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Characterization of a Chinese Hamster Ovary Cell Line with Acquired Resistance to the Bisdioxopiperazine Dexrazoxane (ICRF-187) Catalytic Inhibitor of Topoisomerase II

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ABSTRACT. A Chinese hamster ovary (CHO) cell line highly resistant to the non-cleavable complex-forming topoisomerase II inhibitor dexrazoxane (ICRF-187, Zinecard®) was selected. The resistant cell line (DZR) was 1500-fold resistant (IC_{50} = 2800 vs 1.8 μM) to continuous dexrazoxane exposure. DZR cells were also cross-resistant (8- to 500-fold) to other bisdioxopiperazines (ICRF-193, ICRF-154, and ICRF-186), and somewhat cross-resistant (4- to 14-fold) to anthracyclines (daunorubicin, doxorubicin, epirubicin, and idarubicin) and etoposide (8.5-fold), but not to the other non-cleavable complex-forming topoisomerase II inhibitors suramin and merbarone. The cytotoxicity of dexrazoxane to both cell lines was unchanged in the presence of the membrane-active agent verapamil. DZR cells were 9-fold resistant to dexrazoxane-mediated inhibition of topoisomerase II DNA decatenation activity compared with CHO cells (IC_{50} = 400 vs 45 μM), but were only 1.4-fold (IC_{50} = 110 vs 83 μM) resistant to etoposide. DZR cells contained one-half the level of topoisomerase II protein compared with parental CHO cells. However, the specific activity for decatenation using nuclear extract topoisomerase II was unchanged. Etoposide (100 μM)-induced topoisomerase II–DNA complexes in DZR cells and isolated nuclei were similarly one-half the level found in CHO cells and in isolated nuclei. However, the ability of 500 μM dexrazoxane to inhibit etoposide (100 μM)-induced topoisomerase II–DNA covalent complexes was reduced 4- to 6-fold in both DZR cells and nuclei compared with CHO cells and nuclei. In contrast, there was no differential ability of aclarubicin or merbarone to inhibit etoposide-induced topoisomerase II–DNA complexes in CHO compared with DZR cells and isolated nuclei. It was concluded that the DZR cell line acquired its resistance to dexrazoxane mainly through an alteration in the topoisomerase II target. BIOCHEM PHARMACOL 53;12:1843–1853, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. resistant cell; ICRF-187; dexrazoxane; ICRF-193; bisdioxopiperazine; topoisomerase II

The bisdioxopiperazines ICRF-159 (razoxane), ICRF-154, and ICRF-193 (Fig. 1) are strong inhibitors of mammalian DNA topoisomerase II [1, 2]. This has led to a renewed interest in these compounds as both antitumor agents and probes of the mechanism of topoisomerase II [3–5]. Dexrazoxane (ICRF-187, Zinecard®) is the (+)-(S)-enantiomer of racemic ICRF-159 (razoxane), which was developed originally as an antitumor agent [6]. Dexrazoxane has just been approved in the United States, where it is used for the prevention of doxorubicin-induced cardiotoxicity [7]. Under physiological conditions, dexrazoxane undergoes a slow ring-opening hydrolysis to ADR-925 [8] (Fig. 1), an analog of EDTA. Dexrazoxane likely exerts its cardioprotective effects through its rings-opened hydrolysis product ADR-925 by virtue of its ability to chelate free iron strongly, or to remove iron quickly and efficiently from its complex with doxorubicin [9], thus reducing doxorubicin-induced iron-based oxygen free radical damage.

Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix [10]. A number of antitumor drugs including the anthracycline doxorubicin, the epipodophyllotoxins etoposide and teniposide, and amsacrine are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II–DNA intermediate (the cleavable complex) [10]. However,

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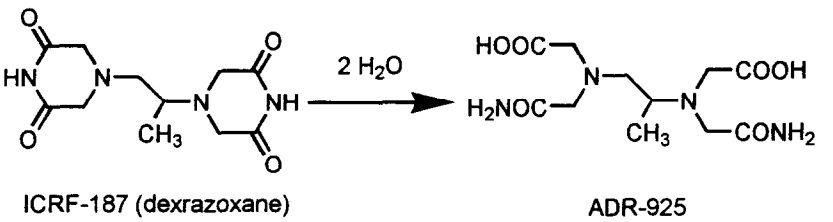

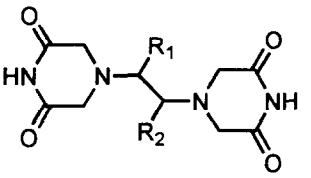

FIG. 1. Structures of the bisdioxopiperazines and ADR-925.

the bisdioxopiperazines [1], like several other cytotoxic topoisomerase II inhibitors, including the hexasulfated napthylurea suramin [11], the thiobarbituric acid merbarone [12], and the anthracycline aclarubicin [13], have been shown to inhibit topoisomerase II [1, 5] without promoting cleavable complex formation. The bisdioxopiperazines can, in fact, reduce protein–DNA cross-links induced by etoposide, amsacrine, daunorubicin, and doxorubicin [1, 5, 14] as well as reduce the growth inhibitory effects of doxorubicin and daunorubicin [5, 15]. It has been proposed that they do this by trapping the enzyme in the form of a closed protein clamp [4], thus preventing the formation or stabilization of cleavable complexes. Dexrazoxane may be promoting an energy-dependent inappropriate binding of topoisomerase II to DNA after the resealing step [16]. We previously showed that the cytotoxicity of twelve bisdioxopiperazines was highly correlated with their ability to inhibit the catalytic activity of topoisomerase II [2]. We also demonstrated that dexrazoxane was more growth inhibitory and more effective than the parental cells in inhibiting etoposide-mediated topoisomerase II–DNA covalent complexes in an etoposide-resistant K562 cell line that contains decreased topoisomerase II protein levels [14]. Hence, we have established that dexrazoxane activity is inversely proportional to topoisomerase II levels.

In this study, we report on the selection and characterization of a dexrazoxane-resistant Chinese hamster ovary cell line that is highly cross-resistant to other bisdioxopiperazines and moderately cross-resistant to other cleavable complex-forming topoisomerase II-directed drugs.

