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Preclinical paper

The one-ring open hydrolysis intermediates of the cardioprotective agent dexrazoxane (ICRF-187) do not inhibit the growth of Chinese hamster ovary cells or the catalytic activity of DNA topoisomerase II

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Dexrazoxane (ICRF-187), which is clinically used to reduce doxorubicin-induced cardiotoxicity, has growth inhibitory properties through its ability to inhibit the catalytic activity of DNA topoisomerase II. Because the bisdioxopiperazine dexrazoxane undergoes significant ring-opening hydrolysis under physiological conditions to form two one-ring open hydrolysis intermediates, a study was undertaken to determine if these two intermediates had either any growth inhibitory or topoisomerase II inhibitory effects. Neither of the one-ring open intermediates exhibited growth inhibitory effects towards Chinese hamster ovary cells nor were they able to inhibit topoisomerase II. Thus, it was concluded that only intact dexrazoxane is able to inhibit the catalytic activity of topoisomerase II. [© 1998 Lippincott-Raven Publishers.]

Key words: Bisdioxopiperazine, CHO, cytotoxicity, dexrazoxane, hydrolysis intermediates, ICRF-187, topoisomerase II.

Introduction

Dexrazoxane (ICRF-187, Zinecard®, Figure 1, A) has recently been approved for clinical use in the USA and Canada, where it is being used to reduce doxorubicin-induced cardiotoxicity.[1-4] We have previously shown that under physiological conditions dexrazoxane undergoes an initial hydrolysis to two one-ring open hydrolysis products (Figure 1, B and C) and a final hydrolysis to its presumably active metal ion chelating form D (ADR-925),[5,6] an analog of EDTA (Figure 1). The one-ring open hydrolysis intermediates B and C have been shown to efficiently displace Fe^{3+} from Fe^{3+}-anthracycline complexes.[7] These hydrolysis intermediates have also been shown to undergo a metal ion-promoted (Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}) hydrolysis to ADR-925.[8,9] We have also previously shown that the enzyme dihydropyrimidine amidohydrolase, which is present in the liver and the kidney, but not the heart, is able to efficiently catalyze the hydrolysis of either of the rings on intact dexrazoxane, but is not able to catalyze the ring-opening hydrolysis of the remaining ring on either B or C.[10-12] Dexrazoxane likely exerts its cardioprotective effects through its rings-opened hydrolysis product ADR-925 by virtue of its ability to strongly chelate free iron[13] or to quickly and efficiently remove iron from its complex with doxorubicin,[17] thus reducing doxorubicin-induced iron-mediated oxygen free radical damage.[14]

Dexrazoxane is the (+)-(*S*)-enantiomer of racemic ICRF-159, which was originally developed as an antitumor agent.[15,16] The bisdioxopiperazines [including dexrazoxane, ICRF-159 (razoxane), ICRF-154 and ICRF-193] are now known to be strong inhibitors of mammalian DNA topoisomerase II.[17-20] 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.[21] Doxorubicin[22] and daunorubicin,[23] like etoposide and amsacrine, are thought to inhibit cell growth by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex).[21,24,25] In contrast, the bisdiox-

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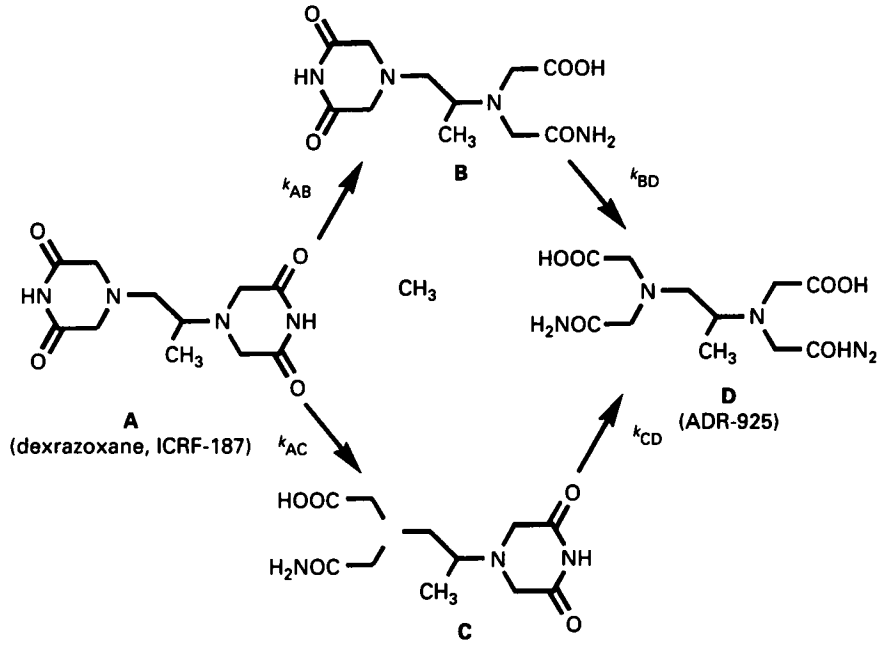

