Topoisomerase IIα-dependent and -independent apoptotic effects of dexrazoxane and doxorubicin

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Abstract
Coadministration of the iron chelator dexrazoxane reduces by 80% the incidence of heart failure in cancer patients treated with anthracyclines. The clinical application of dexrazoxane is limited, however, because its ability to inhibit topoisomerase IIα (TOP2A) is feared to adversely affect anthracycline chemotherapy, which involves TOP2A-mediated generation of DNA double-strand breaks (DSB). Here, we investigated the apoptotic effects of dexrazoxane and the anthracycline doxorubicin, alone and in combination, in a tumor cell line with conditionally regulated expression of TOP2A. Each drug caused apoptosis that was only partly dependent on TOP2A. Unexpectedly, dexrazoxane was found to cause TOP2A depletion, thereby reducing the doxorubicin-induced accumulation of DSB. Despite this latter effect, dexrazoxane showed no adverse effect on doxorubicin-induced apoptosis. This could be explained by the TOP2A-independent apoptotic effects of each drug: those of doxorubicin included TOP2A-independent DSB formation and depletion of intracellular glutathione, whereas those of dexrazoxane were caspase independent. In conclusion, both doxorubicin and dexrazoxane induce apoptosis via TOP2A-dependent and TOP2A-independent mechanisms, the latter compensating for the reduction in cell killing due to dexrazoxane-induced TOP2A depletion. These observations suggest an explanation for the absence of adverse dexrazoxane effects on clinical responses to doxorubicin. [Mol Cancer Ther 2009;8(5):1075–85]

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Introduction
Anthracyclines belong to the most successful drugs used in oncology because they are effective against a wide range of common cancers (1). The most important limitation of their application is the congestive heart failure, which develops in 5% to 20% of patients years to decades after the chemotherapy (2). The bisdioxopiperazine dexrazoxane (ICRF-187) reduces the risk of anthracycline-induced congestive heart failure by ∼80% (3). The cardioprotective effect has been attributed to iron trapping by ADR-925, an EDTA-like product of dexrazoxane hydrolysis. Iron trapping prevents the formation of cardiomyocyte-damaging hydroxyl radicals triggered by “redox cycling” of anthracyclines (4).

Considering the impressive cardioprotective effect of dexrazoxane, its coadministration with anthracyclines to cancer patients has been disappointingly limited. This is mostly due to the persisting fears that combining these drugs may adversely affect outcomes of cancer treatments. Although these fears have been refuted in several randomized clinical studies (3, 5), it is likely that dexrazoxane does indeed interact with anthracyclines in cancer cells. Dexrazoxane (not its cardioprotective derivative ADR-925) binds and inhibits topoisomerase IIα (TOP2A), a major target of anthracyclines. Dexrazoxane locks TOP2A dimers in a “closed clamp” configuration, which differs from the irreversible “poisoning” of the enzyme by anthracyclines (4). The combined effects of dexrazoxane and anthracyclines have been previously tested in several cell and animal cancer models. The results variously indicated synergy, addition, and antagonism between these drugs in their ability to induce cell killing (6, 7). The outcome varied with the cell type, dexrazoxane concentration, addition sequence, and exposure times (6, 8). No satisfactory explanation has been found for these disparate observations.

We are convinced that a better acceptance of dexrazoxane as a cardioprotectant in anthracycline therapies can be achieved only through a more complete understanding of cancer cell-killing effects of these drugs. This issue is of paramount clinical importance, because the application of anthracyclines in oncology shows no signs of abating against congestive heart failure associated with their use (3). We therefore investigated, alone and in combination, the mechanisms of cancer cell killing by dexrazoxane and doxorubicin, the most widely used anthracycline. For several reasons, we focused on the importance of TOP2A. Firstly, both drugs share TOP2A as a major target and the individually variable expression level of TOP2A in breast tumors is being developed as a marker predictive for anthracycline response (9). Further, anthracyclines probably kill cancer cells by several mechanisms independent from TOP2A “poisoning.” In addition to the inhibition of
enzymes such as helicases, they may exert direct DNA-related effects including binding, alkylation, crosslinking, and strand separation (10). Additional DNA and cell membrane damage could be brought about by “redox cycling” of anthracyclines (10). However, the individual contributions of these processes to the antitumor effects of anthracyclines are unknown. Even less understood is the cell-killing effect of dexrazoxane, which occurs in the absence of extensive DNA damage (11, 12). Especially puzzling is the contrast between the cytotoxic effect of dexrazoxane on cancer cells and its protective effect on anthracycline-exposed cardiomyocytes.

