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FLAVONOIDS AS DNA TOPOISOMERASE ANTAGONISTS AND POISONS: STRUCTURE-ACTIVITY RELATIONSHIPS

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ABSTRACT.—Selected flavonoids were tested for their ability to inhibit the catalytic activity of DNA topoisomerase (topo) I and II. Myricetin, quercetin, fisetin, and morin were found to inhibit both enzymes, while phloretin, kaempferol, and 4′,6,7-trihydroxyisoflavone inhibited topo II without inhibiting topo I. Flavonoids demonstrating potent topo I and II inhibition required hydroxyl group substitution at the C-3, C-7, C-3′, and C-4′ positions and also required a keto group at C-4. Additional B-ring hydroxylation enhanced flavonoid topo I inhibitory action. A C-2,C-3 double bond was also required, but when the A ring is opened, the requirement for the double bond was eliminated. Genistein has been previously reported to stabilize the covalent topo II-DNA cleavage complex and thus function as a topo II poison. All flavonoids were tested for their ability to stabilize the cleavage complex between topo I or topo II and DNA. None of the agents stabilized the topo I-DNA cleavage complex, but prunetin, quercetin, kaempferol, and apigenin stabilized the topo II DNA-complex. Competition experiments have shown that genistein-induced topo II-mediated DNA cleavage can be inhibited by myricetin, suggesting that both types of inhibitors (antagonists and poisons) interact with the same functional domain of their target enzyme. These results are of use for the selection of flavonoids that can inhibit specific topoisomerases at specific stages of the topoisomerization reaction.

Several plant-derived flavonoids have been previously reported to inhibit certain regulatory enzymes including protein kinase C (1), reverse transcriptase (2), and DNA topoisomerase (topo) II (3,4). With the exception of genistein, the specific effects of these flavonoids on the two main types of eukaryotic topoisomerases (topo I and topo II) have not been determined. Drugs targeting either topo I or topo II have applications in cancer chemotherapy, but the range of applicable tumors depends on the type of topoisomerase targeted and on their mode of action. For example, the topo I-targeting drugs camptothecin and topotecan, are effective against slow-growing tumors that contain the same levels of topo I as more rapidly growing tumors (5). In contrast, the topo II-targeting etoposide (VP-16) and teniposide (VM-26) are more useful in rapidly proliferating carcinomas expressing high levels of topo II. These clinical considerations necessitate the precise identification of the enzymatic targets of the flavonoids and other compounds that inhibit DNA topoisomerases as well as a clear understanding of their mode of action.

In eukaryotes, DNA topoisomerases are involved in the processes of DNA replication, transcription, and recombination (6), and also play a key role during cell

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proliferation and differentiation (7–12). Inhibitors of eukaryotic topoisomerases, depending on the stage of the catalytic cycle they inhibit, can be distinguished into two classes. In the first class belong cytotoxic agents known as topoisomerase poisons (13), which, by preventing the religation step of the reaction, stabilize the covalent enzyme-DNA complex known as the cleavage complex (14,15). Teniposide, etoposide, ellipticine, doxorubicin, and *m*-AMSA are some representative topo II poisons. In the second class belong agents that do not stabilize the covalent enzyme-DNA complex, but rather prevent formation of this complex and consequently enzymatic turnover; these have been referred to as topoisomerase antagonists because they oppose both the normal topoisomerase strand-passing reaction as well as the action of the poisons (16). Novobiocin (17), merbarone (18), aclarubicin (19), fostriecin (20), *bis*-2,6-dioxopiperazine derivatives (21), and gossypol (22) are some representative topo II antagonists. Although topo I and II poisons are more commonly used as chemotherapeutic agents (11–13,23), some topo-II antagonists that exhibit antitumor effects in animal studies have been reported (24–27).

In the present study, 20 representative flavonoids have been evaluated using specific assays, for their ability to inhibit topo I and topo II activities and to enhance DNA-strand breakage. Structural alterations that drastically change an agent's effectiveness or mode of action have been identified. We report herein that topo II-inhibiting flavonoids can function as topo II poisons, antagonists, or both, depending on the position of hydroxyl groups in the A and B rings of the molecule.