Figure 1. Reaction scheme for the hydrolysis of dexrazoxane (**A**) to the one-ring open hydrolysis intermediates **B** and **C**, and then to ADR-925 (**D**). The rate constants k_{AB}, k_{AC}, k_{BD} and k_{CD} are pseudo-first-order rate constants at a constant pH.

opiperazines and dexrazoxane[17,18] inhibit topoisomerase II catalytic activity *in vitro*[17,26] and in cultured cells,[18] without inducing cleavable complex formation. The bisdioxopiperazines can, in fact, reduce protein-DNA cross-links induced by etoposide, amsacrine, daunorubicin and doxorubicin,[17,18,26] and antagonize the growth inhibitory effects of doxorubicin and daunorubicin.[20,26] The bisdioxopiperazines may act by trapping the enzyme in the form of a closed protein clamp,[27] thus preventing the formation or stabilization of the topoisomerase II-DNA intermediate. The observation that short (approximately 1 h) exposure of cells to dexrazoxane does not result in growth inhibition[20] is suggestive that the hydrolysis intermediates B and C, which are only slowly formed,[5,6] may be the active forms of dexrazoxane. Given the current importance of dexrazoxane as a cardioprotective agent, the utility of bisdioxopiperazines as probes of the mechanism of topoisomerase II and their potential as antitumor agents, a study was undertaken to determine whether the hydrolysis intermediates (B and C, Figure 1) were either wholly or partly responsible for the growth inhibitory effects of dexrazoxane or the inhibition of topoisomerase II. We previously showed that the hydrolysis product ADR-925 had only weak growth inhibitory effects towards CHO cells[19,28] and did not effectively inhibit topoisomerase II catalytic activity.[19]

Materials and methods

Cell culture and growth inhibition assays

Chinese hamster ovary (CHO) cells (type AA8; ATCC CRL-1859), 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 units/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°C (pH 7.4) as previously described.[20]

Cells in exponential growth were harvested and seeded (2000 cells/well) in 96-well microtiter plates (100 μl/well) and allowed to attach for 24 h. Dexrazoxane (Pharmacia & Upjohn, Columbus, OH), B and C were dissolved in α-MEM and were added to give a final volume of 200 μl/well. All growth inhibitory experiments were conducted with continuous exposure to drug for 48 h. The cell growth was determined by MTT assay.[29] Briefly, 20 μl of MTT (2.5 mg/ml in PBS, Dulbecco's phosphate-buffered saline) was added to each well and the plate was incubated for a further 4 h. After careful aspiration of the medium, 100 μl of DMSO was added and the absorbance was read at 490 nm in a plate reader (Molecular Devices, Menlo Park, CA) with reference to the absorbance at 650 nm

and appropriate blanks. 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 (SigmaPlot; Jandel, San Rafael, CA).

