The Dietary Anticancer Agent Ellagic Acid Is a Potent Inhibitor of DNA Topoisomerases In Vitro

Andreas Constantinou, Gary D. Stoner, Rajendra Mehta, Kandala Rao, Constance Runyan, and Richard Moon

Abstract

Ellagic acid and 12 related agents have been tested for their ability to inhibit the activities of human DNA topoisomerase (topo) I and II. Using specific in vitro assays, we found ellagic acid and flavellagic acid to be potent inhibitors of the catalytic activities of the two topoisomerases. The minimum concentration required to inhibit ≥50% of catalytic activity (IC₅₀) of ellagic acid was determined at 0.6 and 0.7 μg/ml for topo I and topo II, respectively. Flavellagic acid’s IC₅₀ was determined at 3.0 and 3.6 μg/ml for topo I and topo II, respectively. Unlike topoisomerase poisons, these two plant phenols did not trap the enzyme-DNA reaction intermediate, known as the cleavable complex. In contrast, ellagic acid prevented other topo I and topo II poisons from stabilizing the cleavable complex, suggesting that the mode of its action is that of an antagonist. Structure-activity studies identified the 3,3'-hydroxyl groups and the lactone groups as the most essential elements for the topoisomerase inhibitory actions of plant phenols. On the basis of these findings and other properties of ellagic acid, a mechanistic model for the documented anticarcinogenic effects of the agent is proposed. (Nutr Cancer 23, 121–130, 1995)

Introduction

Ellagic acid is a naturally occurring phenol found in plants, fruits (especially blackberries, raspberries, and strawberries), and nuts in the free form or in the form of glucoside or ellagitannins (1,2). Ellagic acid has been reported to be effective in inhibiting mutagenesis induced by polycyclic aromatic hydrocarbons, aromatic amines, N-nitrosamines, and aflatoxin (3–5). It has also been found effective in inhibiting lung tumorigenesis induced by 4-(methyl)-nitrosamino)-1-(3-pyridyl)-1-butanone in mice (6,7). The mechanism of ellagic acid’s antimutagenic action has been shown to involve the inhibition of enzyme-mediated binding of [³H]benzo[a]pyrene to calf thymus DNA in vitro and also to inhibit adduct formation between the activated metabolite of the carcinogen and DNA in organ cultures of skin and lung (8,9). However, conflicting results have been reported relating to the effects of ellagic acid in inhibiting tumorigenesis (10–12), and the biochemical mode of action of ellagic acid in inhibiting this process is unknown.

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A variety of antitumor agents currently used in chemotherapy or evaluated in clinical trials are known to inhibit DNA topoisomerase (topo) I and II (reviewed in Reference 13). By altering the conformation of DNA, these enzymes play a role in DNA replication, transcription, and recombination (14) but are also involved in the processes of cell proliferation/differentiation (15-21). Camptothecin, doxorubicin, and etoposide are representative topo I or topo II inhibitors with documented antitumor effects (22). Ellagic acid and related synthetic derivatives have been evaluated for their ability to inhibit topo I and topo II activities with use of specific assays. We report here that ellagic acid and related flavellagic acid are potent topo I and II inhibitors, but synthetic derivatives are less effective or ineffective. These data suggest that the anticarcinogenic effects of ellagic acid may be partially attributed to its action as an inhibitor of DNA topoisomerases.

Materials and Methods

Topo I was purchased from GIBCO-BRL (Gaithersburg, MD), and human topo II was purchased from Topogen (Columbus, OH). Stock solutions of ellagic acid and derivatives were prepared in 50 mM tris(hydroxymethyl)aminomethane (Tris)-Cl (pH 8.5) at 0.5 mg/ml. In some derivatives, dimethyl sulfoxide at 50% was added to increase solubility. Subsequent dilutions were made in distilled water. Etoposide was prepared in dimethyl sulfoxide.

