A Novel Mechanism of Cell Killing by Anti-topoisomerase II Bisdioxopiperazines*

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Bisdioxopiperazines are a unique class of topoisomerase II inhibitors that lock topoisomerase II at a point in the enzyme reaction cycle where the enzyme forms a closed clamp around DNA. We examined cell killing by ICRF-187 and ICRF-193 in yeast cells expressing human topoisomerase II α (htop-IIα). Expression of htop-IIα in yeast cells sensitizes them to both ICRF-187 and ICRF-193, compared with cells expressing yeast topoisomerase II. ICRF-193 is still able to exert growth inhibition in the presence of genes encoding both ICRF-193-resistant and ICRF-193-sensitive htop-IIα enzymes, indicating that sensitivity to bisdioxopiperazines is dominant. Killing by ICRF-193 occurs more rapidly, than the killing in yeast cells due to a temperature-sensitive yeast topoisomerase II incubated at the non-permissive temperature. These results are reminiscent of a top-II poison such as etoposide. However, the killing caused by ICRF-193 and ICRF-187 is not enhanced by mutations in the RAD52 pathway. The levels of drug-induced DNA cleavage observed with htop-IIα in vitro is insufficient to explain the sensitivity induced by this enzyme in yeast cells. Finally, arrest of cells in G1 does not protect cells from ICRF-193 lethality, a result inconsistent with killing mechanisms due to catalytic inhibition of top-II or stabilization of a cleavable complex. We suggest that the observed pattern of cell killing is most consistent with a poisoning of htop-II by ICRF-193 by a novel mechanism. The accumulation of closed clamp conformations of htop-II induced by ICRF-193 that are trapped on DNA might interfere with transcription, or other DNA metabolic processes, resulting in cell death.

There are two well characterized modes of action of drugs acting against eukaryotic topoisomerase II. Anti-cancer topoisomerase II poisons such as etoposide, amssacrine, and doxorubicin stabilize an intermediate in the topoisomerase II reaction in which the two topoisomerase II subunits are covalently bound to DNA via a phosphotyrosine linkage. This covalent intermediate, termed the covalent complex plays a critical role in cell killing by anti-topoisomerase II agents (reviewed in Refs. 1–3). The second class of agents do not stabilize the covalent intermediate of the topoisomerase II reaction, but inhibit the enzyme at other points of the reaction cycle (1, 4). Since blocking the enzyme at other points of the reaction cycle does not result in DNA damage, this second class of agents is thought to kill cells by depriving them of the essential enzyme activity of topoisomerase II. This second class of inhibitors has been termed catalytic inhibitors to distinguish them from agents that act by stabilizing covalent complexes.

A major class of catalytic inhibitors of prokaryotic topoisomerase II inhibits topoisomerase activity by preventing ATP binding (5). These inhibitors include novobiocin and the coumarimycins. Most of these inhibitors have relatively low potency against eukaryotic topoisomerases (5). Other, more potent catalytic inhibitors of eukaryotic topoisomerases have been described, these include anthracyclines such as aclacinomicin that intercalate in DNA and prevent the binding of the enzyme to DNA (6, 7), and mebarone, which inhibits DNA cleavage by the enzyme (8–10).

Wang and colleagues (11) have demonstrated that during the course of the topoisomerase II reaction, the enzyme forms a closed clamp around DNA. ATP binding is required to generate a closed clamp with wild type topoisomerase II, and ATP hydrolysis generates a conformational change that leads to reopening of the clamp (11, 12). Subsequently, Roca et al. (13) showed that bisdioxopiperazines inhibit the re-opening of the closed clamp, and also blocks ATP hydrolysis. Therefore, bisdioxopiperazines would sequester topoisomerase II in the closed clamp conformation, and inhibit enzyme activity inside the cell.

Support for this mode of action of bisdioxopiperazines in vivo has been obtained from both yeast and mammalian cells. Overexpression of topoisomerase II in yeast leads to resistance to ICRF-193, while reducing the activity of the enzyme leads to increased cell killing, suggesting that cell death arises from a lack of topoisomerase II activity. Studies by Andoh and colleagues have shown that ICRF-187 or ICRF-193 exposure results in a failure to complete a normal mitosis, and can generate polyploid cells (4). In vitro, bisdioxopiperazines prevent decatenation of replicated chromosomes by topoisomerase II (4, 14). These results are consistent with the hypothesis that a

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1 The abbreviations used are: ICRF-193, meso-2,3-bis(3,5-dioxopiperazine-1-yl)butane; ICRF-187, (s)-(+)-1,2-bis(3,5-dioxopiperazine-yl)propane; top-IIα, topoisomerase II α; htop-IIα, human topoisomerase II α; ytop-II, yeast topoisomerase II; PAG, poliacrylamide gel electrophoresis; AMPPNP, adenosine 5’-β,γ-imino)triphosphate.
out as described previously (20, 21, 32) The K’/SDS assay was used to determine drug stabilized DNA cleavage, and was performed as described previously (20, 32).

