ORIGINAL ARTICLE

Protection of mouse bone marrow from etoposide-induced genomic damage by dexrazoxane

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Abstract

Purpose The objective of the current investigation is to determine whether non-toxic doses of the catalytic topoisomerase-II inhibitor, dexrazoxane, have influence on the genomic damage induced by the anticancer topoisomerase-II poison, etoposide, on mice bone marrow cells.

Method The scoring of micronuclei, chromosomal aberrations, and mitotic activity were undertaken as markers of cyto- and genotoxicity. Oxidative damage markers such as reduced glutathione and lipid peroxidation were assessed as a possible mechanism underlying this amelioration.

Results Dexrazoxane pre-treatment significantly reduced the etoposide-induced micronuclei formation, chromosomal aberrations, and also the suppression of erythroblast proliferation in bone marrow cells of mice. These effects were dose dependent. Etoposide induced marked biochemical alterations characteristic of oxidative stress including enhanced lipid peroxidation and reduction in the reduced glutathione level. Prior administration of dexrazoxane ahead of etoposide challenge ameliorated these biochemical markers.

Conclusion Based on our data presented, strategies can be developed to decrease the etoposide-induced genomic damage in normal cells using dexrazoxane.

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Introduction

Topoisomerase II (topo II) is a nuclear enzyme that transiently breaks both strands of a DNA segment and passes another double-stranded segment through the transient break, thus changing the DNA linking number and relieving torsional stress generated during DNA metabolism [43]. Since topo II plays an important role in many cellular processes, topo II inhibitors are among the most useful anticancer drugs for many types of cancer [41]. To date, there are two general classes of topo II inhibitors that interfere with enzyme catalysis at distinct points of the enzyme reaction. DNA topo II inhibitors such as etoposide, teniposide and doxorubicin stabilize cleaved DNA-topo II complexes. These drugs generate high levels of enzyme-mediated breaks, thus converting this essential enzyme into a potent cellular toxin. Hence, to distinguish their unique mechanism of action, they are referred to as topo II "poisons". In contrast to the complex-stabilizing topo II inhibitors, merbarone and the bisdioxopiperazines (such as dexrazoxane) block the catalytic activity of the enzyme [3, 43]. Specifically, the bisdioxopiperazines have been reported to stabilize topo II in a closed-clamp configuration around the DNA, whereas agents such as merbarone have been implicated recently in blocking the topo II-mediated DNA cleavage reaction. Because these drugs do not stabilize DNA-topo II complexes (i.e., they do not induce DNA strand breaks), they are termed "catalytic inhibitors" of topo II [3]. A logical consequence of this distinction is that a catalytic inhibitor should be able to inhibit a topo II poison by interfering with

the catalytic cycle in such a way as to reduce the amount of cleavable complex formation, in other words, decrease the available target of the poison.

Etoposide has become one of the most widely used anticancer drugs in the world since its introduction [15]. However, numerous groups have reported that treatment schedules associated with the impressive efficacy of etoposide are also associated with an increased risk of secondary acute myeloid leukemia. This has prompted the removal of this highly effective agent from some treatment regimens. In fact, after application of topo II poisons, damage to DNA may result as DNA fragmentation, chromosomal breaks, and micronuclei (MN) formation causing genomic instability, and may lead to mutagenesis or carcinogenesis. Follow-up studies of patients who received etoposide therapy revealed an increased incidence of acute myeloid leukemia [14]. In animals, etoposide is a somatic and germ-cell mutagen capable of inducing both numerical and structural chromosome aberrations [2, 4, 6, 7, 10, 42]. The majority of the literature has described catalytic inhibitors which produce low levels of topo II-mediated DNA cleavage as having only modest or even no mutagenic activity [9, 12]. In contrast, in a few studies measuring chromosomal alterations, merbarone has been reported to produce significant genotoxic effects both in vitro and in vivo [6–8, 44]. Additionally, in few studies measuring chromosomal damage dexrazoxane has been reported to produce significant genotoxic effects in vitro [8, 44]. However, to our knowledge, the in vivo genotoxic effects of dexrazoxane have never been reported.