RESULTS AND DISCUSSION

The effect of various flavonoids on the catalytic activity of topo I and topo II was evaluated using the relaxation and unknotting assays, respectively. Initially, compounds were evaluated at a concentration of 100 μg/ml. Agents not showing activity at 100 μg/ml were considered ineffective and were not tested further. Agents showing inhibition at 100 μg/ml, were further tested in the 1- through 100-μg/ml range until the minimal concentration necessary to inhibit 50% of the topo I or topo II catalytic activity (IC_{50}) was determined. An example of this approach is shown in Figure 1. In the initial test, quercetin inhibited both topo I and topo II at 100 μg/ml. This compound was then further evaluated at lower concentrations as shown in Figure 1A. When the photographic negative was scanned and the densitometric data plotted as shown in Figure 1C, the IC_{50} value was found to be 10 μg/ml. By averaging the IC_{50} values of three to four such experiments, mean values shown in Tables 1 and 2 were obtained. A similar approach was used for determining the mean IC_{50} values of quercetin for topo II-catalytic activity, except that the photographic negatives of unknotting assays (shown in Figure 1B) were scanned, and the density of the unknotted band was integrated and plotted as illustrated in Figure 1D.

In this manner, the ability of representative flavones to inhibit the catalytic activities of topo I and II was determined, and these results are summarized in Table 1. The flavone derivatives myricetin, quercetin, fisetin, and morin inhibited both topo I-relaxing activity and topo II-unknotting activity. Kaempferol and phloretin inhibited only topo II-unknotting activity. Effective topo II-inhibiting flavones have in common the obligatory C-4 keto group and hydroxyl group substitutions at C-3, C-7, and C-4′. Effective topo I inhibitors exhibited the same structural parameters as topo II inhibitors, with the additional requirement of another hydroxyl group in the B-ring. When this additional group was adjacent to the C-4′ position the flavonoid was more effective in inhibiting topo I. *d*-Catechin, a flavanol with the same hydroxyl group substitutions as the flavone quercetin, did not inhibit either topo I or topo II. Rutin, which also has the same hydroxyl group substitutions as quercetin, with the exception of being a C-3

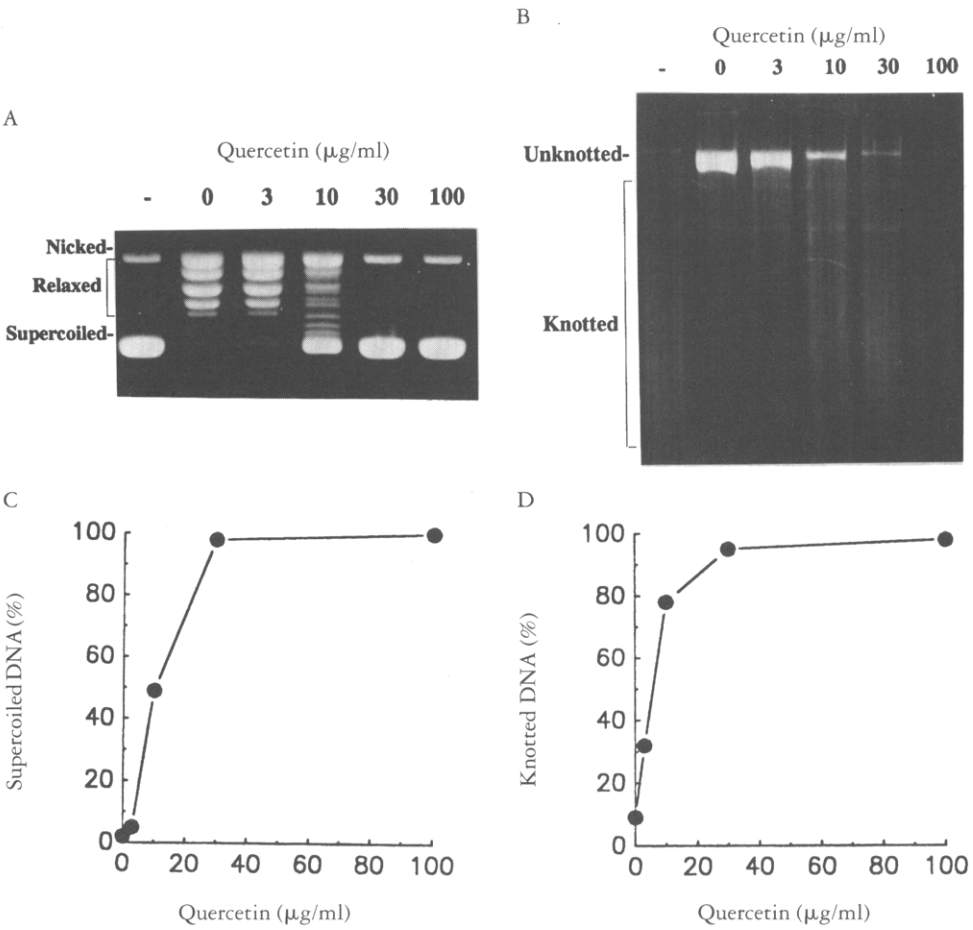
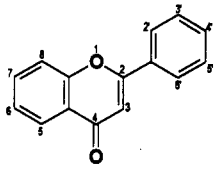
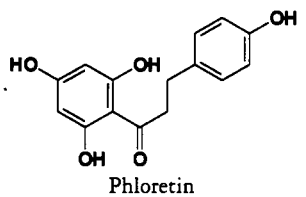
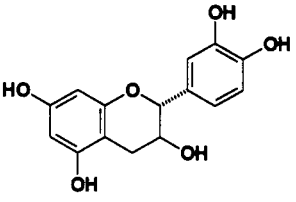


