dithiothreitol (DTT), abrogate their activity against topoisomerase II. Second, although covalent poisons increase the level of DNA cleavage when added to the enzyme−DNA complex, they inhibit topoisomerase II activity when incubated with the enzyme prior to the addition of DNA.

In an effort to discover novel phytochemicals with activity against human type II topoisomerases, we conducted a blind screen of a library of 341 Mediterranean plant extracts to determine whether any of them increased levels of DNA cleavage mediated by topoisomerase IIα. Species in the library were primarily from arid lands or the Tel Aviv University Botanical Garden and included plants used in traditional Bedouin medicine. An extract from *Phillyrea latifolia* L., a member of the Oleaceae family of olive trees, displayed high activity against the human enzyme. Using a metabolomics approach, we identified several polyphenols as potential candidates for topoisomerase II poisons. From these compounds, we determined that hydroxytyrosol, oleuropein, and verbascoside were covalent poisons. The activities of these compounds were accentuated under oxidizing conditions. Finally, commercial olive leaf extract and extra virgin olive oils also poisoned human topoisomerase IIα.

■ EXPERIMENTAL PROCEDURES

Enzymes and Materials. Recombinant wild-type human topoisomerase IIα, topoisomerase IIβ, and Top2$\alpha\Delta$1175 [a deletion mutant (residues 1−1175) of human topoisomerase IIα] were expressed in *Saccharomyces cerevisiae* JEL-1Δtop1 and purified as described previously.[37−40] The catalytic core of human topoisomerase IIα (residues 431−1193) was a gift from J. Deweese and was expressed and purified as described previously.[41−43] Enzymes were stored at −80 °C as a 1.5 mg/mL stock in 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 750 mM KCl, and 5% glycerol. The residual concentration of dithiothreitol was <2 μM in final reaction mixtures.

Negatively supercoiled pBR322 DNA was prepared from *Escherichia coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Analytical grade etoposide, tyrosol (4-hydroxyphenylethanol), 3,4-dimethoxyphenylethanol, 4-hydroxy-3-methoxyphenylethanol, and oleuropein were purchased from Sigma-Aldrich. Analytical grade hydroxytyrosol (3,4-dihydroxyphenylethanol), verbascoside, and caffeic acid (3,4-dihydroxyphenylpropionic acid) were obtained from LKT Laboratories. Tyrosol, hydroxytyrosol, oleuropein, verbascoside, and 4-hydroxy-3-methoxyphenylethanol were prepared as 20 mM stocks in deionized purified water and stored at −20 °C. Caffeic acid and dimethoxyphenylethanol were prepared as 20 mM stocks in 100% dimethyl sulfoxide and stored at room temperature. Potassium ferricyanide [$K_3Fe(CN)_6$] was obtained from Acros and stored at −20 °C as a 50 mM stock solution in deionized purified water. [γ-^{32}P]ATP (5000 μCi) was purchased from PerkinElmer and stored at −20 °C.

A library of methanol/water extracts from 341 native Mediterranean plants was used for the initial screening. Plant species were mainly from arid lands or the Tel Aviv University Botanical Garden and included plants used in traditional Bedouin medicine.[44−46] On the basis of results with the original library, a second library of 36 extracts from the leaf, bark, flowers, or fruit of 11 individual olive tree species was established.

Plant extracts were prepared as described by Kaiser et al.[47] Briefly, samples (1 g) of frozen plant material were ground in a prechilled mortar containing liquid nitrogen. Two milliliters of a methanol/water mixture [50:50 (v/v)] was added, and slurries were mixed and kept on ice for 15 min. The mixtures were centrifuged at 10000 g for 5 min at room temperature using a Hermle Z160M microfuge. Supernatant liquids were stored at −80 °C for analysis. Extract concentrations were determined gravimetrically. Samples were dried *in vacuo*, de-identified, numbered, and stored at −20 °C. The 341 plant extracts were resuspended in deionized purified water at a final concentration of 2 mg/mL and screened in a blind fashion. Samples from the olive tree extract library were prepared as described above, resuspended to a final concentration of 10 or 20 mg/mL in deionized purified water, and stored at −20 °C.

Commercial olive leaf extract (Olive Leaf Plus, ∼30% oleuropein) was purchased from Life-Flo and prepared as a 20 mg/mL stock in deionized purified water. The species of olive leaves used in this preparation was not identified by the manufacturer. Extra virgin olive oils, pressed from the indicated subspecies of *Olea europea*, including Olive Oil Store Ultra Arbosana (arbosana olives), Olive Oil Store Ultra Koroneiki (koroneiki olives), and Lucini Select (a mixture of frantoio, moraiolo, leccino, maurino, and pendolino olives), were stored at room temperature. Soluble extra virgin olive oil extracts were prepared by vigorously vortexing a 1:1 mixture of oil and deionized purified water for 5 min, using 30 s pulses. The oil and water phases were separated by centrifugation at 8000g for 10 min at room temperature, and the aqueous phase was used for subsequent experiments.

Cleavage of Plasmid DNA. DNA cleavage reactions were performed using the procedure of Fortune and Osheroff.[48] Reaction mixtures contained 10 nM negatively supercoiled

DOI: 10.1021/acs.biochem.5b00162
Biochemistry 2015, 54, 4531−4541

pBR322 DNA and 110 nM wild-type topoisomerase IIα, 105 nM topoisomerase IIβ, 78 nM Top2αΔ1175, or 426 nM topoisomerase IIα catalytic core in a total of 20 μL of cleavage buffer [10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol]. DNA cleavage reaction mixtures were incubated at 37 °C for 6 min, and enzyme−DNA cleavage complexes were trapped by the addition of 2 μL of 5% sodium dodecyl sulfate (SDS) followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 μL of agarose loading dye [60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 2 min, and subjected to electrophoresis using 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized by UV light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

DNA cleavage reactions were performed in the absence or presence of plant extracts, purified olive metabolites, compounds, extra virgin olive oils, or aqueous extracts from the oils. In some experiments, DNA cleavage was performed in the presence of etoposide as a control. Unless stated otherwise, extracts, metabolites, compounds, or oils were added last to reaction mixtures. Some reactions were performed in the presence of an oxidant [$K_3Fe(CN)_6$]. In these latter reactions, extracts, compounds, or oils were incubated with the oxidant at room temperature for 10 min prior to their addition to DNA cleavage mixtures.