MATERIALS AND METHODS

Drugs and Chemicals

Doxorubicin, dexrazoxane, ICRF-186, ICRF-159, and ADR-925 were gifts from Pharmacia & Upjohn (Columbus, OH, U.S.A.). ICRF-154 and ICRF-193 [17] and ICRF-201 [18] were prepared essentially as described in the published literature. Daunorubicin (DNR) was a gift from Rhône-Poulenc Pharma (Montreal, Québec, Canada). Sobuzoxane (MST-16) was a gift from Dr. I. Washizawa (Zenyaku Kogyo Co., Tokyo, Japan). CP-115,953 was a gift from Dr. P. R. McGuirk (Pfizer, Groton, CT, U.S.A.). Etoposide and teniposide were gifts from Bristol–Myers Squibb (Saint-Laurent, Québec, Canada). Mafosfamide was a gift from Dr. J. Pohl (ASTA Medica, Frankfurt, Germany). Fostriecin and suramin were gifts from Dr. W. Klohs (Parke–Davis, Ann Arbor, MI, U.S.A.) and the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). Merbarone was a gift from Dr. R. Johnson (SmithKline Beecham, King of Prussia, PA, U.S.A.). DMP 840 was a gift from Dr. M. Kirshenbaum (DuPont Merck Pharmaceutical Co., Wilmington, DE, U.S.A.). Mitoxantrone was a gift from Lederle Laboratories (Pearl River, NY, U.S.A.). Drugs not listed above were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture and Cytotoxicity Assay

CHO[‖] cells (type AA8; ATCC CRL-1859) obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were grown in α-MEM (Gibco BRL, Burlington, Canada) containing 20 mM HEPES (Sigma), 100 U/mL penicillin G, 100 μg/mL streptomycin, 10% calf serum (Gibco, iron supplemented and enriched) in an atmosphere of 5% CO_2 and 95% air at 37° (pH 7.4) as previously described [2]. For the measurement of cytotoxicity by MTT assay [2], cells in exponential growth were harvested and seeded at either 2000 cells/well (CHO) or 5000 cells/well (DZR) in 96-well microtiter plates (100 μL/well) and allowed to attach for 24 hr. The drugs were dissolved in either α-MEM (dexrazoxane, ICRF-186, ICRF-159, ADR-925) or DMSO (all others), and were added to give a final volume of 200 μL/well. When DMSO was used due to

[‖]CHO, Chinese hamster ovary cell line; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DZR, dexrazoxane-resistant cell line derived from the parent CHO cell line; α-MEM, α-Minimum Essential Medium; DMEM, Dulbecco's Modified Eagle's Medium; kDNA, kinetoplast DNA; MDR, multidrug resistant; MRP, multiple drug resistance associated protein.

solubility problems, the final concentration of DMSO did not exceed 0.5% (v/v). This amount of DMSO was shown through the use of appropriate controls to have no significant effect on cell growth. The cells were then allowed to grow for a further 48 hr. Typically, six replicates were measured at each drug concentration. The IC_{50} values for growth inhibition were obtained from a non-linear least squares fit of the absorbance–drug concentration data to either a three- or a four-parameter logistic equation. The number of parameters was reduced to three by setting the limiting MTT absorbance at high drug concentration to zero when high cytotoxicity was observed. In the case of CHO cells, a biphasic survival curve was observed in the presence of dexrazoxane. Accordingly, two IC_{50} values were quantified and designated IC_{50} and IC_{50}' for the low and high concentration IC_{50} values, respectively. The IC_{50} value for each phase is the concentration of the agent at which cell growth is 50% of the maximum observed inhibition of each respective phase.

Characteristics of the Dexrazoxane-Resistant Cell Line

The dexrazoxane-resistant cell line was derived by initially growing the cells in 2 μM dexrazoxane in T-flasks. After the cells grew well at this concentration, the dexrazoxane concentration was increased gradually in a step-wise fashion over a period of 4 months until the cells were able to grow while continuously exposed to 2 mM dexrazoxane. A clone was selected by limiting dilution. The DZR clone used in these studies was found to have a doubling time of 17 hr compared with 13 hr for the parent CHO cell line. The DZR cell line was found to be stably resistant to dexrazoxane in the absence of drug exposure over a period of at least 1 year. The DZR cells used in the experiments reported here had not been exposed to dexrazoxane for at least several months.

Western Blot Analysis of Topoisomerase II

Whole cell lysates were prepared from 2–4 × 10^6 CHO or DZR cells in log phase growth by dissolving cell pellets in SDS–PAGE sample buffer [50 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.5% (v/v) β-mercaptoethanol]. Lysates were sonicated, then boiled for 5 min, and protein content was determined by the BioRad (Hercules, CA) protein assay. Protein (10–20 μg/well) was loaded onto 7% (v/v) SDS–PAGE gels. Resolved proteins were transferred electrophoretically to nitrocellulose and incubated with rabbit polyclonal antisera to human topoisomerase II prepared as described previously [19]. Bound antibody was detected using enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL, U.S.A.). Autoradiographic signals were quantified by densitometric scanning using an LKB densitometer (LKB Pharmacia, Piscataway, NJ, U.S.A.).

Topoisomerase II–DNA Covalent Complexes

Topoisomerase II–DNA covalent complex formation in intact cells and nuclei was measured as previously described [20]. Mid-log growth CHO and DZR cells were labelled for 24 hr with 0.5 μCi/mL [*methyl*-^{3}H]thymidine (0.5 Ci/mmol) and 0.1 μCi/mL [^{14}C]leucine (318 mCi/mmol) in DMEM containing 7.5% (v/v) iron-supplemented calf serum. Cells were then pelleted and resuspended in fresh DMEM/7.5% calf serum and incubated for 1 hr at 37°. Cells were pelleted and resuspended in buffer (pH 7.4) containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 25 mM HEPES, and 10 mM glucose at 37° at a final cell density of 1.0 × 10^6 cells/mL for experimentation. For isolation of nuclei, cells were washed in ice-cold buffer A (1 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, pH 6.4) [20]. Cells were then resuspended in 1 mL of buffer A, lysed by the addition of 9 mL of buffer B [0.3% (w/v) Triton X-100 in buffer A], and incubated on ice for 30 min. After lysis, 40 mL of buffer A was added, and nuclei were pelleted by centrifugation at 150 *g* for 10 min in an IEC model HN-SII tabletop centrifuge. The density of nuclei was adjusted to 1 × 10^6/mL in 37° (pH 7.4) buffer A containing 1 mM ATP for experimentation. Cells or isolated nuclei were then incubated with various concentrations of etoposide alone or in various combinations with dexrazoxane, aclarubicin, or merbarone. Reactions were stopped by adding 1 mL of cell or nuclei suspension to 10 mL of ice-cold PBS. Cells or nuclei were then pelleted and lysed, cellular DNA was sheared, and protein–DNA complexes were precipitated with SDS and KCl as described [20]. Topoisomerase II–DNA covalent complexes were quantified by scintillation counting, and [^{3}H]-DNA was normalized to cell number using the co-precipitated ^{14}C-labelled protein as an internal control.

