ABSTRACT

Anticancer drugs targeted to the nuclear enzyme DNA topoisomerase II are classified as poisons that lead to DNA breaks or catalytic inhibitors that appear to completely block enzyme activity. To examine the effects of the bisdioxopiperazine class of catalytic inhibitors to topoisomerase II, we investigated a Chinese hamster ovary (CHO) subline selected for resistance to ICRF-159 (CHO/159-1). Topoisomerase IIα content in CHO/159-1 cells was reduced by 40–50%, compared to wild-type CHO cells, whereas the β isoform was increased by 10–20% in CHO/159-1 cells. However, the catalytic activity of topoisomerase II in nuclear extracts from CHO/159-1 cells was unchanged, as was its inhibition by the topoisomerase II poison etoposide (VP-16). No inhibition of topoisomerase II catalytic activity by ICRF-187 was seen in CHO/159-1 cells up to 500 μM, whereas inhibition was evident at 50 μM in wild-type CHO cells. VP-16-mediated DNA single-strand breaks and cytotoxicity were similar in the two sublines. ICRF-187 could abrogate these VP-16 effects in the wild-type line but had no effect in CHO/159-1 cells. Western blots of topoisomerase IIα after incubation of CHO cells with ICRF-187 demonstrated a marked band depletion, whereas this effect was completely lacking in CHO/159-1 cells, and an equal effect of VP-16 was observed in both lines. These data imply that the CHO/159-1 topoisomerase IIα lacks sensitivity to bisdioxopiperazines and that the mechanism of resistance in this cell line does not confer cross-resistance to topoisomerase II poisons, suggesting that mutations conferring resistance to bisdioxopiperazines can occur at sites distinct from those responsible for resistance to complex stabilising agents. Accordingly, CHO/159-1 cDNA showed two heterozygous mutations in the proximal NH2-terminal part of topoisomerase IIα (Tyr49Phe and Δ309Gln-Gln-Ile-Ser-Phe313), which is in contrast to those induced by topoisomerase II poisons, which cluster further downstream. Site-directed mutagenesis and transformation of the homologous Tyr50Phe coding mutation in human topoisomerase IIα in a temperature-conditinal yeast system demonstrated a high-level resistance to ICRF-193, compared to cells expressing wild-type cDNA, but none toward the poisons VP-16 or amsacrine, thus confirming that the Tyr49Phe mutation confers specific resistance to bisdioxopiperazines. Thus, these results indicate that the region of the protein involved in ATP-binding also plays a critical role in sensitivity to bisdioxopiperazines, a result consistent with the known requirement for the formation of an ATP-bound closed clamp for bisdioxopiperazine activity. These results may enable a more precise understanding of the interaction of topoisomerase II-directed drugs with their target enzyme.

INTRODUCTION

DNA topoisomerase II is an essential nuclear enzyme that is responsible for the cleavage and religation of double-stranded DNA, thus allowing the topological changes that are required for DNA metabolism. A number of clinically important anticancer drugs, such as the anthracyclines doxorubicin (Adriamycin) and daunorubicin and the epipodophyllotoxins VP-16 and teniposide act on the enzyme by stabilizing a DNA-enzyme covalent complex, which leads to the formation of DNA breaks (1). These drugs are termed topoisomerase II poisons because they convert the enzyme into a potent cellular toxin (2). Recently, it has become evident that other drugs exist that act on the topoisomerase II catalytic cycle without stabilizing cleavable complexes, i.e., they do not lead to DNA breaks. This class of drugs has been termed topoisomerase II catalytic inhibitors (2) and includes such disparate agents as aclarubicin (3), merbarone (4), fostriecin (5), chloroquine (6), and the bisdioxopiperazines (7, 8). The latter appear to have a specific effect on the closed clamp form of topoisomerase II (9, 10) and are, thus, a powerful tool in the elucidation of the actions of various drugs on topoisomerase II (11). There is evidence to suggest that topoisomerase II is itself a significant cellular target for bisdioxopiperazines because its activity and/or level is inversely correlated to sensitivity to these compounds in both yeast (12) and human cells (13, 14), and finally, an ICRF-187-resistant CHO cell line has alterations in its topoisomerase IIα level and response to ICRF-187 (15). Furthermore, although their lack of activity has hitherto precluded their use as primary anticancer agents, they may, in the future, be able to play a clinical role in targeting topoisomerase II poisons, e.g., tumors in the central nervous system (16, 17). In addition, the iron-chelating capability of ICRF-187 is used to protect against the cardiotoxicity induced by doxorubicin (18). To further characterize the interaction between the bisdioxopiperazines and topoisomerase II, we describe mutations in topoisomerase IIα in a bisdioxopiperazine-resistant CHO cell line with specific changes in drug-induced function and activity of the topoisomerase II enzyme.