Relaxation Assay

For the determination of topo I catalytic activity, closed circular pUC8 DNA was used as the substrate. This was prepared with the alkaline lysis method and purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (23). Reaction volumes of 20 μl contained the following: 50 mM Tris-Cl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 30 μg/ml bovine serum albumin, and 2 U of purified calf thymus topo I. The appropriate inhibitor was added, when necessary, and the reaction was started by the addition of the enzyme. Reactions were carried out at 37°C for 30 minutes. Gel electrophoresis was at 4 V/cm for five hours in Tris-borate-EDTA buffer. For the quantitative determination of topo I activity, photographic negatives were densitometrically scanned (Hoefer Scientific Instruments GS300 Scanning Densitometer). The area representing supercoiled DNA, migrating as a single band at the bottom of the gel, was determined. The concentration of the inhibitor at which it prevents 50% of the substrate (supercoiled DNA) from being converted into the reaction product (relaxed DNA) was determined. By averaging three to four such experiments, the IC₅₀ values were determined.

P4 Unknotting Assay

For the determination of topo II catalytic activity, knotted DNA that had been isolated from the tail capsids of the bacteriophage P4 Vrl del10 (24) has been used as the substrate. Reaction mixtures contained 50 mM Tris-Cl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM EDTA, 40 μg/ml bovine serum albumin (nuclease free), and 1 mM ATP. The appropriate inhibitor was added, when necessary, followed by the addition of 2 U of human topo II. Reactions of 20 μl total volume were started by the addition of 0.6 μg of knotted DNA. Reactions were terminated by the addition of 5 μl of a stop solution containing 5% sodium dodecyl sulfate (SDS), 50 mM EDTA, 25% Ficoll, and 0.05 mg/ml bromphenol blue. Samples were loaded on 0.8% agarose gels and electrophoresed at 4 V/cm for five hours in Tris-borate-EDTA buffer. Gels were stained in 1 μg/ml ethidium bromide, destained, and photographed over an ultraviolet light source. For the quantitative determination of topo II activity, photographic negatives were densitometrically scanned. Knotted DNA, migrating as
a single band at the top of the gel, was measured in this manner. The concentration of the inhibitor preventing 50% of the substrate (knotted DNA) from being converted to the reaction product (unknotted DNA) was determined. By averaging three to four such experiments, the IC₅₀ values were determined.

Topo I-mediated DNA cleavage: Topo I-targeting agents with the ability to enhance topo I-mediated DNA cleavage were screened using the pUC8 DNA under the reaction conditions provided by the supplier of the enzyme (Topogen). Briefly, 20 µl of reaction mixtures contained 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1 µl of the test agent (if necessary), 0.5 µg of pUC8 DNA, and 10 U of human topo I (added last). After 30 minutes of incubation at 37°C, SDS-proteinase K was added, and after 15 minutes of incubation at 37°C, samples were extracted with chloroform and electrophoresed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Gels were photographed, and photographic negatives were scanned using a Hoefer GS300 scanning densitometer. After integration of the three bands, the reaction product (nicked DNA) was expressed as percentage of total DNA.

Topo II-mediated DNA cleavage: Topo II-targeting agents with the ability to enhance topo II-mediated DNA cleavage were screened using the pUC8 DNA under the reaction conditions provided by the supplier of the enzyme (Topogen). Briefly, 20 µl of reaction mixtures contained 30 mM Tris-Cl (pH 7.6), 5 mM ATP, 15 mM mercaptoethanol, 8 mM MgCl₂, 60 mM NaCl, 1 µl of the test agent (if necessary), 0.3 µg of pUC8 DNA, and 10 U of human topo II (added last). After 30 minutes of incubation at 37°C, SDS-proteinase K was added, and after 15 minutes of incubation at 37°C, samples were extracted with chloroform and electrophoresed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Gels were photographed, and photographic negatives were scanned using a Hoefer GS300 scanning densitometer. After integration of the three bands, the reaction product (linear DNA) was expressed as percentage of total DNA.

