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Novel and Specific Inhibitors of a Poxvirus Type I Topoisomerase

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ABSTRACT

Vaccinia DNA topoisomerase (vTopo) is a prototypic pox virus family topoisomerase that shares extensive structural and mechanistic properties with the human type IB enzyme (hTopo) and is important for viral replication. Despite their far-reaching similarities, vTopo and hTopo have surprisingly distinct pharmacological properties. To further exploit these differences, we have developed recently the first high-throughput screen for vTopo, which has allowed rapid screening of a 1990-member small-molecule library for inhibitors. Using this approach, 21 compounds were identified with IC_{90} values less than 10 μM, and 19 of these were also found to inhibit DNA supercoil relaxation by vTopo. Four of the most potent compounds were completely characterized and are structurally novel topo I inhibitors with efficacies at nanomolar concentrations. These inhibitors were highly specific for vTopo, showing no inhibition of the human enzyme even at 500- to 2000-fold greater concentrations. We describe a battery of efficient experiments to characterize the unique mechanisms of these vTopo inhibitors and discuss the surprising promiscuity of this enzyme to inhibition by structurally diverse small molecules.

There are nine pox viruses that cause human disease (Fields and Kinpe, 1990). These large DNA viruses encode for many of their own enzymes of DNA metabolism, including a small type IB topoisomerase (vTopo) that is closely related to the human type I topoisomerase (hTopo) (Shuman, 1998). The viral enzyme has been shown to be important for virus replication (Shuman et al., 1989; Da Fonseca and Moss, 2003) and is a potential target for drug treatment against smallpox and molluscum contagiosum virus, a pox virus that infects immunocompromised patients causing painful and disfiguring skin lesions (Hwang et al., 1998). The poxvirus topoisomerases are distinguished by their specificity for forming reversible phosphotyrosine linkages with the backbone of DNA at the pentapyrimidine consensus sequence (T/C)CCTT↓, where the arrow denotes the site of nucleophilic attack (Shuman, 1998).

Although the active sites of the poxvirus and human enzymes are congruous, there are sufficient differences in their modes of DNA recognition that allow selective targeting of small molecules. Examples include the potent poisoning of hTopo by the anticancer drug camptothecin, which is essentially ineffective against the pox enzyme (Gupta et al., 1992; Liu et al., 2000). Likewise, vTopo is competitively inhibited by two coumarin drugs, novobiocin and coumermycin, which show little activity against hTopo (Sekiguchi et al., 1996). Other DNA binding ligands, such as the intercalator nogalomycin, can affect the activity of both enzymes, although the mechanisms are quite distinct (Sim et al., 1997, 2000; Yakovleva et al., 2004). Taking together, these findings indicate that there is significant opportunity for discovery of ligands that interact selectively with these enzymes. Despite this realization, there are no robust technologies available for efficient attainment of this goal.

We reported recently the first continuous fluorometric assay for topoisomerase that allows monitoring of the reaction under multiple-turnover conditions (Kwon et al., 2004). The assay takes advantage of sensitive molecular beacon technology and the surprising ribonuclease reaction of vTopo that allows recycling of the enzyme for multiple turnovers, as shown in Fig. 1. Here, we use this assay to blindly screen a 1990-member small-molecule library and to identify several specific inhibitors of the poxvirus topoisomerase that are orders of magnitude more potent than those known previously. Structural comparison of library hits revealed seven compounds with significant similarity to four known families of topoisomerase IB inhibitors, establishing the validity of the ribonuclease assay in the discovery of ligands that affect the DNA reactions of this enzyme. It is noteworthy that the remaining hits were structurally distinct from any known

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ABBREVIATIONS: vTopo, vaccinia DNA topoisomerase IB; hTopo, human topoisomerase IB; FAM, 6-carboxyfluorescein; topo, topoisomerase; DAB, dabcyl group; NCI, National Cancer Institute; HTP, high throughput; pUC19, pUC19 plasmid DNA purified from *Escherichia coli* strain DH5α.

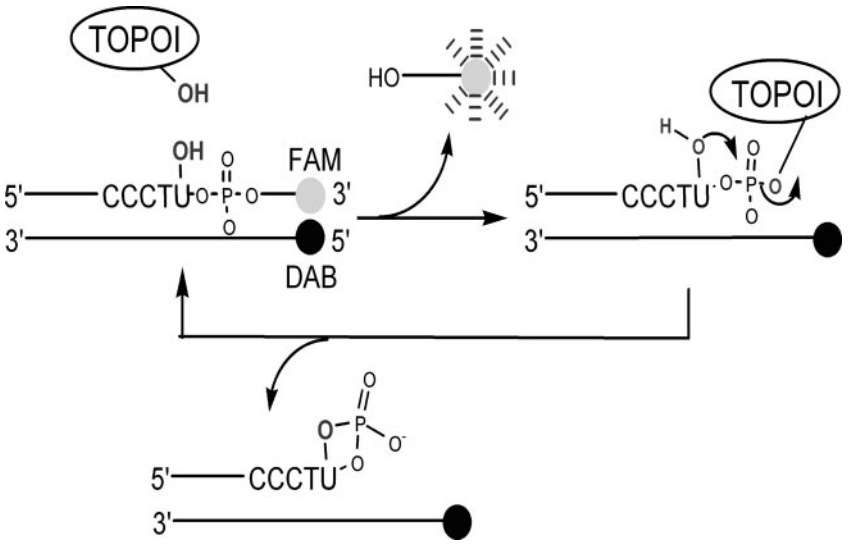


Fig. 1. Molecular beacon assay for topo I. The assay relies on the strong quenching of the 3′ 6-carboxyfluorescein fluorophore (FAM) in the substrate by a dabcyl group (DAB) that is covalently attached to the 5′ end of the complementary strand. Upon cleavage of the scissile strand by topo I, the short strand with the FAM group is released to solution, resulting in an increase in fluorescence. Recycling of the enzyme for multiple turnovers is accomplished by placing the ultimate thymidine of the CCCTT motif with uridine. Attack of the 2′ hydroxyl of the uridine ribonucleotide expels the active site tyrosine from its covalent linkage with the DNA.

topo ligands, and four of these were found to be active in the nanomolar concentration range. A mechanistic characterization of the most potent inhibitors is described.

Materials and Methods

Enzymes and Plasmid DNA. The cloning and purification of wild-type and Y274F vaccinia topoisomerase has been described previously (Kwon and Stivers, 2002; Kwon et al., 2002). The enzyme concentration was determined by UV absorbance using an extinction coefficient of 28,140 $M^{-1}cm^{-1}$ in a buffer containing 20 mM sodium phosphate, pH 6.0, and 6 M guanidinium hydrochloride (Kwon and Stivers, 2002). Human topoisomerase was obtained from Sigma. pUC19 plasmid DNA was purified from *Escherichia coli* strain DH5α (pUC19).

RNA and DNA Substrates with Fluorescent Tags. The sequence of the 18 mer DNA/RNA substrate containing a single uridine ribonucleotide substitution for the 3′ thymidine residue of the consensus cleavage sequence is shown here, where FAM is 6-carboxyfluorescein and DAB is the universal fluorescence quencher, dabcyl[1]: 18U-FAM/18-DAB, 5′ CGTGTCGCCCTUATTCCG-FAM-3′, and 3′ GCACAGCGGGAATAAGGC-DAB-5′.