In reactions that determined whether DNA cleavage by human topoisomerase IIα was reversible, 2 μL of 250 mM EDTA was added to samples prior to treatment with SDS. To determine whether cleaved DNA was protein-linked, proteinase K treatment was omitted. To examine the effects of a reducing agent on the actions of 1 mM hydroxytyrosol, oleuropein, or verbascoside against topoisomerase IIα, 100 μM DTT was added for 5 min before or after establishing enzyme-mediated DNA cleavage complexes.

To assess the effects of hydroxytyrosol, oleuropein, or verbascoside on human topoisomerase IIα prior to the addition of DNA, the enzyme (final enzyme concentration of 110 nM) was incubated in the presence of 1 mM olive metabolite (final concentration) or 10 μM olive metabolite and 10 μM oxidant (final concentration) for 0−3 min at 37 °C in 15 μL of DNA cleavage buffer. DNA cleavage was initiated by the addition of 10 nM negatively supercoiled pBR322 DNA (final concentration) to reaction mixtures (final volume of 20 μL), and samples were incubated at 37 °C for 6 min. Reactions were stopped, and samples were processed and analyzed as described above.

Ligation of Cleaved Plasmid DNA by Human Topoisomerase IIα. DNA ligation mediated by human topoisomerase IIα was monitored according to the procedure of Byl et al.[49] DNA cleavage−ligation equilibria were established for 6 min at 37 °C as described above in the presence of 1 mM hydroxytyrosol, oleuropein, or verbascoside or 10 μM olive metabolites with 10 μM oxidant. Ligation was initiated by cooling samples from 37 to 0 °C. Reactions were terminated at 20 s by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Samples were processed and analyzed as described above. Ligation was monitored by the loss of linear DNA.

Persistence of Cleavage Complexes. The persistence of topoisomerase IIα−DNA cleavage complexes was determined using the procedure of Gentry et al.[50] Initial reaction mixtures contained 50 nM DNA and 550 nM topoisomerase IIα in a total of 20 μL of DNA cleavage buffer. Reactions were conducted in the presence of 1 mM hydroxytyrosol, oleuropein, or verbascoside or 10 μM metabolite with 10 μM oxidant. Reaction mixtures were incubated at 37 °C for 10−20 min and then diluted 20-fold with 37 °C DNA cleavage buffer. Aliquots (20 μL) were removed at times ranging from 0 to 24 h, and DNA cleavage was stopped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Samples were processed as described above for plasmid cleavage assays. The persistence of cleavage complexes was determined by the disappearance of the linear reaction product over time.

DNA Cleavage Site Utilization. DNA cleavage sites were mapped using a modification of the procedure of O'Reilly and Kreuzer.[51] pBR322 DNA was linearized by treatment with *Hin*dIII, and terminal 5′-phosphates were removed and replaced with [^{32}P]phosphate by treatment with calf intestinal alkaline phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled DNA was treated with *Eco*RI, and the 4330 bp singly end-^{32}P-labeled fragment was purified from the short *Eco*RI−*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Reaction mixtures contained 4 nM ^{32}P-labeled 4330 bp DNA substrate and 44 nM human topoisomerase IIα in 50 μL of DNA cleavage buffer. Assays were conducted in the absence of compound or in the presence of 20 μM etoposide or the indicated metabolite at 10 μM in the presence of 10 μM oxidant. Reactions were initiated by the addition of topoisomerase IIα, and mixtures were incubated for 1 min at 37 °C. DNA cleavage intermediates were trapped by adding 5 μL of 5% SDS followed by 3.75 μL of 250 mM EDTA (pH 8.0). Topoisomerase II was digested with proteinase K (5 μL of a 0.8 mg/mL solution) for 30 min at 45 °C. DNA products were precipitated in 100% ethanol and 3 M NaOAc, washed in 70% ethanol, dried, and resuspended in 6 μL of cleavage mapping loading dye (40% formamide, 10 mM NaOH, 0.02% xylene cyanol FF, and 0.02% bromophenol blue). Samples were subjected to electrophoresis in a denaturing 6% polyacrylamide sequencing gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. The gel was dried and exposed to an imaging screen (Bio-Rad). ^{32}P-labeled DNA cleavage products were analyzed on a Pharos Molecular Imager FX (Bio-Rad).

■ RESULTS AND DISCUSSION

An Extract of *P. latifolia* L. Enhances DNA Cleavage Mediated by Human Topoisomerase IIα. A number of dietary polyphenols and isothiocyanates with chemopreventive or anticancer properties have been found to be topoisomerase II poisons.[10−19] In all of the cases mentioned above, studies were initiated by examining the effects of purified compounds on the activity of the type II enzyme. To take a broader and less biased approach to the discovery of naturally occurring topoisomerase II poisons, a library of 341 Mediterranean plant extracts[44,45] was screened for its effects on DNA cleavage mediated by human topoisomerase IIα. Extract 263 prepared from the leaves of *P. latifolia* L., a member of the Oleaceae olive tree family, displayed high activity against topoisomerase IIα and increased levels of DNA cleavage nearly 8-fold at 200 μg/mL (Figure 1).

Previous metabolomic studies of *P. latifolia* L. leaves indicate the presence of several bioflavonoid derivatives, including glucosides and rutinosides of apigenin, quercetin, and

DOI: 10.1021/acs.biochem.5b00162
Biochemistry 2015, 54, 4531−4541

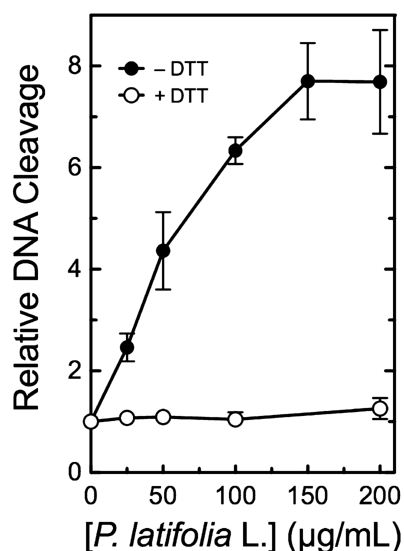


Figure 1. Soluble extract from *P. latifolia* L. leaves enhances DNA cleavage mediated by human topoisomerase IIα. The effects of plant extract 263 (*P. latifolia* L.) on the cleavage of negatively supercoiled DNA by topoisomerase IIα were determined in the absence (●) or presence (○) of 100 μM DTT. DNA cleavage levels were calculated relative to a control reaction mixture that contained no extract. Error bars represent the standard deviation of at least three independent experiments.

