Dissecting the Cell-killing Mechanism of the Topoisomerase II-targeting Drug ICRF-193*

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Topoisomerase II is an essential enzyme that is targeted by a number of clinically valuable anticancer drugs. One class referred to as topoisomerase II poisons works by increasing the cellular level of topoisomerase II-mediated DNA breaks, resulting in apoptosis. Another class of topoisomerase II-directed drugs, the bis-dioxopiperazines, stabilizes the conformation of the enzyme where it attains an inactive salt-stable closed clamp structure. Bis-dioxopiperazines, similar to topoisomerase II poisons, induce cell killing, but the underlying mechanism is presently unclear. In this study, we use three different biochemically well characterized human topoisomerase IIα mutant enzymes to dissect the catalytic requirements needed for the enzyme to cause dominant sensitivity in yeast to the bis-dioxopiperezine ICRF-193 and the topoisomerase II poison m-AMSA. We find that the clamp-closing activity, the DNA cleavage activity, and even both activities together are insufficient for topoisomerase II to cause dominant sensitivity to ICRF-193 in yeast. Rather, the strand passage event per se is an absolute requirement, most probably because this involves a simultaneous interaction of the enzyme with two DNA segments. Furthermore, we show that the ability of human topoisomerase IIα to cause dominant sensitivity to m-AMSA in yeast does not depend on clamp closure or strand passage but is directly related to the capability of the enzyme to respond to m-AMSA with increased DNA cleavage complex formation.

DNA topoisomerase II is an abundant enzyme able to regulate DNA topology by making transient double strand breaks in the DNA helix. It fulfills an essential function in chromosome segregation (1) and is moreover the primary target for many cancer chemotherapeutics and antibiotics. The dimeric topoisomerase II mediates topological changes in DNA by introducing a transient gate in one DNA duplex, the G-segment, whereas another duplex, the T-segment, is transported coordinately through the gated DNA. The topoisomerase II-mediated DNA cleavage reaction involves a transesterification between a pair of phosphodiester bonds in the DNA backbone and the phenolic oxygens from the active site tyrosine of each monomer identified as Tyr-805 in human topoisomerase IIα (2). Besides DNA cleavage, a change in DNA topology catalyzed by topoisomerase II also requires a strand passage step. This is initiated by the closure of an ATP-operated clamp composed of the N-terminal region from each subunit. Upon clamp closure, the T-segment is trapped and transported concomitantly through the cleaved G-segment (3–6). The subsequent ligation of the G-segment fulfills the topoisomerase II-mediated topological change in DNA, leaving the DNA strands intact.

One class of chemotherapeutic agents targeting topoisomerase II, the topoisomerase II poisons, are believed to act primarily by enhancing the concentration of topoisomerase II-mediated double strand breaks in cellular DNA. In this way, the topoisomerase becomes a cellular toxin that eventually will trigger apoptosis (reviewed in Fortune and Osheroff (7)). m-AMSA begins to belong to this class of topoisomerase II-directed drugs and is believed to stimulate the cleavage complex formation by distorting the DNA helix structure.

Another class of topoisomerase II-directed drugs that has been given much attention recently is the bis-dioxopiperazine including ICRF-193. These drugs inhibit topoisomerase II activity by stabilizing the closed clamp form of topoisomerase II and thus generate a non-covalent topological complex between topoisomerase II and DNA (8). It has been anticipated that the lethal effect of ICRF is caused by a lack of topoisomerase II activity during the cell cycle (9). However, several recent studies have questioned the mechanism of action of ICRF (10–15). An ICRF-mediated increase in DNA strand breaks has been observed both in vivo (16) and in vitro (17). Furthermore, the expression of human topoisomerase IIα in yeast confers dominant sensitivity to ICRF in favor of an active poisoning role of topoisomerase II in the cell-killing mechanism of ICRF (15). Finally, it has been demonstrated that cells deficient in non-homologous end joining are hypersensitive to ICRF and to the topoisomerase II poison, etoposide, also suggesting the involvement of DNA strand breaks in the ICRF cell-killing mechanism (12, 18).

