Quinolones Share a Common Interaction Domain on Topoisomerase II with Other DNA Cleavage-Enhancing Antineoplastic Drugs†

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ABSTRACT: Topoisomerase II is the cytotoxic target for a number of clinically relevant antineoplastic drugs. Despite the fact that these agents differ significantly in structure, a previous study [Corbett, A. H., Hong, D., & Osheroff, N. (1993) *J. Biol. Chem. 268*, 14394−14398] indicated that the site of action for etoposide on topoisomerase II overlaps those of other DNA cleavage-enhancing drugs. Therefore, to further define interactions between drugs and the enzyme, the functional interaction domain (i.e., interaction domain defined by drug function) for quinolones on *Drosophila* topoisomerase II was mapped with respect to several classes of antineoplastic agents. This was accomplished by characterizing the effects of ciprofloxacin (a gyrase-targeted antibacterial quinolone) on the ability of etoposide, amsacrine, genistein, and the antineoplastic quinolone, CP-115,953, to enhance topoisomerase II-mediated DNA cleavage. Although ciprofloxacin interacts with the eukaryotic type II enzyme, it shows little ability to stimulate DNA cleavage. Ciprofloxacin attenuated cleavage enhancement by all of the above drugs. Similar results were obtained using a related quinolone, CP-80,080, as a competitor. In addition, kinetic analysis of DNA cleavage indicated that ciprofloxacin is a competitive inhibitor of CP-115,953 and etoposide. Finally, ciprofloxacin inhibited the cytotoxic actions of CP-115,953 and etoposide in mammalian cells to an extent that paralleled its in vitro attenuation of cleavage. These results strongly suggest that several structurally disparate DNA cleavage-enhancing antineoplastic drugs share an overlapping site of action on topoisomerase II. Based on the results of drug competition and mutagenesis studies, a model for the drug interaction domain on topoisomerase II is described.

Topoisomerase II is one of the most important protein targets currently available for the treatment of human cancers (Corbett & Osheroff, 1993; Pommier, 1993; Chen & Liu, 1994; Liu, 1994; Froelich-Ammon & Osheroff, 1995; Pommier et al., 1996). Drugs targeted to this enzyme work in an insidious fashion; rather than inhibiting the overall catalytic activity of topoisomerase II, they act by increasing levels of covalent enzyme-cleaved DNA complexes that are formed during the normal double-stranded DNA cleavage/religation cycle of the enzyme (Osheroff et al., 1991; Corbett & Osheroff, 1993; Chen & Liu, 1994; Liu, 1994; Watt & Hickson, 1994; Froelich-Ammon & Osheroff, 1995; Berger & Wang, 1996). Thus, topoisomerase II-targeted agents convert this essential enzyme into a potent cellular toxin that promotes cell death by introducing breaks into the genetic material (Kreuzer & Cozzarelli, 1979; Corbett & Osheroff, 1993; Chen & Liu, 1994; Liu, 1994; Froelich-Ammon & Osheroff, 1995).

It is likely that DNA cleavage-enhancing drugs act at the topoisomerase II/nucleic acid interface (Corbett & Osheroff, 1993; Chen & Liu, 1994; Liu, 1994; Freudenreich & Kreuzer, 1994; Froelich-Ammon & Osheroff, 1995; Pommier et al., 1996). As a first step toward defining drug−enzyme interactions at the molecular level, drug-resistant mutant type II enzymes have been generated and used to identify specific amino acid residues that confer altered sensitivity to topoisomerase II-targeted agents (Corbett & Osheroff, 1993; Beck et al., 1994; Chen & Liu, 1994; Nitiss, 1994; Nitiss & Beck, 1996). However, interpreting these results in light of a drug interaction domain has been hampered by the lack of detailed structural information for resistant type II topoisomerases and has been further complicated by the fact that many mutant enzymes display unique drug resistance profiles and/or altered enzymatic properties (Danks et al., 1988, 1989; Sullivan et al., 1989; Zwelling et al., 1989, 1991; Lee et al., 1992; Jannatipour et al., 1993; Patel & Fisher, 1993; Liu et al., 1994; Wasserman & Wang, 1994; Elsea et al., 1995; Hsiung et al., 1995). Thus, on the basis of mutagenesis studies alone, no clear picture of a drug interaction domain has emerged.