luteolin.[52,53] Although the three unmodified bioflavonoids are known interfacial topoisomerase II poisons,[11,13] it is not known whether the glycoside derivatives mentioned above retain activity within the soluble extract. Therefore, to determine whether these bioflavonoid glycosides (or other potential interfacial poisons) represented the topoisomerase II-active compounds in *P. latifolia* L., the extract was assayed for activity in the presence of a reducing agent, dithiothreitol (DTT). As seen in Figure 1, the extract lost its activity under reducing conditions. This finding suggests that the active metabolites present in the *P. latifolia* L. extract are covalent poisons rather than bioflavonoid-based interfacial poisons.[32]

Olive Metabolites Poison Human Type II Topoisomerases. Several phenolic compounds with antioxidant activity are abundant in *P. latifolia* L.[52,53] and have the potential to act as covalent topoisomerase II poisons.[10] Among these metabolites are hydroxytyrosol, oleuropein, verbascoside, tyrosol, and caffeic acid. Oleuropein contains an esterified hydroxytyrosol component, and verbascoside contains esterified hydroxytyrosol and caffeic acid components. Tyrosol is a breakdown product of hydroxytyrosol. The structures of these compounds are shown in Figure 2.

To determine whether any of the *P. latifolia* L. metabolites mentioned above contributed to the activity of the extract against human topoisomerase IIα, the ability of individual compounds to enhance enzyme-mediated DNA cleavage was examined (Figure 3). Hydroxytyrosol, oleuropein, and verbascoside all increased levels of DNA cleavage >3-fold. Conversely, tyrosol and caffeic acid displayed virtually no activity against the human type II enzyme.

To ensure that the observed DNA cleavage enhancement was mediated by the type II enzyme, several control experiments were performed (Figure 3B). No DNA scission was seen in the presence of hydroxytyrosol (top), oleuropein (middle), or verbascoside (bottom) when the type II enzyme was omitted from reaction mixtures. Furthermore, enzyme-mediated DNA cleavage induced by the olive metabolites was reversed when the active site Mg^{2+} ions were chelated with EDTA prior to trapping cleavage complexes with SDS. This reversibility is not consistent with an enzyme-independent reaction. Finally, cleaved plasmid products were covalently linked to topoisomerase II. In the absence of proteinase K, the linear DNA band disappeared and was replaced by a band that remained at the origin of the gel. These results demonstrate that the DNA cleavage observed in the presence of the metabolites is mediated by topoisomerase IIα.

Although hydroxytyrosol, oleuropein, and verbascoside are all polyphenols, it is likely that they would have to cycle through a quinone form to become reactive toward topoisomerase IIα.[10,54] A previous study found that the buffer used for topoisomerase II-mediated DNA cleavage reactions does not readily support redox cycling.[17] This is consistent with the high concentrations of

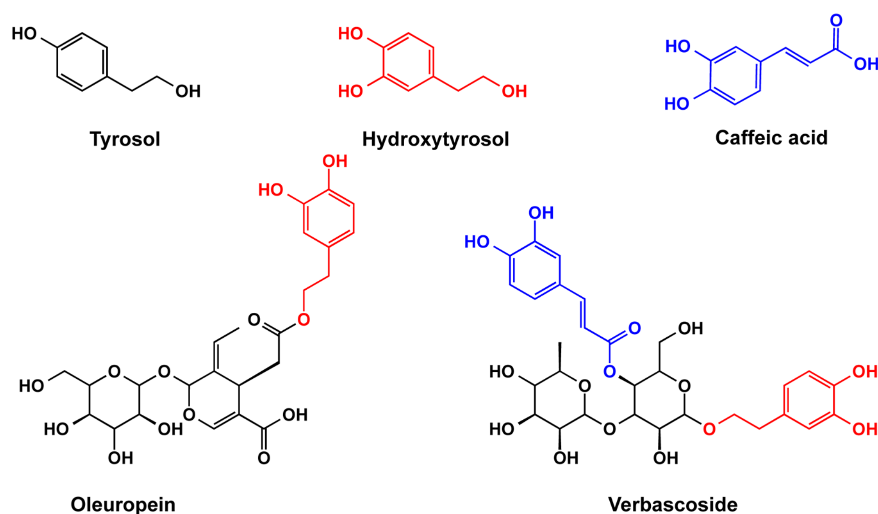


Figure 2. Structures of olive plant metabolites. Polyphenols present in *P. latifolia* L. and other olive species, including hydroxytyrosol, oleuropein, verbascoside, caffeic acid, and tyrosol, are shown. Hydroxytyrosol (red) is a component of oleuropein and verbascoside, and caffeic acid (blue) is a component of verbascoside.

DOI: 10.1021/acs.biochem.5b00162
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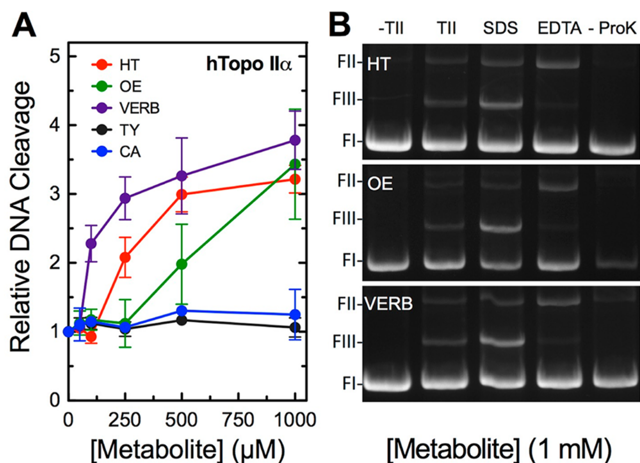

