**Mutagenesis** vol. 26 no. 4 pp. 533–543, 2011
Advance Access Publication 23 March 2011
doi:10.1093/mutage/ger013

# Salubrious effects of dexrazoxane against teniposide-induced DNA damage and programmed cell death in murine marrow cells

**S. A. Bakheet[1], S. M. Attia[1,2,*], N. M. AL-Rasheed[1], M. M. Al-harbi[1], A. E. Ashour[1], H. M. Korashy[1], A. R. Abd-Allah[1], Q. Saquib[3], A. A. Al-Khedhairy[3] and J. Musarrat[3]**

[1]Department of Pharmacology, College of Pharmacy, King Saud University, PO Box 2457, Riyadh, Saudi Arabia, [2]Department of Pharmacology and Toxicology, College of Pharmacy, Al-Azhar University, Cairo, Egypt and [3]Al-Jeraisy Chair for DNA Research, College of Science, King Saud University, PO Box 2457 Riyadh, Saudi Arabia.

[*]To whom correspondence should be addressed. Tel: +966 542927708; Fax: +966 14677200; Email: attiasm@yahoo.com

*Received on December 2, 2010; revised on February 8, 2011; accepted on February 23, 2011*

**The intention of the present study was to answer the question whether the catalytic topoisomerase-II inhibitor, dexrazoxane, can be used as a modulator of teniposide-induced DNA damage and programmed cell death (apoptosis) in the bone marrow cells *in vivo*. The alkaline single cell gel electrophoresis, scoring of chromosomal aberrations, micronuclei and mitotic activity were undertaken in the current study as markers of DNA damage. Apoptosis was analysed by the occurrence of a hypodiploid DNA peak and caspase-3 activity. Oxidative stress marker such as intracellular reactive oxygen species production, lipid peroxidation, reduced and oxidised glutathione were assessed in bone marrow as a possible mechanism underlying this amelioration. Dexrazoxane was neither genotoxic nor apoptogenic in mice at the tested dose. Moreover, for the first time, it has been shown that dexrazoxane affords significant protection against teniposide-induced DNA damage and apoptosis in the bone marrow cells *in vivo* and effectively suppresses the apoptotic signalling triggered by teniposide. Teniposide induced marked biochemical alterations characteristic of oxidative stress including accumulation of intracellular reactive oxygen species, enhanced lipid peroxidation, accumulation of oxidised glutathione and reduction in the reduced glutathione level. Prior administration of dexrazoxane ahead of teniposide challenge ameliorated these biochemical alterations. It is thus concluded that pretreatment with dexrazoxane attenuates teniposide-induced oxidative stress and subsequent DNA damage and apoptosis in bone marrow cells. Based on our data presented, strategies can be developed to decrease the teniposide-induced DNA damage in normal cells using dexrazoxane. Therefore, dexrazoxane can be a good candidate to decrease the deleterious effects of teniposide in the bone marrow cells of cancer patients treated with teniposide.**

## Introduction

Topoisomerase-II is an essential enzyme that alters DNA topology by transiently creating and resealing DNA double-strand breaks to enable the passage of one DNA strand through another (1). DNA topoisomerase-II is a target for a number of clinically useful anti-tumour agents, in part because it is essential for cell survival. To date, there are two general classes of topoisomerase-II inhibitors that interfere with enzyme catalysis at distinct points of the enzyme reaction. DNA topoisomerase-II inhibitors, such as teniposide (VM-26), etoposide and doxorubicin stabilise cleaved DNA–topoisomerase-II complexes. In contrast to the complex-stabilising topoisomerase-II inhibitors, the bisdioxopiperazines [such as dexrazoxane (Dex)], merbarone and aclarubicin block the catalytic activity of the enzyme (2,3). Specifically, the bisdioxopiperazines have been reported to stabilise topoisomerase-II in a closed-clamp configuration around the DNA. Because these drugs do not stabilise DNA–topoisomerase-II complexes (i.e. they do not induce DNA strand breaks), they are termed ‘catalytic inhibitors’ of topoisomerase-II (4). A logical consequence of this distinction is that a catalytic inhibitor should be able to inhibit a topoisomerase-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.

Podophyllotoxins such as VM-26 and etoposide are widely used in the treatment of various malignancies; however, their utilisation is associated with an increased risk of secondary acute myeloid leukaemia and myelosuppression (5). This has prompted the removal of these highly effective agents from some treatment regimens. In fact, after application of topoisomerase-II poisons, damage to DNA may result as DNA fragmentation, chromosomal breaks and micronucleus formation causing genomic instability and may lead to mutagenesis, carcinogenesis or finally to apoptotic cell death (6–8). Follow-up studies of patients who received topoisomerase-II inhibitors therapy revealed an increased incidence of acute myeloid leukaemia characterised by site-specific rearrangements in the mixed multiple leukaemia gene on chromosome 11q23 (9,10). In addition, a significant increase in the frequency of aneuploid sperm during the first 18 months following initiation of podophyllotoxins-including regimen was reported (11). In animals, podophyllotoxins are somatic and germ-cell mutagens capable of inducing both numerical and structural chromosome aberrations (12–15).

Dex is the only well-established and clinically approved cardioprotectant against doxorubicin cardiotoxicity (16). Since Dex 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 CHO cells are exposed to Dex *in vitro* prior to the administration of doxorubicin or daunorubicin, a significant antagonism of the anti-tumour activity occurs. Alternatively, they showed that if Dex is administered simultaneously with or after doxorubicin or daunorubicin, significant additive growth inhibitory effects

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occur (17,18). Dex in combination with etoposide used against a myeloid leukaemia model produced highly synergistic, cytotoxic activity for all schedules (18) and the $IC_{50}$ for Dex plus etoposide were also significantly reduced compared to the $IC_{50}$ for etoposide alone.

