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New peptide inhibitors of type IB topoisomerases: similarities and differences vis a vis inhibitors of tyrosine recombinases

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SUMMARY

Topoisomerases relieve topological tension in DNA by breaking and rejoining DNA phosphodiester bonds. Type IB topoisomerases such as vaccinia topoisomerase (vTopo) and human topoisomerase I are structurally and mechanistically similar to the tyrosine recombinase family of enzymes including bacteriophage lambda Integrase (Int). Previously, our laboratory identified peptide inhibitors of Int from a synthetic peptide combinatorial library. The most potent of these peptides also inhibit vTopo. Here we used the same mixture-based screening procedure to identify peptide inhibitors directly against vTopo using a plasmid relaxation assay. The two most potent new peptides identified, WYCRCK and KCCRCK, inhibit plasmid relaxation, DNA cleavage and Holliday junction (HJ) resolution mediated by vTopo. The peptides tested bind double stranded DNA at high concentration but do not appear to displace the enzyme from its DNA substrate. WYCRCK binds specifically to HJ and perturbs their central base pairing. This peptide also accumulates HJ intermediates when it inhibits Int-mediated recombination, whereas KCCRCK does not. Interestingly, WYCRCK shares four amino acids with a peptide identified against Int, WRWYCR. The octapeptide WRWYCRCK, containing amino acids from both hexapeptides, is more potent than either against vTopo. All peptides are less potent against the type IA *E. coli* topoisomerase I or against restriction endonucleases. Like the Int-inhibitory peptide WRWYCR, WYCRCK binds to Holliday junctions, and both inhibit junction resolution by vTopo. Our results suggest that the newly identified WYCRCK and peptide WRWYCR interact with a distorted DNA intermediate arising during vTopo-mediated catalysis, or interfere with specific interactions between vTopo and DNA.

INTRODUCTION

Topoisomerases release topological tension during replication and transcription and contribute to other essential processes, including chromosome segregation and DNA repair¹. Type I topoisomerases cleave one strand of DNA at a time via a transesterification reaction involving an enzyme-DNA covalent complex. They are divided into two subfamilies designated type IA and type IB: the type IA enzymes form 5' phosphotyrosyl enzyme adducts while the type IB enzymes form 3' phosphotyrosyl enzyme adduct^{1,2}. In both cases, the free DNA end rotates around the continuous strand, changing the linking number (and thus superhelical density) of the substrate. After rotation, the free hydroxyl group nucleophilically attacks the covalent enzyme-DNA linkage to regenerate an intact DNA strand and a noncovalently associated enzyme. Type IB topoisomerases relax either positive or negative supercoils and are stimulated by but do not require magnesium cations^{1,2}. Sequence and structural comparisons have shown

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that vTopo is the simplest of the type IB topoisomerases, consisting largely of the catalytic domain shared among members of the entire family^{3,4}. Although type IB topoisomerases had been thought to be restricted to eukaryotes and their viruses, genes encoding type IB topoisomerases have also been found in *Deinococcus* and *Pseudomonas*, both with demonstrated enzymatic activity^{5,6}.

Structural and mechanistic similarities extend to the catalytic domains of tyrosine recombinases, including those of phage lambda Integrase (Int) and the phage P1 Cre recombinase^{4,7-10}. The type IB topoisomerases and tyrosine recombinases share 4 out of 5 active site residues necessary for catalysis. Both enzyme families cleave double stranded DNA as well as Holliday (4-way) junctions (HJ)⁸⁻¹². The related human topoisomerase I is the target of camptothecin and the related compounds irinotecan and topotecan, clinically important anti-cancer compounds¹³. The crystal structure of topotecan bound to hTopo-DNA complexes shows that the drug intercalates immediately downstream of the cleavage site and inhibits religation¹⁴. Camptothecin traps topoisomerase-DNA complexes resulting in ternary complexes that block the transcription and replication machinery¹⁵, eventually leading to cell death. Camptothecin does not inhibit wild type vTopo, although a point mutation confers some sensitivity¹⁶. Some DNA intercalators such as nogalamycin inhibit DNA cleavage by vTopo with an IC_{50} of 0.7 μM ¹⁷, but would be expected to be quite nonspecific in their enzyme targets. Inhibitors of vTopo are expected to be effective against the topoisomerases of all pox viruses, including small pox, a putative agent of bioterrorism, and may also inhibit human topoisomerase I. Quite recently, very potent small molecules that specifically inhibit vTopo but not human topoisomerase I have been identified¹⁸. Topoisomerase inhibitors, including those specifically targeting the poxvirus enzymes, will thus be useful from both a mechanistic and a therapeutic perspective, since drug resistance will eventually arise against any inhibitor.

Previously, we identified hexapeptide inhibitors of lambda Int-mediated site-specific recombination by screening and deconvoluting synthetic peptide combinatorial libraries (SPCLs)^{19,20}. These inhibitors have provided useful insights into the mechanism of Int site-specific recombination¹⁹⁻²⁴. One class of peptides identified against Int also inhibits DNA cleavage and plasmid relaxation mediated by vTopo, albeit at decreased potency with respect to Int²¹. To further analyze the similarities and differences between the type IB topoisomerases and the tyrosine recombinases, we have re-screened peptide libraries specifically for vTopo inhibitors using a plasmid relaxation assay. We have identified two relatively potent peptide inhibitors and have analyzed their mechanism of inhibition. These peptides inhibit DNA cleavage by vTopo of double stranded DNA and block the resolution of HJ substrates by vTopo without displacing the enzyme from duplex DNA containing its preferred cleavage/binding site. One of these newly identified peptides, WYCRCK, binds HJ specifically and perturbs bases near the crossover region within the vTopo cleavage site, thereby inhibiting DNA cleavage of this substrate. The inhibitors are more potent against enzymes with type IB mechanisms than *E. coli* topoisomerase I (type IA) or restriction enzymes. The peptides may prove to be useful lead compounds for anti-poxviral and/or anti-cancer drug development.

RESULTS

Peptide inhibitors identified against vTopo

To identify amino acids of peptide inhibitors specific for vTopo, we performed a positional scanning screen of an L-amino acid synthetic peptide combinatorial library (SPCLs)²⁵. We used the same procedure as was used earlier to find peptide inhibitors of phage lambda Int-mediated recombination^{19,20}, outlined in (Figure 1(a)). Briefly, mixtures of hexapeptides are added to plasmid relaxation assays containing vTopo and pUC19. During step 1, six different sets of peptide mixtures were screened, each set consisting of 20 mixtures representing all of the naturally occurring amino acid side chains (represented by O, Figure 1(a)) in one of the six

positions in the peptide while the other five positions have an equimolar mixture of the 20 naturally occurring amino acids except cysteine (represented by X, Figure 1(a)). Screening all 120 mixtures tests the contribution of all naturally occurring amino acid side chains within a hexapeptide. To rank the contributions of each amino acid to the inhibitory potential, each mixture was tested at two concentrations (0.1 mg/ml and 0.2 mg/ml). An example of a plasmid relaxation assay testing the 20 peptide mixtures with the second position fixed is shown in Figure 1(b). The amount of remaining supercoiled substrate was compared among the treated reactions (Fig. 1(b), lanes 3-22) and to untreated control reactions (Fig 1(b), lanes 1 and 2). In the example shown, the mixtures containing amino acids C, F, L, M, W and Y at the second position inhibit vTopo activity most. These amino acids were included in the second screening step using dual defined mixtures, which contain two fixed amino acid positions (O) and 4 positions (X) with an equimolar mixture of one of 19 amino acids, again lacking cysteine (Figure 1(a), step 2). Instead of testing all of the 1200 possible combinations of 20 x 20 amino acids at each of three pairs of positions in the peptide, only the best amino acid candidates from the first step of the screen were included. The dual defined mixtures were less complex and thus the effective concentration of each constituent peptide was higher than in the single fixed position mixtures²⁶. Each candidate mixture was tested at four concentrations (25 µg/ml, 6.3 µg/ml, 1.6 µg/ml and 0.4 µg/ml) to find the most potent inhibitory amino acid pairs. An example of a dose response assay testing the first and second position dual defined mixtures is shown (Figure 1(c)). For example, the peptide mixture with the fixed amino acids RC caused nearly complete inhibition of plasmid relaxation at 25 µg/ml (Figure 1C, lane 5). This mixture is more potent than the single position fixed peptide mixture with cysteine at the 2nd position, also added at 25 µg/ml (Figure 1(c), lane 5 versus lane 4). After the screening of 150 dual defined peptide mixtures, eight hexapeptides with defined sequences were synthesized (Figure 1(a), step 3). The amino acids chosen for each fixed position of the eight peptides were: K or W at position 1, C or Y at position 2, C or L at position 3, R, Y, or I at position 4, C or E at position 5, and K or F at position 6. Of the eight specific peptides tested, the two most potent were KCCRCK and WYCRCK. WYCRCK shares amino acid identity with peptide WRWYCR, identified as a potent inhibitor of all Int-mediated recombination *in vitro*²⁰. To investigate the inhibitory contribution of the amino acids in both peptides, the octapeptide WRWYCRCK was synthesized and tested against vTopo in parallel with the hexapeptides.