In this study, we have used three human topoisomerase IIα mutant enzymes to determine the catalytic steps/activities of human topoisomerase IIα through which ICRF and m-AMSA exert cell killing: (i) a mutant enzyme unable to perform DNA strand passage but capable of both clamp closure and DNA cleavage (19); (ii) a mutant enzyme unable to close the clamp and perform strand passage but still capable of DNA cleavage (20); and (iii) a topoisomerase II enzyme defective in DNA cleavage and strand passage but able to close the N-terminal clamp (2).

To investigate the poisoning mechanism of ICRF in combination with human topoisomerase IIα, the ICRF sensitivity of yeast transformants expressing the wild type or one of the different human topoisomerase IIα mutant enzymes was as-

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The abbreviations used are: m-AMSA, 4[prime]-(9-acridinylamino)-methanesulfon-m-aniside; ICRF-193, meso-2,3-bis[2,6-dioxopiperazin-4-y]butane.
sayed. To obtain further knowledge of the mechanism of action of topoisomerase II poisons, the sensitivity of the yeast transformants to m-AMSA also was tested. Moreover, we purified the wild type and mutant enzymes to investigate their response to ICRF-193 and m-AMSA in vitro. Taken together, our results showed that the clamp-closing activity and the DNA cleavage activity as well as both activities together are insufficient for topoisomerase II to cause sensitivity to ICRF-193 in yeast. Rather, the DNA strand passage reaction of the enzyme per se is essentially. In other words, we found that the ability of human topoisomerase IIα to cause dominant sensitivity to m-AMSA in yeast is independent of clamp closure and strand passage. In this case, the sensitivity is related directly to the capability of human topoisomerase IIα to respond to m-AMSA with an increased DNA cleavage complex formation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The Saccharomyces cerevisiae strains BJ201 (Mata ura3 trpl pep4::HIS3 prb1 can1 top2::TRP1) and JEL1Δtop1 (kindly provided by J. C. Wang) were used for complementation analysis and overexpression of topoisomerase II, respectively. Yeast strain YT2409 (BY7472; Mata; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YOR153w::kanMX4) purchased from EUROSCARF, was used for the measurements of drug sensitivity in yeast. Plasmid pBY105 contains the yeast triosephosphate isomerase promoter inserted into the polylinker region of the LEU2/ARS-CEN plasmid pRS315. pRS315 was used as the backbone for pHT300, pHT213, pHT351i (pHT350-2), and polylinker region of the LEU2/ARS-CEN plasmid pRS315. pRS315 was used as a positive control.

Yeast Transformation and Complementation—Yeast cells were transformed by using a modified version of the LiAc method of Ito et al. (21). To test the ability of 351i, 408i, and Y805S to complement the lack of endogenous topoisomerase II in BJ201, the LEU2-based constructs pHT351i, pHT408i, and pHT213 were transformed into BJ201 and the cells were transferred to medium plates containing 5-fluoroorotic acid (1 mg/ml) to select against the URA3 plasmid carrying the Schizosaccharomyces pombe top2* gene (2). pHT300 was used as a positive control.

Measurements of Drug Sensitivity in Yeast—The measurements of drug sensitivity were carried out as described by Nitiss et al. (2). A logarithmically growing culture of yeast cells, YT2409, grown in synthetic dropout medium was diluted to 2 × 10⁸ cells/ml (A₀.1), and drug or Me₅SO (0.8% final) was added. The aliquots were plated to synthetic dropout plates at the indicated time points after proper dilution.