Additionally, Holm *et al.* (19) reported that Dex rescued healthy mice from lethal doses of VM-26. Using an L1210 intracranial inoculation model in mice, Holm *et al.* have shown that the $LD_{10}$ of VM-26 in mice increased 3.4-fold when used together with non-toxic Dex doses. Also, there was a significant increase in lifespan of mice treated with VM-26 and Dex as compared to VM-26 alone. They concluded that tumour cells in the brain were reached by cytotoxic levels of VM-26, whereas normal tissues in the periphery were protected by Dex. This is because the lipophilic drug VM-26 passes the blood-brain barrier to a much greater extent than the hydrophilic drug Dex. Moreover, combining Dex and etoposide synergises with radiotherapy and improves survival in mice with brain tumours (20). The improved survival from radiotherapy following Dex and etoposide is difficult to be explained; however, a lipid solubility-based explanation is attractive.

In preclinical models, Dex reduced myelosuppression and weight loss toxicities from high doses of podophyllotoxins and increased the treatment efficacy and survival, compared with equitoxic doses of podophyllotoxins alone (19,21). Previously, we have shown in our laboratory that the genomic damage induced by etoposide in mouse bone marrow decreased when used together with non-toxic Dex doses (22). Hence, we sought to determine whether Dex in combination with VM-26 can ameliorate VM-26-induced DNA damage and programmed cell death (apoptosis) in mice normal tissues and to elucidate the potential mechanism of this protection. The hypothesis of providing protection against DNA damage and apoptosis in non-tumour 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 (topoisomerase-II poisons) and topoisomerase-II catalytic inhibitors has advanced our knowledge in this area and opens up new therapeutic applications for these drugs. Bone marrow chromosomal analysis, micronucleus test and alkaline single cell gel electrophoresis were used to assess DNA damage. Apoptosis was analysed with the propidium iodide (PI) method and caspase-3 activity. In addition, oxidative stress markers such as bone marrow intracellular reactive oxygen species production, lipid peroxidation, oxidised and reduced glutathione were assessed as a possible mechanism underlying this amelioration.

## Materials and methods

### *Animals*

Adult male Swiss albino mice weighing 25–30 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 ± 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 experiments on animals were carried out according to the Guidelines of the Animal Care and Use Committee at College of Pharmacy, King Saud University.

### *Drugs*

VM-26 and Dex (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA) were dissolved in 10% dimethyl sulfoxide (DMSO) in sterile $dH_2O$, mixed on a magnetic stirrer for at least 30 min prior to administration and administered by intraperitoneal injection within 1 h following preparation. Ethyl methanesulfonate, colchicine and cyclophosphamide (Sigma–Aldrich, St Louis, MO, USA) were dissolved in sterile $dH_2O$. The administrated volume was 0.01 ml/1 g body weight. All other chemicals were of the finest analytical grade.

### *Detection of metaphase chromosomal aberrations*

The influence of Dex on the VM-26-induced chromosomal aberrations in mouse bone marrow cells was assessed by metaphase chromosomal analysis. VM-26 was administered at the doses level of 0.05, 0.5, 2.5, 5 and 10 mg/kg. Dose of 100 mg/kg Dex was administrated 20 min prior to the VM-26 injection. A vehicle-treated control group treated with 10% DMSO was also included. Cyclophosphamide was used at a concentration of 40 mg/kg as a positive control mutagen. Bone marrow cells were sampled 24 h after VM-26 injection. Two hours before sampling, mice were intraperitoneally injected with colchicine at 4 mg/kg and femurs bone marrow cells were collected from the mice killed by cervical dislocation. The slides were prepared and stained as mentioned earlier (23). All slides were coded and scored under ×1000 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 in bone marrow cells. From the same slides, 1000 cells from each animal were taken into consideration for the mitotic activity study. The mitotic index of bone marrow metaphase was evaluated by calculating the number of dividing cells in a population of 1000 cells.

### *Detection of interphase micronuclei formation*

Animal treatment was the same as in the detection of chromosomal aberrations. The animals were killed by cervical dislocation at 24 h after VM-26 treatment, bone marrow cells were collected and two smears were prepared from each mouse. After air-drying, the smears were coded and stained by May-Gruenwald/Giemsa as described earlier (24). From each animal, 1000 polychromatic erythrocytes (PCEs) and 1000 normochromatic erythrocytes (NCEs) were examined for the presence of micronuclei (MN) under ×1000 magnification using a Nikon microscope. In addition, the number of PCEs among 1000 NCE per animal was recorded to evaluate bone marrow suppression, mitotic activity was calculated as %PCE = [PCE/(PCE +NCE)] × 100.

### *Detection of DNA strand breaks*

The influence of Dex on the VM-26-induced DNA strand breaks in mouse bone marrow cells were studied by alkaline single cell gel electrophoresis (comet assay). Four groups consisting of 10 mice each were used. Two groups were administered 10 mg/kg VM-26 and one of these groups received Dex at a dose of 100 mg/kg 20 min prior to VM-26 administration. A vehicle-treated control group and Dex groups were also included. Half of the mice were killed by cervical dislocation at 1 h after VM-26 treatment and the rest of animals were killed at 24 h after VM-26 injection. The bone marrow cells from one femur were collected in tubes containing 1.5 ml foetal calf serum, then centrifuged and washed with ice-cold phosphate-buffered saline (PBS) (pH 7.4). The experiment also included a positive control group orally administered ethyl methanesulfonate at the dose of 100 mg/kg and the bone marrow cells were sampled at 24 h after treatment.

