A Strategy for Identifying Novel, Mechanistically Unique Inhibitors of Topoisomerase I

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A STRATEGY FOR IDENTIFYING NOVEL, MECHANISTICALLY UNIQUE INHIBITORS OF TOPOISOMERASE I[1]

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ABSTRACT.—While the design of molecules that inhibit or antagonize the functions of specific macromolecules is now well precedented, in many cases the structural information requisite to the design process is lacking. The tools of molecular biology can now furnish the target macromolecules for use in mechanism-based exploration; highly defined assays can be devised based upon the known biochemistry of these macromolecules to permit the discovery of novel inhibitors or antagonists present in chemical collections. Presently, we describe a set of assays directed toward the discovery of novel inhibitors of eukaryotic topoisomerase I, an enzyme critical to maintenance of chromosomal DNA topology and therefore essential for normal replication and transcription. The identification of chebulagic acid as an extraordinarily potent and mechanistically novel inhibitor of topoisomerase I illustrates the potential of this approach.

In recent years, the pathogenesis of numerous diseases has become understood in greater detail at the cellular and molecular levels. In parallel with this development, there has been increasing emphasis on the identification of small molecules that inhibit specific biochemical transformations or antagonize the actions of macromolecular loci responsible for controlling cellular functions related to the pathogenic events. The inhibitors and antagonists so identified can be employed to further characterize the biochemical and biological systems involved, and as a starting point for the development of agents of utility for therapeutic intervention.

Where the biochemical system of interest has been characterized in sufficient detail at a molecular level, it is often possible to utilize the derived information for the synthesis of molecules that can act as highly potent inhibitors and antagonists. Knowledge of the structures of molecules possessing some inhibitory activity has provided a traditional starting point for inhibitor design, as exemplified by studies on acetylcholinesterase inhibitors (1). Even in the absence of any information regarding the structure of the macromolecular effector, mechanism-based approaches have yielded novel opioid-based analgesics (2). Inhibitors of nucleic acid biosynthesis have also been obtained in this fashion (3). Alternatively, knowledge of the structure of an endogenous substrate or ligand can suffice to guide molecular design where the assay systems are well developed, as demonstrated convincingly by the design of a series of potent leukotriene B_4 receptor antagonists (4).

Even in cases where no structural data is available for a macromolecular effector and neither a substrate nor inhibitor can be employed as a starting point for inhibitor design, empirical approaches have afforded many species of potential utility as therapeutic agents, notably in the antibiotic (5) and antitumor areas (6,7). The identification of potent inhibitors of hydroxymethylglutaryl-CoA reductase (8,9), which led to the development of clinically useful blood cholesterol lowering agents, is another pertinent example.

[1]An invited review in the series on mechanism-based studies of natural products in drug discovery.

STRATEGIES FOR THE DISCOVERY OF NOVEL INHIBITORS AND ANATAGONISTS.—The foregoing examples, which underscore the feasibility of identifying small molecules that interact specifically with individual enzymes and receptors, illustrate several approaches that can be utilized depending on the amount of information available concerning the structure and function of the macromolecular effector, as well as any small molecule(s) with which it is known to interact. Although no generally applicable scheme can be outlined to guide the identification of potent inhibitors and antagonists, two observations can be made.

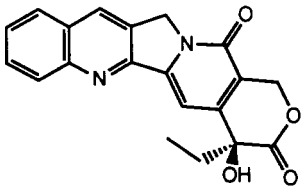
The first is that the key to identifying novel inhibitors/antagonists in a mechanism-based program is to fully exploit the biochemistry of the target macromolecule. Knowledge of enzyme and receptor kinetics specific to the target can be employed not only to identify inhibitors and antagonists, but also to define with a high degree of certainty the mechanisms by which they act. This information can be used to differentiate among structural leads and to focus on those of greater interest. For example, for the design of an enzyme inhibitor of possible utility for therapeutic intervention, one can readily exclude those agents that function by non-specific mechanisms. It may be noted that the experimental tools of molecular biology now provide the wherewithal to perform mechanism-based assays on virtually any macromolecule in well-defined assay systems.

Equally important for the identification of inhibitors/antagonists is the selection of compounds for evaluation. If the natural substrate/ligand of the macromolecule is a small molecule of known structure, the problem may be simplified. However, this is not necessarily the case, as demonstrated by the considerable effort required to identify adrenergic antagonists that were structural analogues of the normal ligands (10). If the substrate itself is a macromolecule, or is unknown, then there exists very little basis upon which to identify a well-defined group of compounds for mechanistic evaluation. Instead, the strategy must be to evaluate a structurally diverse group of molecules in order to discover one that exhibits the desired properties. Since any single compound has only a low probability of functioning in the desired fashion, this approach is feasible only when large numbers of molecules can be evaluated with facility. The synthesis of large numbers of molecules of diverse structure for evaluation in a single assay system is unlikely to prove practical, but the evaluation of samples from a large collection of compounds prepared previously can often be fruitful. Further, the sensitivity of many assay systems permits the evaluation of mixtures of compounds for the presence of a single compound of interest, which can expedite the identification of structures of potential interest for more detailed evaluation. A survey of extracts derived from natural sources (e.g., plants, microorganisms, marine organisms) permits the simultaneous evaluation of the numerous compounds contained in these complex matrixes, and offers the additional advantage that any inhibitor/antagonist discovered may well prove to have a novel structure. The same assay system employed to detect the presence of an inhibitor or antagonist in a complex biological matrix can also be used to guide the fractionation of the extract to provide the purified active principle(s) for structural and biochemical characterization.

Clearly, any strategy that relies on the evaluation of a large number of molecules, either as single species or as mixtures, and whose prospects for success are in direct proportion to the number of materials assayed, will prove most effective when the assay system is configured in a fashion that permits the testing of large numbers of samples with relative facility. The automation of assays can also dramatically increase the throughput of samples, and thereby contribute importantly to the prospects for identifying molecules having the desired properties.