Topoisomerase II inhibition assay

Topoisomerase II-containing nuclear extracts were prepared from human leukemia K562 cells as previously described.[19] The final NaCl concentration of the nuclear extracts was 0.8 M. *Crithidia fasiculata* was labeled with 8 μCi/ml [methyl-^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) and mitochondrial kDNA (kinetoplast DNA) was isolated as previously described.[30] Topoisomerase II catalytic activity was measured by decatenation of kDNA.[28,31,32] 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 and either 0 or 1-100 μM of dexrazoxane, B or C, 1 μg (5000-10000 c.p.m.) of ^{3}H-labeled kDNA and 3 μg nuclear extract protein from K562 cells. After incubation at 30°C for 30 min, reactions were stopped by the addition of 10 μl of 2.5% (w/v) sodium dodecyl sulfate and were then centrifuged for 15 min at 8000 *g* at 25°C. Duplicate 10 μl samples from each tube were counted in a liquid scintillation counter in 3.5 ml of Ecolite (ICN Biochemicals, Irvine, CA). Decatenation was quantitated subsequent to subtraction of counts found in controls in the absence of nuclear extract topoisomerase II. The IC_{50} for dexrazoxane was obtained from a non-linear least squares fit of the topoisomerase II activity-dexrazoxane concentration data to a two-parameter logistic equation.

HPLC quantitation and separation of dexrazoxane and its hydrolysis products B and C

The HPLC apparatus consisted of a programmable Varian 9010 pump, Varian 9050 variable wavelength detector, Varian Star ingetrator software, a Rheodyne injector with a 10 μl loop and a Waters μBondpak C_{18} (10 μm, 3.9 × 300 mm) reversed-phase column. The HPLC quantitations of dexrazoxane and levrazoxane and their hydrolysis products B and C were carried out with a methanol gradient in the presence of 500 μM Na_2EDTA (pH 4.9) as previously described.[5,6,8] The EDTA was necessary to scavenge interfering metal ions from the flow system. Dexrazoxane (A), B, C and ADR-925 (D) (Figure 1) eluted from the column with retention times of 15.6, 6.3, 5.4 and 3.4 min, respectively. Dexrazoxane was detected by its absorbance at 215 nm and the hydrolysis intermediates B and C at 205 nm. The ring-open intermediates B and C were isolated by HPLC in the absence of EDTA in the eluant as previously described.[6,8] B and C were obtained from partially hydrolyzed dexrazoxane, which was prepared by adding 40 μl of 1 M NaOH to 500 μl of 5 mg/ml dexrazoxane. The reaction was allowed to proceed for 40 min at 25°C, was quenched with 40 μl of 1 M HCl and stored at 4°C to prevent further hydrolysis. The peak fractions (approximately 0.4 ml each) of the partially hydrolyzed dexrazoxane sample were collected in 20 mM HCl and pooled. These pooled fractions were freeze-dried and stored at −20°C until needed, at which time stock solutions were made in 20 mM HCl. The B and C so obtained were rechromatographed to check their purity. The purity of B was greater than 99.8% and contained no detectable amounts of C or D; and C was 98.9% pure, with the remaining 1.1% being B, due to a small amount of tailing that occurred at the high sample loadings used. All errors quoted or shown are SEMs unless otherwise indicated.

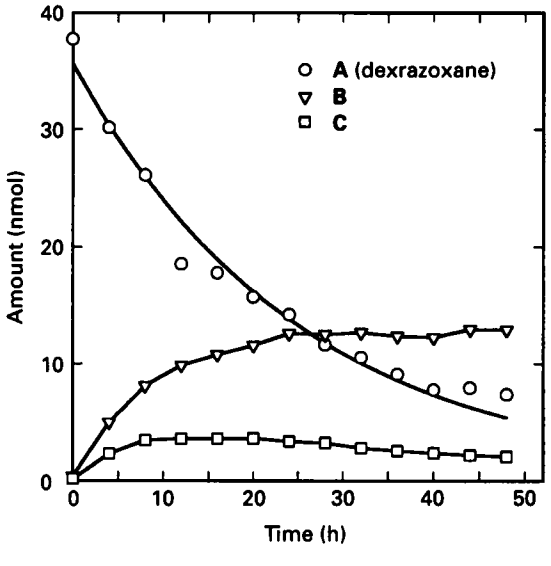

Figure 2. HPLC-determined amounts of dexrazoxane (○), **B** (▽) and **C** (□) upon incubation in 20 mM HEPES buffer (pH 7.4) at 37°C in a CO_2 (5% v/v) incubator as a function of time. The upper smooth solid line is calculated from a non-linear least fit of the dexrazoxane data to a two-parameter exponential decay equation. The solid straight lines for the **B** and **C** data serve only to connect the data points.