The microgel electrophoretic technique was performed essentially by the guidelines of Tice *et al.* (25). Electrophoresis for ~20 000 cells on each slide was conducted at 0.74 1 $Vcm^{-1}$ for 20 min at 4°C. The current was adjusted to 300 mA. After electrophoresis, the slides were washed with cold neutralisation buffer [0.4 M Tris (pH 7.5)] and were stained with ethidium bromide (20 μg/ml). Slides were coded and studied using a fluorescent microscope (Nikon Corporation, Tokyo, Japan) equipped with appropriate filters. The microscope was connected to a computer through a charge-coupled device camera. For all procedures following loading of the cells on glass slides, the slides were shielded from light. Images from 100 cells (50 from each replicate slide) were randomly selected and subjected to image analysis using software Comet Assay IV (Perceptive Instruments, Suffolk, UK). Mean values of the olive tail moment, tail length (micrometres) and tail intensity (%) were separately analysed for statistical significance.

### *Detection of apoptosis and analysis of cell cycle by flow cytometry*

The influence of Dex and/or VM-26 on apoptosis and cell cycle in mouse bone marrow cells was quantified with PI based on the procedure by Nicoletti *et al.* (26) with some modifications as described earlier (27). An aliquot of bone marrow suspension (~1 × $10^6$) from animals treated for 24 h with VM-26 and/or Dex for the detection of DNA strand breaks by comet assay was used. The red fluorescence of 10 000 events of PI-stained cells were acquired in FL4 Log channel through a 675-nm band-pass filter using Beckman Coulter flow

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cytometer. The cell cycle phases and the percentage of sub-$G_1$ (hypodiploid) apoptotic cells were calculated using a computer system Coulter Epics XL/XL-MCL, System II Software, Version 3.0. Events to the left of the $G_1$ peak appear representing the apoptotic cells with a lower DNA content than cells in $G_1$-phase. These peaks are referred to as sub-$G_1$ peaks and the percentage of apoptotic cells was determined from this gated region.

*Detection of caspase-3 activity*

In order to investigate the involvement of caspase-3 in signalling transduction, cleavage products of caspase-3 were detected by enzyme-linked immunosorbent assay (ELISA) using the Caspase-3/CPP32 colorimetric assay kit (BioVision, Mountain View, CA, USA) as described by the supplier. Animal treatment was the same as in the detection of DNA strand breaks by comet assay. Femurs bone marrow cells were collected from the mice killed by cervical dislocation at 24 h after Dex and/or VM-26 treatment (without or pretreated intravenously with 10 mg/kg of the general caspase inhibitor Z-VAD-fmk (Bachem, Bubendorf, Switzerland) (28,29). An aliquot of bone marrow suspension ($\sim 5 \times 10^6$) was centrifuged for 10 min at 4°C. Pellets were then lysed in 50 μl of chilled lysis buffer and then incubated on ice for 10 min. Undissolved protein and nuclei were removed by centrifugation for 1 min, and the supernatant was used to estimate the caspase-3 activity. The equivalent of 100 μg of protein from the cell lysate was diluted to 50 μl with cell lysis buffer. Fifty microlitres of 2× reaction buffer (containing 10 mM dithiothreitol) and 5 μl of the 4 mM DEVD-*p*NA caspase-3 substrate (200 μM final concentration) were added to each sample. After 2 h incubation at 37°C, the formation of *p*-nitroanilide was measured at 405 nm with the use of a microplate reader. Blank reading was subtracted from each sample reading before calculation. Protein quantitative was carried out by the method of Lowry *et al.* (30) using bovine serum albumin as the standard.

*Detection of intracellular reactive oxygen species by flow cytometry analysis*

Intracellular reactive oxygen species were analysed by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma–Aldrich) as a specific dye probe which fluoresces on oxidation by reactive oxygen species to 2′,7′-dichlorofluorescein (DCF) (31). The reaming femora from the same animals used for the detection of DNA strand breaks by the comet assay at 24-h post VM-26 and/or Dex treatment was used. The bone marrow cells were collected in tubes containing 1.5 ml foetal calf serum, then centrifuged and washed with ice-cold PBS (pH 7.4). Cells were harvested by centrifugation, washed twice with cold PBS and resuspended in 500 μl of PBS. Bone marrow cells were then incubated with DCFH-DA (5 μM in DMSO) for 60 min at 37°C in dark. Cells were then washed twice with PBS and finally suspended with 500 μl PBS. The fluorescence of cells (10 000 cells each) was recorded under 488 nm excitation. Green fluorescence from DCF was measured in the FL1 Log channel through a 525-nm band-pass filter on the Coulter EPICS XL/X1-MCL (Beckman Coulter Company, Miami, FL, USA).