To differentiate between TOP2A-dependent and TOP2A-independent effects of dexrazoxane and doxorubicin, we used HTETOP cells, a human tumor cell line that expresses TOP2A exclusively from a tetracycline-regulated transgene (13). This conditionality of TOP2A expression avoids the cellular lethality that would result from a constitutive TOP2A knockout (14). Thus, in the first 2 days following tetracycline addition, HTETOP cells lose up to ~95% of their TOP2A but continue to proliferate without any major changes in morphology or ploidy (13) or, as we show below, in the expression of genes other than TOP2A. Using this unique tool, we were able, for the first time, to clearly differentiate between TOP2A-dependent and TOP2A-independent effects of doxorubicin and dexrazoxane. Based on these results, we suggest an explanation for the somewhat counterintuitive fact that dexrazoxane does not adversely affect doxorubicin-based cancer treatments.

Materials and Methods

Cell Culture

HTETOP cells, derived from the human fibrosarcoma cell line HT1080, were described previously (13). The expression level of TOP2A in HTETOP cells can be reversibly depressed by the addition of tetracycline such as doxycycline. HTETOP cells were cultured in a DMEM supplemented with 10% heat-inactivated fetal bovine serum, 40 units/mL penicillin, 40 μg/mL streptomycin, 20 mM HEPES, 10 mM MEM sodium pyruvate, and 4% (v/v) nonessential amino acids (all from PAA Laboratories). Cells were grown in 10 mmol/L MEM sodium pyruvate, and 4% (v/v) nonessential amino acids and maintained in a CO2 (5%) incubator at 37°C. Dexrazoxane and doxorubicin were from Pfizer.

Cytotoxicity Assay

IC50 values for doxorubicin and dexrazoxane in HTETOP cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Cells were seeded in triplicate at 7,000 per well in a 96-well plate and treated with variable concentrations of doxorubicin or dexrazoxane for 24 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay buffer (20 μL; 5 mg/mL) was added to each well followed by 4 h incubation at 37°C. After a wash with PBS, cells were incubated with DMSO for 10 min and the absorbance was measured at 570 nm. IC50 was calculated by nonlinear regression analysis using GraphPad Prism version 5.01.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting (FACS) assays were done to detect apoptosis in cells stained with Annexin V-FITC (BD Pharmingen) and To-Pro-3 iodide (Invitrogen). Subconfluent cells were gently harvested using accutase. Cell pellet was obtained by low-speed centrifugation (400 × g, 5 min, 4°C). After two washes with PBS, the cell pellet was resuspended in 400 μL of 1× binding buffer. Cell suspension (100 μL) was transferred to a new 5 mL tube in the presence of 2 μL Annexin V-FITC buffer and incubated for 10 min in darkness at room temperature. Finally, 1 μL To-Pro-3 solution was added to the mixture and further incubated for 5 min in darkness followed by the addition of 400 μL of 1× binding buffer. Flow cytometry analysis was done within 1 h.

Reduced Glutathione and Oxidized Glutathione Detection

Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were measured as described previously (15). Briefly, cells were lysed at 4°C for 20 min in a lysis buffer containing 200 mM NaCl, 100 mM Tris-HCl, 1% Triton X-100, and a protease inhibitor cocktail. After centrifugation at 4,000 × g for 5 min, the supernatant was used for protein concentration and total GSH measurement. The supernatant was mixed with 0.73 mM L 5,5-dithiobis(2-nitrobenzoic acid) and 0.24 mM L NADPH in a reaction buffer containing 143 mM L NaH2PO4 and 6.3 mM L EDTA (pH 7.5). Absorbance at 415 nm was immediately recorded after the addition of 1.2 units/mL glutathione reductase. The total amount of GSH was calculated according to the GSH standard curve. GSSG was measured using the same method after trapping of GSH by 2-vinylpyridine. GSH was quantified by subtracting GSSG amount from the total GSH amount.

Caspase-3/7 Activity Measurement

Caspase-3/7 activity was measured with the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer’s instructions on cells seeded in a 96-well plate. At the end of the specified drug treatments, cells were incubated with the Caspase-Glo 3/7 reagent at room temperature for 30 min. Luminescence of each well was recorded in a plate-reading luminometer. The luminescence intensity was expressed as relative light units.

Western Blot

Cells were lysed in a radioimmunoprecipitation assay buffer containing 50 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail. For TOP2A detection, 50 μg/mL DNase I and 5 mM/L MgCl2 (final concentrations) were added to the lysates to release TOP2A protein from the DNA-TOP2A complexes. Protein concentrations were determined by Bradford assay (Bio-Rad Protein Dye Reagent). Protein (20 μg) was separated by SDS-polyacrylamide gels (7.5% for detection of TOP2A and TOP2B and 12% for γ-H2AX and caspase-8) and transferred onto polyvinylidene fluoride membranes. After blocking in TBS-T buffer [10 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 0.05% Tween 20] containing 5% nonfat milk for 1 h at

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room temperature, the membranes were incubated with mouse monoclonal antibody against human TOP2A (1:2,000; Stressgen), TOP2B (1:1,000; BD Pharmingen), γ-H2AX (1:2,000; Abcam), caspase-8 (1:2,000; BD Pharmingen), α-tubulin (1:20,000; Dianova), or GAPDH (1:5,000; Santa Cruz Biotechnology) at 4°C overnight. After washing in TBS-T three times, membranes were incubated with anti-mouse IgG (1:20,000; Sigma) for 1 h at room temperature. Bands were visualized by the ECL+ detection method (Amersham Pharmacia Biotech). Densitometric analysis of the protein bands was done using the NIH Image J freeware.