MATERIALS AND METHODS

Cell Lines. ICRF-159-resistant cell lines were isolated by UV mutagenesis, followed by selection for growth in the presence of drug. Thus, subconfluent monolayers of CHO cells were UV-irradiated for 5 s (200 erg/mm2) to deliver a dose calculated to allow 30% cell survival in a colony-forming assay. Debris was removed after 5 days, and the cells were harvested and resedated at 105–106/50-mm cell culture dish after a further week. The cells were then treated to a single dose (100 μg/ml) of ICRF-159 and grown for a week with medium replacement 3 and 7 days after drug administration. Surviving cells were harvested, replated at 106/dish, and maintained in 100 μg/ml of drug for the first 3 days of each week for three weeks. Distribution in 24-well plates at an

1 The abbreviations used are: VP-16, etoposide; CHO, Chinese hamster ovary; m-AMSA, amssacine; ddH2O, double distilled H2O; SSB, single-strand breaks; NB, nucleus buffer; kDNA, kinetoplast DNA; nt, nucleotide(s); GyrB, gyrase B; URA, synthetic growth medium lacking uracil.
average density of one cell per well yielded resistant clones, including CHO/159-1, which has undergone further characterization (19, 20).

**Drugs.** ICRF-159 and ICRF-193 were synthesized as described previously (21). ICRF-187 (Cardioxane; EuroCetus), vincristine (Lilly), aclacinomycin (Lundbeck), novobiocin (Sigma Chemical Co., St. Louis, MO), daunorubicin (Rhône Poulenc), and doxorubicin (Adrianycin; Pharmacia-Upjohn) were dissolved in sterile water immediately prior to use. m-AMSA (Park-Davis) was delivered in N,N-dimethylacetamide solution and further diluted in acid lactose. VP-16 and cisplatin (Bristol-Myers Squibb) were in solution for infusion. 1,2-D-arabinofuranosylcytosine (Pharmacia Upjohn) was dissolved in benzyl alcohol, camptothecin (Sigma) was dissolved in DMSO, and carmustine [1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU); Bristol-Myers Squibb] was dissolved in 10% (v/v) ethanol in sterile water. Merbarone and foscarnet were gifts from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH (Bethesda, MD), and were dissolved in DMSO and sterile water, respectively. The drugs were diluted with tissue culture medium to 300× final concentrations, portioned into multiple aliquots, frozen in ethanol-dry ice, and stored at −80°C. This procedure has previously been shown to ensure stability (22).

**Clonogenic Assay.** A 3-week clonogenic assay using soft agar on a sheep RBC feeder layer was used with continuous drug incubation (22). Briefly, single-cell suspensions (2 × 10⁵ cells/ml) in RPMI 1640 supplemented with 10% FCS were exposed to the drugs and plated in triplicate in soft agar on top of a feeder layer containing sheep RBCs. The number of cells was adjusted to obtain 2000–3000 colonies in the control dishes.

**Alkaline Elution.** Alkaline elution was performed according to Kohn et al. (23). For measurement of DNA SSBS, L1210 cells were used as internal standard and were exposed to 100 μM H₂O₂ for 60 min on ice, corresponding to an irradiation dose of 300 R, as described (24). CHO or CHO/159-1 cells were incubated in medium supplemented with the indicated drug(s) at 37°C for the specified periods, washed in 10 ml ice-cold PBS, and then lysed on the filter (2.0-μm pore size, Nucleopore) with 5 ml of 0.1 M glycine, 0.025 M Na₂EDTA lysis solution (2% SDS, 0.1 M glycine, and 0.025 M Na₂EDTA) at pH 10, followed by addition of 1.5 ml SDS-EDTA lysis solution supplemented with 0.5 mg/ml proteinase K (Sigma). Mixing of standard and experimental cells was done immediately prior to lysis. DNA was eluted with tetrapropylammoniumhydroxide-EDTA (pH 12.1) containing 1% SDS at a rate of 0.125 ml/min. Fractions were collected at 20-min intervals for 2 h. Filters were treated with 400 μl of 1 N HCl for 1 h at 60°C and cooled, and 0.4 M NaOH was added prior to scintillation counting.

**Western Blot and Band Depletion Assays.** Western blots were performed on both whole-cell and 0.35 or 0.7 M NaCl nuclear extracts for detection of topoisomerase IIα using a monoclonal antibody raised against residues 1513-1593 (Cambridge Research Biochemicals, Cheshire, United Kingdom). For detection of topoisomerase IIβ, the 1851 rabbit polyclonal antibody raised to the COOH-terminal fragment of the human enzyme was kindly supplied by Dr. Caroline Austin (University of Newcastle, Newcastle, United Kingdom). Band depletion assays are Western blots of topoisomerase II isomers on 0.35 M NaCl nuclear extracts after cells have been incubated with drug. Briefly, cells were incubated with the desired drug for 1 h at 37°C in RPMI 1640 supplemented with 10% FCS. Thereafter, crude nuclear extracts were prepared by a modification of a previously described method (25). All steps were performed at 4°C. Exponentially growing cells were harvested and washed twice in NB (2 mM KH₂PO₄, 5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA, 0.2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, pH 6.5). Cells were resuspended in 1 ml of NB and were lysed for 5 min by the gentle addition of 9 ml of NB supplemented with 0.3% (v/v) Triton X-100. Nuclei were isolated by centrifugation at 1000 × g for 10 min and washed with Triton X-100-free NB. Nuclear extracts were prepared by salt extraction for 30 min in NB with either 0.35 or 0.7 M NaCl. Insoluble nuclear material was removed by centrifugation at 17,000 × g for 10 min, and the supernatant was saved. Extracts were adjusted to 50% (v/v) glycerol.