Dexrazoxane was originally developed as an antitumour agent. However, it now is clinically used to reduce doxorubicin-induced cardiotoxicity [40]. Since dexrazoxane is effective in inhibiting doxorubicin's ability to damage cardiac cells, there are concerns that the drug may, as a protective agent, diminish the effectiveness of various chemotherapeutics. There is some clinical and in vitro data supporting this concern. Hasinoff et al. [17] demonstrated that if Chinese hamster ovary cells are exposed to dexrazoxane in vitro prior to the administration of doxorubicin or daunorubicin, a significant antagonism of the antitumour activity occurs. However, if dexrazoxane is administered simultaneously with or after doxorubicin or daunorubicin, significant additive growth inhibitory effects occur [17, 33]. Dexrazoxane in combination with etoposide used against a myeloid leukemia model produced highly synergistic, cytotoxic activity for all schedules [33] and the IC_{50} for dexrazoxane plus etoposide were also significantly reduced compared to the IC_{50} for etoposide alone. Additionally, Holm et al. [20] reported that dexrazoxane rescued healthy mice from lethal doses of etoposide. Using an L1210 intracranial inoculation model in mice, Holm and his colleagues have shown that the LD_{10} of etoposide in mice increased

3.6-fold when used together with non-toxic dexrazoxane doses. Also, there was a significant increase in lifespan of mice treated with etoposide and dexrazoxane as compared to etoposide alone. Moreover, combining etoposide and dexrazoxane synergizes with radiotherapy and improves survival in mice with central nervous system tumors [18]. The improved survival from radiotherapy following dexrazoxane and etoposide is difficult to be explained; however, a pharmacokinetics-based explanation is attractive. In preclinical models, dexrazoxane reduced myelosuppression and weight loss toxicities from high doses of etoposide and increased the treatment efficacy and survival, compared with equitoxic doses of etoposide alone [19].

Considering the widespread use of etoposide in clinical oncology and the ability of dexrazoxane to improve the therapeutic outcome from etoposide prompted us to investigate whether dexrazoxane in combination with etoposide can ameliorate etoposide-induced genomic damage in mice normal tissues. The scoring of MN, chromosomal aberrations, and mitotic activity were undertaken in the current study as markers of cyto- and genotoxicity. Oxidative damage markers such as bone marrow reduced glutathione and lipid peroxidation were assessed as a possible mechanism underlying this amelioration.

Materials and methods

Animals

Adult male white Swiss albino mice, weighing 20–25 g (10–12 weeks old), were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were maintained under standard conditions of humidity, temperature ($25 \pm 2°C$), and light (12-h light/12-h dark). They were fed with a standard mice pellet diet and had free access to water. All animal experimentations described in the manuscript were conducted in accord with accepted standards of humane animal care in accordance with the NIH guidelines and the legal requirements in Kingdom of Saudi Arabia. Each treatment group and vehicle control group consisted of five animals.

Drugs and chemicals

Dexrazoxane and etoposide (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA) were dissolved in 5% DMSO in sterile distilled H_2O, mixed on a magnetic stirrer for at least 30 min prior to administration, and administered by intraperitonial injection within 1 h following preparation. Cyclophosphamide (Sigma-Aldrich St Louis, MO, USA) was dissolved in sterile distilled water and used at a concentration of 40 mg/kg as positive control

Table 1 Frequencies of MNPCE and PCE in bone marrow of mice after treatment with the indicated doses of DMSO, dexrazoxane (Dex) or cyclophosphamide

Chemical (mg/kg)	%MNPCE (mean ± SD)	%PCE (mean ± SD)
Control (H$_2$O)	0.30 ± 0.14	48.2 ± 1.5
DMSO (10%)	0.32 ± 0.10	48.1 ± 2.6
Dex (125)	0.36 ± 0.20	49.2 ± 1.2
Dex (250)	0.38 ± 0.28	48.5 ± 2.1
Cyclophosphamide (40)	1.67 ± 0.25**	42.1 ± 2.2*

PCE:NCE (normochromatic erythrocyte) ratio was calculated as %PCE = [PCE/(PCE + NCE)] × 100

MNPCE micronucleated polychromatic erythrocyte

*$P < 0.05$; **$P < 0.01$ versus control (Mann–Whitney U test)

genotoxic agent [5]. Etoposide was administered at the doses level of 0.5, 1, 10, and 20 mg/kg. The genotoxic doses for etoposide in bone marrow were chosen by reference to earlier studies [2, 4, 6, 10, 42] and the selected doses are within the dose range used for human chemotherapy. Doses of 125 and 250 mg/kg dexrazoxane have previously been shown to be the optimal protective doses against etoposide-induced myelosuppression and weight loss toxicities in mice [19]. All other chemicals were of the finest analytical grade.

Experimental protocol

Preliminary experiment was conducted to assess the in vivo genotoxicity and bone marrow cytotoxicity of dexrazoxane and DMSO. In the preliminary experiment, animals were treated intraperitonially with 10% DMSO or with dexrazoxane at a dose of either 125 or 250 mg/kg, and clinical signs of toxicity and death were recorded within 24 h. The experiment included a positive control group administered cyclophosphamide at the dose of 40 mg/kg. A sterile distilled H$_2$O injected control group was also included. The

animals were killed by cervical dislocation at 24 h after treatment. Preliminary negative cytogenetic results for dexrazoxane and DMSO (Tables 1, 2) led to the use of these two doses for dexrazoxane and DMSO at 10%. Thus, doses of 125 and 250 mg/kg dexrazoxane were injected intraperitonially 20 min before etoposide 0.5, 1, 10, or 20 mg/kg treatment and bone marrow cells were sampled 24 h after etoposide injection. Concurrent solvent controls that received 10% DMSO in sterile distilled H$_2$O were included in every experiment in order to code the slides and avoid scoring biases. The injected volume was 0.01 ml per 1 g body weight.