Figure 3. Olive metabolites enhance DNA cleavage mediated by human topoisomerase IIα. (A) The effects of hydroxytyrosol (HT; red), oleuropein (OE; green), verbascoside (VERB; purple), caffeic acid (CA; blue), and tyrosol (TY; black) on DNA cleavage mediated by topoisomerase IIα are shown. DNA cleavage levels were calculated relative to a control reaction mixture that contained no metabolite. Error bars represent standard deviations for three independent experiments. (B) DNA cleavage induced by hydroxytyrosol (top), oleuropein (middle), or verbascoside (bottom) is reversible and protein-linked. Ethidium bromide-stained agarose gels are shown. Assay mixtures contained DNA with olive metabolites in the absence of enzyme (−TII), topoisomerase IIα with DNA in the absence of olive metabolites (TII), or complete reactions stopped with SDS prior to the addition of EDTA (SDS). To determine whether the reaction was reversible, EDTA was added prior to SDS (EDTA). To determine whether the cleaved DNA was protein-linked, proteinase K treatment was omitted (−ProK). The mobilities of negatively supercoiled DNA (form I; FI), the nicked circular plasmid (form II; FII), and linear molecules (form III; FIII) are indicated. Gels are representative of three independent experiments.

metabolites required to enhance DNA cleavage. Therefore, the effects of an oxidant, $K_3Fe(CN)_6$, on the activity of hydroxytyrosol, oleuropein, and verbascoside toward topoisomerase IIα were examined. This oxidant has little effect on levels of baseline DNA cleavage mediated by topoisomerase IIα but had a dramatic effect on the activity of the metabolites (Figure 4A). DNA cleavage induced by the metabolites plateaued at 10 μM $K_3Fe(CN)_6$ (Figure 4A, left). Consequently, this concentration was used for all subsequent reaction mixtures that contained the oxidant.

The presence of $K_3Fe(CN)_6$ increased the potency of hydroxytyrosol, oleuropein, and verbascoside toward topoisomerase IIα as much as 100-fold (Figure 4A, right). Whereas 250 μM to 1 mM metabolite was required to increase the level of DNA cleavage between 3- and 4-fold in the absence of oxidant (see Figure 3A), a similar DNA cleavage increase in reaction mixtures that contained $K_3Fe(CN)_6$ required only 5−10 μM metabolite. Despite the presence of the oxidant, neither tyrosol nor caffeic acid displayed any significant ability to poison topoisomerase IIα.

The effects of hydroxytyrosol, oleuropein, and verbascoside on DNA cleavage mediated by human topoisomerase IIβ also were assessed (Figure 4B). In the presence of oxidant, all three polyphenols were potent topoisomerase II poisons and increased the level of DNA cleavage >8-fold at a metabolite concentration of 10 μM. The higher relative activity of olive metabolites against topoisomerase IIβ as compared to IIα primarily reflects lower baseline levels of DNA cleavage observed with the β isoform in the absence of poisons.

The effects of hydroxytyrosol, oleuropein, and verbascoside (10 μM) on DNA cleavage site utilization by human topoisomerase IIα were determined in the presence of an oxidant (Figure 5). Similar sites of cleavage were induced by all three metabolites. Several of the sites induced by the metabolite were the same as those induced by etoposide, although some sites were utilized to a different extent. Distinct sites also were observed in the presence of the metabolites compared to the drug. Similar DNA cleavage maps were generated in the presence of 1 mM metabolites in the absence of an oxidant (data not shown).

Although etoposide and other interfacial poisons increase the level of topoisomerase II-mediated DNA scission primarily by

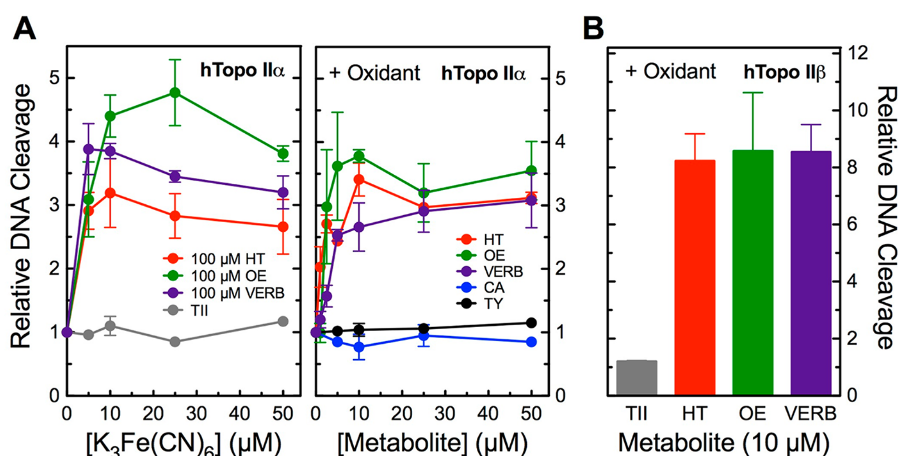

Figure 4. Activity of olive metabolites against topoisomerase IIα and IIβ is enhanced by the presence of an oxidant. (A) The effects of an oxidant, $K_3Fe(CN)_6$, on DNA cleavage mediated by topoisomerase IIα were determined in the presence of 100 μM olive metabolites [hydroxytyrosol (HT; red), oleuropein (OE; green), verbascoside (VERB; purple), caffeic acid (CA; blue), and tyrosol (TY; black)] or in the absence of a metabolite (TII; gray) (left). The effects of olive leaf metabolites on DNA cleavage were determined in the presence of 10 μM $K_3Fe(CN)_6$ (right). (B) The effects of olive metabolites on DNA cleavage mediated by human topoisomerase IIβ were determined in the presence of 10 μM $K_3Fe(CN)_6$. DNA cleavage levels were calculated relative to a control reaction mixture that contained no metabolite and no oxidant. Error bars represent standard deviations for three independent experiments.

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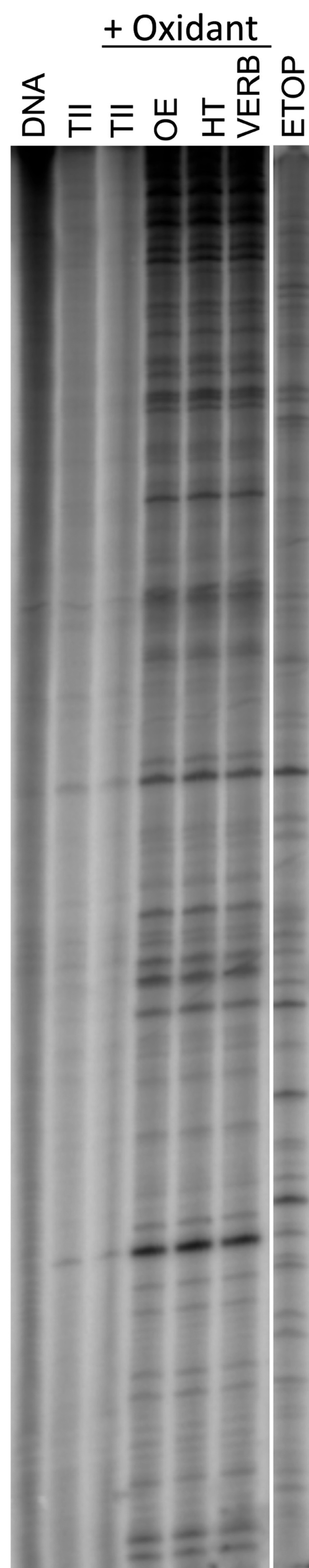