*Measurement of lipid peroxidation, reduced and oxidised glutathione levels*

Animal treatment was the same as in the detection of DNA strand breaks by comet assay. The animals were killed by cervical dislocation at 24 h after VM-26 and/or Dex treatment and bone marrow cells were collected from both femurs. Bone marrow cells were collected in tubes containing saline for estimation of malondialdehyde (MDA), reduced glutathione (GSH) and oxidised glutathione (GSSG) levels. MDA generated by lipid peroxidation was quantified in the bone marrow cells according to the method of Ohkawa *et al.* (32), based on thiobarbituric acid reactivity. The MDA levels of the samples were calculated from the standard curve using the 1,1,3,3-tetramethoxypropane as the standard and expressed as micromoles per gram protein. GSH was assayed with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the protocol described by Ellman (33). GSSG was assayed with DTNB, glutathione reductase and nicotinamide adenine dinucleotide phosphate as described previously (34). The concentrations of GSH and GSSG were calculated from standard curves that were obtained from freshly prepared standard solutions of GSH and GSSG, respectively, and expressed as micromoles per gram protein. Simply, the value obtained for GSH was divided by the GSSG value to get the GSH/GSSG ratio.

*Data analysis*

Data were expressed as the mean ± standard deviation of the means. The analysed parameters were tested for homogeneity of variance and normality and were found to be normally distributed. The data were, therefore, analysed by employing nonparametric tests, Mann–Whitney *U*-test or Kruskal–Wallis test followed by Dunn's multiple comparisons test. Data on apoptosis and oxidative stress parameters were analysed using unpaired *t*-test or analysis of variance (ANOVA) followed by Tukey–Kramer for multiple comparisons. Results were considered significantly different if the *P*-value was $<0.05$.

## Results

*Chromosome analysis*

As shown in Table I, animals treated with the positive control cyclophosphamide showed a high frequency of total chromosomal aberrations in mice bone marrow cells after treatment in comparison with the negative control ($P < 0.01$). Moreover, drastic inhibition in the metaphase mitotic activity was recorded following cyclophosphamide administration ($P < 0.05$). Dex treatment did not exhibit any significant differences in the frequency of total chromosomal aberrations or mitotic activity compared to the solvent control at the tested dose. Significant increase in the frequency of total chromosomal aberrations was observed in animals treated with 5 and 10 mg/kg VM-26. The major two types of aberrations observed in the present study were gaps and breaks. Cells with fragments or polyploidy were also observed frequently in VM-26-treated mice but not statistically significant in comparison to the solvent control (data not shown). Dex pretreatment reduced the total frequency of chromosomal aberrations in VM-26-treated animals in comparison to those treated with VM-26 alone. Moreover, treatment with VM-26 induced significant decreases in the mitotic index of bone marrow cells at the two highest doses, indicating bone marrow suppression. Pretreatment of mice with Dex significantly elevated the reduced mitotic indices to nearly normal level especially in animals treated with 5 mg/kg VM-26.

*MN formation*

The results obtained from the MN test are also presented in Table I. The positive control cyclophosphamide significantly increases in the incidence of MNPCE compared to the control group. A statistically significant decrease in the interphase mitotic index was also observed following treatment with the cyclophosphamide, indicating a reduction in erythroblast proliferation most likely by mitotic arrest. Treatment of mice with Dex did not induce any significant variation in the incidence of MNPCE as compared to the control value. In addition, Dex was not cytotoxic to the bone marrow (i.e. no statistically significant decrease in the interphase mitotic index) at the tested dose level. VM-26 caused significant increases in MN induction at all doses tested. However, an inverse dose response was found between 0.5 and 10 mg/kg. With regard to the animals treated with Dex plus VM-26, a weak protection was observed in animals post-treated with 10 mg/kg of VM-26. However, this protection was not statically significant in comparison to the VM-26 alone. With 0.05–5 mg/kg post-treatment, however, Dex produced a clear significant inhibitory effect on the MNPCE induced by VM-26 in comparison to the VM-26 alone. No effect was observed in NCEs in all groups in comparison with the solvent control (data not shown). The mitotic index at the interphase stage was significantly decreased after treatment with 5 and 10 mg/kg VM-26 compared to the solvent control group. The reduction of mitotic index induced by VM-26 was found to be significantly restored by Dex pretreatment.

*DNA strand breaks*

The results of the single cell gel electrophoresis analysis or comet assay are shown in Table II. The positive control ethyl methanesulfonate significantly increases the level of olive tail moment, tail length and tail intensity compared to the control group ($P < 0.01$). At the 1-h sampling time, the level of these



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**Table I.** Frequencies of total CAs, MI and MNPCE in bone marrow of mice 24 h after treatment with cyclophosphamide, dexrazoxane (Dex) and/or teniposide (VM-26) (mean ± SD)