Micronucleus test (MN test)

In the MN test, polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were scored. Groups of five mice were killed 24 h after treatment with the test chemicals or solvents and both femurs were removed. The bone marrow cells were collected in tubes containing fetal calf serum, centrifuged at 1,100 rpm for 10 min, and the pellet was carefully resuspended in as little supernatant as possible before slide preparation. Two smears of bone marrow were prepared from each mouse. After air drying, the smears were coded and stained by May-Gruenwald/Giemsa [1]. From each animal, 1,000 PCEs and 1,000 NCEs were examined for micronucleated erythrocytes (MNPCE and MNNCE) under ×1,000 magnification using a Nikon microscope. In addition the number of PCEs among 1,000 NCEs per animal was recorded to evaluate bone marrow suppression, PCE:NCE ratio was calculated as %PCE = [PCE/(PCE + NCE)] × 100.

Chromosome analysis

Groups of five mice were intraperitonially injected with colchicine at 4 mg/kg 90 min before killing. The slides for chromosome analysis were prepared and stained as

Table 2 Frequencies of different types of chromosomal aberrations (CA) and mitotic index (MI) in bone marrow of mice 24 h after treatment with the indicated doses of DMSO, dexrazoxane (Dex) or cyclophosphamide (mean ± SD)

Chemical (mg/kg)	Types of chromosomal aberrations					Total CA (%)	MI (%)
	Gaps	Breaks	Fragments	Rings	Polyploidy		
Control (H$_2$O)	0.2 ± 0.4	0.6 ± 0.5	0.4 ± 0.5	0.0 ± 0.0	0.2 ± 0.4	1.2 ± 0.4	3.4 ± 0.3
DMSO (10%)	0.4 ± 0.5	0.6 ± 0.8	0.4 ± 0.8	0.2 ± 0.4	0.2 ± 0.4	1.4 ± 0.8	3.2 ± 0.5
Dex (125)	0.2 ± 0.4	0.8 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	1.4 ± 1.1	3.3 ± 0.5
Dex (250)	0.6 ± 0.5	1.2 ± 0.8	0.4 ± 0.5	0.0 ± 0.0	0.2 ± 0.4	1.8 ± 1.4	3.2 ± 0.4
Cyclophosphamide (40)	1.7 ± 0.5	13.2 ± 1.7	1.7 ± 1.2	0.7 ± 0.9	0.5 ± 1.0	15.7 ± 2.0**	2.0 ± 0.7*

One hundred metaphases were scored for chromosomal aberrations per mouse, for a total of 500 metaphases per treatment. Cells with gaps were not included in the total chromosomal aberrations. The mitotic index per animal was evaluated by calculating the number of dividing cells in a population of 1000 cells

*$P < 0.05$, **$P < 0.01$ versus control (Mann–Whitney U test)

described by Attia [5]. All slides were coded and scored under ×1,000 magnification using a Nikon microscope. One-hundred well-spread metaphase plates per mouse (500 metaphases for each group) were scored for both structural and numerical aberrations (polyploidy) in bone marrow cells. Cells were classified, according to the damage severity, into five categories: cells with gaps only, cells with breaks, acentric fragments, centric rings and polyploidy. Cells with gaps were not included in the percentage of total chromosomal aberrations due to their controversial genetic significance. From the same slides 1,000 cells from each animal were taken into consideration for the mitotic activity study. The mitotic index of bone marrow was evaluated by calculating the number of dividing cells in a population of 1,000 cells.

Determination of bone marrow lipid peroxidation and reduced glutathione levels

To study the effect of dexrazoxane on the oxidative damage induced by etoposide treatment, another five groups consisting of five mice each were used. Two groups were administered etoposide at 20 mg/kg body weight; one of these groups received a single intraperitonial injection of dexrazoxane at a dose of 250 mg/kg 20 min prior to etoposide administration. Two vehicle-treated control (H$_2$O and 10% DMSO in H$_2$O) groups and dexrazoxane (250 mg/kg) group were also included. The animals were killed by cervical dislocation at 24 h after etoposide treatment and bone marrow cells were collected in tubes containing saline for estimation of lipid peroxidation and reduced glutathione (GSH). GSH was assayed with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the protocol described by Ellman [13]. The concentration of GSH (expressed as μmol/g protein) was calculated from a standard curve that was obtained from freshly prepared standard solution of GSH. Total protein was estimated by the method of Lowry et al. [29] using bovine serum albumin as the standard. The extent of lipid peroxidation was assayed by measuring one of the end products of this process, the thiobarbituric acid-reactive substances (TBARS) by the method of Ohkawa et al. [32]. As 99% TBARS is malondialdehyde (MDA), lipid peroxidation levels of the samples were calculated from the standard curve using the 1,1,3,3-tetramethoxypropane and expressed as μmol/g protein.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of the means. The analysis parameters were tested for homogeneity of variance and normality, and were found to be normally distributed. The data were, therefore, analyzed by employing non-parametric tests, Mann–Whitney U test,