**Measurement of Drug Sensitivity in Yeast Cells—**Drug sensitivity in yeast cells was carried out as described previously (21, 32, 35, 36). Briefly, logarithmically growing cultures of yeast cells (grown in either YPDA or synthetic complete dropout medium as indicated) was diluted to 2 × 10^6 cells/ml, and drug or MeSO was added. Aliquots were removed, diluted and plated to either YPDA plates, or to synthetic complete dropout plates. Synthetic complete dropout plates lack one nutrient required for growth of the yeast strains used, and were used to maintain selection for plasmids that complement the auxotrophy. For drug sensitivity determinations, cells growing in logarithmic cell culture having pMJ1, cells were plated to synthetic complete medium lacking uracil. Survival is expressed relative to the number of viable colonies at the time of drug addition. Drug sensitivity determinations were carried out at least three times for each strain, and representative results are shown.

**Immunoprecipitation of hTOP2 Protein from Yeast Cell Lysates—**Cell lysates for immune precipitations were prepared by vortexing cells in the presence of glass beads in ice-cold PEB (200 mM Tris-HCl, pH 8.0, 400 mM (NH₄)_2SO₄, 10 mM MgCl₂, and 10% glycerol (v/v)) containing freshly added 1 mM 4(2-aminoethyl)-benzenesulfonyl fluoride (ICN) and 1 mM diithiothreitol until about 90% of the cells were visibly lysed. The lysate was then spun in an Eppendorf microcentrifuge at 14,000 rpm for 10 min at 4 °C. The cell lysate was then treated with 20 μl of a protein A-Sepharose CL-4B slurry (prepared according to manufacturer’s instructions) for each 400 μl of cell lysate. The cell lysates containing the slurry were incubated with gentle rocking at 4 °C for 30 min, then centrifuged in a microcentrifuge for 1 min at 200 × g. The supernatants were saved, and protein concentration of the supernatants was determined using the Bio-Rad protein determination kit. Appropriate volumes of extract were treated with 10 μl of rabbit polyclonal anti-human topoisomerase II antibody (Topogen; 2.5 units/ml). Samples were incubated at 4 °C with gentle rocking for 2 h, then 100 μl of protein A-Sepharose CL-4B slurry was added to each tube and incubation was continued at 4 °C for another 2 h. The suspension was centrifuged at 200 × g for 5 min, and the pellets were washed successively in 10 μl Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, 0.025% sodium azide; 10 μl Tris-HCI, 150 mM NaCl, 0.025% sodium azide; and 50 μl Tris-HCI, pH 6.8. Finally, the pellets were resuspended in 2× Laemmli buffer (4% SDS, 20% glycerol, 100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, and 2 mg/ml bromophenol blue).

**Gel Electrophoresis and Western Blotting—**Protein samples were subjected to electrophoresis using 6% polyacrylamide gels as described previously (37, 38). Transfer to nitrocellulose membranes used standard procedures, and detection of proteins was performed using an ECL kit (Amersham Pharmacia Biotech). Rabbit polyclonal antibodies directed against yeast or human topoisomerase II were obtained from Topogen.

**Cell Cycle Arrest—**Yeast cells were synchronized in G₁, with a factor described previously (39). Briefly, an overnight culture was diluted to 5 × 10^6 cells/ml, and incubated with 20 μg/ml a factor for 3–4 h. Cells were washed twice with pre-warmed medium and resuspended at 2 × 10^6 cells/ml. Cells were then treated as described in the section describing determination of drug sensitivity.

