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Dexrazoxane mitigates epirubicin-induced genotoxicity in mice bone marrow cells

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Abstract

Dexrazoxane is the only clinically approved cardioprotectant against anthracyclines-induced cardiotoxicity. Thus, detailed evaluation of the genotoxic potential of dexrazoxane and anthracyclines combination is essential to provide more insights into genotoxic and anti-genotoxic alterations that may play a role in the development of the secondary malignancies after treatment with anthracyclines. Thus, our aim was to determine whether non-genotoxic doses of dexrazoxane in combination with the anthracycline, epirubicin can modulate epirubicin-induced genotoxicity and apoptosis in somatic cells. Bone marrow micronucleus test complemented with fluorescence *in situ* hybridization assay and comet assay were performed to assess the genotoxicity of dexrazoxane and/or epirubicin. Apoptosis was analysed by using the annexin V assay and the occurrence of the hypodiploid DNA content. Generation of reactive oxygen species was also assessed in bone marrow by using the oxidant-sensing fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate. Dexrazoxane was neither genotoxic nor apoptogenic in mice at a single dose of 75 or 150 mg/kg. Moreover, it has been shown that dexrazoxane affords significant protection against epirubicin-induced genotoxicity and apoptosis in the bone marrow cells in a dose-dependent manner. Epirubicin induced marked generation of intracellular reactive oxygen species and prior administration of dexrazoxane ahead of epirubicin challenge ameliorated accumulation of these free radicals. It is thus concluded that dexrazoxane can be safely combined with epirubicin and that pre-treatment with dexrazoxane attenuates epirubicin-induced generation of reactive oxygen species and subsequent genotoxicity and apoptosis. Thus, epirubicin-induced genotoxicity can be effectively mitigated by using dexrazoxane.

Introduction

The anthracyclines are among the most effective anticancer treatments ever developed and are effective against more types of cancer than any other class of chemotherapeutic agents (1). The first anthracycline discovered was daunorubicin, which is produced naturally by *Streptomyces peucetius*. Doxorubicin was developed shortly thereafter, and many other related compounds such as epirubicin have followed (2). The primary mechanism of tumour cytotoxicity appears to be the inhibition of topoisomerase II (3). Other mechanisms of tumour cytotoxicity include the inhibition of nuclear helicases, inhibition of DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand and free radical formation (4). Because of these multiple effects, single- and double-strand

breaks are introduced into the DNA. DNA breaks can have a variety of biological effects, including the inhibition of transcription and/or replication and ultimately cell death.

Short-term toxicities of anthracycline including myelosuppression and gastrointestinal toxicity have been reported. Moreover, long-term survivors are at risk of cardiac toxicity and secondary leukemia (5). In fact, therapy-related acute monocytic or myelomonocytic with balanced chromosome translocations to band 11q23 was observed in cancer patients after chemotherapy with epirubicin as single-agent chemotherapy for breast cancer (6). Moreover, therapy-related acute leukemia, often presenting balanced translocations, has been observed following chemotherapy with epirubicin (7,8). Epirubicin-induced gene mutation *in vitro* and both *in vitro* and *in vivo* structural chromosomal aberrations have been reported. *In vitro*, epirubicin was positive for mutagenicity in the Ames test and in the HGPRT assay in V79 Chinese hamster lung fibroblasts in the absence, but not in the presence, of metabolic activation (9,10). Similarly, *in vitro*, epirubicin was clastogenic, producing chromosome aberrations in human lymphocytes, both in the presence and absence of metabolic activation. Epirubicin was also clastogenic *in vivo*, producing structural chromosomal aberrations in a mouse bone marrow (11).

The chemical structure of anthracyclines favours the generation of free radicals, and these compounds can bind to iron, form complexes with DNA and induce DNA damage and cause genotoxicity, and they thus play a key role in genomic damage and secondary leukemia (12). Thus, chemotherapy schedules need to be improved, and other supporting therapies must be investigated to reduce undesirable effects and provide a better quality of life to survivors. In this context, it was hypothesized that dexrazoxane may be useful because dexrazoxane is a derivative of EDTA and chelates iron, thus reducing the number of metal ions complexes with anthracyclines and consequently decreasing the formation of reactive metabolites generated by anthracyclines (13).

Dexrazoxane is the (+)-(S)-enantiomer of the racemic ICRF-159 (razoxane). Dexrazoxane has anti-tumour activity (14), probably by catalytically inhibiting topoisomerase II. Dexrazoxane has been found to reduce the risk of anthracycline-induced cardiotoxicity and promotes anticancer responses to anthracyclines (13,15). Moreover, dexrazoxane is effective against accidental anthracycline extravasation (16). Pre-clinical models showed efficacy when the dose ratio of dexrazoxane:anthracyclines was in the range of 5–20:1, with a clear dose-related effect for cardioprotection. Data from two small phase II studies conducted in 1992 comparing 20:1 and 10:1 dose ratios, respectively were provided. Although no significant differences were seen in either efficacy or safety outcomes, the studies were not adequately powered to determine which ratio has the more favourable benefit-risk balance. Studies conducted with dexrazoxane in the EU selected a dexrazoxane to doxorubicin ratio of 20:1. However, in the US pivotal studies, the initial ratio of 20:1 was reduced to 10:1 following reports of a higher death rate in the dexrazoxane arms (17). Animal studies, have indicated that the cardiac protective window is between 1:10–1:20 for doxorubicin. Since animal studies have shown that epirubicin is less cardiotoxic than doxorubicin at equimolar amounts, a dose ratio of 1:10 was adopted by clinicians to avoid the risk of increased incidence and severity of other tissue toxicities, primarily myelotoxicity (18). This ratio is supported by controlled clinical studies. Therefore, the committee for medicinal products for human use concluded that the 10:1 dexrazoxane to epirubicin ratio should remain unchanged.

Most of the information on the mutagenicity of dexrazoxane was available in the form of unpublished studies conducted by chemical companies solely for registration or marketing purposes. The data showed that the non-mutagenic potency of dexrazoxane in the Ames test but was found to be clastogenic in human lymphocytes *in vitro* and in mouse micronucleus test at a single oral dose of 1000 mg/kg. Data from clinical trials raised concerns that exposure to dexrazoxane may increase the risk of second malignant neoplasms, in particular acute myelogenous leukemia/myelodyspastic syndrome and solid tumours in paediatric patients. Moreover, an increased risk of myelosuppression and infection was also observed in paediatric patients (19).

