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The Catalytic DNA Topoisomerase II Inhibitor Dexrazoxane (ICRF-187) Induces Differentiation and Apoptosis in Human Leukemia K562 Cells

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ABSTRACT

The bisdioxopiperazines ICRF-187 (dexrazoxane), ICRF-193, and ICRF-154 are catalytic noncleavable complex-forming inhibitors of DNA topoisomerase II that do not produce protein-linked DNA strand breaks. In this study, we showed that bisdioxopiperazines induced erythroid differentiation, inhibited human leukemia K562 cell growth, and caused a slow induction of apoptosis. Dexrazoxane treatment caused DNA endoreduplication resulting in large highly polyploid cells. This result suggested the lack of a DNA topoisomerase II activity-based cell cycle checkpoint. The percentage of K562 cells that became apoptotic was much larger than the percentage of cells that stained for hemoglobin, suggesting that prior differentiation was not required for induction of apoptosis. Use of the Bcr-Abl tyrosine kinase inhibitor STI-571 resulted in a reduction in Bcl-xL levels and potentiation of dexrazoxane-induced apoptosis related to an earlier onset and more extensive cleavage of caspase-3. These results indicated that dexrazoxane-induced apoptosis is associated with a caspase-3 activation/cleavage pathway. In addition, these results were consistent with the antiapoptotic signaling function of Bcr-Abl to regulate expression of Bcl-xL. The ability of dexrazoxane to induce differentiation and apoptosis suggests that bisdioxopiperazines may be useful in treating some types of leukemia.

The K562 cell line, which was derived from a patient with chronic myeloid leukemia in blast crisis, is capable of differentiating along erythroid, megakaryocyte, and macrophage lineages, depending upon the inducer used (Sutherland et al., 1986). Consequently, it is widely used as a model of hemopoietic cell differentiation. The K562 cell line has been shown to be refractory to induction of apoptosis by DNA topoisomerase II-targeting anticancer drugs (Kaufmann et al., 1993; Ritke et al., 1994; Dubrez et al., 1995) related to the expression of the product of the Philadelphia chromosome, Bcr-Abl (McGahon et al., 1994), whose constitutively active tyrosine kinase activity maintains high expression of the antiapoptotic protein Bcl-xL (Amarante-Mendes et al., 1998; Horita et al., 2000), thus preventing cytochrome *c* release from mitochondria and activation of caspase-3 (Amarante-Mendes et al., 1998). The role of DNA topoisomerase II and its inhibitors in the induction of leukemia cell differentiation (Constantinou et al., 1992, 1996) has been reviewed (Larsen, 1994) and is probably an important part of their clinical activity. 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 (Corbett and Osheroff, 1993) and has a critical role in DNA processing required for the separation of chromosomes to complete mitosis.

The bisdioxopiperazine dexrazoxane (ICRF-187, Zinecard) and its analogs ICRF-159 (razoxane), ICRF-154 and ICRF-193 (Fig. 1) are potent catalytic inhibitors of mammalian DNA topoisomerase II (Hasinoff et al., 1995) that inhibit without inducing DNA strand breaks. The bisdioxopiperazines have been proposed to act by trapping the enzyme in the form of a closed ATP-modulated protein clamp (Roca et al., 1994), thus preventing the formation or stabilization of cleavable complexes. The inability of bisdioxopiperazines to induce DNA strand breaks is in contrast to the cleavable complex-forming antitumor drugs, which include the anthracycline doxorubicin, the epipodophyllotoxins etoposide and teniposide, and amsacrine. These drugs are thought to be cytotoxic by virtue of their ability to stabilize a cellular toxic covalent topoisomerase II-DNA intermediate (the cleavable complex) and are called topoisomerase II poisons (Corbett

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate buffered saline; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

and Osheroff, 1993). The characterization and the sequencing of topoisomerase IIα from bisdioxopiperazine-resistant cell lines (Hasinoff et al., 1997; Yalowich et al., 1998; Wessel et al., 1999) have identified functional mutations in the amino terminal region, specifically at the clamp portion of the dimer interface of the enzyme (Yalowich et al., 1998) and at the ATP binding site (Wessel et al., 1999).

Dexrazoxane is the (+)-(*S*)-enantiomer of racemic ICRF-159 (razoxane) and was originally developed as an antitumor agent (Edgar and Creighton, 1981). Dexrazoxane is used clinically to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998a). Under physiological conditions, dexrazoxane undergoes a slow ring-opening hydrolysis to ADR-925 (Hasinoff, 1998; Hasinoff et al., 1998a) (Fig. 1), an analog of EDTA. Dexrazoxane probably exerts its cardioprotective effects through its rings-opened hydrolysis product ADR-925 by virtue of its ability to strongly chelate free iron or to quickly and efficiently remove iron from its complex with doxorubicin (Hasinoff, 1998; Hasinoff et al., 1998a), thus reducing doxorubicin-induced iron-based oxygen free radical damage.

We demonstrated previously that dexrazoxane was more growth inhibitory and more effective in inhibiting etoposide-mediated topoisomerase II-DNA covalent complexes in an etoposide-resistant K562 cell line that contains decreased topoisomerase II protein levels compared with parental cells (Fattman et al., 1996), thus establishing that dexrazoxane activity is inversely proportional to topoisomerase II levels. We have also compared the differing abilities of K562 and HL-60 cells to undergo etoposide-mediated apoptosis (Ritke et al., 1994). In this study, we report on the ability of dexrazoxane to induce erythroid-type differentiation and apoptosis in K562 cells.

Materials and Methods

Drugs and Chemicals. Dexrazoxane and ADR-925 were a gift from Pharmacia & Upjohn (Columbus, OH) and were freshly prepared in the media directly before use to avoid hydrolysis (Hasinoff, 1998; Hasinoff et al., 1998a). ICRF-193 and ICRF-154 were synthesized essentially as described previously (Creighton, 1976) and were freshly prepared as stock solutions in DMSO such that the final DMSO concentration was 0.5% (v/v). Hemin (Eastman, Rochester, NY) was prepared as a stock solution in 0.134 M ammonium hydroxide. Agarose (Ultrapure) was obtained from Life Technologies (Burlington, Canada). STI-571 (formerly known as CGP-57148B) was provided by Dr. Elizabeth Buchdunger (Novartis, Basel, Switzerland). All other drugs and chemicals not listed above were obtained from Sigma-Aldrich (Oakville, Canada).

