Phenoxodiol (2H-1-Benzopyran-7-0,1,3-(4-hydroxyphenyl)), a Novel Isoflavone Derivative, Inhibits DNA Topoisomerase II by Stabilizing the Cleavable Complex

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Abstract. Cancer therapeutic drugs that inhibit DNA topoisomerase (topo) II by stabilizing the cleavable complex are collectively known as topo II poisons. Phenoxodiol is a synthetic derivative of the plant isoflavone daidzein and is currently undergoing clinical testing as a cancer therapeutic drug. The development of this agent as an antitumor drug was based on a large extent on its low toxicity in normal tissues but potent topo II inhibitory effects in rapidly dividing tumor cells. To evaluate phenoxodiol as a potential inhibitor of topoisomerases, we used the relaxation and nicking assays that can identify topo I inhibitors, and the unknotted and DNA cleavage assays that can identify topo II inhibitors. Phenoxodiol inhibited the catalytic activity of topo II in a dose-dependent manner and it stabilized the topo II-mediated cleavable complex, demonstrating that this agent is a topo II poison. Phenoxodiol's topo II inhibitory effects were comparable to those of other antitumor agents such as VP-16 and were stronger than those of genistein. Phenoxodiol did not inhibit topo I catalytic activity nor did it stabilize the topo I-mediated cleavable complex. These results demonstrate that phenoxodiol is a topo II-specific poison and suggest that this novel agent may find applications in cancer chemotherapy.

DNA topoisomerases constitute a family of conserved essential enzymes that resolve topological problems during DNA replication transcription and recombination. The mammalian type-I enzyme (or topo I) is an ATP-independent DNA single-strand endonuclease and ligase that functions mainly during transcription. The mammalian type II enzyme (or topo II) is represented by two isoforms (α and β) that are ATP-dependent DNA double-stranded endonucleases and ligases. Topo II α is a major component of the chromosomal matrix that decatenates double-stranded DNA during replication. The expression of topo IIα is cell cycle-regulated and proliferation-dependent, whereas the expression of topo I and topo IIβ are relatively constant throughout the cell cycle and independent of proliferation (1).

Inhibition of topo II may generally take place by either (a) stabilizing a transient reaction intermediate between the topo II enzymes and DNA (called the cleavable complex) or (b) hindering its formation (2). Topo II inhibitors that stabilize the cleavable complex are named topo II poisons and are represented by antitumor drugs such as VP-16 (etoposide) and doxorubicin. Topo II inhibitors that do not stabilize the cleavable complex are named catalytic inhibitors and are represented by agents such as aclarubicin and merbarone that may or may not find applications as cancer therapeutics.

Previously, we and others identified the soy isoflavone genistein as a topo II poison, as it inhibits the catalytic activity of topo II and stabilizes the cleavable complex (2-6). Because of the production of double-strand breakage that may escape the repair process, topo II poisons are cytotoxic. Tumor cells that contain higher levels of topo II are more susceptible to the cytotoxic effects of topo II poisons than normal, non-dividing cells, which generally contain very low topo II levels (8-11). The isoflavone genistein, when introduced at high concentrations, can act as an antitumor drug (12), but, like other antitumor drugs, it may promote human leukemias (13). Here, we report that the isoflavone metabolite phenoxodiol (2H-1-Benzopyran-7-0,1,3-(4-hydroxyphenyl)), is a potent topo II poison. The ability of phenoxodiol to trap the cleavable complex suggests that, like the epipodophyllotoxins, it may also find applications in cancer chemotherapy.

Materials and Methods

Materials. The bacteriophage P4 Viri delTO was isolated as described previously (14). pUC8 DNA was isolated from Escherichia coli by the alkaline lysis method. Reagents, assay buffers, human topo I, human

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topo II, and pRYG DNA were purchased from Topogen (Columbus, OH, USA). Phenoxodiol was provided by Novogen (North Ryde, NSW, Australia). Genstein was purchased from Indofine Chemical Co. (Somerville, NJ, USA). All other reagents, chemicals, and drugs were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Stock solutions were prepared in DMSO at 20 mg/ml, stored at -20°C, and diluted with distilled water just before the assay.

**Topo I-mediated plasmid relaxation assay.** For the determination of topoisomerase (topo I) catalytic activity, pUC8 DNA was used as the substrate in a reaction volume of 20 μl containing the following: 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol, and 2 units of purified human topo I. The inhibitor, when applicable, was added as indicated, and the reaction was initiated by the addition of the enzyme. Reactions were carried out at 37°C for 30 minutes. Gel electrophoresis was performed at 4 V/cm for 5 hours in Tris-borate-EDTA buffer. For the quantitative determination of topo I activity, photographic negatives were scanned. The area representing supercoiled DNA, migrating as a single band at the bottom of the gel, was determined. The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC$_{50}$ values) were determined by averaging the data from at least three experiments.