For the DNA cleavage and religation reactions, a 18/24 mer duplex was synthesized in which the scissile strand contained two modifications. First, the 5′-end was modified with a FAM label, and second, the nucleotide just 3′ of the ultimate T of the pentameric consensus sequence (underlined), was labeled with the fluorescent adenine analog 2-aminopurine (P): FAM-18AP/24, 5′-FAM-CGTGTCGCCCTTPTTCCG-3′, and 3′-GCACAGCGGGAATAAGGCTATCAC-5′.

These two probes allowed measurement of protein binding by monitoring the increase in fluorescein anisotropy of the DNA and DNA cleavage by the increase in 2-aminopurine fluorescence when the 6 mer leaving group rapidly dissociates after strand scission (Kwon and Stivers, 2002). The oligonucleotide strands were synthesized using an ABI 394 synthesizer (Applied Biosystems, Foster City, CA) using nucleoside phosphoramidites obtained from Glen Research (Sterling, VA). The oligonucleotides were purified using anion exchange high-performance liquid chromatography and desalted using disposable gel-filtration columns (G-10). The purity of oligonucleotides was confirmed using electrophoresis through a 20% polyacrylamide gel containing 7 M urea and matrix-assisted laser desorption ionization/time of flight analysis. The DNA duplexes were prepared in buffer A (20 mM Tris-HCl and 200 mM NaCl, pH 9.0) by mixing the two strands in a molar ratio of 1.15:1(the dabcyl-labeled strand was in excess).

Steady-State Fluorescence Anisotropy Measurements for Noncovalent DNA Binding. Samples (150 μl containing 0.1 μM FAM-18AP/24) were excited with vertically polarized light at 492 nm (1-nm bandpass), and both vertical and horizontal emissions were monitored at 517 nm (3-nm bandpass). Three replicate measurements were made for each addition of topoisomerase in the range 0 to 0.15 μM, and the values were then averaged. The G factor was calculated, and its value was used to calculate the anisotropy. To determine whether library compounds displaced the bound DNA from Y274F, a complex was formed using 0.1 μM FAM-18AP/24 and 2 μM Y274F, and the anisotropy was measured as increasing concentrations of compound were added to the solution (0–5 μM). As a positive control, after the addition of compound, the noncovalent DNA complex was completely disrupted by the addition of the competitive inhibitor ATP in the concentration range of 0 to 100 mM. In all cases, anisotropy measurements were carried out in triplicate, and the results were averaged.

High-Throughput Screening. For screening of the 1990-compound NCI Diversity Library, a Fluoromax-3 fluorimeter with a Micro-Max 96-well fluorescence plate reader attachment was used. Each reaction well in the screen contained enough test compound to give a 100 μM concentration after the addition of 100 μl of reaction solution containing enzyme (10 nM) and buffer A. Reactions were then initiated by the addition of 18U-FAM/18-DAB (0.5 μM final concentration in the well). For validation, positive and negative control wells were also included that consisted of enzyme and substrate without inhibitor and substrate without enzyme. The rates of fluorescence change at 517 nm with excitation at 492 nm were followed by taking six fluorescence readings in each well over a 30-min time period.

Inhibition of Steady-State Plasmid Supercoil Relaxation. Supercoil relaxation reactions were performed with 0.5 μg of supercoiled pUC19, 1 nM vTopo, or 2 U of hTopo and various concentrations of inhibitor. The reaction mixtures were incubated for 30 min (20 min for hTopo) at room temperature and were then quenched with one-fifth volume of 5× loading buffer containing 0.4% SDS, 5% glycerol, and Tris-acetate-EDTA. The relaxed DNA and supercoil DNA were resolved on 1% agarose gel at 135 V for 2 h in a Tris-acetate-EDTA running buffer. After ethidium bromide staining and fluorescence imaging using a GelDoc instrument (Bio-Rad, Hercules, CA), the bands corresponding to supercoiled and relaxed DNA in each lane were quantified using the software supplied with the

[1] In previous work, we had screened approximately 500 compounds from this library and thoroughly characterized one hit (NSC 112983) (Kwon et al., 2004). This compound bound exclusively to the enzyme-DNA covalent complex with a modest K_D value of 1.6 μM and did not inhibit DNA cleavage. The inhibitory mechanism involved recycling of the enzyme for catalytic turnover.

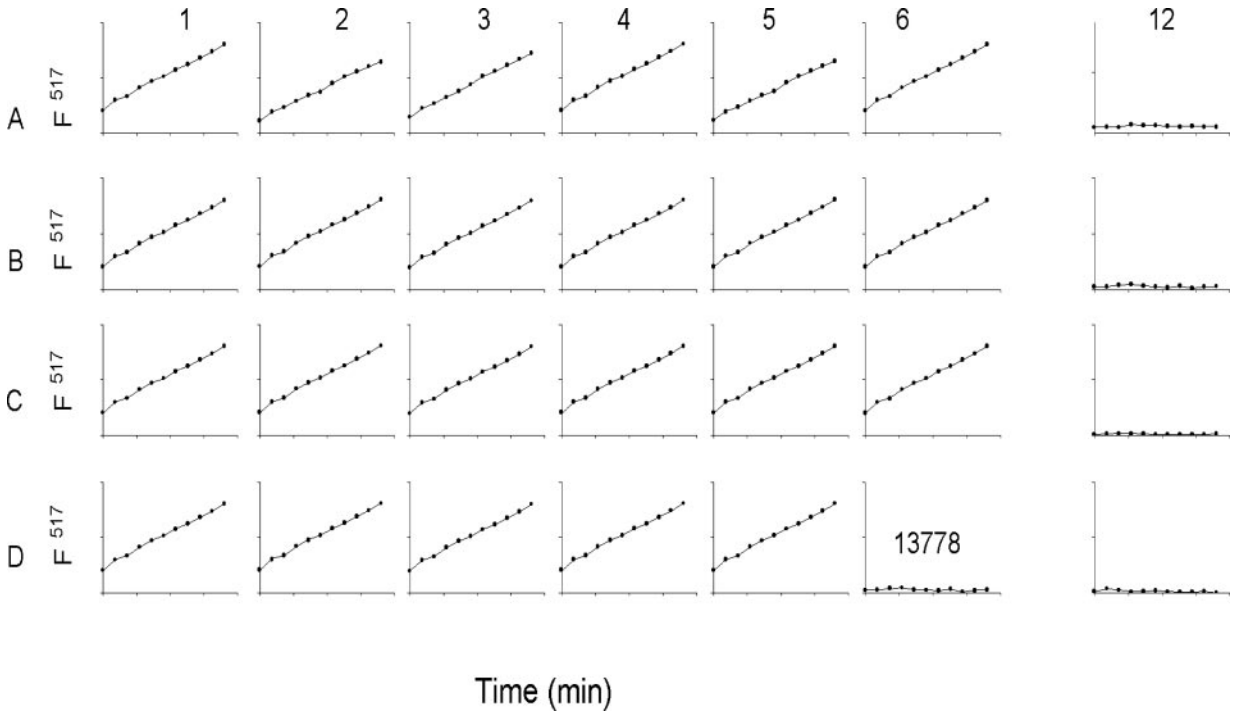


Fig. 2. Representative data from the high-throughput screen. The first four rows and first six columns of plate 3852 from the NCI diversity library are shown. This plate contained one of the most potent hits from the library, compound 13778. Column 1 contains the positive control wells with enzyme and substrate and no added compound, whereas column 12 contains the negative control wells with substrate alone in the absence of vTopo.

instrument. Inhibition results using the supercoiled relaxation assay were repeated three times to estimate the variability in the measurements.