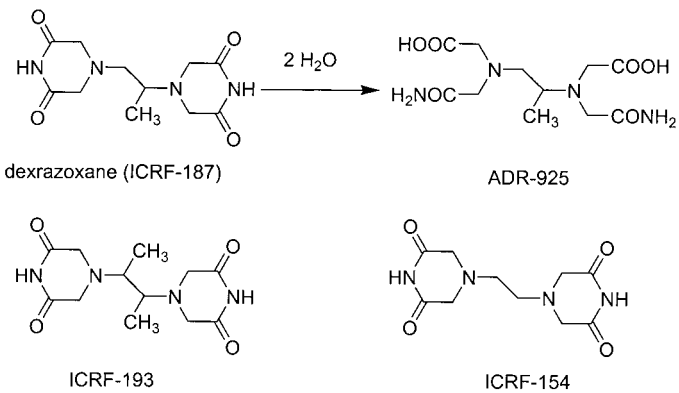

Fig. 1. Structures of the bisdioxopiperazines dexrazoxane (ICRF-187), ICRF-154, ICRF-193, and the dexrazoxane rings-opened hydrolysis product ADR-925.

Cell Culture. Human leukemia K562 cells, obtained from the American Type Culture Collection (Manassas, VA), were maintained as suspension cultures in DMEM (Life Technologies) containing 20 mM HEPES (Sigma, St. Louis, MO), 100 U/ml penicillin G, 100 μg/ml streptomycin, 10% (v/v) fetal bovine serum (Life Technologies) in an humidified atmosphere of 5% CO_2/95% air (v/v) at 37°C (pH 7.1). To avoid cell overgrowth in the experiments that measured the effect of different times of dexrazoxane exposure, cells were collected, counted and resuspended in fresh medium at lower cell density, and the cell numbers were normalized accordingly. Because of the appearance of large numbers of smaller particles at longer times of dexrazoxane exposure (likely apoptotic bodies), a Coulter counter cutoff threshold setting of 12 μm diameter, which is slightly smaller than the mean normal K562 cell size of 16 μm, was used to exclude smaller particles in the cell growth experiments to obtain a meaningful cell count. The ability of the cells to exclude Trypan Blue dye was used to assess cell viability.

Hemoglobin Staining and Determination. The percentage of cells staining for hemoglobin was estimated by staining with benzidine/H_2O_2 essentially as described (Gopalakrishnan and Anderson, 1979). The bright blue-stained hemoglobin-positive cells were counted in a hemacytometer on an inverted microscope. At least 500 cells were counted for each sample. The cellular hemoglobin content was also determined spectrophotometrically essentially as described previously (Cioe et al., 1981). Briefly, PBS-washed cell pellets (1×10^7 cells) were subjected to five freeze-thaw cycles and centrifuged at 16,000*g* for 60 min. The visible absorbance spectrum of the supernatant was taken and the net absorbance (above background) was used to calculate the hemoglobin concentration using a subunit extinction coefficient of 125,000 M^{-1} cm^{-1} (Cioe et al., 1981).

Cell Sizing Analysis. Changes in cell size distribution that occurred after dexrazoxane exposure were followed on a model Z_F Coulter counter (Coulter Electronics, Hialeah, FL) that had been calibrated with 15.0 μm latex beads. Approximately 1×10^7 cells were diluted 1:50 in Isoton II (Coulter Electronics). Replicate counts were made through a range of increasing threshold settings using different amperage and aperture current settings so that the wide range in cell volumes could be accurately measured. The cell counts were expressed in the form of a distribution as a function of cell volume.

Flow Cytometry. Changes in cell cycle progression and DNA ploidy were determined before and after treatment with dexrazoxane. Approximately 8×10^6 cells in Dulbecco's PBS were fixed as a single cell suspension in 70% (v/v) cold ethanol overnight at −20°C. Samples were subsequently washed with Dulbecco's PBS, resuspended in a 0.1% (w/v) Triton X-100 solution containing 0.02 mg/ml propidium iodide and 0.1 mg/ml RNase A followed by incubation for 15 min at 37°C. Analysis was carried out on an EPICS V multiparameter flow cytometer (Coulter Electronics, Hialeah, FL) with an argon laser tuned to 488 nm.

Two-color immunofluorescence flow cytometry was also carried out to examine for the presence of erythroid and megakaryocyte cell surface markers on dexrazoxane-treated cells. All mAbs used were mouse IgG_1 isotypes obtained from Coulter-Immunotech (Burlington, Canada). The 11E4B7.6 (KC16) mAb conjugated FITC was specific for glycophorin A, a major transmembrane sialoglycoprotein expressed on red blood cells and erythroid precursors. The P2 mAb conjugated to PE was specific for CD41 or the GPIIb component of the GPIIb/IIIa complex present on platelets and early promegakaryoblasts (Kanz et al., 1987). The 679.1 Mc7 monoclonal antibodies served as isotype controls (IgG_1-FITC and IgG_1-PE) possessing irrelevant specificity. Each of the mAbs was added in turn to approximately 5×10^5 cells in 150 μl of DMEM/fetal bovine serum, lightly vortexed, and then incubated on ice for 45 min. After dilution with 2 ml of cold buffered DMEM/fetal bovine serum, the cells were

pelletted and the supernatants removed. This was followed by the addition of 350 μl of 2% (w/v) freshly prepared paraformaldehyde in Dulbecco's PBS.

The two-color analysis was also conducted on the Coulter flow cytometer that collected integrated green (FITC) fluorescence, and integrated red (PE) fluorescence on 3-decade logarithmic amplifiers. Bivariate flow cytometric data for untreated and dexrazoxane-treated samples were separated into four regions (I-IV). Region I corresponded to cells that bind anti-CD41 but not anti-glycophorin A; region II to cells that bound both anti-CD41 and anti-glycophorin A; region III to cells that bound neither anti-CD41 nor anti-glycophorin A; and region IV to cells that bound anti-glycophorin A but not anti-CD41. The percentage increase in fluorescence was determined relative to the corresponding control antibody for which the cell count was arbitrarily set to 1% of the total cell count for that region.

Analysis of DNA Fragmentation by Gel Electrophoresis. At various times after dexrazoxane treatment, 6×10^6 cells were pelleted, washed twice with Dulbecco's PBS, and incubated for 1 h at 50°C in 400 μl of lysis buffer [10 mM EDTA, 50 mM Tris, pH 8, 0.5% (w/v) sodium lauryl sarcosine, 0.5 mg/ml proteinase K]. DNase-free RNase A was added (200 μl, 0.5 mg/ml) and the incubation was continued for an additional hour at 50°C. DNA was extracted with 600 μl of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). This was followed by the addition of 0.1 volume of 3 M sodium acetate, 2 volumes of ice-cold 100% ethanol, and incubation overnight at −20°C. Samples were then pelleted by centrifugation at 11,000*g* for 20 min, and resuspended in Tris/EDTA (10/1 mM, pH 8.0) buffer. The samples were loaded onto a 2% (w/v) agarose gel containing ethidium bromide (0.2 μg/ml). A λBstII DNA digest (Sigma) with DNA fragments of known size was used as a reference marker. After electrophoresis at 50 V for 2 h, the gels were photographed under trans-UV illumination.