Topoisomerase II Inhibition Assay

Topoisomerase II-containing nuclear extracts were prepared from CHO and DZR cells as previously described [2]. The final sodium chloride concentration of the nuclear extracts was 0.8 M. *Crithidia fasciculata* was labelled with 8 μCi/mL [*methyl*-^{3}H]thymidine (20 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.), and mitochondrial kDNA was isolated as previously described [21]. Topoisomerase II catalytic activity was measured by decatenation of kDNA [14, 20]. Each 40 μL assay contained 50 mM Tris (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM disodium EDTA, 30 μg/mL bovine serum albumin, 1 mM ATP, 0–1000 μM dexrazoxane or 0–200 μM etoposide (in DMSO), 1 μg (5,000–10,000 cpm) of ^{3}H-labelled kDNA, and 3 μg protein of nuclear extract topoisomerase II from each cell line. After incubation at 30° for 30 min, reactions were stopped by the addition of 10 μL of 2.5% (w/v) SDS, and were then centrifuged at 8000 *g* for 15 min at 25°. Duplicate 10-μL samples from each tube were counted in a liquid scintillation counter in 3.5 mL of Ecolite (ICN

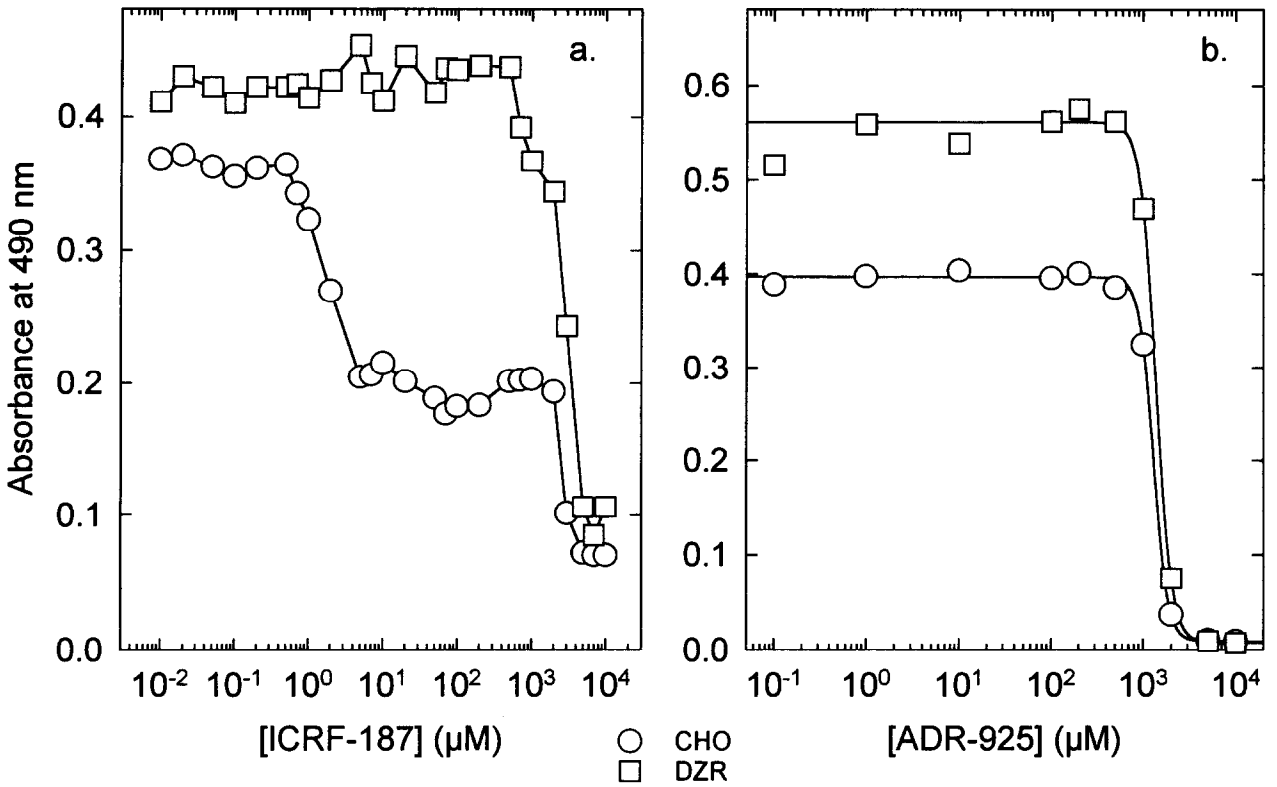

FIG. 2. (A) Inhibition of growth of CHO (○) and DZR (□) cells by dexrazoxane. An IC_{50} of 2800 ± 200 μM was calculated for DZR cells. Two IC_{50} values were calculated from the biphasic CHO survival curve. An IC_{50} value of 1.8 ± 0.2 μM was calculated for dexrazoxane concentrations ≤ 1000 μM, and an IC'_{50} value of 2710 ± 140 μM was calculated for concentrations ≥ 200 μM. The absorbances at 490 nm for the CHO cells have been scaled by a factor of 0.8 to offset the two survival curves. (B) Inhibition of growth of CHO (○) and DZR (□) cells by ADR-925. IC_{50} Values of 1290 ± 30 and 1370 ± 90 μM were calculated for CHO and DZR cells, respectively. The cells were incubated with drug for 48 hr and then assayed with MTT. The errors quoted for the IC_{50} values are SEMs obtained from the non-linear least squares fit to a logistic equation.

Biochemicals, Irvine, CA, U.S.A.). Decatenation was quantitated subsequent to subtraction of counts found in DMSO controls in the absence of nuclear extract topoisomerase II. The IC_{50} concentration for dexrazoxane and etoposide was obtained from a non-linear least squares fit of the percentage inhibition of topoisomerase II activity–drug concentration data to a two-parameter logistic equation. All errors quoted or displayed are SEMs.

RESULTS

Dexrazoxane Cytotoxicity

The cytotoxicity profiles for both the CHO parental and the DZR cell line are compared in Fig. 2a. We have shown before [2] that bisdioxopiperazine survival curves for CHO cells are biphasic, with the survival initially plateauing at about 30%. The reason for this is not known, but it may be related to the fact that even high concentrations of dexrazoxane are unable to inhibit completely formation of etoposide-induced cleavable complexes [14], possibly due to a pool of topoisomerase II in the cell that is inaccessible to dexrazoxane. At higher dexrazoxane concentrations (Fig. 2a), a second drop in the CHO survival curve is seen, with the survival dropping to near zero at the highest dexrazoxane concentrations. Thus, the CHO survival curve gives an IC_{50} value of 1.8 ± 0.2 μM in the low concentration region, and an IC'_{50} value of 2700 ± 140 μM in the higher concentration region. The DZR survival curve was, by contrast, monophasic, with a single IC_{50} of 2800 ± 200 μM. Thus, the DZR cell line is 1500-fold resistant to dexrazoxane.

