Effects of Topoisomerase II-Targeted Drugs on Enzyme-Mediated DNA Cleavage and ATP Hydrolysis: Evidence for Distinct Drug Interaction Domains on Topoisomerase II[†]

Megan J. Robinson,[‡,§] Anita H. Corbett,[§] and Neil Osheroff[*]

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received September 9, 1992; Revised Manuscript Received January 29, 1993

ABSTRACT: Topoisomerase II is the target for two broad groups of clinically relevant drugs. Members of these groups are classically defined by their ability to enhance enzyme-mediated DNA cleavage (such as etoposide and *m*-AMSA) or to inhibit enzyme-catalyzed ATP hydrolysis (such as novobiocin). The above notwithstanding, little is known concerning the interactions of drugs in either mechanistic class with the topoisomerase II·DNA complex. In order to further delineate the mechanism of drug action, the effects of several topoisomerase II-targeted agents on the DNA cleavage and ATP hydrolysis steps of the enzyme's catalytic cycle were determined. Of the drugs examined (genistein, quercetin, quercitrin, etoposide, *m*-AMSA, CP-115,953, and novobiocin), only novobiocin was unable to enhance topoisomerase II-mediated DNA cleavage. Moreover, with the exception of etoposide, all of the drugs were found to inhibit enzyme-catalyzed ATP hydrolysis. This latter finding undercuts the common assumption that DNA cleavage-enhancing drugs are specific for the cleavage/religation activity of topoisomerase II. Finally, by utilizing a series of competition experiments that took advantage of mechanistic differences between drug classes, it was possible to functionally define drug interaction domains on the eukaryotic type II enzyme. Results of this novel approach indicate that the interaction domain for novobiocin on topoisomerase II is distinct from those of the DNA cleavage-enhancing drugs.

Type II topoisomerases modulate the topological state of DNA in vivo by passing an intact nucleic acid helix through a transient double-stranded break generated in a second helix (Wang, 1985; Osheroff, 1989a; Sutcliffe et al., 1989; Huang, 1990a; Osheroff et al., 1991; Reece & Maxwell, 1991). The catalytic double-stranded DNA passage reaction of the enzyme requires a high-energy cofactor and takes place at the expense of ATP hydrolysis.

Both eukaryotic topoisomerase II and its prokaryotic counterpart, DNA gyrase, are essential for cell viability (DiNardo et al., 1984; Goto & Wang, 1984; Uemura & Yanagida, 1984; Holm et al., 1985; Reece & Maxwell, 1991). In addition to their required physiological activities, both are cellular targets for a number of clinically relevant chemotherapeutic drugs (Liu, 1989; Schneider et al., 1990; Hooper & Wolfson, 1991; Reece & Maxwell, 1991). These drugs can be categorized into two broad groups. The first group, which includes agents such as etoposide, *m*-AMSA, and quinolones, converts the type II enzyme into a cellular poison (Kreuzer & Cozzarelli, 1979) by stabilizing covalent enzyme–DNA cleavage complexes (Liu, 1989; Schneider et al., 1990; Hooper & Wolfson, 1991; Reece & Maxwell, 1991). Compounds in this group enhance enzyme-mediated DNA breakage and are used widely for the treatment of human cancers and microbial infections (Liu, 1989; Schneider et al., 1990; Hooper & Wolfson, 1991; Reece & Maxwell, 1991). The second group, which includes coumarin-based drugs such as novobiocin and coumermycin, robs the cell of type II topoisomerase activity by inhibiting the ATP hydrolysis reaction that is essential for enzyme function (Gellert et al., 1976; Sugino et al., 1978; Osheroff et al., 1983). These compounds have been used clinically as antimicrobial agents (Reece & Maxwell, 1991).

Before the potential of topoisomerase II-targeted antineoplastic drugs can be fully realized, it is critical to detail their mechanism of action against the eukaryotic enzyme. However, little is known regarding the interactions of drugs with the topoisomerase II·DNA complex. Genetic studies on DNA gyrase originally identified the B subunit of the enzyme (which contains the site of ATP binding (Tamura & Gellert, 1990; Wigley et al., 1991)) as the target of coumarin-based drugs (Orr et al., 1979). Although this assignment was later confirmed by kinetic and binding studies (Staudenbauer & Orr, 1981; Reece & Maxwell, 1991), the specific site of coumarin interaction on DNA gyrase has not been defined. Corresponding studies have not been carried out with the eukaryotic type II enzyme. However, based on their inhibition of the enzyme's ATPase reaction (Osheroff et al., 1983), novobiocin and coumermycin are believed to interact with the region of topoisomerase II that is homologous to *gyrB*.