Quantification of Apoptosis and Cell Viability. Induction of apoptosis and loss of cell viability after dexrazoxane treatment was assessed by staining with Hoechst 33342 dye (Sigma). Apoptotic cells were identified on the basis of their fragmented nuclei and the condensed chromatin beads around the periphery of the nucleus. The micrographs of DNA-stained K562 cells were obtained by staining with Hoechst 33342 at 5 μg/ml for 30 min at 37°C followed by two washes in growth medium followed by a 30-min incubation at 37°C.

Caspase-3 and Bcl-xL Western Blot Analysis. K562 cells were incubated for various times with STI-571 (0.5 μM) and dexrazoxane (100 μM) used alone or in combination. Whole-cell lysates were then made from 2.5×10^6 cells by the addition of SDS-polyacrylamide gel electrophoresis sample buffer [50 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.5% (v/v) β-mercaptoethanol], followed by boiling for 5 min and brief sonication. Thirty-microgram samples of protein were resolved using 15% (w/v) SDS-polyacrylamide gel electrophoresis then transferred to nitrocellulose. Visual inspection of Ponceau S-stained nitrocellulose membranes was used to assure equivalent protein loading/transfer comparing different samples. Membranes were blocked with nonfat dry milk (3% w/v) in PBS containing Tween 20 [0.05% (w/v)] and then incubated with 1:2500 dilutions of primary rabbit antibodies: either anti-Bcl-xL (Transduction Laboratories, Lexington KY) or anti-caspase-3 (PharMingen, San Diego, CA). The secondary donkey anti-rabbit antibody was purchased from Jackson Immuno-Research Laboratories (Westgrove, PA). Bound antibody was detected using enhanced chemiluminescence (NEN, Boston, MA). Autoradiographic signals were quantified by densitometric scanning using a Molecular Dynamics (Sunnyvale, CA) densitometer.

Results

The Effect of Dexrazoxane on K562 Cell Growth, Differentiation, and Morphology. In the results shown in Fig. 2A K562 cells were incubated with 100 μM dexrazoxane for periods varying from 1 to 7 days. Cells were removed from the growth medium and placed in fresh drug daily. At the indicated times, cells were resuspended in dexrazoxane-free medium. After removal of dexrazoxane, cell growth resumed, although at rates that were progressively slower as the dexrazoxane incubation period was increased. Continuous drug exposure and presumably continuous topoisomerase II inhibition was necessary for dexrazoxane to completely inhibit K562 cell growth. The data plotted in Fig. 2B shows that, as evidenced by the ability to stain for hemoglobin, increased exposure time to dexrazoxane also induced erythroid differentiation in K562 cells. When cells were incubated with dexrazoxane for up to 7 days and then placed in dexrazoxane-free medium, the percentage of cells that stained for hemoglobin dropped rapidly in all cases (Fig. 2B). This was caused by the restoration of cell growth (Fig. 2A) in dexrazoxane-free medium. When this same data was plotted as the absolute number, rather than the percentage of hemoglobin-positive cells (data not shown), the number of hemoglobin-positive cells remained essentially constant, indicating that a portion of dexrazoxane-exposed cells became

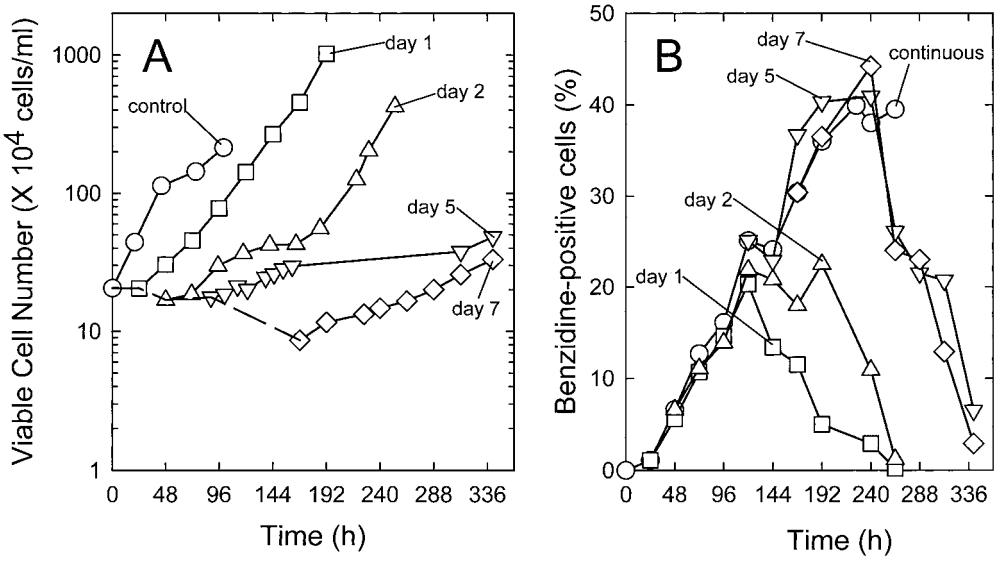

Fig. 2. Effect of different times of exposure to 100 μM dexrazoxane on viable K562 cell growth and percentage of cells that stained for hemoglobin. A, effect of dexrazoxane on viable cell number after no exposure (○), 1 day (□), 2 days (△), 5 days (▽), and 7 days (◇) of continuous exposure to 100 μM fresh daily dexrazoxane and medium. The lower broken line measures the viable cell number occurring upon continuous exposure to dexrazoxane. B, the effect on the percentage of K562 cells that stained for hemoglobin at various times after placing cells in dexrazoxane-free medium (1 day (□), 2 days (△), 5 days (▽), and 7 days (◇) of continuous exposure to 100 μM dexrazoxane. ○, continuous exposure to 100 μM dexrozoxane. Although the results shown are from a single experiment, the results shown are typical of experiments that were carried out a total of four times under similar conditions.

terminally differentiated, whereas newly dividing cells in the absence of dexrazoxane did not undergo differentiation.

Next, the time courses for growth inhibition and for erythroid differentiation were compared among a variety of bisdioxopiperazines, etoposide, hemin, and DMSO (Fig. 3). At the concentrations used, DMSO and the dexrazoxane hydrolysis product ADR-925 did not inhibit growth or induce erythroid differentiation. As expected, hemin induced K562 cell erythroid differentiation but had little effect on growth (Dean et al., 1981). All three bisdioxopiperazines used inhibited growth. Dexrazoxane, ICRF-193 and the topoisomerase II poison etoposide in particular caused a large decrease in the viable cell number. ICRF-193 (5 μM) was a more potent inhibitor of K562 cell growth than dexrazoxane (100 μM), related to its more potent topoisomerase II inhibitory activity (Hasinoff et al., 1995). All the bisdioxopiperazines and etoposide induced a time-dependent increase in benzidine-positive staining indicative of erythroid differentiation (Fig. 3B). The time-dependent increase in hemoglobin content was also examined in dexrazoxane-treated cells and was found to progressively increase (9-fold) over a 120-h incubation period (Fig. 3B). ICRF-154, which was less growth inhibitory, also demonstrated less ability to induce differentiation.

