Inhibition of Flp Recombinase by the Topoisomerase I-targeting Drugs, Camptothecin and NSC-314622*

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Recombinases of the λ-Int family and type IB topoisomerases act by introducing transient single strand breaks in DNA using chemically identical reaction schemes. Recent structural data have supported the relationship between the two enzyme groups by revealing considerable similarities in the architecture of their catalytic pockets. In this study we show that the Int-type recombinase Flp is inhibited by the two structurally unrelated topoisomerase I-directed anti-cancer drugs, camptothecin (CPT) and NSC-314622. The interaction of these drugs with topoisomerase I is very specific with several single amino acid substitutions conferring drug resistance to the enzyme. Thus, the observed interaction of CPT and NSC-314622 with Flp, which is comparable to their interaction with the cleavage complex formed by topoisomerase I, strongly supports a close mechanistic and evolutionary relationship between the two enzymes. The results suggest that Flp and other Int family recombinases may provide model systems for dissecting the molecular mechanisms of topoisomerase I-directed anti-cancer therapeutic agents.

Members of the integrase (Int)1 family of site-specific recombinases (λ-integrase, P1 Cre recombinase, Escherichia coli XerC/XerD recombinase, Saccharomyces cerevisiae Flp, and Zygosaccharomyces rouxii R recombinases among several others) all carry out conservative site-specific recombination using a basic type IB topoisomerase reaction scheme (1, 2). In the first step of recombination, an active site tyrosine nucleophile attacks the target phosphodiester bond in DNA to generate a 3'-phosphotyrosyl linkage and a free 5'-hydroxyl group. In the second step leading to strand rejoining, the 5'-hydroxyl group is the nucleophile, and the 3'-phosphotyrosyl bond is its target.

The nucleophilic attack is directed across partner substrates so that strand ligation occurs in the recombinant configuration, which is opposed to the typical type IB topoisomerase reaction in which ligation restores the original phosphodiester bond (3). However, the two ligation modes are not mutually exclusive, as evident from the fact that some Int recombinases can relax supercoiled DNA in vitro under certain conditions while some type IB topoisomerases can mediate recombination and resolution of Holliday junctions (4–8). Recent structural data have further consolidated the relationship between the Int family recombinases and the type IB topoisomerases. Despite the lack of overall sequence homology between the two enzyme groups, the tertiary folds within the catalytic domains are strikingly similar between the Int-type recombinases, including Flp (9) and Cre (10), and eukaryotic topoisomerase I (topo I) (11–14). Moreover, the critical catalytic moieties include two nearly identical tetrad, RHRH/W in the recombinases and RKRH in the topoisomerases, together with the invariant tyrosine nucleophile (1, 11).

In this study we have further probed the functional relationship between the Int family recombinase and type IB topoisomerase active sites by investigating the sensitivity of Flp toward camptothecin (CPT) (reviewed in Refs. 3, 15, and 16) and the newly synthesized CPT-like agent NSC-314622 (17). CPT and its derivatives are among the most promising anticancer drugs available today and have long been known specifically to target topo I in human cells. Although NSC-314622 is structurally unrelated to the camptothecins, its mode of action appears to be similar to that of CPT (17). Both drugs inhibit the religation step of topo I catalysis, whereas they have no effect on the cleavage reaction (16–19). For the camptothecins, it is well established that the drugs do not interact with either topo I or DNA separately (19). Rather, they form a ternary complex with the cleavage intermediate, blocking the active site of the covalently bound enzyme (18, 20). In the present study we demonstrate that the Int type recombinase, the Flp protein, is inhibited by CPT and NSC-314622. This is the first report of an enzyme other than eukaryotic topo I being sensitive toward these drugs. The results demonstrate several similarities and subtle differences between Flp and topo I in the mode of inhibition by the two drugs and suggest that the simple members of the Int family such as Flp and Cre may be exploited to dissect the molecular action of the topo I-directed anti-cancer drugs.

EXPERIMENTAL PROCEDURES

Materials—CPT was purchased from Sigma-Aldrich (C9911), and NSC-314622 was a kind gift from Dr. Yves Pommier (National Institutes of Health, Bethesda, MD). Both drugs were dissolved and stored in 100% Me2SO.

