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Effects of Novel Fluoroquinolones on the Catalytic Activities of Eukaryotic Topoisomerase II: Influence of the C-8 Fluorine Group

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A previous study (M. J. Robinson, B. A. Martin, T. D. Gootz, P. R. McGuirk, M. Moynihan, J. A. Sutcliffe, and N. Osheroff, J. Biol. Chem. 266:14585–14592, 1991) demonstrated that novel 6,8-difluoroquinolones were potent effectors of eukaryotic topoisomerase II. To determine the contribution of the C-8 fluorine to drug potency, we compared the effects of CP-115,955 [6-fluoro-7-(4-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid] on the enzymatic activities of *Drosophila melanogaster* topoisomerase II with those of CP-115,953 (the 6,8-difluoro parent compound of CP-115,955). Removal of the C-8 fluoro group decreased the ability of the quinolone to enhance enzyme-mediated DNA cleavage ~2.5-fold. Like its difluorinated counterpart, CP-115,955 increased the levels of cleavage intermediates without impairing the DNA religation reaction of the enzyme. Removal of the C-8 fluorine reduced the ability of the quinolone to inhibit topoisomerase II-catalyzed DNA relaxation. In addition, the cytotoxicity of CP-115,955 towards Chinese hamster ovary cells was decreased compared with that of CP-115,953. These results demonstrate that the C-8 fluorine increases the potency of quinolone derivatives against eukaryotic topoisomerase II and mammalian cells. Further comparisons of CP-115,955 with CP-115,953 and CP-67,804 (the N-1 ethyl-substituted derivative of the difluoro parent compound) indicate that the two intrinsic activities of quinolone-based drugs towards topoisomerase II (i.e., enhancement of DNA cleavage and inhibition of catalytic strand passage) can be differentially influenced by alteration of ring substituents. Finally, correlations between the biochemical and cytological activities of these drugs suggest that the ability to inhibit catalytic strand passage enhances the cytotoxic potential of quinolones towards eukaryotic cells.

Topoisomerase II is an essential enzyme (9, 21, 23, 53) that is required for chromosome structure (5, 13, 14, 16, 17), condensation (1, 36, 52, 56), and segregation (9, 23, 47, 54). It also appears to play roles in DNA replication, transcription, and recombination in eukaryotic cells (3, 6, 8, 30, 39, 43, 47, 51, 55).

In addition to its cellular functions, topoisomerase II is the primary target for several classes of antineoplastic drugs (32, 48, 59). These agents are widely used for the treatment of human cancers (32, 48, 59) and their clinical efficacies correlate with their abilities to stabilize covalent enzyme-cleaved DNA complexes that are intermediates in the catalytic cycle of the enzyme (31, 32, 43, 48, 59). Previous studies with etoposide (40, 46) and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) (45, 46) demonstrated that these topoisomerase II-targeted drugs stabilize cleavage complexes primarily by inhibiting the ability of the enzyme to religate cleaved DNA.

Recent work indicates that the DNA cleavage complex of eukaryotic topoisomerase II is also a target for novel 6,8-difluoroquinolone derivatives (4, 44). While quinolone-based drugs have been developed extensively as antimicrobial agents (targeted to DNA gyrase, the prokaryotic counterpart of topoisomerase II) (12, 24, 58), these studies provided evidence that quinolones may have potential as antineoplastic drugs. One of the difluoro compounds examined, 6,8-difluoro-7-(4-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid (CP-115,953) (see Fig. 1), was twice as potent as etoposide at enhancing topoisomerase II-mediated DNA cleavage (44). In addition, the N-1 ethyl-substituted derivative of CP-115,953, CP-67,804, was ~80% as potent as etoposide (46). However, in contrast to previously characterized drugs, neither quinolone inhibited the religation reaction of the enzyme to a significant extent (44). Thus, quinolone-based compounds in the CP-115,953 series may constitute a novel mechanistic class of topoisomerase II-directed drugs.