After the nuclear extracts were heated for 5 min at 50°C, samples were immediately loaded on a 7% SDS-polyacrylamide gel containing 5% glycerol. All following steps were performed at room temperature. The separated proteins were transferred to Trans Blot Nitrocellulose (NEN). A semidry electroblot system (KenEnTec, Copenhagen, Denmark) with 50 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid, pH 11.0) at 100 mA for 1–1.5 h. Membranes were blocked in 1% BSA in TBS-T (10 mM Tris-HCl (pH 8.2), 150 mM NaCl, and 0.05% Tween 20) for 1 h and probed with either topoisomerase IIα (1:1000) or topoisomerase IIβ (1:650) antibodies for 1 h. Secondary antibody (sheep antimouse immunoglobulin or donkey anti-rabbit immunoglobulin, both diluted 1:6250) application and visualization were performed using the Amersham chemiluminescence kit according to the manufacturer’s instructions (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Total protein amount was measured by the Bradford assay (26). Quantitation of immunoreactive bands was done by densitometric scanning.

**Decatenation Assay.** Topoisomerase II catalytic activity was measured using the kDNA decatenation assay from TopoGen (Colombus, OH), according to the manufacturer’s instructions. Briefly, 0.35 M NaCl nuclear extracts of either increasing protein concentration (when comparing CHO and CHO/159-1 baseline catalytic activity) or similar protein concentrations (0.4 μg/ml) with increasing drug concentrations (when comparing activity of different drugs on CHO and CHO/159-1 topoisomerase II activity) were incubated with 0.2 μg of kDNA on CHO/159-1 in a final volume of 20 μl. After the addition of stop buffer-loading dye mix, samples were loaded on 1% agarose-0.5% ethidium bromide gels and run in Tris-boric acid disodium EDTA buffer containing 0.5 μg/ml ethidium bromide at 100 V for approximately 50 min.

**Sequencing of Topoisomerase IIα Segments.** cDNA derived from 10 ng of mRNA was mixed with 25 pmol of primer pairs and 2.5 units of Thermostar plus DNA polymerase (Advanced Biotechnologies, Surrey, United Kingdom) and 0.2 mM dNTP in PCR buffer IV [20 mM (NH₄)₂SO₄, 75 mM Tris-HCl, 0.01% Tween 20, and 1.5 mM MgCl₂; Advanced Biotechnologies]. PCR was carried out in 100 μl using the GeneAmp PCR system 2400 (Perkin-Elmer, Foster City, CA), with an initial denaturation for 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, annealing for 30 s at 58°C, and extension of 45 s at 72°C. To verify that PCR products were of the correct length, 10 μl of PCR products were electrophoresed in TAE buffer (40 mM Tris-acetate-1 mM EDTA) in a 4% agarose gel containing ethidium bromide. When only one band was present, the PCR product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany), and the concentration and purity were determined by spectrophotometry. Genomic DNA was purified using the TRIZol reagent from Life Technologies, Inc. (Gaithersburg, MD).

Sequencing of the PCR products was performed using the ABI Prism Dye Terminator Cycle Sequencing kit (Perkin-Elmer) according to the manufacturer’s instructions using AmpliTaq DNA polymerase (Perkin-Elmer). Sequencing products were run on an ABI PRISM 377 Sequencer. Sequencing was performed from both 5′ and 3′ ends for two or three times each on two different mRNA extractions.

**Sequencing of topoisomerase IIα segments**

**Primer sequences for motifs A and B/dinucleotide binding site, as well as the active tyrosine site, were adapted from the human topoisomerase IIα sequence in Danks et al. (27) to correspond to the CHO topoisomerase IIα sequence published by Chan et al. (28). These are listed in Table 1, together with the primers designed for the sequencing of the rest of the topoisomerase IIα cDNA. A corresponding map of the primer is given in Fig. 1. After cycle sequencing of the entire cDNA of topoisomerase IIα in CHO/159-1 cells had been performed, a heterozygous point mutation at nt 227 and a 15-nt heterozygous deletion at nt 1003–1017 was observed, compared to the published wild-type CHO sequence (Ref. 28; see “Results”). Sequencing of the appropriate stretch of cDNA from the wild-type CHO cells used in this study showed the same result obtained previously (28). To confirm the heterozygosity of the deletion the primer sets designated del1 and del2 in Table 1 were used to amplify a 103-nucleotide segment to check the size of the deletion in an agarose gel. Finally, the forward primer Motif A and reverse primer restr (Table 1) were used to amplify cDNA from nt 487 to nt 1250 for treatment with Tsp5091 restriction enzyme (New England Biolabs), which cuts at 5′-AAAAATTT-3′-TTAA at 5′ (nt 1008) and, thus, would be expected cleave the cDNA from the nondeleted allele only.