To further characterize the ability of dexrazoxane to induce differentiation of K562 cells along either erythroid or megakaryocyte lineages, two-color immunofluorescence flow cytometry was carried out using fluorescent conjugated mAbs that were specific for surface expression of glycophorin A and glycoprotein IIb/IIIa (CD41), respectively (Fig. 4). These results demonstrated that dexrazoxane induced only erythroid differentiation based on induction of the erythroid surface marker glycophorin A but not the megakaryocyte marker. The result that the percentage of benzidine-positive cells is larger at all times studied (Fig. 3B) might reflect cell-to-cell differences in the levels of expression of glycophorin A. The two-color immunofluorescence flow cytometry experiments would only pick up glycophorin A expression above a critical level. The drop in glycophorin A expression seen at 120 h probably reflects the effect of gating out apoptotic cells. Cells that previously expressed glycophorin A and became smaller and/or apoptotic were gated out and thus would not be counted in this analysis.

After 10 days of continuous dexrazoxane (100 μM) treatment, a proportion of K562 cells continued to increase in size up to 100 μm in diameter (Fig. 5A) which corresponds to a 300-fold increase in volume. To better characterize these progressive morphological changes, cell size distribution curves were determined on a Coulter counter. Compared with untreated control cells, there was a progressive increase in the maximum peak diameters up to 48 h, with cell diameters of 15.9, 18.2, 18.8, and 19.4 μm measured for 0, 12, 24, and 48 h, respectively. By 72 and 96 h, there was a large and progressive increase in the number of small particles (Fig. 5) (but also with an increasing number of large cells), which was probably caused by formation of apoptotic cell bodies. The average cellular DNA content increased linearly from 16 to 40 $\mu g/10^6$ cells over 48 h and then slowly decreased to 28 $\mu g/10^6$ cells at 168 h (data not shown). The average protein content also increased from 200 to 320 $\mu g/10^6$ cells over 48 h and then leveled off to 260 $\mu g/10^6$ cells at 168 h (data not shown). The protein/DNA ratio decreased by only about 25% over this time indicating that both DNA and protein continued to be synthesized in near normal proportions. Beyond 48 h, the DNA and protein levels were less reliable because of the increase in small particles (Fig. 5). As can be seen from Fig. 5, not all large cells that were produced after 10 days of continuous dexrazoxane exposure stained for hemoglobin. This indicates that a predisposition to dexrazoxane-induced increase in cell size was not solely a feature of cells that underwent erythroid differentiation.

To further characterize the effect of dexrazoxane on K562 cells, cell cycle analysis was performed on propidium iodide-stained cells (Fig. 6). Results are plotted on a logarithmic propidium iodide fluorescence scale to more clearly demonstrate that there was a trend to a high level of polyploidization with increasing times of dexrazoxane exposure. On a logarithmic DNA fluorescence scale, there is an equidistant separation between peaks that differ in ploidy by a constant factor of 2. Such peaks correspond to cells with 2N, 4N, 8N, 16N, and 32N ploidy (where N is the haploid DNA content). After 216 h of continuous dexrazoxane exposure, some cells with a DNA content as high as 32N were detectable. This result suggested that at least three and possibly four cycles of DNA synthesis or endoreduplication occurred without cell

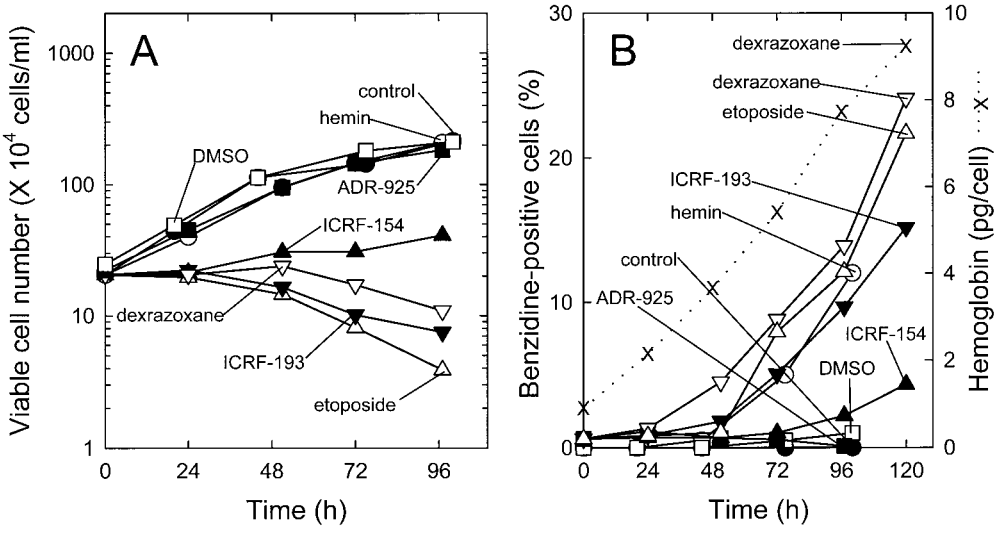

Fig. 3. Effect of topoisomerase II-targeted compounds and other agents on viable K562 cell growth and erythroid differentiation. A, cells were seeded at an initial concentration of 2.1×10^5 cells/ml and were either not treated (●); or were treated once with 30 μM hemin (○), 1.5% (v/v) DMSO (□), or 100 μM ADR-925 (■); or were resuspended daily in fresh medium containing 100 μM dexrazoxane (▽), 5 μM ICRF-193 (▼), 50 μM ICRF-154 (▲), or 10 μM etoposide (△). B, effect of various agents on hemoglobin induction in K562 cells determined by benzidine staining, and spectrophotometrically (×) in the case of dexrazoxane. Incubation conditions were the same as indicted above for Fig. 3A. Although the results shown are from a single experiment, the results shown are typical of experiments that were carried out twice under similar conditions.

division. Care was taken in these experiments to microscopically examine the propidium iodide-stained cell suspensions for aggregated cells that could give a false positive ploidy level. In all cases, cell-doublets were less than 1%, and cell-triplets or larger were not detectable. Back correlation of the cell size light scatter data (not shown) to DNA fluorescence showed that the high ploidy peaks were caused by the large cells. The presence of cells with 2N and 4N ploidy, seen even at long times of dexrazoxane exposure, could be attributable to a population of cells that underwent a complete cell cycle blockage or to some of the cells with higher ploidy having undergone division even in the presence of a high degree of inhibition of topoisomerase II.