Analysis of Protein Expression—The pellet from 2 ml of yeast culture in log phase was lysed, and the lysate was trichloroacetic acid-precipitated. The precipitate was resuspended in SDS-loading buffer (100 mM Tris-HCl, 1% SDS, 140 mM β-mercaptoethanol, 10% glycerol, 0.05% bromophenol blue) and subjected to electrophoresis on an SDS-polyacrylamide gel analysis. Covalent complex formation was revealed by the transfer of the enzyme-DNA catenanes, phenol extraction was performed by adding 1 ml of 800 mM NaCl solution. Upon the removal of the water phase and phenol/water interphase were washed once in 4 M NaCl and once in 0.8 M NaCl solution. Upon the removal of the water phase after the last wash, the remaining material was ethanol precipitated and dissolved in 10 µl of TE buffer containing 1 mg/ml proteinase K. The samples were subjected next to electrophoresis in a 1% agarose gel in TBE (100 mM Tris borate, pH 8.3, 2 mM EDTA) containing 1 mM ethidium bromide, and DNA was visualized by UV light.

Topoisomerase II-mediated DNA Cleavage—Topoisomerase II-mediated DNA cleavage of oligonucleotides was performed by incubating the human topoisomerase II and 1 nM of labeled DNA substrate in a total volume of 20 µl of buffer (2.5 mM MgCl₂, 2.5 mM CaCl₂, 10 mM Tris-HCl, pH 7, 0.1 mM EDTA, 112.5 mM KCl). The substrate was a pBR322-derived 40-mer duplex containing a strong topoisomerase II recognition sequence. The top strand having the sequence 5'-TGC ATG AGG ATG —3' was labeled at the 3'-end with Sequenase (United States Biochemical Corp.). When indicated, the reactions contained 100 µM m-AMSA (Sigma) or 85 µM ICRF-193 in 0.5% Me₅SO. Samples were incubated at 37°C for 10 min, cleavage products were trapped by the addition of SDS to 1%, and samples were subjected subsequently to SDS-polyacrylamide gel analysis. Covalent complex formation was revealed by the transfer of the radiallyabeled oligonucleotide to the topoisomerase polypeptide. Reaction products were visualized and quantified using a Molecular Imager (Bio-Rad). Topoisomerase II-mediated cleavage of circular plasmid DNA was performed by incubating 100 nM topoisomerase II and 6.5 nM of negatively supercoiled pUC19 DNA in a total volume of 20 µl of buffer (50 mM Tris-HCl, pH 8, 140 mM KCl, 1 mM EDTA, 8 mM MgCl₂). The samples were incubated at 37°C for 7 min, and the cleavage products were trapped by the addition of SDS to 1%. After proteinase K digestion (0.8 mg/ml), the samples were subjected to electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris acetate, pH 8.3, 2 mM EDTA) containing ethidium bromide (1 µg/ml). When topoisomerase II-mediated cleavage was carried out in the presence of m-AMSA or ICRF-193, the concentration of the drug was 50 µM in a final concentration of 0.5% Me₅SO.

RESULTS

351i, 408i, and Y805S Are Useful in Defining the Cell-killing Mechanism behind ICRF-193—To investigate the cell-killing mechanism behind ICRF-193, we have taken advantage of the yeast strain JEL1-nitriloacetic acid, in which the active site tyrosine has been substituted with a serine (Y805S), a human topoisomerase II which is able to close the N-terminal clamp. The position of the different enzymes is shown in Fig. 1A. The cDNA encoding 351i, 408i, and Y805S, and wild-type human topoisomerase IIα have been inserted in pRS315 behind a constitutive TPI promoter, generating the plasmids pHT351i, pHT408i, and pHT300, respectively. See Table I for a schematic description of the enzymes expressed from the different plasmids.

To confirm the catalytic incapabilities of the enzymes, complementation analyses were performed using an S. cerevisiae top2 deletion strain. As expected, 351i, 408i, and Y805S all failed to sustain the mitotic growth of the yeast strain in contrast to wild-type human topoisomerase IIα (Fig. 1B). 351i, 408i, and Y805S Do Not Induce Dominant Sensitivity toward ICRF-193 in Yeast—ICRF-193 is a topoisomerase IIα-
in Fig. 2A, both mutant and wild-type enzymes were expressed in the Y12409 yeast cells, although Y805S was expressed consistently at a lower level.