Despite dexrazoxane's increasing use in mitigating epirubicin-induced cardiotoxicity, no data are available in the literature on the potential genotoxicity of drug combination. Therefore, a detailed assessment of the genotoxicity of this combination is essential to provide more insights into genotoxic alterations that may play a role in the development of the secondary tumours from cells that were not originally neoplastic after epirubicin exposure. The concept of providing protection against genotoxicity in normal cells will represent a promising approach of attacking the unavoidable toxicity from cytotoxic chemotherapy; this will permit higher doses of genotoxic drugs to be given.

Materials and Methods

Animals

The widely distributed male Swiss albino mice, largely noninbred, aged 10–14 weeks and weighing 20–25 g were used in our experiments. Mice were obtained from the Experimental Animal Care Center, King Saud University and were maintained in an air conditioned animal house at a temperature of 25–28°C, relative humidity of ~50% and photo-cycle of 12:12 h light and dark periods. The animals were provided with standard diet pellets and water *ad libitum*. All experiments on animals were carried out according to the Guidelines of the Animal Care and Use Committee, King Saud University, Kingdom of Saudi Arabia. Each treatment group and vehicle control group consisted of six randomly assigned animals.

Chemicals and treatment

Epirubicin and dexrazoxane (Sigma-Aldrich, St Louis, MO, USA, >90% pure) were dissolved in 10% dimethyl sulfoxide (DMSO) in saline and were administered by intraperitoneal injection within 1 h following preparation. DMSO up to 15% has previously been shown to be non-genotoxic solvent in several studies carried out *in vivo* (20,21). The intraperitoneal injection was selected because the bone marrow is a well-perfused tissue and the concentrations of the test substance in bone marrow will be similar to those observed in blood after intravenous injection. Moreover, the achievement of drug to bone marrow after intraperitoneal rout is faster than the oral rout. The administrated volume was 0.01 ml per 1 g body weight of each mouse. Epirubicin was administered at the dose level of 3, 6 and 12 mg/kg. The doses of epirubicin were selected on the basis of its effectiveness in inducing genotoxicity in mice (11,22). In human chemotherapy, epirubicin is typically administered at doses up to 135 mg/m^2 as a single agent every 3 to 4 weeks. Mice have a body weight/surface area ratio of ≈ 3 kg/m^2. Thus, the highest dose of 12 mg/kg used in our study corresponds to ≈ 36 mg/m^2 and is within the dose range used for human chemotherapy. Preliminary experiments were conducted to determine the levels at which dexrazoxane has no observable genotoxic effects on mouse somatic cells. In the preliminary experiment, animals were treated intraperitonially with 10% DMSO or with dexrazoxane at single doses of 75, 150, 300 and 600 mg/kg, and clinical signs of toxicity and genotoxicity were recorded. Dexrazoxane caused slight bone marrow suppression at the highest dose as detected by the MN test and increased DNA

strand breaks in bone marrow of mice after treatment with 300 and 600 mg/kg dexrazoxane as detected by the comet assay (Figures 1 and 2); thus the doses have been limited to 600 mg/kg.

The preliminary negative cytogenetic results for dexrazoxane at 75 and 150 mg/kg led to the use of these two doses for dexrazoxane in our experiments. Thus, doses of 75 and 150 mg/kg dexrazoxane were injected intraperitonially 30 min before epirubicin treatment, which modelled the recommended time-course of combining anthracyclines with dexrazoxane in humans. The positive control groups were injected intraperitoneally with either single doses of 50 mg/kg ethyl nitrosourea (ENU; Sigma-Aldrich), 2 mg/kg mitomycin C (MMC; Sigma-Aldrich) or 2 mg/kg colchicine (COL; Sigma-Aldrich) dissolved in saline 3 h (ENU) or 24 h (MMC and COL) before being killed, while the negative control groups received equal amounts of DMSO in saline. All other chemicals were of the finest analytical grade. After drug administration, the animals were maintained with food and water *ad libitum* until being sacrificed (Table 1).

Bone marrow micronucleus test

Twenty-four hours after dexrazoxane and/or epirubicin treatment, six treated and six solvent control mice were killed by cervical dislocation under light ether anaesthesia. Both femurs were removed, and the bone marrow cells were collected. COL and MMC were used as a positive control aneugen and clastogen, respectively and bone marrow cells were sampled 24 h after treatment (23). At least four slides (~1.5×10^6 cells/slide) were made for each animal and allowed to dry overnight. One slide per animal was stained with May-Gruenwald/Giemsa solutions for conventional assessment of the micronuclei (MN) frequencies as described previously (24). The remaining, unstained slides were stored at −20°C to distinguish between clastogenic and aneugenic effects by identifying the origin of MN using the bone marrow fluorescence *in situ* hybridisation (FISH) assay as described previously (25). In addition, the ratio of normochromatic erythrocytes (NCEs) to polychromatic erythrocytes (PCEs) was determined by counting a total of about 2000 erythrocytes per slide such that all NCEs are counted in the field which are scored to count 1000 PCEs. The ratio was recorded to evaluate bone-marrow suppression and mitotic activity was calculated as %PCE = [PCE/(PCE + NCE)] × 100 (24).

Comet assay

In the time-course experiments, groups of six mice each were euthanized at 3 and 24 h following the treatments with dexrazoxane and/or epirubicin. The 3-h time point was chosen to maximise the chances of detecting an early genotoxic response to the dexrazoxane and/or epirubicin treatment. ENU and MMC were used as positive control genotoxins and bone marrow cells were sampled 3 and 24 h, respectively,

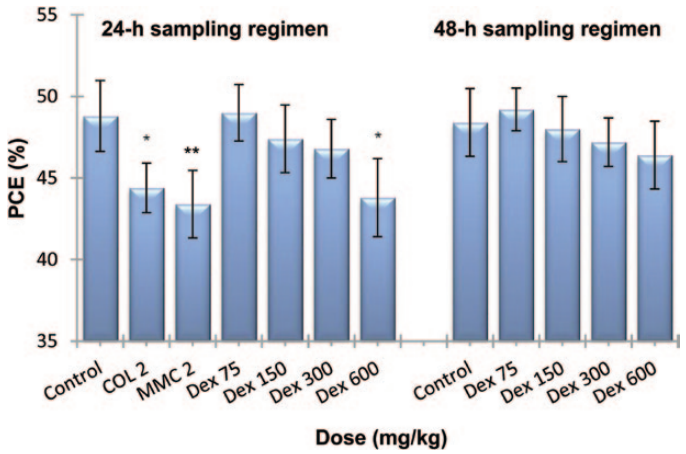

Figure 1. Frequencies of PCE in bone marrow of control mice and mice after treatment with the indicated doses of dexrazoxane (Dex) or with the positive controls MMC (2 mg/kg) and COL (2 mg/kg). Control = 10% DMSO in saline. $*P < 0.05$, $**P < 0.01$, compared with the corresponding control group (Mann-Whitney *U*-test).