**Analytical Ultracentrifugation—**Analytical ultracentrifugation analysis of closed clamp formation was performed as described previously by Hsieh and colleagues (40). Briefly, a 40–μl reaction mixture containing 10 mM Tris-HCI, pH 8.0, 50 mM KCl, 100 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 25 μM pC8SpRbs83 DNA (circular or linearized), and 300 nM topoisomerase II protein was incubated at 30 °C for 15 min. Some reactions also included 0.5 mM nucleoside triphosphate cofactor (ATP or AMPPNP). Experiments were carried out with either wild type yeast topoisomerase II, or with a mutant protein where the active site tyrosine (Tyr²⁶⁵) was mutated to phenylalanine. The reaction was terminated by adding a chilled mixture of 330 μl of saturated CsCl solution and 107 μl of 10 mM Tris-HCl, pH 8.0, to make the final density of the solution 1.65 g/ml. The mixture was spun at 40,000 rpm in an analytical ultracentrifuge (XL-A ultracentrifuge, Beckman Instruments) at 20 °C for 36 h before scanning the DNA concentrations across the gradient at wavelengths of 260 and 280 nm.

**RESULTS**

**Human Topoisomerase II α Is More Sensitive to Bisdioxopiperazines than Yeast Topoisomerase II—**We have been studying the effects of bisdioxopiperazines against eukaryotic...
topoisomerases. Since previous mechanistic characterization of these drugs has relied principally on yeast topoisomerase II (13), we first compared the sensitivity of purified yeast or human topoisomerase II α to bisdioxopiperazines. Nearly complete inhibition of relaxation activity was observed at 2.5 μg/ml ICRF-193 when incubated with 100 ng of human topoisomerase II α (Fig. 1). By contrast, 50–100 μg/ml ICRF-193 was required to achieve the same degree of inhibition with the purified yeast enzyme. Note that both enzymes were purified from a yeast strain lacking topoisomerase I, so that all of the relaxation activity in both enzyme preparations was due to topoisomerase II. Similar results were obtained using a decatenation assay (data not shown). These results indicate that ICRF-193 is about 20-fold more potent as an inhibitor of human topoisomerase II α compared with yeast topoisomerase II α.

**Yeast Cells Expressing Human Topoisomerase II α Are Greatly Sensitized to Bisdioxopiperazines**—We next examined the sensitivity of yeast cells expressing human top-IIα to various bisdioxopiperazines. To assess the cytotoxicity of ICRF-193 on cells depending on human topoisomerase II α for growth, JN362at2–4 cells transformed with pMJ1 were used. We have previously shown that these cells are sensitive to bisdioxopiperazines (28, 41). pMJ1 carries the entire coding sequence of human topoisomerase II α under the control of the yeast TOP1 promoter (25). Bisdioxopiperazine sensitivity was determined at 34 °C, so that the only active topoisomerase was the human enzyme. Cells were exposed to various concentrations of ICRF-193, and aliquots were removed after 8 and 24 h, diluted, and plated to determine cell viability (Fig. 2A).

Yeast cells transformed with pMJ1 were extremely sensitive to ICRF-193. ICRF-193 concentrations of 1 μg/ml were completely growth-inhibitory, while higher drug concentrations were cytotoxic. At 10 μg/ml ICRF-193, cell viability was reduced below 0.1% after 24 h of drug exposure. By contrast, we previously observed that concentrations of greater than 50 μg/ml were required to completely inhibit growth in yeast cells expressing yeast topoisomerase II α (41). These results are consistent with the enhanced in vitro activity of ICRF-193 against the human top-IIα described above.

Previous studies showed that there was only a minor dependence of cell killing by ICRF-193 on the RAD52 recombinational repair pathway (41). This is in marked contrast to complex stabilizing topoisomerase II inhibitors, where mutations in the RAD52 pathway greatly stimulate cell killing (35, 42). The isogenic rad52− strain, JN394at2–4, transformed with pMJ1 was examined for ICRF-193 sensitivity to test the role of the RAD52 pathway in sensitivity to ICRF-193. As shown in Fig. 2B, 1 μg/ml ICRF-193 inhibits growth, and cytotoxicity occurs at higher drug concentrations. The level of cell killing at higher drug concentrations in rad52− cells is very similar to that seen in RAD52− cells. As is the case of yeast cells expressing yeast topoisomerase II α, yeast cell killing mediated by human topoisomerase II α does not show a major dependence on the RAD52 repair pathway.