Inhibition of vTopo-mediated plasmid DNA relaxation by the new peptide inhibitors

The inhibitory potency of the newly identified peptides was evaluated in DNA plasmid relaxation assays, using the conditions described in Klemm *et al.*²¹. The IC₅₀ values of each inhibitor (Table 1) were determined by comparing the supercoiled substrate remaining versus the total amount of DNA in the mock-treated reaction, by densitometry and plotting as % inhibition versus peptide concentration, which agree with inhibitory activity by visual inspection. An example plasmid relaxation assay testing WYCRCK inhibition using 2 different plasmid substrates, pUC19 and pACYC184, is shown (Fig. 2(a) and (b)). KCCRCK was 2-fold less potent than WYCRCK, indicating that the aromatic residues contribute to inhibitory activity. Since all the new peptides contain at least two cysteine residues, we tested the possibility that intermolecular or intramolecular disulfide bonds are important for inhibition. DTT (50 mM) added to 12.5 µM of each peptide abolished their activity, suggesting that the inhibitory form is in fact a multimer (Figure 2(a) and (b), lanes 9). To test directly the importance of the cysteine residues of WYCRCK, hexapeptides with alanine substituted for either cysteine were tested against vTopo-mediated pUC19 plasmid relaxation. Neither WYARCK or WYCRAK, at up to 25 µM, appreciably block the relaxation of pUC19 by vTopo (Figure 2 (c) (lanes 6 and 10 versus lane 2)). WYARAK was also tested but had no inhibitory activity (data not shown). Thus each cysteine residue in WYCRCK is necessary but not sufficient for its maximal inhibitory activity.

The sequence specific DNA cleavage by vTopo allowed us to investigate whether the inhibitory potency of the peptides is related to the number of vTopo sites in the substrate, thereby addressing the specificity of inhibition. We compared the inhibition of vTopo relaxation activity of vTopo on equimolar pUC19 and pACYC184 plasmids. Although pACYC184 is ~60% larger than pUC19, it has only 8 vTopo cleavage sites compared to 17 present in pUC19. Thus pACYC184 has a higher ratio of nonspecific to specific DNA. When peptide potency is normalized to the number of specific cleavages sites present in each plasmid, we observed a reduction in potency of 2.5 fold for WYCRCK and 2.0-fold for KCCRCK (for example, an IC_{50} of 0.3 μ M/site in pUC19 versus 0.8 μ M/site in pACYC184 for WYCRCK) (Table 1). Although the analysis is complicated by possible variations in the context and/or accessibility of each cleavage site within each plasmid, the peptides lose potency in a manner correlated with the increase in nonspecific DNA present in pACYC184 versus pUC19. Using the same analysis, WRWYCR showed a >4-fold decrease in potency against pACYC184 DNA relaxation mediated by vTopo compared to pUC19 (IC_{50} of 0.5 μ M/site in pUC19 versus 2.3 μ M/site in pACYC184). WRWYCRCK, the most potent peptide tested in all *in vitro* assays, showed a 3.5-fold decrease in potency in relaxation assays using pACYC184, indicating that the longer peptide has greater nonspecific inhibitory activity compared to WYCRCK (Table 1). We presume that the extent of nonspecific inhibitory activity of WYCRCK may be due to its DNA binding properties seen in the vTopo binding assays below.

Peptides WYCRCK, KCCRCK and WRWYCRCK inhibit vTopo DNA cleavage

The vTopo catalytic reaction cycle consists of several steps: 1) non-covalent binding to duplex DNA; 2) strand cleavage; 3) strand passage; 4) religation; and 5) dissociation of the enzyme from its substrate⁷. The inhibition of vTopo-mediated DNA cleavage was tested using a double-stranded suicide substrate^{27,28} containing the CCCTT vTopo recognition site placed six nucleotides upstream of the 3' end of the scissile strand, as shown in Figure 3. Upon transesterification by vTopo, the 6-nucleotide section downstream of the cleavage site (denoted by arrow) diffuses away, trapping the vTopo-DNA covalent complex²⁷. The covalent enzyme-DNA complex was separated from uncleaved DNA by electrophoresis through polyacrylamide gels containing SDS. Peptide inhibition of DNA cleavage was tested using conditions that convert 40-50% of the substrate DNA into covalent enzyme-DNA complexes. The extent of cleavage was expressed as a percentage of covalent enzyme-DNA complex formed with peptide treatment normalized to an untreated reaction. Both WYCRCK and KCCRCK inhibited cleavage with an IC_{50} of 0.5 μ M. WRWYCRCK showed an IC_{50} of 0.1 μ M. This peptide was even more potent than the second parent peptide WRWYCR, which inhibited DNA cleavage with an IC_{50} of 0.65 μ M (Table 1). We also tested the inhibitory activity of wycrck, the all D-amino acid stereoisomer of WYCRCK, and found it was 4-fold less potent in the single turnover cleavage assay than the L stereoisomer. Presumably this difference in potency reflects the asymmetric nature of the target. As for plasmid relaxation, 50 mM DTT abolished the activity of 5 μ M peptide, although the cleavage activity of vTopo was unaffected by DTT (data not shown). Thus the peptides inhibit vTopo-mediated cleavage of linear duplex DNA, and the oxidation state of the peptide is important for activity. The inhibitory activity of WYARCK and WYCRACK was compared to that of the parent peptide WYCRCK (Figure 3 (b)). The C>A peptides permitted almost 70% of DNA cleavage by vTopo (Figure 3(b)), while treatment with 5 μ M WYCRCK permitted only 11% of vTopo DNA cleavage. As for inhibition of plasmid relaxation, both cysteine residues within WYCRCK are important for maximal inhibition of vTopo cleavage.

We also explored the possibility that the peptides block vTopo activity by interacting directly with the enzyme. To test this effect, we reversed our previous order-of-addition of reaction constituents and incubated the inhibitory peptides with the enzyme first, then added the DNA substrate. In these conditions, WYCRCK and KCCRCK were found to be at least 10-fold less

active (data not shown); thus, interaction between vTopo and peptide is unlikely to be responsible for inhibition of catalytic activity.

Inhibitory peptides bind ds DNA but do not displace vTopo

DNA binding assays using a 60 bp double-stranded substrate were performed to test if the inhibitory peptides prevent the non-covalent binding of vTopo to linear duplex DNA (Figure 4(a)). When vTopo is incubated with the substrate, approximately 20% of the bound DNA is in the form of covalent complexes while 80% represents non-covalent vTopo-DNA complexes²⁹. The same molar concentration of DNA substrate was added to the binding reactions as was used in the single turnover cleavage assays, and the minimal amount of vTopo necessary to produce a saturated band shift was determined (data not shown). An increasing amount of peptide was added to each reaction, including concentrations above and below the IC₅₀ concentration determined in single turnover cleavage experiments. In no case did peptide addition cause accumulation of free DNA substrate; addition of 0.25-0.5 μ M WYCRCK to the labeled substrate results in more spreading of the substrate throughout the gel compared to a control reaction (Figure 4(a), lanes 5-6 versus lane 2). The addition of 5.0 μ M WYCRCK completely trapped ³²P radiolabeled DNA in the well (e.g., (Figure 4(a), lane 8)). Formation of these aggregates was prevented in the presence of 50 mM DTT and vTopo-DNA complexes migrated into the gel, although they differed in appearance from the complexes made by vTopo alone binding to its substrate (e.g., (Figure 4(a), lane 9)) - presumably because they contain some peptide. Peptides KCCRCK and particularly WRWYCRCK had greater nonspecific dsDNA binding activity than WYCRCK or WRWYCR, evidenced by well-trapped complexes at lower peptide concentrations (Figure 4(a)). The well-trapped complexes may either comprise a distinct peptide-protein-DNA conformation which prevents entry of the complexes into the gel or may reflect peptide multimers, linked via disulfide bridges, binding to different DNA substrates and forming networks too large to enter the gel. The peptide effects are reversed in the presence of 50 mM DTT (Figure 4(a), lane 16). With the exception of KCCRCK, concentrations near the IC₅₀ values did not trap a substantial amount of labeled substrate DNA in the well. The binding results at intermediate peptide concentrations show that the peptides do not displace the enzyme from its target DNA, but the shift of the vTopo-DNA complexes suggest that the peptides may alter the conformation of the complex (see the complex marked with an *). At high peptide concentrations, nonspecific binding to dsDNA is clear. The sensitivity of this nonspecific binding to DTT suggests that the peptides may bind DNA as disulfide-bridged oligomers and this binding probably contributes to inhibition of cleavage by vTopo.

To determine the dsDNA-binding activity of the peptides in the absence of vTopo, we tested binding of the peptide to the 60-bp double-stranded DNA used in the vTopo binding assays [Figure 4(b); (the same peptide concentrations were used as in the single turnover cleavage assays)]. For all peptides, concentrations near the cleavage IC₅₀ resulted in dsDNA-peptide complexes that migrate as a smear throughout the gel lane (Figure 4(b), lanes 3, 11, 20 and 28). WYCRCK and KCCRCK bound to dsDNA with an apparent K_d of 0.25 μ M, WRWYCR had the lowest affinity for dsDNA (K_d = 0.6 μ M), while WRWYCRCK had the highest affinity, with a K_d of 0.15 μ M. The binding of peptide to dsDNA was abolished by 50 mM DTT (Figure 4(b) lanes 8, 16 and 24). We conclude that the peptides bind dsDNA and that binding is DTT-sensitive. The peptides may alter the conformation of the DNA, affecting its migration into the gel in the absence of vTopo, or they may simply bind in sufficient quantity to retard the substrate. The change in mobility is probably not due to a concerted bend, since ligation of short DNA fragments³⁰ did not occur more efficiently in the presence of peptides (data not shown) as it does when bound by a DNA bend-inducing protein^{31,32}.