ADR-925 *Cytotoxicity*

The cytotoxicity of ADR-925 (Fig. 1), the hydrolysis product of dexrazoxane, was determined because it was suspected that the common cytotoxicity seen at high dexrazoxane concentrations for both CHO and DZR cell lines (Fig. 2a) might be due to depletion of free calcium or magnesium from the growth medium by ADR-925. ADR-925 strongly binds calcium and magnesium ions with stability constants of $10^{6.9}$ and $10^{5.1}$ M^{-1}, respectively [22]. At pH 7.4 and 37°, ADR-925 is produced from dexrazoxane with a half-time of 28 hr [8]. As seen in Fig. 2b, the IC_{50} values for ADR-925 were 1290 ± 30 and 1370 ± 90 μM for CHO and DZR cells, respectively. These values compare with very high IC_{50} values of 2700 ± 140 and 2800 ± 200

TABLE 1. Cytotoxicity of various agents to CHO and DZR dexrazoxane-resistant cells*

Cytotoxic agent	CHO IC_{50} (μM)	DZR IC_{50} (μM)	Resistance factor†
Non-cleavable complex-forming topoisomerase II inhibitors			
Aclarubicin	0.027	0.13	4.8
ICRF-154	13	>250‡	>19
ICRF-159	2.7	>250‡	>90
ICRF-186	3	1600	500
Dexrazoxane	1.8	2800	1500
ICRF-193	0.017	0.3	18
ICRF-201	1.7	13.5	7.9
Merbarone	23	31	1.4
Sobuzoxane	24	64	2.7
Suramin	530	490	0.92
Cleavable complex-forming topoisomerase II poisons			
Amsacrine	5.4	8.3	1.5
CP-115,953	7	13	1.8
Daunorubicin	0.11	0.43	3.9
Doxorubicin	0.49	2.3	4.6
Epirubicin	0.23	0.92	4
Etoposide	0.72	6.1	8.5
Genistein	23	46	2
Idarubicin	0.0064	0.089	14
Mitoxantrone	0.041	0.17	4.2
Other cytotoxic agents			
ADR-925	1290	1400	1.1
Bleomycin	27	54	2
Camptothecin	0.019	0.19	10
Cisplatin	10.3	5.3	0.51
Cycloheximide	0.091	0.19	2.1
DMP-840	0.091	0.23	2.5
Fostriecin	28	67	2.4
Mafosfamide	12	9.3	0.76
Methotrexate	0.25	1	4.1
Novobiocin	242	270	1.1
Vinblastine	0.001	0.0018	1.8

*The cytotoxicity was measured by MTT assay with a 48-hr continuous exposure to the cytotoxic agent. The IC_{50} is the concentration of the agent at which cell growth is 50% of the maximum observed inhibition.

† The resistance factor was calculated from the ratio of the IC_{50} for DZR cells to the IC_{50} for CHO cells.

‡ These values are lower limits only from the highest concentration of drug that could be tested due to solubility reasons.

μM determined from the data in Fig. 2a for the cytotoxicity of dexrazoxane towards CHO and DZR cells, respectively. An exact correspondence of IC_{50} values would not be expected, as dexrazoxane is undergoing slow hydrolysis during the 48 hr the cells grow in the presence of dexrazoxane. The IC_{50} values for ADR-925 for both cell lines were similar to the calcium and magnesium ion concentrations in α-MEM of 1800 and 1000 μM, respectively. This suggests that the cytotoxicity for both cell lines seen at high dexrazoxane (Fig. 2a) and ADR-925 (Fig. 2b) concentrations is due to chelation of calcium and magnesium in the medium by ADR-925.

Cross-Resistance towards Other Cytotoxic Agents

The cytotoxicity of a variety of other topoisomerase II-targeted and non-topoisomerase II-targeted agents towards the CHO and DZR cell lines was determined to see if the DZR cell line showed cross-resistance (or increased sensitivity) (Table 1). All of the non-N-substituted bisdioxopiperazines (ICRF-154, ICRF-159, ICRF-186, ICRF-193, and ICRF-201) showed a high degree of cross-resistance (resistance factors of 7.9- to 500-fold). Given the structural similarity to dexrazoxane (Fig. 1), this result is not surprising. Sobuzoxane is an N-substituted ester analog of ICRF-154 [23] that is now being clinically used in Japan as an antitumor agent. Its 2.7-fold cross-resistance is at least 100-fold lower than that of the parent ICRF-154. It has been assumed that ICRF-154 is the active form of the drug produced from the action of esterases on the parent drug [3]. The lack of cross-resistance could be due to DZR cells not containing sufficient levels of esterases to convert sobuzoxane into ICRF-154. Alternatively, the parent or some other hydrolysis product such as formaldehyde [24] may be the active form of the drug.

Three other non-cleavable complex-forming topoisomerase II inhibitors (merbarone [12], suramin [11], and aclarubicin [25]) were examined. Of these only aclarubicin displayed a moderate amount of cross-resistance (4.8-fold).

The four anthracyclines (doxorubicin, daunorubicin, epi-

TABLE 2. Cytotoxicity of dexrazoxane towards the CHO and DZR cell lines in the presence of various concentrations of verapamil

Verapamil (μM)	CHO IC_{50}* (μM)	DZR IC_{50}* (μM)
0	1.9 ± 0.2	2800 ± 200
3	1.5 ± 0.2	2800 ± 300
10	1.4 ± 0.01	2200 ± 200

*Concentration of the drug that causes a 50% reduction of the total growth inhibitory effect on cells with a 48-hr drug incubation as measured by MTT assay. The values shown are means ± SEM from non-linear least squares curve fits of the absorbance–concentration data (N = 25) to a logistic equation as described in Materials and Methods.

rubicin, and idarubicin) and mitoxantrone, which are all cleavable complex-forming drugs [10, 26], all showed a moderate degree of cross-resistance (3.9- to 14-fold). The DZR cell line also displayed a moderate degree of cross-resistance to etoposide. None of the other cleavable complex-forming drugs (genistein, amsacrine, or the fluoroquinolone CP-115,953 [27] showed much cross-resistance.