To assess the cytotoxicity of ICRF-193 in the different transformants, the yeast cells were incubated either in the absence or presence of 85 μM ICRF-193. Aliquots were removed after 4 and 8 h and plated to determine cell survival after proper dilution.

The results confirmed that the expression of wild-type human topoisomerase IIα in yeast confers sensitivity to ICRF-193, because the drug induced severe cell killing (Fig. 2B). Furthermore, the cleavage-deficient enzyme did not confer ICRF sensitivity as expected (11). Yeast cells expressing the clamp-deficient 408i enzyme displayed little or no sensitivity to ICRF-193, showing that the cleavage activity is insufficient to induce ICRF cytotoxicity. The strand passage-deficient human topoisomerase IIα enzyme, 351i, which retains both clamp closure and DNA cleavage activity also induced little or no ICRF cytotoxicity (Fig. 2B). Thus, neither DNA cleavage, clamp closure, nor both activities together are sufficient for ICRF-193 to mediate cell killing via topoisomerase II. Our results strongly suggest that strand transport, which is blocked in all three mutant enzymes, is an absolute requirement for ICRF-mediated cell killing.

ICRF-193 Induces Stable Clamp Formation in Wild-type Topoisomerase IIα, 351i, and Y805S but Not in 408i—To investigate whether the inability of ICRF-193 to cause cytotoxicity in yeast cells expressing 351i, 408i, or Y805S was because of a lack of drug-mediated clamp stabilization with these enzymes, we tested the ability of ICRF-193 to stabilize the closed clamp conformation in the mutant topoisomerase IIα enzymes in vitro. For this purpose, the different enzymes fused to a hexahistidine tail at the C-terminal end were overexpressed in a yeast top2 null strain and purified as described under “Experimental Procedures.”

To test the effect of ICRF-193 in vitro, wild-type and mutant enzymes were preincubated with supercoiled plasmid DNA and ATP before the addition of ICRF-193. The reactions were stopped by adding high concentrations of salt, and the samples were subjected to a clamp-closing analysis modified from Bjergbaek et al. (24). In contrast to 408i, the wild-type enzyme as well as 351i and Y805S caught the circular plasmid DNA in the presence of ATP and ICRF-193 (Fig. 3), in accordance with the previously reported clamp-closing capabilities of the enzymes (11, 19, 20, 23). Thus, the inability of ICRF-193 to cause cytotoxicity in vivo in yeast cells expressing 351i and Y805S does not correlate with an inability of ICRF-193 to stabilize the closed clamp complex in 351i and Y805S. The results further revealed that the general clamp-closing defect of 408i also abolishes the ability of ICRF-193 to induce a salt-stable topological complex between this enzyme and DNA. Also, the results confirm that 351i, 408i, and Y805S are unable to perform the relaxation of supercoiled DNA, because the occurrence of relaxed DNA is only seen when clamp closure is performed in the presence of wild-type topoisomerase IIα.

m-AMSA but Not ICRF-193 Increases the DNA Cleavage Level Obtained with 351i, 408i, and the Wild-type Topoisomerase IIα Enzyme—Traditional topoisomerase II poisons, including m-AMSA, increase the concentration of topoisomerase II-mediated DNA breaks (reviewed by Fortune and Osheroff (7)). Some studies also have reported an ICRF-induced increase in topoisomerase II-mediated DNA cleavage complex formation (17). To test whether the different mutant enzymes as well as wild-type human topoisomerase IIα can respond to m-AMSA and ICRF-193 with increased enzyme-DNA cleavage complex formation, DNA cleavage reactions were performed in the ab-