and Kruskal–Wallis test followed by Dunn's multiple comparisons test. Data on oxidative damage parameters was analyzed using analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons. Results were considered significantly different if the P value was ≤0.05.

Results

During the preliminary experiment, animals dosed with dexrazoxane or DMSO exhibited no clinical signs of toxicity. The preliminary cytogenetic results are presented in Tables 1 and 2. The results of the two solvents (H$_2$O or 10% DMSO in H$_2$O) were not significantly different and were nearly similar. The studied dexrazoxane was neither genotoxic nor cytotoxic for the mouse bone marrow cells at the doses tested compared with control. As expected, animals treated with the positive control cyclophosphamide showed a high frequency of MNPCE and total chromosomal aberrations in mouse bone marrow cells after treatment in comparison with the concurrent negative control (Mann–Whitney U test). Furthermore, cyclophosphamide caused significant decrease in the mitotic activity at both interphase (Table 1) and metaphase (Table 2) stages. The frequencies of MNNCE in all groups were not significantly different in comparison with the solvent control (data not shown).

Effect of dexrazoxane on etoposide-induced MNPCE

The results of the conventional MN test for etoposide and/or dexrazoxane are presented in Table 3. Etoposide caused significant increases in MN induction at all doses tested (Mann–Whitney U test). However, an inverse dose response was found between 1 and 20 mg/kg. With regard to the animals treated with dexrazoxane plus etoposide, a weak protection was observed with 125 mg/kg of dexrazoxane. However, this protection was not statically significant in comparison to the etoposide alone [$P > 0.05$ (Kruskal–Wallis test followed by Dunn's multiple comparisons test)]. With 250 mg/kg pre-treatment, however, dexrazoxane produced a clear significant inhibitory effect on the MNPCE induced by etoposide in comparison to the etoposide alone (Kruskal–Wallis test followed by Dunn's multiple comparisons test). No effect was observed in NCEs in all groups in comparison with the solvent control (data not shown).

Effects of dexrazoxane on etoposide-induced bone marrow suppression at interphase

The results for the mitotic activity at interphase are also presented in Table 3. Etoposide treatment caused significant decreases in the percent PCE only at the two highest

Table 3 Frequencies of MNPCE and PCE in bone marrow of mice after treatment with the indicated doses of etoposide and/or dexrazoxane (Dex)

Chemical (mg/kg)	%MNPCE (mean ± SD)	%PCE (mean ± SD)
Control (10% DMSO)	0.30 ± 0.07	48.7 ± 2.4
Etoposide (0.5)	1.58 ± 0.54**	47.9 ± 1.6
Etoposide (0.5) + Dex (125)	0.82 ± 0.22*	49.6 ± 0.8
Etoposide (0.5) + Dex (250)	0.60 ± 0.16*, a	49.5 ± 0.3
Etoposide (1)	3.06 ± 0.37**	44.7 ± 3.1
Etoposide (1) + Dex (125)	2.16 ± 0.56**	47.8 ± 2.2
Etoposide (1) + Dex (250)	0.98 ± 0.37**, b	48.4 ± 2.2
Etoposide (10)	1.66 ± 0.24**	41.7 ± 3.5*
Etoposide (10) + Dex (125)	1.22 ± 0.37**	45.4 ± 3.1
Etoposide (10) + Dex (250)	0.96 ± 0.32**, a	46.8 ± 2.1
Etoposide (20)	0.88 ± 0.13**	38.1 ± 3.6**
Etoposide (20) + Dex (125)	0.84 ± 0.49	44.1 ± 1.5
Etoposide (20) + Dex (250)	0.40 ± 0.18a	45.7 ± 2.7b

PCE:NCE (normochromatic erythrocyte) ratio was calculated as %PCE = [PCE/(PCE + NCE)] × 100

MNPCE micronucleated polychromatic erythrocyte

$*P < 0.05$; $**P < 0.01$ versus control (Mann–Whitney U test)

a $P < 0.05$

b $P < 0.01$ versus etoposide alone (Kruskal–Wallis test followed by Dunn's multiple comparisons test)

doses [$P < 0.01$ (Mann–Whitney U test)]. Pre-treatment with dexrazoxane was found to protect mouse bone marrow cells against etoposide-induced bone marrow suppression and this protection was not statically significant in comparison with the concurrent control group ($P > 0.05$). Additionally, dexrazoxane 250 mg/kg produced a clear significant inhibitory effect on the bone marrow suppression in comparison with the etoposide alone (20 mg/kg) [$P < 0.01$ (Kruskal–Wallis test followed by Dunn's multiple comparisons test)].