The vast majority of clinically important quinolone antimicrobial agents are fluorinated in the C-6 position (7, 24). This fluorine appears to be critical for potent quinolone action both in vitro and in vivo (7). Although the requirement for the C-6 fluorine has been well established (7), the importance of the C-8 fluoro group in 6,8-difluoroquinolones such as CP-115,953 is not yet known. Therefore, the present study examined the effects of a novel quinolone derivative, CP-115,955, on the catalytic activities of *Drosophila melanogaster* topoisomerase II. This latter compound is identical to CP-115,953 except for the substitution of the C-8 fluorine with a hydrogen atom (see Fig. 1). On the basis of enzymatic and cytotoxicity assays, this substitution decreased the potency of the drug by 2.2- to 3.3-fold. This finding indicates that the presence of a fluorine in the C-8 position enhances quinolone action against eukaryotic topoisomerase II.

MATERIALS AND METHODS

Chemicals. DNA topoisomerase II was purified from the nuclei of 6- to 12-h-old *D. melanogaster* embryos by the procedure of Shelton et al. (49). Negatively supercoiled

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bacterial plasmid pBR322 DNA was obtained from *Escherichia coli* DH1 by Triton X-100 lysis followed by double banding in cesium chloride-ethidium bromide gradients (33). Quinolone derivatives CP-115,955 and CP-115,953 were synthesized by the procedure of Gilligan et al. (18). CP-115,955 and CP-115,953 were dissolved as 25 mM solutions in 0.1 N NaOH, diluted to 5 mM stocks with 10 mM Tris-HCl (pH 8.0), and stored in the dark at −80°C. Etoposide (VePesid; VP-16-23) was purchased from Bristol Laboratories (Evansville, Ind.) 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% (vol/vol) ethanol and was stored at room temperature. Tris and ethidium bromide were obtained from Sigma (St. Louis, Mo.), sodium dodecyl sulfate (SDS) and proteinase K were obtained from E. Merck Biochemicals (West Point, Pa.), and ATP was obtained from Pharmacia LKB Biotechnology (Piscataway, N.J.). All other chemicals were analytical reagent grade.

Topoisomerase II-mediated DNA cleavage. All DNA cleavage reaction mixtures contained 50 to 100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total volume of 20 μl of cleavage buffer (10 mM Tris-HCl [pH 7.9], 25 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5% glycerol). DNA cleavage/religation equilibria were established by incubating samples at 30°C for 6 min. Cleavage products were trapped (15) 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 topoisomerase II. Final products were mixed with 2.5 μl of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, 10 mM Tris-HCl [pH 7.9]), and the mixture was heated at 70°C for 1 min and subjected to electrophoresis in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA in 1% agarose (EM Science, Gibbstown, N.J.) gels. Following electrophoresis, DNA bands were stained in a 1-μg/ml solution of ethidium bromide and visualized by transillumination with UV light (300 nm). DNA bands were photographed through Kodak 23A and 12 filters with Polaroid type 665 positive-negative film. The amount of DNA was quantitated by scanning negatives with an E-C Apparatus model EC910 scanning densitometer using Hoefer GS-370 Data System software. Under the conditions used, the intensity of the bands in the negatives was directly proportional to the amount of DNA present. The effects of drugs were examined over a concentration range of 0 to 150 μM. An amount of diluent equal to that in drug-containing samples was added to all control samples. None of the diluents affected the topoisomerase II-mediated DNA cleavage/religation equilibrium.