INHIBITION OF DNA TOPOISOMERASE I.—Several cellular processes, including DNA replication, and recombination (11,12), require alteration of the topology of DNA. Changes in DNA topology are mediated by enzymes called topoisomerases, which function by transiently cleaving either one (topoisomerase I) or both (topoisomerase II) strands of the DNA duplex (11–14). The essential nature of the foregoing cellular processes suggests that inhibition of topoisomerase function might constitute a useful strategy for the identification of potential antitumor agents. Consistent with this thesis, it has been shown that some existing antineoplastic agents can inhibit DNA topoisomerase II (15,16). Interestingly, cellular topoisomerase II activity is correlated with cell cycle progression and proliferative state (17). Since topoisomerase activity must be present to permit its exploitation as a target for exogenously applied cytotoxic agents, the observed variation in cellular topoisomerase II suggests that agents that act at this locus might sometimes prove less effective, e.g., in the case of slow-growing tumors or where other antineoplastic agents block the progression of tumor cells through the cell cycle. In contrast, cellular topoisomerase I activity remains constant (17), making this enzyme a logical target regardless of the cell cycle kinetics or growth rate of the tumor cell.

In the context of the foregoing discussion concerning the identification of specific enzyme inhibitors, it may be noted that little information is available concerning the structure of mammalian topoisomerase I, and that the substrate for the enzyme (DNA) is a macromolecule. At present, the only well-characterized inhibitor of topoisomerase I is camptothecin [**1**] (18–20; see however, 21), a naturally occurring alkaloid originally identified on the basis of its antineoplastic activity (22,23). The ability of the camptothecins



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to exhibit antitumor activity in animal tumor models (23–25) and clinical trials (26–28), apparently by virtue of their action at the locus of topoisomerase I, prompted us to seek additional types of inhibitors of this enzyme. While this effort could not be guided by knowledge of enzyme or substrate structure (see above), the biochemistry of topoisomerase I is understood in some detail, as is the mechanism of inhibition by camptothecin (18–20). Accordingly, we developed a series of assays based on the known biochemistry of topoisomerase I. A primary assay was used to identify putative inhibitors present as constituents of plant and marine extracts; secondary assays permitted the active principles in the crude extracts to be categorized by mechanism of inhibition even before the active principles were purified and identified. Also described is the use of these assays to guide the identification and isolation of two natural products that specifically inhibit DNA topoisomerase-I-mediated DNA relaxation.

RESULTS AND DISCUSSION

Initially, all extracts were assayed for their ability to stabilize the topoisomerase I-DNA cleavable complex or to inhibit the relaxation of supercoiled plasmid DNA. Reaction mixtures were analyzed by agarose gel electrophoresis. Figure 1 shows the results from a typical experiment in which 32 organic extracts of marine organisms were

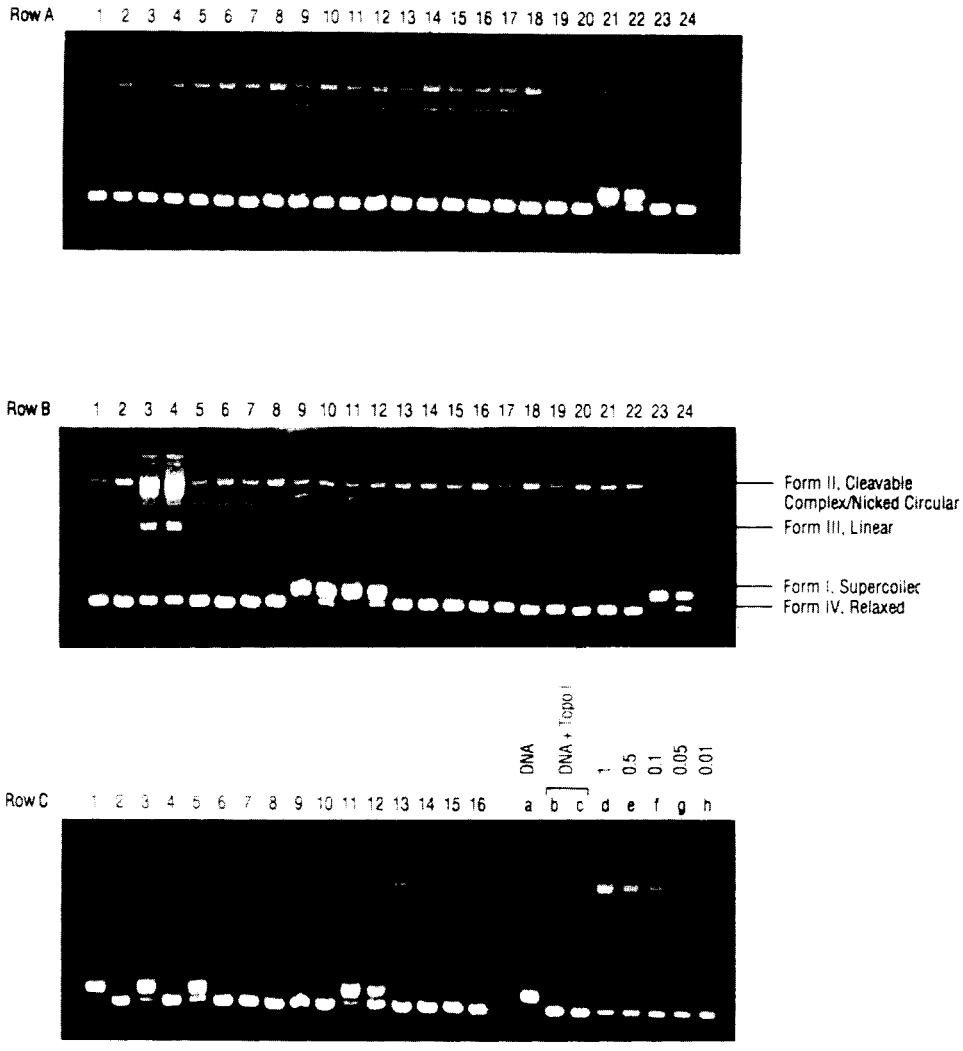
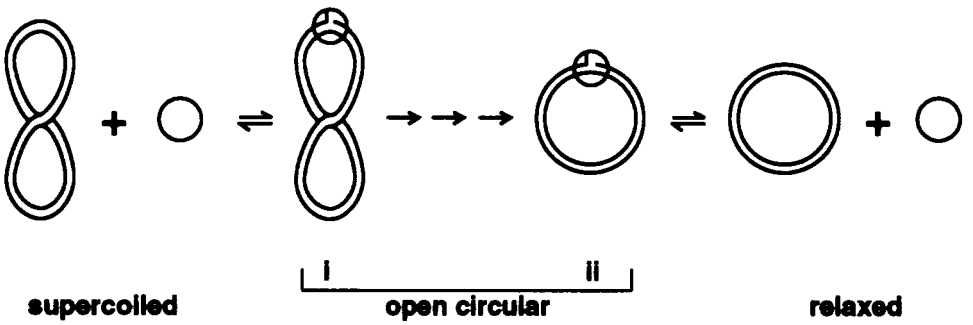