![Fig. 1. 351i, 408i, and Y805S fail to complement growth in a yeast top2 deletion strain. A, schematic representation of human topoisomerase IIα. The numbers indicate amino acid positions. 351i and 408i each have an insertion of two amino acids at position 351 and 408, respectively. In the cleavage-deficient human topoisomerase IIα enzyme, Y805S, the active site tyrosine (Y) at position 805 has been substituted with a serine. The ATP-binding subdomain is shown in white, the core region is in dark gray, and the variable C-terminal region is in black. The transducer domain, which is shown in light gray, is bridging the two catalytic entities. B, a single copy ARS/CEN plasmid carrying 351i (pHT351i), 408i (pHT408i), Y805S (pHT213), or the wild-type human topoisomerase IIα (pHT300) cDNA behind a TPI promoter was transformed into the yeast strain BJ201. As a control, the cells were transformed with the empty vector pRS315. In BJ201, the chromosomal TOP2 gene has been disrupted by the insertion of the structural TRPI gene, whereas the essential topoisomerase II activity is provided by the S. pombe top2+ gene carried on a single copy URA3-based plasmid. After transformation, cells were grown on medium containing 5-氟-2-尿酸 to counterselect against the URA3 plasmid. Directed drug, which stabilizes topoisomerase II in its closed clamp form (8), and until recently, it was anticipated that the cytotoxicity of ICRF compounds simply was attributed to an inhibition of the essential topoisomerase II activity. However, the expression of wild-type human topoisomerase IIα in yeast cells has been demonstrated to confer dominant sensitivity to ICRF, suggesting that the drug acts as a topoisomerase II poison (15). In contrast, the expression of a cleavage-deficient 408i enzyme displayed little or no sensitivity to ICRF-193, because the drug induced severe cell killing (Fig. 2B).

To test which of the enzymatic capabilities of human topoisomerase IIα are important for the enzyme to cause dominant sensitivity to ICRF-193 in yeast cells, we expressed 351i, 408i, Y805S, or wild-type human topoisomerase IIα in the yeast strain Y12409. As a control, the cells were transformed with the empty vector pRS315. To verify the expression of the human topoisomerase IIα enzymes, the Western blot analysis of cell lysates from all of the yeast transformants was carried out using an antibody specific to human topoisomerase IIα.
sence or presence of 100 \mu M m-AMSA or 85 \mu M ICRF-193 using a 3’-end-labeled DNA substrate. Reactions were stopped with SDS and were subjected to electrophoresis in an 8% SDS gel. Cleavage complexes were identified because of the covalent linkage of labeled DNA after DNA cleavage.

As seen in Fig. 4A, upper panel, m-AMSA dramatically increases the DNA cleavage level obtained with the wild-type enzyme as well as that obtained with 351i and 408i. Y805S is unable to cleave DNA to a detectable level even in the presence of drug. Thus, m-AMSA is able to mediate its stimulatory effect on the three cleavage-competent enzymes irrespective of their overall catalytic activity. In contrast, the induction in DNA cleavage complex formation obtained with ICRF-193 is insignificant with all of the enzymes under the reaction condition used in this study (Fig. 4A, lower panel).

To investigate whether ICRF-193 stimulates topoisomerase II-mediated DNA cleavage on plasmid DNA containing multiple DNA cleavage sites, topoisomerase II was incubated with circular plasmid DNA in the presence of 50 \mu M ICRF-193, 50 \mu M m-AMSA, or 0.5% Me\textsubscript{2}SO. Reactions were stopped by the addition of SDS to 1%, and topoisomerase II-mediated double strand cleavage was revealed as linearization of the plasmid DNA after proteinase K digestion (Fig. 4B, upper panel).

As expected, the DNA cleavage activity of wild-type human topoisomerase II as well as that of 351i and 408i is stimulated by m-AMSA. However, even using a substrate containing multiple topoisomerase II recognition sites, neither of the enzymes shows an increased DNA cleavage level in the presence of ICRF-193 under the present conditions (Fig. 4B, lower panel).

The DNA Cleavage-competent Enzymes, 351i and 408i, Both Confer Sensitivity to m-AMSA in Vivo—The cytotoxic effect of m-AMSA is believed to result directly from an increase in the cellular level of DNA double strand breaks, but it has not been investigated whether topoisomerase activity is in fact required for m-AMSA to exert its effect in vivo.