Other cytotoxic agents that act through a variety of mechanisms were also tested for cross-resistance. Of these, camptothecin, which targets topoisomerase I [28], showed the largest cross-resistance (10-fold). Neither of the two cytotoxic imides, DMP-840 or cycloheximide, were very cross-resistant. The antitumor drug DMP-840 is an N-substituted bis-naphthalimide that intercalates DNA and inhibits RNA synthesis [29], while cycloheximide is a protein synthesis inhibitor. Novobiocin, which is an inhibitor of topoisomerase II-catalyzed ATP hydrolysis [27], showed no cross-resistance. Fostriecin, which is now thought to be cytotoxic by inhibiting a phosphatase [30], also showed little cross-resistance. Only cisplatin and mafosfamide showed a small amount of hypersensitivity towards the DZR cell line.

Lack of Effect of Verapamil on Cytotoxicity

A common mechanism by which a cell can acquire multiple drug resistance is through increased expression of the cell surface protein P-glycoprotein [31]. MDR cells that contain elevated levels of P-glycoprotein often regain their sensitivity to cytotoxic drugs in the presence of the membrane-active agent verapamil [31]. Thus, the cytotoxicity of dexrazoxane was measured in both CHO and DZR cells in the presence of 0, 3, and 10 μM verapamil (Table 2). As shown by the data in Table 2, verapamil had no significant effect on the IC_{50} values for either cell line. These results suggest that the DZR cell line does not acquire its resistance through an over-expression of P-glycoprotein.

Topoisomerase II Inhibition by Dexrazoxane and Etoposide

The dexrazoxane-mediated inhibition of the topoisomerase II decatenation activity of nuclear extracts obtained from

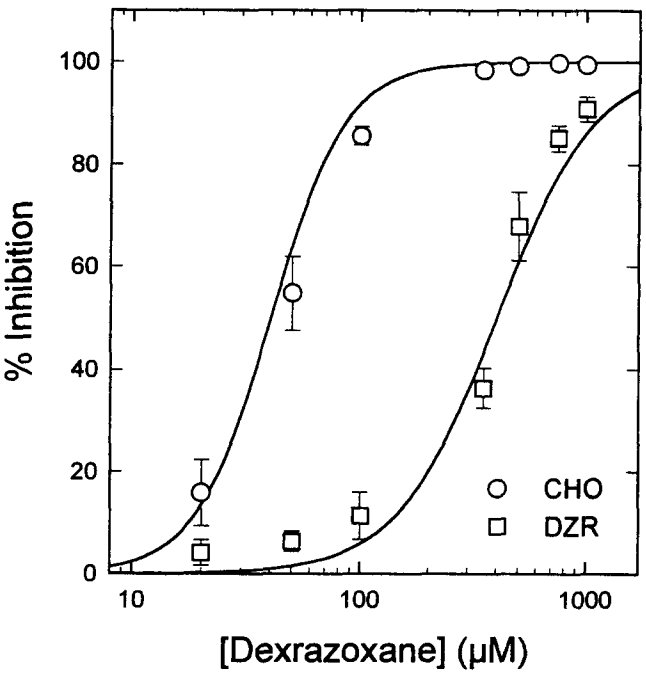

FIG. 3. Dexrazoxane-mediated inhibition of topoisomerase II catalytic activity in nuclear extracts obtained from CHO (○) and DZR (□) cells. Topoisomerase II decatenation activity was assayed as described in Materials and Methods. Under the experimental conditions used, nuclear extracts from CHO and DZR cells decatenated 85–95% of the kDNA. Inhibition of decatenation was expressed relative to decatenation activity observed in the absence of dexrazoxane. Values plotted are means ± SEM for 3–4 experiments. The solid lines are non-linear least squares calculated best fits to a two-parameter logistic equation and yield IC_{50} values of 45 ± 0.5 and 400 ± 20 μM for CHO and DZR cells, respectively.

CHO and DZR cells was measured (Fig. 3) to see if there were qualitative differences in sensitivity to inhibition by dexrazoxane. The topoisomerase II decatenation activity of the DZR-resistant cell line was inhibited by dexrazoxane with an IC_{50} value of 400 ± 30 μM (N = 3) compared with an IC_{50} of 45 ± 1 μM (N = 4) for the parent CHO cell line. Thus, topoisomerase II from DZR cells was 9-fold less sensitive (P = 0.0006 by t-test) to inhibition by dexrazoxane, indicating that the DZR-resistant cells have acquired their resistance, in part, by altering the topoisomerase II target.

The etoposide-mediated inhibition of the topoisomerase II decatenation activity of nuclear extracts was determined for both CHO and DZR cells to see if the decrease in sensitivity to dexrazoxane was paralleled by a decrease in sensitivity to etoposide. These two drugs exert their cytotoxicity through different mechanisms, as etoposide stabilizes a topoisomerase II–DNA covalent intermediate [10], while dexrazoxane does not [1]. As shown in Fig. 4, etoposide inhibited the decatenation activity with an IC_{50} of 83 ± 3 μM compared with 114 ± 3 μM (N = 4) for CHO and DZR cells, respectively. While the 1.4-fold decrease in sensitivity to etoposide for DZR compared with CHO cells was much less than the 9-fold decrease found for

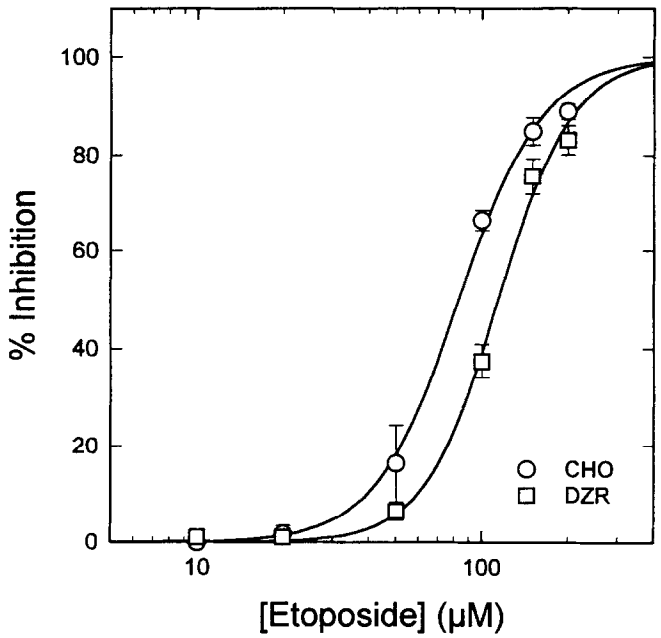

FIG. 4. Etoposide-mediated inhibition of topoisomerase II catalytic activity in nuclear extracts obtained from CHO (○) and DZR (□) cells. Topoisomerase II decatenation activity was assayed as described in Materials and Methods. Under the experimental conditions used, nuclear extracts from CHO and DZR cells decatenated 85–95% of the kDNA. Inhibition of decatenation was expressed relative to decatenation activity observed in the absence of etoposide. Values plotted are means ± SEM for 4 paired experiments. The solid lines are non-linear least squares calculated best fits to a two-parameter logistic equation and yield IC_{50} values of 83 ± 3 and 114 ± 3 μM for CHO and DZR cells, respectively.