Heat-induced topoisomerase II-mediated DNA religation. Reaction mixtures contained 50 to 100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total volume of 20 μl of cleavage buffer. Initial DNA cleavage/religation equilibria were established at 30°C for 6 min. Topoisomerase II-mediated religation of cleaved DNA was induced by rapidly shifting samples from 30 to 55°C (27, 28, 42, 46). Religation was terminated by the addition of SDS (1% final concentration) at various time points up to 20 s. Samples were treated with EDTA and proteinase K as described above. Reaction products were resolved by agarose gel electrophoresis and quantitated as described above. The effects of drugs on topoisomerase II-mediated DNA religation were examined by the addition of 100 μM CP-115,955, 50 μM CP-115,953, or 100 μM etoposide to reaction mixtures. Drugs were added during the initial enzyme-DNA incubation. Control reaction mixtures contained amounts of diluent equivalent to those in drug-containing samples.

Topoisomerase II-mediated DNA relaxation. Assays were carried out as described by Osheroff et al. (41). The effects of drugs were examined over a concentration range of 0 to 250 μM.

Cytotoxicity of quinolone derivatives towards mammalian cells. Drug cytotoxicity was determined by a colony-forming assay (19, 44). Wild-type Chinese hamster ovary (CHO) cells and Vpm^R-5 cells, a mutant CHO cell line selected for resistance against epipodophyllotoxins (19, 22), were used for these studies. Cell lines were the generous gifts of R. Gupta and D. M. Sullivan. Cells were cultured as monolayers at 37°C under 5% CO_2 in alpha-minimal essential medium (GIBCO-BRL, Gaithersburg, Md.) (without antibiotics) supplemented with 5% fetal calf serum.

RESULTS

While most quinolone-based drugs are poor effectors of eukaryotic systems (12, 24, 58), the difluoroquinolone derivative CP-115,953 has potent activity against eukaryotic topoisomerase II and mammalian cell lines (44). Because of the antineoplastic potential of topoisomerase II-targeted agents (32, 48, 59), it is important to understand the relative contributions of ring substituents to drug action. A previous study demonstrated that substitution of an ethyl for the cyclopropyl group in the N-1 position of CP-115,953 decreased its potency against eukaryotic topoisomerase II and mammalian cells by 2.5- to 22-fold (44). To describe the contributions of the C-8 fluoro group to quinolone action, we examined the effects of CP-115,955 (in which the fluorine is substituted by a hydrogen atom) on *D. melanogaster* topoisomerase II. The structures of CP-115,955 and its parent compound, CP-115,953, are shown in Fig. 1.

Effects of CP-115,955 on the DNA cleavage/religation equilibrium of topoisomerase II. The chemotherapeutic efficacies of topoisomerase II-targeted antineoplastic drugs correlate with their abilities to enhance enzyme-mediated DNA cleavage (i.e., to shift the DNA cleavage/religation equilibrium of the enzyme towards the cleavage event) (31, 32, 48, 59). Topoisomerase II establishes cleavage/religation equilibria both before and after its double-stranded DNA passage event (38, 39, 43, 46). Since strand passage requires ATP binding (39, 41, 43, 51, 55), the pre- and post-strand passage equilibria of the enzyme can be separated experimentally. The DNA cleavage/religation equilibrium described here were established in the absence of ATP. Therefore, the following experiments examined the effects of CP-115,955 on the pre-strand passage equilibrium of the enzyme.

As determined by the increase in linear (form III) DNA, CP-115,955 was a less effective enhancer of topoisomerase II-mediated double-stranded DNA cleavage than was CP-115,953 (Fig. 2). No drug-induced DNA breakage was observed in the absence of the enzyme. To quantitate differences in potencies, we examined the effects of these quinolones on the DNA cleavage/religation equilibrium of the enzyme over a range of drug concentrations (Fig. 3). As calculated from the linear portion of the concentration curves (≤50 μM drug), CP-115,955 was ~40% as potent as CP-115,953. Thus, removing the C-8 fluoro group decreased the ability of the quinolone to enhance topoisomerase II-mediated DNA cleavage ~2.5-fold.