FIGURE 1. Assay of marine extracts to identify putative inhibitors of topoisomerase I function. Extracts were dissolved in DMSO and added to reaction mixtures containing supercoiled plasmid DNA. Topoisomerase I, purified from COLO 201 human colon adenocarcinoma cells, was added, and the inhibition mixtures were maintained at 37° for 30 min. The reactions were stopped by addition of sodium dodecyl sulfate and proteinase K, followed by further incubation at 37° for 1 h. The reaction mixtures were then applied to a 1% agarose gel containing 1 μg/ml of ethidium bromide and analyzed by electrophoresis at 40 V for 16 h. The results from 32 extracts are shown, each tested at 100 (odd-numbered lanes) and 10 μg/ml (even-numbered lanes). The various forms of DNA are indicated. The DNA migrating between the form II and form III bands was a supercoiled higher-order concatenane contaminating the plasmid preparation. Camptothecin [**1**] served as a control and was tested at 0.01 to 1 μM (lanes Cd–h). Untreated DNA is shown in lane Ca and relaxed DNA is shown in lanes Cb and Cc.

tested at 100 (odd-numbered lanes) and 10 μg/ml (even-numbered lanes) for their effect on the topoisomerase I-mediated DNA relaxation. Relaxed DNA (form IV; lanes Cb and Cc) had the greatest mobility, followed by supercoiled (form I; lane Ca), linear (form III; lanes B3–4) and open circular (nicked, form II; present in all lanes as a contaminant but enhanced in lanes B3–4) forms. The cleavable complex, an open circular intermediate (Scheme 1) in which the enzyme and DNA are covalently linked, comigrated with form

II DNA as it was assayed following digestion with proteinase K. This complex forms in the presence of topoisomerase I alone, but only at low concentration (e.g., there is no visible difference between lanes Ca and lanes Cb or Cc). However, camptothecin reversibly stabilizes the cleavable complex, raising its steady-state level and thereby increasing its visibility in the gel (lanes Cd–h). Eight extracts (lanes A21–22, B9–12, B23–24, C1–6, and C11–12) clearly inhibited relaxation of supercoiled DNA. One extract (lanes B3–4) caused the appearance of both forms II and III DNA, indicating the presence of both single- and double-strand breaks. Because topoisomerase I does not catalyze double-strand breakage, this extract must have induced DNA breaks independent of an enzyme-mediated process. Therefore, it was concluded that this extract did not contain a specific inhibitor of topoisomerase I.

Many extracts of marine organisms, particularly those prepared by soaking in an



SCHEME 1. Mechanism of relaxation of a supercoiled covalently closed circular DNA by topoisomerase I. The enzyme converts supercoiled (form I) DNA to relaxed (form IV) DNA via the intermediacy of open circular DNA in which one strand of the DNA is broken and covalently attached to topoisomerase I through a phosphorotyrosine linkage. The steady state concentration of this intermediate (the "cleavable complex") is increased in a concentration-dependent fashion by the alkaloid camptothecin, which binds reversibly to the covalent enzyme-DNA binary complex (cf. Figure 1, lanes Cd–Ch).

aqueous medium, increased the amount of DNA migrating as form II DNA. In order to determine if this represented enhancement of cleavable complex formation or merely nicked DNA molecules, extracts that showed this pattern of activity were assayed in the presence and absence of topoisomerase I. Figure 2 shows the results from 12 extracts that were tested at 100 μg/ml in the absence of topoisomerase I, and at 100 and 50 μg/ml in the presence of topoisomerase I. Several different patterns of responses are evident. Three extracts (A, B, and J, in lanes A1–3, A4–6, and B4–6) increased the recovery of both forms II and III DNA in the presence and absence of enzyme. No supercoiled or relaxed circular DNA was evident. These extracts caused substantial enzyme-independent DNA scission, as evidenced by the DNA degradation products apparent below the form III material. Other extracts (E, F, I, K, and L, in lanes A13–15, A16–18, B1–3, B7–9, and B10–12) increased the recovery of forms II and III DNA but produced no DNA degradation products and did not inhibit enzyme-catalyzed relaxation, as form IV DNA was apparent. Extract D (lanes A10–12) caused an increase in form II but not form III DNA (compare to lanes Be–h). This could represent enhancement of cleavable complex; since DNA relaxation was not inhibited, this extract showed the pattern expected of a molecule with camptothecin-like activity. Camptothecin produced a dose-dependent increase in cleavable complex and did not inhibit relaxation (lanes Ba–d). A survey of