**Functional Characterization of Mutant Human Topoisomerase IIα in Yeast.** To confirm that the Tyr49Phe mutation detected in CHO/159-1 topoisomerase IIα plays a role in bisdioxopiperazine resistance, the following transformation to a previously described temperature-sensitive yeast system was performed with human topoisomerase IIα including the heterozygous Tyr50Phe mutation (see Fig. 7). Site-directed mutagenesis was done using the two mutagenic primers Y50F-SN and Y50F-ASN (Table 1) with the yeast
expression vector for human topoisomerase IIa, pMJ1 (29). Oligonucleotide-directed mutagenesis was carried out using a QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). A 50-µl reaction mixture containing 0.40 ng/µl plasmid DNA, 2.5 ng/µl each primer, 0.2 mm dNTP, and 2.5 units of Pfu DNA polymerase in 1 x reaction buffer was exposed to 95°C for 30 s followed by 15 cycles of 95°C for 30 s, 55°C for 60 s, and 68°C for 24 min. Finally, 10 units of DpnI methylation-specific restriction endonuclease were added to digest original template DNA for 2 h at 37°C. One µl of the reaction was used for the transformation of supercompetent XL1-blue Escherichia coli cells (Stratagene). Colonies were selected on 100 µg/liter ampicillin, and plasmid DNA was isolated using the Qiagen tip 100 DNA isolation kit (Qiagen), as described by the manufacturer. The existence of the Tyr50Phe mutation in four independent plasmid clones of the resulting plasmid pMJ1-Y50F was verified by automated dye termination cycle sequencing, using the primer Y50F-SEQ (Table 1).

The pMJ1 and pMJ1-Y50F plasmids were introduced into the haploid yeast strain JN3942-4 (MATa URA-52 leu2 trpl1 his7 ade1-2 ISE2 rad52::LEU2 top2-4) using a modified lithium acetate method using single-stranded herring sperm DNA as carrier, and selection of single colonies and propagation of transformed yeast clones were carried out on URA+ plates at 25°C as described (29). To test for complementation of the top2-4 defect by the Y50F mutant enzyme, pMJ1-Y50F- and pMJ1-transformed JN3942-4 cells, as well as JN3942-4 cells, transformed with the yCPS0 vector used for the construction of pMJ1, were incubated at 25°C and 34°C, respectively, for 5 days.

Clonogenic Assay of Transformed Yeast Cells. Yeast cells taken from URA+ plates were inoculated into 20 ml of YPDA medium containing 2 mg/liter tetracycline and grown overnight at 34°C and 250 rpm. An overnight culture of cells in logarithmic phase was diluted to 2 x 10⁶ cells/ml in prewarmed medium, and 3 ml cultures were exposed to different concentrations of VP-16, m-AMS, and ICRF-193 for 24 h at 34°C and 250 rpm. To assess the colony-forming capacity of drug-treated cells in clonogenic assay, 1-ml samples removed after 0, 8, and 24 h were washed twice in ddH₂O, diluted 10⁻¹⁰ times in ddH₂O, and 200 µl of diluted cells were then plated to URA plates, which were incubated for 6 days at 25°C prior to counting. Plates containing 200–600 colonies were used for counting. Finally, relative cell growth after 8 and 24 h compared to 0 h were calculated for all conditions used. All experiments were performed at least two times.

RESULTS

Cells. A comprehensive characterization of CHO/159-1 was described by Kenwrick (20) and is summarized briefly here. CHO and CHO/159-1 cells had similar doubling times, cell cycle distribution, chromosome content, and morphology. Accumulation of [14C]ICRF-193 was equal in CHO and CHO/159-1 cells. Furthermore, the presence of the permeabilizing detergent Tween 80 did not potentiate killing of CHO/159-1 cells by ICRF-159. Further, interspecific hybrids between CHO/159-1 cells and drug-sensitive mouse L cells were intermediate in their level of drug resistance compared to the parent cells. This codominance indicates that resistance in the hamster line is not due to a deficiency in a cellular component necessary for ICRF-
159 cytotoxicity but rather to the gain of a resistance conferring mutation or amplification of a specific gene product (20). CHO/159-1 are highly cross-resistant to the isomer ICRF-187 and the analogue ICRF-193 (Fig. 2 and Table 2). Slight cross-resistance to VP-16 is seen, which agrees with a reduced topoisomerase IIα amount and with fewer VP-16-induced SSBs in CHO/159 cells in Fig. 5A. Surprisingly, a collateral sensitivity is demonstrated toward intercalating anthracyclines, whether they are topoisomerase II poisons (doxorubicin and daunorubicin) or a catalytic inhibitor (aclarubicin). Furthermore, collateral sensitivity to the tubulin-directed drug vincristine was also observed. However, compared to the resistance to bisdioxopiperazines, variations in sensitivity to other drug types was weak. Of special interest is the finding that cross-resistance to other catalytic inhibitors, such as merbarone, fostriecin, and novobiocin, is negligible or lacking. When CHO/159-1 cells were coincubated with ICRF-187 and VP-16, no protection of VP-16 cytotoxicity was provided by ICRF-187, which is in contrast to both CHO cells (Fig. 3) and human small cell lung cancer NYH cells (30).