Recently, a biochemical approach that complements the above genetic studies has been used to characterize interactions between drugs and topoisomerase II (Corbett et al., 1993; Robinson et al., 1993; Osheroff et al., 1994). This second approach takes advantage of mechanistic differences between drug classes to define drug interaction domains on the enzyme on the basis of competition studies. Since these interaction domains are defined on the basis of drug function,

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they are referred to as *functional interaction domains* (Corbett et al., 1993). Previous studies indicate that the site of action of novobiocin (an ATPase inhibitor) on topoisomerase II is distinct from those of several DNA cleavage-enhancing drugs (Robinson et al., 1993; Osheroff et al., 1994). Furthermore, the site of action of etoposide overlaps those of amsacrine, genistein, and the quinolone CP-115,953 (Corbett et al., 1993; Osheroff et al., 1994). This latter result suggests that many DNA cleavage-enhancing drugs share a common interaction domain on topoisomerase II. However, since etoposide is considerably larger than the other compounds examined, it is possible that its interaction domain encompasses those of the smaller drugs, but that the individual sites of action of these latter agents are nonoverlapping.

Therefore, to further define topoisomerase II−drug interactions, the functional interaction domain for quinolones was mapped. Results indicate that the site of action of quinolones on topoisomerase II overlaps those of etoposide, amsacrine, and genistein. These results support the hypothesis that many DNA cleavage-enhancing drugs share a common interaction domain on the enzyme.

EXPERIMENTAL PROCEDURES

DNA topoisomerase II was purified from the nuclei of *Drosophila melanogaster* Kc tissue culture cells by the procedure of Shelton et al. (1983). Negatively supercoiled bacterial plasmid pBR322 DNA was prepared as previously described (Sambrook et al., 1989). Ciprofloxacin, Tris, and ethidium bromide were obtained from Sigma; SDS was from E. Merck Biochemicals; proteinase K was from United States Biochemicals; CP-115,953 and CP-80,080 were kindly provided by Drs. T. D. Gootz and P. R. McGuirk (Pfizer Central Research); amsacrine was provided by Bristol Myers; genistein was from ICN; and etoposide was purchased from Sigma in powder form or from Bristol Laboratories as a sterile solution (VePesid). All other chemicals were analytical reagent grade.

Topoisomerase II·DNA Binding. The effects of ciprofloxacin on topoisomerase II·DNA binding were determined using an electrophoretic mobility shift assay (Osheroff, 1986). Assay mixtures contained 0−250 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of assay buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5% glycerol). Binding equilibria were established for 6 min at 30 °C in the absence or presence of 400 μM ciprofloxacin. Enzyme·DNA complexes were resolved from free plasmid by electrophoresis in 1% agarose gels in 100 mM Tris−borate, pH 8.3, 2 mM EDTA containing 1 μg/mL ethidium bromide. DNA bands were visualized by transillumination with ultraviolet light (300 nm) and were photographed through Kodak 23A and 12 filters using Polaroid type 665 positive/negative film.

Topoisomerase II-Mediated DNA Cleavage. DNA cleavage assays were carried out as described by Osheroff and Zechiedrich (1987). Reaction mixtures contained 100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of assay buffer. DNA cleavage/religation equilibria were established for 6 min at 30 °C. Cleavage products were trapped by the addition of 2 μL of 10% SDS, followed by 1 μL of 250 mM EDTA and 2 μL of a 0.8 mg/mL solution of proteinase K. Samples were incubated at 45 °C for 30 min to digest topoisomerase II. Final products were mixed with 2 μL of 60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 10 mM Tris-HCl, pH 7.9, heated at 70 °C for 2 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris−acetate, pH 8.3, 2 mM EDTA containing 1 μg/mL ethidium bromide. DNA bands were visualized as described above and quantified by scanning negatives with an E-C Apparatus Model EC910 scanning densitometer using Hoefer GS-370 Data System software. The intensity of the bands in the negative was directly proportional to the amount of DNA present.