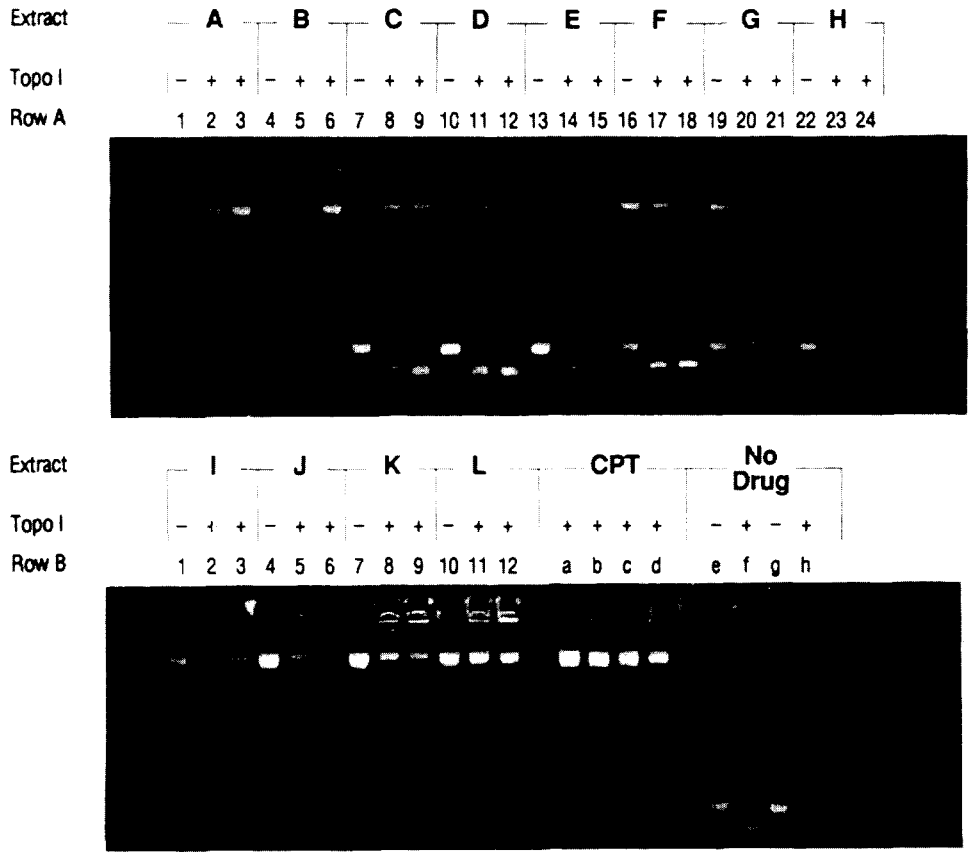


FIGURE 2. Effect of marine extracts on DNA relaxation and form II DNA formation in the presence and absence of topoisomerase I. Reactions were performed as described in the legend of Figure 1, except that each of the 12 extracts was also tested at 100 and 50 μg/ml in the presence of enzyme and at 100 μg/ml in the absence of enzyme. Supercoiled DNA is shown in lanes Be and Bg, relaxed DNA in lanes Bf and Bh. Camptothecin was tested at 10, 5, 1, and 0.5 μM (lanes Ba–d, respectively).

extracts prepared from terrestrial plants also afforded a significant number that exhibited inhibition of topoisomerase I-dependent DNA relaxation (not shown).

Ideally, a novel inhibitor of topoisomerase I should be specific in its effects, interacting only with this enzyme and not interfering with the functions of other enzymes that have DNA as a substrate (e.g., DNA polymerases, nucleases, etc.). Therefore, compounds that bind to DNA in the absence of topoisomerase I are not likely to be good candidates for specific inhibitors. As noted above, other DNA interactive compounds can cause DNA breakage that is both nonspecific and topoisomerase-independent. Therefore, in order to identify authentic inhibitors of topoisomerase I, all DNA-interactive compounds were eliminated. On the other hand, molecules that bind directly to topoisomerase I in the presence or absence of DNA could well be useful lead compounds. While camptothecin acts reversibly to inhibit topoisomerase I, it is not clear a priori whether reversibility is a desirable characteristic for a topoisomerase I inhibitor in the context of potential antitumor activity. Similarly, it is not clear whether stabilization of the cleavable complex is an essential feature of potential antineoplastic agents. It seems possible that any compound which acts to inhibit DNA relaxation may demonstrate potentially useful antitumor activity. Hence a series of assays was devised

binding to DNA, or which produced enzyme-independent DNA strand scission. The emphasis was placed on identifying compounds which acted at the level of topoisomerase I binding, whether in the presence or absence of DNA. The assays also served to characterize certain facets of the process by which inhibition of topoisomerase I function was achieved.

Those extracts found to contain putative inhibitors of topoisomerase I function (Figures 1 and 2) were assayed for the presence of agents which function via DNA binding. Preincubation with DNA-cellulose diminished the ability of some of the crude extracts containing putative topoisomerase I inhibitors to prevent enzyme-dependent DNA relaxation, presumably because the inhibitory activity was actually due to DNA binding agents that bound to the DNA-cellulose prior to the enzyme assay. Incubation with DNA-cellulose also eliminated DNA mobility shifts caused by some extracts, which were attributed to the presence of DNA binding agents. Figure 3 shows the analysis of eight extracts tested at 100, 50 and 0 μg/ml, with and without preincubation with DNA-cellulose. All of the extracts except M and R (lanes A1–6 and lanes B7–12) inhibited enzyme-mediated DNA relaxation. Lanes A22–24 show the results obtained with an extract (P), which effectively inhibited DNA relaxation when not preincubated with DNA-cellulose, but was completely inactive after preincubation with DNA-cellulose (lanes A19–21). A similar, if less dramatic, effect was noted for extract T, while preincubation with DNA-cellulose had no effect on the inhibition of DNA relaxation caused by extracts N, O, Q, or S. None of the extracts in this experiment caused a shift in the mobility of form I DNA in the absence of topoisomerase I. On the basis of this type of analysis, extracts whose effect on topoisomerase I function was judged to be due primarily to DNA binding could be conveniently eliminated from further consideration.