Despite its decreased potency relative to that of CP-115,953, CP-115,955 was still highly active against the *D. melanogaster* enzyme. Indeed, this monofluoroquinolone

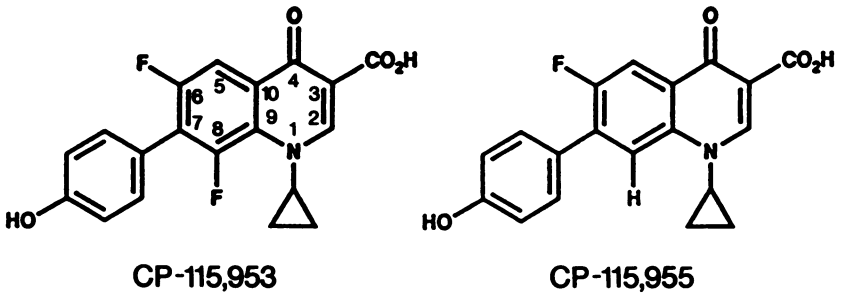

FIG. 1. Structures of CP-115,953 and CP-115,955.

was ~80% as potent as etoposide (a widely used topoisomerase II-targeted antineoplastic drug [32, 48, 59]) at stimulating DNA cleavage (40, 44, 46).

In addition to its activity against the eukaryotic type II enzyme, CP-115,955 was also a potent effector of *E. coli* gyrase (data not shown). The minimal concentration of CP-115,955 required to induce gyrase-mediated DNA cleavage was 0.24 μM, compared with 0.60 μM for ciprofloxacin (4) (the most potent gyrase-targeted antimicrobial agent currently in clinical use [2, 24]). The minimal concentration of the difluoro parent compound, CP-115,953, required to induce gyrase-mediated DNA cleavage was 0.22 μM. Thus, removal of the C-8 fluorine had little effect on the potency of the quinolone towards the prokaryotic enzyme. A similar observation was made previously by comparing the potencies of the C-8 fluoro derivatives of norfloxacin, perfloxacin, and ciprofloxacin with those of their parent compounds (10, 11).

Effects of CP-115,955 on the ability of topoisomerase II to religate cleaved DNA. Although the difluoroquinolone CP-115,953 and its N-1 ethyl-substituted derivative CP-67,804 increase the levels of topoisomerase II-DNA cleavage complexes, they show little ability to inhibit the DNA religation reaction of the enzyme (44). This finding suggests that these compounds enhance the levels of DNA breakage primarily by stimulating the forward rate of DNA cleavage. This is in marked contrast to the results obtained with topoisomerase II-targeted antineoplastic drugs such as etoposide (40, 46) and *m*-AMSA (45, 46), which appear to stabilize cleavage intermediates primarily by inhibiting enzyme-mediated DNA religation. To determine whether the C-8 fluoro group influences the mechanism of quinolone action, we monitored the effects of CP-115,955 on the ability of topoisomerase II to religate cleaved DNA (prior to strand passage) by using a heat-induced DNA religation assay (27, 28, 42, 46). This assay takes advantage of the fact that the religation reaction of topoisomerase II is less sensitive to variations in temperature than is its cleavage reaction (27, 28, 42, 46). Thus, after establishment of the DNA cleavage/religation equilibrium at 30°C (the optimal reaction temperature for *D. melanogaster* topoisomerase II [41]), shifting samples to 55°C leads to the time-dependent conversion of doubly-cleaved linear DNA back to its original supercoiled state (42, 46).