| Treatment (mg/kg) | Total CAs (%) | Metaphase MI (%) | MNPCE (%) | Interphase MI (%) |
|---|---|---|---|---|
| Control | 1.60 ± 0.5 | 3.20 ± 0.8 | 0.28 ± 0.08 | 48.8 ± 2.16 |
| Cyclophosphamide (40) | 14.8 ± 2.5$^{##}$ | 1.80 ± 0.4$^{#}$ | 1.58 ± 0.35$^{##}$ | 42.2 ± 2.9$^{#}$ |
| Dex (100) | 1.40 ± 0.5 | 3.00 ± 1.0 | 0.34 ± 0.05 | 49.0 ± 1.2 |
| VM-26 (0.05) | 2.00 ± 0.7 | 2.80 ± 0.8 | 0.92 ± 0.17** | 45.6 ± 2.4 |
| Dex (100) + VM-26 (0.05) | 2.20 ± 0.4 | 3.00 ± 0.7 | 0.46 ± 0.08$^{b}$ | 46.6 ± 3.7 |
| VM-26 (0.5) | 3.00 ± 0.7 | 2.20 ± 0.8 | 4.48 ± 0.4** | 39.8 ± 2.5 |
| Dex (100) + VM-26 (0.5) | 2.60 ± 0.9 | 3.20 ± 0.8 | 2.60 ± 0.6$^{b}$ | 43.2 ± 6.1 |
| VM-26 (2.5) | 3.20 ± 0.8 | 2.40 ± 0.5 | 1.54 ± 0.26** | 36.6 ± 4.4 |
| Dex (100) + VM-26 (2.5) | 3.20 ± 1.0 | 3.40 ± 0.5 | 0.78 ± 0.17$^{b}$ | 42.2 ± 3.5 |
| VM-26 (5) | 11.8 ± 1.4* | 1.80 ± 0.4* | 1.06 ± 0.35** | 35.0 ± 2.3** |
| Dex (100) + VM-26 (5) | 7.00 ± 1.7$^{a}$ | 3.00 ± 0.7$^{a}$ | 0.56 ± 0.16$^{a}$ | 41.0 ± 3.3$^{b}$ |
| VM-26 (10) | 17.4 ± 2.3** | 1.60 ± 0.5* | 0.94 ± 0.19** | 33.2 ± 3.1** |
| Dex (100) + VM-26 (10) | 10.4 ± 2.5$^{b}$ | 2.40 ± 0.5 | 0.74 ± 0.20 | 39.2 ± 3.5$^{a}$ |

*$P < 0.05$; **$P < 0.01$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test). $^{#}P < 0.05$, $^{##}P < 0.01$ versus control; $^{a}P < 0.05$; $^{b}P <$ 0.01 versus corresponding VM-26 alone (Mann–Whitney *U*-test). CAs, chromosomal aberrations; MI, mitotic index and MNPCE, micronucleated polychromatic erythrocytes.

**Table II.** DNA strand breaks in bone marrow of mice after treatment with dexrazoxane (Dex) and/or teniposide (VM-26) or ethyl methanesulfonate (EMS) detected by comet assay (mean ± SD)

| Treatment (mg/kg) | Tail moment (arbitrary unit) | Tail length (μm) | Tail intensity (%) |
|---|---|---|---|
| 1-h sampling regimen | | | |
| Control | 0.9 ± 0.1 | 17.4 ± 0.81 | 1.90 ± 0.14 |
| Dex (100) | 3.3 ± 0.16** | 50.2 ± 0.52** | 19.2 ± 0.9** |
| VM-26 (10) | 6.8 ± 0.32** | 55.5 ± 1.06** | 34.1 ± 1.4** |
| Dex (100) + VM-26 (10) | 4.3 ± 0.44**$^{,b}$ | 50.5 ± 1.59**$^{,a}$ | 23.6 ± 1.49**$^{,b}$ |
| 24-h sampling regimen | | | |
| Control | 2.47 ± 0.66 | 37.12 ± 1.19 | 13.6 ± 3.07 |
| Dex (100) | 2.53 ± 0.30 | 37.15 ± 1.68 | 9.49 ± 0.72 |
| VM-26 (10) | 8.91 ± 2.09* | 46.07 ± 2.07* | 29.0 ± 6.53* |
| Dex (100) + VM-26 (10) | 3.11 ± 1.11$^{b}$ | 35.00 ± 2.54 | 10.5 ± 2.75$^{a}$ |
| EMS (100) | 17.2 ± 2.6** | 81.38 ± 2.13** | 68.3 ± 1.80** |

*$P < 0.05$ and **$P < 0.01$ versus control (Kruskal–Wallis test followed by Dunn's multiple comparisons test). $^{a}P < 0.05$ and $^{b}P < 0.01$ versus the corresponding VM-26 alone (Mann–Whitney *U*-test).

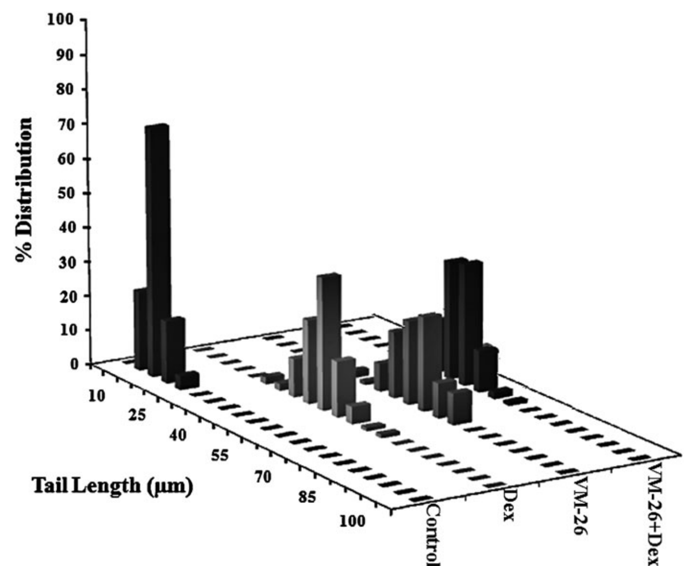


**Fig. 1.** Frequency distribution of tail length of mouse bone marrow cells with varying degree of DNA damage 1 h after treatment with dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg).

parameters observed in Dex-treated animals was significantly increased compared to the solvent control group; however, by 24 h, the level of these parameters was similar to that in the control group. Animals treated with VM-26 showed significant increase in the level of all measured parameters at both sampling regimen in comparison to those of the solvent control groups. Animals pretreated with Dex showed significant decrease in the level of the olive tail moment and tail intensity induced by VM-26 treatment at both sampling time compared to VM-26 alone. Importantly, at 24-h sampling time, the level of all measured parameters in VM-26 post-treated group decreased to close to that in the control groups by Dex administration.