Inhibition of phage lambda Int-mediated recombination and accumulation of Holliday junction intermediates

As discussed above, peptides that inhibit Int-mediated recombination by trapping protein-bound HJ complexes were isolated^{19,20,24}, and some of these peptides, including WRWYCR, inhibited vTopo cleavage^{20,21}. Therefore we tested whether the peptides isolated as vTopo inhibitors can also inhibit Int-mediated recombination via the efficient bent-L recombination pathway³³, which requires Int and IHF. Figure 5(a) shows that WYCRCK causes accumulation of Holliday junction intermediates with 0.8 μM treatment (20% of total DNA). In contrast, KCCRCK does not produce a significant amount of HJ intermediates at any peptide concentration. Addition of WRWYCRCK resulted in a maximum accumulation of 50% of Holliday junction intermediates of the total input substrate at 0.1 μM (Figure 5(a)), compared to 63% accumulation of junctions by WRWYCR at 0.1 μM ²⁰ (Figure 5(b)). The activity of the WRWYCRCK octapeptide could be due to the N-terminal WRWYCR part of its sequence. These data show that WYCRCK accumulates HJ intermediates during Int-mediated recombination, while KCCRCK does not. WYCRCK, WYARCK and WYCRACK were compared for their ability to trap HJ during bent-L Int mediated recombination (Figure 5(b)). Treatment with 1.5 μM peptide produced 14% and 24% trapped Holliday junction intermediates compared to WYCRCK, which accumulated 37% HJ intermediates. Both cysteine residues contribute to accumulation of HJ to some extent, but neither is as necessary for trapping HJ as it is for inhibiting dsDNA cleavage and plasmid relaxation by vTopo. When WRWYCR is added to recombination reactions, very high concentrations of peptide inhibit HJ accumulation because they inhibit even the first DNA cleavage stage of recombination, thereby blocking the progression of substrates through the pathway¹⁹⁻²¹. While WYCRCK does the same at high concentration, neither WYARCK or WYCRACK completely inhibit recombination at 500 μM . These results suggest that, similar to WRWYCR, peptide WYCRCK recognizes the central opening of HJ and led us to test the interactions between the vTopo inhibitor WYCRCK and free HJ substrates.

WYCRCK and wycrck bind specifically to 4-way DNA junctions

Peptide WRWYCR accumulates HJ intermediates during Int-mediated recombination due to its high affinity for protein-free HJ³⁵ (Table 1). Similarly, both WYCRCK and wycrck bind to synthetic Holliday (4-way) junction DNA, and form 2 distinguishable peptide-DNA complexes (Figure 6). We determined the dissociation constants (K_d) for WYCRCK and wycrck by evaluating the amount of unbound HJ DNA in the presence of peptide (Figure 6(a)); the concentration of peptide that left 50% unbound substrate HJ DNA was extrapolated to give affinity constants (K_d) of 35 nM and 25 nM for WYCRCK and wycrck, respectively (Figure 6(a)). We also determined the specificity of peptide-binding to 4-way junctions. Reactions were assembled using radiolabeled HJs DNA and challenged with an excess of unlabeled HJ used in the vTopo resolution assays described below. The competitor HJs branch migrate over a 10-bp region, contain the preferred vTopo binding and cleavage sequence, and have greater 4-way symmetry compared to the radiolabeled HJs showing the distinguishable shift. Addition of 100 nM WYCRCK or wycrck to the labeled junctions resulted in the formation of one predominant complex (Figure 6(b), lanes 2 and 18, cmplx 2). The addition of unlabeled competitor HJ strongly competes for the binding of peptide to the radiolabeled HJ substrate. Addition of 250 nM (435 ng or 250X molar excess) of unlabeled 4-way DNA junctions abolished all or almost all detectable peptide-junction complexes, either with the L-amino acid or d-amino acid peptide (Figure 6(b) lane 7 and lane 23, respectively). Addition of 250 nM non-radiolabeled 60 bp linear double stranded DNA added (equimolar amount compared to the highest amount of HJ competitor) changed the distribution of complexes formed in this assay slightly without producing unbound DNA (Figure 6(b), lanes 12 and 28), showing that peptide-junction interactions are not disrupted by linear double stranded DNA. Addition of an equal mass of salmon sperm DNA (435 ng) versus HJ did compete for peptide

binding the junction complexes more, especially in the case of the WYCRCK peptides, although not completely (Figure 6(b), lanes, 15 and 31 versus lanes, 7 and 23, respectively). Peptide wycrck-junctions were detectable even in the presence of 870 ng salmon sperm DNA (Figure 6(b), lanes 16 and 32). The greater competition of salmon sperm DNA with the junctions compared to the short ds DNA segment could be due to the higher sequence complexity present in salmon sperm DNA, which may have DNA that can extrude hairpins or stem-loop structures that could resemble HJs. Overall, these data indicate that WYCRCK and wycrck bind to 4-way junctions with greater affinity than to linear double stranded DNA, with wycrck having somewhat greater affinity and specificity for junctions. These results lead us to propose that the peptides recognize secondary structure or distorted DNA better than B-form DNA.

WYARCK and WYCRACK were less potent at accumulating Int junctions than WYCRCK. To relate this to their affinity for HJ, we titrated these substituted peptides on HJ using gel-shift assays. Each alanine-substituted peptides bound with 2 to 3-fold less affinity than WYCRCK as judged by the amount of free HJ DNA in lanes with 150 nM compared to 50 nM WYCRCK (Figure 6(c), lanes 4 and 19 versus lanes 11 and 26). The binding of both WYARCK and WYCRACK to HJ DNA was sensitive to DTT (Figure 6(c), lanes 15 and 30). The C>A substituted peptides formed predominantly one complex (Figure 6(c), lanes 7-14 and lanes 21-29) rather than the two complexes formed by WYCRCK (Figure 6(c), lane 3-4 and lanes 18-19), showing a direct correlation between the number of cysteine residues present in each hexapeptide and the peptide-HJ complexes formed. Peptide WYARAK did not shift HJ substrates at all (data not shown).

Peptides block 4-way junction resolution mediated by vaccinia topoisomerase

Holliday junctions are intermediates found in both homologous recombination and site-specific recombination mediated by tyrosine recombinases^{8-10,34}. vTopo can resolve HJ into recombinant products if the junctions contain its preferred cleavage sites oriented head-to-head^{11,12} (Figure 7(a)). Since WYCRCK and WRWYCR cause the accumulation of HJ during Int recombination with potencies in the nanomolar range and binds specifically to HJ DNA, we tested if the peptides inhibit HJ resolution by vTopo. HJ substrates for vTopo were constructed by annealing a 5' ³²P radiolabeled DNA oligonucleotide to three other oligonucleotides to form a partially mobile HJ in which branch migration could occur over the central 10 bp¹¹ (Figure 7(a)). Concerted cleavage and religation by vTopo generates a labeled 59 bp linear duplex product. Equimolar amounts of junction substrate and vTopo were mixed as in the single turnover DNA cleavage assays, and produced 30%-40% 59-bp product when incubated for 10 min at 37°C (Figure 7(b)). Addition of 2.5 μM WYCRCK or wycrck significantly inhibited the formation of the 59-bp recombinant product (Figure 7(b), lanes 13 and 31), while 2.5 μM KCCRCK completely inhibited HJ resolution (Figure 7(b), lane 6). WRWYCRCK was the most potent inhibitor, completely inhibiting resolution at 0.63 μM (Figure 7(b) lane 23). The amount of resolved 59-bp product was quantified, expressed as the percentage of product produced, and normalized to the product produced in the absence of peptide, finally leading to the IC₅₀ value (Table 1). To compare the potency of each peptide in inhibiting junction resolution to its potency at inhibiting single turnover cleavage, the IC₅₀ values for peptide treated reactions were normalized to the number of cleavage sites present in each substrate, which in the case of the HJ is two. This comparison shows that the peptides inhibit the resolution of HJ by vTopo with potencies very close to those observed in single turnover DNA cleavage experiments.

To determine if KCCRCK and WYCRCK inhibit the cleavage of the Holliday junction substrate or block the religation event by vTopo, the continuity of the radiolabeled DNA strand cleaved by vTopo was analyzed. vTopo-mediated junction resolution assays were performed

in the presence and absence of peptide inhibitor, the reactions were quenched with SDS and treated with proteinase K, and then were separated on a DNA-denaturing gel. Processing of junctions by vTopo resulted not only in the 59 nt resolution product but also a 26 nt cleaved product (Figure 7(c), lane 2). The heterogeneity of the cleaved product are probably due to different numbers of residual amino acids remaining linked to it after proteinase K treatment. Reactions in the presence of KCCRCK or WYCRCK resulted in a concomitant decrease in both the 26 nt cleaved product and the 59 nt religated product (Figure 7(c), lanes 3-9 and lanes 10-16, respectively). Thus the peptides block the cleavage event that initiates strand exchange, resulting in reduced Holliday junction resolution. The unligated products may be due to vTopo dissociating from some of the junctions before ligation. WYARCK and WYCRAK were also tested for their effects on vTopo-mediated junction resolution; in this case, 25 μ M peptide resulted in 35% and 50% resolution inhibition, respectively (data not shown). Just as for Int-mediated bent-L recombination resolution, neither of these C>A peptides blocked vTopo junction resolution completely, even at concentrations far beyond their IC₅₀.