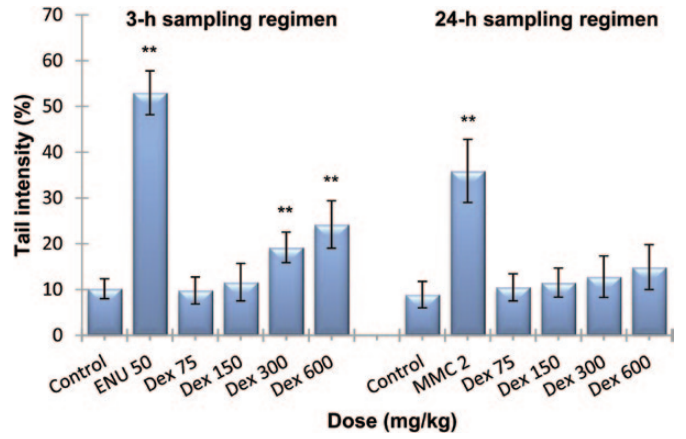

Figure 2. DNA strand breaks as detected by alkaline comet assay in bone marrow of mice 3 h or 24 h after treatment with the positive controls ENU (50 mg/kg) and MMC (2 mg/kg) or 3 h and 24 h after treatment with the indicated doses of dexrazoxane (Dex) (mean ± SD). Control = 10% DMSO in saline. The extent of DNA strand breaking quantified by the use of the percentage of tail intensity. $**P < 0.01$, compared with the corresponding control group (Mann-Whitney *U*-test).

Table 1. Protocol of exposure to the tested compounds, sampling times and measured endpoints for detection of genotoxicity

Drug treatment		Sampling times after exposure			Measured endpoints
30 min	0 h	3 h	24 h	48 h	
–	Dexrazoxane	–	Sacrifice	Sacrifice	1
–	Dexrazoxane	Sacrifice	Sacrifice	–	2
–	Epirubicin	–	Sacrifice	–	1
–	Epirubicin	Sacrifice	Sacrifice	–	2
Dexrazoxane	Epirubicin	Sacrifice	–	–	2
Dexrazoxane	Epirubicin	–	Sacrifice	–	1, 2, 3, 4, 5
–	MMC	–	Sacrifice	–	1, 2, 3
–	COL	–	Sacrifice	–	1, 3
–	ENU	Sacrifice	–	–	2
–	Control	Sacrifice	Sacrifice	Sacrifice	1, 2, 3, 4, 5

1 = Micronucleus test; 2 = Comet assay; 3 = Micronucleus test complemented by fluorescence *in situ* hybridization assay; 4 = Apoptosis assay; 5 = Reactive oxygen species generation assay. Control = 10% DMSO in saline. ENU, ethyl nitrosourea.

after treatment (26). Bone-marrow cells from one femur were collected in tubes containing foetal calf serum then centrifuged and resuspended in ice-cold PBS (Ca^{2+} and Mg^{2+} free, pH 7.4). 10 µl of cell suspension (~10 000 cells) were mixed with 85 µl of 0.5% low melting agarose (LMA), distributed onto the end frosted conventional slides precoated with 1.5% normal melting agarose in PBS. After the agarose solidifies, other 85 µl of LMA was layered and kept over ice for 10 min. The slides were prepared in duplicate. Cell lysis, electrophoresis and slide staining were performed according to the guidelines of OECD (27). The slides were examined using a fluorescent microscope. The microscope was connected to a computer through a charge coupled device camera. Images from 100 scorable cells from each replicate slide for each mouse were randomly selected and subjected to image analysis using software Comet Assay IV (Perceptive Instruments, Suffolk, UK). The extent of primary DNA strand break can be quantified by the use of tail intensity (%). Mean values of the tail intensity (%) were separately analysed for statistical significance.

Detection of apoptosis

To study the impact of dexrazoxane on the epirubicin-induced apoptosis in mouse bone marrow cells, groups of six mice each were treated with 12 mg/kg epirubicin and/or 150 mg/kg dexrazoxane. Animals were killed by cervical dislocation at 24 h after treatment and bone marrow cells from one femur were collected. A series of two related but different methods were used to detect apoptosis. First, the exposure of phosphatidylserine on the surface of early apoptotic cells caused by the test chemicals were assessed by Annexin V/propidium iodide (PI) staining as previously described (28). Second, the DNA content were quantified with PI according to the method described by Nicoletti *et al.* (29). For each mouse the stained cells (~ 5×10^5 in Annexin V/PI staining and ~ 1×10^6 in PI staining) were analysed by a Beckman Coulter flow cytometer. The amount of early apoptosis and the percentage of sub-G_1 apoptotic cells were calculated.

Measurement of reactive oxygen species generation

The remaining femora from the same animals used for the apoptosis detection was used to study the effect of dexrazoxane on oxidative stress induced by epirubicin. The generation of intracellular reactive oxygen species was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA) to form a fluorescent compound, 2′,7′-dichlorofluorescein (DCF), as previously described (30). The fluorescence intensity of ~2×10^5 cells was monitored with a FLUOstar OMEGA microplate reader (BMG LABTECH Ltd, Germany) with excitation wavelength of 485 nm and emission wavelength of 520 nm. Results were expressed as fold-difference with the control.

Statistical analysis

Results were expressed as means ± SD. Data were analysed by Kruskal-Wallis test followed by Dunn's multiple comparisons and Mann-Whitney *U*-test using the software computer program (GraphPad InStat; DATASET1.ISD). Results were considered significantly different if the *P* value was < 0.05.

Results

Effects of dexrazoxane on epirubicin-induced MN formation and bone marrow suppression

As expected, animals treated with the positive controls COL and MMC showed high frequencies of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow cells in comparison with the corresponding negative control (Figure 3). Furthermore, these compounds caused significant decrease in the mitotic activity at the interphase stage (Figure 1). The results of MN test have shown that epirubicin produces dose dependent increase in MN formation in mouse bone marrow (Table 2) and an increase in centromeric-negative and centromeric-positive stained MN, indicating the induction of both clastogenicity and aneugenicity (Figure 3). Additionally, epirubicin treatment caused significant decreases in the percent PCE at the two highest doses, indicating a reduction in erythroblast proliferation. On the other hand, dexrazoxane pre-treatment significantly reduced the epirubicin-induced MN formation however; this amelioration was still significant when compared to the value observed in the control group. Furthermore, both clastogenicity and aneugenicity induced by epirubicin treatment were reduced by dexrazoxane pre-treatment (Figure 3). The genotoxic protection was also directly correlated with bone marrow suppression as an obvious protection was noted with dexrazoxane pre-treated animals when bone marrow suppression was examined (Table 2).