### Table I

| Plasmid     | Enzyme             | Relaxation | DNA cleavage | Clamp closure |
|-------------|--------------------|------------|--------------|---------------|
| pHT300     | Wild-type           | Yes        | Yes          | Yes           |
| pHT213     | Y805S              | No         | No           | Yes           |
| pHT351i    | 351i               | No         | Yes          | Yes           |
| pHT408i    | 408i               | No         | Yes          | No            |
| pRS315     |                    | No         |              |               |

FIG. 2. Expression of 351i, 408i, or Y805S in yeast does not cause increased sensitivity to ICRF-193. A, Western blot analysis of human topoisomerase II expression in extracts from the yeast strain Y12409 transformed with pHT300, pHT351i, pHT408i, and pHT213 expressing the wild-type enzyme, 351i, 408i, and Y805S, respectively, or the empty vector, pRS315. C, purified human topoisomerase II loaded as a control. B, ICRF-193 sensitivity of yeast cells expressing mutant or wild-type human topoisomerase II. Cell survival is plotted as number of cells surviving after 4 and 8 h of ICRF-193 treatment relative to the number of cells at time point zero. Cells were grown in the absence of drug (open circles, pHT300; open triangles, pHT351i; open diamonds, pHT408i; times sign, pHT213; open squares pRS315) or in the presence of 85 \mu M ICRF-193 (closed circles, pHT300; closed triangles, pHT351i; closed diamonds, pHT408i; asterisk, pHT213; closed squares, pRS315).

FIG. 3. 351i, Y805S, and wild-type human topoisomerase II stably close the N-terminal clamp when treated with ICRF-193 \textit{in vitro}. To investigate clamp closure, topoisomerase II and DNA were preincubated for 5 min in the presence of ATP. ICRF-193 then was added to half of the sample and after further incubation for 5 min, the reactions were stopped by the addition of NaCl to 800 mM. The samples were phenol-extracted, and the waterphase was removed and ethanol-precipitated, whereas the remaining phenol/water interphase was washed in high salt before the material was ethanol-precipitated and proteinase K-digested. The samples were subjected to electrophoresis in a 1% agarose gel containing ethidium bromide. Enzyme, nucleotide, and drug used in the samples are indicated above the gel. w and i denote waterphase and interphase, respectively. The positions of supercoiled (SC), dimeric (D), and relaxed (RC) circular plasmid DNA are indicated. C, DNA control; wt, wild-type.
To test this possibility, we have compared the m-AMSA sensitivity of yeast cells expressing 351i or 408i, which are able to cleave DNA in vitro but unable to perform strand passage, to the sensitivity of cells expressing either Y805S, which is incapable of both DNA cleavage and strand passage, or the wild-type enzyme, which is capable of both activities. Yeast cells transformed with the empty vector, pRS315, were used to reveal the sensitivity of the yeast strain expressing only yeast topoisomerase II. The cytotoxicity of m-AMSA in the different transformants was assessed by incubating yeast cells either in the absence or presence of 100 μM m-AMSA. Aliquots were removed after 4 and 8 h and plated to determine cell survival after proper dilution.

The results show that yeast cells expressing either one of the cleavage-competent enzymes, 351i or 408i, or the wild-type enzyme are sensitive to m-AMSA, whereas cells expressing the cleavage-deficient Y805S enzyme are as insensitive as those harboring the empty vector (Fig. 5). These observations demonstrated that the cytotoxicity of m-AMSA is independent of topoisomerase II activity and solely results from the formation of topoisomerase II-DNA cleavage complexes. Furthermore, our data revealed that 351i and 408i, besides being expressed, are capable of mediating DNA cleavage in the yeast cells, in agreement with their in vitro abilities (19, 20).