Single-Turnover Cleavage Reactions. Inhibition of single-turnover cleavage was investigated using the 2-aminopurine-labeled DNA duplex (FAM-18AP/24) (Stivers et al., 2000). The reactions were performed using an stopped-flow fluorescence instrument in the two-syringe mode (Applied Photophysics, Leatherhead, Surrey, UK). Equal volumes of 1 μM vTopo and 50 nM substrate in the presence of varying concentrations of compound were rapidly mixed, and the increase in 2-aminopurine fluorescence was followed using excitation at 315 nm with monitoring at emission wavelengths greater than 360 nm using a cut-on filter (Kwon and Stivers, 2002). On average, five or more kinetic transients were averaged and fitted to a first-order rate equation to obtain the reported rate constants and errors.

Equilibrium Cleavage Measurements. The equilibrium cleavage reactions were performed in the presence and absence of inhibitors 13778, 48300, 14163, and 88915 in the concentration range 0 to 20 μM (Kwon and Stivers, 2002) using a buffer containing 20 mM Tris-HCl and 200 mM NaCl at pH 8. Wild-type topoisomerase (60 nM) and 5′-FAM-labeled 32/32-mer DNA duplex (80 nM) with the scissile strand sequence of 5′-FAM-CGTGTCGCCCTTNTTCCGATAGTGACTACAGC-3′ were incubated for 30 min at room temperature, and the covalent complex present at equilibrium was trapped by the rapid addition of 1 volume of 10% SDS (20 μl). The reactions in the presence of compound were performed by preincubating the compound with the enzyme or DNA for 10 min before initiating the reaction. The free and covalently bound DNA were separated by electrophoresis using a 10% polyacrylamide gel containing 0.1% SDS. The fraction covalent complex was then determined using eq. 1 by phosphorimaging: [Frac complex = counts covalent complex/(counts covalent complex + counts free DNA)] (eq. 1). The equilibrium cleavage measurements were repeated three times for error estimation.

Results

High-Throughput Screening for vTopo Inhibitors. We interrogated the 1990-member Diversity Library available from the NCI using the vTopo high-throughput ribonuclease activity screen. This library consists of small molecules that are selected from the larger 140,000-compound NCI library on the basis of availability, purity, and other diversity criteria. In addition, all of the compounds have been tested for in vitro cell killing using 60 human cancer tumor cell lines (http://www.dtp.nci.nih.gov/branches/btb/ivclsp.html). A 96-well HTP format was used in which each well contained 10 nM vTopo, 0.5 μM substrate, and 100 μM library compound, and representative data acquired from plate 3852 are shown in Fig. 2. Overall, using the primary HTP screening conditions, a total of 247 compounds were identified that inhibited vTopo activity by more than 50%, comprising a high hit rate of 12.4%[2].

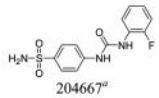
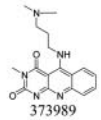
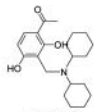
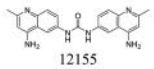
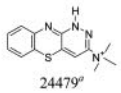
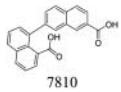
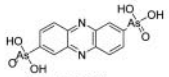
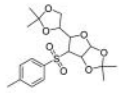
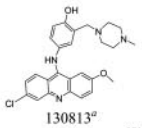
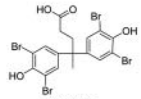
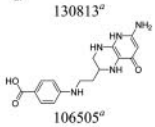
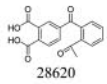
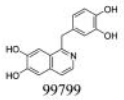
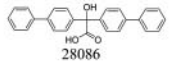
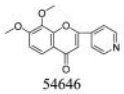
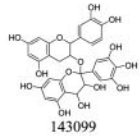
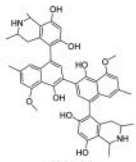
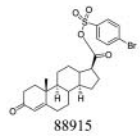
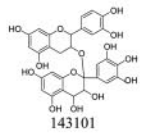
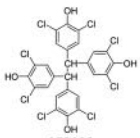
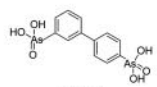
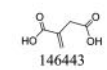
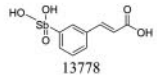
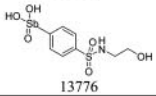
One hundred thirteen "hits" in the primary screen were intensely colored compounds, which is a very large fraction of the total number of colored compounds in the library. One explanation for the promiscuous inhibition by colored compounds is an inner filter effect on the high-throughput fluorescence assay (i.e., an absorption band overlap with the fluorescein fluorophore). To estimate how many false positives were contained within the colored hits, we selected a

[2] In HTP screening, it is important to explicitly define the term *hit*. Here, a hit is simply a compound that gives rise to a decrease in the rate of fluorescence increase in the RNase assay under the conditions of the screen. No presumptions are made as to the mechanism of inhibition. Such mechanisms may include specific inhibition, nonspecific inhibition by compound aggregation or protein denaturation, or even inner filter effects on the fluorescent assay. Many of the uninteresting mechanisms are excluded in subsequent secondary screens and specificity studies.

TABLE 1

Structure and inhibitory potencies of compounds identified in HTP screen

IC_{90} indicates the compound concentration at which 90% or greater inhibition was observed using the high-throughput screen. Relaxation inhibition is that observed in standard plasmid supercoil release assay.

Chemical Structure	IC_{90}	Relaxation Inhibition	Chemical Structure	IC_{90}	Relaxation Inhibition
	μM	%		μM	%
 204667[a]	100	40[b]	 373989	5	70[d]
 109248[a]	100	50[b]	 12155	2	40[d]
 24479[a]	100	50[b]	 7810	2	0[b]
 35682[a]	100	50[b]	 14163	1	50[e]
 130813[a]	100	100[b]	 21588	1	100[b]
 106505[a]	100	100[b]	 28620	1	100[c]
 99799	10	80[b]	 28086	1	100[c]
 54646	10	100[c]	 143099	1	100[c]
 661755	10	40[c]	 88915	0.5	50[e]
$C_{21}H_8Cl_{12}O_5S$ 270718	10	100[b]	 143101	0.5	70[d]
 172033	10	60[b]	 48300	0.1	50[e]
 146443	10	100[b]	 13778	0.1 70[e]	
 13776	5	0[b]			

[a] These compounds were colored at a concentration of 100 μM; [b] compound concentration was 10 μM; [c] compound concentration was 5 μM; [d] compound concentration was 0.5 μM; [e] compound concentration was 6 nM.