Effects of dexrazoxane on epirubicin-induced DNA strand breaks

Statistically significant increases in the incidence of DNA strand breaks over the corresponding control values was observed following treatment with the positive control substances MMC and ENU (Figure 2). The results of comet assay after treatment with dexrazoxane and/or

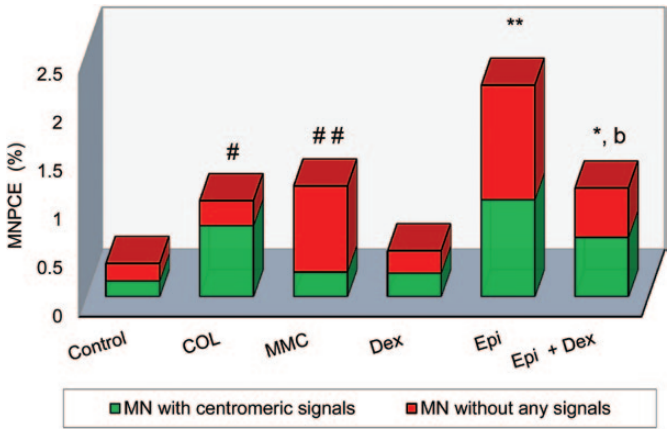

Figure 3. The contribution of clastogenicity (red) and aneugenicity (green) to the induced MN frequencies in mice 24 h after treatment with MMC (2 mg/kg), COL (2 mg/kg), dexrazoxane (Dex; 150 mg/kg) and/or 12 mg/kg of the epirubicin. Control = 10% DMSO in saline. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control (Kruskal-Wallis test followed by Dunn's multiple comparisons test). $^{b}P < 0.01$ compared with epirubicin alone, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the solvent control (Mann-Whitney *U*-test) (available in color online).

Table 2. Frequency of MNPCE and mitotic activity (% PCE) in bone marrow of mice 24 h after treatment with indicated doses of dexrazoxane and/or epirubicin (mean ± SD)

Treatment (mg/kg)	% MNPCE	% PCE
Control	0.32 ± 0.10	49.2 ± 1.30
Dexrazoxane (75)	0.38 ± 0.13	47.8 ± 2.58
Dexrazoxane (150)	0.42 ± 0.14	47.2 ± 4.38
Epirubicin (3)	0.96 ± 0.15**	43.4 ± 4.21
Epirubicin (6)	2.58 ± 0.44**	38.6 ± 3.50**
Epirubicin (12)	2.18 ± 0.29**	36.2 ± 4.54**
Dexrazoxane (75) + Epirubicin (3)	0.62 ± 0.13[a]	46.6 ± 2.30
Dexrazoxane (75) + Epirubicin (6)	1.32 ± 0.36*,[b]	44.4 ± 5.31
Dexrazoxane (75) + Epirubicin (12)	1.38 ± 0.29*,[a]	42.8 ± 2.16
Dexrazoxane (150) + Epirubicin (3)	0.52 ± 0.10[b]	48.0 ± 2.91
Dexrazoxane (150) + Epirubicin (6)	1.06 ± 0.25*,[b]	46.4 ± 2.96[b]
Dexrazoxane (150) + Epirubicin (12)	1.12 ± 0.24*,[b]	45.8 ± 4.02[a]

Control = 10% DMSO in saline.
[a]$P < 0.05$ compared with epirubicin alone (Mann-Whitney *U*-test).
[b]$P < 0.01$ compared with epirubicin alone (Mann-Whitney *U*-test).
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

epirubicin are shown in Table 3. Dexrazoxane treatment did not result in any significant difference in the level of DNA strand breaks at 75 and 150 mg/kg compared to the solvent control. Animals treated with epirubicin alone showed significant increase in the level of DNA strand breaks at both sampling time in comparison to those of the solvent control groups. Conversely, animals pre-treated with dexrazoxane showed significant decrease in the level of DNA strand breaks induced by epirubicin treatment only at 24-h sampling time compared to epirubicin alone. Importantly, at 3-h sampling time, the levels of DNA strand breaks in dexrazoxane pre-treated animals were decreased however; this amelioration was weak and still significant when compared to the values observed in the corresponding control group.

Effects of dexrazoxane on epirubicin-induced apoptosis

As shown in Figure 4, the average percentage of early apoptotic cells in bone marrow did not show any significant variation in dexrazoxane-treated animals compared with the solvent control. The number of early apoptotic cells observed in animals treated with epirubicin alone was significantly increased compared with the solvent control group. On the other hand, animals pre-treated with dexrazoxane showed a significant decrease in the percentage of early apoptotic cells in comparison with those treated with epirubicin alone. Similarly, the percentage of sub-G_1 peak did not show significant variation in dexrazoxane-treated animals compared to the solvent control (Figure 5). Compared to the control group, the percentage of cells with sub-G_1 peak was significantly increased by epirubicin treatment. Pre-treatment with dexrazoxane was found to protect mouse bone marrow cells against epirubicin-induced apoptosis and produces a noticeable decrease in the level of the hypodiploid DNA content induced by epirubicin in comparison to epirubicin alone.

Preventive potential of dexrazoxane against epirubicin-induced reactive oxygen species generation

Reactive oxygen species generation in mouse bone marrow was evaluated by determining the fluorescence intensity of DCF. As shown in Figure 6, DCF fluorescence did not show any significant variation after treatment of mice with dexrazoxane compared with the solvent control. A significant rise in bone marrow DCF fluorescence was observed in the epirubicin-treated mice compared to the solvent control. On the other hand, the epirubicin-induced increase in bone marrow DCF fluorescence was markedly suppressed in dexrazoxane pre-treated animals in comparison with epirubicin alone.