Chemical Derivatives of Ellagic Acid

Ellagic acid, purchased from Aldrich Chemical (Milwaukee, WI), was further purified with pyridine to 99% purity. Agents 2, 3, and 11 were chemically synthesized, and their purity was >98%, 99.8%, and 97%, respectively. The synthesis of the remaining agents, the purity of which exceeded 90%, was carried out in the laboratory of Dr. Thomas Kinstle (Dept. of Chemistry, Bowling Green State University, Bowling Green, OH). The structures of all agents were confirmed by mass spectrometry.

Results

The effect of ellagic acid and related agents on the catalytic activity of topo I and topo II was evaluated using the relaxation and unknotting assays, as described in Materials and Methods. Initially, agents were evaluated at 100 µg/ml. Interestingly, agents inhibited both topoisozerases or none. As shown in Figure 1, 3 of 13 agents tested positive. Agents not showing effect at this concentration were considered ineffective and were not tested further.

Effective inhibitors of topo I and II require hydroxyl group substitutions on Positions 3 and 3' (Figure 1). An additional hydroxyl group on Position 5, as in Agent 2 (flavellagic acid), reduced but did not eliminate the inhibitory effect. When other groups were introduced in Positions 3 and 3', such as H for Agent 4, glucopyranose for Agent 7, hexanoyl for Agent 5, and lipid for Agent 6, the resulting derivatives became inactive. Agent 3, containing only one lactone group, was also able to inhibit topo II and, to a lesser degree, topo I.

Positive agents were further tested at lower concentrations to define the minimum concen-
Figure 1. Overall effect of ellagic acid and related agents on topo I and topo II enzymatic activities when tested at a final concentration of 100 μg/ml. He, hexanoyl; Ac, acetyl; R1, β-D-glucopyranosyl; R2, glucosyl; R3, tetraacetyl glucosyl.

Formulations that were necessary to inhibit ≥50% of the topo I or topo II catalytic activities (IC₅₀). An example of the two assays used is shown in Figure 2. In this example, final concentrations of ellagic acid ranging from 0.3 to 10 μg/ml were used in the relaxation and unknotting assays.

In the relaxation assay, supercoiled plasmid DNA, migrating on agarose gels as a single band, is relaxed by topo I, resulting in a population of bands with lower mobility on agarose gels. The unknotting assay is specific for measuring topo II activity, because it is based on the conversion of knotted DNA to its unknotted form, which requires double-strand breakage followed by strand rotation and ligation, qualities that are unique to topo II (13,14). The removal of these knots can be visualized with agarose gel electrophoresis, because P4-knotted DNA migrates as a smear whereas unknotted DNA migrates as a single band (Figure 2B).
A. Topo I Assay

![Graph showing the effect of ellagic acid on Topo I activity.](image)

B. Topo II Assay

![Graph showing the effect of ellagic acid on Topo II activity.](image)

Figure 2. Representative experiment showing effect of ellagic acid on topo I (A) and topo II (B) activities. All reactions contain 2 U of purified topo I (A) or topo II (B) and ellagic acid at concentrations shown. Mean minimum concentrations necessary to inhibit ≥50% of catalytic activity (IC_{50}) were determined in this manner from ≥3 independent expts.
Table 1. Determination of IC₅₀ for Ellagic Acid and Related Agents Toward Topo I and Topo II Activities Evaluated With Relaxation and Unknotting Assays, Respectively

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC₅₀, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Topo I</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>0.6</td>
</tr>
<tr>
<td>Flavellagic acid</td>
<td>3.0</td>
</tr>
<tr>
<td>3,8,9,10-Tetrahydro dibenzo[b,d]pyran-6-one</td>
<td>50</td>
</tr>
</tbody>
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*a* IC₅₀, minimum concn necessary to inhibit ≥50% of catalytic activity.

The concentration of the agents required to inhibit these topological conversions was calculated from scanning the photographic negatives of gels similar to the one shown in Figure 1. In this example, ellagic acid's IC₅₀ was 0.6 µg/ml for topo I and 0.7 µg/ml for topo II.

