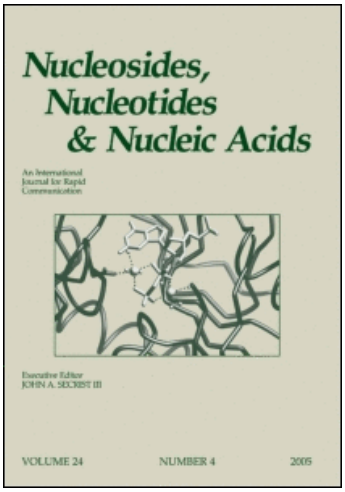

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Structural Basis for Topoisomerase I Inhibition by Nucleoside Analogs

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ABSTRACT

Nucleoside analogs such as 1-β-D-arabinofuranosyl cytidine (AraC) and 2′, 2′-difluoro deoxycytidine (dFdC) are important components of the anticancer chemotherapeutic arsenal and are among the most effective anticancer drugs currently available. Although both AraCTP and dFdCTP impede DNA replication through pausing of DNA polymerases, both nucleoside analogs are ultimately incorporated into replicated DNA and interfere in DNA-mediated processes. Our laboratories are investigating the structural basis for the poisoning of topoisomerase I (top1) due to antipyrimidine incorporation into duplex DNA. We recently reported that both AraC and dFdC induce formation of top1 cleavage complexes, and poisoning of top1 contributes to the anticancer activities of both these drugs. Recent NMR and thermodynamic studies from

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our laboratories provide insight into the mechanism by which AraC and dFdC poison top 1. NMR studies from our laboratories have revealed that the arabinosyl sugar of AraC adopted a C2′-endo conformation. Although this is a B-type sugar pucker characteristic of duplex DNA, the conformation is rigid, and this lack of flexibility probably contributes to inhibition of the religation step of the top 1 reaction. In contrast to AraC, NMR studies revealed dFdC adopted a C3′ endo sugar pucker characteristic of RNA, rather than DNA duplexes. dFdC substitution enhanced formation of top1 cleavage complexes, but did not inhibit religation. The enhancement of top1 cleavage complexes most likely results from a combination of conformational and electrostatic effects. The structural effects of dFdC and AraC are being further investigated in duplex DNA with well-defined top1 cleavage sites to analyze more specifically how these structural perturbations lead to enzyme poisoning.

Key Words: Topoisomerase 1; Antimetabolite; DNA; Cancer chemotherapy; Ara-C; Gemcitabine.

top1 AS A TARGET FOR CANCER CHEMOTHERAPY

Topoisomerases are ubiquitous enzymes that regulate the topological state of supercoiled DNA.[1] Regulation of DNA superhelicity is necessary for DNA replication, transcription, recombination, repair, and other essential processes. There are two principal classes of topoisomerases that interact with nuclear DNA in eukaryotic cells. Topoisomerases I (top1) alter the linking number of supercoiled DNA by single integral units (+/−1),[2] while type II topoisomerases (top2) alter the linking number of DNA by two units (+/−2).[3] Topoisomerases are targets for several of the most widely used classes of anticancer drugs, including the camptothecins, which interfere with top1, and the anthracyclines and epipodophyllotoxins, that interfere with top2.[4] The mechanism for top1 enzymes involves formation of transient cleavage complexes in which a reactive tyrosine (Y723 in human top1) nucleophilically attacks the phosphodiester backbone of one strand of the DNA double helix.[5] This reaction results in covalent bond formation between top1 and the 3′-phosphate of the cleaved DNA strand. The 5′-OH of the cleaved DNA strand serves as the leaving group. The top1 : DNA cleavage complex undergoes concerted rotation(s) that alters the linking number of the supercoiled DNA. The 5′-OH of the cleaved DNA strand then displaces the tyrosine hydroxyl group to regenerate the intact DNA double helix with altered linking number and free top1. There are two classes of compounds that interfere with top1 function. Top1 poisons, such as camptothecins, stabilize top1 cleavage complexes.[6] DNA damage results when replication forks collide with top1 cleavage complexes that are stabilized by top1 poisons.[7] Top1 inhibitors, such as the DNA minor groove binder distamycin, inhibit top1 function, but do not transform top1 complexes into entities capable of causing potentially cytotoxic DNA damage.[4] Recent data from our laboratories have shown that the nucleoside analogs AraC and dFdC can induce top1-DNA cleavage complex formation in vitro and in treated cells, and thus could mimic top1 poisons following their incorporation into DNA.[8,9] The following sections summarize recent published studies demonstrating that AraC and dFdC are top1 poisons, as well as structural and