DNA cleavage was monitored by quantitating the conversion of negatively supercoiled plasmid substrate to unit length linear molecules. Under the reaction conditions employed, no detectable levels of sub-unit length linear molecules were generated during the course of assays. This indicates that at any given time in the cleavage/religation equilibrium of the enzyme, only a single molecule of topoisomerase II was being trapped in a cleavage complex on any given plasmid substrate.

The effects of drugs were examined over a concentration range of 0−800 μM. For these studies, ciprofloxacin was dissolved as a 40 mM solution in 0.1 N NaOH and diluted to 8 mM with 10 mM Tris-HCl, pH 8.0; CP-80,080 and CP-115,953 were dissolved as 25 mM solutions in 0.1 N NaOH and diluted to 8 mM and 5 mM solutions, respectively, with 10 mM Tris-HCl, pH 8.0; etoposide was purchased as a 34 mM solution in 2 mg/mL citric acid, 30 mg/mL benzyl alcohol, 80 mg/mL polysorbate 80−Tween 80, 650 mg/mL polyethylene glycol 300, and 30.5% (v/v) ethanol; amsacrine was dissolved as a 5 mM solution in 50% DMSO; and genistein was dissolved as a 20 mM solution in 100% DMSO. An amount of diluent equal to that in drug-containing samples was added to all control samples. No drug-induced DNA cleavage was observed in the absence of topoisomerase II. Finally, unless stated otherwise, antineoplastic drugs and competing quinolones (ciprofloxacin or CP-80,080) were both present in the reaction mixture prior to the addition of topoisomerase II, so that the enzyme was exposed simultaneously to both compounds.

Drug Cytotoxicity Studies. Wild-type Chinese hamster ovary cells were cultured as monolayers at 37 °C under 5% CO_2 in F-12 nutrient mixture supplemented with 10 μg/mL gentamicin and 10% fetal bovine serum. Drug cytotoxicity was determined using a colony-forming assay (Glisson et al., 1986; Elsea et al., 1993); Assays employed log-phase, trypsinized cells that were seeded at ∼250 cells/plate (35 mm) for 18 h prior to drug treatment. All drugs used in cytotoxicity assays were dissolved as 20 mM solutions in 100% DMSO. Cells were treated with either ciprofloxacin or DMSO (control) for 30 min followed by a 1 h incubation at 37 °C that also included either etoposide or CP-115,953. Cells were rinsed twice with phosphate-buffered saline and incubated in fresh medium for 5 days. Culture medium was removed, cells were rinsed, and colonies were stained with 2% crystal violet in methanol and counted. Plating efficiencies were ∼95%.

RESULTS

The quinolone CP-115,953 (Figure 1) is a potent enhancer of topoisomerase II-mediated DNA cleavage (Robinson et al., 1991; Elsea et al., 1993). In contrast, some related

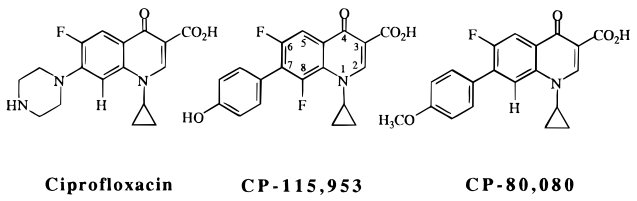

FIGURE 1: Structures of the quinolones ciprofloxacin, CP-115,-953, and CP-80,080.

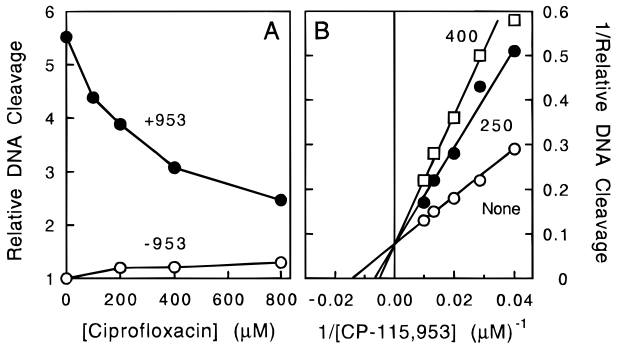

FIGURE 2: Effects of ciprofloxacin on the enhancement of topoisomerase II-mediated DNA cleavage by the antineoplastic quinolone, CP-115,953. Data represent the averages of 2−3 independent experiments. Panel A shows a ciprofloxacin titration in the presence (closed circles) or absence (open circles) of 50 μM CP-115,953. Panel B shows a double-reciprocal plot for the attenuation of CP-115,953-induced enhancement of DNA cleavage by ciprofloxacin. Assays were carried out in the absence of ciprofloxacin (None, open circles) or in the presence of 250 (closed circles) or 400 (open squares) μM ciprofloxacin.