By averaging the results of at least three independent experiments, mean IC₅₀ values were obtained (Table 1). Ellagic acid and flavellagic acid were the most potent inhibitors of topo I and topo II. The potency of ellagic acid is comparable to that of the most potent inhibitors reported to date (see Discussion).

A group of topoisomerase (I and II) inhibitors, known as targeting agents or poisons, stabilizes a covalent complex between the enzyme and the DNA (reviewed in Reference 22). Treatment of this complex with denaturing agents reveals single- (topo I) or double- (topo II) strand breakage. Another group of topoisomerase inhibitors, known as antagonists, do not stabilize this complex (25). We have used specific assays designed to reveal the mode of topoisomerase inhibition. The production of linear plasmid DNA by an agent in the presence of topo II and after proteinase K-SDS digestion indicates the ability of the agent to stabilize the cleavage complex (Figure 3). Using this assay, we have found that ellagic acid, at ≤50 µg/ml, did not stabilize the cleavage complex (Lanes 3–5). Genistein, which is a known topo

![Figure 3](image)

Figure 3. Effects of ellagic acid on topo II-mediated cleavage of plasmid pUC8 DNA. Lane 1, control (no topo II, no drug); Lane 2, control (no drug); Lanes 3–5, ellagic acid at 1, 10, and 50 µg/ml final concentration, respectively; Lanes 6–8, genistein at 50 µg/ml (Lane 6) plus ellagic acid at 10 µg/ml (Lane 7) or 50 µg/ml (Lane 8). All reactions (except Lane 1) containing 10 U of purified human topo II were performed as described in Materials and Methods. OC, open circular (nicked) DNA; Lin, linear DNA; CCC, covalently closed circular DNA.

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Figure 4. Effects of ellagic acid on topo I-mediated cleavage of plasmid pUC8 DNA. Lane 1, control (no topo I, no drug); Lane 2, control (no drug); Lanes 3–5, ellagic acid at 1, 10, and 50 μg/ml final concentration, respectively; Lanes 6–8, camptothecin at 50 μg/ml (Lane 6) plus ellagic acid at 10 μg/ml (Lane 7) or 50 μg/ml (Lane 8). All reactions (except Lane 1) containing 10 U of purified human topo I were performed as described in Materials and Methods. See Figure 3 legend for definition of abbreviations.

II-targeting agent, was used as a positive control (Lane 6). Ellagic acid, however, competed against genistein's effect, as determined by the reduced levels of the linear form of plasmid DNA when the two agents were used in combination (Lanes 7 and 8). Camptothecin is known to stabilize the topo I-DNA complex. This can be demonstrated by increases in the levels of the nicked form of plasmid DNA (Figure 4, Lane 6). Unlike camptothecin, ellagic acid at 10–50 μg/ml did not trap the topo I-DNA cleavage complex (Lanes 3–5). In contrast, at 50 μg/ml, ellagic acid entirely prevented the camptothecin-induced topo I-mediated DNA cleavage (Lane 8). These results demonstrate that ellagic acid inhibits the catalytic activity of the two topoisomerases as a true antagonist.

Discussion

We have demonstrated that ellagic acid and flavellagic acid are potent topo I and topo II antagonists. With respect to topo II, ellagic acid is equally potent to the mechanistically similar antitumor agent ICRF-193 (26), a bis(2,6-dioxopiperazine) derivative. Both agents demonstrated an IC₅₀ of about 2 μM, which is far more potent than those of other commonly used topo II antagonists; for comparison, novobiocin is known to inhibit the catalytic activity of eukaryotic topo II, with an IC₅₀ of 200–500 μM (27).