The only information concerning sites on type II topoisomerases that interact with DNA cleavage-enhancing agents comes from the characterization of mutant drug resistant enzymes. Initial studies on DNA gyrase defined a "quinolone resistance-determining region" in the A subunit of the enzyme (which contains the active site tyrosine that mediates DNA cleavage) (Yoshida et al., 1990; Reece & Maxwell, 1991; Maxwell, 1992). While most clinical isolates of resistant bacterial strains contain mutations in this region, mutations resulting in quinolone resistance also have been found in the B subunit of gyrase (Yamagishi et al., 1986). Sites that confer resistance to DNA cleavage-enhancing drugs in the bacte-

---

[†] This work was supported by National Institutes of Health Grant GM33944 and American Cancer Society Research Grant NP-812. M.J.R. and A.H.C. were trainees under National Institutes of Health Grant 5 T32 CA09582. N.O. was supported by American Cancer Society Faculty Research Award FRA-370.

[*] To whom correspondence should be addressed.

[‡] Present address: Department of Biochemistry, University of Texas, Southwestern Medical Center, Dallas, TX 75235-9050.

[§] The order of these two authors is arbitrary.

0006-2960/93/0432-3638$04.00/0 © 1993 American Chemical Society

riophage T4 and eukaryotic type II enzymes also have been defined. However, none of the mutations described to date map within the equivalent quinolone resistance-determining region of topoisomerase II. Surprisingly, most are located in the *gyrB* domain of the enzyme in the vicinity of a consensus sequence for ATP binding (Huff et al., 1989; Huang, 1990b; Bugg et al., 1991; Hinds et al., 1991; Chan et al., 1992; Lee et al., 1992). Moreover, several of these mutant type II topoisomerases display a decreased ability to utilize ATP (Danks et al., 1989; Zwelling et al., 1989; Yalowich et al., 1992). Thus, despite the mechanistic differences between coumarins and DNA cleavage-enhancing drugs, it is not yet obvious that the interaction domains on the type II enzyme for these two drug classes are distinct from one another.

To further define relationships between drug classes, an enzymological approach was employed to categorize drug interaction domains on the eukaryotic type II topoisomerase. This was accomplished by coupling the differential effects of drugs on individual enzyme-mediated reaction steps with a series of competition experiments. Results indicate that there are mechanistic differences between etoposide and a number of other topoisomerase II-targeted antineoplastic agents and that the interaction domain for novobiocin on the enzyme does not overlap those of several DNA cleavage-enhancing drugs.

EXPERIMENTAL PROCEDURES

DNA topoisomerase II was purified from the nuclei of *Drosophila melanogaster* Kc tissue culture cells or 6–12-h-old embryos by the procedure of Shelton et al. (1983). Negatively supercoiled bacterial plasmid pBR322 DNA was prepared from *Escherichia coli* DH1 by a Triton X-100 lysis followed by a double banding in cesium chloride–ethidium bromide gradients (Sambrook et al., 1989). Genistein, quercetin, and quercitrin were purchased from ICN. These drugs were stored at –20 °C as 10 mM solutions in DMSO. Novobiocin was purchased from Sigma and was stored at –20 °C as a 10 mM solution in distilled water. Etoposide (VePesid, VP-16-23) was purchased from Bristol Laboratories as a sterile 20 mg/mL 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. The drug was stored at room temperature. *m*-AMSA (NSC-249992) was the generous gift of Dr. Y. Pommier (NCI). The drug was stored at –20 °C as a 10 mM solution in DMSO. The quinolone CP-115,953 was synthesized at Pfizer Central Research by the procedure of Gilligan et al. (1986) and was the generous gift of Dr. T. D. Gootz and Dr. P. R. McGuirk. CP-115,953 was dissolved as a 25 mM solution in 0.1 N NaOH, diluted to a 5 mM stock with 10 mM Tris-HCl, pH 8.0, and stored in the dark at –80 °C. Tris and ethidium bromide were obtained from Sigma; SDS and proteinase K were from E. Merck Biochemicals; ATP was from Pharmacia LKB Biotechnology Inc.; and [γ-^{32}P]ATP (~3000 Ci/mmol) was from Amersham. All other chemicals were analytical reagent grade.

Topoisomerase II-Mediated DNA Cleavage. All DNA cleavage reactions contained 100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total volume 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). Samples were incubated at 30 °C for 6 min. Cleavage products were trapped (Gale & Osheroff, 1990; Andersen et al., 1991) by the addition of 2 μL of 10% SDS. One microliter of 250 mM EDTA and 2 μL of a 0.8 mg/mL solution of proteinase K were added, and samples were incubated at 45 °C for 30 min to digest the topoisomerase II. Final products were mixed with 2.5 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 1 min, and subjected to electrophoresis in 1% agarose (MCB) gels in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA. Following electrophoresis, DNA bands were stained in a 1 μg/mL solution of ethidium bromide and visualized by transillumination with ultraviolet light (300 nm). DNA bands were photographed through Kodak 23A and 12 filters using Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with an E-C Apparatus Model 910 scanning densitometer in conjunction with Hoefer GS-370 software. Under the conditions employed, the intensity of the bands in the negative was proportional to the amount of DNA present. Control assays always contained an amount of drug diluent equivalent to that present in drug-containing reactions. In the absence of topoisomerase II, no drug-induced DNA cleavage was observed.