The effects of CP-115,955 on topoisomerase II-mediated

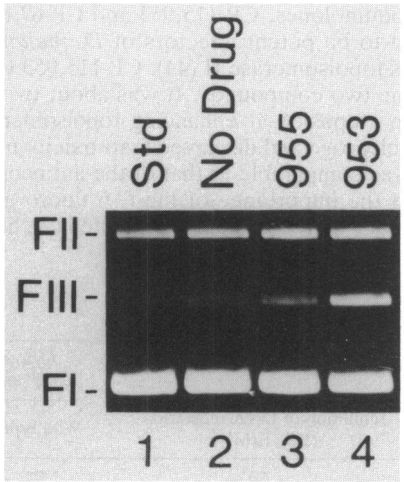

FIG. 2. Enhancement of topoisomerase II-mediated DNA cleavage by CP-115,955. An agarose gel is shown. Lanes: 1, DNA standard; 2, reaction mixture containing *D. melanogaster* topoisomerase II but no drug; 3, reaction mixture containing 100 μM CP-115,955; 4, reaction mixture containing 100 μM CP-115,953. The positions of negatively supercoiled DNA (form I, FI), nicked circular plasmid molecules (form II, FII), and linear molecules (form III, FIII) are indicated.

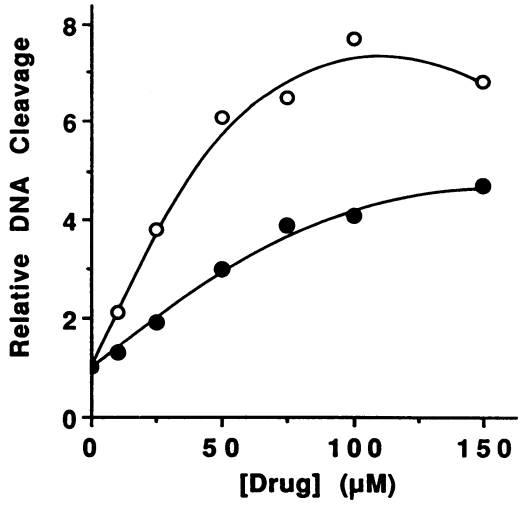

FIG. 3. Effects of quinolone concentration on the pre-strand passage DNA cleavage/religation equilibrium of topoisomerase II. Assays were carried out as described in Materials and Methods. Results are plotted as the relative level of DNA cleavage versus the quinolone concentration. The relative level of DNA cleavage in the absence of the drug was set arbitrarily to 1. Reactions were carried out in the presence of CP-115,955 (●) or CP-115,953 (○). Data represent the average of two independent experiments. The average standard error for the data shown was less than 0.6.

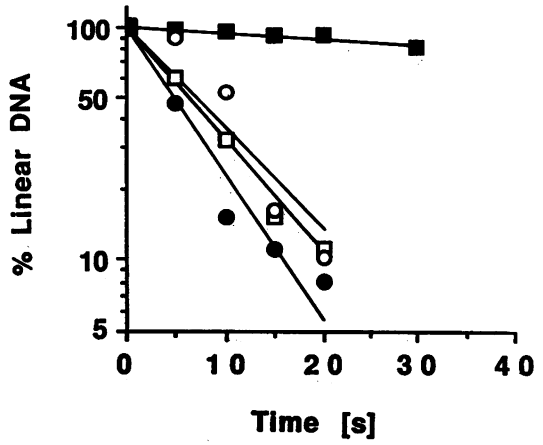

FIG. 4. Effects of CP-115,955 on topoisomerase II-mediated DNA religation. Religation was initiated by shifting assay mixtures from 30 to 55°C as described in Materials and Methods. Reaction mixtures contained no drug (□), 100 μM CP-115,955 (●), or 50 μM CP-115,953 (○). Results with 100 μM etoposide (■) are shown for comparison. Data are plotted in a semilogarithmic fashion as the loss of linear DNA versus time. The percentage of linear DNA for each assay was set arbitrarily to 100% at time zero. Plots represent the average of two or three independent experiments. The average standard deviation (or standard error, when appropriate) for the data shown was less than 5%.

DNA religation are shown in Fig. 4. Data for CP-115,953 and etoposide are shown for comparison. Clearly, CP-115,955 did not impair the DNA religation reaction of the enzyme. In contrast, etoposide inhibited this reaction ~9-fold at the same drug concentration. Therefore, the substitution of hydrogen for fluorine at the C-8 position decreases quinolone potency but does not alter the mechanism of drug action.