RNA Microarray Analysis

Expression changes induced by tetracycline or dexrazoxane were evaluated by high-density oligonucleotide RNA microarrays as described previously (16). Briefly, RNA was extracted from triplicates of untreated or tetracycline-treated cells and cDNA was synthesized by reverse transcription for in vitro cRNA synthesis. After biotin labeling, purification, and fragmentation, cRNA was hybridized to Human Genome U133 Plus 2.0 GeneChips (Affymetrix). The raw data obtained from scanned chips were analyzed by robust multiarray analysis (17) for background adjustment and normalization. The microarray data are available at the Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/(GSE14886).

TaqMan Assay

Total RNA was isolated from the cells with the peqGOLD Total RNA Kit according to the manufacturer’s instructions. Total RNA (1 µg) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a total volume of 20 µL. cDNA (1 µL) was mixed with a TaqMan Universal PCR Master Mix (Applied Biosystems) and TOP2A Gene Expression Assay (Hs00172214_m1; Applied Biosystems) according to the manufacturer’s recommendations and real-time PCR was conducted in a 7900HT iCycler from Bio-Rad Laboratories. 18S rRNA (Hs99999901_s1; Applied Biosystems) was used for normalization. The results were calculated using the ΔΔCt method.

Statistical Analysis

All experiments were conducted at least three times. Results are expressed as mean ± SE. Between groups, comparisons were made with Student’s t test or ANOVA; statistical significance was defined by \( P < 0.05 \).

Results

We wanted to characterize the contribution of TOP2A to apoptosis induced by doxorubicin and dexrazoxane. To this end, HTETOP cells were incubated for 24 h with 1 µg/mL of the tetracycline doxycycline. In agreement with the original publication (13), tetracycline depleted the TOP2A (e.g., Fig. 1A) but not TOP2B (data not shown) protein. This was due to the ∼100-fold reduction in the expression of mRNA derived from the tetracycline-dependent TOP2A transgene as confirmed by TaqMan (data not shown) and by expression profiling of the entire transcriptome using RNA microarrays (Fig. 2A). The latter experiment showed an extraordinarily specific effect on TOP2A expression: the only mRNAs with significantly changed levels following addition of tetracycline for 24 h were TOP2A transcripts.

TOP2A-depleted (tetracycline-treated) and control (tetracycline-untreated) cells were then exposed to doxorubicin and dexrazoxane at concentrations corresponding to the range measured in blood of patients treated with either drug. In addition, we tested a supraclinical doxorubicin concentration of 10 µmol/L. For comparison, the IC50 values of the cytotoxicity/viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for doxorubicin
and dexrazoxane in HTETOP cells were 0.52 μmol/L and 7.45 mmol/L, respectively. Doxorubicin induced apoptosis in an exposure time-dependent manner (Fig. 2B) and concentration-dependent manner (Fig. 2C) in both untreated and tetracycline-pretreated HTETOP cells. However, the apoptosis in tetracycline-pretreated (TOP2A-depleted) cells was abolished at 0.1 μmol/L doxorubicin and reduced by ~50% at 1 and 10 μmol/L doxorubicin (Fig. 2C). Dexrazoxane also induced apoptosis in a concentration-dependent manner (Fig. 2D) and exposure time-dependent manner (Fig. 3B, bottommost). Depletion of TOP2A by tetracycline reduced by ~50% the fold increase in apoptosis induced by ≥25 μmol/L dexrazoxane (Fig. 2D).