**DISCUSSION**

Recently, several studies have focused on the topoisomerase II-directed ICRF compounds, and accumulating evidence that these drugs work as a type of topoisomerase II poison has been presented (10–15). However, it is presently not clear how the ICRF compounds exert their poisoning action.

In this study, we have taken advantage of three biochemically well characterized human topoisomerase IIα enzymes to test this possibility, we have compared the m-AMSA sensitivity of yeast cells expressing 351i or 408i, which are able to cleave DNA in vitro but unable to perform strand passage, to the sensitivity of cells expressing either Y805S, which is incapable of both DNA cleavage and strand passage, or the wild-type enzyme, which is capable of both activities. Yeast cells transformed with the empty vector, pRS315, were used to reveal the sensitivity of the yeast strain expressing only yeast topoisomerase II. The cytotoxicity of m-AMSA in the different transformants was assessed by incubating yeast cells either in the absence or presence of 100 μM m-AMSA. Aliquots were removed after 4 and 8 h and plated to determine cell survival after proper dilution.

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**FIG. 4.** m-AMSA but not ICRF-193 increases the DNA cleavage complex formation with wild-type human topoisomerase IIα, 351i, and 408i. A, upper panel, the effect of m-AMSA and ICRF-193 on DNA cleavage complex formation was tested by incubating human topoisomerase IIα with a 3'-end-labeled 40-mer DNA substrate in the absence (MeSO, DMSO) or presence of either m-AMSA (100 μM) or ICRF-193 (85 μM). Reactions were stopped by the addition of SDS to 1% and subjected subsequently to electrophoresis in an 8% SDS gel. CI, cleavage complexes; S, substrate; C, DNA control. Lower panel, histogram of the DNA cleavage experiment presented in the upper panel. A Molecular Imager was used for quantification of the labeled cleavage complexes obtained with wild-type human topoisomerase IIα, 351i, and 408i. The level of DNA cleavage in the absence of drug for each enzyme is set to one. Results are the means ± S.D. of three independent experiments. B, upper panel, DNA cleavage was further tested by incubating human topoisomerase IIα and circular plasmid DNA in the absence (MeSO) and presence of m-AMSA (50 μM) or ICRF-193 (50 μM). Reactions were stopped by the addition of SDS to 1%, treated with proteinase K, and analyzed subsequently on a 1% agarose gel containing ethidium bromide. D, L, and SC represent dimeric, linear, and supercoiled plasmid DNA, respectively. Lower panel, histogram of the DNA cleavage experiment described in the upper panel. The level of cleavage in the presence of m-AMSA for each enzyme is set to one. Results are the means ± S.D. of five independent experiments. wt, wild-type.

**FIG. 5.** Wild-type human topoisomerase IIα, 351i, and 408i are able to cause sensitivity to m-AMSA when expressed in yeast. m-AMSA sensitivity of yeast cells expressing mutant or wild-type human topoisomerase IIα. Cell survival is plotted as the number of cells surviving after 4 and 8 h of m-AMSA treatment relative to the number of cells at time point zero. Cells were grown in the absence of drug (open circles, pHT300; open triangles, pHT351i; open diamonds, pHT408i; times sign, pHT213; open squares, pRS315) or in the presence of 100 μM m-AMSA (closed circles, pHT300; closed triangles, pHT351i; closed diamonds, pHT408i; asterisk, pHT213; closed squares, pRS315)
determine the steps in the catalytic cycle of human topoisomerase IIα required for the enzyme to cause sensitivity to ICRF-193 in yeast cells. In agreement with previous findings (11, 15), we have shown that yeast cells expressing wild-type human topoisomerase IIα in trans are hypersensitive to ICRF-193. In contrast, the expression of neither one of the strand passage-deficient human topoisomerase IIα enzymes, 351i, 408i, and Y805S, conferred sensitivity to ICRF-193 in yeast cells, although both 351i and Y805S were found to undergo stable closure of the N-terminal clamp in the presence of ATP and ICRF-193 in vitro. The lack of sensitivity in cells expressing 408i can be explained by the inability of the enzyme to perform clamp closure. Y805S would also be expected to be unable to cause ICRF sensitivity if topoisomerase II-mediated DNA cleavage was a requirement for ICRF cytotoxicity. However, the fact that 351i, capable of both clamp closure and DNA cleavage, still does not confer sensitivity to ICRF-193 demonstrates that neither the DNA cleavage activity of topoisomerase II nor the ability of the drug to induce stable clamp closure can be the main cause of cell killing by ICRF-193. Rather, the strand transport activity of topoisomerase II, which is the only activity lacking in all of the mutant enzymes, seems to be necessary for the cell-killing mechanism of ICRF-193.

