Application of Topoisomerase Assays in the Evaluation of Natural Products as Antitumor Agents

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ABSTRACT

Initially, DNA topoisomerase (topo) inhibitors found clinical applications as antibiotics and cancer chemotherapeutic agents. Recently, we demonstrated that plant flavonoids that inhibit mammalian topo I or topo II might be useful as cancer chemopreventive agents (Constantinou et al., 1995b). Phytochemicals can inhibit DNA topoisomerases in different ways; depending on the mode and the type of enzyme, these can be classified as topo I poisons, topo II poisons, topo I antagonists, or topo II antagonists. Correctly classifying topo inhibitors is critical because it provides an important lead as to whether the plant agent can be useful in chemoprevention or in chemotherapy. We outline below a strategy that was designed to identify and classify topo I and II inhibitors.

Some of the most potent antitumor agents currently being used in cancer chemotherapy or being evaluated as chemopreventive agents inhibit DNA topoisomerase (topo) I or II. In eukaryotes, topoisomers are involved in the processes of DNA replication, transcription, and recombination, and they play key roles in cell proliferation and differentiation (Heck and Earnshaw, 1986; Constantinou et al., 1989; Kiguchi et al., 1990). Transient DNA strand breakage is an integral component of both topo I and topo II normal enzymatic activities. In the presence of certain inhibitors known as topo I or topo II poisons, a reaction intermediate between the enzyme and DNA (known as the "cleavable complex") becomes stabilized, giving rise to DNA breaks (Liu, 1989; Robinson and Osheroff, 1991; Osheroff, 1989). Cancer cells characterized by increased topo II activity are especially susceptible to the DNA-damaging and cytotoxic effects of topo II poisons (Constantinou et al., 1990; Shin et al., 1990; Tanabe, et al., 1991). Paradoxically, not only topo II poisons but also poisons of topo I (whose levels are generally not higher in cancer cells) have found applications in cancer chemotherapy. Antitumor drugs such as teniposide (VM-26), etoposide (VP-16), ellipticine, doxorubicin, and amsacrine are mechanistically topo II poisons. Drugs such as irinotecan and topotecan (both camptothecin derivatives) are mechanistically topo I poisons.

A different class of topo inhibitors includes agents that do not stabilize the covalent enzyme-DNA complex but rather hinder its formation. These inhibitors have been named catalytic inhibitors or topo antagonists, because they oppose the DNA cleavage reaction of the target enzyme (Shin et al., 1990). Novobiocin, merbarone, aclarubicin, fostriecin, bis(2,6-dioxopiperazine) derivatives, and gossypol are some representative topo II catalytic inhibitors exhibiting antitumor effects (Creighton et al., 1969; Witia et al., 1978; Pedersen-Bjergaard et al., 1984; Rao et al., 1985; Adlakha et al., 1989; Tanabe et al., 1991). Catalytic inhibitors often
present growth-inhibitory and/or differentiation-inducing properties, and, because of these properties, they can find applications as chemopreventive agents. In a recent study, dextrazoxane (ICRF-187), a topo II catalytic inhibitor, given shortly after a high dose of etoposide in a central nervous system tumor model, rescued normal tissue from the cytotoxic effects of etoposide (Holm et al., 1998).

Phytochemicals that inhibit DNA topoisomerases (e.g., flavonoids) may act as topo I poisons, topo II poisons, topo I antagonists, or topo II antagonists. Dual topo I/topo II catalytic inhibitors are also found, as are dual topo I/topo II poisons. We previously characterized the flavonoid myricetin as a dual catalytic inhibitor of topo II and topo I (Constantinou and Huberman, 1995). We showed that genistein inhibits topo II by stabilizing the cleavable complex; this quality classifies it as a topo II poison (Constantinou, et al., 1990, 1995). Based on this background, the correct classification of topo inhibitors is critical because it provides an important lead as to whether the agent should be evaluated as a chemopreventive, cytoprotective, or chemotherapeutic drug. We outline here an approach that we have used successfully in the past several years to identify new topo inhibitors and classify them.

RESULTS AND DISCUSSION

Initially, purified natural agents are tested at two concentrations (50 and 100 μg/ml) in the four topoisomerase assays illustrated in Figure 1. These are the relaxation (A), unknotting (B), nicking (C), and linearization (D) assays.

![Diagram](image)

**FIG. 1.** Agarose gel assays for evaluating topo I and topo II inhibitors as described in the text: (A) relaxation assay, (B) unknotting assay, (C) nicking assay, and (D) linearization assay. Diagrammatic representations of DNA topology are shown on each side of the panels. Numbers on the left of panel A represent the number of negative supercoils. Numbers on the bottom of panel D represent the genistein concentration in micrograms per milliliter. SC, supercoiled DNA; REL, relaxed DNA; NIC, nicked DNA; L/N, linear DNA; EA, ellagic acid; CAM, camptothecin.
Agents that are active in one or more of these topo assays are subsequently tested at lower concentrations. If an agent is found to be active in assays A or B but not C or D, it is considered a catalytic inhibitor. By testing lower concentrations, we can determine the concentration required to prevent 50% of the substrate from being converted to the reaction product (IC₅₀). If an agent is found to be effective in assays C or D, it is considered a poison. In that case, the concentration that provides the maximum reaction intermediate is determined. Some agents can be effective in more than one assay. For example, it is possible that an agent can be effective in both A and B but with a different IC₅₀ in each assay. An agent also might be effective in both A and C or in both B and D, but again at different concentrations. Agents not showing effect at 50 μg/ml are considered ineffective and are not tested further.