WYCRCK perturbs the crossover point of 4-way junctions

WYCRCK inhibits Holliday junction resolution by vTopo, accumulates Holliday junction intermediates in lambda Int-mediated recombination, and binds HJ. To test how WYCRCK influences HJ structure, KMnO₄ was used to probe the sensitivity of thymine bases near the junction crossover point when bound with WYCRCK in the presence and absence of competitor DNA. The synthetic HJ contained only one radiolabeled strand of 66 nucleotides (Figure 8 (a)). The HJ was subjected to KMnO₄ in the presence or absence of WYCRCK at concentrations above and below the IC₅₀ values determined for DNA cleavage and HJ resolution by vTopo. The addition of 0.5 μ M WYCRCK produced over a 2-fold increase in the sensitivity of the + 2T compared to untreated 4-way junctions, indicating that WYCRCK distorts the conformation of this base (Figure 8 (c)). The +1T is similarly affected by the presence of peptide, although to a lesser extent. The differential cleavage was quantified by densitometry and graphed as the relative amount of cleaved product in the presence of peptide compared to cleaved product in the absence of peptide. The addition of 50X molar excess of 60-bp competitor DNA over ³²P-radiolabeled HJ resulted in a small decrease in +2T sensitivity compared to the non-peptide treated control (Figure 8b, lanes 6-10 and Figure 8 (c) open symbols). These results show that WYCRCK perturbs thymine bases near the crossover region and alters the conformation of the bases within the vTopo cleavage site in the presence or absence of exogenous double stranded DNA.

Peptides inhibit a type IA topoisomerase I or restriction enzymes less than type IB topoisomerases

Type IA topoisomerases have a distinct structure and catalytic mechanism compared to type IB topoisomerases^{1,2}. Both use a tyrosine nucleophile during transesterification reactions. Type IA topoisomerases form transient 5' phosphotyrosyl intermediates, producing a free 3' hydroxyl, which then nucleophilically attacks the phosphotyrosyl bond, displacing the covalently-bound enzyme and reforming a continuous DNA strand. In contrast, type IB topoisomerases form a transient 3' phosphotyrosyl intermediate producing a free 5' hydroxyl upon cleavage of DNA. Furthermore, type IA enzymes prefer binding to single stranded regions of DNA, while type IB enzymes require a dsDNA substrate. The peptides were tested against the *E. coli* type IA topoisomerase I, using reaction conditions similar to the vTopo plasmid relaxation assays (see Materials and Methods). These reactions were incubated for 15 minutes, the minimal amount of time necessary to achieve 80-90% relaxation of pUC19 (Figure 9 and data not shown). Peptide WYCRCK did not substantially inhibit plasmid relaxation by *E. coli* topo I until reaching 25 μ M (Figure 9, lane 2 versus lane 8, compare the amount of completely supercoiled DNA remaining in the reaction), whereas at this concentration vTopo-mediated plasmid relaxation was blocked completely (Figure 2(a), lane 8). WYCRCK and

KCCRCK inhibited *E. coli* topo I with similar potencies, with an IC₅₀ of 21 μ M and 24 μ M, respectively. WRWYCRCK had a similar IC₅₀ of 5.5 μ M against both vTopo and *E. coli* topoisomerase I in assays measuring the relaxation of pUC19. WYCRCK and KCCRCK are 4-fold and 2-fold less potent against *E. coli* topoisomerase I, respectively, than against vTopo (Table 1).

To test the specificity of the peptides further, inhibition of cleavage by three different restriction enzymes (EcoRI, PstI and XbaI) was determined. Restriction enzymes cleave DNA by hydrolysis rather than by transesterification. The enzymes chosen each restrict at a single site within pUC19. Peptide inhibition assays were performed using buffer conditions and pUC19 concentrations identical to those employed in the plasmid relaxation assays. One unit of restriction enzyme was added to reactions followed by incubation at 37°C for a time period that produced 50-80% digested product. The reactions were quenched by the addition of SDS and samples were analyzed by agarose gel electrophoresis. The amount of cleaved product in the presence of peptide was normalized to the amount of product in the absence of peptide and expressed graphically as % inhibition with respect to peptide concentration. The IC₅₀ values for the peptides tested are shown in Table 1. WYCRCK inhibits restriction enzymes with IC₅₀ values of 18-26 μ M, KCCRCK with IC₅₀ values of 16-20 μ M, and WRWYCRCK with IC₅₀ values of 9-16 μ M. When the IC₅₀ values determined in vTopo plasmid relaxation assays are normalized to the number of preferred vTopo cleavage sites present in pUC19 (14 sites), WYCRCK, KCCRCK and WRWYCRCK are at least 45-fold, 22-fold and 36-fold more potent against vTopo than restriction enzymes. By comparison, WRWYCR is 50-fold more potent against vTopo than restriction enzymes.

DISCUSSION

Our laboratory previously identified peptide inhibitors against λ Int-mediated recombination by screening mixture-based combinatorial peptide libraries^{19,20}. Type IB topoisomerases, including human topoisomerase I and vTopo, are structurally and mechanistically related to Int^{1-4,8-10}. Our most potent peptides isolated as Int inhibitors, WRWYCR and KWWCRW, also blocked DNA cleavage and plasmid relaxation by vTopo and human topoisomerase I^{20, 21}. This was surprising because the peptide inhibitors do not appear to interact directly with Int's active site^{19-21,24} but rather stabilize the Holliday junction recombination intermediate by interacting with branched DNA molecules^{35,36} (K. Kepple, N. Patel, and A. Segall, ms. in preparation). We set out to identify and characterize the structure of inhibitors screened directly for inhibiting vTopo-mediated plasmid relaxation, and to determine how similar or different they are from the previously identified inhibitory peptides. The two most potent peptides identified by the screen described herein are WYCRCK and KCCRCK. Because WYCRCK shares amino acid identity with WRWYCR, we also characterized the octapeptide WRWYCRCK. The shared amino acid sequence between WYCRCK and WRWYCR suggests that the peptides inhibit Int and vTopo via a common mechanism. On the other hand, peptide KCCRCK with its three dispersed positive charges resembles the structure of polyamines, spermidine in particular. In fact, hexamine compounds that aggregate also inhibit DNA cleavage by human topoisomerase I³⁷.

The newly identified peptides inhibit vTopo plasmid relaxation with similar potency as the Int-inhibitors KWWCRW and WRWYCR. All these peptides bind duplex DNA and block vTopo-mediated DNA cleavage (the first transesterification step) without displacing vTopo from its substrate (Table 1; Fig. 5). The KCCRCK peptide showed no significant difference in potency between inhibiting relaxation of pUC19 (14 vTopo sites, 192 bp of nonspecific DNA per site) versus pACYC184 (8 vTopo sites, 530 bp nonspecific DNA per site), indicating that the number of vTopo sites in the substrate or the total number of base pairs in the substrate was irrelevant. We conclude that KCCRCK's affinity for dsDNA is the determining factor in its

inhibition of vTopo and that a ~2-fold difference in DNA or sites does not affect its potency. The WYCRCK peptide was about 2X as potent at inhibiting relaxation by vTopo when we calculated the IC_{50} per single vTopo site, while its overall inhibition of relaxation in the two plasmids was roughly equal. The peptide is probably titrated among vTopo sites, or more likely, vTopo-DNA complexes. In the case of WRWYCR, relaxation of the longer plasmid is inhibited roughly half as well as the shorter plasmid when simply considering total nucleotides, and is ~4X less potent when considering the 2.8X difference in ratio of nonspecific DNA to vTopo recognition site. This suggests that WRWYCR is titrated more by nonspecific DNA than WYCRCK. However, WRWYCR has less ds DNA binding activity than peptide WYCRCK (in fact, has the least nonspecific ds DNA binding activity of all the peptides in this study; Fig. 5). Peptide WRWYCR may bind more weakly to vTopo-DNA complexes than WYCRCK. While WRWYCRCK appears to be the most specific for vTopo-DNA complexes when comparing relaxation of pUC19 and pACYC184, this probably reflects that it has the highest affinity for non-specific DNA.

We discovered that WYCRCK is more potent when inhibiting Int-mediated recombination ($IC_{50} = 0.085 \mu M$) than vTopo-mediated plasmid relaxation ($IC_{50} = 5.5 \mu M$ or $0.4 \mu M/site$) and concomitantly traps junction intermediates, despite the fact that our Int recombination assays include nonspecific DNA. WYCRCK binds free Holliday junctions and is able to block their resolution by vTopo. Peptide WRWYCR inhibits junction resolution almost twice as well as peptide WYCRCK, although their affinities for junctions differ by only 30%. In contrast, KCCRCK inhibits Int recombination less potently ($IC_{50} = 0.3 \mu M$) than the other peptides and does not trap junction intermediates appreciably. We did not measure the affinity of peptides KCCRCK or WRWYCRCK for junctions directly, but these peptides do not appear to distinguish between the different contexts of the vTopo recognition sequence (Table 1). The comparison between inhibition of vTopo single turn-over cleavage and HJ resolution showed that WRWYCR, KCCRCK, and WRWYCRCK have almost the same IC_{50} value when inhibiting these two vTopo activities, while WYCRCK inhibits cleavage of suicide substrates better than resolution of junctions. The different readouts we obtain from different assays suggests that the peptides differ in their affinity for and/or mode of binding to double stranded oligo substrates, supercoiled plasmids, and 4-way junctions.

