Cytotoxicity of Quinolones toward Eukaryotic Cells

IDENTIFICATION OF TOPOISOMERASE II AS THE PRIMARY CELLULAR TARGET FOR THE QUINOLONE CP-115,953 IN YEAST*

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The quinolone CP-115,953 (6,8-difluoro-7-(4-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid) represents a novel mechanistic class of drugs with potent activity against eukaryotic topoisomerase II in vitro (Robinson, M. J., Martin, B. A., Gootz, T. D., McQuirk, F. R., Moynihan, M., Sutcliffe, J. A., and Osheroff, N. (1991) J. Biol. Chem. 266, 14585–14592). Although the quinolone is highly toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known. Therefore, yeast was used as a model system to determine whether topoisomerase II is the primary target responsible for the in vivo effects of CP-115,953. The quinolone was equipotent to etoposide at enhancing DNA breakage mediated by the Saccharomyces cerevisiae type II enzyme. Moreover, at concentrations as low as 5 μM, CP-115,953 was cytotoxic to yeast cells that carried wild type topoisomerase II (TOP2+). By utilizing a yeast strain that expressed the top2-1 temperature-sensitive mutant, the effect of topoisomerase II activity on quinolone cytotoxicity was determined. At the permissive temperature of 25 °C, cells were highly sensitive to CP-115,953. However, at the semipermissive temperature of 30 °C (where in vivo enzyme activity is present but is greatly diminished), cells displayed only marginal sensitivity to the quinolone at concentrations as high as 50 μM. These results strongly suggest that topoisomerase II is the primary physiological target responsible for quinolone cytotoxicity and that CP-115,953 kills cells by converting the type II enzyme into a cellular poison.

Topoisomerase II plays important roles in a number of fundamental nuclear processes (1–3) and is essential for the survival of eukaryotic cells (4–6). Beyond its required physiological functions, the enzyme is a target for some of the most active compounds currently employed for the treatment of human cancers (7, 8). Among the topoisomerase II-targeted antineoplastic agents in clinical use are etoposide, amsacrine (m-AMSA), adriamycin, and mitoxantrone. The activities of these drugs correlate with their ability to stabilize covalent enzyme-cleaved DNA complexes (7, 8) that are intermediates in the catalytic cycle of topoisomerase II (3, 9). Mechanistic studies on etoposide and amsacrine indicate that these compounds stabilize DNA cleavage complexes primarily by inhibiting enzyme-mediated religation of cleaved nucleic acids (10–12). Since topoisomerase II-targeted drugs act by converting the enzyme into a cellular poison (7, 8, 13), antineoplastic potential is a reflection of the physiological level of the type II enzyme (7, 8). Thus, cells that overexpress topoisomerase II are hypersensitive to these compounds (14–16), while cells with diminished enzyme levels display resistance (17–21).

Recent evidence strongly suggests that topoisomerase II is also a target for a variety of quinolone-based drugs (23–27). Thus far, most of the compounds that show high activity against the eukaryotic type II enzyme contain aromatic substituents at their C-7 positions (22–27). This is in marked contrast to clinically relevant quinolones that target DNA gyrase, the prokaryotic homologue of topoisomerase II (28, 29), all of which contain an aliphatic group at C-7 (30–32). Of the quinolones examined to date, CP-115,953 (see Fig. 1, inset) displays the highest activity against the eukaryotic enzyme. This compound stimulates DNA cleavage mediated by Drosophila (24), CHO cell, or calf thymus topoisomerase II (24) with a potency that is approximately 2, 7, or 40 times greater than that of etoposide. Although CP-115,953 is a potent enhancer of enzyme-mediated nucleic acid breakage, it (as well as other related quinolones) shows little ability to inhibit the DNA religation reaction of topoisomerase II (24, 25). Therefore, CP-115,953 represents a novel mechanistic class of drugs that appears to alter topoisomerase II function by enhancing the forward DNA cleavage reaction of the enzyme. The cytotoxicity of CP-115,953 toward wild type CHO cells is approximately equipotent to that of etoposide (24). However, the quinolone also displays high toxicity toward Vpmp5-5 cells (24), a CHO line that expresses a mutant form of topoisomerase II with resistance to a wide variety of antineoplastic drugs (33, 34). Indeed, while Vpmp5-5 cells (as compared with wild type CHO cells) are ~12-fold resistant to etoposide, they are only ~1.3-fold resistant to CP-115,953 (24). This disparity in cytotoxic potential can be explained by two possibilities; either CP-115,953 has an interaction domain on topoisomerase II that differs from that of etoposide, or the type II enzyme is not the major cytotoxic target for quinolones in eukaryotic cells. While the former possibility is supported by the mechanistic studies discussed above, the latter is