The advantage of comet assay is that it is capable of analysing population of cells with various degrees of DNA damage. Thus, some differences exist in the distribution of damage in cell population. The heterogeneity in the distribution of DNA damage as a consequence of 1- and 24-h drug exposure is shown in Figures 1 and 2. Among the solvent control >90% of cells have exhibited tail length in the range of 5–15 μm. However, in case of Dex and VM-26 treatment, >60% of cells have exhibited tail length in the range of 40–65 μm and 50–75 μm of tail length, respectively. However, combined exposure of both drugs has resulted in the reduction of tail length, as 70% of cells exhibited tail length in the range of 35–45 μm (Figure 1). On the other hand, animals exposed to Dex or VM-26 for 24 h have resulted in the appearance of 27% cells having the tail migration between 45 and 85 with Dex and 29% of cells with tail migration of 65–95 μm with VM-26 treatment as compared to only 13% cells of vehicle control that have exhibited the high tail length in between 65 and 95 μm. Nonetheless, the combined exposure of animals with both drugs has resulted a remarkable reduction of tail length as evident by the appearance of only 13% cells that have tail migration between 65 and 95 μm, which was equal to the solvent control (Figure 2).

### *Apoptosis and cell cycle analysis*

The effects of Dex and/or VM-26 on apoptosis and cell cycle progression have been investigated using flow cytometry by



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measuring the DNA content of the cell population. Cells undergone apoptosis characterised by the appearance of Sub-$G_1$ peak. The percentage of sub-$G_1$ peak did not show significant variation in Dex-treated animals compared to the solvent control (Figure 3). Moreover, the levels of cells in $G_1$ and S phases in animals treated with Dex were near to those in the control group (Figure 4). On the other hand, these animals have more cells in $G_2$/M phase as compared to cells in the solvent control (Figure 4, panel C); however, this value (66.92 $\pm$ 10.8) was not statistically significant in comparison to the solvent control (51.86 $\pm$ 11.5). Compared to the control group, the percentage of cells with sub-$G_1$ peak was significantly increased by VM-26 treatment (Figure 3). Pretreatment with Dex was found to protect mouse bone marrow cells against VM-26-induced apoptosis and produces a noticeable decrease in the level of the hypodiploid DNA content induced by VM-26 in comparison to VM-26 alone ($P < 0.05$).

*Caspase-3 activity*

Activation of caspases plays a critical role in the execution stage of apoptosis. Therefore, we asked whether caspase-3 is activated during VM-26-induced apoptosis in bone marrow cells. In order to investigate the involvement of caspase-3 in signalling transduction, cleavage products of caspase-3 were detected by ELISA. As shown in Figure 5, Dex induced 1.15-fold increases in caspase-3 activity in comparison to control. However, this increase was not statistically significant in comparison to control. The hydrolytic enzyme activity of caspase-3 towards DEVD *p*-NA was significantly elevated by ~1.9-fold in animals treated with VM-26 in comparison to control. Pretreatment of mice with Dex significantly reduced the elevated caspase-3 activity relative to the values obtained after treatment with VM-26 alone ($P < 0.05$). To elucidate whether caspase-3 activation is required for VM-26-induced apoptosis and to clearly show that Dex blocks VM-26-induced apoptotic cell death, additional groups of mice were pretreated with Z-VAD-fmk before Dex and/or VM-26 treatment. Apoptosis was measured as described in Materials and methods by counting nuclei with hypodiploid DNA content. Caspase inhibition led to complete inhibitions of VM-26 and VM-26 plus Dex-induced apoptosis (data not shown), proving the importance of caspase-3 in these processes. Likewise, the activity of caspase-3 in all groups as detected by the Caspase-3/CPP32 colorimetric assay kit was nearly similar to those in the control group (data not shown).

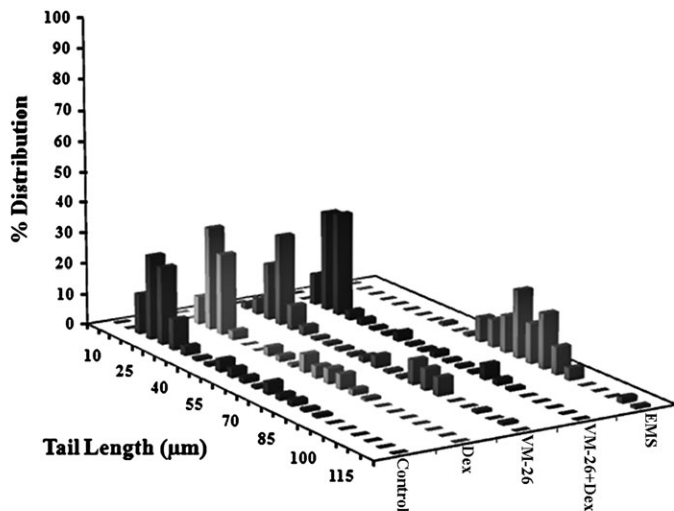


**Fig. 2.** Frequency distribution of tail length of mouse bone marrow cells with varying degree of DNA damage 24 h after treatment with dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg) or ethyl methanesulfonate (EMS; 100 mg/kg).

