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Antihelicase action of CI-958, a new drug for prostate cancer

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Abstract CI-958, a new DNA-intercalating drug derived from a series of substituted 2*H*-[1] benzothiopyrano[4,3,2-*cd*]indazoles, is being tested in clinical trails because of its curative properties against murine solid tumor models and because it has demonstrated activity in a pilot phase II study of patients with hormone-refractory prostate cancer. However, the mechanism of anticancer action of CI-958 has not been established. Because CI-958 binds to DNA and DNA helicases are profoundly affected by DNA-binding drugs, we examined the effects of CI-958 on human DNA helicase action. DNA helicase activity was measured by strand dissociation of double-stranded (ds) DNA with a gel electrophoresis assay, and ATPase activities were determined on thin-layer chromatography by measurement of the conversion of ATP to ADP. For human helicase blockade, CI-958 is slightly more potent than doxorubicin (EC_{50} values 0.17 and 0.26 μM, respectively). We observed no difference in helicase-blockade EC_{50} values recorded for three helicase substrates containing A-T rich, G-C rich, and both types of oligonucleotide sequences. The effects of CI-958 helicase blockade and DNA-dependent ATPase activities were similar for the two reactions. The kinetics of the blockade by CI-958 of the human DNA helicase indicates that it involves a reversible ternary complex of helicase-drug-dsDNA. CI-958 produces potent blockade of human DNA helicases with no apparent strong DNA sequence-binding preference. Similar potency against helicase strand dissociation and DNA-dependent ATPase suggests that the mechanism against these reactions is the same. The blockade of DNA helicases by CI-958 may be central in its mechanism of action as an anticancer drug.

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Introduction

A new anticancer DNA intercalator, CI-958 (Fig. 1), derived from 2*H*-[1] benzothiopyrano [4,3,2-*cd*] indazoles, has structural similarities to mitoxantrone (Fig. 1). These agents are designed to reduce the cardiotoxicity potential seen with doxorubicin and other anthracyclines (Fig. 1), important drugs for the treatment of cancer [1]. The benzothiopyranoindazoles display excellent anticancer activity against a broad spectrum of tumors in vitro and in vivo [2–4]. As a DNA intercalator, CI-958 is a potent inhibitor of DNA and RNA synthesis, similar to doxorubicin, and causes both single- and double-strand breaks in DNA that are not readily repaired [5]. Several of the more active anthrapyrazoles, compounds similar to CI-958, are active against doxorubicin-resistant cell lines [6] and multidrug-resistant cell lines [7].

In a recent phase II study of CI-958 against hormone-refractory prostate cancer this drug produced a 20% objective response rate and a median survival (Kaplan-Meier) of 15 months in 33 patients [8]. In the light of previous experience showing that cytotoxic chemotherapies have minimal activity against prostate cancer [9, 10], this clinical activity is quite encouraging.

Because of the unique clinical activity of CI-958 against prostate cancer and its DNA interaction, we were interested in determining if this agent affected DNA helicase activity. We and other investigators have shown that several important DNA-binding anticancer drugs are potent blockers of DNA helicases [11–15]. DNA helicases are essential for DNA replication, RNA transcription, and DNA repair in that they prepare the single-stranded DNA template for these processes [16]. DNA helicases dissociate double-stranded (ds) DNA in a reaction coupled to the hydrolysis of nucleoside 5′-

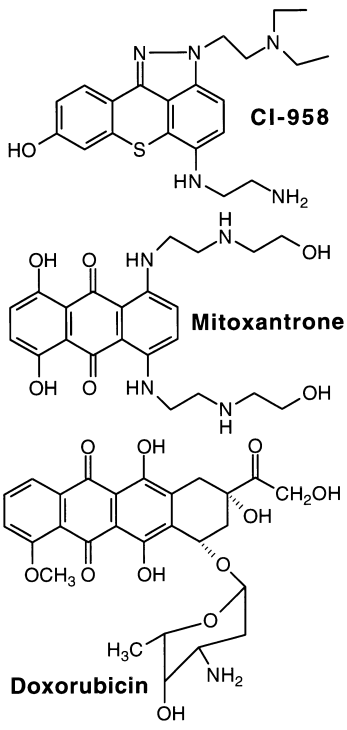

Fig. 1 Structures of CI-958, mitoxantrone, and doxorubicin

triphosphate (NTP) and are therefore also DNA-dependent nucleoside 5′-triphosphatases (NTPases) [17].