We describe here four of the most commonly employed topo assays for the characterization of natural or synthetic agents. This description is tailored to the newcomer in the field.

Relaxation assay

To determine topo I catalytic activity, pUC8 supercoiled DNA is used as the reaction substrate, but any plasmid DNA of 3–4 Kb or smaller is appropriate. In a reaction volume of 20 μl, we add the following: 10 mmol/L Tris-HCl (pH 7.9), 1 mmol/L EDTA, 150 mmol/L NaCl, 0.1% bovine serum albumin, 0.1 mmol/L spermidine, 5% glycerol, and 2 units of purified human topo I. The test agent is added, when necessary, and the reaction is started by the addition of 2 units of the enzyme. Reactions are carried out at 37°C for 30 minutes. After the reactions are stopped with 5 μl of loading buffer (5% sodium dodecylsulfate [SDS] mmol/L EDTA, 25% ficoll, and 0.05 mg/ml bromophenol blue), samples are loaded on 1% agarose gels and electrophoresed at 4 V/cm for 5 hours in Tris-borate-EDTA (TBE) buffer. Ethidium bromide must not be present in the gel or the buffer during electrophoresis, because the topoisomers will not be separated. For the quantitative determination of topo I activity, photographic negatives are scanned. The area representing highly supercoiled DNA, migrating as a single band at the bottom of the gel, is measured. In this manner, the concentration of the inhibitor that prevents 50% of the substrate (supercoiled DNA) from being converted into the reaction product (relaxed DNA) is determined. In the example given, this is represented by the middle lane of Fig. 1A. The IC₅₀ values are determined by averaging the data from at least three independent experiments. Topo II can also relax supercoiled DNA, but topo II (unlike topo I) requires adenosine triphosphate (ATP). Therefore, the relaxation assay can be used to measure topo II catalytic activity. This assay, however, should be employed only for identifying topo II inhibitors when one is certain that the enzyme is highly purified and free of topo I activity.

Unknotted assay

The unknotted assay is specific for evaluating topo II catalytic activity because it is based on the conversion of knotted DNA to its unknotted form, which requires DNA double-strand breakage, strand-rotation, and religation—activities uniquely performed by topo II. The removal of these knots by topo II can be visualized in agarose gels. The substrate, knotted DNA is isolated from the tailless capsids of the bacteriophage P4 Viral del1O. After electrophoresis, under low voltage, P4-knotted DNA migrates as a smear, whereas unknotted DNA migrates as a single band (Fig. 1B). Reaction mixtures of 20 μl contain 50 mmol/L Tris-HCl (pH 8.0), 120 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L ATP, and 0.5 mmol/L dithiothreitol. The test agent is added, when necessary, followed by 2 units of human topo II. Reactions are started by the addition of 0.6 μg of knotted DNA and are carried out at 37°C for 30 minutes. Reactions are terminated by the addition of 5 ml of a stop solution containing 5% SDS, 50 mmol/L EDTA, 25% ficoll, and 0.05 mg/ml bromophenol blue. Samples are located on 0.8% agarose gel and electrophoresed at 4 V/cm for 5 hours in TBE buffer in the absence of ethidium bromide. Gels are stained in ethidium bromide, destained, and photographed over a ultraviolet light source. For the quantitative determination of topo II activity, photographic negatives are
densitometrically scanned. Unknotted DNA, migrating as a single band at the top of the gel, is 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) is determined from a standard curve. By averaging the data from at least three experiments, we can determine the IC<sub>50</sub> value.

**Nicking assay**

Topo I poisons (or targeting agents) with the ability to enhance topo I-mediated DNA cleavage can be identified using the pUC8 DNA under the following reactions. In 20 μl of reaction mixtures, we add 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 100 mmol/L NaCl, 1 μl of the test agent if necessary, 0.5 μg of pUC8, and at least 10 units of human topo I (added last). It is critical that the enzyme is of high specific activity and free of nucleases. After a 30-minute incubation at 37°C, SDS-proteinase K was added, and after another 30-minute incubation at 37°C, samples were extracted with CHCl<sub>3</sub>-isopropanol and electrophoresed on a 1% agarose gel containing ethidium bromide. Gels were photographed, and photographic negatives were scanned. In Figure 1D, the effects of increasing concentrations of genistein are shown as an example.

**CONCLUSION**

In summary, depending on the mode of topo inhibition, natural products (including phytoestrogens) can find applications as chemopreventive agents or chemotherapeutic drugs. Considering that some of the most promising cancer therapeutic drugs are plant derived and inhibit topos, the assays described here can provide important leads in the selection of natural products that may be effective antitumor agents. Phytochemicals that are present in the diet can promote or prevent DNA damage. With the described assays, agents that either cause DNA damage or prevent DNA damage, through the natural action of topos, can be identified. The results of these studies should be interpreted with caution, because the inhibition of topos usually requires higher concentrations of agents than those reached through dietary means in the blood or tissues.

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**REFERENCES**


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