The results shown in Fig. 6 also indicated the presence of a subdiploid population of particles with increasing times of dexrazoxane exposure suggesting that K562 cells began to undergo apoptosis. The appearance of small particles in the sizing data after 48 h of dexrazoxane was also suggestive of apoptosis. Both the forward and right angle light scattering data were also greatly increased upon the treatment of K562 cells with dexrazoxane (data not shown) again suggesting dexrazoxane-induced apoptosis.

Induction of Apoptosis by Dexrazoxane. The appearance of a subdiploid population in Fig. 6 after dexrazoxane exposure and previous reports of bisdioxopiperazine-induced apoptosis in CEM cells (Khelifa and Beck, 1999a; Morgan et al., 2000) and K562 cells (Synold et al., 1998) prompted examination of other indicators of dexrazoxane-induced apoptotic pathway activation in K562 cells (Figs. 5B, 7–10). Time-dependent denaturation of DNA suggestive of internucleosomal fragmentation was observed in the presence of dexrazoxane (100 μM), ICRF-193 (5 μM), and etoposide (10 μM) (Fig. 7). All of these topoisomerase II inhibitors (catalytic and cleavable complex forming) caused DNA laddering that was particularly pronounced after 96 h of dexrazoxane exposure but without the sharp banding patterns usual upon internucleosomal fragmentation. Cells, such as the K562 cell line, that are refractory to apoptosis are also typically associated with a smear of denatured DNA on the gel (as seen in Fig. 7) (Dubrez et al., 1995). This occurs because of the slow induction of apoptosis in this apoptotic-refractory cell line and the combination of apoptosis and secondary necrosis (Dubrez et al., 1995).

In addition to DNA fragmentation, DNA-stained dexrazoxane-treated cells showed the distinct morphological features (fragmented nuclei and condensed chromatin) of apoptotic cells (Fig. 5B). The percentage of apoptotic cells slowly and

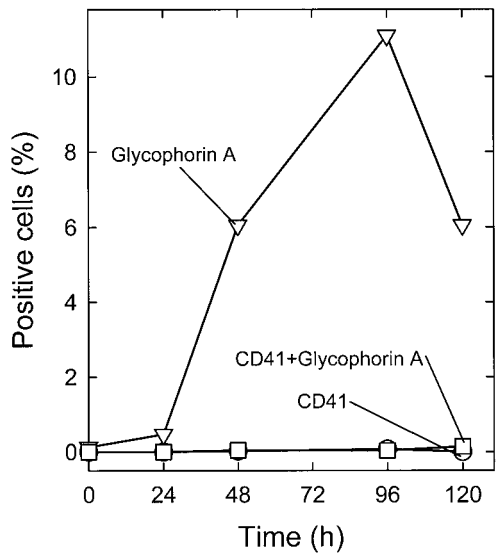

Fig. 4. Expression of erythroid (glycophorin A, ▽) and megakaryocyte (CD41, ○) cell surface markers, or both markers (□) simultaneously, as determined by immunofluorescence flow cytometry of K562 cells continually exposed to 100 μM dexrazoxane for various times. The percentage of cells that stained positive for the marker indicated is relative to isotype control conjugated mAbs (IgG_1-FITC and IgG_1-PE) possessing irrelevant specificity. Although the results shown are from a single experiment, the results shown are typical of experiments that were carried out twice under similar conditions.

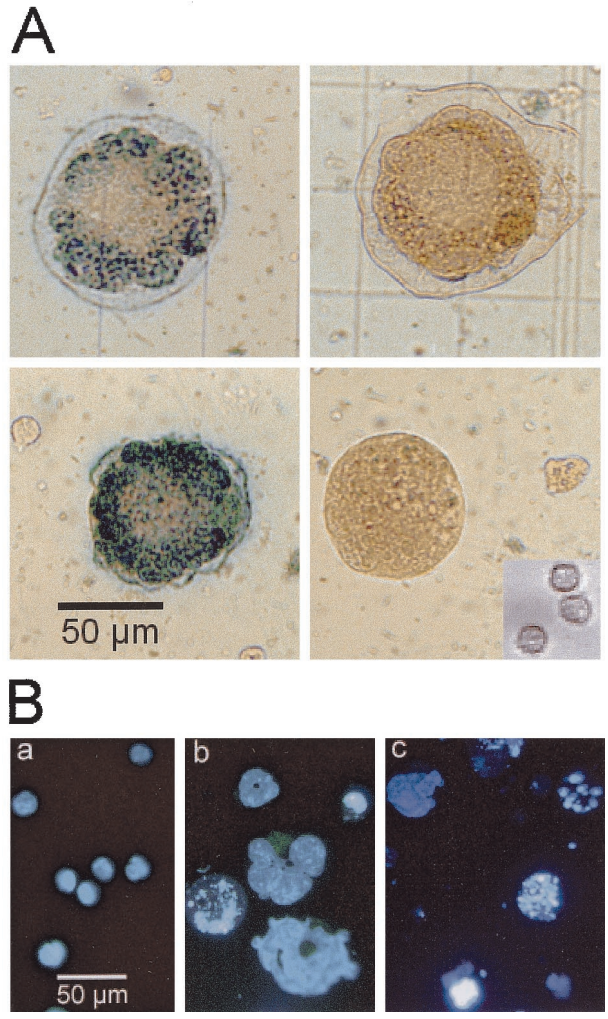

Fig. 5. A, bright-field photomicrographs of benzidine-stained K562 cells in suspension after 10 days of continuous exposure to 100 μM dexrazoxane. After 10 days of exposure, the diameter of some cells approached 100 μm. Untreated cells are shown in the bottom right inset for comparison. The dark stained regions of the two leftmost photomicrographs are from the blue benzidine product produced by reaction with hemoglobin. As shown in the two rightmost micrographs, other large cells did not stain for hemoglobin. The numerous small particles, which are produced after extended dexrazoxane exposure, are probably apoptotic debris. B, epifluorescence photomicrographs of DNA-stained (with Hoechst 33342) K562 cells after 0 h (a), 96 h (b), or 120 h (c) continuous exposure to 100 μM dexrazoxane. The cells were resuspended daily in fresh medium containing fresh dexrazoxane. The apoptotic cells were identified by fragmented nuclei, and bright, uniformly fluorescent spherical beads of condensed chromatin around the periphery of the nucleus. Apoptotic bodies in the late stages of apoptosis can also be seen in c.

continuously increased (to greater than 60%) with dexrazoxane exposure times up to 120 h (Fig. 8). This result agreed with the slow appearance of the subdiploid population of cells seen in the flow cytometry results (Fig. 6). In addition, the time dependence of dexrazoxane-induced apoptosis correlated with the time-dependent induction of caspase-3 cleavage (Fig. 9, top).