Figure 5. Effects of olive leaf metabolites on the sites of DNA cleavage generated by topoisomerase IIα. An autoradiogram of a polyacrylamide gel is shown. Reaction mixtures contained no enzyme (DNA), enzyme in the absence of metabolite (TII), or enzyme in the presence of 10 μM hydroxytyrosol (HT), oleuropein (OE), or verbascoside (VERB) in the presence of 10 μM $K_3Fe(CN)_6$. A control DNA cleavage reaction mixture that contained 20 μM etoposide also is shown. The autoradiogram is representative of three independent experiments.

inhibiting the ability of the enzyme to ligate cleaved molecules, covalent poisons often induce DNA cleavage without displaying large effects on rates of ligation.[10,20,30,32] As seen in Figure 6A, hydroxytyrosol, oleuropein, and verbascoside had relatively little effect on DNA ligation mediated by topoisomerase IIα in the absence or presence of an oxidant. In contrast, no ligation was observed in the presence of etoposide. These findings are consistent with the olive metabolites acting as covalent poisons and suggest that they may increase levels of DNA cleavage complexes primarily by enhancing the forward rates of DNA cleavage.

Because covalent poisons adduct topoisomerase II, they cannot dissociate from the enzyme.[10,20,31] Thus, once DNA cleavage complexes are formed in the presence of covalent poisons, they can remain intact for hours.[18] To address the stability of cleavage complexes formed in the presence of hydroxytyrosol, oleuropein, or verbascoside, DNA cleavage complexes were diluted 20-fold and their decay was monitored (Figure 6B). In the absence of poisons, topoisomerase IIα−DNA cleavage complexes undergo a rapid decay and display a half-life of <1 min.[18] In contrast, cleavage complexes formed in the presence of 10 μM metabolite with an oxidant or 1 mM metabolite without an oxidant were extremely stable and remained intact for at least 24 h.

Hydroxytyrosol, Oleuropein, and Verbascoside Are Covalent Topoisomerase IIα Poisons. The results described above, together with the finding that *P. latifolia* L. extracts lost their ability to poison topoisomerase IIα in the presence of a reducing agent, strongly suggest that hydroxytyrosol, oleuropein, and verbascoside are covalent topoisomerase II poisons. Therefore, a series of experiments was conducted to address the basis for the actions of the olive metabolites against topoisomerase IIα.

First, if the olive metabolites are covalent poisons, their ability to cycle through an activated quinone form should be critical to their activity.[10,13,17] Therefore, to inhibit redox cycling, 1 mM hydroxytyrosol, oleuropein, and verbascoside were incubated with 100 μM DTT prior to their addition to DNA cleavage reaction mixtures. As seen in Figure 7A, treatment with the reducing agent abrogated the activity of the olive metabolites (empty bars). Levels of DNA scission were reduced to baseline cleavage levels generated by the type II enzyme.

Second, once covalent poisons have adducted topoisomerase II, their redox state no longer affects their activity.[10,20,32,55] Thus, the addition of reducing agents to reaction mixtures after DNA cleavage−ligation equilibria have been established in the presence of a covalent poison should not reverse the cleavage enhancement. As seen Figure 7A (stippled bars), the addition of DTT after cleavage complexes had been formed in the presence of hydroxytyrosol, oleuropein, or verbascoside had no significant effect on levels of DNA scission mediated by the type II enzyme.

Third, to further examine the requirement for the conversion of olive metabolites to an activated quinone, one or both of the hydroxyl moieties of hydroxytyrosol were converted to methoxyl groups (4-hydroxy-3-methoxyphenylethanol and 3,4-dimethoxyphenylethanol). The loss of the hydroxyl groups should prevent these compounds from being converted to a quinone during redox cycling.[17] Even in the presence of an oxidant, neither compound increased the level of DNA cleavage mediated by topoisomerase IIα above baseline levels (Figure 7B). These findings provide further evidence that olive metabolites require redox cycling to enhance topoisomerase II-mediated DNA cleavage.

DOI: 10.1021/acs.biochem.5b00162
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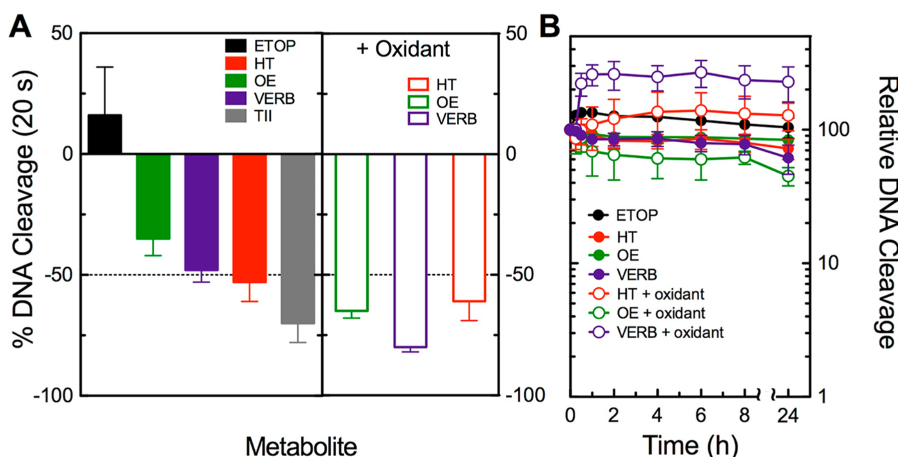

Figure 6. Effects of olive leaf metabolites on DNA cleavage complex stability. (A) The ability of human topoisomerase IIα to ligate cleaved DNA is shown. Reactions (20 s) were conducted in the presence of no metabolite (TII; gray), hydroxytyrosol (HT; red), oleuropein (OE; green), verbascoside (VERB; purple), or 100 μM etoposide (ETOP; black). Reaction mixtures contained 1 mM metabolite and no oxidant (left) or 10 μM metabolite in the presence of 10 μM $K_3Fe(CN)_6$ (right). (B) The effects of olive metabolites on the persistence of topoisomerase IIα−DNA cleavage complexes in the absence or presence of oxidant are shown. Assays were conducted in the presence of 1 mM metabolite (empty circles) or 10 μM metabolite with 10 μM $K_3Fe(CN)_6$ (filled circles). Colors are as described above. For the ligation and persistence reactions, DNA cleavage levels at time zero were set to 100% to allow a direct comparison. Error bars represent the standard deviation of at least three independent experiments.