We have proposed that the antihelicase action of DNA-binding anticancer drugs may be central to their anticancer action [11, 12]. In the present study we investigated whether CI-958 would block human DNA helicase in vitro and we compared the potency of its helicase blockade with that of doxorubicin. CI-958 had no apparent DNA sequence-binding specificity in helicase blockade, and the blockade of DNA helicase by CI-958 involved a reversible ternary complex. From these observations we propose that DNA helicase blockade by CI-958 may be involved in its mechanism of action against prostate cancer.

Materials and methods

The drugs tested were obtained from several sources. CI-958 was kindly provided by Warner-Lambert/Parke-Davis Pharmaceutical Research. Doxorubicin was supplied by Farmitalia (Milan, Italy). The drug stock solutions were made in dimethylsulfoxide and stored at – 20 °C. All other chemicals and reagents were of the highest grade commercially available, and all solutions were made in ultrapure deionized water unless noted otherwise.

The preparation of helicase substrate, single-stranded (ss) phage M13mp19 (+) circular DNA annealed to radioactively labeled oligonucleotide sequences has previously been described [11]. DNA oligonucleotide sequences (6210-17) 5′-TCATGGTCATAGCTGTT, (6486-17)5′-CGCACTCCAGCCAGCTT, and (1634-24)5′-CTAAACAACTTTCAACAGTTTCAG were synthesized by the Biopolymer Core Facility, University of Maryland at Baltimore, and were used to provide different oligonucleotide sequences for drug binding and helicase assay. The unlabeled (Bluescript) plasmid ds DNA was supplied by Stratagene Cloning System (La Jolla, Calif.).

Human DNA helicases were purified by modified methods described by Tuteja et al. [18]. The purified human DNA helicases retained full enzymatic activity at physiological potassium chloride concentration. These purified helicases had DNA-dependent ATPase activity and showed no evidence of nuclease activity.

Helicase assay

The helicase assay was a modification of a previously described method [11], whereby we measured the amount of ss DNA dissociated from ds DNA substrate. The final reaction mixture (total 20 µl) contained 20 m*M* TRIS-HCl (pH 7.4), 5 m*M* dithiothreitol, 140 m*M* potassium chloride, 2 m*M* ATP, 50 ng bovine serum albumin, 2 m*M* magnesium chloride, approximately 1 fmol ^{32}P end-labeled partial duplex DNA substrate, and human DNA helicase preparation at an optimal concentration for the enzyme reaction. Reactions were run at 37 °C for 30 min unless otherwise stated. CI-958 and other drugs were added to the DNA-containing reaction mixture at room temperature at concentrations ranging from 10^{-8} to 10^{-5} *M* and the mixture was preincubated for 15–30 min. The reactions were started with the addition of purified human DNA helicase. Reactions were terminated by the addition of 2 µl stop buffer containing 0.2% bromophenol blue, 1.5% sodium dodecyl sulfate (SDS), 25% glycerol, and 100 m*M* ethylenediamine tetraacetic acid (EDTA). After the helicase reaction had been terminated the reaction mixture was electrophoresed in a 16 × 18 cm 12% nondenaturing polyacrylamide gel at constant 12-mA current for 10–20 V/cm with water cooling for 2 h. The gel was dried under vacuum and the amounts of ds DNA and ss DNA in each reaction were quantified with a radioimage analyzer (Molecular Dynamics, Sunnyvale, Calif.). We saw no evidence of DNA strand reannealing after strand separation in our experiments.