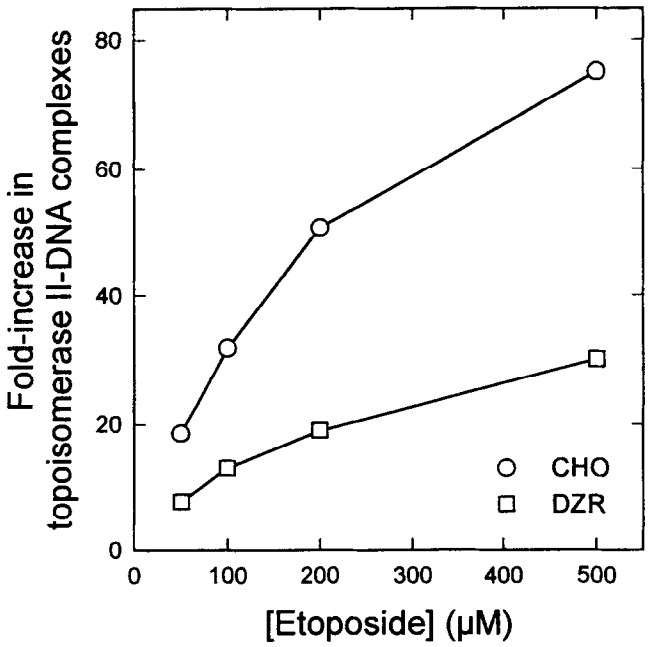

FIG. 5. Formation of topoisomerase II–DNA covalent complexes in CHO (○) and DZR (□) cells treated with etoposide. Cells were prelabelled with [*methyl*-^{3}H]thymidine and [^{14}C]leucine for 18–24 hr. Cells were incubated for 30 min in the presence of various concentrations of etoposide. KCl-SDS-precipitable complexes were isolated, and the ^{3}H counts were normalized using ^{14}C as an internal standard for cell number as described in Materials and Methods. Results are expressed as fold-increase in etoposide-induced topoisomerase II–DNA complexes relative to complexes isolated from cells incubated in the absence of etoposide. Results shown are from a single representative experiment typical of two experiments using the same range of etoposide concentrations. Ten to seventeen separate experiments were performed (with similar results) using one or more of the etoposide concentrations shown. All experiments were performed under conditions in which etoposide was incubated with both CHO and DZR cells.

dexrazoxane (Fig. 3), the difference was, nonetheless, significant ($P < 0.01$).

Inhibition of Etoposide-Induced Topoisomerase II–DNA Covalent Complexes by Dexrazoxane, Aclarubicin, and Merbarone in CHO and DZR Cells and Isolated Nuclei

Using various concentrations of etoposide, topoisomerase II–DNA complexes were decreased in resistant DZR cells compared with parental CHO cells (Fig. 5). Etoposide (100 μM)-induced topoisomerase II–DNA complexes were reduced to one-half the level in DZR cells and isolated nuclei compared with CHO cells and isolated nuclei, respectively (Fig. 6). This reduction in etoposide activity parallels the reduction in topoisomerase II protein quantified in mid-log growth phase DZR compared with CHO cells by immunoblot analysis (Fig. 6). Topoisomerase II protein in the DZR cell line was 0.48 ± 0.07 (N = 8) of that in the CHO cell line (Fig. 6). This difference was significant by the paired Student's *t*-test ($P = 0.006$). The decatenation activity of the two cell lines (on a nuclear extract protein basis) was also compared (data not shown) using nuclear extracts. Averaging the results from four experiments performed on separate days yielded 50% decatenation values of 0.52 ± 0.03 and 1.11 ± 0.04 μg nuclear extract protein for CHO and DZR cells, respectively. Hence, the amount of nuclear extract protein from the DZR cell line that was required to produce 50% decatenation was 0.50 ± 0.04 (N = 4) of that of the CHO cell line. This difference was significant by the paired Student's *t*-test ($P = 0.003$). This ratio is very close to that found (Fig. 6) for the level of the topoisomerase II protein comparing CHO and DZR cells. Together these results indicate that the DNA decatenation activity of topoisomerase II from DZR cells was unchanged, even though the topoisomerase II levels in DZR cells were decreased compared with CHO cells. It is unlikely that the 1500-fold dexrazoxane resistance observed in DZR cells is associated with a 2-fold decrease in expression of topoisomerase II protein. In fact, a decrease in the level of topoisomerase II would be expected to result in cells that were hypersensitive rather than resistant to dexrazoxane, based on previous reports indicating that bisdioxopiperazine cytotoxicity was related inversely to the level of topoisomerase II [14, 32].

Pretreatment of CHO and DZR cells with various con-

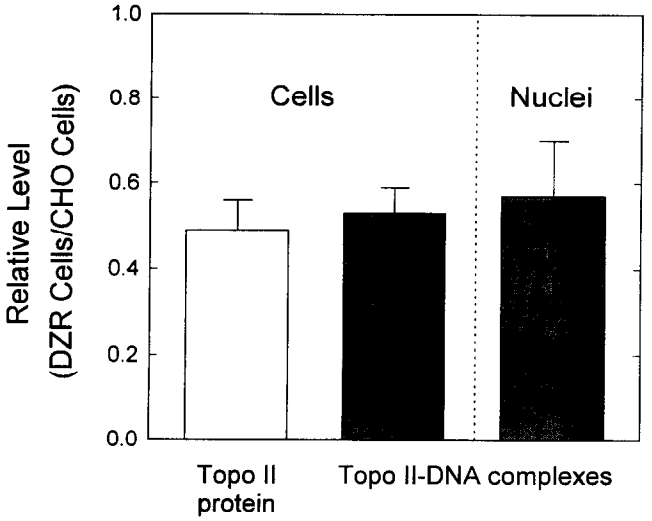

FIG. 6. Relative levels of cell topoisomerase II protein and etoposide-induced topoisomerase II–DNA covalent complexes in CHO and DZR cells and isolated nuclei. Topoisomerase II protein was measured by immunoblot analysis from log phase cells as described in Materials and Methods. Cells and nuclei were incubated for 30 min in the presence or absence of 100 μM etoposide, following which KCl-SDS-precipitable complexes were isolated as described in Materials and Methods. The fold-increase in etoposide-induced topoisomerase II–DNA complexes relative to complexes isolated from cells incubated in the absence of etoposide was calculated for each cell line, and results are presented as the ratio of etoposide effects in resistant DZR compared with sensitive CHO cells. Bars represent the mean ± SEM from 4 to 8 experiments performed on separate days.