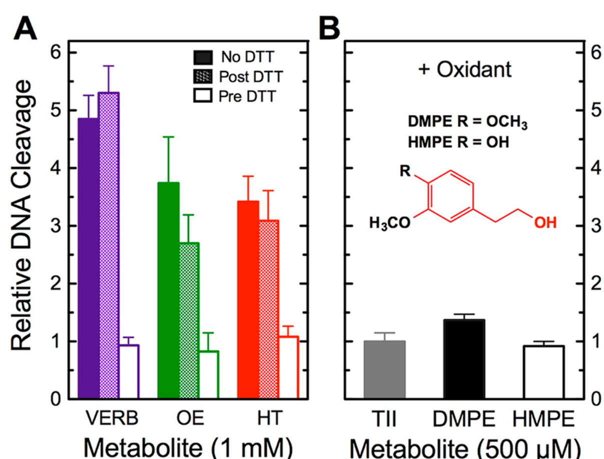

Figure 7. Olive metabolites are covalent topoisomerase II poisons. (A) Effects of DTT on the ability of olive metabolites to enhance DNA cleavage mediated by topoisomerase IIα. DNA cleavage reactions were performed in the absence of DTT (filled bars, No DTT), in the presence of 100 μM DTT that was added after the cleavage−ligation equilibrium was established (stippled bars, Post DTT), or in the presence of 100 μM DTT that was added at the start of the reaction (empty bars, Pre DTT). Reaction mixtures contained 1 mM hydroxytyrosol (HT; red), oleuropein (OE; green), or verbascoside (VERB; purple). DNA cleavage levels were calculated relative to a control reaction mixture that contained no metabolite. (B) Effects of 3,4-dimethoxyphenylethanol and 4-hydroxy-3-methoxyphenylethanol on topoisomerase IIα-mediated DNA cleavage. The effects of 500 μM 3,4-dimethoxyphenylethanol (black bar) or 4-hydroxy-3-methoxyphenylethanol (white bar) on the cleavage of negatively supercoiled plasmid DNA by topoisomerase IIα were determined in the presence 10 μM $K_3Fe(CN)_6$. Data for reaction mixtures that contained no compounds are colored gray. DNA cleavage levels were calculated relative to a control reaction mixture that contained no compounds or oxidant. In all cases, error bars represent standard deviations for three independent experiments.

Fourth, although covalent poisons enhance DNA scission when added to cleavage complexes, they irreversibly inhibit topoisomerase IIα when they are incubated with the enzyme prior to the addition of DNA. This inhibition is a hallmark characteristic of covalent poisons and is not seen with interfacial poisons.[10,31−33,56] Hydroxytyrosol, oleuropein, and verbascoside (1 mM) all inhibited the DNA cleavage activity of topoisomerase IIα when added to reaction mixtures prior to the addition of DNA (Figure 8, left). Rates of enzyme inactivation were increased by the presence of $K_3Fe(CN)_6$ (right), despite the fact that the concentration of the olive metabolites (10 μM) was 100-fold lower than that used in the absence of the oxidant.

Fifth, covalent topoisomerase II poisons are believed to enhance enzyme-mediated DNA cleavage, at least in part, by affecting the N-terminal protein clamp.[33,36] Consequently, they require the presence of the N-terminal domain (but not the C-

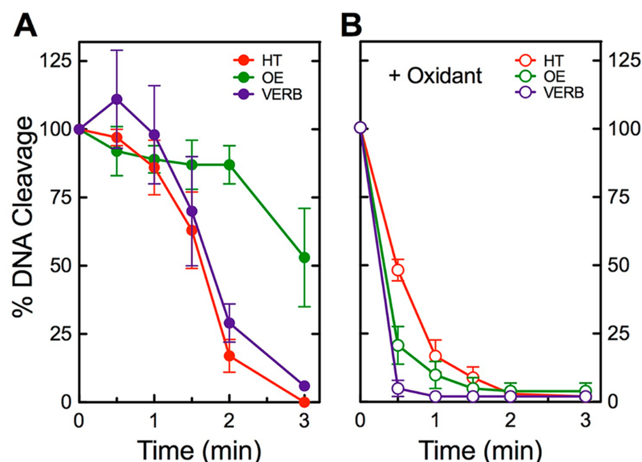

Figure 8. Olive metabolites inhibit topoisomerase IIα when incubated with the enzyme prior to DNA. The effects of hydroxytyrosol (HT; red), oleuropein (OE; green), and verbascoside (VERB; purple) are shown. Metabolites were incubated with the human enzyme in the absence of oxidant (1 mM metabolite, filled circles, left) or in the presence of 10 μM $K_3Fe(CN)_6$ (10 μM metabolite, empty circles, right). DNA cleavage levels were calculated relative to a control reaction mixture to which the metabolite was added after the addition of DNA to assay mixtures. Error bars represent standard deviations of at least three independent experiments.

DOI: 10.1021/acs.biochem.5b00162
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terminal domain) of the protein to exert their effects. Interfacial topoisomerase II poisons, such as etoposide, require neither the N-terminal nor the C-terminal protein domain and enhance DNA cleavage even in a protein construct that contains only the catalytic core.[34] To determine which protein domains are required for hydroxytyrosol, oleuropein, and verbascoside to poison topoisomerase II, their effects on DNA cleavage mediated by topoisomerase IIα constructs lacking the C-terminal domain or both the N- and C-terminal domains (catalytic core) were assessed (Figure 9). Etoposide displayed high activity against both constructs. Although the olive metabolites retained activity against Top2$\alpha\Delta$1175 (panel A), they lost their ability to enhance DNA cleavage in the absence of the N-terminal domain (catalytic core, panel B). The presence of oxidant did not alter this latter result.