**Topo I-mediated plasmid nicking assay.** Topo I poisons enhance topo I-mediated pUC8 DNA cleavage under the reaction conditions provided by the supplier of the enzyme (Topogen, Inc.). Briefly, 20 μl of reaction mixtures contained 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 μl of the test agent (or solvent), 0.5 mg of pUC8, and 10 units of human topo I (added last). After a 30-minute incubation at 37°C, SDS-proteinase K was added, and, following a 30-minute incubation at 37°C, samples were extracted with CHCl3-isopropanol and electrophoresed on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. The gels were photographed, and the photographic negatives were scanned.

**Topo II-mediated P4 unknotting assay.** To determine topoisomerase (topo II) catalytic activity, knotted DNA that had been isolated from the tailless capsids of the bacteriophage P4 Vir1 del10 was used as the substrate. Reaction mixtures contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl2, 0.5 mM ATP, and 0.5 mM dithiothreitol. The topo II inhibitor was added prior to the addition of 2 units of human topo II. Reactions (20 μl final volume) were initiated by adding 0.5 μg of knotted DNA and carried out at 37°C for 30 minutes. Reactions were terminated by the addition of 5 μl of a stop solution containing 5% SDS, 50 mM EDTA, 25% Ficoll, and 0.05 mg/ml bromphenol blue. Samples were loaded on 0.8% agarose gels, and electrophoresis was performed at 4 V/cm for 5 hours in Tris-borate-EDTA buffer. The gels were stained with ethidium bromide, destained, and photographed under a UV light source. For the quantitative determination of topo II activity, photographic negatives were densitometrically scanned. Unknotted 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 into the reaction product (unknotted DNA) was determined from a standard curve. By averaging three to four such experiments, the IC$_{50}$ values were determined.

**Topo II-mediated plasmid linearization assay.** Topo II poisons enhance topo II-mediated DNA cleavage and can be identified with the linearization assay under the reaction conditions provided by the supplier of the enzyme (Topogen, Inc.). Briefly, 20-μl reaction mixtures contained 30 mM Tris-HCl, pH 7.6, 3 mM ATP, 15 mM β-mercaptoethanol, 8 mM MgCl$_2$, 60 mM NaCl, 1 μl of the test agent (or solvent), 0.3 μg of pRYG, and 10 units of human topo II (added last). After a 15-minutes incubation at 37°C, SDS-proteinase K was added, and following a 15-minutes incubation at 37°C, samples were extracted with CHCl3-isopropanol and electrophoresis was performed on a 1% agarose gel containing ethidium bromide. The gels were photographed, and the photographic negatives were scanned.

**Results**

**Inhibition of the catalytic activity of topo II but not topo I by phenoxodiol.** The stepwise removal of DNA knots (unknotting) requires transient double-strand breakage followed by strand passage and religation. Type II topoisomerases uniquely catalyze this reaction. The effect of phenoxodiol (see Figure 1 for chemical structure) on topo II catalytic activity is displayed in Figure 2. Unknotted DNA from a mutant bacteriophage (P4 Vir1 del10) is used as a reaction substrate that migrates as a smear (due to the variable number of knots). In the presence of topo II, topological DNA knots are removed, and the reaction product (unknotted DNA) migrates as a single band. Phenoxodiol inhibited this reaction in a dose-dependent manner as shown in Figure 2. Complete inhibition was evident at 100 μg/ml phenoxodiol. It was determined from densitometric measurements of the unknotted band that 50% inhibition (IC$_{50}$) was at about 20 μg/ml phenoxodiol. The effect of phenoxodiol was comparable to that of VP-16, which was used as a positive control.

To determine if phenoxodiol is a selective inhibitor of topo II, we evaluated its effect in the topo I-mediated relaxation of plasmid DNA in the absence of ATP. Topo II can also relax supercoiled plasmid DNA, but it requires ATP. Figure 3 shows that purified human topo I relaxes supercoiled plasmid DNA (lane 2). Camptothecin, a known topo I inhibitor, prevents pUC8 DNA relaxation (lane 3), but phenoxodiol at concentrations up to 100 μg/ml did not inhibit this topo I-catalyzed reaction (lanes 4-8). These results show that phenoxodiol does not inhibit topo I and therefore is a topo II-specific inhibitor.

**Induction of topo II-mediated double-strand DNA cleavage but not topo I-mediated DNA single-strand breakage by pheno-xodiol.** We employed the linearization assay to determine if
“pure” topo II poison (15). In the present study, we found phenoxodiol to produce detectable topo II-mediated linear plasmid DNA at a concentration of 20 µg/ml, which is lower than the concentration of genistein (30 µg/ml) that produced comparable DNA cleavage. The effect of phenoxodiol was similar to that of VP-16.