Discussion

The bisdioxopiperazine dexrazoxane has been found to reduce the risk of anthracycline-induced cardiotoxicity and promotes anticancer responses to anthracyclines (13,15). However, the detailed *in vivo* genotoxicity of dexrazoxane and the influence of dexrazoxane on anthracyclines-induced genotoxicity in somatic cells have not been reported yet. Therefore, a detailed evaluation of the genotoxic potential of this combination is essential to provide more insights into genotoxic/anti-genotoxic alterations that may play a role in the development of the secondary malignancies observed in cancer patients after chemotherapy with epirubicin. Hence, the objective of the present study was to determine the levels at which dexrazoxane has no observable genotoxic effects on mouse somatic cells and to

Table 3. DNA strand breaks as detected by alkaline comet assay in bone marrow of mice 3 h or 24 h after treatment with the indicated doses of dexrazoxane and/or epirubicin (mean ± SD)

Treatment (mg/kg)	Tail intensity (%)	
	Sampling time (3 h)	Sampling time (24 h)
Control	11.8 ± 2.94	9.4 ± 2.30
Dexrazoxane (75)	12.2 ± 4.02	9.2 ± 1.64
Dexrazoxane (150)	12.4 ± 3.84	11.8 ± 2.68
Epirubicin (3)	23.6 ± 4.87*	24.2 ± 4.38*
Epirubicin (6)	33.6 ± 3.36**	31.4 ± 3.57**
Epirubicin (12)	39.2 ± 3.96**	40.8 ± 5.21**
Dexrazoxane (75) + Epirubicin (3)	21.4 ± 3.97*	16.2 ± 2.16[a]
Dexrazoxane (75) + Epirubicin (6)	30.2 ± 5.54**	23.0 ± 3.53*,[b]
Dexrazoxane (75) + Epirubicin (12)	35.2 ± 2.48**	27.2 ± 5.42*,[a]
Dexrazoxane (150) + Epirubicin (3)	20.2 ± 2.77*	12.4 ± 3.50[b]
Dexrazoxane (150) + Epirubicin (6)	27.6 ± 4.87**	16.6 ± 3.20[a]
Dexrazoxane (150) + Epirubicin (12)	31.2 ± 4.38**	18.4 ± 2.88*,[b]

The extent of DNA strand break quantified by the use of the percentage of tail intensity. Control = 10% DMSO in saline.

[a]$P < 0.05$ compared with epirubicin alone (Mann-Whitney *U*-test).

[b]$P < 0.01$ compared with epirubicin alone (Mann-Whitney *U*-test).

*$P < 0.05$ and **$P < 0.01$ compared with the control (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

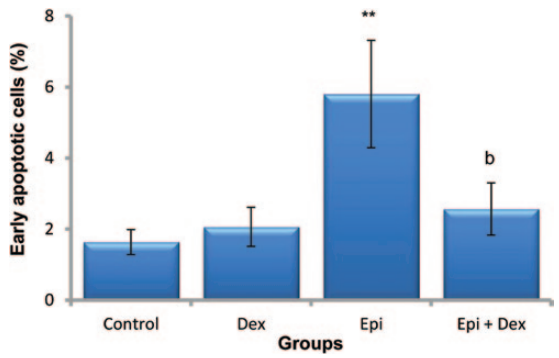

Figure 4. The percentage of early apoptotic cells in bone marrow of control mice and mice treatment with dexrazoxane (Dex; 150 mg/kg) and/or 12 mg/kg of epirubicin (Epi) (mean ± SD). Control = 10 % DMSO in saline. **$P < 0.01$ compared with the control (Kruskal-Wallis test followed by Dunn's multiple comparisons test). [b]$P < 0.01$ compared with epirubicin alone (Mann-Whitney *U*-test).

determine whether non-genotoxic doses of dexrazoxane in combination with epirubicin can modulate epirubicin-induced genotoxicity and apoptosis in somatic cells.

A series of related but different methods were used to detect genotoxicity of dexrazoxane in somatic cells of mice. Dexrazoxane caused slight bone marrow suppression and increased DNA strand breaks after treatment with 300 and 600 mg/kg. Thus, 75 and 150 mg/kg dexrazoxane were used to determine whether dexrazoxane in combination with epirubicin can ameliorates epirubicin-induced genotoxicity and apoptosis in bone marrow cells. These results confirm the literatures that have described the catalytic topoisomerase

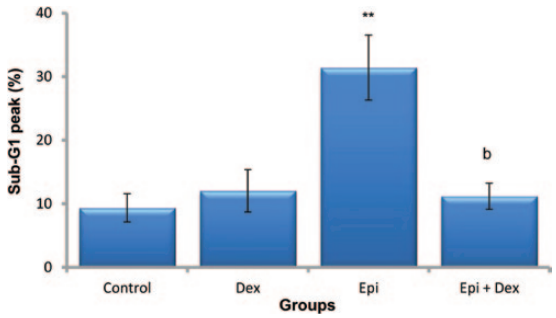

Figure 5. The percentage of apoptotic cells in bone marrow of control mice and mice treatment with dexrazoxane (Dex; 150 mg/kg) and/or 12 mg/kg of epirubicin (Epi) (mean ± SD). % Sub-G1 peak denote the percentage of cells with subdiploid DNA content (apoptotic cells), control = 10% DMSO in saline. **$P < 0.01$ compared with the control (Kruskal-Wallis test followed by Dunn's multiple comparisons test). [b]$P < 0.01$ compared with epirubicin alone (Mann-Whitney *U*-test).

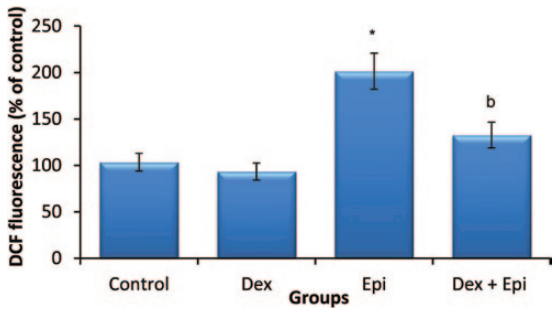

Figure 6. Effects of dexrazoxane (Dex; 150 mg/kg) on epirubicin (Epi; 12 mg/kg)-induced generation of intracellular reactive oxygen species in the bone marrow cells of mice (mean ± SD). Control = 10% DMSO in saline. *$P < 0.05$ compared with the control (Kruskal-Wallis test followed by Dunn's multiple comparisons test). [b]$P < 0.01$ compared with epirubicin alone (Mann-Whitney *U*-test).

II inhibitors which produce low levels of topoisomerase II-mediated DNA cleavage as having only modest or even no genotoxic activity (31,32). The positive control genotoxins MMC, COL and ENU were used in this study and the results of these compounds were in the same range as those of the earlier studies (26,33,34). These data confirmed the sensitivity of the experimental protocol followed in the detection of DNA damaging effects.

