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# A QSAR STUDY COMPARING THE CYTOTOXICITY AND DNA TOPOISOMERASE II INHIBITORY EFFECTS OF BISDIOXOPIPERAZINE ANALOGS OF ICRF-187 (DEXRAZOXANE)

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**Abstract**—A series of twelve structurally related bisdioxopiperazines that included ICRF-187 (dexrazoxane), ICRF-159 (razoxane), ICRF-193, and ICRF-154 were examined both for their ability to inhibit the growth of Chinese hamster ovary (CHO) cells and their ability to inhibit the catalytic activity of mammalian DNA topoisomerase II. The bisdioxopiperazines exhibited a wide range in both growth inhibitory effects (30,000-fold), and in their ability to inhibit the catalytic activity of topoisomerase II (150-fold). The cytotoxicity of the bisdioxopiperazines toward CHO cells was highly correlated (correlation coefficient $r = 0.86$, $P = 0.0003$) with their inhibition of the catalytic activity of DNA topoisomerase II. This result strongly suggests that DNA topoisomerase II is the functional target of the bisdioxopiperazines. The stereoisomers (+)-ICRF-187 and (−)-ICRF-186 were observed to be equally cytotoxic and equally inhibitory toward DNA topoisomerase II. This result indicates that the bisdioxopiperazine binding site on DNA topoisomerase II is large enough or flexible enough to accommodate either form of the drug. The strongly metal-ion binding fully rings-opened hydrolysis product of ICRF-187, ADR-925, demonstrated no measurable inhibitory activity toward DNA topoisomerase II or cytotoxicity toward CHO cells.

*Key words:* ICRF-187; dexrazoxane; ICRF-193; bisdioxopiperazine; topoisomerase II; inhibition

The recent discoveries that the bisdioxopiperazines ICRF-159 (razoxane), ICRF-154 and ICRF-193 (Fig. 1) are strong inhibitors of mammalian DNA topoisomerase II [1, 2] have led to a renewed interest in these compounds as antitumor agents [3–5]. Sobuzoxane (MST-16), an ICRF-154 derivative, has undergone phase II clinical trials recently with promising results [4]. ICRF-187 (dexrazoxane, Zinecard®) is the (+)-(*S*)-enantiomer of racemic ICRF-159, which was developed originally as an antitumor [6, 7] and antimetastatic agent [8]. ICRF-187 is currently in clinical use in Europe and is in late phase III clinical trials in the U.S., where it is being used for the prevention of doxorubicin-induced cardiotoxicity [9, 10]. Under physiological conditions, ICRF-187 undergoes ring-opening hydrolysis to ADR-925 [11, 12], an analog of EDTA. ICRF-187 likely exerts its cardioprotective effects through its rings-opened hydrolysis product by virtue of its ability to strongly chelate free iron [13], or to quickly and efficiently remove iron from its complex with doxorubicin [14], thus reducing doxorubicin-induced iron-based oxygen free radical damage [15].

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 [16]. A number of antitumor drugs including doxorubicin [17], the epipodophyllotoxins VP-16‖ and VM-26, and *m*-AMSA are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex) [16, 18, 19]. However, the bisdioxopiperazines [1, 2], like several other cytotoxic topoisomerase II inhibitors, including fostriecin [20], suramin [21], merbarone [22] and aclarubicin [23], have been shown to inhibit topoisomerase II *in vitro* [1, 5] and in cultured cells [2], without inducing cleavable complex formation. The bisdioxopiperazines can, in fact, reduce protein–DNA cross-links induced by VP-16, *m*-AMSA, daunorubicin and doxorubicin [1, 2, 5]. They may do this by trapping the enzyme in the form of a closed protein clamp [24], thus preventing the formation or stabilization of cleavable complexes.

This study was undertaken to see if a series of structurally related bisdioxopiperazines with a wide range of cytotoxicities could be correlated with their ability to inhibit the catalytic activity of topoisomerase II, and thus to determine if these compounds exert their cytotoxic, and thus presumably their antitumor activity as well, through their ability to inhibit topoisomerase II.

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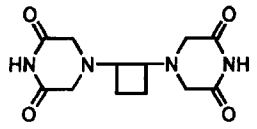
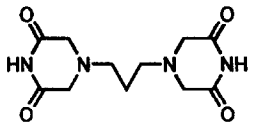
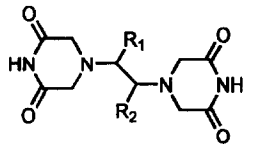
‖ Abbreviations: VP-16, etoposide; VM-26, teniposide; *m*-AMSA, amsacrine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CHO, Chinese hamster ovary; and α-MEM, α-minimum essential medium.