Effect of dexrazoxane on etoposide-induced chromosomal aberrations

The results of the chromosomal aberrations are presented in Table 4. Etoposide treatment caused significant increases in total frequency of chromosomal aberrations only at the highest dose [$P < 0.01$ (Mann–Whitney U test)]. The major two types of aberrations observed in the present study were gaps and breaks. Cells with fragments rings or polyploidy were also observed frequently in etoposide-administered groups but not statistically significant in comparison to the solvent control. Dexrazoxane pre-treatment reduced the total frequency of chromosomal aberrations in etoposide-treated animals in comparison with those treated with etoposide alone, and the higher dose of dexrazoxane gave

Table 4 Frequencies of different types of chromosomal aberrations (CA) and mitotic index (MI) in bone marrow of mice 24 h after treatment with the indicated doses of etoposide and/or dexrazoxane (Dex) (mean ± SD)

Chemical (mg/kg)	Types of chromosomal aberrations					Total CA (%)	MI (%)
	Gaps	Breaks	Fragments	Rings	Polyploidy		
Control (10% DMSO)	0.2 ± 0.4	0.6 ± 0.5	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	1.2 ± 0.8	3.4 ± 0.6
Etoposide (0.5)	0.6 ± 0.5	0.8 ± 0.8	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.4	1.6 ± 0.5	3.0 ± 0.5
Etoposide (0.5) + Dex (125)	0.2 ± 0.5	0.5 ± 0.5	0.2 ± 0.5	0.0 ± 0.0	0.2 ± 0.5	1.0 ± 0.8	3.5 ± 0.5
Etoposide (0.5) + Dex (250)	0.2 ± 0.5	1.0 ± 1.4	0.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 1.2	3.2 ± 0.3
Etoposide (1)	0.6 ± 0.5	0.8 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	0.6 ± 0.8	1.8 ± 0.8	2.8 ± 0.7
Etoposide (1) + Dex (125)	0.6 ± 0.5	0.8 ± 0.8	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	1.4 ± 1.1	3.0 ± 0.2
Etoposide (1) + Dex (250)	0.8 ± 0.8	0.8 ± 0.8	0.4 ± 0.5	0.4 ± 0.5	0.2 ± 0.4	1.6 ± 0.8	3.6 ± 0.4
Etoposide (10)	0.6 ± 0.8	2.2 ± 1.7	0.6 ± 0.5	0.6 ± 0.5	0.4 ± 0.5	3.6 ± 2.6	1.7 ± 0.3**
Etoposide (10) + Dex (125)	0.4 ± 0.5	0.8 ± 0.8	0.6 ± 0.8	0.4 ± 0.5	0.2 ± 0.4	2.0 ± 1.2	2.4 ± 0.7
Etoposide (10) + Dex (250)	0.6 ± 0.5	1.2 ± 1.3	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	1.8 ± 1.3	3.1 ± 0.4a
Etoposide (20)	2.8 ± 1.9	14.2 ± 3.3	1.8 ± 1.4	1.2 ± 0.8	1.8 ± 2.0	19.0 ± 3.9**	1.3 ± 0.4**
Etoposide (20) + Dex (125)	0.6 ± 0.5	7.6 ± 3.3	2.0 ± 1.8	0.8 ± 1.3	0.4 ± 0.5	10.8 ± 5.2**	2.6 ± 0.4
Etoposide (20) + Dex (250)	0.8 ± 0.8	3.8 ± 1.3	0.8 ± 1.3	0.6 ± 0.5	0.6 ± 0.5	5.8 ± 2.30**b	3.0 ± 1.2a

One hundred metaphases were scored for chromosomal aberrations per mouse, for a total of 500 metaphases per treatment. Cells with gaps were not included in the total chromosomal aberrations. The mitotic index per animal was evaluated by calculating the number of dividing cells in a population of 1000 cells

$**P < 0.01$ versus control (Mann–Whitney U test)

a $P < 0.05$

b $P < 0.01$ versus etoposide alone (Kruskal–Wallis test followed by Dunn's multiple comparisons test)

the more effective reduction in the total chromosomal aberrations [$P < 0.01$ (Kruskal–Wallis test followed by Dunn's multiple comparisons test)].