These results showed both TOP2A-dependent and TOP2A-independent apoptosis induced by either drug. To better delineate the apoptotic pathways involved, we determined the expression or activity of several cellular parameters previously implicated in the cytotoxic effects of doxorubicin and dexrazoxane, beginning with double-strand breaks (DSB), TOP2A expression, and oxidative stress parameters. DSB arise through TOP2A “poisoning” by doxorubicin and are detectable as γ-H2AX foci (18) following the proteasome-mediated degradation of doxorubicin-TOP2A-DNA complexes (10). As shown in Fig. 1A, clinically relevant doxorubicin concentrations of 0.1 and 1 μmol/L (19) indeed increased DSB levels. In agreement with previous observations (20), the DSB increase at the supraclinical doxorubicin concentration of 10 μmol/L was much smaller. Although incompletely understood, this latter effect may reflect doxorubicin toxicity toward the proteasome, which is necessary for γ-H2AX foci detection (21). In the absence of tetracycline, doxorubicin also resulted in a statistically nonsignificant trend toward a moderate (<2-fold) accumulation of the TOP2A protein (Figs. 1A and 3A), most likely reflecting the diminished degradation of the doxorubicin-“poisoned” enzyme. TOP2A depletion reduced the DSB formation by 0.1 and 1.0 μmol/L doxorubicin (Fig. 1A). This result indicated that, although doxorubicin induces DSB via TOP2A “poisoning,” DSB may also arise via mechanisms independent from TOP2A. DSB formation was also detected following dexrazoxane treatment (Fig. 1B). In contrast to doxorubicin, dexrazoxane-induced DSB formation was entirely dependent on TOP2A as shown by the absence of DSB in TOP2A-depleted cells.

**Figure 2.** Involvement of TOP2A in doxorubicin- and dexrazoxane-induced apoptosis in HTETOP cells. A, scatter plot of the comparison between genome-wide gene expression profiles, expressed as log2, of untreated and tetracycline-treated (1 μg/mL tetracycline for 24 h) HTETOP cells. Genes with <2-fold expression differences are contained between the two diagonal lines. Arrows, two probe sets targeting TOP2A. B, time course and TOP2A dependency of apoptosis induced by doxorubicin as assessed by Annexin V-FITC staining and FACS analysis. Untreated or TOP2A-depleted (1 μg/mL tetracycline for 24 h) cells were incubated with 1 μmol/L doxorubicin for the indicated periods. Results are expressed as fold change in apoptosis in comparison with cells without doxorubicin treatment at 0 h. #, P < 0.05, compared with untreated cells at 0 h; *, P < 0.05, compared with untreated cells at 0 h; **, P < 0.05, compared with tetracycline-untreated cells at the same time point. C, effect of tetracycline pretreatment (1 μg/mL, 24 h) on apoptosis induced by 24 h of subsequent incubation with the indicated doxorubicin concentrations. #, P < 0.05, compared with untreated cells (0 μmol/L); **, P < 0.01, compared with cells treated with the same doxorubicin concentration but without tetracycline. D, dexrazoxane-induced apoptosis detected by Annexin V-FITC staining and FACS analysis in cells incubated for 24 h at the indicated concentrations of dexrazoxane with or without tetracycline pretreatment (1 μg/mL, 24 h). *, P < 0.05, compared with cells treated with the same dexrazoxane concentration but without tetracycline.

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**Topoisomerase IIα in Anticancer Drug-Induced Apoptosis**

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Unexpectedly, we found that dexrazoxane decreased, in a concentration-dependent manner, the intracellular TOP2A protein content of HTETOP cells (Fig. 4A, top; see Supplementary Figure 3 for the quantitative results of all Western blots from Fig. 4). This effect is unlikely to reflect the well-known phenomenon of "band depletion," which is caused by the rapid formation of dexrazoxane-DNA-TOP2A complexes that remain in sample wells during electrophoresis (22), because all samples were treated with DNase I to release TOP2A from the complexes with DNA before analysis. Furthermore, TOP2A protein decreased slowly, over a period of 24 h (Fig. 4A, bottom; Supplementary Fig. B), whereas "band depletion" is detectable within minutes after the incubation onset (22). Because dexrazoxane has been recently shown to trigger a proteasome-mediated degradation of TOP2B (20),