**Expression of Human Topoisomerase II α in Yeast Cells Confers Dominant Sensitivity to ICRF-193**—Since the yeast enzyme is much less sensitive to ICRF-193 than the human top-IIα, we had available both a drug-sensitive and a drug-resistant form of topoisomerase II. It has previously been shown that resistance to complex stabilizing drugs such as etoposide is recessive, i.e. a drug-sensitive allele of the enzyme will confer drug sensitivity regardless of the presence of a drug-resistant allele (20, 21, 43). If bisdioxopiperazines kill cells by depriving them of an essential catalytic activity, then drug sensitivity should be recessive, i.e. a bisdioxopiperazine-resistant enzyme will confer drug resistance even if a drug-sensitive enzyme is present. We tested this hypothesis by transforming JN394, a yeast strain with a wild type topoi-
Fig. 3. Expression of human top-IIα confers dominant sensitivity to ICRF-193. Yeast cells carrying the plasmid pMJ1 (wild type top-IIα under the control of the yeast TOP1 promoter) were exposed to different concentrations of ICRF-193 for the indicated times. Unlike the experiments shown in Fig. 2, the yeast strain JN394 expresses a wild type yeast topoisomerase II (as well as wild type htop-IIa). JN394 is also rad52−. As in Fig. 2, aliquots were removed, and diluted samples were plated to synthetic medium lacking uracil. Cells (relevant genotype top2−4 rad52−) were treated with no drug (open squares), 1 μg/ml ICRF-193 (open diamonds), 5 μg/ml ICRF-193 (open circles), or 10 μg/ml ICRF-193 (open triangles).

Fig. 4. TyrA805 → Phe mutant of human top-IIα does not confer ICRF-193 sensitivity. JN394 cells were transformed with plasmid pMJ1Y805F. This plasmid expresses a mutant htop-IIa, in which the active site tyrosine has been changed to phenylalanine. A shows the ICRF-193 sensitivity of JN394 cells that carry pMJ1Y805F. Cells were plated to SC-URA to confine the survival data to cells that still carried pMJ1Y805F. B shows an immunoprecipitation that compares the level of human top-IIa polypeptide in cells carrying either pMJ1 or pMJ1Y805F. Cell extracts were prepared from both strains as described under “Experimental Procedures,” and 500 μg of total protein from each extract was used for immune precipitation. Precipitates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with antibody directed against htop-IIa polypeptide.

Fig. 5. Yeast topoisomerase II does not detectably heterodimerize with human topoisomerase II. Cell extracts were prepared from JEL11- cells carrying pYX113pGALhTOP2 grown in galactose. Extracts were immunoprecipitated with antibody directed against human topoisomerase II α, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was probed with antibody directed against either htop-IIa polypeptide or yeast topoisomerase II. Lane 1, 1 mg of extract; lane 2, 2 mg of extract probed with antibody directed against htop-IIa polypeptide; lane 3, 1 mg of extract; lane 4, 2 mg of extract probed with antibody directed against yeast topoisomerase II.

A potential explanation for the dominant sensitivity conferred by the expression of human top-IIα in yeast cells also expressing wild type yeast topoisomerase II is that the two proteins heterodimerize, resulting in a holoenzyme that is either bisdioxopiperazine-sensitive or enzymatically inactive. To
test this possibility, we carried out immune precipitations with antibody directed against human top-IIα. We have previously observed that the antibody we used directed against human top-IIα minimally cross-reacts with yeast topoisomerase II (25). Therefore, if the yeast and human proteins can heterodimerize, immune precipitation with antibody directed against the human enzyme will also bring down the yeast enzyme. Immune precipitation was carried out as described under “Experimental Procedures,” and the precipitated proteins were electrophoresed in acrylamide gels. After transfer to nylon membranes, the blots were probed with antibody directed against either yeast Top2p or human top-IIα protein. As shown in Fig. 5, probing of the immunoprecipitate with anti-human top-IIα antibody detects the 170-kDa human top-IIα polypeptide, but the antibody directed against the yeast protein fails to detect a band at the expected 165-kDa position. This result argues that human and yeast topoisomerase II do not form stable heterodimers when they are co-expressed in yeast.

A second possible explanation for the observed dominant sensitivity to bisdioxopiperazines by expression of human topoisomerase II is that expression of human topoisomerase II reduces the level of yeast topoisomerase II. This could happen in at least two ways. Expression of human topoisomerase II could reduce the transcription or translation of the yeast Top2p message. Alternately, abortive heterodimerization of the human and yeast proteins could result in unstable holoenzyme that is targeted for degradation. Both possibilities would result in a reduction in the level of yeast Top2 poly peptide, leaving primarily the bisdioxopiperazine-sensitive human topoisomerase II protein. We examined this possibility using two different approaches. First, we directly compared the level of Top2 poly peptide in yeast cells expressing human top-IIα, or in cells that did not express the human protein. Extracts were prepared from the two strains and electrophoresed in acrylamide gels. After transfer to nylon membranes, the blots were probed with antibody directed against yeast Top2p (Fig. 6). The levels of Top2p were essentially indistinguishable whether human top-IIα was expressed or not. This result strongly suggests that expression of human topoisomerase II does not affect the level of yeast Top2p.