FIGURE 1. Agarose gel assays for determining the effect of quercetin on topo I (A) and topo II activities (B). Two units of purified topo I or topo II were incubated, under the conditions described in the Experimental, with increasing concentrations of quercetin as shown in the top of panels A and B. The amount of the reaction substrate, evaluated by scanning densitometrically the supercoiled form of pUC8 DNA for topo I (panel C), or the unknotted form of P4 DNA for topo II (D), is plotted against quercetin concentration. The IC_{50} value was determined by extrapolating the concentration of quercetin required to prevent conversion of 50% of the substrate to the reaction product. This is a representative experiment; mean IC_{50} values were determined in this manner from a total of three independent experiments.

glycoside, lacked topoisomerase inhibitory activity. Galangin was ineffective in inhibiting topoisomerases, apparently because it lacks hydroxyl groups in the B ring. Flavone and all monohydroxyflavones were found in this study to be ineffective as enzyme inhibitors.

From the five isoflavones tested, only genistein (4′,5,7-trihydroxyisoflavone) and 4′,6,7-trihydroxyisoflavone inhibited topo II-unknotting activity (Table 2). Genistein has been previously shown to inhibit topo II-unknotting activity, while it is unable to inhibit topo I-relaxing activity at concentrations lower than 1 mM (28,29). In the present study, genistein was used as a positive control. Daidzein, which differs from genistein in that it has no hydroxyl group at the C-5 position, did not inhibit topo II- (or topo I-) unknotting activity. Prunetin, with a C-7 methoxyl group was also ineffective in this assay. From these results, it was concluded that hydroxyl groups at C-

TABLE 1. Effect of Flavones, a Dihydrochalcone, a Flavanol, and Derivatives on Topo I-Relaxing and Topo II-Unknotting Activities.

Compound	Position of Hydroxyl Substitution										Mean[a] IC_{50} (μg/ml)	
	A ring					B ring					Topo I	Topo II
												
Flavone											—[b]	—[b]
Myricetin	3		5		7			3′	4′	5′	11.9	11.9
Quercetin	3		5		7			3′	4′		12.8	6.9
Fisetin	3				7			3′	4′		20.6	8.2
Morin	3		5		7	2′			4′		42.1	40.8
Kaempferol	3		5		7				4′		—[b]	8.1
Apigenin			5		7				4′		—[b]	—[b]
Rutin[c]			5		7			3′	4′		—[b]	—[b]
Galangin	3		5		7						—[b]	—[b]
3-OH Flavone	3										—[b]	—[b]
5-OH Flavone			5								—[b]	—[b]
6-OH Flavone				6							—[b]	—[b]
7-OH Flavone					7						—[b]	—[b]
 Phloretin												
 *d*-Catechin												
Phloretin			5		7				4′		—[b]	46.1[b]
d-Catechin	3		5		7			3′	4′		—[b]	—[b]

[a]Values represent the mean from at least three independent experiments, the variance was less than 25% of the mean value.