Figure 3. Effect of coapplication of dexrazoxane and doxorubicin on DSB formation, TOP2A protein level, and apoptosis. A, DSB, assessed as γ-H2AX foci, and TOP2A protein level following 0.1 or 1 μmol/L doxorubicin and/or 100 μmol/L dexrazoxane administered for the indicated periods schematically depicted in B, that is for 0.5 h before doxorubicin (0.5 h), for 0.5 h before and subsequently together with doxorubicin (24.5 h), together with doxorubicin (24 h), and during the last 6 h of the incubation with doxorubicin (6 h). *, P < 0.05, compared with cells treated with the same doxorubicin concentration but without dexrazoxane. B, apoptosis detected by Annexin V-FITC staining and FACS analysis in cells treated under the same conditions as in A. *, P < 0.05, compared with cells treated with the same concentration of doxorubicin but without dexrazoxane.
we investigated the importance of proteasome in the dexrazoxane-induced TOP2A depletion. Dexrazoxane-induced TOP2A depletion could not be prevented by the proteasome inhibitor MG-132 (0.25 μmol/L; Fig. 4B). In most experiments, MG-132 also increased TOP2A level in dexrazoxane-untreated cells (Fig. 4B), casting doubts on the specificity of its antagonistic effect on dexrazoxane. In search of an alternative explanation for TOP2A depletion, we measured mRNA expression level of TOP2A following dexrazoxane (100 μmol/L, 24 h) exposure. Dexrazoxane clearly reduced TOP2A mRNA (Fig. 4C). This effect was also observed in the parental, nontransgenic cell line HT1080 (data not shown). In contrast to HTETOP cells, TOP2A expression in HT1080 cells is driven by an normal, endogenous TOP2A promoter. The results from both cell lines taken together suggest that dexrazoxane decreases TOP2A mRNA level via a promoter-independent mechanism. Regardless of its precise mechanism, the reduction of TOP2A mRNA was remarkably specific as shown by genome-wide RNA microarray analysis of HTETOP cells exposed to 100 μmol/L dexrazoxane for 24 h. In this analysis, TOP2A was one of only two genes with expression level altered by dexrazoxane (3-fold decrease), the other being the activating transcription factor 3 (2.5-fold increase). In agreement with a recent report (20), dexrazoxane also caused TOP2B depletion in a concentration- and time-dependent manner (Fig. 4D). This effect was observed in the absence of any changes of TOP2B transcript level as assessed by genome-wide RNA microarrays. Taken together, these results indicate that the dexrazoxane-induced depletion of TOP2A and TOP2B occur via different mechanisms.

Further differences in the cellular responses to doxorubicin and dexrazoxane were revealed by measurements of glutathione content following drug exposure. Glutathione [specifically the ratio between its oxidized (GSSG) and its reduced (GSH) forms] is an indicator of oxidative stress, which has been inconclusively implicated in doxorubicin response (10). The proportion of total glutathione (GSSG + GSH) that was GSSG remained unchanged (8-11%) following exposure to doxorubicin (Fig. 5A). On the other hand, up to 60% of the total glutathione content in HTETOP cells was depleted, in a concentration-dependent manner, following treatment with doxorubicin. As a positive control, an inhibitor of glutathione synthesis (buthionine sulfoximine; ref. 23) was found to deplete total glutathione by >95% (Fig. 5A). The compensation of glutathione loss by coincubation with the cell membrane-permeable glutathione ethyl ester reduced the apoptosis rate by 46% (Fig. 5B). This indicated that the depletion of total glutathione contributed to apoptosis by doxorubicin. Strikingly, glutathione ethyl ester had no effect on DSB formation by 1 μmol/L doxorubicin, although it prevented DSB evoked by H2O2 (Fig. 5C). This suggested that the portion of doxorubicin-induced apoptosis associated with loss of total glutathione is not mediated by DSB. In contrast to doxorubicin, dexrazoxane (100 μmol/L) affected neither the total glutathione nor the ratio between its oxidized and reduced forms (data not shown). Accordingly, supplementation with glutathione ethyl ester had no effect on dexrazoxane-induced apoptosis (data not shown).

Responses to doxorubicin and dexrazoxane showed more similarities with respect to the involvement of p53 and
caspases. Although these factors play central roles in apoptosis triggered by DNA damage, the importance of p53 to the doxorubicin response is controversial (24, 25) and to the dexrazoxane response unknown. Apoptosis induced in HTETO cells by 0.1 and 1 μmol/L doxorubicin or by 100 μmol/L dexrazoxane was totally abolished by the recently developed p53 inhibitor PFT-μ (Fig. 6A and B). PFT-μ inhibits p53 binding to mitochondria via reducing its affinity to anti-apoptotic proteins Bcl-xL and Bcl-2 without affecting the p53-dependent transactivation (26). In contrast, the inhibitor of the p53 transcriptional pathway PFT-α had no effect on apoptosis (Fig. 6A and B). Furthermore, doxorubicin and dexrazoxane each activated caspase-3/7, and activation was attenuated by the pan-caspase inhibitor Z-VAD-FMK (Fig. 6C). TOP2A depletion using tetracycline almost halved caspase-3/7 activation by 1 μmol/L doxorubicin (Fig. 6C, top) and abolished that evoked by dexrazoxane (Fig. 6C, bottom). Furthermore, doxorubicin (Fig. 6D), but not dexrazoxane (data not shown), cleaved the upstream activator of caspase-3/7, caspase-8. This effect was abolished following tetracycline pretreatment (Fig. 6D), indicating that caspase-8 acts downstream of TOP2A in response to doxorubicin.