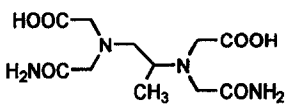


ICRF-197



ICRF-161





(*S*)-ADR-925, (±)-ICRF-198

ICRF-154 $R_1 = R_2 = H$
ICRF-159 $R_1 = H$, $R_2 = CH_3$ (racemic)
ICRF-187 $R_1 = H$, $R_2 = CH_3$ ((*S*)-(+)-enantiomer of ICRF-159)
ICRF-186 $R_1 = H$, $R_2 = CH_3$ ((*R*)-(-)-enantiomer of ICRF-159)
ICRF-192 $R_1 = H$, $R_2 = C_2H_5$ (racemic)
ICRF-193 $R_1 = R_2 = CH_3$ (meso)
ICRF-201 $R_1 = R_2 = C_2H_5$ (meso)
ICRF-202 $R_1 = CH_3$, $R_2 = C_2H_5$ (racemic, erythro)
ICRF-215 $R_1 = CH_3$, $R_2 = C_3H_7$ (racemic)
ICRF-220 $R_1 = CH_3$, $R_2 = CH_2OCH_3$ (racemic)

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

## MATERIALS AND METHODS

### *Cell culture and cytotoxicity assay*

CHO cells (type AA8) obtained from the American Type Culture Collection (Rockville, MD) were grown in α-MEM (Gibco BRL, Burlington, Canada) containing 20 mM HEPES (Sigma, St. Louis, MO), 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). Cells in exponential growth were harvested and seeded at 2000 cells/well in 96-well microtiter plates (100 μL/well) and allowed to attach for 24 hr. The drugs were dissolved either in α-MEM (ICRF-187, 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 poor solubility, 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. The cell growth was determined by the MTT assay, basically as described in Ref. 25. Briefly, 20 μL of MTT (2.5 mg/mL in 150 mM NaCl or PBS) was added to each well, and the plate was incubated for a further 4 hr. After careful aspiration of the medium, 100 μL of DMSO was added, and the plates were read at 490 nm with reference to the absorbance at 650 nm and appropriate blanks in a Molecular Devices (Menlo Park, CA) plate reader. 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 a four-parameter logistic equation. In cases where less than 100% cytotoxicity was achieved due to problems with low cytotoxicity, or poor drug solubility, the number of parameters was reduced to three by setting the limiting MTT absorbance at high drug concentration to zero. The regression analysis was carried out using SigmaStat (Jandel Scientific, San Rafael, CA).

### *Drugs*

ICRF-187, ICRF-186, ICRF-159, and ADR-925 were gifts from Adria-SP Inc. (Columbus, OH). The remaining compounds were prepared essentially as described in the published literature: ICRF-154, ICRF-193, and ICRF-197 [26]; ICRF-201, ICRF-202, ICRF-215, and ICRF-220 [27]; ICRF-192 [13]; and ICRF-161 [28].

### *Topoisomerase II inhibition assay*

Topoisomerase II-containing nuclear extracts were prepared from $1–2 \times 10^8$ human leukemia K562 cells as previously described [29]. The final sodium chloride concentration of the nuclear extracts was 0.8 M. *Crithidia fasiculata* was labelled with 8 μCi/mL [*methyl*-$^3$H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA), and kinetoplast mitochondrial DNA (kDNA) was isolated as previously described [30]. Topoisomerase II catalytic activity was measured by decatenation of kDNA [31]. Each 40-μL assay contained 50 mM Tris (pH 7.5), 85 mM potassium chloride, 10 mM magnesium chloride, 0.5 mM dithiothreitol, 0.5 mM disodium EDTA, 30 μg/mL bovine serum albumin, 1 mM ATP, a 0–100 μM concentration of the bisdioxopiperazine drug (in DMSO), 1 μg (5,000–10,000 cpm) of $^3$H-labelled kDNA and 3 μg of nuclear extract topoisomerase II from K562 cells. After incubation at 30° for 30 min, reactions were stopped by the addition of 10 μL of 2.5% (w/v) SDS, and the reaction mixtures were then centrifuged for 15 min at 8000 *g* at 25°. Duplicate 10-μL samples from each tube were counted in a liquid scintillation spectrometer in 3.5 mL of Ecolite (ICN Biochemicals, Irvine, CA). 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 each bisdioxopiperazine tested was obtained from a non-linear least squares fit of the percentage inhibition of the topoisomerase II activity–drug concentration data to a two-parameter logistic equation.