Ellagic acid, because of its flat planar structure, demonstrated by crystallography (28), is expected to intercalate to DNA. The hydroxyl and lactone groups of ellagic acid, which are believed to be the electron acceptor or donor sites during its interaction with the nucleic acid bases, are essential for hydrogen bonding (29). Thus, ellagic acid is known to inhibit complex formation between N₇ or O₆ of guanine and carcinogens such as aflatoxin, N-methyl-N-nitrosourea, or N-nitrosobenzylmethyamine (4,5). It is expected that ellagic acid, because of its ability to intercalate to DNA and to form new hydrogen bonds, will reduce the flexibility of the double helix. Strand rotation, one of the components of the topoisomerization reaction (30,31), may be curtailed because of this reduced flexibility of the double helix.
On the basis of the proposed model of ellagic acid action in inhibiting DNA topoisomerases, the following predictions can be made. First, ellagic acid derivatives lacking both lactone groups or hydroxyl group substitution at the 3,3' positions will be inactive as topo inhibitors. The hydroxyl and lactone groups have been proposed to be the sites of ellagic acid’s interaction with the phosphate oxygen atom of DNA or the electron acceptor or donor sites in the nucleic acid bases during the formation of hydrogen bonds (26). The structure-activity data of this study identified the same groups as being the most critical for the inhibition of the two topoisomerases. Irrespective of whether hydrogen or hexanoyl or hydrocarbon side chains are the substituents on these positions, the result is the same: the derivatives cannot form hydrogen bonds and cannot inhibit topoisomerases (see structures of Agents 4–7). Second, the suggested hindering effect of ellagic acid on strand rotation (due to the hydrogen bonding between ellagic acid and DNA) is not expected to interfere with the cleavage event of the catalytic cycle but rather with a subsequent event.

On the basis of a model of the topo II catalytic cycle proposed by Osheroff and co-workers (32), DNA passage and religation will not be blocked, but possibly the regeneration of the enzyme will be hindered. This kind of inhibition predicts reduced drug-stabilized topo II-mediated double-strand DNA breakage in the presence of ellagic acid. This is consistent with our observations shown in Figure 3. A similar effect may be exerted by ellagic acid on the camtotheacin-induced topo I-mediated single-strand DNA breakage (Figure 4). Third, other plant phenols with planar structures, size, and polar oxygen groups similar to that of ellagic acid are expected to inhibit DNA topoisomerases. Indeed, quercetin, a flavonoid meeting the above criteria (33), also inhibits topo I and topo II activities (34,35). Interestingly, quercetin also exhibits anticancerogenic activity (36,37). From these data, it is becoming evident that the above structural elements are essential components for the inhibition of topo activities by plant phenols.

Ellagic acid, when consumed in the form of nuts and fruits, may protect against carcinogenesis as a natural anti-initiator by blocking carcinogen binding to DNA (4,5). It may also inhibit tumor cell growth by inhibiting two enzymes (topo I and II) that are essential for DNA replication and cell proliferation (14,15,22). The effectiveness of ellagic acid as an antitumorogen is limited, however, by its poor absorption and rapid elimination (12). Although synthetic lipophilic derivatives are expected to be absorbed more easily than ellagic acid, their effectiveness as anticancerogens has not been determined. Two such derivatives, however, have been shown to be ineffective as inhibitors of benzo[a]pyrene in the lungs of strain A/J mice (38).

Our data provide evidence for two enzymatic activities that can be blocked by ellagic acid and flavellagic acid, two naturally occurring phenols. The enzymes involved in this biochemical pathway, besides being the targets of antitumor agents, are also essential for cell cycling and proliferation. Concentrations of about 10–50 μM ellagic acid have been measured in the blood and lung tissues of mice six to eight hours after intraperitoneal administration of 120 μM ellagic acid (12). Although this concentration is well within the range required to inhibit DNA topoisomerases in vitro, it is unknown whether these ellagic acid levels can be reached in persons consuming diets rich in fruits and nuts, especially because poor absorption of dietary ellagic acid in mice has been reported (12). More studies are necessary to determine the biodistribution of ellagic acid in humans and the ability of this agent to inhibit topoisomerases intracellularly during normal physiological processing.

Acknowledgments and Notes

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References