**Western Blots and Band Depletion.** CHO/159-1 cells had a 40–50% reduced topoisomerase IIα and a 10–20% elevated topoisomerase IIβ content (Table 3). In band depletion assays, a major difference was observed between CHO/159-1 and wild-type cells. In CHO cells, increasing concentrations of ICRF-187 caused a decrease of topoisomerase IIα, as also previously demonstrated in NYH cells (11), indicating that the drug causes the enzyme to bind to DNA, leaving less available extractable enzyme to show up in the Western blot (Fig. 4). This phenomenon of ICRF-187-induced band depletion was, however, completely lacking in CHO/159-1 cells, where even a concentration of 500 μM had no effect (Fig. 4). This was also the case when incubation with ICRF-187 was extended to 2 h or when 100 μM ICRF-193 was used, the latter creating an even more marked band depletion in CHO cells than 500 μM ICRF-187 (data not shown). However, when cells were incubated with VP-16, an equal reduction in topoisomerase IIα was observed (Fig. 4). Finally, when blots were probed with anti-topoisomerase IIβ antibodies, equal band depletion was observed with ICRF-187 in both CHO and CHO/159-1 cells (Fig.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CHO</th>
<th>CHO/159-1</th>
<th>RR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICRF-159</td>
<td>47</td>
<td>920</td>
<td>19.6</td>
</tr>
<tr>
<td>ICRF-187</td>
<td>57</td>
<td>718</td>
<td>12.6</td>
</tr>
<tr>
<td>ICRF-193</td>
<td>&lt;1</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.52</td>
<td>0.22</td>
<td>0.43</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.60</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.23</td>
<td>0.114</td>
<td>0.49</td>
</tr>
<tr>
<td>Aclarubicin</td>
<td>0.069</td>
<td>0.023</td>
<td>0.34</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>0.0086</td>
<td>0.0172</td>
<td>2.0</td>
</tr>
<tr>
<td>Ara-C</td>
<td>0.152</td>
<td>0.120</td>
<td>0.79</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.443</td>
<td>0.417</td>
<td>0.94</td>
</tr>
<tr>
<td>Carmustine</td>
<td>10.1</td>
<td>7.57</td>
<td>0.75</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.22</td>
<td>0.037</td>
<td>0.20</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>0.22</td>
<td>0.15</td>
<td>0.7</td>
</tr>
<tr>
<td>Merbarone</td>
<td>5.5</td>
<td>6.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Fostriecin</td>
<td>4.2</td>
<td>5.9</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*RR, relative resistance; Ara-C, 1-β-D-arabinofuranosylcytosine.
Table 3 Relative levels of topoisomerase IIα and topoisomerase IIβ in CHO (set to 1.0) and CHO/159-1 cells in repeated experiments.

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>CHO</th>
<th>CHO/159-1</th>
<th>CHO</th>
<th>CHO/159-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>head</td>
<td>0.35 M NaCl</td>
<td>11</td>
<td>5.06</td>
<td>0.60</td>
</tr>
<tr>
<td>head</td>
<td>0.70 M NaCl</td>
<td>4</td>
<td>0.52</td>
<td>0.45</td>
</tr>
<tr>
<td>whole cell</td>
<td>3</td>
<td>0.55</td>
<td>0.53</td>
<td>0.52-0.59</td>
</tr>
<tr>
<td>head</td>
<td>0.35 M NaCl</td>
<td>6</td>
<td>1.38</td>
<td>1.15</td>
</tr>
<tr>
<td>head</td>
<td>0.70 M NaCl</td>
<td>1</td>
<td>1.08</td>
<td>1.08</td>
</tr>
<tr>
<td>whole cell</td>
<td>1</td>
<td>1.23</td>
<td>1.23</td>
<td></td>
</tr>
</tbody>
</table>

DNA Sequences. Comparison of the complete cDNA sequence for topoisomerase IIα from CHO/159-1 cells with the published CHO wild-type sequence (28), as well as with the CHO cells used in this study, revealed two changes, both in the NH2-terminal region of the enzyme, namely, a A → T point mutation at nt 227 leading to an amino acid change from Tyr to Phe at residue 49 and a deletion of nt 1003–1017, resulting in a deletion of residues 309Gln-Gln-Ile-Ser-Phe311 (Fig. 7). Both changes were in highly conserved residues in eukaryote topoisomerases (Fig. 7). It was obvious from the sequencing analysis that both changes were heterozygous (see Fig. 8 for the nt227 mutation). This was confirmed for the deletion by PCR analysis of a 103-nucleotide fragment spanning the deletion (Fig. 9), as well as by gel electrophoresis, following digestion with a restriction enzyme designed to cleave the nonmutated allele only (data not shown). Furthermore, to investigate whether the deletion had occurred at a splice site, genomic DNA from both CHO and CHO/159-1 spanning nt 1003–1017, resulting in a deletion of residues 309Gln-Gln-Ile-Ser-Phe311 (Fig. 7).