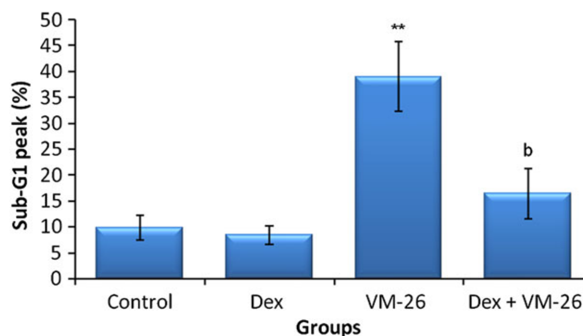


**Fig. 3.** Effects of dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg) on the percentage of apoptotic cells (mean $\pm$ SD). % Sub-$G_1$ peak denote the percentage of cells with subdiploid DNA content (apoptotic cells). $^{**}P < 0.01$ versus control, $^{b}P < 0.01$ versus VM-26 alone (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test).

*Reactive oxygen species generation*

To assess the role of Dex on VM-26-induced reactive oxygen species accumulation, we have measured the fluorescence of DCFH-DA-stained bone marrow cells by flow cytometry. DCFH-DA is a permeable dye, which cleaved to form non-fluorescent dichlorofluorescein (DCFH) in the cells. Upon oxidation, DCFH convert to fluorescent DCF by reactive oxygen species. As shown in Figure 6, after 24 h of treatment, only VM-26-treated animals show a noticeable shift in the DCF fluorescence peak. DCF fluorescence level did not show significant variation after treatment of mice with Dex as compared to the solvent control (Figure 7). The DCF fluorescence level in mice treated with VM-26 was significantly increased by ~1.8-fold as compared to the control animals ($P < 0.01$). However, VM-26-induced production of DCF fluorescence was profoundly abrogated by Dex and decreased to the level significantly different from the level of DCF fluorescence in the VM-26-treated alone ($P < 0.01$).

*Lipid peroxidation, oxidised and reduced glutathione levels*

As shown in Figures 8, 9 and 10, bone marrow GSSG, GSH and lipid peroxidation levels did not show significant variation in Dex-treated animals compared to the solvent control. Dex-treated animals showed a significant increase in GSH/GSSG ratio over the control group ($P < 0.05$). The GSH level observed in VM-26-treated animals was significantly decreased, together with increase in GSSG level compared to the control animals. So that GSH/GSSG ratio fell from 3.19 $\pm$ 0.91 to 0.62 $\pm$ 0.21 ($P < 0.01$), indicating increased oxidative stress. Animals pretreated with Dex showed a significant increase in GSH level over the VM-26-treated group and increased to the level significantly different from the level of GSH in the VM-26-treated alone ($P < 0.05$). The GSSG level was also significantly decreased in Dex pretreated animals compared to VM-26-treated group ($P < 0.01$). Consequently, the GSH/GSSG ratio was increased in Dex pretreated animals and was statistically significant when compared to the VM-26-

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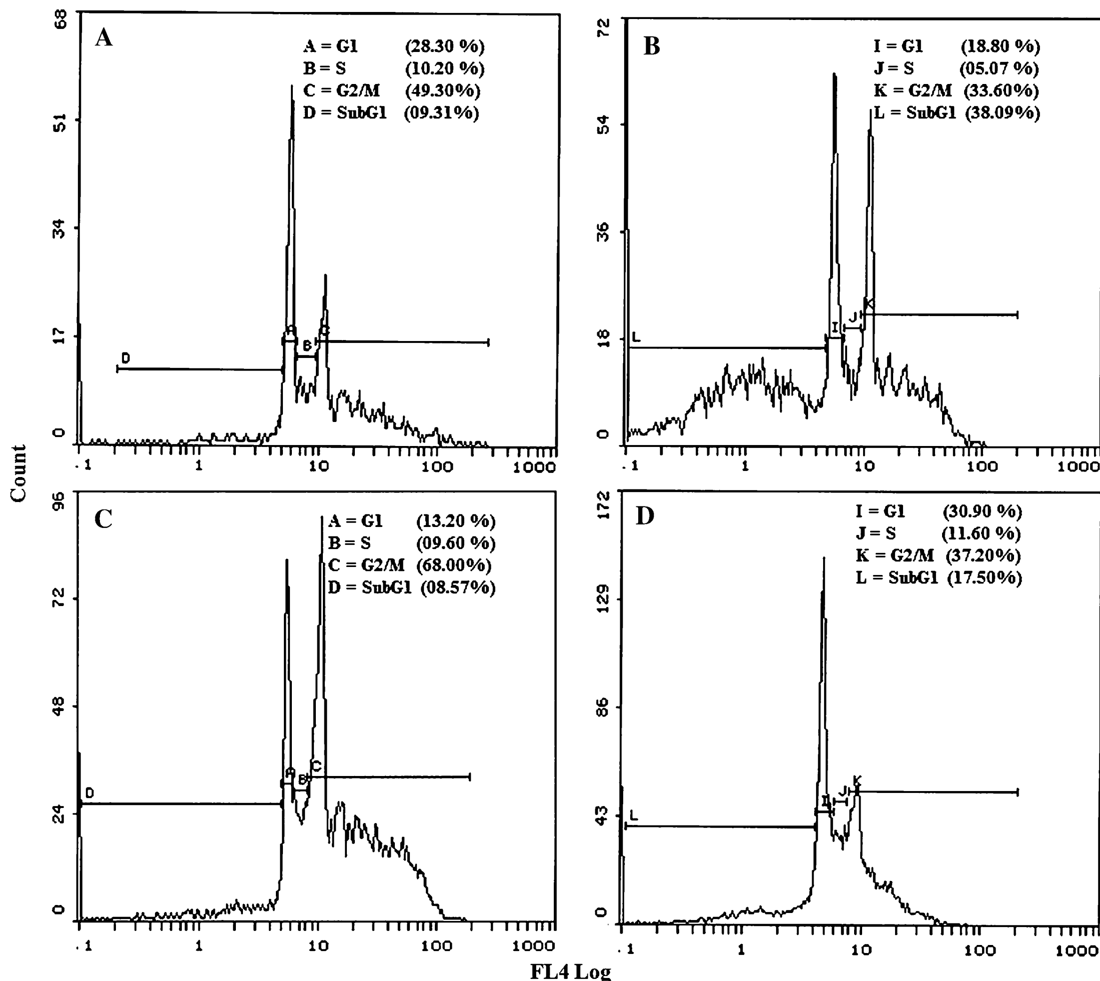