quinolones show little ability to stimulate DNA scission mediated by the eukaryotic enzyme, even though they inhibit overall catalytic activity (Barrett et al., 1989; Elsea et al., 1993). Two such compounds, ciprofloxacin and CP-80,080, are shown in Figure 1. Ciprofloxacin is notable because it is the most active oral antibacterial agent currently in clinical use and is targeted to the prokaryotic type II topoisomerase, DNA gyrase (Hooper & Wolfson, 1991; Reece & Maxwell, 1991; Maxwell, 1992).

The present study took advantage of these two compounds to determine whether the interaction domain for quinolones on *Drosophila* topoisomerase II overlaps those of other antineoplastic agents. As described below, this was accomplished by determining the ability of ciprofloxacin and CP-80,080 to block the DNA cleavage-enhancing properties of several topoisomerase II-targeted drugs.

Attenuation of CP-115,953-Enhanced DNA Cleavage by Ciprofloxacin. In the absence of competing quinolone, 50 μM CP-115,953 increased topoisomerase II-mediated cleavage of negatively supercoiled pBR322 DNA >5-fold. As shown in Figure 2A, ciprofloxacin attenuated this stimulation. While this result implies that ciprofloxacin and CP-115,953 share a common site of action on the enzyme, it is possible that ciprofloxacin blocks drug-induced DNA scission by inhibiting the ability of the enzyme to bind or cleave DNA rather than by competing with the stimulatory quinolone. Two control experiments were carried out to address this critical point.

First, the effects of ciprofloxacin on topoisomerase II·DNA binding were assessed. As determined by gel electrophoretic mobility shift assays (Osheroff, 1986), this drug did not impair the ability of the enzyme to bind negatively supercoiled pBR322 DNA (Figure 3).

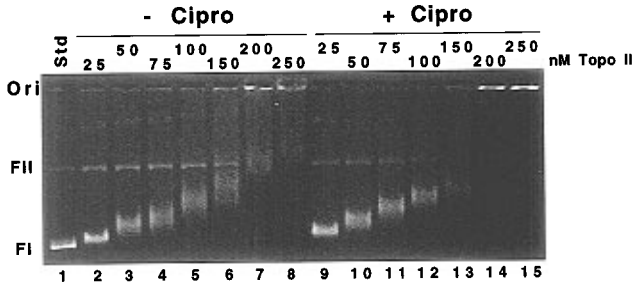

FIGURE 3: Effects of ciprofloxacin on the binding of topoisomerase II to DNA. An ethidium bromide stained agarose gel of an electrophoretic mobility shift assay is shown. Samples contained 5 nM negatively supercoiled pBR322 in the absence of enzyme (Std, lane 1), or in the presence of 25−250 nM topoisomerase II (as indicated). Assays were carried out in the absence (lanes 2−8) or presence (lanes 9−15) of 400 μM ciprofloxacin. The positions of negatively supercoiled (form I, FI) and nicked (form II, FII) plasmid as well as the origin (Ori) are indicated.

Second, the effects of ciprofloxacin on DNA scission mediated by *Drosophila* topoisomerase II were determined (Figure 2A). Over the concentration range employed (0−800 μM), ciprofloxacin enhanced (albeit weakly) DNA cleavage (maximal stimulation was ~1.3-fold). This demonstrates that ciprofloxacin does not inhibit the DNA cleavage step of the topoisomerase II catalytic cycle.