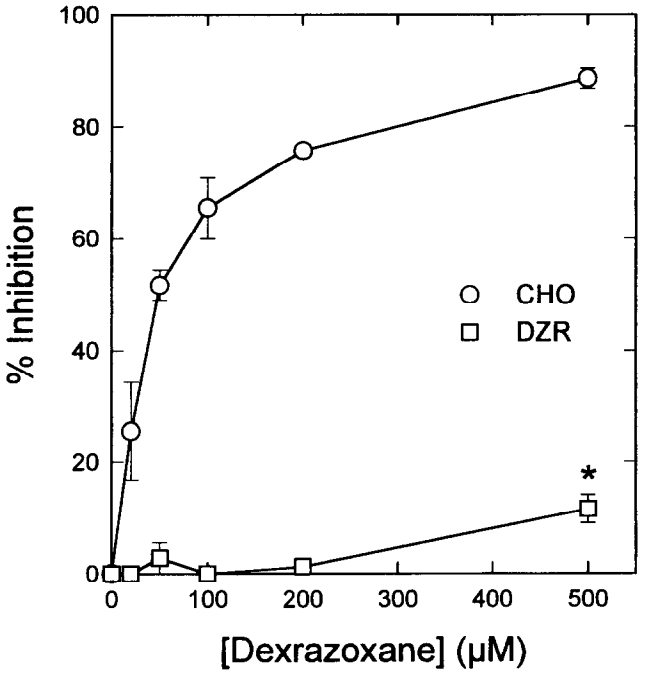

FIG. 7. Inhibition of etoposide-induced topoisomerase II–DNA covalent complexes in CHO (○) and DZR (□) cells following a 10-min preincubation with dexrazoxane. CHO and DZR cells were incubated for 10 min with the indicated concentrations of dexrazoxane and then incubated for an additional 30 min in the presence of 100 μM etoposide. KCl-SDS-precipitable complexes were isolated as described in Materials and Methods. Data shown are the mean ± range or SEM from 2 to 9 separate experiments performed on separate days. The inhibitory effects of dexrazoxane on etoposide activity in both cell lines were determined by comparing the fold-increase in etoposide-induced topoisomerase II–DNA complex formation (as in Fig. 5) in the absence and presence of dexrazoxane. Key: (*) averaging the results from 9 separate experiments showed that the inhibitory effect of dexrazoxane on DZR cells was significantly different from the dexrazoxane effect on CHO cells ($P < 0.0001$, paired Student's *t*-test).

centrations of dexrazoxane resulted in significantly less inhibition of etoposide-induced topoisomerase II–DNA complexes in DZR cells compared with CHO cells (Fig. 7). Unlike our previous results in etoposide-resistant K562 cells, where reduced topoisomerase II levels were associated with greater dexrazoxane-mediated inhibition of etoposide activity compared with parental K562 cells [14], these results indicate that dexrazoxane inhibition of etoposide activity in DZR cells is not related to topoisomerase II levels. Rather, the attenuated activity of dexrazoxane in DZR cells is likely due to an altered form of topoisomerase II present in these cells that limits the ability of this drug to inhibit etoposide activity. Averaging results from six separate experiments using aclarubicin rather than dexrazoxane to inhibit etoposide-induced topoisomerase II–DNA complexes, there was a similar concentration-dependent inhibition of etoposide activity in both CHO and DZR cells (data not shown), consistent with the low cross-resistance profile for aclarubicin (Table 1). Similarly, using merbarone at 750 and 1000 μM, there was no differential inhibition of etoposide activity in CHO cells compared with DZR cells (not shown), consistent with a lack of cross-resistance to merbarone (Table 1). When dexrazoxane, aclarubicin, and merbarone were incubated with nuclei isolated from CHO and DZR cells, only dexrazoxane showed a decreased ability to inhibit etoposide activity in DZR nuclei (Fig. 8). Hence, in dexrazoxane-resistant DZR cells there was decreased dexrazoxane-mediated inhibition of etoposide activity in cells and isolated nuclei, whereas a lack of differential inhibition of etoposide activity in DZR compared with CHO cells and nuclei was observed for both aclarubicin and merbarone, consistent with low cross-resistance to both these agents.

DISCUSSION

A CHO cell line was selected for resistance by gradually increasing the concentration of dexrazoxane in the growth medium over a period of several months followed by cloning sublines. The cloned DZR cell line was 1500-fold resistant to dexrazoxane compared with parental CHO cells. The DZR cell line displayed a high degree of cross-resistance with other structurally similar bisdioxopiperazine analogs. The other non-cleavable complex-forming inhibitors displayed only moderate (aclarubicin) or no (merbarone and suramin) cross-resistance, consistent with the idea

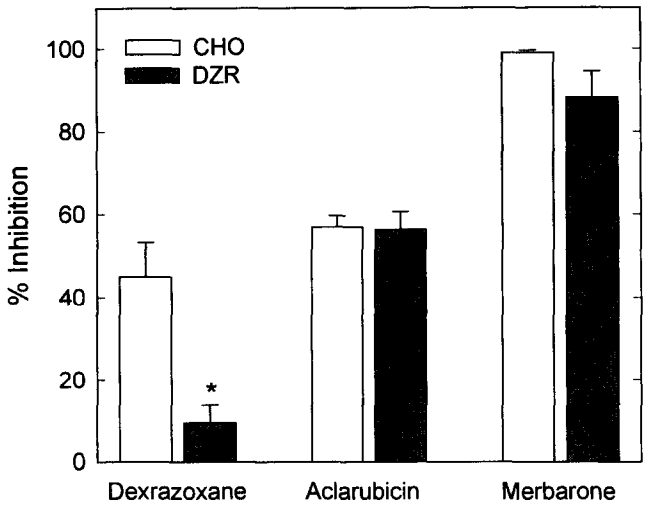

FIG. 8. Inhibition of etoposide-induced topoisomerase II–DNA covalent complexes in isolated nuclei from CHO and DZR cells following preincubation with dexrazoxane, aclarubicin, or merbarone. Isolated nuclei from CHO and DZR cells were preincubated for 10 min with dexrazoxane (500 μM) or aclarubicin (5 μM), and 30 min with merbarone (1000 μM) and then incubated for an additional 30 min with 100 μM etoposide. KCl-SDS-precipitable complexes were isolated as described in Materials and Methods. The inhibitory effects of dexrazoxane, aclarubicin, and merbarone on etoposide activity were determined as in Fig. 7. Bars are the mean ± SEM from 3 to 4 experiments performed on separate days. The inhibitory effects of these drugs on etoposide activity in nuclei from both cell lines were determined by comparing the fold-increase in etoposide-induced topoisomerase II–DNA complex formation in the absence (4.30 ± 0.50 for CHO cells; 2.42 ± 0.16 for DZR cells; mean ± SEM averaging four separate paired experiments) and presence of these drugs. Key: (*) averaging the results from 4 separate experiments showed that the inhibitory effect of dexrazoxane on DZR nuclei was significantly different from the dexrazoxane effect on CHO nuclei ($P < 0.05$, paired Student's *t*-test).

that these agents inhibit topoisomerase II by interacting with the enzyme in a manner different from that of dexrazoxane. From earlier studies on an etoposide-resistant cell line [14], it was also concluded that merbarone and dexrazoxane exert their effects through different topoisomerase II inhibitory mechanisms.