Topo II poisons, including VM-26, VP-16, doxorubicin, amascrine, and several dietary bioflavonoids, represent a class of topo II inhibitors that convert a normal enzyme (topo II) into a cellular poison. The ternary complexes, formed between topo II, DNA, and the drug, are initially reversible by DNA religation or DNA repair (2). Cellular processing of the accumulating ternary complexes activates an irreversible step that leads to protein-associated DNA fragments 300-600 kb in size (16). Following this irreversible step, caspase 3 becomes activated, which produces endonucleolytic DNA cleavage characteristic of apoptosis. Thus, following DNA replication or transcription, these topo II poisons convert cleavable complexes into lethal lesions (15, 17). The sensitivity of tumor cells to topo II inhibitors is strongly associated with intranuclear topo II levels (8, 10, 11). Since rapidly dividing lung cancer, breast cancer, ovarian cancer, and malignant lymphoma cells generally express much higher levels of topo II than normal non-dividing cells, the former are more susceptible to the deleterious effects of topo II poisons. Furthermore, reduced topo II activity has been associated with cell differentiation (2, 3). Based on the effects of phenoxodiol on topo II activity, this novel agent is expected to induce tumor cell differentiation and activate the apoptotic pathway. These biological effects of phenoxodiol are consistent with its ability to inhibit topo II and produce double-strand DNA breaks.

We have shown in this manuscript that phenoxodiol is a topo II-specific poison. The agent did not inhibit the topo I catalytic activity nor did it trap the topo I cleavable complex. The specificity of phenoxodiol towards topo II places it in the same category as the most widely prescribed antineoplastic drugs that target topo II (18). The topo I levels are relatively similar between normal and tumor cells. Contrary to that, the topo II levels are much higher in rapidly dividing tumor cells. Consequently, agents that act as topo II poisons direct their cytotoxic effects mainly against tumor cells, while those that act as both topo I and II poisons may also be cytotoxic to normal cells. This observation may well explain the observed low toxicity of phenoxodiol in normal healthy tissues (Novogen Ltd, unpublished data).

The catalytic cycle of topo II can be divided into six discrete steps. These are: 1) binding of topo II to DNA, 2) double-stranded DNA cleavage, 3) double-stranded passage through the break, 4) religation of the cleaved DNA, 5) ATP hydrolysis, and 6) enzyme turnover (19). The clinical applications of topo II poisons depend on the exact steps of the catalytic cycle that are inhibited. Although we have established in the present study that phenoxodiol traps the cleavable complex, we do not know if this is done by enhancing the cleavage step or by inhibiting the religation step. Topo II poisons such as daunorubicin, doxorubicin, amascrine, ellipticine and mitoxantrone are DNA intercalators (20). Other topo II poisons such as VP-16, VM-26, ceroidin and salvinorine do not intercalate to the DNA (20, 21). Future studies will apply specific assays to identify the exact step(s) of the catalytic cycle that are being inhibited, and to determine whether phenoxodiol binds to topo II, the DNA, or the topo II/DNA complex. This information is crucial in identifying the precise clinical applications of phenoxodiol. For example, if phenoxodiol binds on a different topo II site than VP-16, it may find applications in small cell lung carcinomas expressing mutant forms of topo II that do not bind VP-16 and escape its cytotoxic effects.

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phenoxodiol is a topo II poison. Double-strand breakage results in the appearance of linear DNA. Phenoxodiol in the presence of topo II, followed by treatment with proteinase K/SDS, effectively produced the linear form of plasmid DNA (Figure 4, lanes 2-4), indicating that it stabilizes the cleavable complex. This effect, which was evident at 10 μg/ml, peaked at 30 μg/ml. In the absence of topo II (lane 1) or proteinase K/SDS (not shown), phenoxodiol did not produce linear DNA. The effect of phenoxodiol on topo II-mediated DNA strand breakage is comparable to that of genistein (lane 5) and VP-16 (lane 6), which were used as positive controls. Since enzyme denaturation and digestion are necessary to release the DNA cleavage, these data demonstrate that phenoxodiol-induced DNA breakage is mediated by topo II.

Topo I poisons trap the enzyme-DNA reaction intermediate and, following the digestion of the enzyme, produce single-strand DNA breaks (nicks). Under the electrophoretic conditions that we used in the experiment shown in Figure 5, covalently closed circular (supercoiled or relaxed) plasmid DNA migrates on the bottom of the gel. In the presence of a topo I poison that stabilizes the cleavable complex, and following denaturation and degradation of the enzyme with proteinase K/SDS, the resulting nicked DNA migrates on the top of the gel. Phenoxodiol at 20 and 100 μg/ml failed to produce nicked DNA (lanes 3 and 4). Camptothecin, a known topo I poison, produced single-strand DNA cleavage indicated by an increase in the nicked form of DNA, as expected (lanes 5 and 6). These results demonstrate that phenoxodiol is devoid of topo I inhibitory effects and therefore is a topo II-specific poison.

Discussion

The novel isoflavone metabolite phenoxodiol is presently undergoing evaluation as an antitumor drug in phase I/II clinical studies in patients with a range of solid tumors, excluding breast cancer. The ability of phenoxodiol to stabilize the cleavable complex and produce topo II-mediated DNA cleavage in vitro is comparable to that of other topo II poisons that are currently used in cancer chemotherapy. One such drug is VP-16, used in the treatment of small cell lung carcinoma, which yields remissions in 70% of patients and is a

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