## RESULTS

Representative cytotoxicity profiles for ICRF-159 and ICRF-193 are shown in Fig. 2a. The $IC_{50}$ values of the bisdioxopiperazines tested are given in Table 1. The ring-opened form of ICRF-187, ADR-925 (Fig. 1) displayed no measurable cytotoxicity from 0.02 to 50 μM ADR-925 (data not shown). Representative inhibitory profiles for the inhibition of topoisomerase II by ICRF-159 and ICRF-193 are shown in Fig. 2b. ADR-925 also showed no significant topoisomerase II inhibition up to a concentration of 100 μM. The $IC_{50}$ values for topoisomerase II inhibition by the bisdioxopiperazines are also given in Table 1.

Linear regression was carried out on the data in Table 1 to determine the correlation between the cytotoxicity of the bisdioxopiperazines and their ability to inhibit topoisomerase II (Fig. 3, a and b). In the case of CHO cells, the regression of the log of $IC_{50}$ for the cytotoxicity versus the log of $IC_{50}$ for the inhibition of topoisomerase II catalytic activity yielded a regression line (correlation coefficient $r = 0.860$, $P = 0.0003$, $N = 12$) with a slope (±SEM) of 1.72 ± 0.32. A similar analysis of $IC_{50}$ cytotoxicity data for mouse-L cells obtained in a clonogenic assay (obtained from data in Ref. 32) gave a best fit regression line ($r = 0.883$, $P = 0.0003$, $N = 11$) with a slope of 1.73 ± 0.31. A regression analysis was also carried out to correlate the cytotoxicity of the bisdioxopiperazines toward the two cell lines and gave a best fit regression line ($r = 0.911$, $P < 0.0001$, $N = 11$) with a slope of 0.84 ± 0.10 and a constant term of 0.10 ± 0.16. The fact that the coefficient of the independent variable is close to one, and the constant term is close to zero, indicates that the bisdioxopiperazines have a very similar cytotoxicity towards these two cell lines. This result suggests that the functional target of the bisdioxopiperazines on the two cell lines is the same. A regression analysis was also carried out on both the CHO cell and mouse-L cytotoxicity data of Table 1 with the addition of log $P_{oct}$ and $(\log P_{oct})^2$ terms, individually and together, and in the absence of the log $IC_{50}$ terms for CHO cells or mouse-L cells, where $P_{oct}$ is the bisdioxopiperazine octanol-water partition coefficient. None of the additional coefficients obtained were significant at the $P < 0.05$ level.

## DISCUSSION

The highly significant correlations obtained between the cytotoxicity of the bisdioxopiperazines towards both cell types (CHO and mouse-L) with topoisomerase II inhibition strongly suggest that the bisdioxopiperazines primarily exert their cytotoxicity through their ability to inhibit topoisomerase II. The $IC_{50}$ values for topoisomerase II inhibition from Table 1 for ICRF-193, ICRF-154 and ICRF-159 of 0.6, 2.9 and 9.6 μM, respectively, compare reasonably well to values of 2, 13 and 30 μM, respectively [1], for inhibition of calf thymus topoisomerase II catalytic activity that were determined by densitometry scanning of photographs of gels. The fact that inclusion of linear and square log $P_{oct}$ terms in the regression analysis did not yield coefficients that were significant indicates that the activity of the bisdioxopiperazines is not significantly dependent on their hydrophobicity. However, it must be noted that the addition of either one or two new independent variables to the regression analysis results in an over-parameterized regression equation for the number of available observations.

ICRF-187 is the (+)-(*S*)-enantiomer of racemic ICRF-159, while ICRF-186 is the corresponding (−)-(*R*)-enantiomer (Fig. 1). As can be seen from the data in Table 1, these drugs differ very little in either their cytotoxicity or their inhibition of topoisomerase II. These results indicate that the bisdioxopiperazine binding site on topoisomerase II is large enough or flexible enough to accommodate the methyl group in either stereo configuration.