We performed an additional functional test to determine whether expression of human top-IIα affects the level of active yeast Top2p. Gasser and colleagues (45) previously reported that expression of an active site tyrosine mutant (Tyr282 → Phe) of yeast TOP2 confers resistance to etoposide. Presumably, the resistance arises from forming an inactive heterodimeric protein, and that the etoposide resistance occurs due to the reduced level of drug-sensitive enzyme. If the yeast and human proteins can heterodimerize, then expression of a catalytically inactive human top-IIα protein will reduce the etoposide sensitivity arising from yeast Top2p. However, if human Top2 does not affect the level of yeast Top2 protein, then etoposide sensitivity will be unchanged. Table I shows the etoposide sensitivity after 24 h of ICRF-193 exposure in cells expressing human Top2 (Tyr805 → Phe). Sensitivity to etoposide in cells expressing an inactive human top-IIα is identical to cells carrying a control plasmid that does not carry the hTOP2 α gene. Taken together, these results demonstrate that expression of human topoisomerase II α does not affect the level of yeast Top2p or activity. Changes in yeast topoisomerase II cannot explain the dominant sensitivity to bisdioxopiperazines that is conferred by expression of human TOP2 α.

Dominant Sensitivity Is Also Observed When Two Different Alleles of Human top2 Are Co-expressed—We recently described the isolation of a mutant in Chinese hamster ovary topoisomerase II that results in resistance to bisdioxopiperazines (28). The amino acid substitution found in the Chinese hamster ovary mutant was constructed in pMJ1, and found to confer high levels of resistance to ICRF-193 in yeast cells expressing this mutant human top-IIα. We constructed a yeast strain by transforming JN394/2–4 sequentially with pMJ1(Tyr50 → Phe) and pKN9 (wild type), where the information in parentheses indicates the allele of human top-IIα. The plasmid pMJ1 is maintained by selection in media lacking uracil, while pKN9 is selected in media lacking leucine, thus selection for both plasmids is maintained in media lacking both supplements. In addition we constructed strains that carry both pMJ1(Tyr50 → Phe) and yCPlac111 (no TOP2 gene) and pMJ1(Tyr50 → Phe) and pKN9(Tyr805 → Phe). Sensitivity to ICRF-193 was determined for all three strains. Cells were grown in synthetic medium lacking uracil and leucine, and plated to SC-Leu-Ura. The results of this experiment are shown in Fig. 7. The strain bearing both pMJ1(Tyr282 → Phe) and yCPlac111 (no TOP2 gene) also has no sensitivity to ICRF-193. Results shown above indicated that the Tyr805 → Phe mutant by itself does not confer ICRF-193 sensitivity. Furthermore, although the expression of both an active and an inactive topoisomerase II should reduce topoisomerase II activity by 50% (because of the Tyr805 → Phe:Ty/282 → Phe heterodimers, which will be inactive), the cells are still able to grow well in the presence of ICRF-193, i.e. reduction of the level of drug-resistant enzyme does not make the cells ICRF-193-sensitive. However, when the cells express both the Tyr50 → Phe top-IIα as well as the wild type enzyme, ICRF-193 causes substantial growth inhibition. The cell titer after 24 h of exposure to ICRF-193 is 10–20-fold lower than in the two control strains. Therefore, co-expression of both a wild type and bisdioxopiperazine-resistant human top-IIα results in yeast cells that are sensitive to the drug, and the sensitivity is not due to a reduction in the level of drug-resistant enzyme.

ICRF-193-mediated Cell Killing of Yeast Cells Expressing Human Topoisomerase II α Differ From Cell Kallng Arising From a Lack of Topoisomerase II Activity—If bisdioxopiperazines kill yeast cells by inhibiting the catalytic activity of human topoisomerase II α, then the kinetics and extent of cell killing by exposure to the drug should be similar to that ob-

| Drug concentration | yCP50 | pMJ1(Tyr805 → Phe) |
|--------------------|-------|-------------------|
| 20 µg/ml           | 7.47  | 7.75              |
| 80 µg/ml           | 5.17  | 3.01              |