This strategy for identifying putative DNA-interactive molecules present in crude extracts was validated by the use of a variety of structurally dissimilar molecules whose ability, or lack of ability, to bind to DNA had been documented previously. The agents employed included doxorubicin and echinomycin, both of which are known to bind strongly to DNA (29,30). As anticipated, both agents caused pronounced DNA mobility shifts which were greatly diminished by preincubation with DNA-cellulose (data not shown). Preincubation of authentic DNA binding drugs with cellulose, rather than DNA-cellulose, had no effect on their ability to affect DNA relaxation by topoisomerase I.

Once the DNA binding compounds had been eliminated, virtually all of the remaining extracts that inhibited DNA relaxation in a topoisomerase I-dependent fashion represented attractive leads. In order to obtain additional information concerning the probable mechanism of inhibition by individual extracts, further assays were utilized. For example, it has been shown that the cleavage of double-stranded DNA by topoisomerase I is a fully reversible process (18,20). The cleavage of single-stranded DNA, on the other hand, is not, because hydrogen bonding between base-pairs does not maintain the free ends in apposition after cleavage has occurred (12,31). This observation formed the basis for an assay to determine if inhibitory principles exerted their activity by binding to topoisomerase I. The only extracts tested were those that had been shown not to be affected by preincubation with DNA-cellulose; therefore, if they could inhibit single-strand DNA cleavage, they must do so by binding to topoisomerase I either before or after the enzyme is bound to the single-stranded DNA. Figure 4 shows that single-strand ϕX174 DNA was not only linearized by topoisomerase I but also cleaved at multiple sites to yield additional bands on the gel. This reaction was topoisomerase I-dependent and unaffected by camptothecin (data not shown), the latter of which is known to bind only to a binary complex of topoisomerase I and DNA (20). Figure 4 shows the effects of 11 extracts on this reaction; each was tested at 100 (odd-numbered

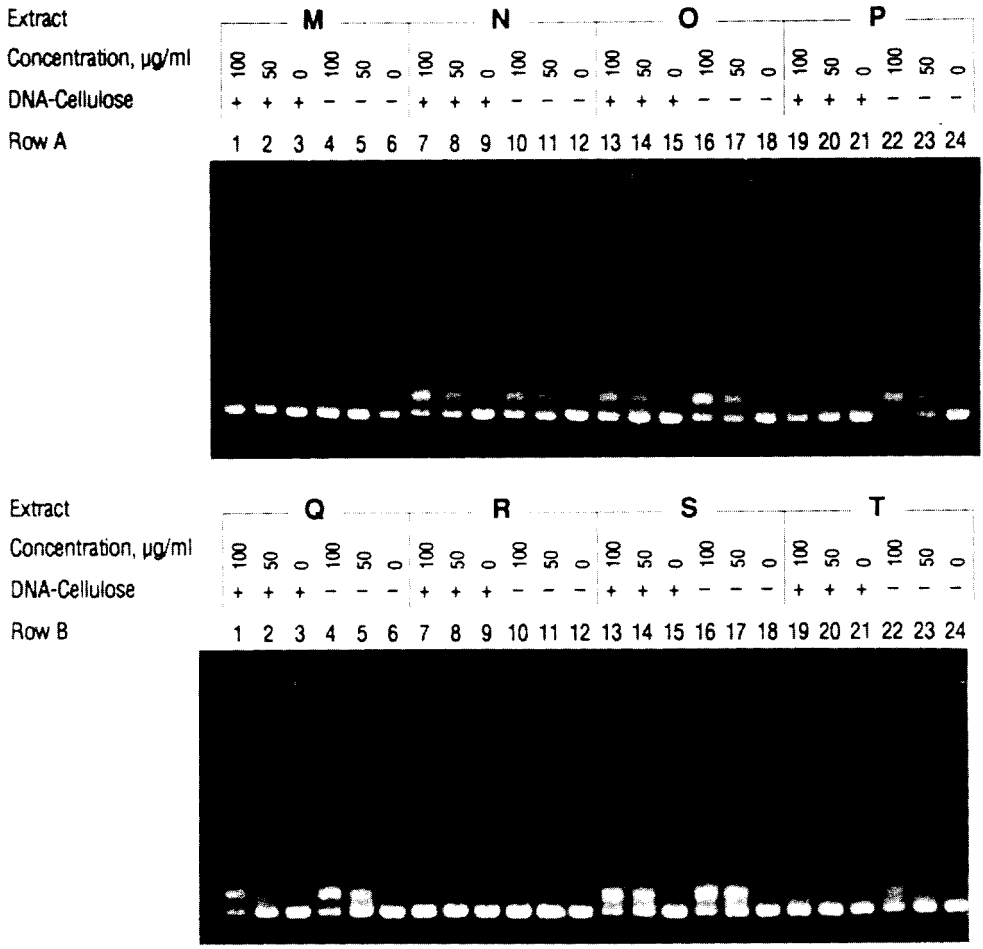


FIGURE 3. Effect of preincubation with DNA-cellulose on the ability of marine organism extracts to inhibit topoisomerase I function. Extracts were dissolved in DMSO, and part of each sample was incubated with DNA-cellulose (1 mg/ml DNA concentration) with gentle mixing for 1 h. The suspensions were centrifuged to remove the DNA/cellulose, and aliquots of the supernatants were introduced into topoisomerase I-DNA relaxation assays. Other aliquots of the same extracts were not treated with DNA-cellulose; they were subsequently added to separate DNA relaxation assays. Aliquots of each extract were assayed at 100, 50, and 0 µg/ml as described in Figure 1 to determine their effects on topoisomerase I-mediated DNA relaxation.

lanes) and 50 µg/ml (even-numbered lanes). In the absence of any extract, circular DNA was converted to linear DNA by topoisomerase I (lane b), and a set of smaller molecules was apparent (compare to lane a). One extract prevented this conversion. Lanes 15–16 show that the extract tested inhibited linearization at 100 µg/ml (lane 15) but not at 50 µg/ml (lane 16). Other extracts tested completely inhibited the reaction at both concentrations (not shown).