ICRF-187 undergoes ring-opening hydrolysis with a

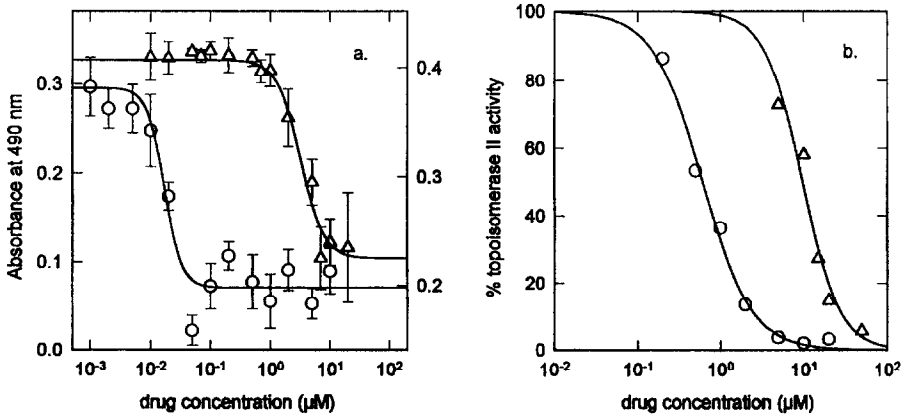



Fig. 2. (a) Inhibition of growth of CHO cells by ICRF-193 (○) and ICRF-159 (△). The CHO cells were incubated with drug for 48 hr and then assayed with MTT. The errors shown are the standard deviations of absorbance values at 490 nm of six wells. The solid lines are non-linear least-squares calculated best fits to a four-parameter logistic equation and yield $IC_{50}$ values of 3.2 ± 0.5 and 0.017 ± 0.003 μM, respectively. For clarity, the ordinate of ICRF-159 is on the left and the ordinate of ICRF-193 on the right. (b) Inhibition of topoisomerase II catalytic activity by ICRF-193 (○) and ICRF-159 (△). Topoisomerase II decatenation activity was assayed as described in Materials and Methods. The solid lines are non-linear least-squares calculated best fits to a two-parameter logistic equation and yield $IC_{50}$ values of 9.6 ± 1.0 and 0.61 ± 0.03 μM, respectively. All errors of the $IC_{50}$ values quoted are SEM obtained from the non-linear least-squares fitting.

Table 1. Bisdioxopiperazine cytotoxicity and topoisomerase II inhibition

| Drug | CHO $IC_{50}$* (μM) | Mouse-L $IC_{50}$† (μM) | Topo II $IC_{50}$‡ (μM) | log $P_{oct}$§ |
|---|---|---|---|---|
| ICRF-193 | 0.017 | 0.09 | 0.6 | −0.8 |
| ICRF-202 | 0.067 | 0.045 | 0.9 | −0.4 |
| ICRF-220 | 1.1 | 0.11 | 4.1 | −2.2 |
| ICRF-201 | 1.7 | 0.90 | 6.9 | −0.1 |
| ICRF-186 | 2.6 | 3.0 | 10 | −1.8 |
| ICRF-187 | 3.0 | 3.0 | 13 | −1.8 |
| ICRF-159 | 3.2 | 3.0 | 9.6 | −1.8 |
| ICRF-215 | 7.4 | 1.9 | 3.1 | −0.6 |
| ICRF-197 | 11 | 55 | 30 | −1.5 |
| ICRF-154 | 13 | 7.3 | 2.9 | −2.3 |
| ICRF-192 | 340 | 720 | 91 | −1.0 |
| ICRF-161 | 500 | ⩾800 | 33 | −1.8 |
| ADR-925 | ⩾50 | ND‖ | ⩾100 | ND |
| ICRF-198 | ND | ⩾400 | ND | ND |

* This study. The drug concentration at which CHO cell growth (as measured by MTT assay) is 50% of the maximum observed inhibition.

† The $IC_{50}$ for 50% inhibition of growth of mouse-L cells measured by clonogenic assay. The data are from Refs. 32 and 33.

‡ This study. $IC_{50}$ for inhibition of topoisomerase II activity.

§ Log of octanol–water partition coefficient from Refs. 32 and 33.

‖ ND = not determined.