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1 The abbreviations used are: CP-115,953, 6,8-difluoro-7-(4-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid; CHO, Chinese hamster ovary.
suggested by studies that demonstrated mitochondrial function to be impaired by high concentrations of quinolones or other drugs that are targeted to topoisomerase II (35-37).

Before quinolones can be exploited as potential antineoplastic agents, it is necessary to establish their mechanism of action in the cell. Therefore, to resolve the above issues, the physiological target of CP-115,953 was determined using a novel yeast system that allows the ready manipulation of topoisomerase II activity in vivo. Results indicate that the type II enzyme is the target primarily responsible for the cytotoxicity of quinolones toward eukaryotic cells.

EXPERIMENTAL PROCEDURES

Materials and Yeast Strains—Yeast topoisomerase II was isolated by the procedure of Worland and Wang (38). Negatively supercoiled pBR322 plasmid DNA was prepared as previously described (24). Quinolone CP-115,953 was synthesized at Pfizer Central Research by the procedure described in Ref. 39 and was the generous gift of Dr. P. R. McGuirk and Dr. T. D. Gootz. The drug was dissolved as a 25 mM solution in NaOH, diluted to a 5 mM stock with 10 mM Tris-HCl, pH 8.0, and stored in the dark at −25°C. Etoposide (VePesid) was obtained from Bristol Laboratories. The yeast strains employed in the present study were Saccharomyces cerevisiae JN394, whose genotype is ura3-52, leu2, his7, ade1-2, ISF2, rad52::LEU2; and JN394/t-1, whose genotype is isogenic to JN394 except for the replacement of the wild type topoisomerase II gene (TOP2+) with the top2-1 mutant allele (5, 40).

Topoisomerase II-Mediated Reactions—DNA cleavage reactions contained 100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 plasmid DNA in a total volume of 20 μl of 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 2.5% glycerol. Assays were at 30°C for 6 min as described by Robinson et al. (24). The effects of drugs were examined over a concentration range of 0–100 μM. In the absence of drugs, levels of DNA cleavage were set arbitrarily to 100%, and levels of DNA cleavage were set arbitrarily to 1.0. The structure of CP-115,953 is shown in the inset of panel B.

FIG. 1. Effects of CP-115,953 (●) on the DNA relaxation (panel A) and DNA cleavage (panel B) activities of yeast topoisomerase II. Effects of etoposide (○) are shown as a control. In the absence of drug, levels of DNA relaxation were set arbitrarily to 100%, and levels of DNA cleavage were set arbitrarily to 1.0. The structure of CP-115,953 is shown in the inset of panel B.

Effect of CP-115,953 on Yeast Topoisomerase II—As a prelude to cellular studies, the activity of CP-115,953 against yeast topoisomerase II was characterized. As monitored by a DNA relaxation assay, the quinolone inhibited the catalytic DNA strand passage reaction of the enzyme (Fig. 1A). CP-115,953 decreased DNA relaxation by 50% at a concentration of ~10 μM. While the ability of the quinolone to inhibit catalytic strand passage was 3-4-fold lower than that observed for etoposide, CP-115,953 was equipotent to the antineoplastic drug at enhancing the DNA breakage activity of the enzyme (Fig. 1B). The quinolone increased levels of double-stranded DNA cleavage mediated by yeast topoisomerase II by 2-fold at a concentration of ~10 μM and by over 10-fold in the presence of 100 μM drug. Since the antineoplastic potential of topoisomerase II-targeted agents appears to correlate with their ability to enhance enzyme-mediated DNA breakage (7, 8), this latter result strongly suggests that CP-115,953 should be toxic to yeast cells.