Hydrolysis of ATP by Topoisomerase II. ATPase assays were carried out as described by Osheroff et al. (1983). Reactions contained 10 nM topoisomerase II and 250 nM negatively supercoiled pBR322 DNA in a total of 20 μL of assay buffer that included 1 mM [γ-^{32}P]ATP (2 μCi/reaction). Mixtures were incubated at 30 °C. Two-and-one-half-microliter samples were removed at various time intervals up to 20 min, spotted onto thin layer cellulose plates impregnated with polyethyleneimine (Polygram CEL 300 PEI, Brinkmann), and chromatographed in freshly made 400 mM NH_4HCO_3. Reaction products were visualized by autoradiography. Radioactive areas corresponding to inorganic phosphate released by ATP hydrolysis were cut out and placed in scintillation vials with 10 mL of Ecolume (ICN) aqueous counting scintillant. Radioactivity was quantitated with a Beckman LS-7500 liquid scintillation counter. Control reactions contained drug diluent as described above.

RESULTS

The underlying premise of the present work is that mechanistic differences between classes of topoisomerase II-targeted agents may provide tools to probe drug interaction domains on the enzyme. Therefore, the effects of several agents on the DNA cleavage and ATP hydrolysis steps of the topoisomerase II catalytic cycle were analyzed. These two reaction steps were chosen as the focus of this study because they are the classically defined targets of DNA cleavage-enhancing drugs, on the one hand, and coumarins, on the other (Osheroff et al., 1983; Liu, 1989; Schneider et al., 1990).

Effects of Drugs on DNA Cleavage Mediated by Drosophila Topoisomerase II. Etoposide, *m*-AMSA, and the quinolone CP-115,953 are potent enhancers of DNA cleavage mediated by *Drosophila* topoisomerase II (Osheroff, 1989b; Robinson & Osheroff, 1990; Robinson et al., 1991) (Table I). However, the effects of isoflavones, flavones, and coumarins on DNA cleavage mediated by this enzyme have not been established. Therefore, as a prelude to further mechanistic studies, representative members of these drug classes were examined to categorize their mode of action against *Drosophila* topoisomerase II.

The isoflavone genistein and the flavones quercetin and quercitrin, originally were isolated as tyrosine kinase inhibitors (Graziani et al., 1983; Srivastava, 1985; Akiyama et al., 1987). Moreover, the first two compounds act by competitively blocking interactions between tyrosine kinases and their ATP substrates (Akiyama et al., 1987). Since ATP stimulates DNA

Table I: Effects of Drugs on Topoisomerase II-Mediated Reactions

drugs	inhibition of ATPase, IC$_{50}$ (μM)[a]	stimulation of DNA cleavage, EC$_2$ (μM)[b]
novobiocin	40	>1000
genistein	7	5
quercetin	8	40
quercitrin	40	105
etoposide	>1000	10
m-AMSA	50	20
CP-115,953	110	5

[a] Drug concentration required to inhibit the rate of ATP hydrolysis by 50%. [b] Effective drug concentration required to stimulate enzyme-mediated DNA cleavage (i.e., to increase levels of enzyme–DNA cleavage complexes) by 2-fold.

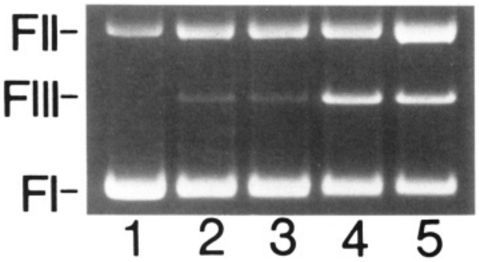

FIGURE 1: Effects of genistein and novobiocin on the DNA cleavage/religation equilibrium of *Drosophila* topoisomerase II. An agarose gel is shown. Lane 1, DNA standard; lane 2, DNA cleavage carried out in the absence of drug; lanes 3–5, DNA cleavage carried out in the presence of 100 μM novobiocin, genistein, or etoposide, respectively. The positions of negatively supercoiled DNA (form I, FI), nicked circular molecules (form II, FII), and linear molecules (form III, FIII) are indicated.