4). These data suggest that an alteration in topoisomerase IIα may play a role in the observed bisdioxopiperazine resistance.

Alkaline Elution Assay. Using the topoisomerase II poison VP-16, fewer DNA SSBs were detected in CHO/159-1 cells compared with CHO cells (Fig. 5A), which agrees with the reduced enzyme content shown in Table 3. However, the most qualitative difference lies in the complete lack of inhibition of VP-16-induced DNA SSBs by ICRF-187 in CHO/159-1 cells, which is in contrast to both wild-type CHO cells (Fig. 5A) and to other previously described cell lines (30). When another catalytic inhibitor, aclarubicin, was used, it was able to inhibit VP-16-induced DNA SSBs in both sublines (Fig. 5B), attesting to the ability of these VP-16-induced SSBs to be antagonized in CHO/159 cells and confirming that the mechanism of inhibition of VP-16-mediated damage is different for these two drugs.

kDNA Decatenation Assay. The enzymatic activity as measured by decatenation assay was unchanged in CHO and CHO/159-1 cells (Fig. 6A). However, it was also possible to inhibit the topoisomerase II catalytic activity by ICRF-187 in CHO/159-1 cells, although this was readily attained in the wild-type cells (Fig. 6B). VP-16 had a similar inhibitory effect in both cell lines (Fig. 6C), attesting to the fact that the enzyme can still be inhibited by a topoisomerase II poison in CHO/159-1 cells. Thus, Fig. 6 clearly demonstrates that the topoisomerase II activity in CHO/159-1 cells is selectively resistant to bisdioxopiperazine inhibition.

Fig. 5. A, alkaline elution showing inhibition of VP-16-induced DNA SSBs by ICRF-187 in CHO, but not in CHO/159-1 cells. Cells were incubated with the indicated drug(s) at 37°C for 1 h before lysis and filter elution. X and Y axes, percentage filter retention. B, alkaline elution showing inhibition of VP-16-induced DNA SSBs by aclarubicin (ACLA) in both CHO and CHO/159-1 cells. Cells were incubated with the indicated drug(s) at 37°C for 1 h. X and Y axes, percentage filter retention.

Fig. 4. Top, band depletion assay demonstrating a decrease in topoisomerase IIα content in 0.35 M NaCl nuclear extracts induced by ICRF-187 in CHO cells but not in CHO/159-1 cells. Cells were incubated with drug at 37°C for 1 h. Lanes 1–4, CHO cells; Lanes 5–8, CHO/159-1 cells. Lanes 1 and 5, no drug control; Lanes 2 and 6, 50 μM ICRF-187; Lanes 3 and 7, 200 μM ICRF-187; Lanes 4 and 8, 500 μM ICRF-187. Middle, band depletion assay demonstrating a similar decrease in topoisomerase IIβ content in 0.35 M NaCl nuclear extracts induced by ICRF-187 in CHO, as well as in CHO/159-1 cells. Cells were incubated with drug at 37°C for 1 h. Lanes 1–4, CHO cells; Lanes 5–8, CHO/159-1 cells. Lanes 1 and 5, no drug control; Lanes 2 and 6, 50 μM ICRF-187; Lanes 3 and 7, 200 μM ICRF-187; Lanes 4 and 8, 500 μM ICRF-187. Bottom, band depletion assay demonstrating a similar decrease in topoisomerase IIα content in 0.35 M NaCl nuclear extracts induced by the topoisomerase II poison VP-16 in CHO cells, as well as in CHO/159-1 cells. Cells were incubated with drug at 37°C for 1 h. Lanes 1–4, CHO cells; Lanes 5–8, CHO/159-1 cells. Lanes 1 and 5, no drug control; Lanes 2 and 6, 50 μM VP-16; Lanes 3 and 7, 100 μM VP-16; Lanes 4 and 8, 200 μM VP-16.
Fig. 6. A, decatenation assay demonstrating equal topoisomerase II catalytic activity in 0.35 M NaCl nuclear extracts from wild-type CHO (Lanes 1–4, WT) and CHO/159-1 (Lanes 5–8, /159) cells. kDNA was treated with 0.35 M NaCl nuclear extracts from the two cell lines at the following protein concentrations: Lanes 1 and 5, 0.8 μg/μl; Lanes 2 and 6, 0.4 μg/μl; Lanes 3 and 7, 0.2 μg/μl; Lanes 4 and 8, 0.1 μg/μl. Lane C, control catenated kDNA marker; Lane D, control decatenated kDNA marker. B, ICRF-187 inhibits topoisomerase II-mediated decatenation in wild-type CHO (Lanes 1–4, WT) but not in CHO/159-1 (Lanes 5–8, /159) cells. kDNA was treated with 0.35 M NaCl nuclear extracts (0.4 μg/μl protein) coincubated with increasing concentrations of ICRF-187. Lanes 1 and 5, no drug control; Lanes 2 and 6, 50 μM ICRF-187; Lanes 3 and 7, 100 μM ICRF-187; Lane C, control catenated kDNA marker; Lane D, control decatenated kDNA marker. C, VP-16 inhibits topoisomerase II-mediated decatenation equally in wild-type CHO (Lanes 1–5, WT) and CHO/159-1 (Lanes 6–10, /159) cells. kDNA was treated with 0.35 M NaCl nuclear extracts (0.4 μg/μl protein) coincubated with increasing concentrations of VP-16. Lanes 1 and 6, no drug control; Lanes 2 and 7, 25 μM VP-16; Lanes 3 and 8, 50 μM VP-16; Lanes 4 and 9, 100 μM VP-16; Lanes 5 and 10, 200 μM VP-16; Lane C, control catenated kDNA marker; Lane D, control decatenated kDNA marker.