Effects of dexrazoxane on etoposide-induced bone marrow suppression at metaphase

Mitotic index data recorded in the bone marrow cells at metaphase stage are also presented in Table 4. Treatment with etoposide induced significant decreases in the mitotic index of bone marrow cells only at the two highest doses (Mann–Whitney U test). The bone marrow suppression produced by the etoposide was weak protected by the use of 125 mg/kg of dexrazoxane. However, the combinations of 250 mg/kg dexrazoxane with etoposide produced responses which were close to the one observed with the solvent control alone, and statistically different from the results obtained in the etoposide alone treated animals [$P < 0.05$ (Kruskal–Wallis test followed by Dunn's multiple comparisons test)].

Effect of dexrazoxane on etoposide-induced oxidative stress

The effect of dexrazoxane on the etoposide-induced oxidative stress in mice was assessed by measuring bone marrow GSH and MDA levels. The bone marrow levels of GSH in the two solvent controls were not significantly different and were very similar (Fig. 1). Bone marrow GSH level did not show significant variation in dexrazoxane-treated animals compared with the two solvent controls. The GSH level observed in etoposide-treated animal was significantly decreased compared to the solvent controls ($P < 0.01$). Animals pre-treated with dexrazoxane showed a significant increase in GSH level over the etoposide-treated group [$P < 0.01$ (one-way ANOVA followed by Tukey–Kramer multiple comparisons test]. In the control groups, the values of MDA were not significantly different and were also
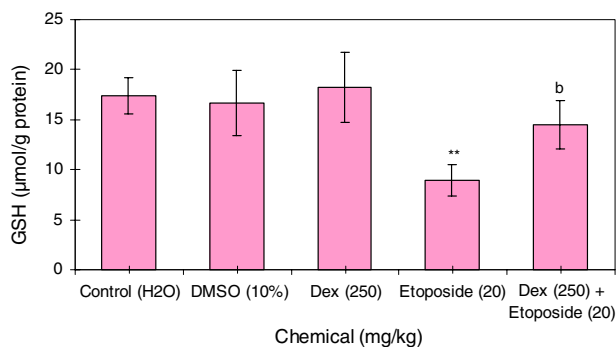

Fig. 1 Effects of dexrazoxane (Dex) and/or etoposide on the bone marrow contents of reduced glutathione (GSH) in mice (mean ± SD). ** $P < 0.01$ versus control or DMSO, b $P < 0.01$ versus etoposide alone (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)
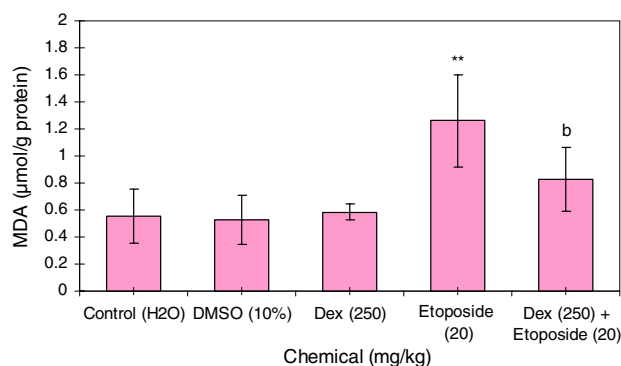

Fig. 2 Effects of dexrazoxane (Dex) and/or etoposide on the bone marrow lipid peroxidation levels (MDA) in mice (mean ± SD). ** $P < 0.01$ versus control or DMSO, b $P < 0.01$ versus etoposide alone (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

similar. The treatment with dexrazoxane failed to induce any significant changes in the levels of MDA at the tested dose. A significant rise in bone marrow MDA level was observed in etoposide-treated group ($P < 0.01$). Pre-treatment with dexrazoxane was found to significantly [$P < 0.01$ (one-way ANOVA followed by Tukey–Kramer multiple comparisons test] decrease the MDA concentration as compared to the values obtained after treatment with etoposide alone (Fig. 2).

Discussion

The hypothesis of providing protection against genomic damage in non-tumor tissues will represent a promising approach to counteract the unwanted toxicity from conventional cytotoxic chemotherapy; this will allow the safe use of increased drug doses for the benefit of future cancer patients. The mechanistic differentiation of DNA cleavage-enhancing drugs (topo II poisons) and topo II catalytic inhibitors has advanced our knowledge in this area and opens up new therapeutic applications for these drugs. Our results demonstrated for the first time that dexrazoxane was neither genotoxic nor cytotoxic in vivo in mouse bone marrow cells with the doses tested. Moreover, it is able to protect mouse bone marrow cells against the etoposide-induced genomic damage and decline in the cell proliferation as observed by reduction in the MN frequencies, chromosomal aberrations, and increases in mitotic activities, respectively. The positive control mutagen cyclophosphamide was used and this compound produced the expected responses and the results of this compound were in the same range as those of earlier studies [5, 10]. These data confirmed the sensitivity of the experimental protocol followed in the detection of genotoxic effects.