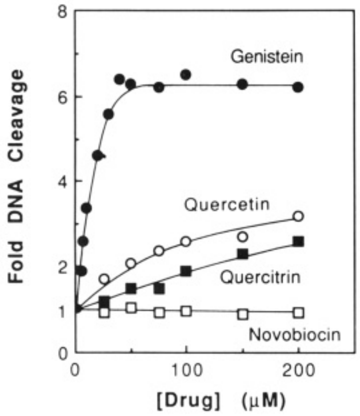

FIGURE 2: Effects of isoflavone-, flavone-, and coumarin-based drugs on the DNA cleavage/religation equilibrium of *Drosophila* topoisomerase II. Titrations of topoisomerase II-mediated DNA cleavage in the presence of genistein (○), quercetin (■), quercitrin (□), and novobiocin (▲) are shown. The level of DNA cleavage in the absence of drug was set to 1. Data represent the average of three to six independent experiments, and the average standard deviation was less than 0.7-fold. The effect of etoposide (●) on DNA cleavage is shown for comparison.

breakage mediated by topoisomerase II (Sander & Hsieh, 1983; Osheroff, 1986; Robinson & Osheroff, 1991), DNA cleavage assays reported in this paper were carried out in the absence of the nucleoside triphosphate. This precaution was taken so that effects of drugs on nucleic acid breakage could be distinguished from potential effects on enzyme·ATP interactions.

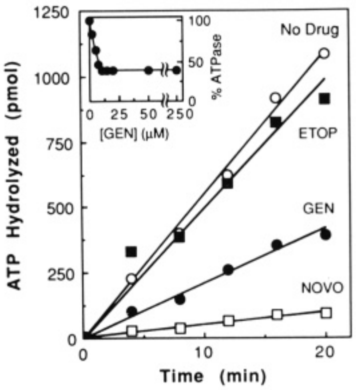

FIGURE 3: Effects of topoisomerase II-targeted compounds on enzyme-catalyzed ATP hydrolysis. The main figure shows time courses for ATP hydrolysis in the absence of drug (○) or in the presence of 500 μM etoposide (■), 250 μM genistein (●), or 250 μM novobiocin (□). The inset shows the effect of genistein concentration on topoisomerase II-catalyzed ATP hydrolysis. The amount of ATP hydrolysis in the absence of drug was set to 100%. The data shown are typical of three independent experiments.

The effects of genistein, quercetin, and quercitrin on the DNA cleavage/religation equilibrium of *Drosophila* topoisomerase II are shown in Figures 1 and 2 and Table I. All three compounds stimulated DNA cleavage. Genistein was the most potent of the three and displayed an activity comparable to that of etoposide. These results are similar to those reported for the calf thymus enzyme (Markovits et al., 1989; Yamashita et al., 1990; Austin et al., 1992) and confirm the classification of these compounds as topoisomerase II-targeted DNA cleavage-enhancing agents.

Since the effects of coumarin-based drugs on DNA cleavage mediated by type II topoisomerases have not been well characterized, the ability of novobiocin to alter the DNA cleavage/religation equilibrium of the *Drosophila* enzyme was determined. As shown in Figures 1 and 2, novobiocin did not stimulate enzyme-mediated DNA breakage. Furthermore, no enhancement of nucleic acid cleavage was observed even at drug concentrations as high as 1 mM (Table I). Thus, at least with regard to DNA cleavage, the mechanism of novobiocin is distinct from that of the other drugs employed in this study.

Effects of Topoisomerase II-Targeted Drugs on Enzyme-Mediated ATP Hydrolysis. Virtually nothing is known about the effects of DNA cleavage-enhancing drugs on any step of the topoisomerase II catalytic cycle other than nucleic acid cleavage/religation (Liu, 1989; Schneider et al., 1990). Since genistein and flavone-based compounds that enhance DNA breakage also interact with the ATP site of tyrosine kinases (Graziana et al., 1983; Srivastava, 1985; Akiyama et al., 1987), it is possible that these or other topoisomerase II-targeted agents alter the interaction of the enzyme with its high-energy cofactor. Therefore, the effects of DNA cleavage-enhancing agents on the ATPase activity of topoisomerase II were examined.

The first experiments focused on genistein. At a concentration of 250 μM, the drug inhibited the rate of enzyme-catalyzed ATP hydrolysis ~60% (Figure 3). To more fully characterize this inhibition, the effect of genistein on the rate of ATP hydrolysis was examined over a concentration range of 0–250 μM drug (Figure 3, inset). The isoflavone was a potent effector of the topoisomerase II-catalyzed reaction and inhibited ATP hydrolysis 50% at a concentration of ~7 μM (Table I). This value is severalfold lower than that determined for novobiocin (Table I). However, in contrast to the coumarin, which inhibited ATP hydrolysis ~95% at 250 μM drug (Figure 3), genistein inhibition plateaued at a drug concentration of 10 μM. This inhibition plateau indicates that the isoflavone may impair ATP hydrolysis by a mechanism that differs from that of novobiocin.