subset of 43 on the basis of their conformance with Lipinski's Rule of Five and assayed these in a secondary plasmid relaxation assay using 20 μM compound (Lipinski, 2003) (see also below). At this reduced concentration, 6 compounds (15%) showed complete inhibition, 26 (60%) showed partial inhibition, and 11 (25%) showed no inhibition at all. Of the six compounds showing complete inhibition of plasmid supercoil relaxation at 20 μM compound concentration, only one of these (327447) showed significant activity at lower concentrations ($IC_{50} \sim 2$ μM; Table 1). We conclude that the promiscuous inhibition by colored compounds does not solely arise from an inner filter effect but may also be primarily caused by a propensity of these extensively aromatic compounds to form nonspecific inhibitory aggregates (Seidler et al., 2003; Feng et al., 2005). In fact, a recent study estimated that 19% of randomly selected small molecules commonly found in chemical libraries are nonspecific, aggregation-based inhibitors at 30 μM concentrations (Feng et al., 2005). This percentage is somewhat higher than the 12.4% hit rate we observed with the Diversity Library at the 100 μM initial screening conditions but strongly suggests that many of these initial hits are nonspecific. In any event, the high hit rate under the primary screening conditions is not especially problematic for a library of this size because follow-up studies can quickly discriminate between weak and strong inhibitors (see below). For larger libraries, it would be advantageous to use more stringent screening conditions (i.e., concentrations of compound less than 100 μM) and to include a low concentration of detergent in the RNase assay buffer to disperse aggregates (Seidler et al., 2003; Feng et al., 2005).

Secondary Screening Using Ribonuclease and Plasmid Supercoil Relaxation Assays. To ascertain relative potencies of the most effective initial hits, we further assessed the activities of 42 colorless compounds that showed >90% inhibition of the ribonuclease reaction at the initial concentration of 100 μM (Table 1). Secondary screening was performed in two steps. First, all 42 compounds were screened using the ribonuclease assay at a concentration of 10 μM. Then, the 21 compounds that showed >90% inhibition in the first step were further screened using concentrations in the range of 0.1 to 10 μM (data not shown). For this abbreviated set of 21, the lowest compound concentration that gave >90% inhibition is reported in Table 1.

We brought forward these 21 compounds for testing in a DNA supercoil release assay (Fig. 3). These compounds were initially tested at 10 μM concentration, and representative data from 13 of these are shown in Fig. 3A. Although 19 compounds showed similar inhibitory potencies as observed in the RNase assay (Table 1), 2 did not inhibit supercoil relaxation at 10 μM concentration (13776 and 7810) (Fig. 3A). Further testing of the active compounds at serial 10-fold dilutions revealed that four compounds (13778, 48300, 14163, and 88915) showed significant inhibition of plasmid supercoil relaxation even at 100 nM concentrations (Fig. 3, B and C). These final four compounds had IC_{50} values around 10 nM (data not shown) and were subjected to a thorough mechanistic analysis to determine the selectivity and mode of inhibition as described below.

Selectivity of Inhibitors. We were interested whether the four most potent vTopo inhibitors showed selectivity for the pox family enzyme. Thus, inhibition of supercoil relaxation by the human enzyme was tested (Table 1). For 13778 and 88915, partial or complete inhibition of hTopo was only observed at relatively high compound concentrations in the range 5 to 20 μM (Fig. 4, A and B). For 48300 and 14163, no inhibition was observed even at compound concentrations as high as 20 μM (Fig. 4, C and D). Because vTopo shows approximately 50% inhibition using 10 nM concentrations of these compounds, these results indicated selectivity in the range of 500- to >2000-fold compared with hTopo.

Assessing Inhibition of DNA Binding by Active Compounds. We investigated whether these compounds inhibited noncovalent DNA binding by Y274F vTopo, which lacks the active site tyrosine nucleophile but binds to DNA containing the specific recognition sequence, just like wild-type vTopo (Kwon and Stivers, 2002). In this analysis, the

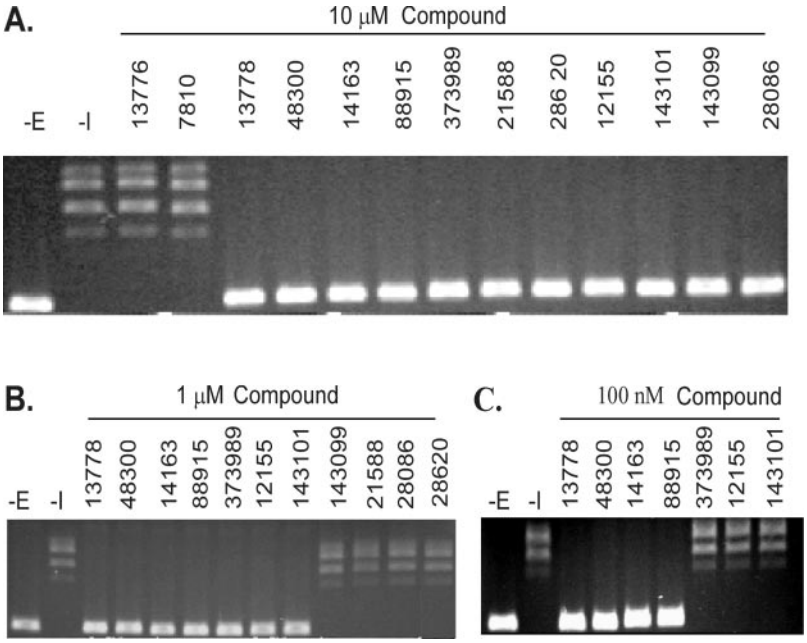


Fig. 3. Inhibition of DNA supercoil relaxation by vTopo. Plasmid pUC19 and enzyme were incubated for 30 min at room temperature in the presence and absence of compound in a buffer consisting of 20 mM Tris-HCl, 0.1 M NaCl, and 20 μg/ml bovine serum albumin, at pH 7.5. A, DNA relaxation in the presence of 10 μM compound. Of the 13 tested compounds, 11 showed complete inhibition. B, DNA relaxation in the presence of 1 μM compound. Of the 11 compounds tested, 7 showed complete inhibition. C, DNA relaxation in the presence of 100 nM compound. Of the seven compounds tested, four showed significant inhibition at that concentration.

change in fluorescence anisotropy upon Y274F binding to the 5′-FAM 18AP/24 duplex is followed (Fig. 5). The addition of increasing concentrations of Y274F to a solution of 5′-FAM 18AP/24 resulted in a saturable increase in the steady-state fluorescence anisotropy of the DNA (Fig. 5A), as would be expected upon the formation of a high molecular weight enzyme-DNA complex.

To determine whether binding of the inhibitory compounds was competitive with DNA, we then monitored the anisotropy as each compound was titrated into the solution containing the noncovalent Y274F-DNA complex (representative data for 13778 and 14163 are shown in Fig. 5, A–C). If binding of the compound displaces the bound DNA, the anisotropy should return to the level of the free DNA in a concentration-dependent manner. In each case, the anisotropy was unchanged from that of the enzyme-DNA complex alone using concentrations of compound 50 times greater than the IC_{90} values for inhibition of supercoil release (Fig. 5B). Changing the order of addition of enzyme, compound, or DNA did not result in a decrease in anisotropy, excluding the possibility that slow-onset binding by the inhibitor was influencing the results (data not shown). As a positive control, we then titrated each complex with the competitive inhibitor ATP and observed that the anisotropy returned to the level of the free DNA (Fig. 5C)[3]. These data clearly establish that these four compounds do not inhibit noncovalent substrate binding by vTopo.