Fig. 7. Comparison of amino acid sequences between human topoisomerase Ila (Hs Topo Ila), human topoisomerase IIß (Hs Topo IIß), CHO topoisomerase Ila (CHO Topo Ila), Saccharomyces cerevisiae topoisomerase II (S. cer. Topo II), and E. coli GyrB. The changes found in CHO/159-1 topoisomerase Ila are indicated.

956-1059 was sequenced. This analysis showed that the deletion had occurred entirely within a single exon.

Functional Studies in Yeast. The Tyr49Phe mutation identified in the CHO/159-1 cell line was reconstructed in human topoisomerase Ila in the yeast expression vector pMJ1, which includes the entire coding region of human topoisomerase Ila under the control of the yeast topoisomerase 1 promoter. In addition, it includes an URA3 marker, a yeast origin of replication and a yeast centromere for the introduction and maintenance of the plasmid in yeast. The human topoisomerase Ila residue homologous to CHO Tyr49 is Tyr50 (Fig. 7). Both pMJ1 and pMJ1-Tyr50Phe transformed cells were able to grow at 34°C, whereas cells transformed with the vector plasmid yCP50 used for the construction of pMJ1 were only able to grow at 25°C, demonstrating complementation of the nonfunctional endogenous yeast TOP2 allele at the nonpermissive temperature by the mutant human enzyme (data not shown).

The JN3942-4 carries a dominant drug permeability mutation ISE2, which greatly increases the sensitivity toward a number of topoisomerase II-directed drugs, including the bisdioxopiperazines (12, 31). At 34°C, JN3942-4 cells expressing the mutant enzyme displayed an extremely high level of resistance to ICRF-193 compared to cells expressing the wild-type enzyme (Fig. 10). In contrast, cells expressing wild-type and mutant enzyme were equally or slightly hypersensitive to VP-16 (Fig. 10) and m-AMSA (data not shown), whereas cells transformed with the vector plasmid yCP50 used for the construction of pMJ1 were only able to grow at 25°C.
Fig. 9. PCR analysis of a 103-nt cDNA from nt 956-1059 in topoisomerase Ila from CHO and CHO/159-1, demonstrating two alleles in CHO/159-1, one of which is 15 nt shorter, corresponding to the deletion of residues 309–313 shown in Fig. 7.

demonstrating both that the mutation is specific for bisdioxopiperazines and that the mutant enzyme is catalytically active. Thus, the functional significance of the Tyr49Phe mutation identified in the CHO/159-1 cell line for the acquired bisdioxopiperazine resistance is demonstrated.

DISCUSSION

The interaction of topoisomerase II catalytic inhibitors with the enzyme-DNA complex has received considerable attention in recent years. Thus, it is now considered that intercalaters such as aclarubicin and chloroquine act early in the catalytic cycle by preventing the enzyme from reaching its DNA substrate (3, 6, 32), whereas the bisdioxopiperazines, such as ICRF-159, ICRF-187, and ICRF-193, act on the closed-clamp step of the catalytic cycle in an ATP-dependent manner (9, 10). That the bisdioxopiperazines have a unique mechanism of action on topoisomerase II is indicated by the lack of cross-resistance to the other catalytic inhibitors in Table 2. Further evidence that DNA topoisomerase Ila is a, if not the, major drug target in mammalian cells is based on the very marked differences between CHO and CHO/159-1 cells with respect to the inhibitory effect of ICRF-187 in several different assays (Figs. 3–6). Thus, lack of band depletion of topoisomerase Ila by ICRF-187 (Fig. 4), together with the lack of protection by ICRF-187 on VP-16-induced cytotoxicity (Fig. 3) and DNA breaks (Fig. 5), and, finally, the lack of ICRF-187 effect on enzymatic activity in nuclear extracts from CHO/159-1 cells.
(Fig. 6) strongly indicates that the resistance to bisdioxopiperazines in these cells is due to an alteration in topoisomerase IIa. This is further confirmed in the temperature-conditional yeast system, in which transformation of the Tyr→Phe mutation found in CHO/159-1 topoisomerase IIa DNA (Fig. 7) is sufficient to cause high-level resistance (Fig. 10).