To further confirm this finding, we analyzed the effects of CP-115,955 by using a second DNA religation system (data not shown). This second system relies on the fact that calcium can be used to trap topoisomerase II-DNA cleavage complexes in a kinetically active form (40, 42, 45, 57). Once again, CP-115,955 showed little ability to inhibit enzyme-mediated DNA religation (compared with etoposide, which inhibited DNA religation ~3-fold in this system [40]).

Inhibition of topoisomerase II-catalyzed DNA relaxation by CP-115,955. A number of quinolone-based drugs inhibit the catalytic DNA strand passage reaction of eukaryotic topoisomerase II (4, 20, 25, 26, 29, 34, 35, 37, 41). The most potent compound reported to date is CP-115,953, which inhibits the DNA relaxation activity of *D. melanogaster* topoisomerase II by 50% (IC_{50}) at a concentration of ~60 μM drug (44). As shown in Table 1, ~130 μM CP-115,955 inhibited DNA relaxation by 50%. Thus, removal of the C-8 fluorine decreased quinolone potency ~2.2-fold.

Cytotoxicity of CP-115,955 towards CHO cell lines. To further assess the contributions of the C-8 position to quinolone action, we examined the cytotoxicity of CP-115,955 towards mammalian cells. Two tissue culture lines were used for this study. The first was a wild-type CHO cell line. The second was Vpm^R-5, a CHO cell line selected for resistance against epipodophyllotoxins (22). Compared with CHO cells, Vpm^R-5 cells are 10- to 20-fold resistant to etoposide (a representative epipodophyllotoxin) (19, 22, 44), and they show broad cross-resistance to a number of topoisomerase II-targeted antineoplastic drugs (19, 22). Drug resistance in this line results from the presence of a mutated, resistant type II enzyme, rather than a decrease in drug uptake (19, 22, 50).

Substitution of a hydrogen atom for the C-8 fluorine decreased quinolone cytotoxicity towards wild-type CHO cells ~2.2-fold (Table 1). The effective concentration of CP-115,955 required to kill 50% of the cells (EC_{50}) was ~20 μM; that of the parent compound, CP-115,953, was ~9 μM (44).

In a previous study, the Vpm^R-5 line showed only low (~1.3-fold) cross-resistance to CP-115,953 (EC_{50} ≈12 μM) (46). Removal of the C-8 fluoro group increased the resistance of Vpm^R-5 cells to this quinolone series (EC_{50} of CP-115,955, ≈40 μM) to ~2.0-fold (Table 1). The cross-resistance of Vpm^R-5 cells to CP-115,955, albeit low, suggests that topoisomerase II is a physiological target for this quinolone in mammalian cells.

DISCUSSION

Quinolone-based drugs represent some of the most effective antimicrobial agents currently in clinical use (2, 12, 24, 58). Most members of this drug class show little potency against eukaryotic systems (12, 24, 58). However, two novel 6,8-difluoroquinolones, CP-115,953 and CP-67,804, recently were found to be potent effectors of *D. melanogaster* and calf thymus topoisomerase II (44). CP-115,953 was the more active of the two compounds. It was about two times more potent than etoposide at enhancing topoisomerase II-mediated DNA cleavage and displayed cytotoxicity towards CHO cells that was comparable to that of the antineoplastic drug.

Although the importance of the C-6 fluoro group to quinolone potency has been well documented (7), little is known

TABLE 1. Properties of novel quinolones

Compound	Substituent		Activity against topoisomerase II[a]		Cytotoxicity (EC_{50} [μM])[b] against CHO cells	
	C-8 position	N-1 position	Stimulation of DNA cleavage (relative potency)[c]	Inhibition of DNA relaxation (IC_{50} [μM])[d]	Wild type	Vpm^R-5
CP-115,953	Fluorine	Cyclopropyl	1.00	60	9[e]	12[e]
CP-115,955	Hydrogen	Cyclopropyl	0.38	130	20	40
CP-67,804[e]	Fluorine	Ethyl	0.39	325	70	265