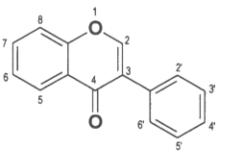
[b]No effect at up to 100 μg/ml final concentration.

[c]Contains a rutinose sugar unit attached to C-3.

5, C-7, and C-5′ are required for the inhibition of topo II-unknotting activity by isoflavonoids. However, C-6 instead of C-5 hydroxyl substitution is permissible. None of these isoflavones inhibited topo I at concentrations below 100 μg/ml.

To further characterize the stage of the topoisomerization reaction initially blocked by the agent, we used a linearization assay. Topo II poisons stabilizing the cleavage complex can be evaluated using the pUC8 plasmid linearization assay. 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 2). Using this, or other similar assays, genistein has been shown to stabilize the cleavage complex (3, 4, 28, 29). The flavonoids listed in Tables 1 and 2 were evaluated for their ability to induce DNA-breakage in the presence of topo II. Positive agents are listed in Table 3; quercetin, kaempferol, apigenin, and prunetin enhanced by at least 60% the

TABLE 2. Effect of Isoflavones on Topo I-relaxing and Topo II-Unknotting Activities.

Compound	Position of Hydroxyl Substitution		Mean[a] IC_{50} (μg/ml)	
	A ring	B ring	Topo I	Topo II
				
Genistein	5 7	4′	—[b]	30
Daidzein	7	4′	—[b]	—[b]
3′,4′,7-Trihydroxyisoflavone	7	3′ 4′	—[b]	—[b]
4′,6,7-Trihydroxyisoflavone	6 7	4′	—[b]	43.7
Prunetin	5 7-OMe	4′	—[b]	—[b]

[a]Values represent the mean from at least three independent experiments; the variance was less than 25% of the mean value.

[b]No effect up to 100 μg/ml final concentration.

topo II-mediated DNA cleavage as determined by increases in the linear form of plasmid DNA in the presence of topo II, but these agents were considerably less potent than genistein. In two experiments, fisetin, morin, 5-hydroxyflavone, and 6-hydroxyflavone weakly enhanced the cleavage complex (by less than 50%). These agents, however, were considered marginally effective and are not listed in Table 3. None of the effective agents produced linear DNA in the absence of the enzyme, indicating that the effect is topo II-mediated.

It is currently unknown whether flavonoid antagonists of topo II bind on the same site as flavonoid topo II poisons. To address this issue, we evaluated the effect of myricetin, which is a strong inhibitor of topo II-unknotting activity (IC_{50} 11.9 μg/ml), on the DNA cleavage enhancing-effect of genistein. The results are shown in Figure 2. Genistein, at concentrations of 100 μg/ml (lane 3) and 30 μg/ml (lane 6) effectively produced linear DNA (form III), in the presence of topo II. Myricetin, at concentrations ranging from 3 to 100 μg/ml, did not produce linear DNA. Only the highest myricetin concentration is shown in this figure (lane 4). Furthermore, myricetin prevented