* This work was supported by the Danish Cancer Society (Grants 9710032, 9910012, and 9910013), the Alfred Benzon Foundation, the Danish Research Councils, the Biotechnological Research Program (Biotec III), and the Danish Center for Molecular Gerontology. Support for the Jayaram laboratory was provided by the Robert F. Welch Foundation, The Texas Board for Coordinating Higher Education, and the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: Int, integrase; CPT, camptothecin; NSC-314622, 5,6-dihydro-5,11-diketo-2,3-dimethoxy-6-methyl-8,9-methylenedioxy-11H-indenol1,2-cisoquinoline; topo I, topoisomerase I.
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Purification of FLP—Wild type FLP and FLP(Y343F) were purified as described by Prasad et al. (21). The concentrations of the enzymes were estimated according to the procedure of Lee and Jayaram (22).

Synthetic DNA Substrates—Oligonucleotides for the construction of the half-sites were synthesized in an Applied Biosystems model 380A DNA synthesizer using phosphorylamine chemistry (23) and purified as described previously (24). The sequence of the half-site DNA substrate was: 5'-taacctgagcaagctctcatcagctta/3'lttctagagaataggaacttcggg. The assembly of substrates containing a 5'-radioabeled scissile strand and a 5'-cold phosphorylated uncleaved strand was performed as described by Knudsen et al. (25).

Strand Cleavage and Ligation by FLP—To investigate the effect of CPT or NSC-314622 on DNA cleavage mediated by wild type FLP, -0.5 pmol of enzyme was preincubated for 5 min at room temperature in a standard FLP reaction buffer (100 mM Tris (pH 7.5), 10% polyethylene glycol, 80 mM NaCl, 10 mM mercaptoethanol, and 2 mM EDTA) with or without added drugs as stated in the figure legends. Me2SO was added to a final concentration of 10% to control reactions to make them comparable with the samples containing drugs (which were applied as 10× stock solutions dissolved in 100% Me2SO). Subsequent to preincubation, DNA cleavage was initiated by the addition of 0.02 pmol of the radiolabeled half-site, and incubation was continued for 15 min at 30 °C in a 20-μl reaction volume.

To assay the effect of the drug on wild type FLP-mediated DNA ligation, active cleavage complexes were generated by incubating 0.5 pmol of the enzyme with 0.02 pmol of the half-site in 20 μl of the standard reaction buffer for 30 min at 30 °C. The active cleavage complexes were pretreated with indicated concentrations of drugs for 5 min as described above. Subsequently, ligation was initiated by the addition of 0.00625 pmol of ligator strand (unless otherwise stated), and incubation was continued in a 30-μl reaction volume for another 15 min at 30 °C. All reactions were terminated by the addition of SDS (0.1% final concentration) and were treated with proteinase K (100 μg reaction for 1 h at 37 °C) prior to ethanol precipitation. The reaction products were fractionated by electrophoresis in 12% denaturing (5% bis-acrylamide) polyacrylamide gels, and the reaction products were visualized by phosphorimaging. Studies of the drug effects on FLP(Y343F)-mediated reactions were carried out as described above except that the wild type enzyme was replaced with 0.5 pmol of FLP(Y343F) plus 30 mM tyramine.

Quantification of FLP Reaction Products—The amount of FLP-mediated cleavage and ligation was quantified on a model SF Molecular Dynamics PhosphorImager by integrating the area under the curve for each radioactive band using ImageQuant software (Molecular Dynam-ics). The cleavage activity was calculated as C(1 + S), and the ligation activity was calculated as L(L + C), where C is the amount of cleavage product, S is the amount of substrate, and L is the amount of ligation product.