The IC_{50} values of peptides inhibiting vTopo-mediated plasmid relaxation or DNA cleavage either on a ds oligo or a Holliday junction are all within a factor of 4 of each other, and relatively close to the apparent affinity for ds DNA. In contrast, peptides WRWYCR and WYCRCK bind to HJ substrates and inhibit Int-mediated recombination significantly better, by at least a factor of 5 (Table 1). We conclude that the inhibition of vTopo most likely depends on the peptides' interaction with ds DNA. In contrast, the inhibition of Int-mediated recombination depends on the ability of the peptides to bind to 4-way junctions. Kinetic analysis of vTopo single turnover DNA cleavage led to proposals that this step is partially rate-limiting during vTopo catalysis^{38,39}. Our demonstration that the peptides inhibit the DNA cleavage step independently supports this, because the rate-limiting step would be expected to be highly sensitive to inhibitors.

As an indicator of specificity, we compared how effectively the inhibitors blocked DNA recognition and cleavage by three restriction enzymes and plasmid relaxation by the structurally and mechanistically unrelated type IA topoisomerase encoded by *E. coli*, EcTopo. Given that each of the restriction enzymes has a single recognition sequence in pUC19 while EcTopo acts at many more sites within pUC19, the difference in IC_{50} concentrations suggests that WRWYCR inhibits EcTopo with more specificity than it does the restriction enzymes (Table 1). Most likely, the basis of the inhibition of restriction enzymes by all the peptides is their nonspecific DNA binding activity. WRWYCRCK appears to inhibit all of these enzymes

at lower concentration, and has characteristics combining those of WRWYCR and WYCRCK, as expected.

WYCRCK, KCCRCK, WRWYCR, and WRWYCRCK all contain cysteine residues. DTT severely curtails all of their activities, strongly suggesting that monomer peptides assemble into at least dimers and in some cases into higher multimers via disulfide bonds. Binding of the WRWYCR to Holliday junctions and inhibition of Int recombination is severely reduced by DTT^{20,35}. Both cysteines within peptide WYCRCK are very important to its activity. WYARCK and WYCRAK did not inhibit vTopo-mediated plasmid relaxation and both had greatly reduced inhibitory activity against vTopo-mediated DNA cleavage of suicide substrates. In addition, these peptides had reduced activity in blocking vTopo-mediated junction resolution. WYARCK and WYCRAK modestly accumulated HJ intermediates during Int recombination and partially inhibited HJ resolution by vTopo. These peptides probably do not completely block resolution of HJ processing enzymes due to their lower affinity for HJ. WYARAK does not inhibit vTopo in any assay (data not shown). WYARCK and WYCRAK are restricted to assembling dimers via a single disulfide bridge, whereas WYCRCK could potentially form greater-order multimers or networks. Alternatively, WYCRCK may form two intermolecular disulfide linkages between two hexapeptides, resulting in a small ring that is presumably more rigid than an unconstrained peptide dimer. HPLC traces of peptide WYCRCK compared to those of the single cysteine derivatives or of the cysteine-lacking peptide indicate one predominant conformation that is probably a peptide dimer, with only small amounts of larger molecular weight species, and do not support the presence of high molecular weight networks or aggregates (data not shown).

Modes of inhibition by WYCRCK and WRWYCR underline differences between type IB topoisomerases and tyrosine recombinases

The fact that both WYCRCK and WRWYCR exhibit specific binding to Holliday junctions suggests that a similar DNA intermediate is formed during vTopo catalysis, for example a transiently denatured duplex DNA resembling the branch point of a Holliday junction. Kwon and colleagues³⁹ showed that both non-covalent and covalent interactions of vTopo with DNA induce unstacking within the vTopo recognition site. This vTopo-DNA structure may share some character of the branch point of a Holliday junction. KMnO₄ footprinting experiments of Holliday junctions containing the vTopo 5'-(C/T)CCTT⁺ cleavage site near the crossover point show that the +2T and, to a lesser extent, +1T is perturbed by WYCRCK. The +2T base is very important for DNA cleavage - its removal decreases the rate of cleavage 60-fold⁴⁰. Furthermore, the addition of the covalent adduct +2R diastereomeric benzo[c]phenanthrene between the +2 and +1 base pairs abolishes DNA cleavage by disrupting interactions between vTopo and specific bases at the cleavage site⁴¹. The +1T base of the 5'-(C/T)CCTT⁺ cleavage site is in equilibrium between stacked and unstacked conformations when bound by vTopo, independently of DNA cleavage^{29,42}. This observation is consistent with our proposal that WYCRCK inhibits cleavage during Holliday junction resolution by perturbing the position of +1T and +2T bases. Perturbation of the junction center has also been shown for peptide WRWYCR (or KWWCRW)³⁵.

Our biochemical analyses of peptide-DNA interactions indicate the peptides most likely bind to DNA deformed by the binding of vTopo and induce further conformational changes in the DNA structure. The recent cocrystal structures of the variola topoisomerase with DNA in either covalent or noncovalent complexes do not show any topoisomerase-induced deformation in the double helix⁴³. Unlike the case of the tyrosine recombinase tetramers in which such deformations are seen even in noncovalent complexes⁹, type IB topoisomerases act as monomers and such DNA deformations may be too transient and unstable for capture in the crystals since they would not be stabilized by protein-protein interfaces. An alternate possibility

is that the peptides interfere with the network of hydrogen bonds which appear to determine specific interactions between the topoisomerase and the T/CCCTT site, which evidently is necessary for positioning of the Arg130 catalytic residue and thus for efficient cleavage.⁴³ As the enzyme does not rely on specific interactions to bind ds DNA, the peptides may interfere with such interactions and still permit nonspecific binding to DNA. We propose that the difference in the potency of peptide inhibition of different enzymes is related to their affinity for different DNA conformations, either pre-existing or induced by specific enzymes³⁵ (K. Kepple, N. Patel and A. Segall, submitted). These peptides have a distinct mechanism of DNA-based inhibition of DNA transacting enzymes.

Vaccinia virus has at least two potential targets of the peptides: the topoisomerase itself, which is necessary for normal levels of early transcription⁴⁴, and the A22R protein, a Holliday junction resolvase similar to the *E. coli* RuvC protein and involved in the maturation of unit-length viral genomes^{45,46}. Our peptides may inhibit poxvirus growth but those comprised of L amino acids are expected to be unstable due to degradation. Previously, we showed that the d-amino acid stereoisomer of WRWYCR is almost as active at inhibiting recombination as the L stereoisomer²⁰; in the present study, both d-amino acid peptides wrwycr and wycrcr are effective inhibitors of vTopo-mediated plasmid relaxation and Holliday junction resolution, and bind to free junctions. These d-peptides should be resistant to peptidases and, if effective at blocking poxvirus growth, may prove to be interesting leads for a new class of anti-poxvirus compounds.

MATERIALS AND METHODS

Preparation of DNA substrates

Synthetic oligonucleotides were 5' end-labeled with T4 polynucleotide kinase (New England Biolabs) with γ -³²P-ATP (New England Nuclear). Linear duplex DNA substrates (shown in results) were annealed by heating two complementary oligonucleotides to 95°C in the presence of 0.2 M NaCl followed by cooling to room temperature slowly. Double-stranded DNAs were gel-purified. Supercoiled plasmid DNA was isolated from *E. coli* DH5 α using the midi-prep kit from Promega Inc. (Madison WI).

Preparation of HJ DNA used in HJ resolution assays was performed as described in Sekiguchi *et al.*⁸. Preparation of the HJ used in gel retardation assays was as follows: one oligonucleotide was radiolabeled, then the kinase reaction was heated to 95°C in the presence of 0.2 M NaCl. The three remaining oligonucleotides were added to an equimolar concentration and all were annealed by slow cooling to room temperature, followed by gel purification as described³⁵. The sequences of the four oligonucleotides are: 5'-

CGCGGTACGGCCGGAAGCCTGCTTTTTTATACAAGCTTAACTCCC-3', 5'-
GGGAGTTAAGCTTGTATAAATGAGGTACTGTTACCAATTCTCCAAGTGACTACG
G-3', 5'-
CCGTAGTCACTTGGAGAATTCCTAACAGTACCTCATTTATACCTGCAGCCGACG
C-3', 5'-GCGTCGGCTGCAGGTATAAAAAGCAGGCTTCCGGCCGTACCGCC-3'.

Proteins

Vaccinia virus topoisomerase was the generous gift of Dr. Stewart Shuman, while *E. coli* topoisomerase I was the generous gift of Dr. Ken Mariani. Phage lambda integrase and excisionase, and *E. coli* IHF were purified as described²⁰. Restriction enzymes were obtained from New England Biolabs (Ipswich, MA).

Peptides and concentration determination

Peptides with defined amino acid sequences were purchased from Sigma Genosys (Sigma-Genosys Corp. Woodlands, TX) all oligopeptides were modified by C-terminal amidation. Peptide concentrations were determined by measuring absorbance at 280 nm and calculating the concentration using the extinction coefficients $5690 \text{ M}^{-1}\text{cm}^{-1}$ for tryptophan, $1289 \text{ M}^{-1}\text{cm}^{-1}$ for tyrosine, and $120 \text{ M}^{-1}\text{cm}^{-1}$ for cysteine, as described in Gill et al.⁴⁷ For peptides that do not contain tryptophan or tyrosine, molar concentration corresponds to the dry weight of lyophilized peptide suspended in a known volume of 10% DMSO. Mixture-based combinatorial peptide libraries were synthesized and provided by Torrey Pines Institute for Molecular Studies (La Jolla, CA).