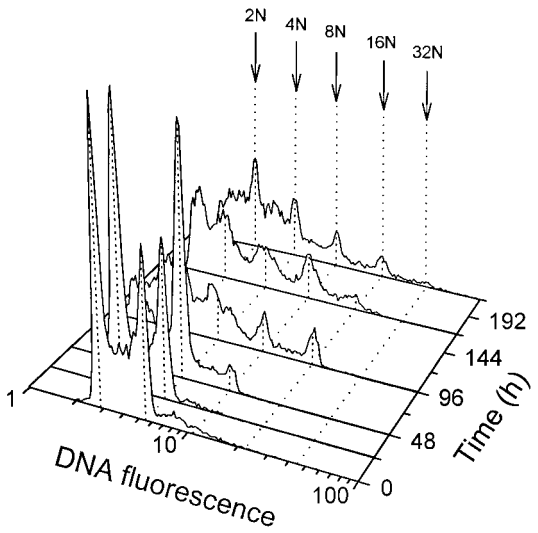

Fig. 6. Cell cycle analysis measuring DNA content of K562 cells continuously exposed to 100 μM dexrazoxane. Cells were stained with propidium iodide and then analyzed by flow cytometry. The integrated red fluorescence, which is a measure of DNA content, is plotted on a 3-decade logarithmic scale versus the cell number. The leftmost peak in the control (0 h) culture represents cells in G_1 with diploid (2N) DNA content, and the next highest peak represents cells in G_2/M with tetraploid (4N) levels of DNA. At longer times, equally spaced peaks corresponding to higher multiples of the G_1 peak can be seen, indicating the increasing appearance of higher ploidies (8N, 16N, 32N, respectively). After 48 h, subdiploid apoptotic bodies (left of G_1) increasingly appear in the population because of the induction of apoptosis.

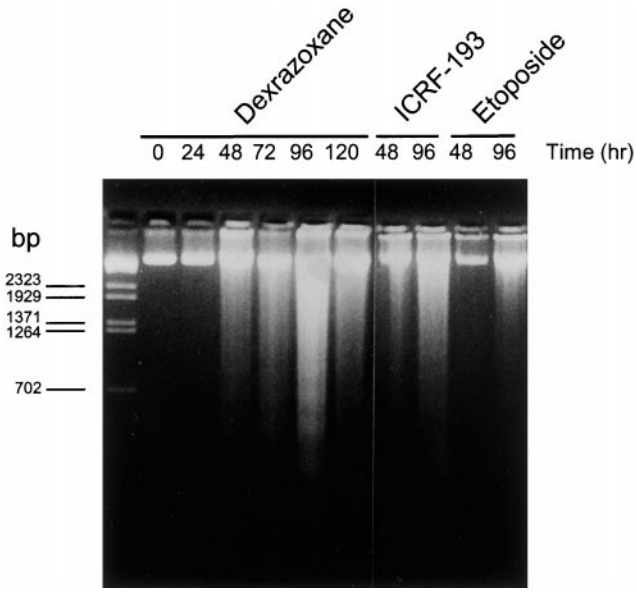

Fig. 7. DNA fragmentation of K562 cells visualized by ethidium bromide-stained agarose gel electrophoresis. The K562 cells were continuously exposed to 100 μM dexrazoxane, 5 μM ICRF-193, and 10 μM etoposide for the times indicated. The leftmost lane is marker DNA of known DNA base pair size (bp). The progressive appearance of a DNA laddering pattern with increasing time of drug exposure is indicative of apoptosis.

To further establish that dexrazoxane-induced apoptosis involved a caspase-3 dependent pathway, the Bcr-Abl tyrosine kinase inhibitor STI-571 (Druker et al., 1996) was

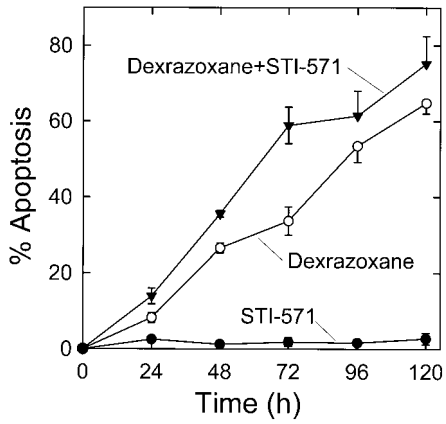

Fig. 8. Percentage of apoptotic K562 cells produced after continuous exposure to 100 μM dexrazoxane in the absence (○) and the presence (▼) of the BCR-Abl tyrosine kinase inhibitor STI-571. The effect of STI-571 alone on percentage apoptosis is also shown (●). The cells were resuspended daily in fresh medium containing 100 μM dexrazoxane and every other day with medium also containing 0.5 μM STI-571. Results shown are the mean ± S.E. from four separate experiments.

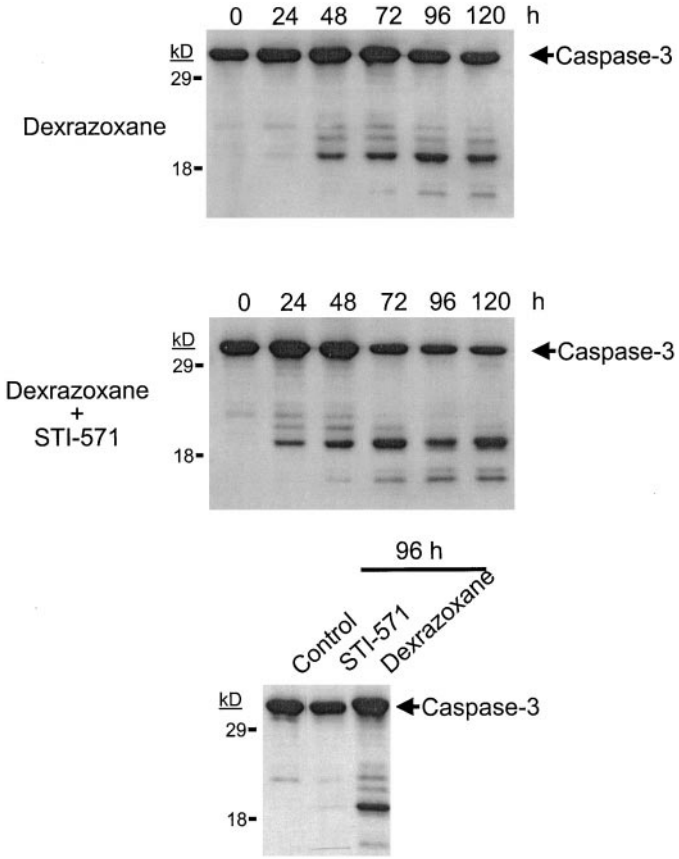

Fig. 9. Caspase-3 cleavage activity in K562 cells after exposure to 100 μM dexrazoxane in the absence (top) and presence (middle) of 0.5 μM STI-571. The bottom panel indicates the lack of caspase-3 activation at 96 h after addition of 0.5 μM STI-571 alone compared with the robust caspase-3 cleavage demonstrated in the presence of 100 μM dexrazoxane. The treatment protocol was as described in the legend to Fig. 8. The Western blots were obtained as described under *Materials and Methods*. Results shown are representative of three identical experiments yielding similar results.