Competitive Inhibition of Drug-Induced DNA Cleavage by Ciprofloxacin. A kinetic analysis of CP-115,953-induced DNA scission was carried out to determine if the attenuation of drug action by ciprofloxacin was due to a competitive interaction between this antibacterial quinolone and CP-115,-953. To this end, double-reciprocal plots for the enhancement of topoisomerase II-mediated DNA cleavage by CP-115,953 were generated in the presence of 0, 250, or 400 μM ciprofloxacin (Figure 2B). Drug-induced DNA scission appears to be amenable to this type of analysis despite the fact that cleaved DNA is never released by the enzyme (Osheroff et al., 1991; Corbett & Osheroff, 1993; Watt & Hickson, 1994; Berger & Wang, 1996). Most likely, this is because all components of the cleavage reaction are in equilibrium with one another. Consequently, the level of DNA scission, which is a direct measurement of the concentration of the topoisomerase II−DNA cleavage intermediate, reflects the rates of all forward reactions (*i.e.*, binding and cleavage) minus the rates of all backward reactions (*i.e.*, DNA religation and dissociation) and hence is a value that can be equated to the velocity of the cleavage reaction at steady-state. The fact that the drugs employed are reversible effectors of topoisomerase II (Corbett & Osheroff, 1993; Liu, 1994; Froelich-Ammon & Osheroff, 1995; Pommier et al., 1996) and are present in large molar excess over the enzyme contributes to the steady-state assumption (Segel, 1975).

As calculated from the intercepts for the *y*- and *x*-axes generated in the absence of ciprofloxacin, CP-115,953 maximally stimulated topoisomerase II-mediated DNA cleavage ~12-fold with an apparent $K_{enhancement}$ (i.e., level of drug required to induce 50% of maximal DNA cleavage) of ~70 μM (Figure 2B). Both of these values are consistent with previously published drug titration curves (Robinson et al., 1991, 1992; Elsea et al., 1993).

In the presence of ciprofloxacin, the maximal level of DNA cleavage remained unchanged while the apparent $K_{enhancement}$ increased (Figure 2B). These effects on CP-115,-

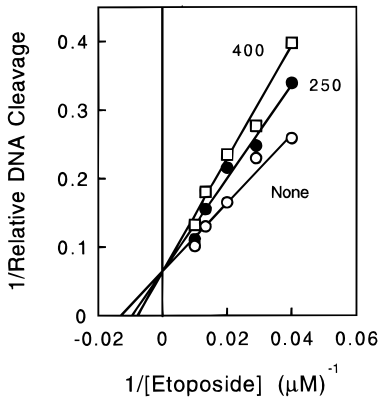

FIGURE 4: Effects of ciprofloxacin on the enhancement of topoisomerase II-mediated DNA cleavage by etoposide. A double-reciprocal plot for the attenuation of etoposide-induced enhancement of DNA cleavage by ciprofloxacin is shown. Data represent the averages of 2−3 independent experiments. Assays were carried out in the absence of ciprofloxacin (None, open circles) or in the presence of 250 (closed circles) or 400 (open squares) μM ciprofloxacin.

953-induced DNA cleavage are hallmarks of competitive inhibition (Segel, 1975). The apparent K_i value for ciprofloxacin was ~300 μM. The competitive kinetic inhibition observed for ciprofloxacin indicates that this quinolone diminishes the enhancement of DNA cleavage by CP-115,-953 by competing for a common site of action on topoisomerase II.

To provide further support for this conclusion, the inhibition of etoposide-enhanced DNA cleavage by ciprofloxacin was analyzed (Figure 4). In the absence of inhibitor, etoposide maximally stimulated DNA cleavage ~13-fold with an apparent $K_{enhancement}$ of ~70 μM. Once again, these kinetic constants are consistent with previously published drug titration curves (Robinson & Osheroff, 1991). As determined by double-reciprocal analysis, ciprofloxacin appeared to be a competitive inhibitor of etoposide. The apparent K_i value of the antibacterial quinolone calculated from its attenuation of etoposide-induced DNA cleavage was ~400 μM.

Defining the Functional Interaction Domain for Quinolones on Topoisomerase II. The functional interaction domain for quinolones on topoisomerase II was defined by characterizing the effects of ciprofloxacin and CP-80,080 on the ability of several antineoplastic drugs to stimulate enzyme-mediated DNA cleavage. Data for competition with CP-115,953, etoposide, amsacrine, and genistein are shown in Figures 5 and 6. In these experiments, drugs and competing quinolones were both present in the reaction mixture prior to the addition of topoisomerase II.