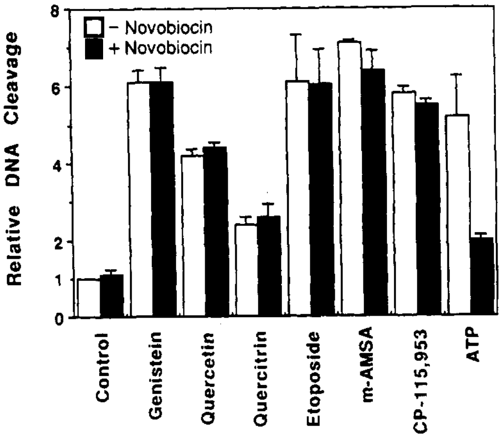

FIGURE 4: Effects of novobiocin on drug-enhanced topoisomerase II-mediated DNA cleavage. Assays were carried out in the absence (open bars) or the presence (black bars) of 250 μM novobiocin. Control reactions were carried out with topoisomerase II in the absence of DNA cleavage-enhancing drugs. Other reactions were carried out in the presence of topoisomerase II and 100 μM genistein, 200 μM quercetin, 200 μM quercitrin, 100 μM etoposide, 100 μM m-AMSA, 50 μM CP-115,953, or 1 mM ATP as indicated. Results are the average of three to six independent experiments. Standard deviations are indicated by the error bars.

The effects of various topoisomerase II-targeted agents on the ATPase activity of the enzyme are shown in Figure 3 and Table I. Compounds were selected to represent several structurally and mechanistically diverse drug classes. In addition to genistein, the flavones, and novobiocin, the epipodophyllotoxin etoposide, the anilinoacridine *m*-AMSA, and the quinolone CP-115,953 were employed. While etoposide and *m*-AMSA enhance topoisomerase II-mediated nucleic acid breakage primarily by inhibiting DNA religation (Osheroff, 1989b; Robinson & Osheroff, 1990, 1991), CP-115,953 appears to act by stimulating the forward rate of DNA cleavage (Robinson et al., 1991).

Genistein and the flavones were the most potent inhibitors of the topoisomerase II-catalyzed ATPase reaction. Additionally, both *m*-AMSA and CP-115,953 impaired ATP hydrolysis. As found with genistein, inhibition plateaued at ~40% residual activity for all of these DNA cleavage-enhancing compounds. In contrast, etoposide showed little ability to inhibit the ATPase activity of topoisomerase II (Table I, Figure 3). This is despite the fact that etoposide shares a common mechanism of action with *m*-AMSA for enhancing enzyme-mediated DNA cleavage (Osheroff, 1989b; Robinson & Osheroff, 1990, 1991).

The Enzyme Interaction Domain of Novobiocin is Distinct from Those of Topoisomerase II-Targeted Drugs That Enhance DNA Cleavage. The finding that some of the topoisomerase II-targeted compounds that enhance DNA breakage also impair ATP hydrolysis raises the possibility that the enzyme interaction domain for these antineoplastic agents overlaps that of novobiocin. Therefore, relationships between drug interaction domains were determined by a series of competition experiments. Initial studies examined the ability of novobiocin to attenuate the stimulation of topoisomerase II-mediated DNA cleavage by other drugs. If DNA cleavage-enhancing agents share a common interaction domain with the coumarin, high concentrations of novobiocin should displace these compounds from the enzyme·DNA complex and consequently block the drug-induced stimulation of DNA breakage.

As seen in Figure 4, 250 μM novobiocin did not diminish the enhancement of DNA cleavage induced by 100 μM genistein. Even at 500 μM novobiocin, no attenuation was observed (not shown). Furthermore, the coumarin was unable to block the DNA cleavage-enhancing activities of quercetin, quercitrin, etoposide, *m*-AMSA, or CP-115,953 (Figure 4). These results strongly suggest that the interaction domain for novobiocin on topoisomerase II is distinct from those of several DNA cleavage-enhancing drugs.

Another possible interpretation of the competition data is that the binding affinity of novobiocin for the enzyme·DNA complex is so weak compared to those of the other drugs examined that the coumarin would not be able to displace the DNA cleavage-enhancing agents, even if both drugs shared a common interaction site. This latter interpretation is unlikely at least for quercitrin, etoposide, *m*-AMSA, and CP-115,953, given the fact that their relative abilities to inhibit ATP hydrolysis were comparable to or less than that of the coumarin (see Table I). Furthermore, novobiocin was always in molar excess of the DNA cleavage-enhancing agents employed in competition assays, and the two drugs were always added to the enzyme simultaneously.

Despite the above precautions, three additional experiments were carried out to confirm the conclusion that the enzyme interaction domain for novobiocin is distinct from those of the other drugs examined. First, to demonstrate that the coumarin can displace an effector molecule from the topoisomerase II·DNA complex, the effect of novobiocin on the enhancement of DNA cleavage by ATP was determined (Figure 4). Consistent with previous reports (Sander & Hsieh, 1983; Osheroff, 1986), 1 mM ATP enhanced double-stranded nucleic acid breakage ~5-fold. As expected for a drug that interferes with ATP binding to the type II enzyme, 250 μM novobiocin decreased DNA cleavage ~80% of the way toward the baseline (ATP-free) level. An even greater attenuation (~95%) was observed in the presence of 500 μM novobiocin (not shown). Thus, the coumarin is able to displace compounds that share its interaction domain on the enzyme.