Cytotoxicity of CP-115,953 toward Yeast—The cytotoxicity of CP-115,953 toward yeast cells that contain wild type topoisomerase II was determined at 25°C (Fig. 2). The yeast strain employed (JN394) carries the ISF2 drug permeability mutation as well as the rad52 mutation that enhances the sensitivity of cells to DNA damaging agents (40). Under growth conditions in which cells multiplied by ~2 orders of magnitude in the absence of quinolone, cell proliferation was blocked by as little as 5 μM CP-115,953. Following a 25-h exposure to 50 μM quinolone, nearly 99.99% cell death was observed. The cytotoxicity of the quinolone is greater than or equal to that found for the topoisomerase II-targeted antineoplastic drugs etoposide or amsacrine (40). Therefore, CP-115,953 appears to be highly toxic to yeast cells that express...
a normal complement of wild type topoisomerase II.

Effect of Cellular Topoisomerase II Activity on the Cytotoxicity of CP-115,953 toward Yeast—Three possibilities exist for the physiological mechanism of quinolone toxicity. The first is that topoisomerase II is the primary target responsible for drug action, and quinolone cytotoxicity correlates with the ability to increase levels of enzyme-mediated DNA breakage. Such a mechanism has been demonstrated for topoisomerase II-targeted antineoplastic drugs that enhance DNA breakage and convert the type II enzyme into a cellular poison by impairing its DNA religation activity (7, 8, 10–13). The second is that CP-115,953 acts through topoisomerase II, but toxicity correlates with the ability to block the DNA strand passage activity of this essential enzyme (1, 2, 4–6, 28). Such a mechanism has been demonstrated for coumarin-based drugs that impair the overall catalytic activity of topoisomerase II without stimulating DNA breakage (1, 2, 28). Since quinolones in the CP-115,953 series enhance enzyme-mediated DNA cleavage with little effect on rates of religation (24, 25), its mode of action may more closely resemble those of drugs that do not stabilize enzyme-DNA cleavage complexes. The third possibility is that topoisomerase II is not the physiological target predominantly responsible for quinolone cytotoxicity.

The possibilities described above can be distinguished from one another by decreasing the cellular activity of the type II enzyme. (Since topoisomerase II is essential for survival (4–6), its activity cannot be deleted completely from the cell.) If topoisomerase II is the primary in vivo target for CP-115,953 and drug toxicity correlates with the ability to enhance enzyme-mediated DNA cleavage (i.e., the quinolone converts topoisomerase II into a cellular poison; Refs. 7, 8, and 13), a reduction in enzyme activity should greatly diminish quinolone-induced cell death. In fact, in this case, cell proliferation might continue even at relatively high drug concentrations. Conversely, if topoisomerase II is the major target but quinolone toxicity correlates with the ability to impair the overall catalytic function of the enzyme, cells with decreased levels of topoisomerase II activity should become hypersensitive to CP-115,953. Finally, if quinolone action is mediated by targets other than the type II enzyme, reduced levels of topoisomerase II should have little effect on the cytotoxicity of CP-115,953.

A novel yeast system was developed to manipulate cellular levels of topoisomerase II activity. The strain employed for these studies (JN394t2-1) contains the ISE2 and rad52 mutations described earlier and expresses the temperature-sensitive top2-1 mutation (5) in place of the wild type TOP2+ topoisomerase II gene. While the top2-1 protein shows wild type activity at 25 °C, it shows less than 10% activity at 37 °C and at best 10–20% activity at 30 °C (5). Consequently, yeast top2-1 strains proliferate like wild type cells at the permissive temperature (25 °C), are non-viable at 37 °C, and are viable but grow slowly at the semipermissive temperature (30 °C) (5). Therefore, by comparing the cytotoxicity of CP-115,953 toward yeast JN394t2-1 cells at 25 °C and 30 °C, it is possible to address the physiological mechanism of quinolone action.