Topoisomerase plasmid DNA relaxation assays

Screening of mixture-based combinatorial peptide libraries was performed essentially as described in Cassell et al.¹⁹ Peptide mixtures were screened using plasmid relaxation assays in the presence of 5 mM MgCl_2 . Ten μl reactions containing 50 mM Tris-HCl (pH 7.5), 0.1 μg of pUC19, 5 mM MgCl_2 were initiated by adding 0.25 pmol vaccinia topoisomerase along with peptide mixtures at the concentrations indicated. The reactions were incubated for 1 hour at 30°C , quenched with a mixture of 5% SDS, 25% glycerol, 0.025% xylene cyanol/bromophenol blue and separated on 1% agarose gels electrophoresed in 1X TBE (90 mM Tris, 90 mM borate and 2.5 mM EDTA). Gels were stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide for 20 min, washed with water and visualized by UV light. To determine peptide potency at inhibiting vTopo-mediated plasmid relaxation, 20 μl reactions contained 50 mM Tris (pH 7.5), 5 mM MgCl_2 , 0.3 μg supercoiled pUC19 and varying concentrations of peptide as indicated. Peptide(s) were pre-incubated with the DNA premix before addition of 0.5 pmol vTopo, and reactions were incubated at 37°C for 5 min. Reactions using pACYC184 as the DNA substrate were identical to the reactions containing pUC19 except 0.47 μg (equimolar concentration) supercoiled plasmid was added to the reaction prior to incubation at 37°C for 1 min. The amount of supercoiled DNA left in each reaction was determined by densitometry using the ImageQuant program (GE Healthcare Corp.). Percent relative relaxation was expressed as the % supercoiled substrate present in peptide-treated reactions normalized to input of DNA (DNA-only control). IC_{50} concentration values were extrapolated from graphs of % relative relaxation versus peptide concentration.

Peptide inhibition of *E. coli* topoisomerase I plasmid relaxation

Reactions (20 μl) containing 0.3 μg pUC19, 50 mM Tris-HCl (pH 7.5) and 5 mM MgCl_2 were pre-incubated with peptide at 37°C for 5 min. *E. coli* topoisomerase I (0.5 pmol) was added and reactions were incubated for 15 min at 37°C , then stopped with 5 μl of 5% SDS, 50% glycerol, 0.025% bromophenol blue/xylene cyanol. Reaction products were separated on 1% agarose gels in 1X TBE and stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. The IC_{50} values were calculated as described above and shown in Table 1.

Peptide inhibition of vTopo single-turnover cleavage

Single turnover reactions were performed as described in Klemm et al.²¹ The 18-mer scissile strand was 5' labeled with polynucleotide kinase and annealed to the 30-mer complementary strand (Figure 6). Reactions (20 μl) contained 50mM Tris (pH 7.5), 0.1 pmol 18-mer/30-mer annealed DNA substrate. Reactions were pre-incubated with peptide at 37°C for 5 min, initiated by the addition of 0.25 pmol vaccinia topoisomerase, and incubated at 37°C for 15 sec. Reactions were quenched by the addition of glycerol, SDS and bromophenol blue/ xylene cyanol and separated using 10% polyacrylamide gels (29:1 acrylamide:bis-acrylamide) containing 0.1% SDS and 0.5X TBE. Percent relative cleavage was determined by amount of

covalent protein DNA complex formed normalized to the amount of the complex formed in reactions without peptide.

Vaccinia topoisomerase DNA-binding assays

Reactions (20 μ l) contained 50 mM Tris (pH 7.5), 0.1 pmol 60-bp duplex DNA substrate (pre-incubated for 10 min at 37°C with the appropriate amount of peptide unless otherwise stated) and initiated by adding 0.25 pmol vTopo followed by incubation at 37°C for 5 min. Samples were adjusted to 10% glycerol and separated by native 6% (29:1) polyacrylamide gel electrophoresis containing 0.25 $\times$ TBE for 2 hours at 4°C. Gels were dried and viewed using a Molecular Dynamics Storm instrument (GE Healthcare Corp.).

Peptide inhibition of Int-mediated recombination

Bent-L recombination reactions (10 μ l) were assembled as described²⁴ and contained 1 nM ³²P end-labeled *attLtenP'1* 179 bp fragment, 4 nM unlabeled *attLtenP'1*, 50 ng salmon sperm DNA, 5 mM spermidine, and 50 ng/ml BSA. Reactions were initiated by the addition of IHF and lambda integrase and incubated at 37°C for 1 hour. Reactions were quenched with 0.5% SDS with xylene cyanol. Recombinant products were separated on 5% polyacrylamide (29:1 acrylamide:bisacrylamide) Tris-tricine-SDS gels at 100 mA constant power. Gels were dried and the amount of Holliday junction DNA accumulated in each reaction was determined by densitometry and expressed as the percentage of total input DNA.

Peptide binding to Holliday junction DNA and competition assays

Reactions (20 μ l) contained 50 mM Tris (pH 7.5), 5% glycerol, 5 mM NaCl, and 1 nM ³²P labeled synthetic HJ which can branch-migrate over the central 7 bp and had arms of different lengths³⁵. Reactions were initiated by the addition of peptide and were incubated for 20 min at 37°C. The amount and type of each competitor DNA is shown above the gel image (Figure 6). Complexes were separated on 8% (29:1) acrylamide:bisacrylamide polyacrylamide gels run for 10 hrs at 4°C; gels were dried and visualized on a Storm phosphorimager.

KMnO₄ footprinting of WYCRCK bound to Holliday junctions

The footprinting reactions of HJ bound to WYCRCK the same HJ as described in Sekiguchi *et al.*¹¹. Reactions contained 50 mM Tris (7.5), 5 nM ³²P-radiolabeled HJ DNA and various concentrations of WYCRCK as shown (Figure 6). Peptides were added to the DNA premix and allowed to bind at 37°C for 10 minutes. Peptide-HJ complexes were oxidized with 11 mM KMnO₄ reduced with 2-mercaptoethanol and reactions were treated with piperidine at 90°C for 30 min³⁵. Products were electrophoresed on 10% (19:1) acrylamide: bisacrylamide gels containing 7M urea. Gels were dried and visualized by phosphorimaging (GE Molecular Dynamics Storm instrument). Cleaved thymines in or near the vTopo site were quantified using ImageQuant software and normalized to thymines not affected by peptide treatment in the same lane.

Inhibition of vTopo-mediated Holliday junction resolution

Reactions (20 μ l) contained 50 mM Tris-HCl (pH 7.5), 0.1 pmol synthetic Holliday junction prepared as described by Sekiguchi *et al.*¹¹ and 0.5 pmol vTopo. Reactions were incubated at 37 °C for 10 min, quenched with SDS added to 1%, digested with 1.0 μ g of proteinase K, and extracted with phenol/chloroform. Samples were separated by 5% native polyacrylamide (19:1) gel electrophoresis and visualized using a Storm phosphorimager. To determine the strand continuity of the peptide inhibition of vTopo-mediated Holliday junction resolution, reactions were also separated on 12% (19:1) gels containing 7M urea¹¹, followed by exposure of the wet gel on a phosphorimager.

Peptide inhibition of restriction endonuclease cleavage

Reactions (20 μ l) contained 0.3 μ g pUC19, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1U of restriction enzyme, and were incubated at 37 ° C for a time period that produced 60-90% digested plasmid. Reaction products were separated by electrophoresis on 1% agarose gels containing 90mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA, and stained with 0.5 μ g/ml ethidium bromide. Densitometry measurements reflecting the cleaved product in the presence and absence of peptide were expressed as arbitrary densitometry units (A.U). A ratio was generated by dividing A.U. corresponding to cleaved product in the presence of peptide by cleaved product without peptide treatment and subtracted from 1 (1-cleaved product with peptide/ cleaved product without peptide) $\times$ 100, and designated % inhibition. The % inhibition was plotted with respect to peptide concentration and used to extrapolate the IC₅₀ values (Table 1).