ATPase assay

DNA-dependent ATPase activity was determined by the thin-layer chromatography method of Hübscher and Stalder [19]. For the formation of [^{3}H]-ADP from [^{3}H]-ATP the reaction mixture was the same as that used in the helicase assay except that the final concentration of the reaction mixture contained 1 m*M* ATP/[^{3}H]-ATP (0.1 mCi/ml). Reaction samples were spotted on cellulose PEI-F paper (J.T. Baker Inc., Phillipsburg, N.J.) and placed in 0.5 *M* formic acid/0.5 *M* LiCl solvent for separation of the individual nucleotides. The ADP spots were cut out and analyzed in a liquid scintillation counter (Beckman LS 5801, Beckman Corporation).

Hill analysis

The Hill equation was a transformation of the data to assess the cooperativity of ligand binding with the helicase blockade process. Thus, the Hill coefficient was a measure of ''cooperativity'' as described previously [15, 20].

Results

Blockade of human DNA helicase by CI-958

The effect of CI-958 on human DNA helicase was measured with the partial dsDNA substrate (6210-17), 5′-TCATGGTCATAGCTGTT, ^{32}P-labeled M13/17 mer (Fig. 2A). CI-958 blocked human helicase potently, its effective concentration for 50% helicase blockade (EC_{50}) being 1.7×10^{-7} *M* (Fig. 2B). In accompanying helicase assays, doxorubicin was an effective helicase

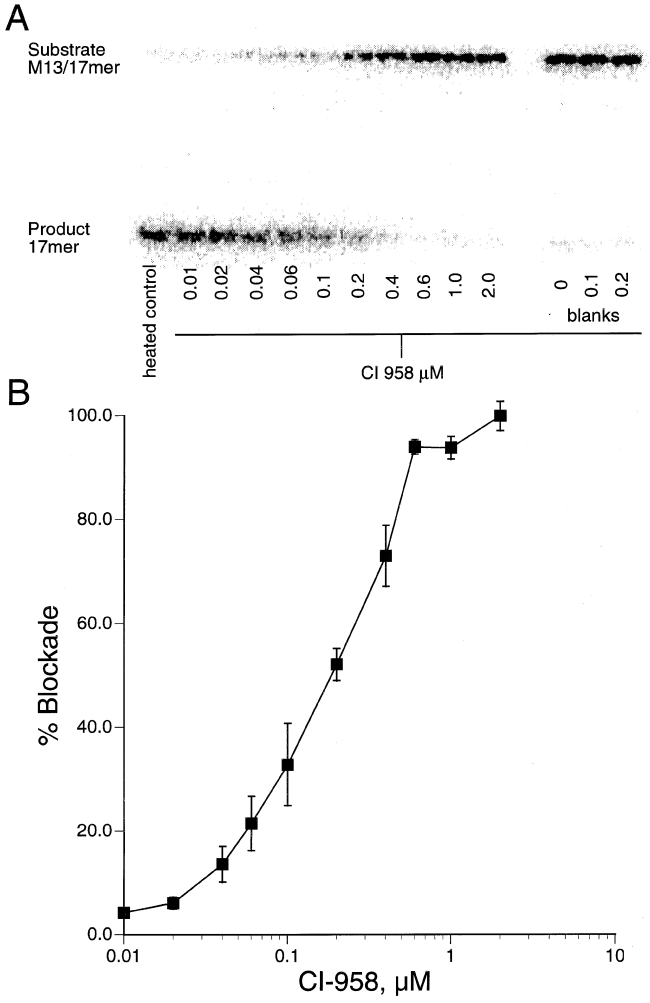

Fig. 2 **A** Radioimage of human DNA-helicase assay separation on polyacrylamide gel electrophoresis (see Materials and methods). Substrate ds DNA is shown at the *top* and dissociated 17mer, at the *bottom*. Lanes are identified as heated control, helicase reactions containing CI-958, and blanks containing no helicase enzyme. The concentration of CI-958 (μM) in each reaction is indicated. **B** Helicase blockade by CI-958. Data represent mean values ± SD for 3 separate series of reactions