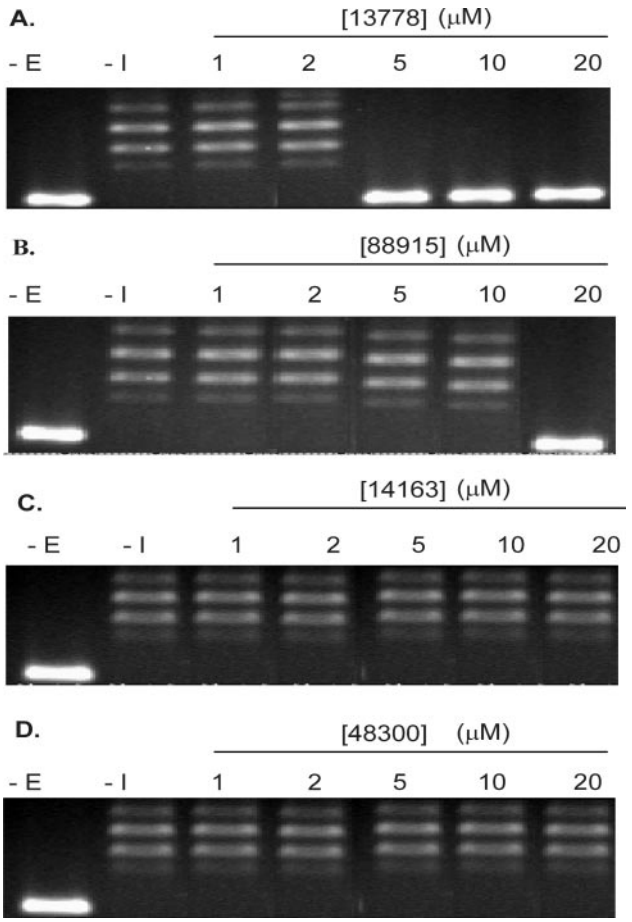


Fig. 4. Inhibition of DNA supercoil relaxation by hTopo. pUC19 (0.5 μg) was incubated with 2 U of hTopo at room temperature for 20 min with increasing amounts of compounds 13778 (A), 88915 (B), 14163 (C), and 48300 (D).

Effect of Active Compounds on DNA Transesterification. A second potential mechanism of inhibition involves the reversible DNA transesterification step catalyzed by topo I. This step involves DNA cleavage (k_{cl}) to form a 3′-phosphotyrosyl linkage between the enzyme and DNA, and DNA religation (k_r) in which the expelled 5′-hydroxyl of the DNA attacks the phosphotyrosyl linkage to reform the intact DNA backbone. The forward-cleavage reaction may be studied under irreversible cleavage conditions using "suicide substrates" that have weakly base-paired DNA strands 3′ to the site of cleavage that dissociate to solution after cleavage (Shuman, 1991). If the leaving strand contains a 2-aminopurine fluorescent label on the 5′ end, rapid strand dissociation leads to an increase in the 2-aminopurine fluorescence that is a measure of the preceding rate-limiting cleavage reaction (Kwon and Stivers, 2002). Otherwise, reversible conditions may be used in which the covalent complex accumulates to its equilibrium level dictated by the ratio of forward and reverse rate constants $k_{cl}/k_r = K_{cl}$ = [covalent complex]/[noncovalent complex](Kwon and Stivers, 2002). In this case, larger substrates are used in which the strands remain paired after DNA cleavage.

We first performed stopped-flow fluorescence analysis of the irreversible cleavage reaction of the FAM-18AP/24 suicide substrate by vTopo in the presence of 13778, 48300, 14163, and 88915. In this assay, compound concentrations as high as 100 times the IC_{90} value for supercoil release were used. Representative stopped-flow data are shown in Fig. 6 in the presence of 0, 1, and 10 μM concentrations of compound 13778. The cleavage rate in the absence of 13778 was 1.4 s^{-1}, and was found to be unaffected by the presence of 13778. Identical results were obtained for 48300, 14163, and 88915 (data not shown). Therefore, these data establish that inhibition of the steady-state RNase and supercoil release reactions involves a step *after* formation of the covalent complex. As observed for DNA binding, the results were the same regardless of whether the compound was preincubated with the enzyme or DNA (data not shown).

We then investigated the effect of each compound on the equilibrium level of covalent complex using reversibly cleaved substrates (Fig. 7). In these experiments, a stoichiometric complex between a 5′-fluorescein-labeled 32/32 mer CCCTT-containing DNA (60 nM) and vTopo (80 nM) was formed, and then increasing amounts of each inhibitor were added. The equilibrium amount of covalent complex can be assessed by quenching the reaction with SDS, which traps the covalently bound enzyme on the DNA (Kwon and Stivers, 2002). The covalent complex is then separated from the free DNA using denaturing polyacrylamide gel electrophoresis, and the fluorescence in each species was quantified by fluorescence imaging. In the absence of inhibitor, 18% of the total DNA fluorescence migrates in a covalent complex with the

[3] Under certain conditions, ATP has been reported to accelerate the rate of supercoil release by vTopo (Sekiguchi and Shuman, 1994). We were surprised to find that this nucleotide is an inhibitor of the RNase reaction and noncovalent DNA binding by Y274F vTopo. These apparently contradictory findings may indicate that the rate of supercoil relaxation is limited by product release, which is in turn facilitated by ATP. This nucleotide could bind to an allosteric site on the enzyme and give rise to these effects.

enzyme (Fig. 7, A–D). Compounds 13778, 48300, and 88915 had no discernible effect on the equilibrium amount of covalent complex (Fig. 7, A, B, and D). In stark contrast, compound 14163 produced a concentration-dependent decrease in the amount of covalent complex (Fig. 7C), such that at concentrations greater than 2 μM, no covalent complex could be detected. The decrease in the amount of covalent complex observed with 14163 cannot arise from inhibition of noncovalent DNA binding (Fig. 5B). Although this effect of 14163 could, in principle, arise from an increase in the rate of religation relative to cleavage, we favor a mechanism involving hindrance of the cleavage chemistry, either by direct or indirect interactions with active site groups. Even though 14163 showed no effect on the single turnover cleavage rate, it is quite possible that the cleavage rate is affected in the equilibrium substrate because the binding site for this compound may be created after cleavage and/or requires the presence of a paired DNA strand 3′ to the cleavage site. On the other hand, the binding rate of 14163 could be slow relative to the single turnover cleavage rate, making it an ineffective inhibitor under this type of assay condition. Whatever the detailed mechanism, these findings accentuate the importance of measuring inhibitory activities in multiple assay formats. The distinct inhibition mechanisms for these four active compounds are discussed further below.

Discussion

Inhibitory Compounds Fall into Known and Novel Structural Families. Small molecules that target topoisomerases may be placed in two general categories: those that inhibit DNA binding or transesterification, and those that stabilize the covalent enzyme-DNA complex (Hwang et al., 2000). Ligands that fall into the latter category are called "topoisomerase poisons" because they stabilize single-strand nicks in duplex DNA that in turn give rise to toxic double-strand breaks when encountered by replication forks during DNA replication. This is the mode of action of all clinically useful drugs that target topoisomerases (Chen and Liu, 1994).