Using a MN assay, etoposide caused significant increases in MN induction at all doses tested. However, an

inverse dose response was found between 1 and 20 mg/kg. Furthermore, etoposide caused a dose-dependent suppression of erythroblast proliferation indicating an inhibition of erythroblast proliferation most likely by mitotic arrest. These results are similar to the one obtained by conventional-staining of etoposide-induced MN in mouse bone marrow cells [4] and contrasts the results of the MN assay with etoposide in mouse bone marrow by Choudhury et al. [10]. In the mouse bone marrow MN test, etoposide caused a dose-dependent increase in MN induction up to 1 mg/kg [4]. Twenty-four hours following intraperitonial injection and in the tested dose range of 0.01–15 mg/kg, the lowest effective dose was 0.1 mg/kg. Turner et al. [42] confirmed the sensitivity of the mouse bone marrow MN test to etoposide. An inverse dose response was found between 1 and 15 mg/kg [4]. The decline of the MN yields with increasing doses was accompanied by a significant reduction of PCE frequencies so that at low PCE rates hardly any MNPCE could be seen. On the other hand, Choudhury et al. [10] determined the presence of MN in bone marrow 30 h after intraperitonial treatment of mice with 10, 15, and 20 mg/kg of etoposide. They found that the MN induction was not significant with the lowest dose, but it was significant in mice that received 15 mg/kg etoposide and was highly significant in mice that received 20 mg/kg etoposide. The differences between these results can be attributed to different animal species, sampling times, and technical features of the test procedures.

In vivo genotoxicity studies have shown that etoposide induced statistically significant increase in chromosomal aberrations and sister chromatid exchange in mouse bone marrow after treatment with etoposide [2, 10]. There was a dose-dependent significant increase in chromosomal aberrations with etoposide at concentrations of 5, 10, 15, and 20 mg/kg of mice [2]. However, in the current study 10 mg/kg of etoposide failed to induce chromosomal aberrations significantly at 24 h after treatment. Only the highest dose tested (20 mg/kg) induced a statistically significant increase in the total chromosomal aberrations. A similar observation was found in the bone marrow mitotic chromosomal aberrations assay carried out by Choudhury et al. [10]. Barring few fragments, rings, and polyploidy the etoposide-induced chromosomal aberrations recorded were mostly gaps and breaks, which is in harmony with earlier studies [2, 10]. However, in contrast to our result, the dose response effects of etoposide and the induction of significant chromosomal aberrations with lower doses (5–15 mg/kg) reported by Agarwal and colleagues [2] might have been due to the earlier harvesting time at 6 and 12 h post-treatment, whereas the harvesting of cells in the present study was at 24 h after treatment. The present MN study showed that exposure to 0.5 mg/kg etoposide yielded 5.2 fold increases on MNPCE. However, in the chromosome analysis assay, we found that the lowest genotoxic dose, which caused chromosomal aberrations, was 20 mg/kg of etoposide. These observations suggest that MN are induced at lower doses of etoposide than chromosomal aberrations; hence, the MN test is the more sensitive than metaphase chromosome analysis assay. It must of course be noted that the assays measure different end-points. Chromosome loss and damage is measured in the MN test, and true chromatid discontinuities and gaps are detected in the metaphase chromosome analysis assay. Therefore, the present data confirm the general idea, that the genotoxicity of etoposide should be detected by different methods [42].

Inhibition of topo II function at S phase by topo II inhibitors, such as etoposide [38], teniposide, and merbarone [9] slows down cell cycle progression through the S phase and causes cells to arrest at the G_2 phase, which delays entry into mitosis. Treatment of HL-60 cells with etoposide in subcytotoxic concentrations resulted in a massive accumulation of the cells in the G_2/M phase of the cell cycle [21]. In vivo, etoposide was shown to significantly prolong the cell cycle time in mouse bone marrow [2] and in mouse sperm [7]. In agreement with the above-cited reports, the present experiment showed that exposure to etoposide interfered with the cell cycle progression manifested as significant bone marrow suppression compared to the values obtained after treatment with solvent control. Pre-treatment with dexrazoxane was found to protect mice against genotoxic and bone marrow suppressive effects of etoposide. However, in regard to this response it is worthwhile to emphasize the observed dose-dependent antigenotoxic effect of dexrazoxane, with the stronger inhibitory effect produced with 250 mg/kg; this suggests the possibility of using an even higher amount of the chemical with good results. It should be noted that in the MN test the frequency of MNPCE in animals pre-treated with 250 mg/kg dexrazoxane was between 0.4 and 0.9/100 PCEs in compared to 0.88 and 3.06/100 PCEs in animals treated with etoposide alone (Table 3). Since the increase of the dose of etoposide by 10- and 20-folds leads to the decreases of MNPCE level to two and fourfolds, respectively, the interpretations of antigenotoxic effects of dexrazoxane obtained in the MN test are difficult to explain. However; there is no doubt that pre-treated with 250 mg/kg dexrazoxane significantly decreased the genotoxic effects of etoposide on mouse bone marrow cells.