blocker (EC_{50} 2.6×10^{-7} M; data not shown). Applying our CI-958 data to the Hill model, we obtained Hill coefficients ranging from 1.4 to 1.6, indicating that there was no "cooperativity" of CI-958 binding in the helicase blockade process. Thus, a single molecule of CI-958 was sufficient to block the helicase action upon binding to the ds DNA substrate.

DNA sequence specificity of helicase blockade by CI-958

We examined the helicase blockade by CI-958 for base sequence specificity and found no apparent sequence specificity in DNA helicase blockade with three different DNA oligomer sequences. DNA oligomer 6210-17 contains both sequential G-C and A-T bases, DNA oligomer 6486-17 contains no sequential A-T base and is a G-C-rich sequence, and DNA oligomer 1634-24 contains no sequential G-C or G-G base but has sequential A-T runs. The EC_{50} values recorded for these sequences ranged from 0.17 to 0.24 μM (Table 1), indicating no high degree of base sequence-binding preference for CI-958.

Table 1 DNA helicase blockade by CI-958 and substrate DNA base sequences

DNA sequences[a]	EC_{50} 10 M[b] (95% CI)
6210-17	0.17 (0.15–0.18)
6486-17	0.24 (0.20–0.26)
1634-24	0.18 (0.12–0.40)

[a] DNA sequences are described in Materials and methods
[b] EC_{50} values are the concentrations of drug producing 50% blockade of helicase activity relative to the control value. Data represent mean values and 95% CI (in parentheses) obtained from the Hill model for 2–3 independent experiments

Kinetics of CI-958 helicase blockade

In kinetics studies of human helicase blockade using CI-958 concentrations increasing from 2×10^{-8} to 2×10^{-6} M, we observed (1) a reduction in the initial rate of the ds DNA dissociation, (2) a cessation of the reaction

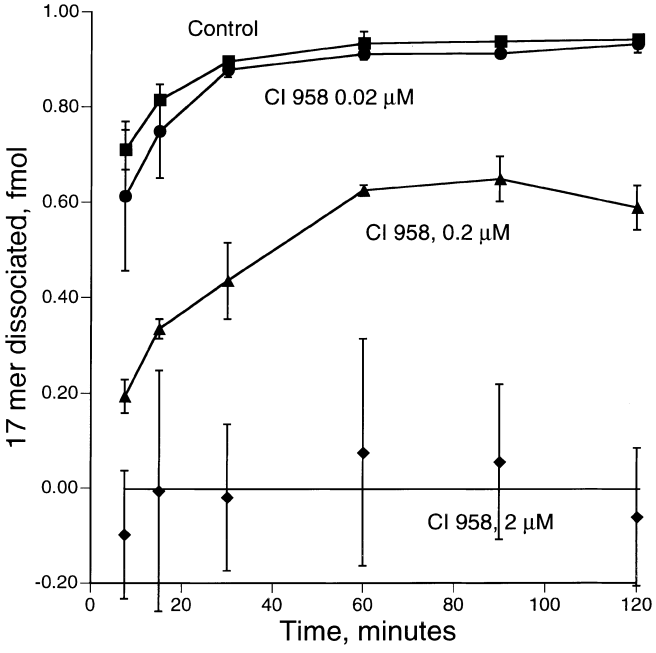

Fig. 3 Time and concentration kinetics of CI-958 helicase blockade. The partial ds DNA substrate 6210-17 was preincubated with increasing concentrations of CI-958 – (●) 0.02 μM, (▲) 0.2 μM, and (◆) 2 μM – and with no drug – (■) control – for 15 min at room temperature in reaction mixtures (final volume 120 μl). DNA helicase was added to start the reactions, and the reactions were run at 37 °C for 2 h, with 20-μl samples of reaction mixture being removed and reactions being stopped at the indicated times. The activities are shown as the total amount of 17mer dissociated from the ds DNA substrate at the sampling times. Data represent mean values ± SD for 3 experiments