The MN assay has been widely used to measure chromosome damage. The assay, when performed properly, detects clastogenicity arising chromosome breakage (an important event in carcinogenesis) and aneugenicity arising chromosome lagging resulting from dysfunction of the mitotic apparatus (35). The results of MN test have shown that epirubicin produce dose dependent increases in the MN formation and an increase in centromeric-negative and centromeric-positive stained MN, indicating the induction of both clastogenicity and aneugenicity. The results of MN test confirm the findings of previous *in vivo* studies, where increases in clastogenicity and the aneugenicity in mouse bone marrow cells were observed after epirubicin treatment (11,22). Both the clastogenic and the aneugenic potential of epirubicin in somatic cells can give rise to the development of secondary tumours. On the other hand, dexrazoxane pre-treatment significantly reduced the epirubicin-induced MN formation however; this amelioration was still significant when compared to the values observed in the control group. The genotoxic protection was directly correlated with mitotic activity as an obvious protection was

noted with dexrazoxane pre-treated animals when bone marrow suppression was examined, which is in harmony with our previous study (21) and the study by Hofland *et al.* (36), where a reduction in etoposide-induced myelosuppression in mice pre-treated with dexrazoxane was observed. In addition, both clastogenicity and aneugenicity induced by epirubicin treatment were also reduced by dexrazoxane pre-treatment as detected by FISH assay with centromeric DNA probe.

In addition to genotoxicity, DNA repair and susceptibility to apoptosis are factors with an emerging role in cancer. Therefore, it was interesting to study the impact of dexrazoxane on these processes in normal cells. In order to investigate the impact of dexrazoxane on the capacity of repair of DNA damage induced by epirubicin, DNA strand breaks in animals treated with epirubicin and/or dexrazoxane were evaluated by sacrificing groups of exposed mice at 3 h or 24 h after the exposure to the epirubicin. At specific time, bone marrow cells were collected and comet assay was performed. Comet assay is capable of detecting the early stages of DNA strand breaks that frequently lead to genotoxicity in various organs. This assay has been reported to be sensitive to genotoxic carcinogens and relatively insensitive to non-genotoxic rodent carcinogens (37).

At the 3-h sampling time, animals treated with epirubicin showed significant increase in the level of DNA strand breaks at 3 and 24 h sampling regimen in comparison to those of the solvent control groups. Animals pre-treated with dexrazoxane showed significant decrease in the level of DNA strand breaks induced by epirubicin treatment only at 24-h sampling time compared to epirubicin alone. The antigenotoxic results of dexrazoxane confirm the findings of previous *in vitro* studies of the inhibition of topoisomerase-II poisons-induced DNA damage by dexrazoxane. Using alkaline elution assays, dexrazoxane in a dose-dependent manner inhibited the formation of DNA single-strand breaks as well as DNA-protein cross-links induced by the topoisomerase-II poisons etoposide and daunorubicin which are known to stimulate DNA-topoisomerase II cleavable complex formation (38). Importantly, at 3-h sampling time, the levels of DNA strand breaks in dexrazoxane pre-treated animals were decreased however, this amelioration was weak and not significant when compared to the values observed in the epirubicin alone groups. This means that dexrazoxane has a weak protective effect on the incidence of early DNA strand breaks induced by epirubicin and indicates that DNA damage observed by epirubicin at 24-h sampling time was weak and easily mitigated by dexrazoxane pre-treatment.

It is generally accepted that there is a threshold level of exposure below which the toxic effect is not expected to occur and that safe exposure levels can be defined for which an adequate margin of exposure exists between clinical exposure levels and the no effect level (NOEL) for the observed effect (39). For example, topoisomerase inhibitors are expected to have a threshold and the evidence has been shown by Lynch *et al.* (40). The present results with dexrazoxane indicate that the observed genotoxic effects have a threshold and dexrazoxane treatment did not result in any significant difference in the MN formation and the level of DNA strand breaks at 75 and 150 mg/kg. Moreover, these doses afford significant protection against epirubicin-induced MN formation and DNA strand breaks. However, the reduction is not complete (i.e. presence of some genotoxic effects compared with the corresponding solvent control) thus, the potential genotoxic risk still exists but weak.

As with many other anticancer drugs, high doses of anthracyclines disrupt the inner mitochondrial membrane potential and induce apoptosis. However, several lines of evidence indicate that low doses of various anthracyclines are capable of inducing mitotic catastrophe as the result of abnormal mitotic events that produce improper chromosomal segregation and cell division and lead to the formation of mutant cells (41). The average percentage of spontaneous apoptosis in bone marrow cells did not show any significant variation in dexrazoxane-treated animals compared with the solvent controls. The number of early apoptotic cells observed in mice treated with epirubicin was significantly increased compared with the untreated mice. On the other hand, animals pre-treated with dexrazoxane showed a significant decrease in the percentage of early apoptotic cells in comparison with those treated with epirubicin alone. Moreover, compared to the control group, the percentage of cells with sub-G_1 peak was significantly increased by epirubicin treatment. Pre-treatment with dexrazoxane was found to protect mouse bone marrow cells against epirubicin-induced apoptosis and produces a noticeable decrease in the level of the hypodiploid DNA content induced by epirubicin in comparison to epirubicin alone, which correlated well with the level of fragmented cells detected by comet assay. Information on the modulatory effects of dexrazoxane on topoisomerase-II poisons-induced apoptosis is limited, but certain catalytic inhibitor of topoisomerase-II, such as aclarubicin has been shown to inhibit etoposide-mediated apoptosis and toxicity in small intestine (42). Moreover, several results had shown that dexrazoxane reduced doxorubicin-induced apoptosis which are consistent with its ability to prevent daunorubicin-induced apoptosis of myocytes (43,44).

Epirubicin and the other anthracyclines are thought to exert their toxicity through iron-based oxygen free radicals (45). It is believed that oxygen free radicals can induce mutations and inhibit the DNA repair process, which results in the inactivation of certain tumour suppressor genes and leads to cancer (46,47). Junjing *et al.* (48) demonstrated that dexrazoxane was an effective antioxidant that could scavenge hydroxyl radicals in *in vitro* solution systems. Moreover, dexrazoxane has a potent intrinsic scavenging activity against not only hydroxyl radicals, which are the typical free radical produced from the redox reaction of iron complexes, but also peroxynitrite and peroxyl radicals when compared with the classic antioxidants glutathione, uric acid and trolox. These results indicate that the antioxidant properties of dexrazoxane are not solely dependent on iron chelation, although iron chelation may contribute to the decrease in iron-based free radical generation (48,49). In the present work, in order to evaluate whether the observed antigenotoxic effect was due to an enhancement of the scavenger of free radicals generated by epirubicin, generation of reactive oxygen species was determined after the animals were treated with epirubicin. The present study demonstrates that dexrazoxane pre-treatment reduced the epirubicin induced reactive oxygen species accumulation. Thus, scavenging of free radicals by dexrazoxane seems to be an important mechanism against the epirubicin-induced genotoxicity and apoptosis.