**FIG. 6. Expression of human top-IIα does not change levels of yeast Top2p.** Cell extracts were prepared from strain JN394 carrying either yCP50 or pMJ1. Duplicate samples containing 400 µg of protein were subjected to electrophoresis, transferred to nitrocellulose membranes, and probed with either antibodies directed against either yeast topoisomerase II or human topoisomerase II α as indicated on the figure.
served when cells carrying a temperature-sensitive top2 mutation are grown at a non-permissive temperature. To test this, we compared cell killing of cells expressing human top-IIα when exposed to 25 μg/ml ICRF-193 with cell killing that occurred with cells carrying the top2–4 allele. JN394t2–4 cells were transformed with either pMJ1 or yCP50. Both strains were pre-grown at 25 °C. At the start of the experiment, both strains were shifted to 34 °C, and ICRF-193 was added to cells carrying pMJ1. Thus, both strains were exposed to an identical heat shock. Growth of the cells was in synthetic medium lacking uracil to maintain plasmids. At various times, aliquots were removed and plated to SC-URA plates to determine viable titer. The results are shown in Fig. 8. Cell killing of pMJ1-transformed JN394t2–4 cells in the presence of 25 μg/ml ICRF-193 was much faster and to a higher level, than yCP50-transformed JN394t2–4 cells exposed to a temperature where the endogenous yeast top2 mutant is inactive. These results provide further support for the notion that bisdioxopiperazines do not kill yeast cells expressing human top-IIα solely through depriving the cells of an essential enzyme activity.

G1 Cell Cycle Arrest or Inhibition of DNA Replication Does Not Prevent ICRF-193 Cytotoxicity in Yeast Cells Expressing Human Topoisomerase II—Since killing by a lack of topoisomerase II occurs specifically at mitosis (16, 46), and since ICRF-193-treated cells lose viability more quickly than cells that carry a temperature-sensitive allele incubated at the non-permissive temperature, it seemed likely that the timing of cell killing differed from that occurring due to a lack of topoisomerase II activity. Previous studies indicated that arrest with α factor, which blocks cells in G1, protected cells from cytotoxicity due to topoisomerase II poisons, while DNA replication inhibitors provided only partial protection (39). Thus, an examination of cell killing by ICRF-193 in α factor-arrested cells or in cells treated with replication inhibitors should clearly distinguish between topoisomerase II poisons and topoisomerase II catalytic inhibitors.

Fig. 9 illustrates the effects of α factor arrest and treatment with hydroxyurea on cells expressing human top-IIα exposed to ICRF-193. In this experiment, cells were synchronized with α factor, as described under “Experimental Procedures,” and then either maintained in α factor or released from α factor arrest. For some samples released from α factor arrest, hydroxyurea, a ribonucleotide reductase inhibitor, was added. Unlike the results obtained with a topoisomerase II poison, α factor arrest did not protect cells appreciably from ICRF-193-mediated cell killing. The degree of cell killing was the same whether α factor was present or not. Treatment with hydroxyurea slightly increased cell killing by ICRF-193.

Because neither α factor arrest nor hydroxyurea treatment blocked cell killing, we considered the possibility that the ICRF-193 was incompletely washed out of the cells prior to plating. Although, this possibility could not be rigorously excluded, we tested this possibility by synchronizing cells with α factor, treating cells with ICRF-193 plus α factor for 3 h, then washing the cells again, and resuspending the cells in medium with α factor but no ICRF-193. Cells were incubated with only α factor for another 3 h, then the cells were washed and plated. This additional 3-h drug washout did not change cell survival, compared with cells that did not have the 3-h drug-free incubation prior to plating (data not shown). Taken together, these results indicate that cells arrested in either G1 or S phase can still be killed by ICRF-193, a result distinct from that expected...
for either a topoisomerase II poison or inhibition of topoisomerase II activity.

Cleavage with Human top-IIa and ICRF-193—The results described in the previous section differ from results we have previously obtained for the effect of cell cycle inhibitors on a drug that stabilizes a covalent complex. Furthermore, topoisomerase II covalent complexes in cells have not been observed in cells treated with bisdioxopiperazines (4). Nonetheless, we directly examined covalent complex formation with ICRF-193 with purified human topoisomerase II α using a K+/SDS assay. The results, shown in Fig. 10, indicate a slight increase in covalent complex formation with human topoisomerase II and ICRF-193. The increase in cleavage at the highest concentration tested, 100 μg/ml, is statistically above cleavage in the absence of drug. Induction of covalent complexes, however, is very weak compared with canonical topoisomerase II poisons. For comparison, a doubling of the level of covalent complex with etoposide is seen at 0.3 μM etoposide (data not shown). Comparing the drug concentration required to achieve a doubling of DNA cleavage, etoposide is about 750 times more potent than ICRF-193. Since the concentration required to kill yeast cells expressing human topoisomerase II is much less than the concentration required to significantly increase covalent complex formation, DNA cleavage stimulated by ICRF-193 is unlikely to be responsible for the dominant cell killing of yeast cells expressing human topoisomerase II.