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BIBLIOGRAPHY

1. Wang JC. DNA topoisomerases. Annu. Rev. Biochem 1996;65:635–692. [PubMed: 8811192]
2. Champoux JJ. DNA Topoisomerases: Structure, Function, and Mechanism. Annu. Rev. Biochem 2001;70:369–413. [PubMed: 11395412]
3. Redinbo MR, Champoux JJ, Hol WG. Structural insights into the function of type IB topoisomerases. Curr. Opin. Struct. Biol 1999;9:29–36. [PubMed: 10047584]
4. Cheng C, Kussie P, Pavletich N, Shuman S. Conservation of structure and mechanism between eukaryotic topoisomerase I and site-specific recombinases. Cell 1998;92:841–850. [PubMed: 9529259]
5. Krogh BO, Shuman S. A poxvirus-like type IB topoisomerase in bacteria. Proc. Natl Acad. Sci. USA 2002;99:1853–1858. [PubMed: 11830640]
6. Raymond, A.; Burgin, AB. Personal communication.
7. Shuman S. Vaccinia virus DNA topoisomerase: a model eukaryotic type IB enzyme. Biochim. Biophys. Acta 1998;1400:321–337. [PubMed: 9748643]
8. Nash, HA. Site-specific recombination: integration, excision, resolution, and inversion of defined DNA segments. In: Neidhardt, FC., editor. Escherichia coli and Salmonella: Cellular and Molecular Biology. ASM Press; Washington, D.C.: 1996. p. 2363-2367.
9. Van Duyne, GD. A structural view of tyrosine recombinase site-specific recombination. In: Craig, NL.; Craigie, R.; Gellert, M.; Lambowitz, AM., editors. Mobile DNA II. ASM Press; Washington, D. C.: 2002. p. 93-117.
10. Azaro, MA.; Landy, A. Lambda Integrase and the lambda Int family. In: Craig, NL.; Craigie, R.; Gellert, M.; Lambowitz, AM., editors. Mobile DNA II. ASM Press; Washington, D. C.: 2002. p. 111-148.
11. Sekiguchi J, Seeman N, Shuman S. Resolution of Holliday junctions by eukaryotic DNA topoisomerase I. Proc. Natl. Acad. Sci. USA 1996;93:785–789. [PubMed: 8570635]
12. Sekiguchi J, Cheng C, Shuman S. Resolution of a Holliday junction by vaccinia topoisomerase requires a spacer DNA segment 3' of the CCCTT cleavage sites. Nuc. Acids Res 2000;28:2658–2663.
13. Pommier Y, Pourquier P, Urasaki Y, Wu J, Laco GS. Topoisomerase I inhibitors: selectivity and cellular resistance. Drug Resist. Updat 1999;2:307–318. [PubMed: 11504505]

14. Staker BL, Hjerrild K, Feese MD, Behenke CA, Burgin AB, Stewart L. The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc. Natl. Acad. Sci. USA* 2002;99:15387–15392. [PubMed: 12426403]
15. Hsiang Y-H, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem* 1985;260:14873–14878. [PubMed: 2997227]
16. Gupta M, Zhu CX, Tse-Dinh YC. Mutations of vaccinia virus DNA topoisomerase I that stabilize the cleavage complex. *J. Biol. Chem* 1994;269:573–578. [PubMed: 8276853]
17. Yakovleva L, Hardy CJ, Sayer JM, Pirrung M, Jerina DM, Shuman S. Benzo[c]phenanthrene adducts and nogalamycin inhibit DNA transesterification by vaccinia topoisomerase. *J. Biol. Chem* 2004;279:23335–23342. [PubMed: 15044474]
18. Bond A, Reichert Z, Stivers JT. Novel and specific inhibitors of a poxvirus type I topoisomerase. *Mol. Pharmacol* 2006;69:547–557. [PubMed: 16267207]
19. Cassell G, Klemm M, Pinilla C, Segall A. Dissection of bacteriophage lambda site-specific recombination using synthetic peptide combinatorial libraries. *J. Mol. Biol* 2000;299:1193–1202. [PubMed: 10873445]
20. Boldt JL, Pinilla C, Segall AM. Reversible inhibitors of λ integrase-mediated recombination efficiently trap Holliday junction intermediates and form the basis of a novel assay for junction resolution. *J. Biol. Chem* 2004;279:3472–3483. [PubMed: 14625310]
21. Klemm M, Cheng C, Cassell G, Shuman S, Segall AM. Peptide inhibitors of DNA cleavage by tyrosine recombinases and topoisomerases. *J. Mol. Biol* 2000;299:1203–1216. [PubMed: 10873446]
22. Bankhead T, Segall AM. Characterization of a mutation of bacteriophage lambda integrase: putative mechanistic role for a conserved residue. *J. Biol. Chem* 2000;275:36949–36956. [PubMed: 10938278]
23. Bankhead TM, Etzel BJ, Wolven F, Bordenave S, Boldt JL, Larsen TA, Segall AM. Mutants at residues 282, 286 and 293 of phage λ integrase exert pathway-specific effects on synapsis and catalysis in recombination. *J. Bact* 2003;185:2653–2666. [PubMed: 12670991]
24. Cassell GD, Segall AM. Mechanism of inhibition of site-specific recombination by the Holliday junction-trapping peptide WKHYNY: insights into phage λ Integrase-mediated strand exchange. *J. Mol. Biol* 2003;327:413–429. [PubMed: 12628247]
25. Dooley CT, Spaeth CG, Berzetei-Gurske IP, Craymer K, Adapa ID, Brandt SR, Houghten RA, Toll L. Binding and in vitro activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL1. *J. Pharmacol. Exp. Ther* 1997;283:735–741. [PubMed: 9353393]
26. Houghten RA, Pinilla C, Appel JR, Blondelle SE, Dooley CT, Eichler J, Nefzi A, Ostresh J. Mixture-based synthetic combinatorial libraries. *J. Med. Chem* 1999;42:3743–3778. [PubMed: 10508425]
27. Shuman S. Site-specific DNA cleavage by vaccinia virus DNA topoisomerase I. *J. Biol. Chem* 1991;266:1796–1803. [PubMed: 1846364]
28. Kho SH, Landy A. Dissecting the resolution reaction of lambda integrase using suicide Holliday junction substrates. *EMBO J* 1994;13:2714–2724. [PubMed: 8013469]
29. Wittschieben J, Shuman S. Mechanism of DNA transesterification by vaccinia topoisomerase: catalytic contributions of essential residues Arg-130, Gly-132, Tyr-136 and Lys-167. *Nucl. Acids Res* 1997;25:3001–3008. [PubMed: 9224599]
30. Shore D, Langowski J, Baldwin RL. DNA flexibility studied by covalent closure of short fragments into circles. *Proc Natl Acad Sci USA* 1981;78:4833–4837. [PubMed: 6272277]
31. Kotlarz D, Fritsch A, Buc H. Variations of intramolecular ligation rates allow the detection of protein-induced bends in DNA. *EMBO J* 1986;5:799–803. [PubMed: 3011427]
32. Pil PM, Chow CS, Lippard SJ. High-mobility-group 1 protein mediates DNA bending as determined by ring closures. *Proc Natl Acad Sci USA* 1993;90:9465–9469. [PubMed: 8415724]
33. Segall AM, Nash HA. Architectural flexibility in lambda site-specific recombination: three alternate conformations channel the attL site into three distinct pathways. *Genes Cells* 1996;1:453–463. [PubMed: 9078377]
34. Sharples GJ. The X philes: structure-specific endonucleases that resolve Holliday junctions. *Mol. Micro* 39:823–834.
35. Kepple KV, Boldt JL, Segall AM. Holliday junction-binding peptides inhibit distinct junction-processing enzymes. *Proc. Natl. Acad. Sci. USA* 2005;102:6867–6872. [PubMed: 15867153]

36. Ghosh K, Lau CK, Guo F, Segall AM, Van Duyne GD. Peptide trapping of the Holliday junction intermediate in Cre-loxP site-specific recombination. *J. Biol. Chem* 2005;280:8290–8299. [PubMed: 15591069]
37. Sukhanova A, Devy J, Pluot M, Bradley JC, Vigneron JP, Jardillier JC, Lehn JM, Nabiev I. Human DNA topoisomerase I inhibitory activities of synthetic polyamines: relation to DNA aggregation. *Bioorg. Med. Chem* 2001;9:1255–1268. [PubMed: 11377184]
38. Stivers JT, Shuman S, Mildvan AS. Vaccinia DNA topoisomerase I: single-turnover and steady-state kinetic analysis of the DNA strand cleavage and ligation reactions. *Biochemistry* 1994;33:327–339. [PubMed: 8286354]
39. Kwon K, Jiang YL, Song F, Stivers JT. ¹⁹F NMR studies of vaccinia type IB topoisomerase. Conformational dynamics of the bound DNA substrate. *J. Biol. Chem* 2002;277:353–358. [PubMed: 11689573]
40. Tian L, Sayer JM, Jerina DM, Shuman S. Individual nucleotide bases, not base pairs, are critical for triggering site-specific DNA cleavage by vaccinia topoisomerase. *J. Biol. Chem* 2004;279:39718–39726. [PubMed: 15252055]
41. Yakovleva L, Handy CJ, Sayer JM, Pirrung M, Jerina DM, Shuman S. Benzo[c]phenanthrene adducts and nogalomycin inhibit DNA transesterification by vaccinia topoisomerase. *J. Biol. Chem* 2004;279:23335–23342. [PubMed: 15044474]
42. Sekiguchi J, Shuman S. Covalent DNA binding of vaccinia topoisomerase results in unpairing of the thymine base 5' of the scissile bond. *J. Biol. Chem* 1996;271:19436–19442. [PubMed: 8702632]
43. Perry K, Hwang Y, Bushman FD, Van Duyne GD. Structural basis for specificity in the poxvirus topoisomerase. *Mol. Cell* 2006;23:343–354. [PubMed: 16885024]
44. Da Fonseca F, Moss B. Poxvirus DNA topoisomerase knockout mutant exhibits decreased infectivity associated with reduced early transcription. *Proc. Natl Acad. Sci. USA* 2003;100:11291–11296. [PubMed: 12972642]
45. Garcia AD, Aravind L, Koonin EV, Moss B. Bacterial-type DNA Holliday junction resolvases in eukaryotic viruses. *Proc Natl Acad Sci USA* 2000;97:8926–8931. [PubMed: 10890916]
46. Garcia AD, Moss B. Repression of vaccinia virus Holliday junction resolvase inhibits processing of viral DNA into unit-length genomes. *J. Virol* 2001;75:6460–6471. [PubMed: 11413313]
47. Gill SC, von Hippel PH. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem* 1989;182:319–332. [PubMed: 2610349]

Figure 1.