As expected, CP-115,953 was a potent toxin toward JN394t2-1 yeast cells at 25 °C, the temperature at which topoisomerase II activity is similar to wild type (Fig. 3, left panel). Greater than 99.9% of the cells were killed following an ~25-h incubation with 50 μM drug. However, a markedly different result was obtained when quinolone cytotoxicity was examined at 30 °C, the temperature at which enzyme activity is greatly reduced (Ref. 3, right panel). At best, CP-115,953 displayed marginal cytotoxicity toward JN394t2-1 cells at the semipermissive temperature. In fact, at a concentration of 50 μM drug, an ~25% increase in cell proliferation (compared with cultures grown in drug-free medium) was observed. This lack of quinolone toxicity did not result from the slower growth rate of JN394t2-1 cells at the elevated temperature. Indeed, at 30 °C this yeast strain is still highly sensitive to camptothecin, a topoisomerase I-targeted drug that requires cell proliferation and DNA replication for its cytotoxic action (7, 8). Finally, quinolone resistance did not result from a difference in the cellular efflux or metabolism of CP-115,953, because the sensitivity of JN394 (TOP2+) cells at 30 °C was similar to that found at 25 °C (not shown). The lack of quinolone toxicity toward cells that contained decreased levels of topoisomerase II activity strongly suggests that the type II enzyme is the primary physiological target for CP-115,953 and that quinolone cytotoxicity results from the ability to enhance enzyme-mediated DNA breakage.

DNA Recombination Induced by CP-115,953 in Yeast (TOP2+) Cells—Several lines of evidence indicate that drugs targeted to topoisomerase II produce DNA recombination in treated cells (7, 8, 40). For example, as determined by a HIS gene conversion assay, 250 μM amssacine increases levels of recombination in yeast 5–20-fold following a 24-h exposure (40). In a similar assay, 10, 20, and 50 μM CP-115,953 increased gene conversion at the HIS locus 2-, 6-, and 13-fold, respectively, over that observed in drug-free cells (recombination frequency ≈ 2.9 ± 0.1 HIS+ colonies/106 viable cells). Thus, CP-115,953 induces DNA recombination similarly to other drugs that act by enhancing topoisomerase II-mediated DNA breakage.
DISCUSSION

Although CP-115,953 is highly active against eukaryotic topoisomerase II in vitro and displays potent toxicity toward mammalian cells in culture (24, 25), the primary molecular target of the quinolone and its mode of cytotoxicity have not been established previously. The present study, which employed a novel yeast system that allowed the manipulation of topoisomerase II activity in vivo, indicates that CP-115,953 exerts its cytotoxic action by converting the type II enzyme into a cellular poison.

CP-115,953 was at least as cytotoxic and recombinogenic toward yeast as were the topoisomerase II-targeted antineoplastic drugs etoposide and amsacrine (40). Although all of the above compounds stimulate topoisomerase II-mediated nucleic acid breakage (7, 8, 24, 25), the quinolone apparently does so by enhancing the forward rate of DNA cleavage (24, 25) while the other drugs act primarily by inhibiting the DNA religation activity of the enzyme (10–12). Thus, the physiological consequences of drug action appear to be dictated by the ability of the compound to increase levels of topoisomerase II-DNA cleavage complexes irrespective of the mechanism used to stabilize these enzyme intermediates.

Quinolones represent an important class of medically relevant antimicrobial agents (30–32). The recent discovery of novel quinolones with potent activity against eukaryotic systems (22–27) suggests that some members of this drug class eventually may be useful for the treatment of human cancers. However, before the clinical value of quinolones as antineoplastic agents can be fully evaluated, it is imperative to define the physiological site of action of these drugs. The identification of topoisomerase II as the primary cellular target responsible for the cytotoxicity of CP-115,953 provides the foundation necessary for the future development of quinolone-based drugs with antineoplastic potential.

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