As seen in Figure 5, ciprofloxacin decreased the activity of all drugs examined. Over the concentration range of ciprofloxacin employed, drug-induced enhancement of DNA cleavage was reduced by ~60−85%. Similar results were obtained when antineoplastic drugs or ciprofloxacin was incubated with the enzyme·DNA complex prior to the addition of the second compound (not shown). These data indicate that ciprofloxacin is a reversible inhibitor of topoisomerase II and that its site of action on the enzyme overlaps those of CP-115,953, etoposide, amsacrine, and genistein.

To test the generality of this conclusion, the effects of the quinolone CP-80,080 on drug-induced DNA cleavage were

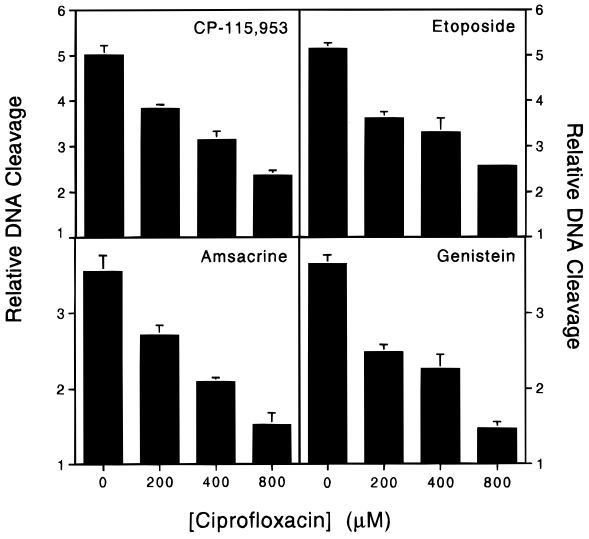

FIGURE 5: Effects of ciprofloxacin on the enhancement of topoisomerase II-mediated DNA cleavage by 50 μM CP-115,953, etoposide, amsacrine, or genistein. Data represent the averages of 3 independent experiments. Standard errors are shown.

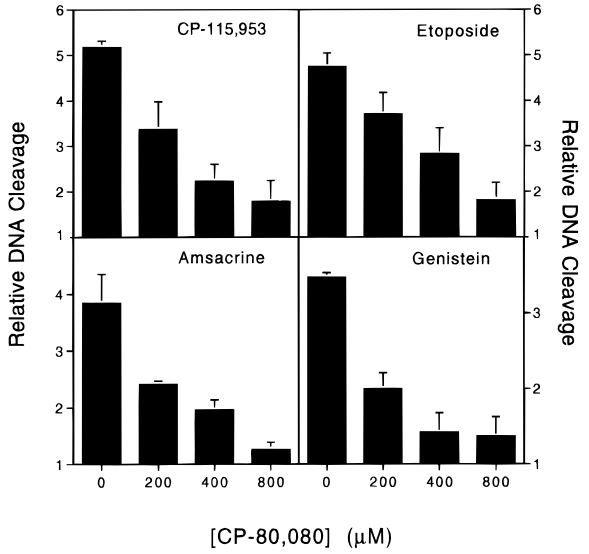

FIGURE 6: Effects of CP-80,080 on the enhancement of topoisomerase II-mediated DNA cleavage by 50 μM CP-115,953, etoposide, amsacrine, or genistein. Data represent the averages of 3 independent experiments. Standard errors are shown.

examined.[1] Like ciprofloxacin, CP-80,080 does not impair enzyme function prior to DNA scission and only weakly enhances topoisomerase II-mediated DNA cleavage (not shown). As seen in Figure 6, CP-80,080 decreased the stimulation of DNA cleavage by the other drugs tested by ~75−90%. Together with the above findings, these data indicate that quinolones share an interaction domain on topoisomerase II with other classes of antineoplastic drugs.

Competition between Ciprofloxacin and Topoisomerase II-Targeted Antineoplastic Drugs in Mammalian Cells. To extend the in vitro results described above, the effects of 400 μM ciprofloxacin (which is the concentration that decreased drug-induced DNA cleavage enhancement by ~50%) on the cytotoxicity of CP-115,953 or etoposide were

[1] Preliminary data for some of the results shown for the inhibition of drug-induced DNA cleavage by the quinolone CP-80,080 were reported previously (Osheroff et al., 1994).