These results clearly showed commonalities but also differences in cell-killing mechanisms by doxorubicin and dexrazoxane. Common elements included TOP2A targeting resulting in DSB formation, involvement of mitochondrial p53, and activation of caspase-3/7. Doxorubicin-specific elements comprised GSH depletion and caspase-8 activation. The dexrazoxane-specific ones included the TOP2A depletion and the caspase-3/7-independent fraction of apoptosis in TOP2A-depleted cells (compare bottom of Fig. 6C with Fig. 2D). We therefore investigated the effects of both drugs, applied in various combinations of 100 μmol/L dexrazoxane, 0.1 or 1 μmol/L doxorubicin, and four incubation time schedules. Thus, in comparison with the 24 h incubation with doxorubicin (or control vehicle), dexrazoxane was applied during the preceding 0.5 h followed by washout (5 × 5 min with PBS), simultaneously with doxorubicin (24 h), during the last 6 h of doxorubicin incubation (6 h), or throughout the entire experiment (24.5 h). As shown in Fig. 3A, addition of dexrazoxane clearly decreased DSB induced by 0.1 μmol/L doxorubicin (dexrazoxane for 24.5, 24, and 6 h) and 1 μmol/L doxorubicin (dexrazoxane for 24.5 and 6 h) but not dexrazoxane (data not shown), cleared the upstream activator of caspase-3/7, caspase-8. This effect was abolished following tetracycline pretreatment (Fig. 6D), indicating that caspase-8 acts downstream of TOP2A in response to doxorubicin.

Figure 5. Role of intracellular glutathione in the doxorubicin response of HTETOP cells. A, GSH and GSSG content in cells following 24 h treatment with the indicated concentrations of doxorubicin or with buthionine sulfoximine (2 mmol/L). *, P < 0.01, compared with untreated cells. B, effect of coincubation with 10 mmol/L GSH ester on the apoptosis in response to 1 μmol/L doxorubicin (24 h) assessed by Annexin V-FITC staining and FACS. #, P < 0.05, compared with doxorubicin-untreated cells; *, P < 0.05, compared with cells treated with doxorubicin but without GSH ethyl ester. C, effect of 10 mmol/L GSH ethyl ester on DSB formation assessed by Western blot as γ-H2AX foci in response to 100 μmol/L H2O2 (2 h) or 1 μmol/L doxorubicin (24 h) treatment. *, P < 0.05, compared with cells treated with H2O2 but without GSH ethyl ester.
dexrazoxane did not reduce apoptosis at any combination of doxorubicin concentration or incubation schedule (Fig. 3B). Quite the contrary, the extent of apoptosis in cells cotreated with 0.1 \( \mu \)mol/L doxorubicin plus dexrazoxane was actually greater than in cells treated with 0.1 \( \mu \)mol/L doxorubicin alone (Fig. 3B, middle). Similar results were also observed in two other tumor cell lines, A549 and DLD1 (data not shown). Finally, TOP2A depletion by tetracycline pretreatment of HTETOP cells reduced by 30% (\( P < 0.05 \)) the apoptotic response to the combination of 1 \( \mu \)mol/L doxorubicin and 100 \( \mu \)mol/L dexrazoxane over 24 h (data not shown). This indicated that apoptosis was mediated by both TOP2A-dependent and TOP2A-independent mechanisms as was observed when each drug was applied alone (Fig. 2C and D).

**Discussion**

The replacement in HTETOP cells of the endogenous TOP2A gene by a tetracycline-responsive transgene allows for bypassing the cellular lethality of TOP2A deletion. Phenotypic differences between tetracycline-treated and untreated cells reflect chiefly the effects of TOP2A, because its depletion occurs in the absence of any changes in the expression of other genes (Fig. 2A) and in cell cycle distribution within the first 48 h (13). This allowed, for the first time, to clearly differentiate between TOP2A-dependent and TOP2A-independent effects of doxorubicin. Using this tool, we also assessed the cancer cell-killing mechanisms of dexrazoxane, which is administered to cancer patients to prevent the cardiotoxic side effects of doxorubicin and other anthracyclines. The results suggest an explanation for the absence of adverse dexrazoxane effects on cancer treatments with doxorubicin despite these drugs sharing TOP2A as their major molecular target. The explanation lies in the TOP2A-independent component of the apoptosis triggered by each drug, which compensates for the dexrazoxane-induced TOP2A depletion and associated reduction in doxorubicin-induced DSB. To arrive at this conclusion, we first had to characterize the importance of TOP2A in the apoptotic effects of each drug.
**Doxorubicin-Induced Apoptosis Involves TOP2A Inhibition and GSH Depletion**

DSB formation and apoptosis are mediated exclusively by TOP2A only at low doxorubicin concentrations (0.1 μmol/L). At higher doxorubicin concentrations, roughly half of the doxorubicin-induced apoptosis is TOP2A independent (Fig. 2C). This finding is important in the light of current efforts to develop TOP2A expression level as a predictor of doxorubicin response in breast cancer (9). Such a predictive value of TOP2A is at present controversial, because some studies found only weak correlations between tumor response to doxorubicin and TOP2A expression levels (28). Our data indicate that this may be caused, at least in part, by the confounding contribution of TOP2A-independent apoptotic pathways.