While formation of the covalent topoisomerase I-DNA intermediate can be visualized on gels as the cleavable complex, it can also be quantitated by its adsorption to membrane filters. Figure 5 (panel a) shows that [^{14}C]DNA was not retained on nitrocellulose filters either alone or in the presence of 10 µM camptothecin. Incubation with topoisomerase I increased DNA adsorption to the filters three-fold, but retention was stimulated tenfold when camptothecin was included. Figure 5b shows the results

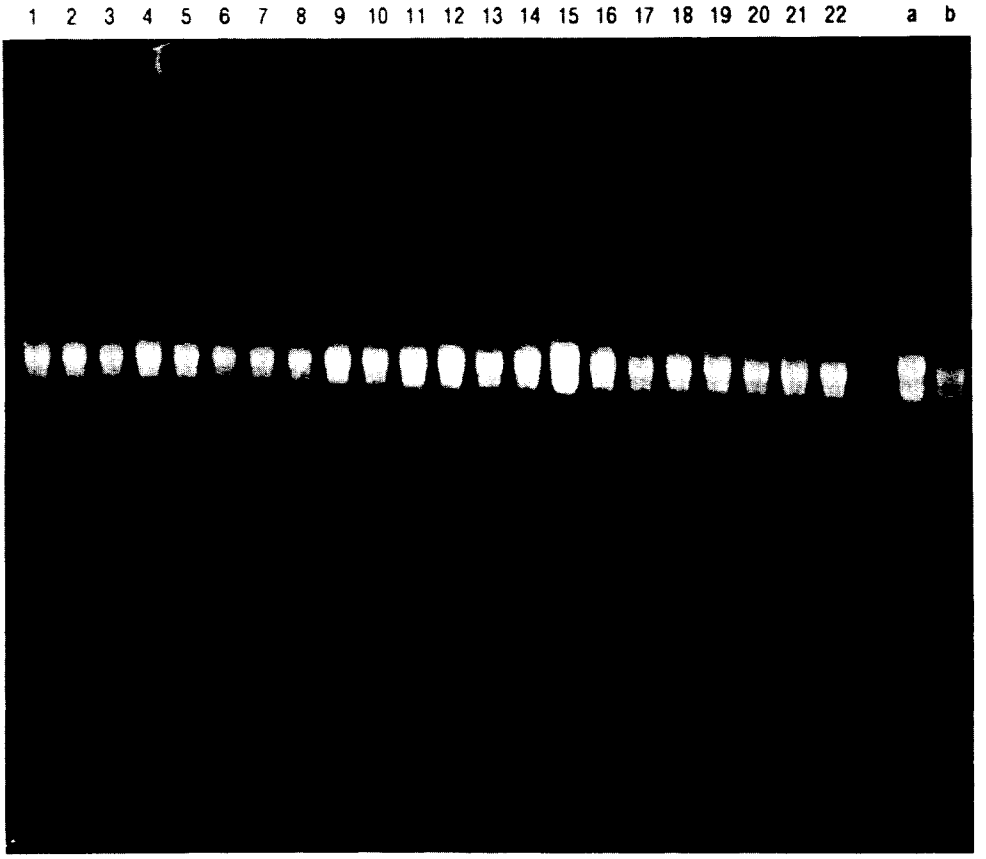


FIGURE 4. Effect of crude extracts on the cleavage of single-stranded DNA by topoisomerase I. φX174(+) single-stranded DNA was incubated with topoisomerase I and crude extracts at 37° for 20 min. Final concentrations were 100 (odd-numbered lanes) and 50 μg/ml (even-numbered lanes). Reactions were terminated by the addition of sodium dodecyl sulfate and proteinase K and then applied to a 1% agarose gel for electrophoretic analysis. Lane a contained DNA alone; lane b contained DNA and topoisomerase I, but no extract.

obtained in the same experiment when crude extracts of marine organisms were assayed for their ability to stabilize the topoisomerase I-DNA complex. In the absence of enzyme, extracts U, V, and W did not affect the retention of DNA, while in the presence of enzyme, extracts U and V, and to a lesser extent W, caused a concentration-dependent increase in DNA retention. Extract V was less potent than extract U, and extract W had less effect still. Extract X efffected little, if any, retention of DNA in an enzyme-dependent fashion. Therefore, extracts U, V, and possibly W appeared to stabilize the cleavable complex in a manner similar to camptothecin while extract X did not. It may be noted that this assay system, while more convenient experimentally than agarose gel analysis (Figure 1), cannot distinguish between compounds that stabilize the DNA-topoisomerase I cleavable complex and species which simply have a high affinity for the enzyme or enzyme-DNA complex. Reversible inhibitors could be further characterized by variation of the salt and detergent employed in this assay to provide information on affinity (data not shown). This assay also revealed that substances which inhibited relaxation but did not stabilize the cleavable complex sometimes decreased the enzyme-mediated retention of DNA on filters (not shown).