On the basis of studies in yeast (12) and mammalian cells (13, 14), in which topoisomerase II levels were inversely correlated to sensitivity to bisdioxopiperazines, one would expect to find an increased enzyme level and activity in a resistant cell line. It is, therefore, unexpected that the level of topoisomerase IIa is decreased in CHO/159-1 cells. However, this may be explained by the specificity of the alterations that appear to render the enzyme completely insensitive to ICRF-187 and could also be due to the closed-clamp form of the enzyme induced by bisdioxopiperazines (9, 10) interfering with DNA metabolism and, thus, acting as a type of poison. Furthermore, the only other published bisdioxopiperazine-resistant cell line until now also has a 50% reduction in topoisomerase IIa content, despite an equal catalytic activity in its nuclear extract (15) and, thus, a similar situation to CHO/159-1 cells. The described point mutation and deletion in CHO/159-1 topoisomerase IIa are both in highly conserved regions of the enzyme, and based on the sensitivity of the enzyme to changes in these regions (33), it is possible that the 5-residue deletion is lethal. Furthermore, as the functional topoisomerase IIa enzyme in CHO/159-1 cells appears to be fully resistant to the bisdioxopiperazines (Figs. 4–6), it is possible that these cells have one allele with a nonfunctioning gene product with the 5-residue deletion and one allele with the Y49F mutation giving rise to the specific bisdioxopiperazine resistance. This would again fit in with the approximately 50% decrease in topoisomerase IIa level in these cells (Table 3). This lack of functional protein, despite the mRNA expression of the putative allele with the 5-residue deletion, could be due to rapid degradation or faulty processing of the mRNA. The elucidation of the possible importance of the deletion of residues 309–313 will have to await side-directed mutagenesis and transformation to yeast. That the enzyme containing the Tyr49Phe mutation is both catalytically active, as well as being extremely resistant to bisdioxopiperazines, is demonstrated by the transformation of the homologous human Top50Phe enzyme to the temperature-conditional yeast system (Fig. 10).

Mutations in cells exposed to bisdioxopiperazines have not been described previously. Furthermore, mutations have not been reported previously in the proximal part of NH2-terminal eukaryote topoisomerase IIa because cell lines resistant to topoisomerase II poisons carry mutations clustered around either the Walker B/dinucleotide binding β/α/β units at residues 449–494 or near the catalytic Tyr805, reviewed in (34, 35). The Tyr49Phe mutation described here is thus, unique. Although it obviously remains to be proven, it is a reasonable hypothesis that Tyr49 is involved in the bisdioxopiperazine-binding site.

Eukaryote topoisomerase IIa has been crystallized only from residue 410 to 1202 (36), which does not include the mutations found in CHO/159-1 cDNA. However, the crystal structure of residues 2–392 in E. coli GyrB, which is the bacterial counterpart of eukaryote topoisomerase II, has been determined (37). Tyr49 in mammalian topoisomerase IIa corresponds to Tyr26 in GyrB (Fig. 7). Computer-assisted modeling using the known structure and function of GyrB (37, 38) revealed that Tyr26 in GyrB is very close (OH-to-OH distance, 4.91 Å) to the symmetry related tyrosine on the other monomer.6 The hydrogen bond formed between the OH of Tyr26 and His99 of the other monomer (which is Lys in the human enzyme) may be important in stabilizing the monomer-monomer interaction. Thus, removal of this H bond by the Tyr to Phe mutation could destabilize clamp formation and, thereby, abrogate the proposed specific closed-clamp formation induced by the bisdioxopiperazine drugs (9, 10), a hypothesis that may also fit in with this site being the drug-binding site of the enzyme. It is certainly remarkable that such a very minor change in the protein as the loss of a single OH group can lead to so marked and specific a resistance to bisdioxopiperazines. The currently accepted model of eukaryote topoisomerase II has multiple joints, constructed such that the alternating events of breaking and reforming contact surfaces at opposite ends of the enzyme are linked mechanically to several hinge segments (36). The location of the functional Tyr49Phe mutation in CHO/159-1 topoisomerase IIa, thus, indicates that the bisdioxopiperazine class of compounds act via the NH2-terminal ATPase clamp and not on the B‘ or A‘ subfragments of the enzyme.

The biochemistry of the bisdioxopiperazine-enzyme clamp interaction using purified wild-type and mutant human enzyme propagated in yeast is currently being studied and will hopefully lead to a better understanding of the molecular pharmacology of this important cellular anticancer drug target.

ACKNOWLEDGMENTS

We are indebted to Drs. A. Maxwell and N. A. Gormley (University of Leicester, Leicester, United Kingdom) for their kind assistance in comparing the mutations in CHO/159-1 topoisomerase IIa to the GyrB crystal structure and function and to Dr. Caroline Austin (University of Newcastle, Newcastle, United Kingdom) for supplying us with topoisomerase IIβ antibody. We are also much obliged to the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH (Bethesda MD), for the gifts of merbarone and fostriecin. The technical assistance of Susanne Rasmussen and Annette Nielsen is highly appreciated.

REFERENCES


6 A. Maxwell and N. A. Gormley, personal communication.


Chinese Hamster Ovary Cells Resistant to the Topoisomerase II Catalytic Inhibitor ICRF-159: A Tyr49Phe Mutation Confers High-Level Resistance to Bisdioxopiperazines


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/7/1460

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.