Dexrazoxane and the other bisdioxopiperazines interact with topoisomerase II without forming topoisomerase II–DNA cleavable complexes [1]. It has been shown that in the presence of ATP, ICRF-193 (Fig. 1) converts topoisomerase II to a form incapable of binding circular DNA [4]. These results were interpreted in terms of an ATP-modulated protein-clamp model [33] in which ICRF-193 binds to the closed clamp form of the enzyme and prevents its conversion to the DNA-binding open-clamp form. Based on this model of dexrazoxane binding to topoisomerase II, bisdioxopiperazine resistance in DZR cells could be related to an altered form of topoisomerase II in which the putative dexrazoxane binding site is altered. Our results are consistent with this hypothesis of an altered topoisomerase II in DZR cells. First, the ability of dexrazoxane to inhibit the topoisomerase II-mediated DNA decatenation activity of a nuclear extract from the DZR cell line was 9-fold less than for the parent CHO cell line. Inhibition of topoisomerase II catalytic activity by etoposide was only 1.4-fold different in DZR and CHO cells. Second, dexrazoxane was significantly less effective in inhibiting etoposide-induced topoisomerase II–DNA complex formation in both intact cells and isolated nuclei from resistant compared with sensitive cells. Neither aclarubicin nor merbarone showed any differential ability to inhibit etoposide activity in CHO compared with DZR cells or nuclei, consistent with a lack of cross-resistance to these agents. These results again suggest a different topoisomerase II inhibitory mechanism for these agents than for dexrazoxane.

Since dexrazoxane is limited in its ability to inhibit etoposide activity in isolated nuclei from DZR cells, it is unlikely that alterations in plasma membrane drug transport play a role in bisdioxopiperazine resistance. Also, the lack of a verapamil effect on dexrazoxane-mediated cytotoxicity in either the DZR or CHO cell line suggests that *mdr*1-mediated P-glycoprotein over-expression is not responsible for resistance to dexrazoxane. Finally, there is no cross-resistance to vinblastine in DZR cells, consistent with the absence of a P-glycoprotein mechanism of resistance.

We previously showed that an etoposide-resistant human leukemia K562 cell line with decreased topoisomerase II protein levels was more sensitive to dexrazoxane than the parent cell line [14]. This result is consistent with suggestions [32, 34] that decreased topoisomerase II levels should result in increased sensitivity to bisdioxopiperazines. Since it was demonstrated that resistant DZR cells contain one-half the level of topoisomerase II compared with parental CHO cells, we conclude that the DZR cell line does not acquire its resistance to dexrazoxane through a change in the level of topoisomerase II protein. The reduced level of topoisomerase II protein in DZR cells, however, is related to a reduced level of etoposide-induced topoisomerase II–DNA complex formation in both cells and isolated nuclei. Cross-resistance to etoposide, therefore, is due, in part, to a reduction in the level of topoisomerase II and may also be due to the observed 1.4-fold decrease in the ability of etoposide to inhibit topoisomerase II catalytic activity using nuclear extracts from sensitive compared with resistant cells. Currently, we do not understand the basis for a reduction in the level of topoisomerase II protein in DZR cells.

Reports of cells that acquire resistance to various cleavable complex-forming topoisomerase II-targeted drugs have been reviewed [35, 36]. Stable resistance can be: (1) due to decreased topoisomerase II protein expression or altered localization [37, 38], alone or together with the P-glycoprotein or MRP [39] phenotype; (2) associated with a change in the phosphorylation status of topoisomerase II [20, 40–42]; and (3) the result of a mutation(s) resulting in a qualitative change in topoisomerase II activity. There are, however, few reports of cells that acquire resistance to non-cleavable complex-forming topoisomerase II inhibi-

tors. A suramin-resistant (10-fold) lung fibrosarcoma cell line has been described [43]. Similar to the DZR cell line, this cell line displayed a 2-fold cross-resistance to the cleavable complex-forming drugs doxorubicin, etoposide, and amsacrine. The suramin-resistant cell line had unaltered levels of topoisomerase II and was unchanged in its etoposide-induced cleavable complex-forming ability. The catalytic activities of nuclear extracts of both topoisomerases I and II were 2-fold higher in the suramin-resistant cell line.

An ICRF-159 (razoxane) resistant (300-fold) CHO cell line (CHO/159-1) produced by UV-mutagenesis has been described [24, 44, 45] that is also highly cross-resistant to other bisdioxopiperazines. The topoisomerase II levels in CHO/159-1 were decreased only slightly, and the inhibition of topoisomerase II catalytic activity by etoposide was similar [45]. Similar to observations in the DZR cells, dexrazoxane was limited in its ability to inhibit etoposide-induced DNA single-strand breaks in CHO/159-1 cells. A line of BHK 21S cells has also been described [46, 47] that showed 40-fold resistance to racemic ICRF-159 (razoxane). This cell line also displayed a high degree of cross-resistance (25- to 32-fold) to several other bisdioxopiperazines [46, 47], and more moderate cross-resistance only to doxorubicin (4-fold) and daunorubicin (2.6-fold). While the dexrazoxane-resistant BHK 21S cell line is significantly less resistant than the DZR cell line, it is similar in that it shows a high level of cross-resistance to other bisdioxopiperazines and a similar level of cross-resistance to doxorubicin and daunorubicin.

Taken together, our results and those of others are consistent with acquired resistance to bisdioxopiperazines arising as a result of expression of an altered form of topoisomerase II. Topoisomerase II from DZR cells could contain point mutations that change critical regions of the enzyme required for bisdioxopiperazine binding and/or activity. Current studies are underway to determine whether DZR cells contain a mutant topoisomerase II. Because dexrazoxane and doxorubicin are used together clinically, the possibility arises that cancer cells that acquire resistance to dexrazoxane may become collaterally resistant to doxorubicin as well.

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