Second, since etoposide had little effect on the ATPase reaction of topoisomerase II, the ability of this DNA cleavage-enhancing drug to overcome the inhibition of ATP hydrolysis by novobiocin was determined. If these two drugs have separate interaction domains, etoposide should have little effect on the inhibition induced by novobiocin. Conversely, if these two drugs share a common interaction domain on the enzyme and the DNA cleavage-enhancing agent binds considerably more tightly than the coumarin to the topoisomerase II·DNA complex, etoposide should readily reverse the inhibition by novobiocin. As shown in Figure 5, etoposide was unable to overcome the diminution of ATPase activity by novobiocin. Together with the competition assays that demonstrate that novobiocin does not block etoposide-enhanced DNA cleavage (Figure 4), this latter result provides strong evidence that novobiocin and etoposide interact with topoisomerase II at distinct sites.

Third, since the inhibition of ATP hydrolysis by genistein plateaus at ~40% residual activity (Figure 3, inset) and the DNA cleavage competition assays demonstrate that a 5:1 molar excess of novobiocin is unable to displace genistein from the enzyme·DNA complex, experiments were carried out to determine whether the coumarin and the isoflavone had additive effects on ATPase inhibition. If these two drugs have different interaction domains on topoisomerase II, the addition of novobiocin to genistein-containing assays should further decrease rates of ATP hydrolysis, even at saturating concentrations of the isoflavone. In contrast, if the two drugs share an overlapping interaction domain (and as shown above

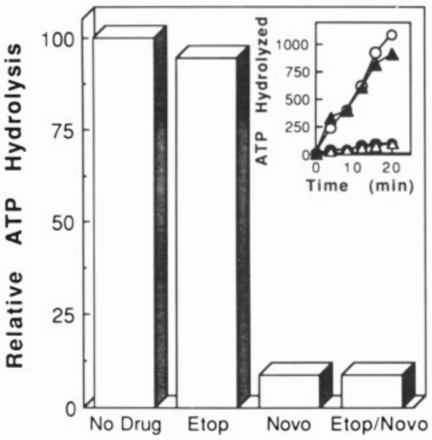

FIGURE 5: Effect of etoposide on novobiocin-induced inhibition of topoisomerase II-catalyzed ATP hydrolysis. Time courses (pmol/min) and relative rates of ATP hydrolysis are shown in the inset and by the bars, respectively. Experiments were carried out in the absence of drug (○, No Drug) or in the presence of 500 μM etoposide alone (▲, Etop), 250 μM novobiocin alone (●, Novo), or 500 μM etoposide and 250 μM novobiocin together (△, Etop/Novo). The relative rate of ATP hydrolysis in the absence of drug was set to 100%.

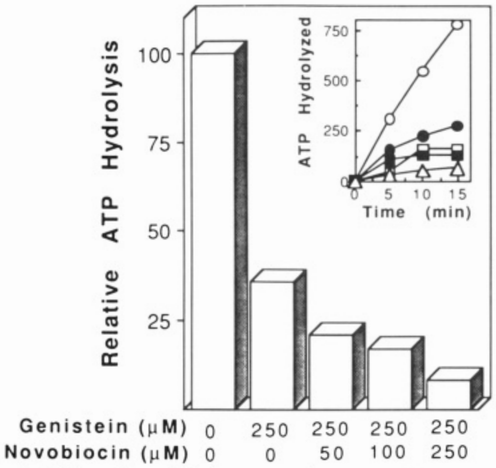

FIGURE 6: Effect of novobiocin on topoisomerase II-catalyzed ATP hydrolysis carried out in the presence of a saturating concentration of genistein. Time courses (pmol/min) and relative levels of ATP hydrolysis are shown in the inset and by the bars, respectively. Reactions were carried out in the absence of drug (○, column 1) or in the presence of 250 μM genistein alone (●, column 2), 250 μM genistein plus 50 μM novobiocin (□, column 3), 100 μM novobiocin, (■, column 4), or 250 μM novobiocin (△, column 5). The relative level of ATP hydrolysis in the absence of drug was set to 100%.

the coumarin cannot displace the isoflavone), no additional inhibition of ATPase activity beyond the plateau level should be observed. The concentration of genistein employed for these experiments (250 μM) was ~25-fold greater than that required for saturation of ATPase inhibition. As seen in Figure 6, the inclusion of novobiocin in genistein-containing ATPase assays decreased enzyme activity well below the 40% plateau level. A significant diminution of ATP hydrolysis was observed even in the presence of 50 μM novobiocin. This result provides further proof that the enzyme interaction domains of novobiocin and DNA cleavage-enhancing agents do not overlap, even though many antineoplastic drugs share the ability to inhibit topoisomerase II-mediated ATP hydrolysis.