proportional to the CI-958 concentration, and (3) a sustained cessation of ds DNA dissociation (Fig. 3). The helicase assays were performed with DNA sequence 6210-17, and the reactions were followed over a 2-h period. At 2×10^{-6} *M* CI-958 the helicase blockade was complete, showing no ds DNA dissociation for up to 2 h. However, we found increased variations in dissociation of the 6210-17 substrate at this concentration. We detected a nonenzymatic, partial dissociation of the 17mer DNA substrate at higher drug concentrations (10^{-5} *M*). However, this phenomenon did not occur at lower CI-958 concentrations, with the longer ds DNA substrate 1634-24, or with a 40-bp oligonucleotide (data not shown). We observed this artifact with other DNA-binding drugs and small (17mer or less) ds DNA.

To determine if CI-958 were interacting directly with the helicase protein we preincubated the helicase with 2×10^{-7} *M* drug prior to the dilution of the helicase in the reaction mixture. The diluted helicase-drug mixture was competent for dissociation of the dsDNA, and no blockade was seen.

Because the observed characteristics of DNA helicase blockade by CI-958 are similar to those produced by daunorubicin [15], we wished to test the reversibility of the CI-958-induced blockade. If the drug-induced helicase blockade were reversible, the addition of excess helicase or excess DNA to the blocked reaction could compete for binding to the DNA or drug, respectively, of the blocked reaction. We tested the binding of the human helicase to phage M13mp 19(+) circular ss DNA and to plasmid (Bluescript) ds DNA. In standard human helicase reactions, either excess ss DNA (0.5–5 μg) or ds DNA (1–10 μg) was added immediately before and after the start of the reactions. When ss DNA was added, product formation decreased from 88% to 14% of the control helicase activity, indicating that excess ss DNA competed with the helicase substrate for the human helicase binding. However, added excess ds DNA did not affect product formation relative to control helicase activities, indicating that Bluescript ds DNA does not bind the helicase competitively. Therefore, we could use the Bluescript ds DNA to assess the stability of the CI-958 binding in the helicase blockade.

Addition of excess unlabeled Bluescript plasmid ds DNA to CI-958-blocked helicase reactions changed the kinetics of helicase blockade established by CI-958 (Fig. 4). The blockade was released by added excess ds DNA in a dose-dependent manner, indicating that the DNA-drug complex can be reversed even after the enzyme blockade has occurred.

Similarly, we examined the effects of the addition of excess human helicase to CI-958 blockade reactions to see if excess helicase could overcome an established helicase blockade. To a helicase blockade established by CI-958 at 2.5×10^{-7} *M*, the addition of 2-fold the amount of DNA helicase in the reaction at 15, 30, or 45 min did not overcome the helicase blockade. The total duplex DNA dissociated on the addition of a 2-fold excess of helicase was 44 ± 11% as compared with 36 ± 6% in the absence of excess helicase for four separate comparisons. Helicase assays were performed with DNA sequence 6210-17, and the total reaction time was 60 min.

Because helicases contain an ATPase as part of their enzymatic machinery, we analyzed the effects of CI-958 on the ATPase activity and we compared the effects of the drug on the simultaneous dissociation of ds DNA and the DNA-dependent ATPase activities of human DNA helicase. CI-958 inhibits both the ATPase activity of human helicase and the strand dissociation of ds DNA. The blockade of the dissociation of ds DNA and the inhibition of the ATPase by CI-958 were similar at several drug concentrations (Fig. 5).