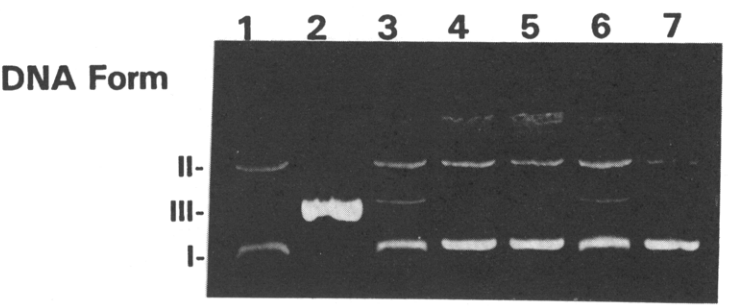


FIGURE 2. Effects of myricetin on topo II-mediated cleavage of plasmid pUC8 DNA. Lane 1, control (no drug); lane 2, linear pUC8 (marker for form III DNA), lane 3, genistein (100 μg/ml), lane 4, myricetin (100 μg/ml), lane 5, genistein (100 μg/ml) plus myricetin (100 μg/ml); lane 6, genistein (30 μg/ml); lane 7, genistein (30 μg/ml) plus myricetin (30 μg/ml). All reactions (except lane 3) containing 10 units of purified human topo II were performed as described in the Experimental under "Plasmid Linearization Assay."

TABLE 3. Flavonoids Effective in Enhancing Topo II-Mediated DNA Cleavage.

Compound[a]	Linear DNA[b] (% of total DNA)	Linear DNA (times control value)
None (control)	5.3	1
Quercetin	11.5	2.2
Kaempferol	8.3	1.6
Apigenin	8.7	1.6
Genistein	16.2	3.1
Prunetin	9.2	1.7

[a]All flavonoids were tested, as shown in Figure 2, at a final concentration of 100 μg/ml.

[b]Values are the means from at least triplicate experiments; the variance was less than 25% of the mean value.

genistein's DNA cleavage-promoting action as determined by the absence of linear DNA in lanes 5 and 7. Myricetin antagonized the genistein-induced DNA breakage only when it was provided at the same concentration as the isoflavone. Myricetin, when introduced at lower concentrations than genistein, was only partially effective and when genistein was at 10-fold excess myricetin became ineffective in reducing the linear form of DNA (data not shown). This antagonistic action of myricetin was not limited to genistein, since it also prevented the DNA cleavage action of prunetin (data not shown). One possible explanation of these results is that flavonoids inhibiting different stages of the topoisomerization reaction may bind on the same site of the enzyme or the DNA enzyme-complex.

The C-4 carbonyl group was found to be essential for the inhibition of both topo I and topo II activities. This is apparent by comparing the active quercetin with the inactive *d*-catechin. Comparison of these two compounds also suggested that the C-2,C-3 double bond in the pyrone ring is essential for inhibiting both topoisomerases. This requirement, however, is not necessary when the A ring is cleaved, as in the case of phloretin. Hydroxyl group substitution in the A and B rings provides additional specificity. These critical structural observations are discussed below.

Myricetin, quercetin, and fisetin were the most potent inhibitors of topo I and topo II catalytic activities among the flavonoids investigated; these have in common hydroxyl group substitutions at positions C-3, C-7, C-3′, and C-4′. The inability of rutin (in which the C-3 position is glycosylated) to inhibit either one of the two enzymes studied further demonstrates the requirement for hydroxyl group substitution at C-3. Phloretin was an apparent exception to this structural requirement, although this is based on a dihydrochalcone rather than a flavone structure. The three flavones, quercetin, kaempferol, and apigenin that functioned as topo II poisons have as a common characteristic C-5, C-7, and C-4′ hydroxyl group substitutions.

Similar but not identical structural requirements have been reported for flavonoid inhibitors of other enzymes. For example C-7, C-3′, and C-4′ hydroxyl group substitution was required for the inhibition of protein kinase C, while C-3, C-5, C-7, C-3′, and C-4′ hydroxylation was required for the inhibition of reverse transcriptase (1,2). These data suggest that the above enzymes may contain a common amino acid sequence serving as the flavonoid recognition site.

It has been postulated previously that the ATP binding domain of topo II may also serve as the binding site for genistein (29). The ability of quercetin and genistein to inhibit the ATPase component of the topoisomerization reaction (30) provides some support to this hypothesis. In addition, the demonstration of antagonism between myricetin and genistein provides further support. Based on the observation that

genistein-induced (topo II-mediated) DNA breakage is prevented by equimolar concentrations of myricetin, we propose that the two flavonoids bind on the same site of the target enzyme; different stages of the topoisomerization reaction, however, are blocked due to spatial differences between the two flavonoids. Genistein, which is planar, stabilizes the cleavable complex, while the nonplanar myricetin prevents enzymatic turnover.