Results

HPLC quantitation of the hydrolysis of dexrazoxane

As shown in Figure 2, incubation of dexrazoxane (2 mg/ml) in HEPES buffer (20 mM), pH 7.4 in air and 37°C in a CO_2 (5% v/v) incubator under conditions identical to those used in the growth inhibition determinations, resulted in the loss of dexrazoxane from the incubation mixture and an initial slow build up of B and C, and the slow decrease of C over the 48 h of the experiment. The differential equations for the reaction scheme in Figure 1 have been solved.[5,6] The loss of A from the reaction mixture is a first-order process with an observed first-order rate constant $k_{obs} = k_{AB} + k_{AC}$. A fit of the concentration-time data for A to a two-parameter exponential decay equation yielded a k_{obs} of 0.039 $\pm$ 0.002 h^{-1} ($t_{½}$ 17.6 h). This value compares to a k_{obs} of 0.074[5] and 0.079 h^{-1} [6] at the same pH and temperature but in Tris buffer (100 mM) and phosphate buffer (50 mM), respectively. The hydrolysis of dexrazoxane has been previously shown to be very sensitive to both pH and buffer.[5,6]

The effect of preincubated dexrazoxane on CHO cell growth

In the experiments shown in Figure 3(a) the growth inhibitory effects of dexrazoxane that had been preincubated in a 5% CO_2 incubator at 37°C and 20 mM HEPES buffer (pH 7.4) for the times indicated (0-48 h) prior to drug treatment of the cells were measured. The IC_{50} values were observed to steadily increase from 3.6 $\pm$ 0.3 μM with no buffer preincubation of dexrazoxane, to 15.1 $\pm$ 1.4 μM with 48 h preincubation of dexrazoxane in HEPES buffer before drug addition to attached cells (Figure 3b). The decrease in growth inhibition with time suggests that dexrazoxane is the active growth inhibitory species, since at times up to 48 h the amounts of B and C have achieved or passed their maxima, while dexrazoxane is increasing with time. The IC_{50} value is plotted as a percentage dexrazoxane remaining (as determined by HPLC) in the preincubated dexrazoxane stock solution (Figure 3c). The IC_{50} was observed to vary linearly with the percentage dexrazoxane remaining ($r^2 = 0.96$), again strongly suggesting that it is dexrazoxane that is the active growth inhibitory species.