DISCUSSION

Although the stimulation of topoisomerase II-mediated DNA cleavage by antineoplastic agents has been well documented (Liu, 1989; Schneider et al., 1990), little is known concerning the effects of these drugs on other steps of the enzyme's catalytic cycle. The present study characterized the effects of DNA cleavage-enhancing drugs on the ATPase reaction of topoisomerase II. Results indicate that a number of structurally diverse drugs impair the enzyme-catalyzed hydrolysis of ATP. This finding undermines the common assumptions that the actions of topoisomerase II-targeted antineoplastic agents are confined to enzyme-mediated DNA cleavage/religation events and that the ATPase reaction of topoisomerase II is the exclusive domain of coumarin-based drugs (Liu, 1989; Schneider et al., 1990; Reece & Maxwell, 1991). While the mechanism by which DNA cleavage-enhancing drugs inhibit ATP hydrolysis is not well understood, recent work suggests that these agents impair the ability of ATP to induce the DNA strand passage event of topoisomerase II (Corbett et al., 1992). Finally, of the drugs examined, only etoposide showed little ability to inhibit ATPase activity. This finding provides further evidence that mechanistic differences exist even among topoisomerase II-targeted drugs that enhance DNA cleavage.

At the present time, it is not known whether the effects of DNA cleavage-enhancing agents on ATP hydrolysis stem from direct drug interactions with topoisomerase II or from drug-induced alterations in the structure of DNA. However, two observations argue against the latter. First, the mode of DNA binding varies for members of different drug classes. For example, *m*-AMSA and the quinolone CP-115,953 have similar effects on enzyme-catalyzed ATPase activity, but the former intercalates into the double helix (Wilson et al., 1981), while the latter is nonintercalative in nature (Robinson et al., 1991). Second, genistein inhibits the ability of topoisomerase II to hydrolyze ATP even in the absence of DNA (not shown).

Despite a wealth of genetic information, studies on mutant drug-resistant type II topoisomerases have yet to establish definitive relationships between sites of drug action on the enzyme. However, by coupling mechanistic studies with a series of competition experiments, it was possible to define functional drug interaction domains on wild-type topoisomerase II. Results of these experiments indicate that the site of action on the enzyme for novobiocin is distinct from those of a number of DNA cleavage-enhancing agents. Thus, the enzymological approach described in the present study establishes a novel and alternative technique for characterizing interactions between drugs and topoisomerase II.

ACKNOWLEDGMENT

We are grateful to Dr. P. R. McGuirk and Dr. T. D. Gootz (Pfizer Central Research) for kindly providing quinolone CP-115,953, to Dr. Y. Pommier (NCI) for kindly providing *m*-AMSA, to C. Brewer for assistance with cell culture and the preparation of topoisomerase II and plasmid DNA, to Dr. J. L. Nitiss for helpful discussions, to J. Rule for expert photography, and to S. Heaver for the conscientious preparation of the manuscript.

REFERENCES

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., & Fukami, Y. (1987) *J. Biol. Chem. 262*, 5592–5595.

Andersen, A. H., Sørensen, B. S., Christiansen, K., Svejstrup, J. Q., Lund, K., & Westergaard, O. (1991) *J. Biol. Chem. 266*, 9203–9210.

Austin, C. A., Patel, S., Ono, K., Nakane, H., & Fisher, L. M. (1992) *Biochem. J. 282*, 883–889.

Bugg, B. Y., Danks, M. K., Beck, W. T., & Suttle, D. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7654–7658.

Chan, V. T.-W., Ng, S., Eder, J. P., & Schnipper, L. E. (1992) *J. Biol. Chem. 268*, 2160–2165.

Corbett, A. H., Robinson, M. J., Froelich-Ammon, S. J., Hong, D., & Osheroff, N. (1992) *Fourth Conference on DNA Topoisomerases in Therapy, New York*, p 26, Abstract 10.

Danks, M. K., Schmidt, C. A., Deneka, D. A., & Beck, W. T. (1989) *Cancer Commun. 1*, 101–109.

DiNardo, S., Voelkel, K., & Sternglanz, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2616–2620.

Gale, K. C., & Osheroff, N. (1990) *Biochemistry 29*, 9538–9545.

Gellert, M., Mizuuchi, K., O'Dea, M. H., & Nash, H. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3872–3876.

Gilligan, P. J., McGuirk, P. R., & Witty, M. J. (1986) U.S. Patent 4,623,650.

Goto, T., & Wang, J. C. (1984) *Cell 36*, 1073–1080.

Graziani, Y., Erikson, E., & Erikson, R. L. (1983) *Eur. J. Biochem. 135*, 583–589.