used to down-regulate Bcl-xL protein expression (Fig. 10), thus permitting earlier onset and more extensive dexrazoxane-induced caspase-3 cleavage (Fig. 9) and greater apoptosis (Fig. 8). Under the experimental conditions used here (STI-571 added every other day to achieve 0.5 μM; cells washed free of drug 24 h after each addition), Bcr-Abl autophosphorylation was reduced to 14.7 $\pm$ 2.8% of control 1 h after addition as measured by antiphosphotyrosine immunoblotting, and this inhibition persisted throughout the course of the experiment (results not shown). STI-571 used alone caused a decrease in Bcl-xL protein expression at 96 h (Fig. 10, bottom). In addition, when STI-571 was combined with dexrazoxane, there was a time-dependent decrease in Bcl-xL protein levels (Fig. 10). These results are consistent with previous reports of STI-571-mediated down-regulation of Bcl-xL expression (Yalowich et al., 1999; Horita et al., 2000).

After accounting for the low level of apoptosis induced by STI-571 alone (Fig. 8), STI-571 was found to increase the rate of dexrazoxane-induced apoptosis by more than 27% (Fig. 8). For each of four paired experiments, slopes for the apoptosis-time curves were calculated by linear regression. After subtraction of the STI-571 linear regression line from that of the dexrazoxane+STI-571 condition for each experiment, comparison of dexrazoxane alone versus dexrazoxane+STI-571 indicated that addition of STI-571 potentiated dexrazoxane-induced apoptosis ($p = 0.039$, paired Student's t test). STI-571 addition to dexrazoxane-treated K562 cells also resulted in an earlier onset and more extensive cleavage of caspase-3 (compare Fig. 9, top and middle) but did not induce caspase-3 cleavage when used alone (Fig. 9, bottom). Results using STI-571 further established that dexrazoxane-induced apoptosis involved a caspase-3 dependent pathway activation that is regulated in part by Bcl-xL expression. The slow induction of apoptosis in K562 cells in response to bisdioxopiperazines and etoposide was probably caused by Bcr-Abl mediated maintenance of Bcl-xL protein levels (Horita et al., 2000).

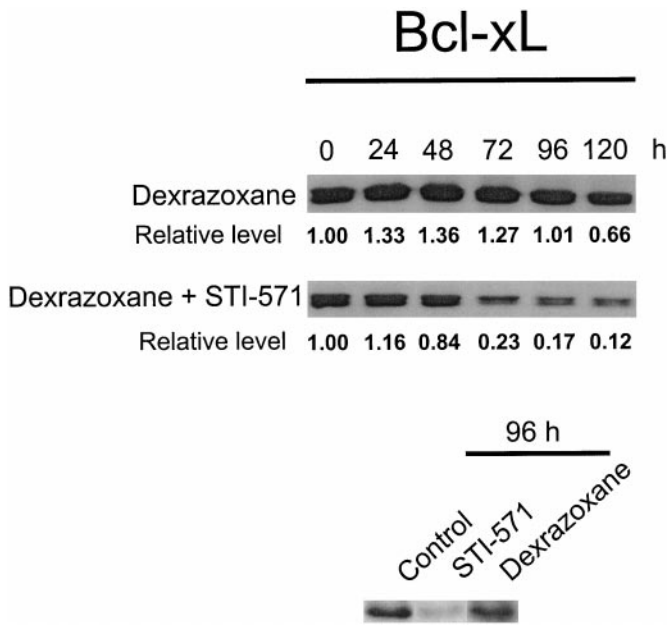

Fig. 10. Bcl-xL protein expression in K562 cells at various times after exposure to 100 μM dexrazoxane in the absence (top) or presence (middle) of 0.5 μM STI-571. The bottom panel indicates that 0.5 μM STI-571 used alone is responsible for a decrease in Bcl-xL protein expression after a 96 h incubation period. The treatment protocol was as described in the legend to Fig. 8. The Western blots were obtained as described under *Materials and Methods*.

Discussion

The ability of bisdioxopiperazines or other noncleavable complex-forming topoisomerase II inhibitors to induce cellular differentiation has not been well studied. ICRF-193 can induce differentiation of a leukemic U-937 promonocytic cell line after continuous exposure (Perez et al., 1997). As we observed, continuous exposure of K562 cells to ICRF-193 was also required to induce differentiation (Fig. 2B). Aclarubicin, a DNA intercalating topoisomerase II catalytic inhibitor, induces erythroid differentiation of K562 cells (Larsen, 1994), indicating that this is not a property unique to bisdioxopiperazines.

Generally, topoisomerase II inhibitors induce K562 cells along an erythroid lineage (Larsen, 1994). It has been shown in a number of studies that topoisomerase II inhibitors (and other agents) greatly reduce topoisomerase II activity, protein levels (Constantinou et al., 1992; Larsen, 1994), and phosphorylation status (Constantinou et al., 1996). It has not been established whether these changes in activity are required for differentiation or are a result of it, although one study has suggested that decreased topoisomerase II phosphorylation permits erythroid differentiation (Constantinou et al., 1996).

In this study, we have shown that the bisdioxopiperazines dexrazoxane, ICRF-154, and ICRF-193 induce differentiation of K562 cells along an erythroid pathway similar to hemin (Fig. 3B). After removal of dexrazoxane from the medium, the decrease in the percentage of benzidine-stained cells (Fig. 2B) was similar to that seen after removal of hemin (Yumoto et al., 1990). Dexrazoxane undergoes a slow hydrolysis (Hasinoff et al., 1998b) (Fig. 1) under physiological conditions with a loss of dexrazoxane from the medium occurring with a half-life of 18 h. The dexrazoxane hydrolysis product ADR-925 (Fig. 1), which is a strong iron chelator (Hasinoff et al., 1998a), however, was unable to induce erythroid differentiation (Fig. 3B), indicating that it is not the metal ion-binding hydrolysis product that is the active form of dexrazoxane. We have also previously shown that neither the one-ring open dexrazoxane intermediates nor ADR-925 are topoisomerase II inhibitors (Hasinoff et al., 1998b), suggesting that the hydrolysis products were not able to induce differentiation or apoptosis.