Finally, human topoisomerase IIα was treated with hydroxytyrosol, and the resulting peptides generated by tryptic digestion were analyzed by mass spectrometry (data not shown). A change in a peptide containing cysteine 104, positioned in the ATPase domain of the enzyme, was observed following treatment with hydroxytyrosol. No significant changes were observed in peptides that did not contain cysteine residues.

Taken together, the findings mentioned above provide strong evidence that hydroxytyrosol, oleuropein, and verbascoside are covalent topoisomerase II poisons.

Extracts from Olive Tree Species Enhance DNA Cleavage Mediated by Topoisomerase IIα. Hydroxytyrosol, oleuropein, and verbascoside (and other polyphenols) have been reported in several members of the olive tree family.[57−60] Therefore, it is possible that other olive tree species may also produce topoisomerase II poisons.

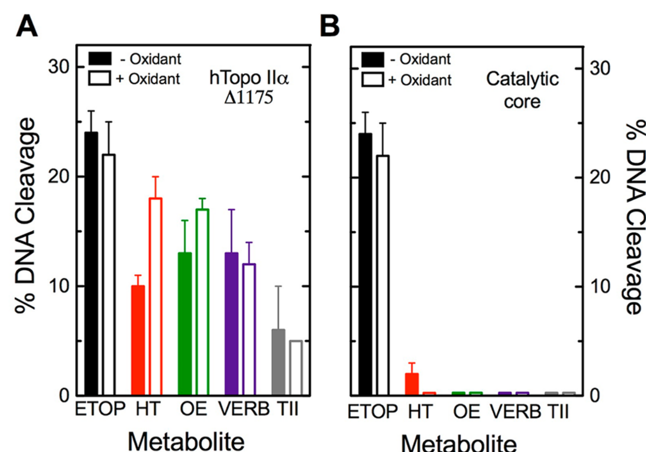

Figure 9. Olive metabolites require the N-terminal domain to enhance DNA cleavage mediated by topoisomerase IIα. The effects of olive metabolites on DNA cleavage mediated by topoisomerase IIα lacking the C-terminal domain (Δ1175) or both the C-terminal and N-terminal domains (Catalytic core) are shown in panels A and B, respectively. DNA cleavage reactions were performed using 1 mM metabolite [hydroxytyrosol (HT; red), oleuropein (OE; green), or verbascoside (VERB; purple)] in the absence of an oxidant (filled bars) or 10 μM metabolite in the presence of 10 μM $K_3Fe(CN)_6$. Results with no metabolite (TII, gray) or 100 μM etoposide (ETOP, black) in the absence or presence of an oxidant are shown as controls. DNA cleavage levels were calculated relative to scission generated by restriction endonucease *Eco*RI, which was set to 100%. Error bars represent the standard deviation of at least three independent experiments. Baseline levels of DNA cleavage generated by the catalytic core are lower than those generated by full-length topoisomerase IIα.

Eleven different species of plants from the *Oleaceae* family grow in Israel. Leaf, bark, and fruit samples were harvested from these species, and 36 extracts were prepared and tested for activity against human topoisomerase IIα (Figure 10). A number of extracts (at 2 mg/mL) increased the level of enzyme-mediated DNA cleavage. Activity against topoisomerase IIα was observed across multiple species and was found in the leaf, bark, and fruit of the trees. Generally, the highest levels of activity were seen in bark extracts. However, several fruit extracts also displayed high levels of DNA cleavage enhancement. These results suggest that topoisomerase II poisons are widely produced by members of the olive tree family.

To further explore the presence of topoisomerase II poisons in olive species, the ability of a commercial olive leaf extract (species not identified by the manufacturer) to enhance enzyme-mediated DNA cleavage was assessed (Figure 11). The herbal supplement enhanced DNA cleavage mediated by topoisomerase IIα nearly 5-fold at 20 mg/mL. Thus, olive metabolites can poison the type II enzyme, even in more complex formulations intended for human consumption.

Effects of Extra Virgin Olive Oils on DNA Cleavage Mediated by Topoisomerase IIα. Hydroxytyrosol, oleuropein, and verbascoside all are present in the fruit of the olive tree and have been reported in olive oil.[57−60] Therefore, the effects of three commercial extra virgin olive oils on DNA cleavage mediated by topoisomerase IIα were assessed. Oils were pressed from a variety of subspecies of *O. europea*, including arbosana, koroneiki, or a mixture of frantoio, moraiolo, leccino, maurino, and pendolino olives. Olive oils were added to DNA cleavage assay mixtures at a final concentration of 10% by volume.

In the absence of an oxidant, no DNA cleavage enhancement was observed (data not shown). However, in the presence of 10 μM oxidant, the extra virgin olive oils increased the level of DNA scission 2−4-fold (Figure 12, left). Because polyphenols are water-soluble, aqueous extracts of each olive oil were tested for

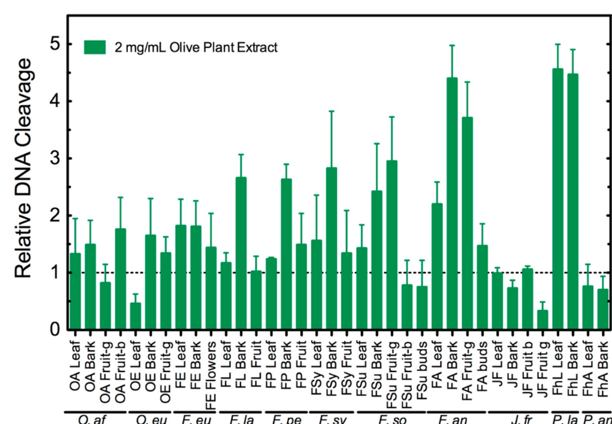

Figure 10. Effects of soluble olive tree extracts on DNA cleavage mediated by topoisomerase IIα. Thirty-six leaf, bark, and fruit extracts were prepared from 11 different species of olive trees that are indigenous to Israel. DNA cleavage reactions were performed in the presence of 2 mg/mL extract. Abbreviations: *O. af*, *Olea africana*; *O. eu*, *O. europea*; *F. eu*, *Forsithia europaea*; *F. la*, *Fraxinus latifolia*; *F. pe*, *Fraxinus pennsylvanica*; *F. sy*, *Fraxinus syriaca*; *F. so*, *Fraxinus sogdiana*; *F. an*, *Fraxinus angustifolia*; *J. fr*, *Jasminium fruiticans*; *P. la*, *Phillyrea latifolia*; *P. an*, *Phillyrea angusifolia*. DNA cleavage levels were calculated relative to a control reaction mixture (dashed line) that contained no extract. Error bars represent standard deviations of at least three independent experiments.