Hinds, M., Deisseroth, K., Mayes, J., Altschuler, E., Jansen, R., Ledley, F. D., & Zwelling, L. A. (1991) *Cancer Res. 51*, 4729–4731.

Holm, C., Goto, T., Wang, J. C., & Botstein, D. (1985) *Cell 41*, 553–563.

Hooper, D. C., & Wolfson, J. S. (1991) *N. Engl. J. Med. 324*, 384–394.

Huang, W. M. (1990a) in *DNA Topology & Its Biological Effects* (Cozzarelli, N. R., & Wang, J. C., Eds.) pp 265–284, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Huang, W. M. (1990b) in *DNA Topology & Its Biological Effects* (Cozzarelli, N. R., & Wang, J. C., Eds.) pp 409–457, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Huff, A. C., Leatherwood, J. K., & Kreuzer, K. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1307–1311.

Kreuzer, K. N., & Cozzarelli, N. R. (1979) *J. Bacteriol. 140*, 424–435.

Lee, M.-S., Wang, J. C., & Beran, M. (1992) *J. Mol. Biol. 223*, 837–843.

Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351–375.

Markovits, J., Linassier, C., Fosse, P., Couprie, J., Pierre, J., Jacquemin-Sablon, A., Saucier, J. M., Le Pecq, J. B., & Larsen, A. K. (1989) *Cancer Res. 49*, 5111–5117.

Maxwell, A. (1992) *J. Antimicrob. Chemother. 30*, 409–414.

Orr, L., Fairweather, N. F., Holland, I. B., & Pritchard, R. H. (1979) *MGG, Mol. Gen. Genet. 177*, 103–112.

Osheroff, N. (1986) *J. Biol. Chem. 261*, 9944–9950.

Osheroff, N. (1989a) *Pharmacol. Ther. 41*, 223–241.

Osheroff, N. (1989b) *Biochemistry 28*, 6157–6160.

Osheroff, N., Shelton, E. R., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9536–9543.

Osheroff, N., Zechiedrich, E. L., & Gale, K. C. (1991) *BioEssays 13*, 269–275.

Reece, R. J., & Maxwell, A. (1991) *Crit. Rev. Biochem. Mol. Biol. 26*, 335–375.

Robinson, M. J., & Osheroff, N. (1990) *Biochemistry 29*, 2511–2515.

Robinson, M. J., & Osheroff, N. (1991) *Biochemistry 30*, 1807–1813.

Robinson, M. J., Martin, B. A., Gootz, T. D., McGuirk, P. R., Moynihan, M., Sutcliffe, J. A., & Osheroff, N. (1991) *J. Biol. Chem. 266*, 14585–14592.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sander, M., & Hsieh, T.-S. (1983) *J. Biol. Chem. 258*, 8421–8428.

Schneider, E., Hsiang, Y.-H., & Liu, L. F. (1990) *Adv. Pharmacol. 21*, 149–183.

Shelton, E. R., Osheroff, N., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9530–9535.

Srivastava, A. K. (1985) *Biochem. Biophys. Res. Commun. 131*, 1–5.

Sugino, A., Higgins, N. P., Brown, P. O., Peebles, C. L., & Cozzarelli, N. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4838–4842.

Sutcliffe, J. A., Gootz, T. D., & Barrett, J. F. (1989) *Antimicrob. Agents Chemother. 33*, 2027–2033.

Staudenbauer, W. L., & Orr, E. (1981) *Nucleic Acids Res. 9*, 3589–3603.

Tamura, J. K., & Gellert, M. (1990) *J. Biol. Chem. 265*, 21342–21349.

Uemura, T., & Yanagida, M. (1984) *EMBO J. 3*, 1737–1744.

Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665–697.

Wigley, D. B., Davies, G. J., Dodson, E. J., Maxwell, A., & Dodson, G. (1991) *Nature 351*, 624–629.

Wilson, W. R., Baguley, B. C., Wakelin, L. P. G., & Waring, M. J. (1981) *Mol. Pharmacol. 20*, 404–414.

Yalowich, J. C., Allan, W. P., & Ritke, M. K. (1992) *Proc. Am. Assoc. Cancer Res. 33*, 450, Abstract 2686.

Yamagishi, J., Yoshida, H., Yamyoshi, M., & Nakamura, S. (1986) *MGG, Mol. Gen. Genet. 205*, 367–373.

Yamashita, Y., Kawada, S., & Nakano, H. (1990) *Biochem. Pharmacol. 39*, 737–744.

Yoshida, H., Bogaki, M., Nakamura, M., & Nakamura, S. (1990) *Antimicrob. Agents Chemother. 34*, 1271–1272.

Zwelling, L. A., Hinds, M., Chan, D., Mayes, J., Sie, K. L., Parker, E., Silberman, L., Radcliffe, A., Beran, M., & Blick, M. (1989) *J. Biol. Chem. 264*, 16411–16420.