In our blind screen of the NCI library, we detected inhibitors of vTopo that are structurally related to four known classes of mammalian topo I poisons and structurally novel inhibitors (Fig. 8). One library member, camptothecin, is a clinically used topo I poison that showed partial inhibition of vTopo using the primary screening conditions. In addition,

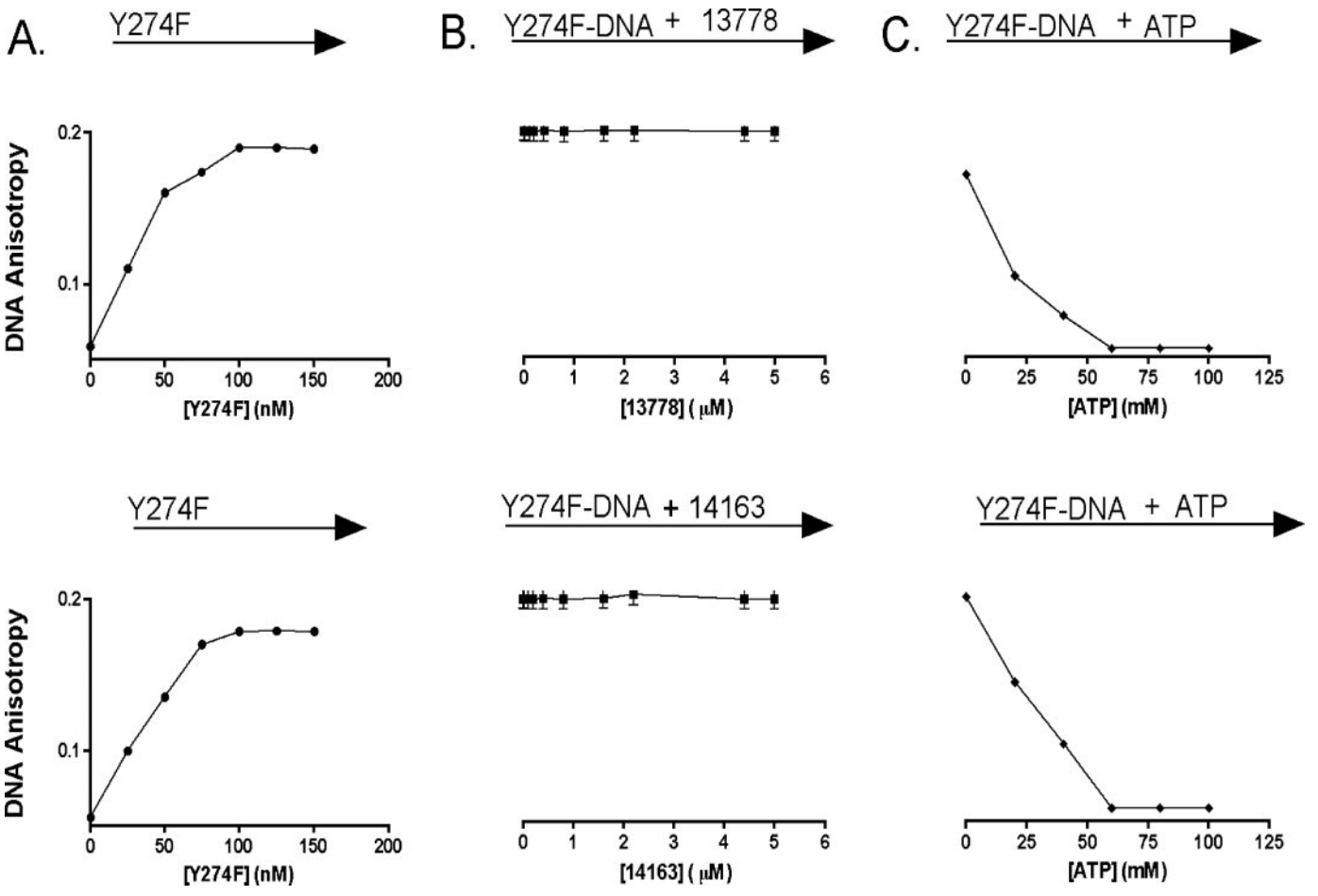


Fig. 5. Noncovalent DNA binding by Y274F vTopo in the absence and presence of compounds 13778 and 14163 as determined by DNA fluorescence anisotropy. A, 100 nM 18/24 duplex was titrated with Y274F to a final concentration of 150 nM, at which point a maximum steady-state anisotropy value was obtained, indicating saturation of the DNA with enzyme. B, compound 13778 or 14163 was titrated into the Y274F-DNA complex to a final concentration of 5 μM. No change in anisotropy was observed, indicating that these compounds do not competitively displace the DNA. C, After titration with each compound, increasing concentrations of the competitive inhibitor ATP were added, which ultimately returned the anisotropy to the level of the free DNA. All reactions were performed in a buffer containing 20 mM Tris and 0.1 M NaCl at pH 7.5.

two other structural congeners of camptothecin were also detected as inhibitors (Fig. 8). In this respect, it is interesting to note that camptothecin is widely considered a highly specific poison of mammalian type I enzymes. Previous work has shown that camptothecin does not affect wild-type vTopo DNA relaxation activity at concentrations ≤100 μM, but it can inhibit relaxation by a mutant enzyme (D221V) that more closely matches the coding sequence in a highly conserved region of eukaryotic topo I enzymes (Gupta et al., 1992). The sensitive ribonuclease high-throughput screen has uncovered that camptothecin is weakly active against vTopo, which may indicate that its efficacy is greater for the ribonuclease activity than for the DNA unwinding action of vTopo, or that the ribonuclease assay is simply more sensitive.

Although we have generally found that inhibitors of the ribonuclease reaction are also comparable or even better inhibitors of the DNA reactions of vTopo (Table 1), this is not always true. In this study, two compounds that potently inhibited the ribonuclease reaction were not found to inhibit DNA relaxation in the same concentration range (13776 and 7810; Fig. 3A). The detection of novel inhibitory scaffolds that do not initially show an effect on DNA relaxation is not necessarily a detriment. In terms of the development of new classes of topoisomerase poisons, a viable strategy may be to identify interesting inhibitory scaffolds using the RNase assay and then to make chemical modifications that transform these into clinically useful poisons. We are especially interested in discovering allosteric topoisomerase poisons that trap the covalent complex by binding to an enzyme site distant from the covalent chemistry. Such compounds would be structurally and mechanistically distinct from the planar, intercalative molecules that dominate the current repertoire of topoisomerase poisons.