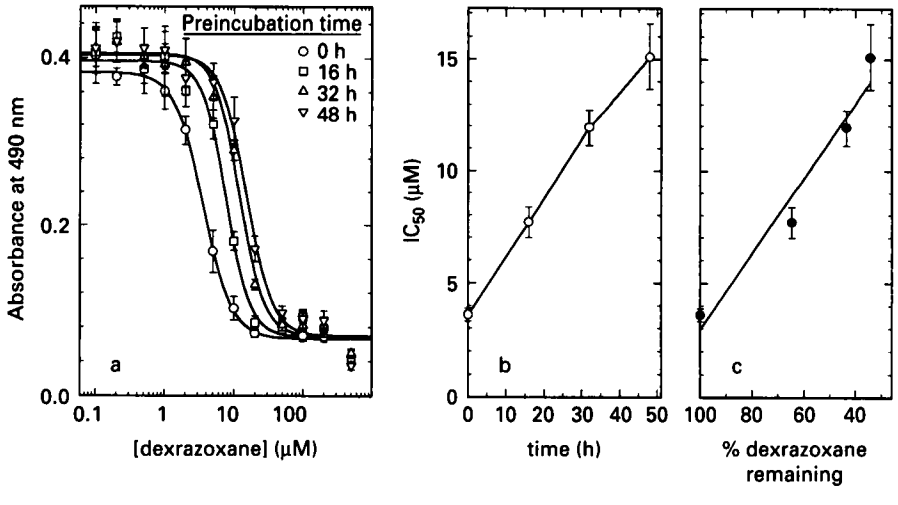

Figure 3. The growth inhibitory effects on CHO cells of continuous exposure of dexrazoxane that had been preincubated in buffer for various times. (a) Dexrazoxane was preincubated in 20 mM HEPES buffer (pH 7.4) at 37°C for 0 (○), 16 (□), 32 (△) or 48 (▽) h before addition to attached cells. The CHO cells were continuously incubated with drug for 48 h and then assayed with MTT. The solid curves are calculated from four-parameter non-linear least squares fits to a logistic equation. The error bars shown are SDs from six replicate determinations on the multiwell plate. (b) Effect of time of preincubation of dexrazoxane on IC_{50} (○) obtained from the growth inhibition curves in (a) to the left. The straight lines serve only to connect the data points. (c) Effect of percentage of dexrazoxane remaining in the incubation mixture of dexrazoxane on the IC_{50} values (●) obtained from the growth inhibition curves in (a). The percentage dexrazoxane remaining was determined by HPLC. The straight line is calculated by linear least squares. The error bars in (b) and (c) are SEMs calculated from the non-linear least squares fit to a logistic equation.

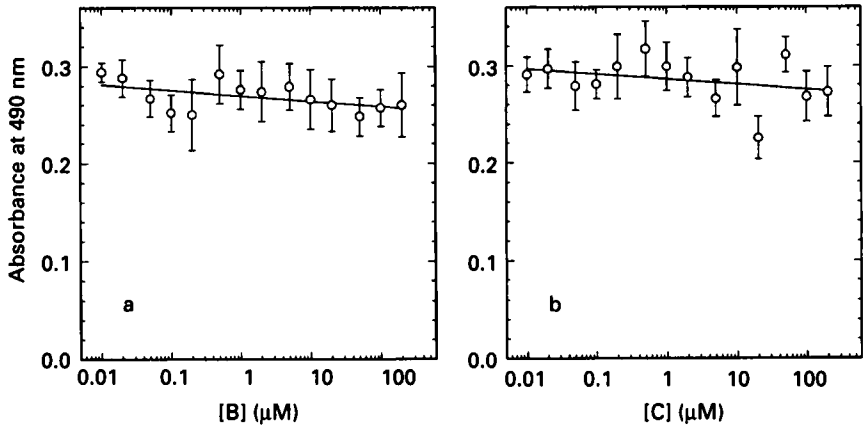

Figure 4. The effect of hydrolysis intermediates (a) **B** and (b) **C** on growth of CHO cells. The CHO cells were continuously incubated with **B** or **C** for 48 h and then assayed with MTT at 490 nm. The straight lines are linear least squares calculated.

Lack of growth inhibitory effects of dexrazoxane hydrolysis intermediates B and C

The effect of B and C on the growth of CHO cells was determined as shown in Figure 4(a and b). These results show that neither B and C displayed any significant effect on the growth of CHO cells. These results also suggest that neither B nor C are the active growth inhibitory species.

Lack of effect of B and C on the decatenation activity of topoisomerase II

Under conditions which cell culture was carried out, both B and C would be expected to be anionic species, and thus may not be easily taken up by CHO cells. Thus, these experiments cannot rule out that B and C inhibit cellular topoisomerase II after they are formed by hydrolysis in the cell, subsequent to their uptake as neutral dexrazoxane. In order to explore this possibility, the effect of B and C on the decatenation activity of nuclear extract topoisomerase II was investigated in a cell-free system (Figure 5). As shown in Figure 5, neither B nor C exhibited any significant inhibition of topoisomerase II decatenation activity. The IC_{50} value for the inhibition of topoisomerase II decatenation activity by dexrazoxane itself was determined to be 13.5 ± 0.2 μM. This result agrees closely with a previous determination of 13 μM.