In summary, this study demonstrates that 75 and 150 mg/kg dexrazoxane can be safely combined with epirubicin. Moreover, dexrazoxane significantly mitigates epirubicin-induced genotoxicity and apoptosis. However, this amelioration was still statistically significant in comparison to the solvent control groups. The improvement in mitotic activity of bone marrow cells of animals pre-treated with dexrazoxane in epirubicin toxicity may focus attention on the beneficial effect of dexrazoxane to overcome one of the most serious problems in cancer chemotherapy, which is the bone-marrow suppression and related immunosuppression. The genotoxic effects of epirubicin might be, at least in part, mediated by an oxidative stress mechanism that may be prevented by dexrazoxane. Thus,

Oncologists who prescribe dexrazoxane as a cardioprotectant against anthracyclines-induced cardiotoxicity should also consider its additional, beneficial effect—a possible decrease in genotoxicity, which seems especially important for patients receiving epirubicin. Thus, epirubicin-induced genotoxicity can be effectively mitigated by using dexrazoxane and therefore it may be an effective drug to be used in combination with epirubicin to minimize the genotoxic effects of epirubicin in the bone marrow cells. Conclusively, dexrazoxane can be safely combined with epirubicin and might be useful to attenuate epirubicin insult in bone marrow and decrease bone marrow genotoxicity and associated health risks without decreasing the effectiveness of cancer therapy as evidenced by its clinical application to prevent both anthracyclines-induced cardiotoxicity and extravasation (13,15).

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Reference

1. Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. (2004) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev*., 56, 185–229.
2. Hortobágyi, G. N. (1997) Anthracyclines in the treatment of cancer. An overview. *Drugs*, 54, 1–7.
3. Gewirtz, D. A. (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem. Pharmacol*., 57, 727–741.
4. Ames, M. M. and Spreafico, F. (1992) Selected pharmacologic characteristics of idarubicin and idarubicinol. *Leukemia*, 6, 70–75.
5. Attia, S. M. (2008) Mutagenicity of some topoisomerase II-interactive agents. *Saudi Pharm. J*., 16, 1–24.
6. Kim, Y. G., Cho, S. Y., Park, T. S., Oh, S. H. and Yoon, H. J. (2011) Therapy-related myelodysplastic syndrome/acute myeloid leukemia with del(7)(q22) in a patient with de novo AML. *Ann. Clin. Lab. Sci*., 41, 79–83.
7. Hoffmann, L., Möller, P., Pedersen-Bjergaard, J., Waage, A., Pedersen, M. and Hirsch, F. R. (1995) Therapy-related acute promyelocytic leukemia with t(15;17) (q22;q12) following chemotherapy with drugs targeting DNA topoisomerase II. A report of two cases and a review of the literature. *Ann. Oncol*., 6, 781–788.
8. Andersen, M. K., Christiansen, D. H., Jensen, B. A., Ernst, P., Hauge, G. and Pedersen-Bjergaard, J. (2001) Therapy-related acute lymphoblastic leukaemia with MLL rearrangements following DNA topoisomerase II inhibitors, an increasing problem: report on two new cases and review of the literature since 1992. *Br. J. Haematol*., 114, 539–543.
9. El-Mahdy Sayed Othman, O. (2000) Cytogenetic effect of the anticancer drug epirubicin on Chinese hamster cell line in vitro. *Mutat. Res*., 468, 109–115.
10. Brumfield J. M. and Mackay W. J. (2002) Mutagenic activity of idarubicin and epirubicin in the bacterium Salmonella typhimurium. *Tex. J. Sci*., 54, 249–260.
11. Sen, A. K., Karakas, E., and Bilaloglu, R. (2010) Genotoxic effect of epirubicin in mouse bone marrow in vivo. *Z. Naturforschung. C*., 65, 211–217.
12. Luch, A. (2009) On the impact of the molecule structure in chemical carcinogenesis. *EXS*, 99, 151–179.
13. Jones, R. L. (2008) Utility of dexrazoxane for the reduction of anthracycline-induced cardiotoxicity. *Expert Rev. Cardiovasc. Ther*., 6, 1311–1317.
14. Von Hoff, D. D., Howser, D., Lewis, B. J., Holcenberg, J., Weiss, R. B. and Young, R. C. (1981) Phase I study of ICRF-187 using a daily for 3 days schedule. *Cancer Treat. Rep*., 65, 249–252.
15. Pearlman, M., Jendiroba, D., Pagliaro, L., Keyhani, A., Liu, B. and Freireich, E. J. (2003) Dexrazoxane in combination with anthracyclines lead to a synergistic cytotoxic response in acute myelogenous leukemia cell lines. *Leuk. Res*., 27, 617–626.
16. Conde-Estévez, D. and Mateu-de Antonio, J. (2014) Treatment of anthracycline extravasations using dexrazoxane. *Clin. Transl. Oncol*., 16, 11–17.
17. Swain, S. M., Whaley, F. S., Gerber, M. C., *et al*. (1997) Cardioprotection with dexrazoxane for doxorubicin-containing therapy in advanced breast cancer. *J. Clin. Oncol*., 15, 1318–1332.
18. Marty, M., Espie, M., Llombart, A., Monnier, A., Rapoport, B.L., Stahalova, V., and Dexrazoxane Study, G. (2006) Multicenter randomized phase III study of the cardioprotective effect of dexrazoxane (Cardioxane) in advanced/metastatic breast cancer patients treated with anthracycline-based chemotherapy. *Ann. Oncol*., 17, 614–622.
19. EMA. (2011) Assessment report dexrazoxane-containing medicinal products. www.ema.europs.eu.
20. Attia, S. M., Kliesch, U., Schriever-Schwemmer, G., Badary, O. A., Hamada, F. M. and Adler, I. D. (2003) Etoposide and merbarone are clastogenic and aneugenic in the mouse bone marrow micronucleus test complemented by fluorescence in situ hybridization with the mouse minor satellite DNA probe. *Environ. Mol. Mutagen*., 41, 99–103.
21. Attia, S. M., Al-Anteet, A. A., Al-Rasheed, N. M., Alhaider, A. A. and Al-Harbi, M. M. (2009) Protection of mouse bone marrow from etoposide-induced genomic damage by dexrazoxane. *Cancer Chemother. Pharmacol*., 64, 837–845.
22. Attia, S. M., Ahmad, S. F., Okash, R. M. and Bakheet, S. A. (2014) Aneugenic effects of epirubicin in somatic and germinal cells of male mice. *PLoS One*, 9, e109942.
23. Attia, S. M. (2009) Use of centromeric and telomeric DNA probes in in situ hybridization for differentiation of micronuclei induced by lomefloxacin. *Environ. Mol. Mutagen*., 50, 394–403.
24. Adler, I.-D. (1984) Cytogenetic tests in mammals. In Venitt, S., and Parry, JM. (ed.), *Mutagenicity Testing: A Practical Approach*. IRI Press, Oxford, pp. 275–306.
25. Attia, S. M. (2007) Chromosomal composition of micronuclei in mouse bone marrow treated with rifampicin and nicotine, analyzed by multicolor fluorescence in situ hybridization with pancentromeric DNA probe. *Toxicology*, 235, 112–118.
26. Attia, S. M., Ahmad, S. F., Zoheir, K. M., Bakheet, S. A., Helal, G. K., Abd-Allah, A. R., Al-Harbi, N. O., Al-Hosaini, K. A., and Al-Shabanah, O. A. (2014) Genotoxic evaluation of chloroacetonitrile in murine marrow cells and effects on DNA damage repair gene expressions. *Mutagenesis*, 29, 55–62.
27. *OECD Guidelines for Testing of Chemicals; Guideline no. 489: In Vivo Mammalian Alkaline Comet Assay*. Adopted, September 26, 2014.
28. Attia, S. M. (2010) The impact of quercetin on cisplatin-induced clastogenesis and apoptosis in murine marrow cells. *Mutagenesis*, 25, 281–288.
29. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F. and Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, 139, 271–279.
30. Attia, S. M. (2012) Influence of resveratrol on oxidative damage in genomic DNA and apoptosis induced by cisplatin. *Mutat. Res*., 741, 22–31.
31. Drake, F. H., Hofmann, G. A., Mong, S. M., Bartus, J. O., Hertzberg, R. P., Johnson, R. K., Mattern, M. R. and Mirabelli, C. K. (1989) In vitro and intracellular inhibition of topoisomerase II by the antitumor agent merbarone. *Cancer Res*., 49, 2578–2583.
32. Chen, M. and Beck, W. T. (1995) Differences in inhibition of chromosome separation and G2 arrest by DNA topoisomerase II inhibitors merbarone and VM-26. *Cancer Res*., 55, 1509–1516.
33. de Lima, P. L., Delmanto, R. D., Sugui, M. M., da Eira, A. F., Salvadori, D. M., Speit, G. and Ribeiro, L. R. (2001) Letinula edodes (Berk.) Pegler (Shiitake) modulates genotoxic and mutagenic effects induced by alkylating agents in vivo. *Mutat. Res*., 496, 23–32.
34. Dobrzyńska, M. M. (2005) The effects in mice of combined treatments to X-rays and antineoplastic drugs in the Comet assay. *Toxicology*, 207, 331–338.