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thermodynamic studies that have begun to elucidate the mechanism of top1 poisoning by these nucleoside analogs.[10–13]

NUCLEOSIDE ANALOGS AS top1 POISONS

Biological evidence that AraC and dFdC are top1 poisons comes from studies demonstrating reduced cytotoxicity for these nucleoside analogs towards top1-deficient cells. This cell line was established through clonal expansion of P388 mouse leukemia cells and selected for in the presence of camptothecin.[8] The resulting P388/CPT45 cells were deficient in top1 activity, and were about 10,000-fold resistant to CPT, consistent with top1 being the cellular target of CPTs. The P388/CPT45 cells were about 7-fold resistant to AraC[8] and displayed similar resistance to dFdC.[9] Both AraC and dFdC affect several distinct cellular processes, but both are ultimately incorporated into DNA.[13] The observation of cross-resistance of P388/CPT cells to both AraC and dFdC indicates that a portion of the cytotoxicity of each of these nucleoside analogs is due to poisoning of top1. Further evidence that both AraC and dFdC are top1 poisons was established by isolation of top1 cleavage complexes from CEM cells exposed to either AraC or dFdC. Top1 cleavage complexes were isolated from drug-exposed CEM cells using the immuno complex of enzyme (ICE) bioassay.[14] The levels of top1 : DNA complexes detected correlated with the concentration of AraC or dFdC that the CEM cells were exposed to.[8,9] Neither AraC nor dFdC exposure resulted in formation of top2 : DNA complexes in these cells.

MECHANISM OF top1 POISONING

The mechanism by which AraC and dFdC incorporation into DNA causes poisoning of top1 was investigated using a synthetic 36 base pair DNA duplex containing a single, strong cleavage site for top1.[8,15] Oligodeoxynucleotides were synthesized with site-specific incorporation of either AraC or dFdC for each dC in the duplex. Top1 cleavage complex formation was enhanced about 5-fold when AraC was substituted for dC at the +1 position of the non-scissile strand. Substitution of AraC for dC at other positions of the duplex did not affect top1 cleavage complex formation. A similar enhancement of cleavage complex formation was observed upon substitution of dFdC for dC at the +1 position of the non-scissile strand. In addition to enhancing top1 cleavage complex formation at the +1 position, one new top1 cleavage site was observed adjacent to dFdC when it was substituted at the −5 position of the non-scissile strand. This second site was observed only in the presence of CPTs. Thus, dFdC incorporation affects the rate of top1 cleavage complex formation and results in formation of new CPT-stabilized top1 cleavage sites.

The enhancement of top1-mediated DNA cleavage due to AraC and dFdC substitution apparently arise due to interference in different aspects of the top1 reaction. Enhanced top1-mediated DNA cleavage can arise either due to an increased rate of top1 cleavage complex formation, as was observed for dFdC, or due to a decrease in

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the rate of religation. To distinguish between these processes, the rate of cleavage complex formation and the rate of religation were measured separately for the AraC-substituted duplexes. The kinetics of top1-mediated DNA cleavage were measured by incubating the model duplex with recombinant human top1, quenching the reaction with SDS, and visualizing the intensity of the cleaved DNA by gel electrophoresis as a function of time. The rate of religation for the AraC-substituted duplex was measured using a modified duplex in which top1-mediated cleavage released a 5 mer ODN while religation to a 23 mer generated the thermodynamically more stable 37 mer duplex. These studies showed that AraC substitution enhanced top1-mediated DNA cleavage by decreasing the rate of religation.[8] The structural basis for the distinct mechanistic effects of AraC and dFdC on top1 cleavage complex formation and religation is currently under investigation in our laboratories. The following section describes recent NMR structures from our laboratories that provide insight into the structural basis for these mechanistic differences.