TOP2A-independent apoptosis pathways may nevertheless be useful for optimizing doxorubicin treatments and so deserve further investigation. A major portion of doxorubicin-induced apoptosis in HTETOP cells appears to be triggered by a pronounced depletion of total cellular glutathione. Glutathione depletion by doxorubicin has been reported in a recent study (29) and intracellular glutathione levels are remarkably specific predictors of doxorubicin-induced cell growth inhibition (30). The glutathione depletion could be caused by outward cotransport of doxorubicin and glutathione by the multidrug resistance protein 1 (31) or by inhibition of glutathione synthesis (23). Irrespective of the exact mechanism, the depletion contributes to, rather than results from, doxorubicin-induced apoptosis in HTETOP cells, because cell death is reduced by glutathione supplementation (Fig. 5B). The TOP2A and glutathione depletion most likely represent nonoverlapping apoptotic pathways. Indeed, although both TOP2A depletion and GSH supplementation diminish doxorubicin-induced apoptosis (Figs. 2C and 5B), only the former reduces the associated DSB (Figs. 1A and 5C). We detected no evidence for oxidative stress due to “redox cycling” of doxorubicin as judged from the remarkably constant proportion of GSSG in the total intracellular glutathione pool following doxorubicin treatment (Fig. 5A). This is consistent with the growing evidence that oxidative stress due to doxorubicin “redox cycling” plays no role in cancer cell killing at clinically relevant concentrations of the drug (10).

A portion of DSB induced by ≥1 μmol/L doxorubicin was refractory to TOP2A depletion (Fig. 1A) and/or glutathione supplementation (Fig. 5C), indicating the existence of further mechanisms of DSB generation. Likely candidates include the multifarious direct interactions between doxorubicin and DNA (10) or the doxorubicin poisoning of TOP2B, which is expressed in HTETOP cells, albeit at a much lower level than TOP2A (13). TOP2B could also mediate a portion of TOP2A-independent apoptosis in response to doxorubicin. Oxidative stress was probably not involved in DSB formation, because, as already stated, glutathione ethyl ester did not reduce doxorubicin-induced DSB accumulation.

**Dexrazoxane Decreases TOP2A Transcript Level**

The dexrazoxane-induced formation of DSB was fully tetracycline sensitive (Fig. 1B), suggesting that the DSB are exclusively TOP2A derived. This supports previous reports of dexrazoxane and other bisdioxopiperazines being TOP2A “poisons” in addition to the inhibition of the catalytic activity of the enzyme (12, 32). The molecular mechanism of TOP2A “poisoning” could involve TOP2A targeting beyond the ATPase-bearing NH2-terminal domain as shown for a Drosofila TOP2 and the related bisdioxopiperazine ICRF-159 (33). Dexrazoxane-induced apoptosis in HTETOP cells is in agreement with several previous studies of cancer-derived cells (34-36), but its mechanism remains poorly understood. Our data clearly show the involvement of TOP2A, because apoptosis was partly reduced by tetracycline (Fig. 2D).

Like cardiomyocytes (20), HTETOP cells respond to dexrazoxane by degradation of the TOP2B protein (Fig. 4D), indicating that this effect is not heart exclusive. At the mRNA level, dexrazoxane affected the expression of only two genes, activating transcription factor 3 and TOP2A. The remarkable specificity of dexrazoxane-induced gene expression changes contrasts with effects of doxorubicin and other oncologic drugs, which usually affect hundreds of genes (37). This is important in the context of further development of dexrazoxane as simultaneously anticancer and organ-protective agent. Activating transcription factor 3 is proapoptotic in several cancer cell lines (38, 39), but it protects cardiomyocytes against doxorubicin-induced cell death (40). Activating transcription factor 3 may be a specific and central determinant of the disparate and cell type-specific outcomes of dexrazoxane exposure such as cell death and cell protection.

The reduced expression of TOP2A mRNA in response to dexrazoxane was reflected by reduced levels of TOP2A protein. Dexrazoxane-induced depletion of TOP2A transcripts was observed in both HTETOP and parental HT1080 cells, which transcribe TOP2A mRNA from totally different promoters. It seems unlikely, therefore, that this new and unexpected effect of dexrazoxane involves reduced transcriptional initiation, but transcript degradation, repression of transcriptional elongation, or termination may be responsible. TOP2A depletion following dexrazoxane treatment in HTETOP cells contrasts, at least at a first glance, with the absence of such an effect in cardiomyocytes (20). However, the effects of dexrazoxane on TOP2A in cardiomyocytes were investigated over short periods of time (≤60 min), whereas, in HTETOP, the decrease in TOP2A cells was only detectable 12 h after dexrazoxane addition (Supplementary Fig. B).3 It should also be emphasized that we detected the dexrazoxane-associated TOP2A decrease in HTETOP cells at both protein and mRNA levels using three different methods (Western blot, TaqMan, and RNA chips). The exact mechanisms of dexrazoxane-induced TOP2A depletion require more detailed studies, which are currently ongoing. Because dexrazoxane binds and inhibits the TOP2A protein, the simplest hypothesis is that the enzyme regulates its own transcript level.