DOI: 10.1021/acs.biochem.5b00162
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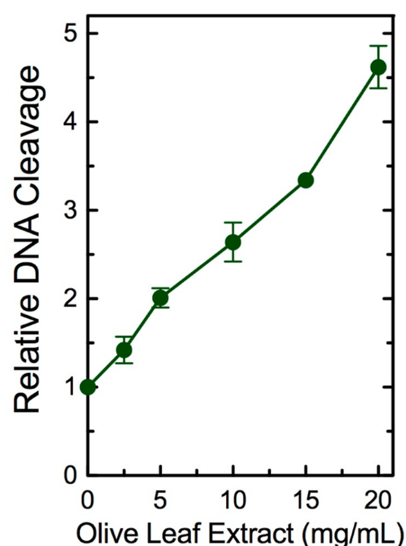

Figure 11. Commercial olive leaf extract enhances DNA cleavage mediated by topoisomerase IIα. The effects of a commercial olive leaf extract (Olive Leaf Plus) on DNA cleavage mediated by the human type II enzyme are shown. DNA cleavage levels were calculated relative to a control reaction mixture that contained no extract. Error bars represent standard deviations of at least three independent experiments.

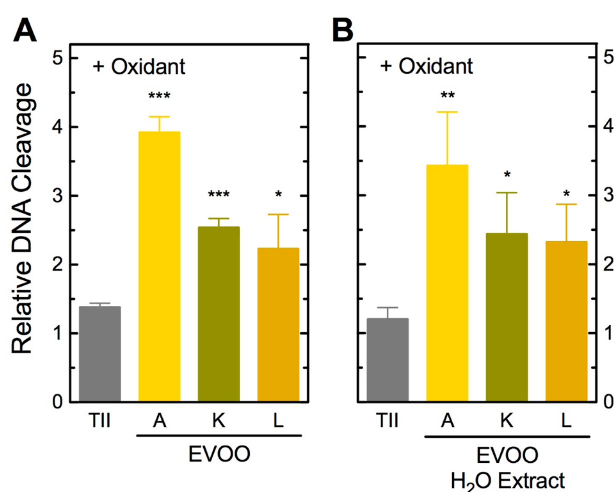

Figure 12. Extra virgin olive oils enhance DNA cleavage mediated by topoisomerase IIα. The effects of extra virgin olive oils (EVOOs) or an EVOO H_2O extract on DNA cleavage mediated by the human type II enzyme are shown in panels A and B, respectively. Olive oils [Ultra Arbonsana (A, yellow), Ultra Koroneiki (K, green), or Lucini Select (L, brown)] or extracts from these oils were included in reaction mixtures at final concentrations of 10% (v/v) in the presence of 10 μM $K_3Fe(CN)_6$. Results for reaction mixtures that contained no oils or extracts but contained oxidant (TII; gray) are shown. DNA cleavage levels were calculated relative to a control reaction mixture that contained no olive oil or oxidant. Error bars represent the standard deviation of at least three independent experiments. Statistically significant differences are noted with asterisks (*$p < 0.05$; **$p < 0.001$; ***$p \leq 0.0001$).

activity against the type II enzyme (Figure 12, right). The extracts increased the level of enzyme-mediated DNA cleavage to an extent that was similar to those of each individual extra virgin olive oil. Thus, olive products that are part of the human diet are capable of acting as topoisomerase II poisons.

■ SUMMARY

In an effort to identify natural products that function as topoisomerase II poisons, a library of 341 extracts from Mediterranean plants was screened for the ability to enhance DNA cleavage mediated by human topoisomerase IIα. An extract from *P. latifolia* L., a member of the olive tree family, displayed high activity against the human enzyme. Further studies led to the identification of hydroxytyrosol, oleuropein, and verbascoside as covalent topoisomerase II poisons. An herbal supplement from olive leaf extracts, as well as extra virgin olive oils pressed from a variety of *O. europea* subspecies, also enhanced DNA cleavage mediated by human topoisomerase IIα. Thus, olive metabolites appear to act as topoisomerase II poisons in complex formulations intended for human dietary consumption.

Hydroxytyrosol, oleuropein, and verbascoside are well-established antioxidants. All of them induce cell cycle arrest, display antiproliferative effects, and show activity against *in vivo* tumor models.[61−66] Furthermore, hydroxytyrosol is believed to have chemopreventative properties and currently is in clinical trials as a preventative agent for women at high risk for breast cancer.[67,68] Olive oil is a key component of the Mediterranean dietary pattern, and epidemiological observations indicate that this diet has great potential for cancer prevention.[2,58,69,70]

Following consumption of 25 mL of virgin olive oil, the concentration of hydroxytyrosol in blood is estimated to be as high ∼1 μM.[71] Thus, at least under oxidizing conditions, physiological levels of hydroxytyrosol are in a range at which the compound displays activity against human type II topoisomerases.

Although phenolic olive metabolites display complex cellular activities, it has been proposed that they exert at least some of their biological effects by redox-induced oxidation to quinones and subsequent protein adduction.[54] The finding that hydroxytyrosol, oleuropein, and verbascoside are covalent topoisomerase II poisons and that their activity is enhanced by oxidation is consistent with this hypothesis. Results of this study suggest that the ability of these olive leaf metabolites to poison topoisomerase II may contribute to their therapeutic properties.

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Funding

This research was supported by Grant GM033944 (N.O.) from the National Institutes of Health and funding from ICA in Israel, the Deutsche Forschungsgemeinschaft, and the Richard H. Holzer Foundation (A.G.-G. and J.G.). K.R.V. was a trainee under Grants R25-GM062459 and T32-GM08320 from the National Institutes of Health. She also was supported in part by an Administrative Research Supplement to Grant GM033944 to Promote Diversity in Health-Related Research.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Jo Ann Byl for her help with the preparation of human type II topoisomerases, to Joseph Deweese for the preparation of the topoisomerase IIα catalytic core, to Ahuva Vonshak for technical assistance, and to MaryJean Pendleton, Rachel Ashley, Lorena Infante, and Jo Ann Byl for critical reading of the manuscript.

DOI: 10.1021/acs.biochem.5b00162
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DOI: 10.1021/acs.biochem.5b00162
Biochemistry 2015, 54, 4531−4541