We also used the yeast system to investigate the cell-killing mechanism of a traditional topoisomerase II poison, m-AMSA. The cleavage-competent topoisomerase IIα enzymes, wild type, 351i, and 408i, were all found to cause hypersensitivity to m-AMSA when expressed in yeast, in agreement with their in vitro capability to respond to m-AMSA with increased levels of DNA cleavage complex formation. In contrast, the expression of the cleavage-deficient Y805S did not elevate the m-AMSA sensitivity of the yeast cells. This strongly suggests that m-AMSA solely works via the DNA cleavage ability of topoisomerase II and that the m-AMSA-induced increase seen in cleavage complex formation in vitro directly reflects the cell-killing mechanism of m-AMSA. The ability of 351i and 408i to cause sensitivity to m-AMSA in yeast further confirms that these enzymes are present in the yeast nucleus and have retained their ability to cleave DNA, in agreement with the previous in vitro characterizations (19, 20).

The fact that ICRF cytotoxicity depends on the strand passage reaction of topoisomerase II strongly indicates that the drug traps a cellular topoisomerase II reaction intermediate that is toxic to the cell. The inability of 351i to create this toxic intermediate suggests that it arises during the strand passage reaction or at a later step in the catalytic cycle of topoisomerase II. The newly published structure of an N-terminal fragment of yeast topoisomerase II shows that the cavity formed by the N-terminal clamp of eukaryotic topoisomerase II is considerably smaller than that of DNA gyrase and thus cannot hold a T-segment after the clamp has been closed (6). Consequently, the strand passage-deficient 351i and Y805S enzymes only interact with one DNA segment, the G-segment, once the clamp has been stably closed by the binding of ICRF-193 to the N-terminal domain. This provides an obvious explanation for the lack of ICRF sensitivity displayed by the yeast cells expressing these mutant enzymes compared with those expressing the wild-type human topoisomerase IIα, namely the toxic intermediate being topoisomerase II stably bound to two DNA segments.

The lack of cytotoxicity observed with 351i and Y805S can be explained by a model predicting that topoisomerase II clamped around one DNA segment can slide freely on DNA and therefore does not interfere with DNA-tracking machineries during transcription and replication. In contrast, the complex formed between the wild-type topoisomerase II and two DNA segments may be more restricted in its movements along DNA and in this way inhibits both the transcription and replication processes. In support of this model, it has been shown that ICRF-193 inhibits transcription and it has been suggested that collision with the transcription machinery is responsible for the efficient degradation of topoisomerase IIβ that occurs upon ICRF treatment of human cell cultures (25). The elevated sensitivity to ICRF displayed by chicken cells deficient in non-homologous end joining indicates that DNA strand breaks do occur upon ICRF treatment (12). Along with the presented model, these breaks could occur upon collision of the complex of topoisomerase II bound to two DNA segments with the replication or transcription machinery. Alternatively, the DNA breaks could result from intolerable strain in the DNA created by the topoisomerase II complex.

In conclusion, we have found that ICRF-193 and m-AMSA work by distinct mechanisms where ICRF-193 depends on the strand passage activity of topoisomerase II, most probably the result of the simultaneous interaction of the enzyme with two DNA segments. This is different from m-AMSA that works directly via the DNA cleavability of topoisomerase II.

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