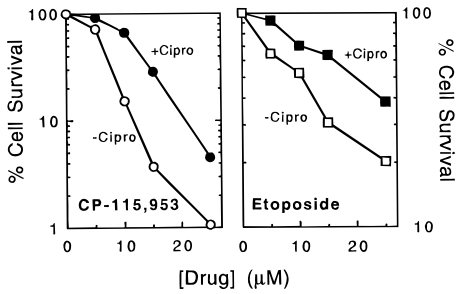

FIGURE 7: Effects of ciprofloxacin on the cytotoxicity of CP-115,-953 or etoposide in Chinese hamster ovary cells. Cytotoxicity assays were carried out in the presence (closed symbols) or absence (open symbols) of 400 μM ciprofloxacin. Data represent the averages of 2−3 independent experiments.

examined in Chinese hamster ovary cells. Previous studies indicate that topoisomerase II is the primary cellular target for both of these drugs and that cell death correlates with levels of enzyme-mediated DNA breakage (Elsea et al., 1992, 1993; Corbett & Osheroff, 1993; Nitiss et al., 1993; Chen & Liu, 1994).

The ability of ciprofloxacin to compete with antineoplastic drugs in cells paralleled the competition observed in vitro. As seen in Figure 7, ciprofloxacin doubled the concentrations of CP-115,953 and etoposide required to decrease cell survival by 50% (from ~6 μM to ~12 μM and from ~10 μM to ~20 μM, respectively). Thus, ciprofloxacin appears to be an antagonist of DNA cleavage-enhancing drugs in cultured mammalian cells. Although the concentration of ciprofloxacin employed in this study was well beyond its therapeutic range (Hooper & Wolfson, 1991), this result implies that some quinolone-based antibacterials are capable of diminishing the cellular efficacy of topoisomerase II-targeted antineoplastic drugs in chemotherapeutic regimens.

DISCUSSION

Defining the interaction domain for antineoplastic drugs on topoisomerase II represents an important step in the delineation of drug−enzyme interactions. The present study utilized competition assays as a basis for determining whether drugs share a common site of action on topoisomerase II. Results indicate that the functional interaction domain for quinolones overlaps those of etoposide, amsacrine, and genistein.

The compounds used in the present work come from structurally dissimilar drug classes (Corbett & Osheroff, 1993; Chen & Liu, 1994; Liu, 1994; Froelich-Ammon & Osheroff, 1995). Furthermore, they enhance topoisomerase II-mediated DNA breakage by two different mechanisms. While etoposide and amsacrine act primarily by inhibiting DNA religation, CP-115,953 and genistein act primarily by stimulating the forward rate of DNA cleavage (Osheroff, 1989; Robinson & Osheroff, 1990, 1991; Robinson et al., 1992; Sørensen et al., 1992; Corbett & Osheroff, 1993). Despite the structural and mechanistic differences between these drugs, their sites of action on topoisomerase II appear to overlap. Together with the previous study that mapped the functional interaction domain for etoposide on topoisomerase II (Corbett et al., 1993), this finding strongly suggests that a variety of DNA cleavage-enhancing drugs act within a common domain on the enzyme.

Most drug resistance-conferring mutations in topoisomerase II have distinct phenotypes, ranging from broad resistance to resistance directed against specific drug classes (Zwelling et al., 1991; Corbett & Osheroff, 1993; Nitiss, 1994; Elsea et al., 1995; Hsiung et al., 1995; Nitiss & Beck, 1996). These findings imply that interactions between different antineoplastic agents and the enzyme are nonidentical. In light of the present study, we propose that (1) many DNA cleavage-enhancing drugs interact within a common domain on topoisomerase II, (2) sites of drug action within this domain overlap one another, and (3) different specific amino acid residues within this domain may be involved in binding individual drugs.

It is clear from the above model that point mutations generated within the common drug interaction domain on topoisomerase II can produce dramatically different resistance profiles. Depending on whether a mutation alters an amino acid that interacts with several drug classes or a single compound, numerous phenotypes (ranging from broad to highly specific resistance) can be predicted. Thus, this drug interaction domain model is compatible with the varied phenotypes previously reported for mutant drug-resistant type II topoisomerases and provides a novel framework in which to interpret the results of future mutagenesis studies.

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