35. Krishna, G. and Hayashi, M. (2000) In vivo rodent micronucleus assay: protocol, conduct and data interpretation. *Mutat. Res.*, 455, 155–166.
36. Hofland, K. F., Thougaard, A. V., Sehested, M. and Jensen, P. B. (2005) Dexrazoxane protects against myelosuppression from the DNA cleavage-enhancing drugs etoposide and daunorubicin but not doxorubicin. *Clin. Cancer Res.*, 11, 3915–3924.
37. Collins, A. R. and Ferguson, L. R. (2012) DNA repair as a biomarker. *Mutat. Res.*, 736, 2–4.
38. Sehested, M., Jensen, P. B., Sørensen, B. S., Holm, B., Friche, E. and Demant, E. J. (1993) Antagonistic effect of the cardioprotector (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187) on DNA breaks and cytotoxicity induced by the topoisomerase II directed drugs daunorubicin and etoposide (VP-16). *Biochem. Pharmacol.*, 46, 389–393.
39. Thybaud, V., Macgregor, J. T., Müller, L., *et al.* (2011) Strategies in case of positive in vivo results in genotoxicity testing. *Mutat. Res.*, 723, 121–128.
40. Lynch, A., Harvey, J., Aylott, M., Nicholas, E., Burman, M., Siddiqui, A., Walker, S. and Rees, R. (2003) Investigations into the concept of a threshold for topoisomerase inhibitor-induced clastogenicity. *Mutagenesis*, 18, 345–353.
41. Roninson, I. B., Broude, E. V., and Chang, B. D. (2001) If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist. Updat.*, 4, 303–313.
42. Holm, B., Jensen, P. B., Sehested, M. and Hansen, H. H. (1994) In vivo inhibition of etoposide-mediated apoptosis, toxicity, and antitumor effect by the topoisomerase II-uncoupling anthracycline aclarubicin. *Cancer Chemother. Pharmacol.*, 34, 503–508.
43. Sawyer, D. B., Fukazawa, R., Arstall, M. A. and Kelly, R. A. (1999) Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. *Circ. Res.*, 84, 257–265.
44. Popelová, O., Sterba, M., Hasková, P., *et al.* (2009) Dexrazoxane-afforded protection against chronic anthracycline cardiotoxicity in vivo: effective rescue of cardiomyocytes from apoptotic cell death. *Br. J. Cancer*, 101, 792–802.
45. Grankvist, K. and Henriksson, R. (1987) Doxorubicin and epirubicin iron-induced generation of free radicals in vitro. A comparative study. *Biosci. Rep.*, 7, 653–658.
46. Ravi, D. and Das, K. C. (2004) Redox-cycling of anthracyclines by thioredoxin system: increased superoxide generation and DNA damage. *Cancer Chemother. Pharmacol.*, 54, 449–458.
47. Attia, S. M. (2010) Deleterious effects of reactive metabolites. *Oxid. Med. Cell. Long.*, 3, 238–253.
48. Junjing, Z., Yan, Z. and Baolu, Z. (2010) Scavenging effects of dexrazoxane on free radicals. *J. Clin. Biochem. Nutr.*, 47, 238–245.
49. Galetta, F., Franzoni, F., Cervetti, G., *et al.* (2010) In vitro and in vivo study on the antioxidant activity of dexrazoxane. *Biomed. Pharmacother.*, 64, 259–263.