One possible mode of helicase inhibition may be the direct inhibition of ATPase activity by CI-958. To determine whether CI-958 would directly affect the DNA-dependent ATPase activities we tested the effect of CI-958 (2×10^{-6} *M*) on ATP hydrolysis with the ss phage M13mp 19(+) circular DNA. We observed no difference in ATP hydrolysis (data not shown), indicating that CI-958 does not directly inhibit the DNA-dependent ATPase activities of these helicases when ss DNA is provided as the substrate for the reaction.

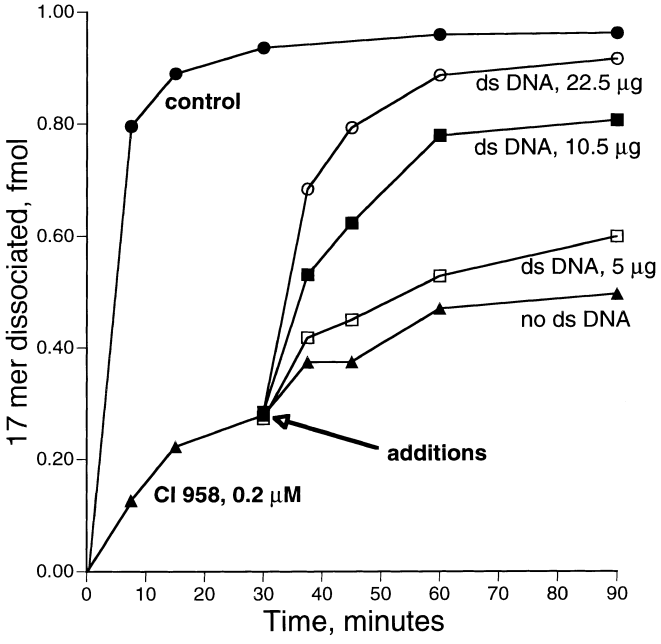

Fig. 4 Effect of competitive binding with excess ds DNA on CI-958 blockade of human DNA helicase activity. The partial ds DNA substrate 6210-17 was preincubated with 0.2 μ*M* CI-958 for 15 min at room temperature in reaction mixtures (final volume 140 μl). DNA helicase was added to start the reactions, and the reactions were incubated at 37 °C for 30 min to achieve a partially blocked helicase reaction mixture. Excess (Bluescript) ds DNA was added at 30 min [no ds DNA added (▲), 5 μg (□), 10.5 μg (■), and 22.5 μg (○)]. The helicase control activity is shown (●). The reaction was continued for 90 min, with 20 μl aliquots of reaction mixture being removed at the indicated times