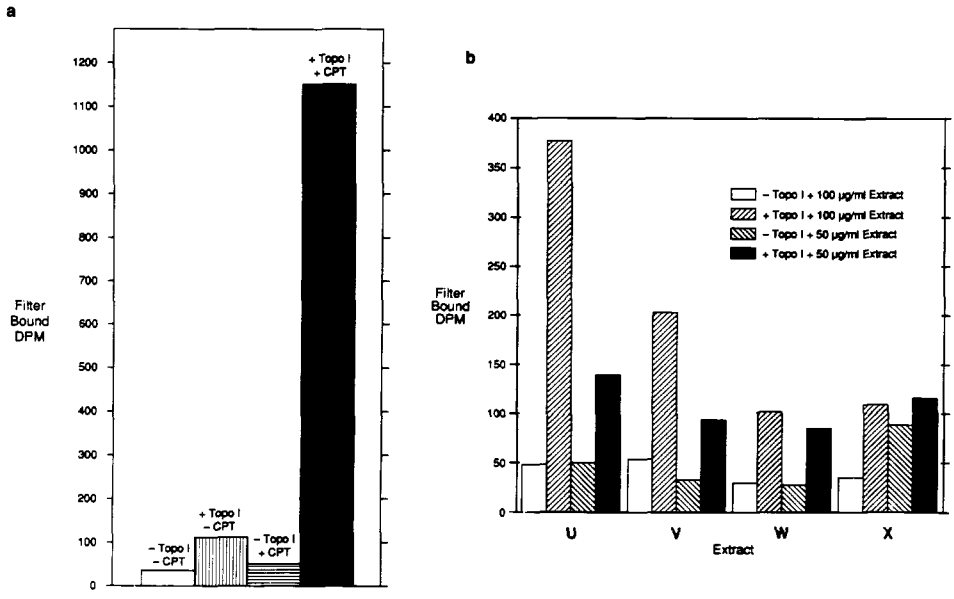


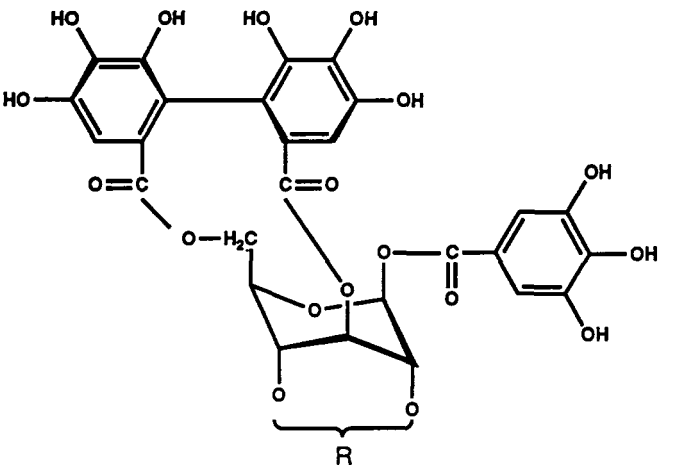
FIGURE 5. Topoisomerase-I-mediated DNA filter binding. (a) The cleavage assay was performed as described in Figure 1, except that *Escherichia coli* [^{14}C]DNA was substituted for plasmid DNA. Camptothecin (CPT) was included at 10 µM concentration with and without topoisomerase I. Reactions were stopped by addition of sodium dodecyl sulfate and EDTA to final concentrations of 1% and 1 mM, respectively. Samples were applied to polyvinyl chloride filters and washed with EDTA/NaCl as described in the Experimental. Radioactivity remaining on the filters was quantitated. (b) In the same experiment, extracts were tested at 100 and 50 µg/ml, with and without topoisomerase I.

One criterion for evaluating the utility of an assay system designed to identify novel inhibitors of a target anticipated to have few selective inhibitors is that the occurrence of putative inhibitors in the collection of compounds tested should be relatively low. This criterion was satisfied for the series of assays presented here. Only 7% of 241 marine extracts tested displayed reproducible inhibition of DNA relaxation that did not result primarily from DNA binding per se. The active extracts included 2% that stimulated filter binding by more than fourfold in the presence of enzyme (see Figure 5), thereby mimicking the action of camptothecin in this assay. In fact, some of the extracts were able to block the binding of camptothecin to the topoisomerase-I-DNA binary complex (data not shown). Two extracts were observed to inhibit the cleavage of single-stranded ϕX174 DNA by topoisomerase I, presumably by binding directly to the enzyme. A survey of approximately 1100 plant extracts indicated a similar frequency of occurrence of active constituents, which also displayed the range of putative mechanisms of inhibition noted for the marine extracts.

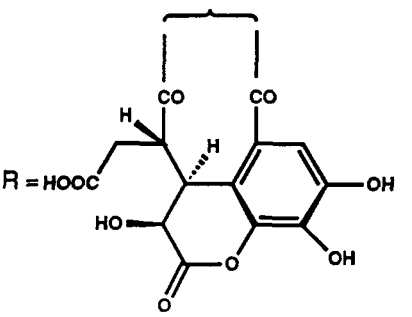
The foregoing assays have permitted the identification of specific extracts whose behavior suggested that they contained topoisomerase I inhibitors of special interest. For example, an MeOH extract of the plant *Erodium stephanianum* was found to inhibit the topoisomerase-I-dependent relaxation of plasmid DNA. Unlike camptothecin, the extract did not stabilize the cleavable complex (Scheme 1). However, the potency of the extract in mediating inhibition of DNA relaxation was exceptional, increased in proportion to the amount of extract employed, and could be shown not to be due to DNA binding by the active principle(s). In order to permit structural characterization of the active species, this extract was fractionated, and individual fractions were assayed for their ability to inhibit topoisomerase I-mediated relaxation of plasmid DNA.