NMR STUDIES OF AraC AND dFdC

The NMR structures of three model Okazaki fragments were solved to determine how substitution of dC with either AraC or dFdC altered duplex nucleic acid structure.[10,12] The dC nucleotide that was the site for AraC or dFdC substitution (dC_{20}) was in the DNA-duplex region of the model Okazaki fragment, and was part of a stretch of six consecutive pyrimidines. The DNA-duplex region of the control model Okazaki fragment adopted largely B-form duplex structure. The sugar pucker of dC_{20}, however, differed somewhat from the C2′-endo conformation characteristic of B-form DNA ($P \sim 120°$; C2′-endo: $P \sim 150°$). One consequence of this altered sugar pucker was that very efficient base overlap occurred among the purine bases for the nucleotides in the complementary strand. This efficient base overlap contributed substantially to the global stability of the model Okazaki fragment.[10,11] Substitution of AraC for dC_{20} decreased the melting temperature for the model Okazaki fragment by about 4°C. Changes to the structure of the AraC-substituted model Okazaki fragment were limited to near the site of substitution. The most substantial difference was that the conformation for the arabinosyl sugar of AraC was more nearly prototypical C2′-endo ($P \sim 160°$). Adoption of this sugar pucker by AraC resulted in less efficient overlap among the purine bases in the complementary strand. Thus, the stereoelectronic preference for AraC to adopt a strongly C2′-endo sugar pucker resulted in decreased global stability for the AraC-substituted model Okazaki fragment. The strong stereoelectronic preference of the arabinosyl sugar for a C2′-endo conformation likely contributes to the reduced rate of religation observed for top1 cleavage complexes substituted with AraC.[8]

In contrast to what was observed for AraC, dFdC adopted a C3′-endo sugar pucker ($P \sim 20°$). The decrease in base stacking among adjacent purines in the complementary strand was not as pronounced as was observed for AraC substitution. Changes in base stacking were, however, observed in the junction region of the dFdC-substituted model Okazaki fragment relative to the native structure. These changes in base stacking likely contributed to the decreased stability of the dFdC-substituted model Okazaki fragment. One substantial difference between the

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dFdC-substituted and the native model Okazaki fragments was that the electrostatic surface of dFdC was considerably more negative than dC.[12] This difference in the electrostatic surface potential probably contributes to the decreased stability of the dFdC-substituted model Okazaki fragment, and also may be responsible for the increased top1-mediated cleavage of dFdC-substituted DNA.[9,13] Thus, dFdC substitution electrostatically favors top1 cleavage complex formation by favorable interactions between the positively charged DNA recognition site of top1 and the partial negative charge of the geminal difluoro group of dFdC. The lack of an effect of dFdC on the religation step of the top1 cleavage reaction indicates that either a C3′-endo conformation for the nucleotide at the +1 site is preferred, or that dFdC is sufficiently flexible so that its conformation does not pose an energy barrier for the reaction.

SUMMARY

Recent studies from our laboratories have shown that the cytotoxicity of AraC and dFdC is due, in part, to the poisoning of the ubiquitous top1 enzyme. This poisoning was due to incorporation of the nucleoside analog into nascent DNA. These studies are useful for rationalizing the effectiveness of current chemotherapy protocols and drug combinations. In order to further exploit the top1 poisoning properties of nucleoside analogs such as AraC and dFdC for therapeutic benefit, the structural basis for enhancing top1 cleavage complex formation, and/or inhibiting religation, must be understood. Recent NMR structures determined in our laboratories have shown that AraC adopts a rigid C2′-endo sugar pucker that likely contributes to the slow rate of religation for AraC-substituted DNA. In contrast, dFdC adopts a C3′-endo sugar pucker that does not impede the top1-mediated religation reaction. The more highly negative electrostatic surface of the geminal difluoro group of dFdC results in greater electrostatic attraction to the DNA-binding surface of top1, resulting ultimately in enhanced top1-mediated DNA cleavage of dFdC-substituted DNA. Additional studies using NMR spectroscopy and other biophysical methods are in progress to investigate further the structure/function relationships involved in top1 cleavage of DNA substituted with nucleoside analogs.

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