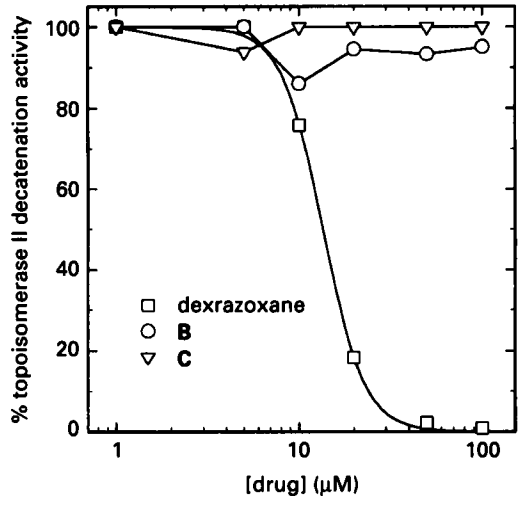

Figure 5. Inhibition of DNA topoisomerase II decatenation activity by dexrazoxane (□), **B** (○) or **C** (▽). Topoisomerase II decatenation activity was assayed as described in Materials and methods. The solid curve for dexrazoxane is a non-linear least squares calculated best fit to a two-parameter logistic equation and gives an IC_{50} value of 13.5 ± 0.2 μM. The straight lines for the **B** and **C** data serve only to connect the data points. While the results are from a single experiment, they are typical of those obtained from three experiments conducted on different days.

Discussion

The results of this study clearly show that only the parent compound dexrazoxane has the ability to inhibit topoisomerase II and inhibit the growth of CHO cells. Over the time (48 h) that the CHO cells were incubated with dexrazoxane, it was shown by HPLC that significant amounts of dexrazoxane had hydrolyzed to produce the one-ring open intermediates B and C (Figure 2). The loss of the growth inhibitory properties of dexrazoxane that had been preincubated at 37°C at pH 7.4 for various times closely correlated

with the loss of dexrazoxane from the preincubation mixture (Figure 3c). In a previous report, the biological half-life of ICRF-159 (the racemate of dexrazoxane) was shown to be about 12 h in complete culture medium (pH 6.8–7.0).[34] This is somewhat shorter than what was observed for dexrazoxane in this study but the difference could be due to differences in solution composition or pH. The one-ring open hydrolysis products B and C were shown to have no growth inhibitory effects on CHO cells and were also unable to inhibit topoisomerase II decatenation activity. Consistent with bisdioxopiperazine hydrolysis to rings-opened inactive forms, Ishida *et al.* reported that complete growth inhibition of RPMI 8402 cells treated with ICRF-154 or ICRF-193 required a daily change of media and addition of fresh drug.[18]

We previously showed that the two-ring open hydrolysis product ADR-925 (D) showed no growth inhibitory effects up to 1000 μM[28] and did not inhibit topoisomerase II.[19] The IC_{50} value of 1300 μM previously measured[28] for ADR-925 (D), which is a strong metal chelator, is probably due to ADR-925 causing significant depletion of Ca^{2+} and Mg^{2+} from the culture media, rather than inhibition of topoisomerase II. These results taken together indicate that of the four compounds in Figure 1, only dexrazoxane has the ability to inhibit topoisomerase II. The fact that B and C lack the ability to inhibit topoisomerase II may indicate that two intact imide rings are required to inhibit topoisomerase II. Our structure–activity study[19] using 12 structurally related bisdioxopiperazines, all of which had two intact imide rings, demonstrated a strong correlation of CHO growth inhibition with strength of topoisomerase II inhibition, indicating that bisdioxopiperazine-mediated cell growth inhibition occurs through the inhibition of topoisomerase II.[19] This study also showed that the strength of topoisomerase II inhibition was very sensitive to both the number and types of substituents on the main alkane chain, as well as the alkane central chain length. Our recent finding that the highly conformationally constrained bisimide mitindomide that has nearly co-planar imide rings also acted as a catalytic inhibitor of topoisomerase II at the bisdioxopiperazine binding site also suggests that two intact immide rings are required for topoisomerase II inhibitory activity.[33] However, it cannot be ruled out that B and C fail to inhibit topoisomerase II because of their high polarity or their negative charge.

Conclusion

This study has shown that the two one-ring open hydrolysis intermediates of the bisdioxopiperazine dexrazoxane are unable to either inhibit the growth of CHO cells or inhibit the catalytic activity of DNA topoisomerase II. Thus, intact dexrazoxane is the active form of the drug that is responsible for inhibition of topoisomerase II.

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