An alternative explanation of the above observations requires the flavonoids to exert their effects non-specifically, that is, through intercalation into DNA. Three lines of evidence oppose this possibility. First, Yamashita *et al.* reported that neither myricetin nor genistein intercalate into pBR322 DNA even at concentrations of 250 μM (3). Second, no correlation between DNA intercalation and induction of DNA breakage has been shown in any studies with flavonoids or other topo II-targeting agents (3,4). Third, if the effect were non-specific, one would expect myricetin to also inhibit camptothecin-mediated topo I nicking. We have found that myricetin had no effect on camptothecin-induced topo I-mediated DNA breakage (unpublished results). Our data, in conjunction with those from the above studies, suggest that the competition between genistein and myricetin is due to the binding of drugs with comparable affinity for the same pharmacophore (topo II) and not due to nonspecific intercalation of myricetin into DNA. Thus, flavonoid antagonists may bind with a spatial orientation that neither interferes with the DNA cleavage/religation equilibrium, nor opposes the DNA strand-passage step of the reaction (31); but rather they inhibit enzymatic turnover through a mechanism requiring ATP hydrolysis. Flavonoid poisons, on the other hand, because of a different spatial arrangement, may stabilize the (normally transient) DNA-enzyme complex and favor the DNA cleavage component of the reaction. We have demonstrated here that the effects of flavonoids on the topoisomerization reaction are varied, and, depending on minor structural alteration, these agents can function either as topo II antagonists, or as topo II poisons.

EXPERIMENTAL

CHEMICALS AND REAGENTS.—The sources of the chemicals used in the present study were as follows: apigenin, kaempferol, quercetin, myricetin, *d*-catechin, phloretin, and morin (Sigma Chemical Co., St. Louis, MO); flavone, 3-hydroxyflavone, galangin, fisetin, and rutin (Aldrich Chemical Co., Milwaukee, WI); 5-hydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, genistein, and daidzein (Indofine Chemical Co, Somerville, NJ); *m*-AMSA from the National Cancer Institute; etoposide and teniposide (Bristol-Myers Squibb Co., Wallingford, CT). Topo I was purchased from Gibco-BRL, Gaithersburg, MD, and human topo II was purchased from Topogen, Inc., Columbus, OH. Stock solutions of all flavonoids, etoposide, and teniposide were prepared in DMSO; *m*-AMSA was dissolved in EtOH.

TOPOISOMERASE I 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: 50 mM Tris-Cl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 30 μg/ml bovine serum albumin and 2 units 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° for 30 min. Gel electrophoresis was performed at 4 V/cm for 5 h 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 prevented 50% of the substrate (supercoiled DNA) from being converted into the reaction product (relaxed DNA) was determined by plotting the data as shown in Figure 1. By averaging three to four such experiments, the IC_{50} values were determined.

TOPOISOMERASE II P4 UNKNOTTING ASSAY.—For the determination of 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, basically as described by Liu *et al.* (32), but with some modifications. Reaction mixtures contained 50 mM Tris-Cl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 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 units 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% SDS, 50 mM EDTA, 25% Ficoll, and 0.05 mg/ml bromophenol blue. Samples were loaded on 0.8% agarose gels and electrophoresed at 4 V/cm for 5 h in Tris-borate-EDTA buffer. Gels were stained in 1 μg/ml ethidium bromide, destained, and photographed over 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 (Figure 1). By averaging three to four such experiments, the IC_{50} values were determined.

PLASMID LINEARIZATION ASSAY.—Topo II-targeting agents having the ability to enhance topo II-mediated DNA cleavage were screened using 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-Cl, pH 7.6, 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$, 60 mM NaCl, 1 μl of the test agent if necessary, 0.3 μg of pUC8 DNA, and 10 units of human topo II (added last). After a 30-min incubation at 37°, SDS-proteinase K was added and following a 15-min incubation at 37°, samples were extracted with $CHCl_3$ 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, linear DNA was expressed as percentage of total DNA.

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