Top2p Does Not Require the Ability to Cleave DNA to Form a Stable Closed Clamp in the Presence of ICRF-193—Lindsley and colleagues have recently demonstrated that mutation of the tyrosine involved in forming a covalent linkage with DNA is still able to form a stable closed clamp in the presence of a non-hydrolyzable ATP analog (47). Since mutating the homologous amino acid in human Top2α results in a protein that does not confer sensitivity to bisdioxopiperazines, we were interested in determining whether these compounds can stabilize a closed clamp formed by an active site tyrosine mutant. For these experiments, an active site mutant of yeast Top2 was used (47). Closed clamp formation was monitored using analytical ultracentrifugation as described by Hsieh and colleagues (40, 48). In this assay, Top2p is incubated with DNA and a non-hydrolyzable ATP analog (47). Since mutating the homologous amino acid in human TOP2α (48) and indicating that ICRF-193 can stabilize a salt-stable complex by Top2p even if the enzyme cannot cleave DNA. Similar results were also obtained using the filter binding assay described by Roca and Wang (13). In addition, a salt-stable complex was not observed with circular DNA in the presence of ICRF-193 in the absence of ATP (data not shown). Taken together, our results indicate that Top2p does not require the ability to cleave DNA to form a salt-stable complex, but that the ability to cleave DNA is required for bisdioxopiperazines to induce cytotoxicity in yeast (Fig. 4A). Therefore, a closed clamp appears necessary, but not sufficient for bisdioxopiperazine-induced cytotoxicity.

DISCUSSION

Previous studies have indicated that bisdioxopiperazines are specific inhibitors of topoisomerase II (4, 41). Several lines of evidence suggested that these compounds are catalytic inhibitors of topoisomerase II rather than agents that stabilize topoisomerase II covalent complexes, including a lack of dependence on the RAD52 pathway for cell killing in yeast (41), and phenotypes in mammalian cells that are consistent with a failure to decatenate replicated chromosomes (4). In addition, it has also been shown that bisdioxopiperazines can suppress the formation of covalent complexes by topoisomerase II poisons such as etoposide (49). This work demonstrates that there is an additional aspect to cell killing by bisdioxopiperazines, i.e. the potential to act as a novel type of topoisomerase II poison.
Wang and colleagues (13) have shown that bisdioxopiperazines trap topoisomerase II at a unique intermediate form, where the enzyme has formed a closed clamp. The topological state of the enzyme is the same as when the enzyme turnover is inhibited by non-hydrolyzable ATP analogs (11, 50, 51). Under both of these conditions, the enzyme and covalently closed circular DNA forms a catenane, with one loop comprising DNA and one loop comprising protein. We suggest that topoisomerase II in this state acts as a DNA lesion, which could inhibit DNA metabolic processes such as replication, transcription, or chromatin assembly or disassembly.

The hypothesis that the closed clamp form of topoisomerase II can act as a type of DNA “lesion” is based on the demonstration in this work that bisdioxopiperazines are able to kill yeast cells even in the presence of a bisdioxopiperazine-resistant topoisomerase II. A drug that kills cells because of a lack of topoisomerase II should confer recessive drug sensitivity, i.e., a drug-resistant topoisomerase II confers drug sensitivity regardless of whether a drug-sensitive form of the enzyme is present (20, 43).

Interpretation of dominant drug sensitivity is complicated by the fact that topoisomerase II is a stable dimer (52). Co-expression of a drug-sensitive and a drug-resistant enzyme can result in heterodimers of one sensitive and one resistant subunit. Under these circumstances, the drug sensitivity could depend on the drug sensitivity of the heterodimer. However, we have shown that potential heterodimers are not relevant to the dominant drug sensitivity conferred by bisdioxopiperazines. In experiments where active human and yeast enzymes are expressed, we were unable to detect heterodimers with both yeast and human monomers. In experiments where different alleles of human top-IIα are expressed, we showed that the drug sensitivity of the heterodimer is irrelevant if bisdioxopiperazines act only by depriving cells of topoisomerase II activity. We showed that co-expression of a bisdioxopiperazine-resistant human top-IIα along with an active site tyrosine mutant still results in yeast cells that are very resistant to ICRF-193. If
these two subunits can freely heterodimerize, then there will be a 50% reduction in active topoisomerase II. Expression of both the active site tyrosine mutant and the Tyr50 → Phe mutant resulted in cells that were completely resistant to ICRF-193. However, expression of wild type human top-IIα along with the Tyr50 → Phe mutant reduces cell growth below that observed with the active site mutant. The level of Tyr50 → Phe heterodimers will be the same in both experiments. Nonetheless, only expression of wild type drug-sensitive topoisomerase II reduced cell growth. Therefore, the wild type:wild type homodimers and/or the wild type:Y50 → Phe heterodimers confer drug sensitivity, i.e., dominant drug sensitivity.