[a] *D. melanogaster* topoisomerase II was used for all experiments.
[b] Values represent the average of three independent experiments.
[c] The relative potency of CP-115,953 was set arbitrarily to 1.00. The potency of etoposide relative to CP-115,953 was 0.48. Values represent the average of two independent experiments.
[d] Values represent the average of three or four independent experiments.
[e] From Robinson et al. (44).

concerning the contribution of the C-8 fluorine to the actions of these 6,8-difluoro compounds. To clarify the role of this ring substituent, we compared the activities of the 6-monofluoroquinolone, CP-115,955, towards *D. melanogaster* topoisomerase II and CHO cells with those of its 6,8-difluoro parent compound, CP-115,953. In all cases, substitution of a hydrogen atom for the C-8 fluorine decreased quinolone potency 2.2- to 3.3-fold (Table 1). As observed previously with CP-115,953 and CP-67,804 (44), CP-115,955 stimulated topoisomerase II-mediated DNA cleavage without inhibiting the DNA religation reaction of the enzyme. Therefore, CP-115,955 belongs to the same mechanistic class as the difluoro compounds described above.

The presence of the C-8 fluorine enhanced quinolone activity towards both eukaryotic topoisomerase II and mammalian cells. However, it did not affect the potency of the drug towards DNA gyrase. In other studies (10, 11), this fluoro group has been correlated with decreased quinolone activity against gyrase and/or bacterial cells. Thus, the C-8 fluorine appears to be specific for eukaryotic systems. This finding makes the C-8 position a potential target for the future development of quinolone-based antineoplastic agents.

The cytotoxic effects of topoisomerase II-targeted antineoplastic agents and gyrase-targeted antimicrobial agents have been correlated to their enhancement of enzyme-mediated DNA cleavage (2, 12, 24, 31, 58). However, CP-115,955 was 3.5 times more cytotoxic towards CHO cells than was CP-67,804, despite the fact that both were equally potent at increasing levels of enzyme-DNA cleavage complexes (Table 1). This finding suggests that other drug actions may contribute to cytotoxicity. To this point, CP-115,955 was 2.5 times more potent at inhibiting enzyme-catalyzed DNA relaxation than was CP-67,804 (Table 1). Since the double-stranded DNA passage reaction of topoisomerase II is essential to cell survival (9, 21, 23, 39, 43, 51, 53, 55), it is tempting to speculate that the ability of quinolones to inhibit this activity contributes to their cytotoxic potential. It will be necessary to fully characterize the pharmacokinetic properties of these quinolone-based drugs, however, before correlations between this inhibitory activity and cell death can be firmly established.

The two intrinsic activities of quinolone-based drugs (i.e., enhancement of DNA cleavage and inhibition of catalytic strand passage) (12, 24, 58) can be differentially influenced by the alteration of ring substituents. Although substitutions at the C-8 and N-1 positions (Table 1) had similar effects on DNA cleavage, the N-1 substitution increased the IC_{50} of the quinolone for DNA relaxation to a greater extent. Given the relationships between DNA cleavage, DNA strand passage, and cytotoxicity, the ability to differentially alter drug activities by specific ring substitutions may significantly affect future quinolone design.

Gyrase-targeted quinolones have been developed exclusively as antimicrobial agents (2, 12, 24, 58). Even the most potent gyrase-targeted drug in clinical use, ciprofloxacin, shows only limited activity towards eukaryotic topoisomerase II (4). The series of fluoroquinolones described in Table 1 are novel in their high potency towards both gyrase and the eukaryotic type II enzyme. Considering the antineoplastic potential of these compounds, it is important to establish structure-activity relationships for different ring substituents within the series. The results of the present study indicate that the C-8 fluoro group contributes to the potency of these compounds against eukaryotic topoisomerase II and mammalian cells.

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