(a) Deconvolution strategy used to identify peptide inhibitors from a mixture-based combinatorial library. The fixed positions in each mixture are denoted by O, and the equimolar mixtures containing all natural amino acids except cysteine are denoted by X. In step 1, 120 mixtures with single position fixed amino acids are screened (20 mixture per fixed position) in all mixtures there are 2.47 million peptides in each mixture. In step 2, a subset of the 1200 peptide mixtures with 2 positions fixed are screened, which include fixed amino acids identified in step 1. These mixtures are less complex, 132,000 peptides per mixture, and their effective concentration is higher than the single fixed position peptide mixtures. In step 3, peptides with defined sequences are synthesized based on amino acids in the dual defined peptide mixtures that significantly inhibit enzyme activity. (b) Positional scanning of the peptide library against vTopo plasmid relaxation by peptide mixtures with second position fixed amino acids. Reactions contained 0.1 μg of pUC19, 0.2 mg/ml of each single fixed position peptide mixture, and were initiated with 0.25 pmol vTopo and incubated at 30°C for 60 min. Lane 1, untreated supercoiled pUC19; lane 2, control reaction with only vTopo and pUC19; lanes 3-22, reaction

products treated with the second position fixed peptide mixtures, each mixture tested is represented by the one letter amino acid symbol shown above the gel image. R = relaxed plasmid DNA, S = supercoiled plasmid DNA. (c) Example of a dose response assay testing peptide mixtures with the first and second position fixed. Reaction conditions were identical to those used above. Lane 1, control with supercoiled plasmid pUC19; lane 2, vTopo and pUC19. For comparison, peptide mixtures with the second position fixed were tested in parallel with dual-defined peptide mixtures. Lanes 3 and 4 are reactions containing mixtures with the second position fixed as cysteine. Similarly, reactions separated in lanes 9 and 10 were treated with mixtures with the second position fixed as tyrosine. Lanes 5-8, dual defined peptide mixtures with arginine and cysteine at positions 1 and 2 of the peptides. Lanes 11-14, dual defined peptide mixtures with tryptophan and tyrosine residues at positions 3 and 4. S = supercoiled pUC19; vTopo = reaction with no added peptide.

Figure 2.

Peptide inhibition of plasmid DNA relaxation mediated by vaccinia topoisomerase. (a) pUC19 substrate. (b) pACYC184 substrate. DTT (50 mM) was added when specified. (c) Inhibition of pUC19 relaxation by vTopo with WYARCK or WYCRAK. R = relaxed DNA, S = supercoiled DNA.

Figure 3.

Peptide inhibition of single-turnover DNA cleavage mediated by vaccinia topoisomerase. The sequence of the suicide substrate used in this assay is shown with the cleavage site depicted by the arrow and the vTopo recognition site, CCCTT, boxed. (a) Dose response assay testing the inhibition of DNA cleavage by WYCRCK (solid triangles), KCCRCK (open triangles), and WRWYCRCK (open circles). WYCRCK and KCCRCK were tested for cleavage inhibition potency at 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, and 25.6 μM final concentrations. WRWYCRCK was tested at 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 μM final concentrations. The amount of covalent vTopo-DNA complex was determined by densitometry and expressed as a percentage of covalent enzyme-DNA complex formed in

mock-treated reactions (% relative DNA cleavage). (b) Inhibition of vTopo-mediated DNA cleavage by WYARCK and WYCRAK, compared to WYCRCK, tested at 5 μ M.

Figure 4.

(a) Peptide effects on vaccinia topoisomerase binding. peptide-duplex DNA binding assays. The sequence of the 60-bp linear DNA binding target is shown with the vTopo CCCTT-binding site bolded and boxed. Complexes were separated on a 6% (29:1) polyacrylamide gel with 0.25X TBE. DTT (50 mM) was added as specified. (b) Peptide binding to free duplex DNA. Reaction conditions were as above, except no vTopo was added.

Figure 5.

Effect of peptides on the accumulation of Holliday junctions of lambda integrase mediated recombination (bent-L pathway). (a) WYCRCK or KCCRCK were added to the following final concentrations: 1.0, 5.0, 25, 125, 625 nM, 3.1 μM and 15.6 μM. The amount of Holliday junctions formed was expressed as a percentage of total input radiolabeled DNA (% Holliday junctions). (b) Inhibition of bent-L recombination by WRWYCR, WYCRCK, WYCRAK and WYARCK. Peptides were tested at the following concentrations: 0.005, 0.05, 0.5, 1.5, 5.0, 15, 50, and 500 μM.

Figure 6.

Binding of WYCRCK and wycrck to Holliday junctions. (a) Graphical representation of a peptide-HJ titration. Reactions (20 μ l) contained 50 mM Tris (pH 7.5), 5 mM NaCl, 5% glycerol and 1 nM radiolabeled HJ. Reactions were initiated with the addition of increasing amounts of WYCRCK or wycrck, followed by incubation at 37°C for 20 min. The bound and unbound HJs were separated by native PAGE and K_d values defined as the concentration of peptide resulting in 50% bound HJ. Peptide WYCRCK or wycrck were added to the following concentrations: 3.1, 6.3, 12.5, 25, 50, 100, 250, 500 nM. (b) Affinity of peptides for HJ in the face of challenge by various unlabeled competitor DNAs. Reaction conditions were identical to those described above except competitor DNAs were added to reactions followed by the

addition of 100 nM WYCRCK or wycrck. The amount of unlabeled competitor HJ DNA, 60-bp DNA and salmon sperm DNA per reaction is shown above each gel image. (c) Binding of WYARCK and WYCRAK to HJ. As controls, WRWYCR and WYCRCK were tested at the concentrations specified.

Figure 7.

Peptide inhibition of Holliday junction resolution by vTopo. (a) Schematic illustrating vTopo-mediated Holliday junction resolution. The asterisk denotes the 5' radiolabeled end of strand 2; strands 1, 3, and 4 of the Holliday junction are unlabeled. Arrows depict the position of the vTopo cleavage sites. Ovals represent vTopo covalently linked to the substrate during resolution and the products are shown as duplex DNA products. (b) Inhibition of vTopo-mediated resolution of HJ. (c) Effect of peptides on strand continuity (cleavage versus cleavage followed by ligation) during vTopo-mediated Holliday junction resolution.

Figure 8.

Potassium permanganate footprinting of Holliday junction DNA bound with WYCRCK. (a) A schematic of ^{32}P radiolabeled (asterisk) HJs used in this experiment showing the sequence of the nucleotides present near the crossover region. The position of each nucleotide is numbered with respect to the preferred vTopo cleavage site (+1) cleaved nucleotide. Thymidine nucleotides perturbed by peptide are highlighted by boxes and correspond to the data in Figure 8(b). (b) The amount of peptide added to each reaction in the presence and absence of competitor DNA is shown above the gel image; 0.25, 0.5, 1.0 and 2.0 μM (lanes, 2-5) and (lanes, 7-10). Competitor DNA (1 μM of 60 bp ds DNA) was added to peptide-HJ reactions (lanes 7-10). (c) Piperidine cleavage sensitivity was determined by densitometry for WYCRCK and untreated reactions. The amount of cleaved product in the presence of peptide was

normalized to the amount in the absence of peptide (% relative cleavage sensitivity). +CD denotes reactions in the presence of competitor DNA.

Figure 9. WYCRCK inhibition of DNA plasmid relaxation catalyzed by *E. coli* topoisomerase I. R = relaxed pUC19 DNA, SC = supercoiled pUC19 DNA.

Table 1Activities of peptide inhibitors (IC₅₀ values, μ M)

Assay ^a	WYCRCK	WRWYCRCK	KCCRCK	WRWYCR
vTopo plasmid relaxation (pUC19; 14 vTopo sites) ^b	5.5/0.4 ^b	3.5/0.25	10/0.7	8.0/0.6
vTopo plasmid relaxation (pACYC184; 8 vTopo sites)	6.5/0.8	6.0/0.1	8.0/0.7	18.0/2.3
vTopo DNA cleavage ^c	0.5	0.1	0.5	0.65
ds DNA binding (estimated from Fig. 4b)	0.25	0.15	0.25	0.6
vTopo-mediated HJ resolution	1.8/0.9	0.2/0.1	1.0/0.5	1.0/0.5
Int-mediated recombination ^d	0.085	0.015	0.3	0.015
HJ binding	0.035	NT ^e	NT ^e	0.025 ^f
EcTopo plasmid relaxation (pUC19)	21.0	5.5	24.0	10.5
XbaI restriction (TCTAGA)	26	10	16	29
EcoRI restriction (GAATTC)	20	16	20	34
PstI restriction (CTGCAG)	18	9	16	30

^a Inhibition of vTopo activity, unless specified otherwise.^b The value before the slash is the overall IC₅₀ for inhibiting relaxation of the plasmid; the value after the slash is the IC₅₀ calculated per vTopo site (*i.e.*, 5.5 μ M : 14 vTopo sites = 0.4 μ M / site).^c DNA cleavage measured in the single turnover assay shown in Figure 3.^d Int recombination assays were performed in the presence of 100 ng of salmon sperm DNA, as described in Boldt et al.²⁰.^e NT = not tested^f Data taken from Kepple et al.³⁵.