As described previously (21), chromatography on a Sephadex LH-20 column, followed by further fractionation of the active principles by C_{18} reversed-phase hplc, provided two highly active compounds as off-white solids. The first of these compounds **2** had a mol wt of 634, as judged by fabms. Consistent with the ir spectrum, which had a broad absorption at 3600–3100 cm^{-1} indicative of the presence of OH groups, peracetylation afforded a derivative having M_r 1096. Of the eleven OH groups whose presence was inferred from this experiment, nine were methylated in the presence of CH_2N_2, suggesting that these OH groups were phenolic in nature. The presence of resonances in the ^1H- and ^{13}C-nmr spectra of **2** attributable to galloyl and 4,4′,5,5′,6,6′-hexahydroxydiphenoyl esters, and to a glucose moiety, suggested that **2** might be corilagin. In fact comparison of the ^1H- and ^{13}C-nmr spectra of **2** with those reported (32,33) for authentic corilagin indicated that the newly isolated compound was indeed corilagin. Compound **3** was analyzed by ms and found to have M_r 954. ^1H- and ^{13}C-nmr analysis of **3** indicated that this compound contained all of the structural elements present in **2** and additional highly functionalized aliphatic and aromatic moieties. Comparison with nmr spectra reported for chebulagic acid, chebulinic acid, and chebulic acid derivatives (34–37) permitted the assignment of **3** as chebulagic acid.

Studies of the mechanism of inhibition of topoisomerase-I-mediated DNA relaxation by **2** and **3** indicated that neither would stabilize cleavable complex formation, i.e., that neither acted in the same fashion as camptothecin. Further, while camptothecin [**1**]



2 R=H



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inhibited DNA relaxation only when topoisomerase I was present at very low concentration, corilagin [2] and chebulagic acid [3] inhibited DNA relaxation even when relatively high concentrations of topoisomerase I were employed. As would be expected, the extent of inhibition of DNA relaxation by **2** and **3** was directly proportional to the amount of inhibitor used, and inversely proportional to topoisomerase I concentration. For example, the IC_{50} values for inhibition of DNA relaxation by chebulagic acid was 50 nM in the presence of 14 units of topoisomerase I, and 100 nM when 70 units of enzyme was used in the assay. Corilagin [2] exhibited analogous behavior, although the extent of inhibition was less at all tested concentrations (e.g., **2** had an IC_{50} of 40 μM when 70 units of topoisomerase I was present). That compounds **2** and **3** did not act simply by binding to DNA may be judged from their lack of effect on the gel mobility of the DNA.

Although it did not stabilize formation of the cleavable complex, chebulagic acid [3] was about 10–50-fold more potent than camptothecin in inhibiting DNA relaxation mediated by topoisomerase I. Compound **3** was also >800-fold more potent than **1** in inhibiting the initial enzyme-mediated nicking of DNA. As such, it is the most potent inhibitor of topoisomerase I yet reported. The selectivity of inhibition by chebulagic acid was also studied; compound **3** was very much less potent as an inhibitor of DNA topoisomerase II and avian myeloblastosis virus reverse transcriptase (data not shown).

The successful isolation and identification of a potent topoisomerase I inhibitor as a constituent of a plant extract illustrates the way in which a mechanistically focused survey of a large collection of compounds can be used to identify a mechanistically unique inhibitor of an enzyme essential to cell function. It is clear that mechanism-based assays can provide an elegant, powerful, and efficient way in which to identify novel inhibitors and antagonists, including those of interest as potential therapeutic agents. If the biochemistry of the target is understood in sufficient detail, assays can be designed for biochemical systems that vary from highly purified cell-free systems to cell- and tissue-based protocols. Mechanisms can be explored in some detail, and techniques or tests can be implemented to avoid interference by substances expected to produce nonspecific inhibition.

EXPERIMENTAL

DNA RELAXATION ASSAY.—The assay for relaxation/cleavage of supercoiled DNA was adapted from that of Liu and Miller (38). Plasmid DNA (100 ng pDPT2789, 6.4 kb, covalently closed supercoils) was incubated with 260 units of topoisomerase I from COLO 201 human colon adenocarcinoma cells in a reaction buffer (20 μl) containing 40 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 μg/ml bovine serum albumin, and pure compounds or crude extracts (dissolved in DMSO to 10 mg/ml, final DMSO concentration 1%). After 30 min at 37°, the reaction was stopped by incubation for 1 h with sodium dodecyl sulfate (SDS) and proteinase K at final concentrations of 2.5% and 0.75 mg/ml, respectively. Reaction products were then analyzed by electrophoresis on 1% agarose gels containing 1 μg/ml of ethidium bromide.

BINDING TO DNA-CELLULOSE.—Samples were added to reaction buffer containing native DNA-cellulose (Pharmacia) so that the extract and DNA concentrations were 1 mg/ml. After gentle mixing for 1 h, the suspensions were centrifuged for 3 min at 12,000 g in a Beckman Microfuge 12, and aliquots of the supernatants were withdrawn for use in the DNA relaxation assay described above.

CLEAVAGE OF SINGLE-STRANDED DNA.—Cleavage of ϕX174(+) single-stranded DNA (ssDNA, Bethesda Research Laboratories) was measured as described by Halligan *et al.* (31). ϕX174(+) DNA (28 μg/ml) was incubated with topoisomerase I and extract (dissolved in DMSO and diluted to achieve a final solvent concentration of 1%) for 20 min at 37° in 10 mM Tris-HCl (pH 7.8), 50 mM KCl, and 1 mM $MgCl_2$. Reactions were terminated by the addition of SDS and proteinase K as above. Products were analyzed on 1% agarose gels and visualized by staining with ethidium bromide.

DNA FILTER BINDING.—The method of Minford *et al.* (39) was modified to permit measurement of the retention of topoisomerase I-DNA complexes on membrane filters. The cleavage reaction was performed as described above, except that *Escherichia coli* ^{14}C-DNA (New England Nuclear) was substituted for plasmid DNA. The reactions were quenched with 100 μl of 2% SDS and 2 mM EDTA (pH 10) and applied to

polyvinyl chloride filters (Millipore type BS, 2 μm pore size, 24 mm diameter). Filters were rinsed with 3 ml of 1 mM EDTA, 3 ml of 2 M NaCl/1 mM EDTA, and then another 3 ml of 1 mM EDTA. Radioactivity retained on the filters was then determined.

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