$T_{1/2}$ of 9.3 hr (pH 7.4, 37°) [11, 12] to yield its one-ring open intermediates. Due to the obligatory formation of these intermediates, the strongly metal-ion binding fully hydrolyzed form of the drug ADR-925 (Fig. 1) lags behind, and is formed with a $T_{1/2}$ of 28 hr [11, 12]. ADR-925 itself is unlikely to be cellular permeable due to its negative charge. The fact that ADR-925 does not inhibit topoisomerase II indicates that it is not cytotoxic through any action on topoisomerase II in the cell. This conclusion is also supported by the observation that the cytotoxicity of ICRF-159 toward HEp/2 cells is lost with a $T_{1/2}$ of 12 hr [34] when the drug is preincubated in the culture medium (pH 6.8 to 7.0, 37°). ADR-925 is such a strong chelating agent [13] that it may be able to remove metal ions from the active sites of metalloenzymes that are involved in cell growth and replication. Thus, it cannot be ruled out that the ability of the bisdioxopiperazines to form strong chelating agents intracellularly may also contribute, in some part, to their overall cytotoxicity.

The bisdioxopiperazines are not unique in their ability to inhibit topoisomerase II without stimulating DNA–topoisomerase II cleavable complex formation [1, 2, 5]. Other drugs that act similarly include aclarubicin [23], suramin [21], merbarone [22] and fostriecin [20]. Since the cytotoxicity of the cleavable complex-stabilizing drugs is thought to be due to cellular responses to the stabilized complex [16], the bisdioxopiperazines may have the potential to antagonize the cytotoxicity and possibly the antitumor activity of these drugs when they are administered together. It has been shown [5] that ICRF-187 antagonizes both VP-16 and daunorubicin (but not

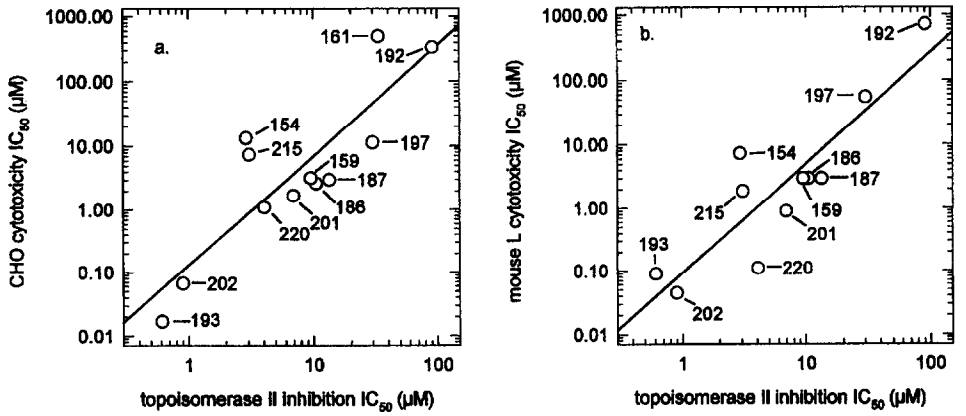



Fig. 3. (a) Plot of $IC_{50}$ for cytotoxicity of bisdioxopiperazines toward CHO cells versus $IC_{50}$ for inhibition of topoisomerase II catalytic activity. (b) Plot of $IC_{50}$ for cytotoxicity of bisdioxopiperazines toward mouse-L cells versus $IC_{50}$ for inhibition of topoisomerase II catalytic activity. The solid straight lines are best fit regression lines calculated as described in Results. The individual drugs are identified on the plots with a label with the last three numbers of their name.

doxorubicin) cytotoxicity towards OC-NYH cells in a clonogenic assay in a dose-dependent manner. However, doxorubicin and ICRF-187 have also been shown to synergistically inhibit the proliferation of murine sarcoma S180 cells [35]. ICRF-193 also reverses VP-16-induced growth inhibition of RPMI 8402 cells [2]. Similarly, aclarubicin also antagonizes the cytotoxicity of VP-16 [23, 36], as does fostriecin for VM-26 [20]. It has been shown that in the presence of ATP, ICRF-193 converts topoisomerase II to a form incapable of binding circular DNA [24]. These results were interpreted in terms of an ATP-modulated protein-clamp model [37] in which ICRF-193 binds to the closed clamp form of the enzyme and prevents its conversion to the DNA-binding open-clamp form.

The myelosuppressive toxicity that is seen when ICRF-187 is used clinically as an antitumor drug [38] may be due to topoisomerase II inhibition. If this is so, then a bisdioxopiperazine analog that did not inhibit topoisomerase II might not be as toxic, and would also not interfere with the action of anthracyclines on topoisomerase II. The results of this study firmly establish that the bisdioxopiperazines are cytotoxic through their ability to inhibit topoisomerase II catalytic activity, and thus support further investigation of bisdioxopiperazines for their development as potential antitumor agents.

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