Our antigenotoxic results confirm the findings of previous studies of the inhibition of topo 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 topo II poisons etoposide, amsacrine, daunorubicin, and doxorubicin which are known to stimulate DNA-topo II cleavable complex

formation [36]. The antagonistic effect of dexrazoxane on teniposide-induced DNA damage reported by Mo and Beck [31], where in culture HeLa cells, an inhibition in the DNA strand breaks, using alkaline elution assays, was also observed. In the current study, the genotoxicity protection was also directly correlated with mitotic activity as more protection was noted with 250 mg/kg pre-treated animals when bone marrow suppression was examined at both interphase and metaphase stages. A similar protection was also reported by Hofland and colleagues [19]. They observed a reduction in etoposide-induced myelosuppression in mice pre-treated with dexrazoxane.

The exact mechanism by which the catalytic inhibitors dexrazoxane protected against etoposide-induced genomic damage in the form of MN and chromosomal damage is not well known. However, the mechanism of protection could be the result of reduction in the amount of cleavable complex formation [16] or simultaneous treatment with dexrazoxane that would allow interception of free radicals generated by etoposide before they reach DNA and induce damages. 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 etoposide, oxidative stress markers such as lipid peroxidation and GSH were done after the animals were treated with etoposide, compared with the simultaneous treatment with dexrazoxane and the solvent control groups. The present study demonstrates that dexrazoxane pre-treatment reduced the etoposide induced lipid peroxidation and prevented the reduction in GSH significantly. The increased GSH level suggests that protection by dexrazoxane may be mediated through the modulation of cellular antioxidant levels. These observations confirm earlier studies in which dexrazoxane was reported to scavenge free radicals and lipid peroxides [24, 28, 45].

Generation of etoposide phenoxyl radicals in the redox reaction was reported by Kagan's group [22, 23] and was confirmed by Kapiszewska et al. [25, 26]. These radicals have been shown to deplete antioxidant cellular sulfhydryl compounds, the oxidation of which can lead to superoxide and H_2O_2 formation, leading to iron-based oxygen radical damage. It is believed that accumulation of these radicals may cause damage to cellular genome and also the cell membrane leading to lipid peroxidation [22, 23, 35, 37]. The latter can be inhibited by the presence of an antioxidant, e.g., vitamin C [23, 35]. In addition, the presence of quercetin, a dietary antioxidant flavonoid abundant in food of plant origin, reduces the cellular DNA damaging activity of etoposide in human neutrophils [25] and in bone marrow cells of rats [11, 26]. Moreover, this effect was associated with a concomitant alteration of the antioxidant potential. Dexrazoxane has been reported to elevate reduced glutathione, glutathione peroxidase, superoxide dismutase and to reduce lipid peroxidation [24, 28, 45]. Scavenging of free radicals by dexrazoxane seems to be an important mechanism against the etoposide-induced genomic damage.

A crucial consideration of coadministration of topo II catalytic inhibitors and DNA cleavage-enhancing drugs is how it will possibly affect the anticancer treatment efficacy; there are, however, important differences between these two processes, which suggest that a reduction in side effects does not necessarily go hand-in-hand with a reduction in the antitumor effects. Dexrazoxane reduced myelosuppression [19], ameliorated intestinal cell damage in mice [34] and prevented skin ulceration following experimental extravasation [27] from several different anthracyclines: The mechanism of action on these preventions are unclear, whereas the current understanding is that dexrazoxane protects the heart [39] from iron-mediated oxidative damage through the iron-chelating properties of the ring-opened molecule rather than through its topo II inhibitory properties [30, 39]. In conclusion, a critical point of this study is the possibility that there may be a therapeutic window for the use of etoposide in combination with dexrazoxane, so that its genotoxic side effects in normal cells are minimized. The genotoxic effects of etoposide might be, at least in part, mediated by an oxidative stress mechanism that may be prevented or reduced by radical scavengers. Etoposide has a direct inhibitory effect on topo II, an important component of its antitumor activity, and this will be unchanged by any manipulations that alter the redox reaction. Apart from its well-known anti-topo II effect, the antigenotoxic effect of dexrazoxane could be possibly ascribed to its radical scavenger effect that modulated the genotoxic responses and erythroblast proliferation changes induced by etoposide. Based on our data presented, strategies can be developed to decrease the genotoxic and carcinogenic potentials of etoposide in normal cells using dexrazoxane.

Conflict of interest statement None.

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