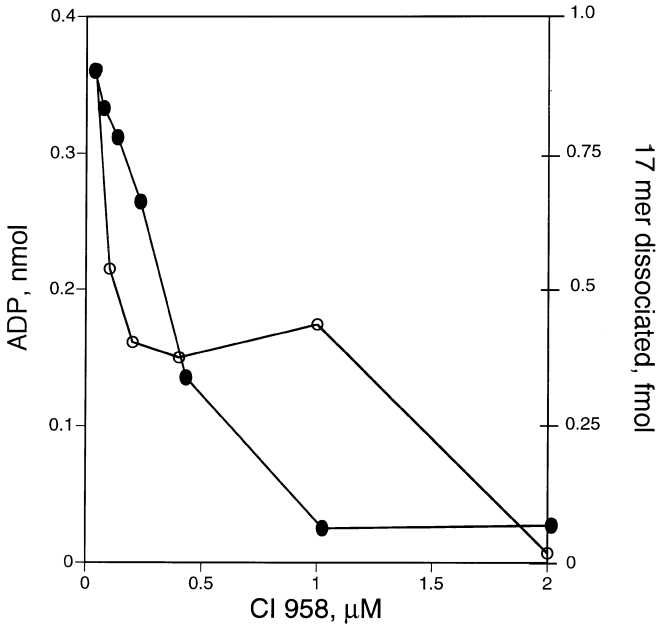

Fig. 5 Effect of CI-958 on helicase and DNA-dependent ATPase activities of human DNA helicase. The 6210-17 partial ds substrate was preincubated with increasing concentrations of CI-958 for 15 min at room temperature in helicase reaction mixtures (as described in Materials and methods, containing 1m*M* [^{3}H]-ATP). Human DNA helicase was added, and the reactions were incubated at 37 °C for 1 h. Reaction mixtures (20 μl) were analyzed for the production of [^{3}H]-ADP (5 μl) and the displacement of the 17mer DNA fragment (15 μl). The data for both helicase (●) and ATPase (○) activities are shown as a function of the concentration of CI-958. In the control reaction, human DNA helicase displaced 0.86 fmol 17mer DNA and hydrolyzed 0.4 nmol ATP. All data points represent mean values for 2 independent experiments

Discussion

Our studies show that CI-958, a DNA-intercalating drug that has activity against prostate cancer, is a potent blocker of purified human DNA helicase. This mechanism involves the binding of CI-958 to ds DNA, resulting in the blockade of the helicase translocation, inhibition of the dissociation of the ds DNA, and the concomitant inhibition of the DNA-dependent ATPase activity of helicase.

In previous studies we found DNA-intercalating agents to be potent blockers of SV40 T antigen helicase [11, 15] and several eukaryotic DNA helicases [13, 14]. We showed a correlation between anthracycline DNA-binding affinities and their helicase-blockade EC_{50} values [15]. Comparing our human helicase EC_{50} measurements for CI-958 and doxorubicin with published DNA-binding constants to calf-thymus DNA as determined by a spectrophotometric titration method, we find a paradox. The helicase-blockade EC_{50} value recorded for doxorubicin (2.6×10^{-7} *M*) is slightly less potent than that of CI-958 (1.7×10^{-7} *M*) for the human helicase, whereas doxorubicin has about a 4-fold higher apparent binding constant as compared with CI-958 (17×10^5 and 3.9×10^5 M^{-1}, respectively) [5]. This suggests that other factors must be involved in the helicase blockade. The example of CI-958 is not unusual in this apparent paradox. Other examples are the anthracyclines doxorubicin and daunorubicin, echinomycin, and other DNA-binding drugs, which have spectrally determined DNA-binding constants that are not equivalent to their helicase-blocking potencies. This may result from binding of the drug to high-affinity binding sites where the helicase is blocked. High-affinity binding sites have been documented for the anthracyclines by DNA footprinting [23].

We have proposed that the antihelicase action of naturally occurring anticancer antibiotics may be central to their mechanisms of action and may be related to their reported DNA-binding specificity for guanosine-cytidine (dG-dC)-containing base-pair sites [12, 22]. Other investigators have shown that natural DNA-intercalating antibiotics such as daunorubicin and actinomycin D have DNA-binding preference for dG-dC-containing base-pair sites [21, 23, 24]. The dG-dC base-pair binding preference of intercalating antibiotics may result from evolutionary selection because of the higher dG-dC binding stability as compared with dA-dT binding stability. Thus, the combination of the higher base-pair stability at dG-dC regions and the increased duplex DNA stability induced by an intercalating antibiotic yields a total additive stability of the intercalator-dG-dC base-pair (DNA-drug) complex that resists helicase action. However, CI-958 is not a naturally occurring substance and in our examination of DNA sequence specificity for CI-958 helicase blockade we observed no apparent DNA sequence specificity. Nevertheless, because of the limitation of our substrate sequences and the potential of other DNA interactions with CI-958, we have not ruled out other possible sequence-specific binding in our system.