The K562 cell line is well known to be pluripotent and to be inducible along either erythroid or megakaryocytic lineages (Sutherland et al., 1986; Rowley et al., 1992). Some of the morphological features (large size and large multilobulated nucleus) and DNA content (high ploidy) of dexrazoxane-treated K562 cells were similar to those of megakaryocytes. However, we were unable to find any increased expression of the CD41 megakaryocyte surface marker in immunofluorescence flow cytometry experiments. Hematopoietic cell lines that have undergone differentiation often display dramatic changes in nuclear size and shape because of chromatin reorganization (Larsen, 1994), which may allow for selective gene expression associated with implementation of differentiation programs (Larsen, 1994).

Bisdioxopiperazine-mediated growth inhibition and the re-

versibility of dexrazoxane effects upon drug washout have been described (Edgar and Creighton, 1981; Wheeler et al., 1983). These earlier results and the reversibility of dexrazoxane-mediated growth inhibition in K562 cells (Fig. 2A) suggests that continuous inhibition of topoisomerase II is required to maintain growth inhibition of K562 cells by preventing complete chromosomal separation in anaphase as demonstrated previously in dexrazoxane-treated PtK1 rat kangaroo kidney cells (Gorbsky, 1994). The dexrazoxane concentration used (100 μM) in Fig. 2A is, in fact, sufficient to maintain a high degree of inhibition of topoisomerase II (Hasinoff et al., 1995) over the 24-h period before replacement of the medium with fresh dexrazoxane, even after taking into account the rate of dexrazoxane hydrolysis to inactive metabolites (Hasinoff et al., 1998a). We previously measured an IC_{50} of 13 μM for inhibition of topoisomerase II (Hasinoff et al., 1995).

The fact that removal of dexrazoxane from the medium allows K562 cells to resume growth (Fig. 2A) but halts further differentiation (Fig. 2B) suggests that continuous catalytic inhibition of topoisomerase II (and perhaps alteration in chromosomal organization) is also required for erythroid differentiation. This is in contrast to the cleavable complex-forming topoisomerase II poisons such as etoposide or doxorubicin that produce protein-associated DNA strand breaks and induce terminal differentiation (Larsen, 1994; Perez et al., 1997). This result suggests that it is the inhibition of topoisomerase II catalytic activity alone that is sufficient to commit K562 cells to erythroid differentiation.

Razoxane (ICRF-159) has previously been shown to increase the size and ploidy (up to 8N) of a number of cell lines (Stephens and Creighton, 1974; Edgar and Creighton, 1981) similar to what we observed for dexrazoxane-treated K562 cells (Fig. 5, A and B). These ploidy levels were lower than what we observed (Fig. 6), which is probably related to shorter incubation times and/or use of a linear scale for recording fluorescence output from the flow cytometer, which can flatten and obscure peaks of high ploidy.

The fact that cell ploidies as high as 32N were seen in the flow cytometry results indicates that catalytic inhibition of topoisomerase II does not result in prominent cell cycle blockage, suggesting that K562 cells lack a topoisomerase II activity-based cell cycle checkpoint. This conclusion is at odds with the results of microscopic studies on synchronized cells that concluded that mammalian cells have a G_2 checkpoint sensitive to the decatenation activity of topoisomerase II or the catenation of DNA (Downes et al., 1994).

In addition to bisdioxopiperazine-induced differentiation, the ability of catalytic topoisomerase II inhibitors to induce apoptosis is another area that has not been well studied. Exposure of K562 cells to constant levels of dexrazoxane in the 20 to 100 μM range for 96 h produced subdiploid peaks that were thought to be produced from induction of apoptosis (Synold et al., 1998). Dexrazoxane and merbarone, a nonbisdioxopiperazine catalytic inhibitor of topoisomerase II, have been shown to induce apoptosis in leukemic CEM cells (Khelifa and Beck, 1999a,b; Morgan et al., 2000).

In this study, we have shown that the bisdioxopiperazines are potent but slow inducers of apoptosis in K562 cells as has been demonstrated previously in response to topoisomerase II poisons such as etoposide (Ritke et al., 1994; Dubrez et al., 1995). Dexrazoxane-induced apoptosis is linked temporally to the cleavage of caspase-3. The refractoriness of K562 cells to drug-induced apoptosis has been clearly related to expression of the anti-apoptotic protein Bcl-xL (Amarante-Mendes et al., 1998) coupled to the constitutive tyrosine kinase activity of Bcr-Abl (McGahon et al., 1994; Amarante-Mendes et al., 1998; Horita et al., 2000). Using the potent and specific Abl tyrosine kinase inhibitor, STI-571, formerly called CGP-57148b (Druker et al., 1996), we have demonstrated an increased rate and extent of dexrazoxane-induced apoptosis in K562 cells linked to an earlier onset of caspase-3 cleavage/activation and a reduction in the level of Bcl-xL protein (Figs. 8 to 10). Together our results further establish the caspase-3 mediated pathway for bisdioxopiperazine-induced apoptosis and connect this pathway to Bcl-xL expression and its regulation by downstream signaling of Bcr-Abl tyrosine kinase.

The stronger apoptosis and differentiating properties of ICRF-193 compared with dexrazoxane shown in Figs. 3 and 7 are in accord with our structure-activity study (Hasinoff et al., 1995), in which we showed that ICRF-193 inhibited topoisomerase II with an IC_{50} value of 0.6 μM compared with 13 μM for dexrazoxane. These results further indicate that topoisomerase II-mediated DNA strand breakage is not an absolute requirement for the induction of apoptosis in the presence of topoisomerase inhibitors. Although apoptosis has been considered a normal endpoint after differentiation of some leukemic cell lines (Larsen, 1994), the fact that we observed large cells (Fig. 5A) that did not stain for hemoglobin and had not undergone apoptosis suggests that this is not true for all dexrazoxane-treated K562 cells. In addition, it can be seen from a comparison of the data in Figs. 3 and 8 that the percentage of cells that become apoptotic upon dexrazoxane-treatment was always more than 2-fold greater than the percentage of cells that stained for hemoglobin. Hence, dexrazoxane can induce K562 cell apoptosis without undergoing prior differentiation, suggesting that these processes need not be mechanistically linked.

The concentration of dexrazoxane (100 μM) used in this study is pharmacologically relevant, because dexrazoxane dosing of 600 mg/m^2 yields a peak plasma concentration of 340 μM in patients with an elimination $t_{1/2}$ of 4.2 $\pm$ 2.9 h (Hochster et al., 1992). Hence, the ability of the bisdioxopiperazines to induce growth inhibition, associated differentiation, and late onset apoptosis suggests that these compounds should be reinvestigated for their efficacy in some types of leukemia.

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