RESULTS

Flp-mediated DNA Cleavage and Ligation Are Inhibited by CPT and NSC-314622—The sensitivity of Flp toward CPT and NSC-314622 in the cleavage and ligation steps of catalysis was investigated using a synthetic DNA “half-site” substrate containing a single Flp binding sequence (Fig. 1, A and B, right panels). This substrate supports cleavage, but the concomitant ligation is prevented because of 5'- phosphorylation of the non-cleaved DNA strand and release of the short oligonucleotide containing the 5'-OH end. However, ligation of the cleavage product to free 5'-OH ends can be effected by adding excess ligator DNA strands that are able to form base pair with the noncleaved strand (25).

The cleavage assays were performed by incubating the enzyme with a radiolabeled half-site substrate in the presence of increasing concentrations of CPT or NSC-314622 and the products analyzed by electrophoresis in denaturing polyacrylamide gels followed by phosphorimaging (an example of a gel picture is shown in Fig. 1A, inset). The extent of cleavage was deter-
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The inhibition by the drugs of Flp(Y343F)-mediated reactions argues against their action being directed to the dimeric interface between Flp monomers. More likely, CPT and NSC-314622 interfere with Flp catalysis by interacting with the pro-active site (the active site that has to acquire the tyrosine nucleophile in trans to become cleavage competent) harbored by the Flp monomer. This mode of action would agree with what has previously been established for eukaryotic topo I (31). The difference between the two enzymes in drug sensitivity during cleavage may be explained by the catalytic pocket of Flp having a more open conformation than that of topo I. Such a conformation would be required to permit the entrance of the tyrosine nucleophile in trans from a second Flp monomer and could facilitate interaction with the active portion of the drug molecules. This notion is supported by the crystal structure of Flp showing that within a single monomer the active site tyrosine points away from the RHRW tetrad, leaving the catalytic pocket in an open conformation (9).

CPT and NSC-314622 Inhibit Flp Transesterification in a Competitive Manner—In an attempt to verify the suspected drug interaction with the catalytic pocket of Flp, we tested whether inhibition by CPT and NSC-314622 was competitive with the cleavage and ligation nucleophiles. For this purpose, DNA cleavage and ligation were assayed in the presence of varying concentrations of substrates (the nucleophile for transesterification) and drugs relative to each other. To vary the concentration of the cleavage nucleophile without changing enzyme concentration, we utilized tyramine-assisted cleavage mediated by Flp(Y343F) of a half-site substrate. A range of tyramine concentrations (from 5 to 70 mM) was employed in the presence of different concentrations of either CPT (from 0 to 1 mM) or NSC-314622 (from 0 to 0.1 mM). The results are depicted as Lineweaver-Burk plots in Fig. 2, A and B. The mode of inhibition of ligation was investigated in a similar manner using the strand joining reaction in a cleaved half-site substrate as described above. Because the natural nucleophile during ligation is a 5′-OH end of DNA, it was possible to assay this step using the wild type enzyme. The concentrations of the ligator strand ranged from 0.005 to 1 pmol in the presence of 0–1 mM CPT or 0–0.1 mM NSC-314622. The Lineweaver-Burk plots of the data are shown in Fig. 2, C and D. The intercepts on the ordinates of the plots for both cleavage (Fig. 2, A and B) and ligation (Fig. 2, C and D) were nearly the same regardless of the concentrations of either CPT or NSC-314622. Thus, the inhibitions of cleavage and ligation were competitive in nature and could be overcome by a sufficiently high concentration of either tyramine or the ligator strand, respectively. We therefore believe that the sites of occupancy of the drugs and the incoming nucleophile (tyramine or the 5′-OH DNA end) are the same, partially overlapping or at least in close enough proximity to elicit mutual competition. Such a drug interaction mode with Flp is consistent with previously published results demonstrating that CPT inhibits topo I-mediated ligation in a competitive manner (31). Moreover, the similarity in the inhibition of Flp-mediated cleavage and ligation suggests that the two reactions are catalyzed by similar active site conformations. In both instances, the pro-active site of the Flp monomer orients the phosphodiester (the DNA phosphodiester or the phosphotyrosine bond formed by strand cleavage) so that it can be targeted by the attacking nucleophile supplied in trans (the active site tyrosine or the 5′-OH from the cleaved strand).