Aside from camptothecin, three other known structural classes of topo I poisons were also detected in the primary screen at 100 μM compound concentration. These compounds are related to the protoberberine, nogalamycin, and bibenzimidazole chemical scaffolds (Fig. 8). The five-ring protoberberines and the closely related benzo[*i*]phenanthridine derivatives are potent hTopo poisons with activity against several human tumor cell lines (Li et al., 2000, 2003; Ruchelman et al., 2004). Their mechanism of action involves DNA intercalation by the C and D rings (Fig. 8) (Li et al., 2000) and enzyme-specific interactions with the methylenedioxybenzene A ring. The A and D ring features are recapitulated in the smaller two- and three-ring system inhibitors detected

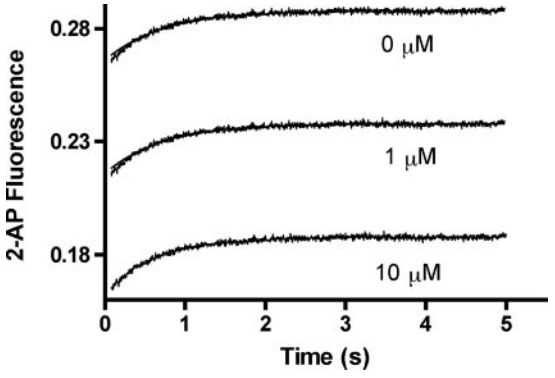


Fig. 6. Effect of compound 13778 on single-turnover DNA cleavage. vTopo was rapidly mixed with a suicide substrate in a stopped-flow fluorescence device, and the increase in fluorescence of the 2-AP label in the leaving strand was followed. The cleavage rate was k_{cl} = 1.38 ± 0.02 s^{-1}, 1.4 ± 0.02 s^{-1}, and 1.39 ± 0.02 s^{-1} in the presence of 0, 1, and 10 μM [13778], respectively.

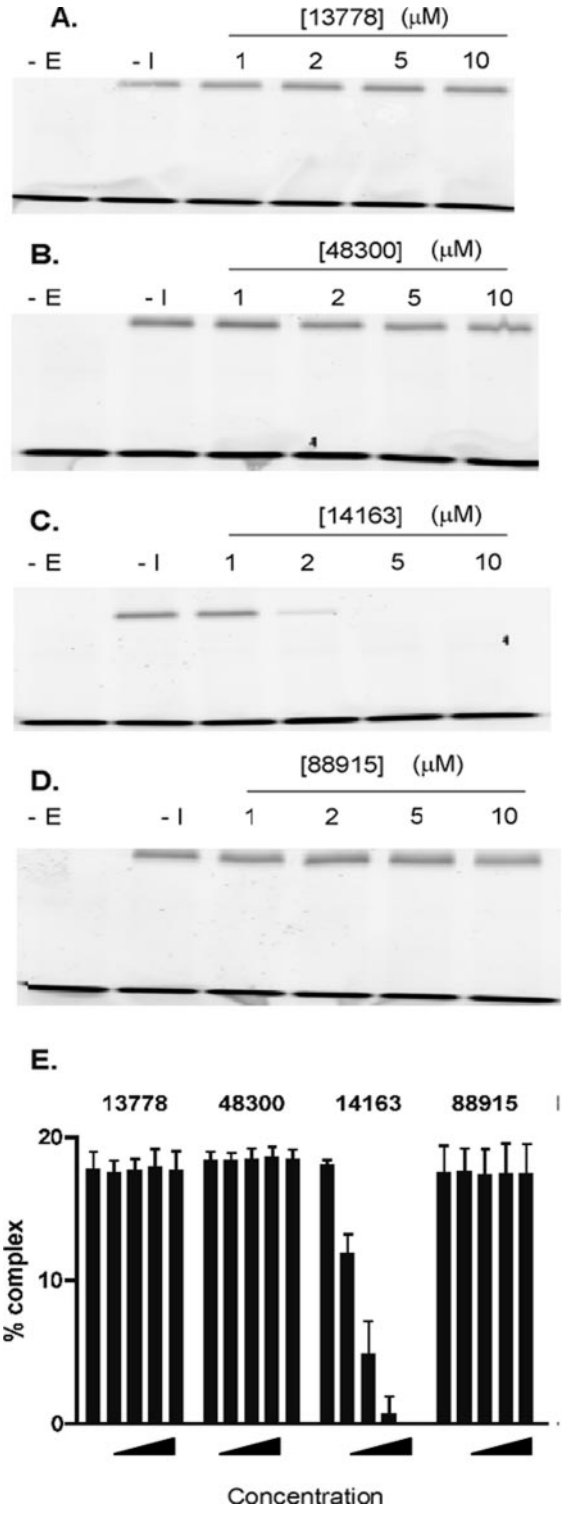


Fig. 7. Equilibrium cleavage measurements with vTopo (80 nM) and 5′-FAM labeled 32/32 mer (60 nM) in a buffer containing 20 mM Tris and 200 mM NaCl at pH 8.0. Enzyme and substrate were incubated with increasing amounts of compounds 13778, 48300, 14163, and 88915 for 30 min before quenching with one volume of 10% SDS. The percentage of covalent complex was determined after separation by gel electrophoresis using a 10% polyacrylamide gel containing 0.1% SDS and imaged.

here, suggesting a shared mechanism of inhibition between the vaccinia and human enzymes. Nogalomycin has been shown to intercalate into DNA using its four-ring aglycone ring system, which positions its nogalose and amino glucose sugar rings into the minor and major grooves, respectively (Fig. 8) (Sim et al., 1997). Its mode of action with hTopo seems to involve induced DNA bending that stabilizes the covalent complex (Sim et al., 2000). Recent studies have also examined nogalomycin inhibition of vTopo and concluded that it does not stabilize the covalent complex but instead inhibits DNA cleavage with an IC_{50} value of 0.7 μM (Yakovleva et al., 2004). The most obvious feature of the nogalomycin congeners detected here is their lack of the nogalose sugar substituent (Fig. 8), indicating that this minor groove binding group is not essential for vTopo inhibition. Finally, bi- and terbenzimidazoles have been shown to be DNA minor groove binders acting by stabilizing the covalent complex between hTopo and DNA via a long range conformational change rather than direct intercalation at the cleavage site, as with camptothecin (Xu et al., 1998). The ribonuclease assay readily detected the mono- and bibenzimidazole library compounds shown in Fig. 3, and the bibenzimidazole compound has been reported previously as a potent hTopo inhibitor (Jin et al., 2000). The related terbenzimidazole derivatives of this library member have been shown to increase the potency as an hTopo poison, and the activity is strongly correlated with the greater binding affinity of the terbenzimidazole for the DNA minor groove. We conclude that the ribonuclease assay robustly detected all of the known topoisomerase inhibitors and poisons present in the Diversity Library and that this screen has significant potential to quickly reveal structure-activity relationships relevant for development of specific inhibitors of pox family topoisomerases.

The high-throughput screen has also uncovered 21 structurally novel inhibitors of vTopo that had IC_{90} values for the ribonuclease reaction of less than 10 μM. Only 2 of these 21 compounds failed to show comparable inhibition in the supercoil relaxation assay (Fig. 3A). The remaining 19 compounds showed potencies of inhibition against DNA relaxation that were comparable with or even significantly enhanced compared with their potencies in the ribonuclease screen. It is not surprising that there are differences between the inhibitory effects of the same compound in the two assays. If the compound interacts with the covalent complex and not the free enzyme, as we have generally found (see below), the presence of a 2′ hydroxyl with the ribonuclease substrate could have a differential effect on the interaction of the inhibitor compared with the 2′ hydrogen in DNA. Nevertheless, the results here show that any differences in inhibitor interactions between these two substrates do not prevent the detection of both novel and established topo I ligands that act by widely different mechanisms.