The level of drug sensitivity conferred by wild type human topoisomerase II when the drug-resistant topoisomerase is a human enzyme is considerably less than what was observed when the drug-resistant enzyme is yeast topoisomerase II. We suggest that this is due to the level of drug-sensitive enzyme present in the two circumstances. Because the two forms of the human enzyme can heterodimerize, while the yeast and human forms cannot, expression of drug-resistant human top-II can reduce the level of drug-sensitive enzyme by 50%, provided that the heterodimers are fully drug-resistant. In agreement with this hypothesis, van Hille and Hill (53) have shown that expression of high levels of wild type human top-II in yeast result in higher levels of bisdioxopiperazine sensitivity than lower levels of the human enzyme.

If bisdioxopiperazines act as topoisomerase poisons, then one could hypothesize that these drugs are complex stabilizing topoisomerase II poisons. We detected a small increase in covalent complex formation with purified human topoisomerase II and ICRF-193. However, the potency of covalent complex formation is inconsistent with the cytotoxicity observed. It is probably not surprising that a small increase in covalent complex-stabilizing drugs such as etoposide or amrasarcine (35, 36). The experiments using agents that block cell cycle progression also demonstrated a phenotype distinct from what has been previously observed with complex stabilizing topoisomerase II poisons.

An alternate explanation that would be consistent with bisdioxopiperazines acting as topoisomerase II poisons is that the formation of stable covalent complexes by human Top2p (in yeast) is more cytotoxic than stabilized covalent complexes formed with yeast Top2p. One way this could happen is if Top2p:DNA complexes are relatively efficient substrates for repair processes, while the human enzyme is not efficiently recognized. We do not favor this possibility, because we have previously shown that yeast cells expressing human Top2p are not more sensitive to complex-stabilizing drugs such as etoposide than cells expressing yeast Top2 (25).

How might bisdioxopiperazines kill cells? Although these drugs do not lead to high levels of covalent complexes between topoisomerase II and DNA, the closed clamp form of the enzyme might impede DNA metabolic events. If topoisomerase II cannot freely slide along DNA when trapped as a closed clamp, then it might block transcriptional elongation, DNA replication, repair, and other DNA metabolic events. Although we do not think that bisdioxopiperazines act as complex stabilizing topoisomerase II poisons, our results indicate that the enzyme must be able to cleave DNA in order to act as a poison. A possible interpretation is that the ability of Top2p to cleave DNA impedes the ability of the closed clamp form of the enzyme to slide along DNA. Perhaps an enzyme that cannot cleave DNA freely slides along DNA as a washer on a string, and does not impede other DNA metabolic events.

The ability of ICRF-193 and other bisdioxopiperazines to kill cells by converting topoisomerase II into a “non-covalent poison” probably depends on the level of topoisomerase II that is expressed. van Hille and Hill (53) showed that the level of expression of hTop2p in yeast is proportional to the degree of cell killing induced by bisdioxopiperazines. Whether mammalian cells are strongly affected may depend on the their level of expression of different topoisomerase II isoforms. In this regard, it will be important to quantitate the sensitivity of topoisomerase II β to bisdioxopiperazines, since this isoform is expressed in both proliferating cells and quiescent cells (54).

Finally, the results presented here suggest that caution is needed in the use of bisdioxopiperazines as probes of topoisomerase function in eukaryotic cells. It is well established that topoisomerase II poisons such as etoposide can exert effects on cells due to the generation of covalent complexes. The effect of etoposide on specific cell processes may not be due to a requirement for topoisomerase II in that process. Results presented here suggest that a trapped non-covalent complex of topoisomerase II on DNA may also produce effects on cells that are not specifically due to a normal involvement of topoisomerase II. Nonetheless, because bisdioxopiperazines are in clinical use as cardioprotectants, it will be important to understand the effects of this novel type of poisoning of topoisomerase II.

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