Inhibition of Flp by CPT and NSC-314622 Is Reversible—It is well documented that CPT interaction with topo I is transient and that the effect of high drug concentrations can be reversed by dilution (19). To further compare Flp and topo I
with respect to drug interaction, we investigated whether inhibition of the Flp recombinase is also reversible upon drug dilution. Active cleavage complexes (obtained as described earlier) were split into three samples of 60 μl each and incubated for 5 min without added drug or in the presence of 2.0 mM CPT or 0.25 mM NSC-314622. At these drug concentrations DNA ligation was abolished (see Fig. 1). Each sample was then split into halves. To the first half of each sample, the ligator strand was added directly to a final concentration of 200 nM, and incubation was continued for 60 min. The other halves were diluted to a final volume of 1200 μl (drug concentrations diluted to 0.05 mM CPT or 0.006 mM NSC-314622) and then incubated with 200 nM ligator strand. The ligation results are depicted by a bar chart (Fig. 3). The almost complete inhibition by the drugs in the undiluted samples (Undiluted, compare white and gray bars with black bar) is in contrast with the nearly complete recovery of ligation activities upon drug dilution (Diluted, compare white and gray bars with black bar).

DISCUSSION AND CONCLUSIONS

In the present study we have demonstrated that CPT and NSC-314622 inhibit Flp recombinase by blocking the active site from the incoming nucleophile for strand cleavage or ligation. The fact that two structurally distinct topo I-directed drugs also inhibit Flp recombinase argues that their interaction with Flp is unlikely to be mere coincidence. Rather, the active site of Flp may share sufficient structural similarities to that of eukaryotic topo I to be able to bind the two drugs specifically, although Flp differs from topo I regarding drug affinity. Flp is less sensitive than topo I toward CPT (26), but the effect of NSC-314622 on the two enzymes appears comparable (17). Drug interaction with topo I is very specific as revealed by several single amino acid mutations conferring drug resistance to the enzyme (32–36) and by topo I from vaccinia virus being drug-resistant because of a single amino acid difference compared with the cellular forms of topo I (37, 38). The extent of structural similarities and differences between Flp and human topo I seen in recently published crystal structures is comparable with those between Flp and other members of the λ-Int recombinase family (9, 12, 13). Our data are consistent with the notion that at least some recombinases of the Int family, including Flp, may have evolved in parallel with the type IB topoisomerases from a common, and possibly drug-sensitive, ancestral topoisomerase.

Studies with eukaryotic topo I have shown that CPT interacts with the enzyme only after formation of the covalent cleavage intermediate (19) and that the inhibitory effect is enhanced by a G-positioned 3' to the cleavage site (39–41). Moreover, affinity labeling experiments have indicated that the drug interacts with both the enzyme and the DNA within the cleavage complexes (18, 20). It has therefore been proposed that the drug forms a ternary complex with the cleavage intermediate, with the base 3' to the cleavage site playing a direct role in drug binding (18, 20). Our results with Flp can be reconciled with the topo I data if drug accessibility is facilitated by an open post-cleavage conformation of the topo I active site.
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This transition may be required for the entry of the DNA nucleophile (5'-OH) for the strand joining reaction. Because of the trans cleavage mechanism of Flp, the pro-active site of the Flp monomer has an inherent open conformation (9), allowing drug binding both in the pre- and post-cleavage states of the enzyme. Thus, unlike topo I, Flp is drug sensitive in both the strand cleavage and ligation steps. Although the conformation of the catalytic pocket of the enzyme may determine the initial interaction with the drug, this interaction could be further reinforced by additional drug-DNA interactions. Consistent with such a mechanism, the presence of DNA downstream to the cleavage site in topo I-DNA complexes has been found not to be a prerequisite for, even though it stimulates, the action of camptothecins (26).

Our findings suggest that Flp and other related recombinases that utilize the type IB topoisomerase mechanism can be exploited successfully to study the mechanism of action of an important class of anti-tumor drugs.

Acknowledgment—We are grateful to Dr. Yves Pommier (National Institutes of Health, Bethesda, MD) for kindly providing the NSC-314622 for these studies and to Kirsten Andersen for skillful technical assistance.

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