The structural features of the 21 most potent vTopo inhibitors are varied (Table 1). Several compounds contain pentavalent antimony or arsenic, and two of these (48300 and 13778) are specific inhibitors of vTopo, with IC_{50} values in the nanomolar range against DNA relaxation. It is noteworthy that compound 13778 has been identified recently as a human immunodeficiency virus-1 entry inhibitor that disrupts the binding of the viral gp120 with the CD4 receptor (Yang et al., 2005) and is also an inhibitor of B-ZIP transcription factor DNA binding (Rishi et al., 2005). Three others compounds with IC_{50} values in the 0.5 to 10 μM range

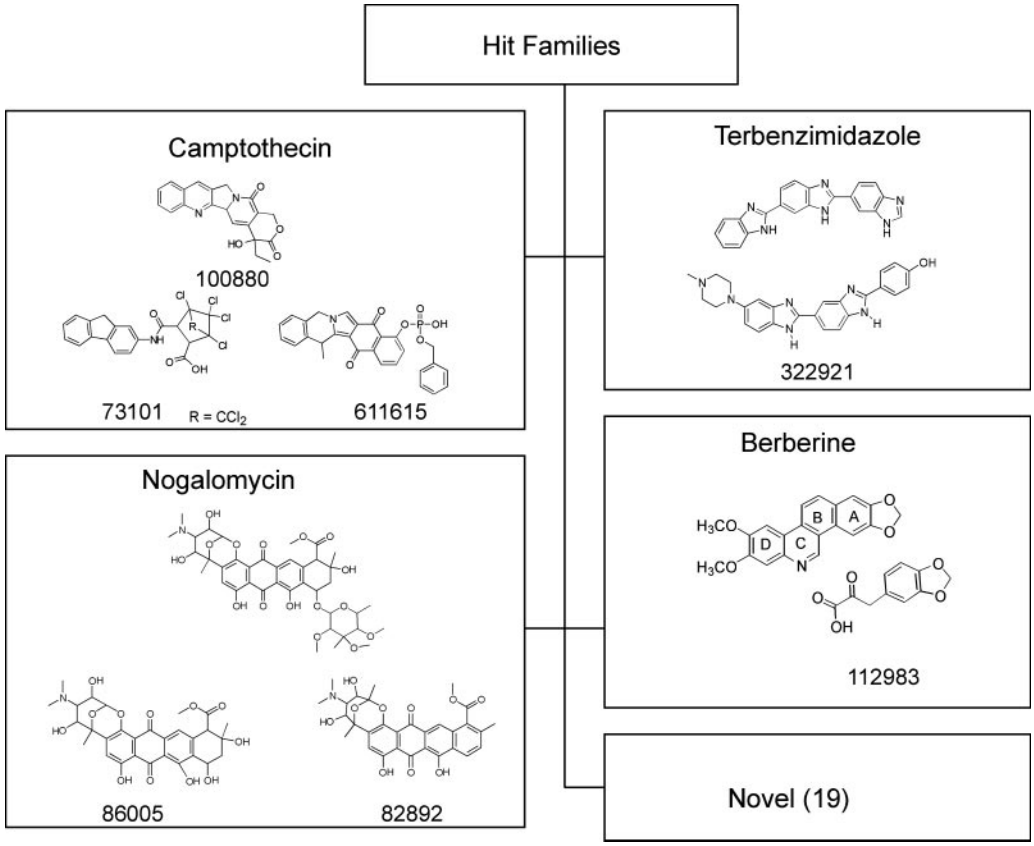


Fig. 8. Inhibitory drug classes observed in screening of the NCI diversity library using the RNase assay. Seven compounds are related to known families of topo I inhibitors. Nineteen novel compounds with IC_{90} values less than or equal to 10 μM were also discovered (Fig. 9).

(373989, 12155, and 7810) have two or three fused aromatic rings suggestive of an intercalative mode of action. Finally, compound 14163, which shows nanomolar potency in DNA relaxation assays, is an entirely novel topoisomerase inhibitor with an unusual substituted furan ring structure (Table 1). Given the structural diversity of these compounds, it is unlikely that they use similar binding modes, suggesting that the vTopo-DNA target displays a promiscuous array of potentially inhibitory binding sites.

Inhibitory Mechanism of Highly Active vTopo-Specific Inhibitors. We used three efficient assays to ascertain the mode of inhibition for supercoil relaxation brought around by the four potent vTopo specific compounds 13778, 48300, 88915, and 14163. DNA anisotropy measurements established that none of these compounds bind to vTopo competitively with DNA (Fig. 5). Likewise, single-turnover suicide DNA cleavage measurements revealed no effect of these compounds on any step leading to the formation of the covalent enzyme-DNA adduct (Fig. 6). Finally, compounds 13778, 48300, and 88915 had no discernible effect on the equilibrium level of covalent complex (Fig. 7), but increasing concentrations of 14163 completely prevented the formation of the covalent complex (Fig. 7C). Thus, all four inhibitors bind noncompetitively or uncompetitively to the noncovalent or covalent complex. Although an intercalative mechanism for inhibition has not been directly studied here, the structures of 88915, 13778, 14163, and 48300 are not suggestive of such a mechanism, and these compounds are equally active in the ribonuclease and supercoil release assays. Compound 14163 is unique in that its binding to the enzyme-DNA complex does not significantly interfere with DNA binding but totally disrupts covalent chemistry.

It is intriguing that no compounds have yet been identified that act as vTopo poisons (i.e., act by stabilizing the covalent enzyme-DNA complex). To date, vTopo inhibitors have been characterized as 1) competitive inhibitors of DNA binding (novobiocin, coumermycin, and ATP) (Sekiguchi et al., 1996)[3], 2) noncompetitive inhibitors by binding to the noncovalent complex (nogalamycin) (Yakovleva et al., 2004), or 3) uncompetitive inhibitors by binding to the noncovalent or covalent complex, as found for the inhibitors identified here. It is possible that there are unique structural features of the covalent complex between vTopo and DNA that prevent extensive intercalation of a compound into the strand break, as observed in crystal structures of camptothecin bound to hTopo and cleaved DNA (Staker et al., 2002; Chrencik et al., 2004). Instead, the uncompetitive inhibitors discovered here and previously (Kwon et al., 2004) act by inducing an alternative form of the covalent complex that is unproductive for DNA relaxation or steady-state turnover of the ribonuclease substrate. The frequency of this mode of inhibition suggests that it may turn out to be the rule, and not the exception, with vTopo.

Antiviral Applications of vTopo Inhibitors. Although an early study indicated that the poxvirus topoisomerase was essential (Shuman et al., 1989), a recent study has established that the deletion mutant is viable but has low infectivity because of reduced early transcription (Da Fonseca and Moss, 2003). Thus, vTopo acts very early in viral infection, and drugs that merely inhibit the enzyme may not totally prevent infectivity. It has been suggested that a vTopo poison that stabilizes the covalent intermediate might block infectivity more efficiently than the null mutant because of the accumulation of toxic nicks in the DNA (Da Fonseca and Moss, 2003). Although the compounds characterized here are not classic topoisomerase poisons that increase the level of covalent species, three of these do serve to trap the enzyme in a nonproductive covalent complex on DNA that could be just as effective in producing therapeutic effects.

Acknowledgments

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