Our studies suggest that CI-958 develops a helicase-blockade ternary complex and show that a single molecule of drug is sufficient to block the helicase. With increasing drug concentrations, CI-958 stops ss DNA product formation at a level proportional to the drug concentration, similar to our findings with anthracyclines [15]. This blockade is reversed in a concentration-dependent manner by excess ds DNA but not by the addition of excess DNA helicase. Finally, we detect no direct interaction between helicase and CI-958. Therefore, we propose that helicase/ds DNA/CI-958 are linked in a ternary complex that is reversible under these conditions. This differs from our findings for the anthracycline daunorubicin [15], which produces an irreversible ternary complex and blockade.

The inhibition of both the helicase and ATPase activities may be explained by the coupling effects of these reactions. The binding of CI-958 to the ds DNA is necessary for the inhibition of ATP hydrolysis since ss DNA-stimulated ATPase is not inhibited by the drug. Also, the concentration of CI-958 required to inhibit the DNA-dependent ATPase activity approximates that

observed for the blockade of the helicase action, although there are large differences in the absolute amounts of ATPase and helicase products. This, however, is a well-known but little understood property of DNA helicase [17]. Because CI-958 bound in a ds DNA-drug complex blocks the translocation of the DNA helicase, the helicase does not consume ATP; therefore, we observe an apparent inhibition of ATPase.

CI-958 has broad-spectrum activities against solid tumors in vitro and in vivo [2–4]. In clinical trials, CI-958 is active against hormone-refractory prostate cancer [8], whereas doxorubicin is less effective [25]. Since there are DNA helicases of various types and functions in eukaryotic cells [17], different blockade specificity and DNA-binding specificities may explain these differences in the tumor specificity of CI-958 and doxorubicin. DNA helicases are crucial components of the DNA replication complex [26] and of the RNA transcription complex [27]. Therefore, blockade of DNA helicases by CI-958 may be the cause of its potent inhibition of DNA and RNA synthesis [5]. Similar to doxorubicin ad daunorubicin, CI-958 inhibits both processes to the same extent at similar concentrations [5, 28]. This pattern of inhibition is quite distinct from that of anthrapyrazoles [29], which inhibit DNA synthesis at much lower concentrations than they do RNA synthesis, or that of actinomycin D [28] and aclacinomycin [30], which preferentially inhibit RNA synthesis. This selectivity for DNA or RNA inhibition may reside in different DNA helicases involved with DNA and RNA synthetic processes.

Although CI-958 has been associated with ds and ss DNA breaks in cells in a time- and concentration-dependent manner, the precise cause for the breaks has not been explained [5]. Of the various mechanisms proposed, the inhibition of topoisomerase II by CI-958 is not clear, and the concentration of CI-958 at which ds breaks occur in cells is a log-order of magnitude higher than that which causes cytotoxicity in L1210 cells [5]. Second, there is no correlation of cytotoxicity with the amount of ds breaks in cells caused by the drug [29]. The cytotoxicity of CI-958 may be related to the inability of a cell to recover from or repair the DNA breaks induced by the drug rather than to the quantity of these lesions. DNA strand breaks induced by CI-958 are not readily repaired, even after the removal of the drug, and additional lesions can occur thereafter [5]. Repair mechanisms involving DNA helicases in the nucleotide excision-repair pathway may be impaired by this drug. Because the disruption of a topoisomerase-DNA cleavage complex by a DNA helicase may be necessary for ds breaks to occur [31], the blockade of DNA helicases may actually decrease the DNA breaks observed at higher concentrations of CI-958. However, high concentrations of CI-958 are nonetheless highly cytotoxic.

CI-958 is currently undergoing clinical trial for the treatment of cancer. We propose a new mechanism of action for this promising drug. The biochemical effects of CI-958 are similar to those of doxorubicin and daunorubicin in that all suppress both DNA and RNA synthesis and cause DNA ss and ds breaks and all are potent blockers of purified human DNA helicases. Because cancer cells contain numerous DNA helicases of varying type, specificity, and abundance, the efficacy and selectivity of CI-958 as an anticancer agent and, specifically, as an antiprostate cancer drug may be a function of specific helicase blockade.

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