**No Adverse Effect of Dexrazoxane on Doxorubicin-Induced Apoptosis**

TOP2A depletion by dexrazoxane could reasonably be expected to reduce the TOP2A-dependent portion of apoptosis
by doxorubicin. This is indeed the case in respect of doxorubicin-induced DSB, which are clearly reduced, along with the TOP2A level, in cells coexposed to dexrazoxane (Fig. 3A). Dexrazoxane-dependent reductions in DSB induction and TOP2A levels are especially pronounced in HTETOP cells exposed to 0.1 μmol/L doxorubicin. Considering that dexrazoxane depletes TOP2A (Fig. 4A) and that apoptosis induced by 0.1 μmol/L doxorubicin is completely dependent on TOP2A (Fig. 2C), the majority of apoptosis in response to 0.1 μmol/L doxorubicin combined with dexrazoxane must be induced by dexrazoxane. In support of this conclusion, dexrazoxane combined with 0.1 μmol/L doxorubicin induces levels of apoptosis similar to, or greater than, those induced by 0.1 μmol/L doxorubicin alone (Fig. 3B) despite the much lower accumulation of DSB (Fig. 3A). Thus, dexrazoxane-induced apoptosis fully compensates for the reduction of 0.1 μmol/L doxorubicin-induced apoptosis due to dexrazoxane-induced TOP2A depletion. When applied with 1 μmol/L doxorubicin, dexrazoxane still reduces doxorubicin-induced DSB, especially significant for coinoculation periods of 24.5 h (Fig. 3A). The depletion of TOP2A by dexrazoxane is, however, less pronounced under these conditions than on coinoculation with 0.1 μmol/L doxorubicin. This can be explained by the accumulation of doxorubicin-“poisoned” TOP2A, which is higher at 1 μmol/L than 0.1 μmol/L doxorubicin, and may partly offset the TOP2A-depleting effect of dexrazoxane. Thus, as for 0.1 μmol/L doxorubicin, apoptosis induced by 1 μmol/L doxorubicin is not diminished by dexrazoxane in any of the tested incubation schedules (Fig. 3B).

This apparent contradiction between dexrazoxane-dependent reductions in TOP2A and doxorubicin-induced DSB on one hand and the absence of any dexrazoxane-dependent reductions in doxorubicin-induced apoptosis on the other hand can be explained by the TOP2A-independent apoptosis effects of each drug. Those of doxorubicin clearly involve glutathione depletion as well as DSB formation via an unknown mechanism. TOP2B is unlikely to contribute, because it is depleted by dexrazoxane (Fig. 4D), in agreement with previous studies (20). The TOP2A-independent component of dexrazoxane-induced apoptosis is at present more enigmatic, but it is dependent on the mitochondrial p53 and may involve caspase-independent apoptotic pathways. Indeed, unlike the caspase-3/7 activity itself, the TOP2A-independent fraction of apoptosis evoked by dexrazoxane was insensitive to the pan-caspase inhibitor Z-VAD-FMK. The involvement of mitochondrial p53 is in agreement with previous reports of dexrazoxane-induced apoptosis being modulated by Bcl-xL (36).

Taken together, doxorubicin and dexrazoxane may interact in cancer cells such as HTETOP via several different mechanisms. The overlapping component comprises inhibition of TOP2A by both drugs, in each case resulting in DSB formation and apoptosis. Dexrazoxane interacts with the doxorubicin response via degradation of the doxorubicin target TOP2A, which explains the reduction in total DSB accumulation observed on combination of these drugs. Despite this interaction, dexrazoxane does not compromise the apoptotic activity of doxorubicin. This is caused by the compensating contribution of TOP2A-independent apoptosis evoked by both drugs. These results suggest a mechanistic explanation for the absence of adverse effects of dexrazoxane on anthracycline treatments, recently confirmed in a metanalysis conducted by the Cochrane Collaboration (3). Under circumstances exemplified by low doxorubicin availability, dexrazoxane seems actually to enhance the net extent of HTETOP cell killing. This is in agreement with early observations in several animal cancer models (7) as well as with dexrazoxane having doubled the mean survival time of breast cancer patients who responded to doxorubicin (41).

Disclosure of Potential Conflicts of Interest

L. Wojnowski: Dexrazoxane Advisory Board of Novartis. No other potential conflicts of interest were disclosed.

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