**Fig. 4.** Fluorescence-activated cell sorter analysis of PI-stained nuclei of untreated animals (**A**), animals treated 10 mg/kg teniposide (VM-26; **B**), 10 mg/kg dexrazoxane (Dex; **C**) or Dex plus VM-26 (**D**). Histograms demonstrate the distribution of nuclei according to their DNA content. Counts left of the $G_1$-peak (gated) indicate the appearance of nuclei with subdiploid DNA content (Sub-$G_1$).

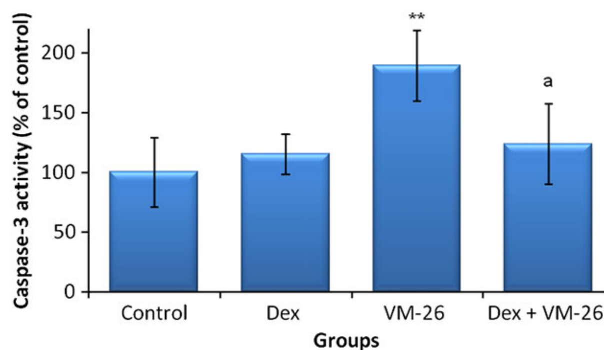


**Fig. 5.** Effects of dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg) on caspase-3 activity in the bone marrow cells of mice (mean ± SD). $^{**}P < 0.01$ versus control, $^{a}P < 0.05$ versus VM-26 alone (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test).

treated group ($P < 0.01$). A significant rise in bone marrow lipid peroxidation was observed in the VM-26-treated group compared to the solvent control ($P < 0.01$). Pretreatment of mice with Dex was found to significantly decrease the lipid peroxidation relative to the values obtained after treatment with VM-26 alone ($P < 0.05$). However, this amelioration was still significant when compared to the values observed in the control group ($P < 0.01$).

## Discussion

The influence of Dex on VM-26-induced DNA damage and apoptosis in non-tumour cells *in vivo* has not been reported yet. Thus, the main objective of this study was to answer the question whether Dex can be used as a modulator of VM-26-induced DNA damage and apoptosis in the bone marrow cells *in vivo*. A single dose of 100 mg/kg Dex was given intraperitoneally and bone marrow genotoxicity was assessed at 1, 24 (Tables I and II), 48 and 72 h (data not shown) after treatment. Dex treatment did not exhibit any significant genotoxicity at 24-, 48- and 72-h treatment periods as compared to the solvent control. The positive control mutagens cyclophosphamide and ethyl methanesulfonate were used in this study and these compounds produced the expected responses and the results of these compounds were in the same range as those of the earlier studies (23,35). These data confirmed the sensitivity of the experimental protocol followed in the detection of DNA damaging effects. The current results demonstrate that Dex was neither genotoxic nor apoptogenic at the dose tested. Moreover, it is able to protect mouse bone



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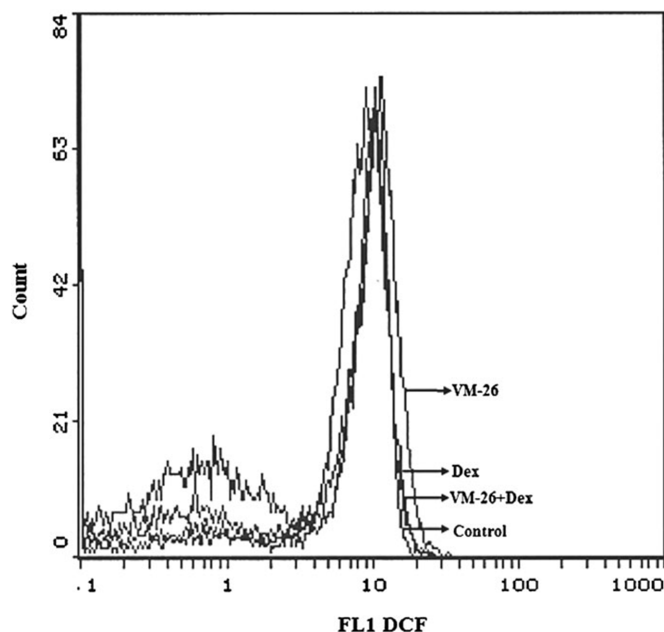


**Fig. 6.** Flow cytometric spectra of fluorescence probe DCF in bone marrow cells of mice treated with dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg).

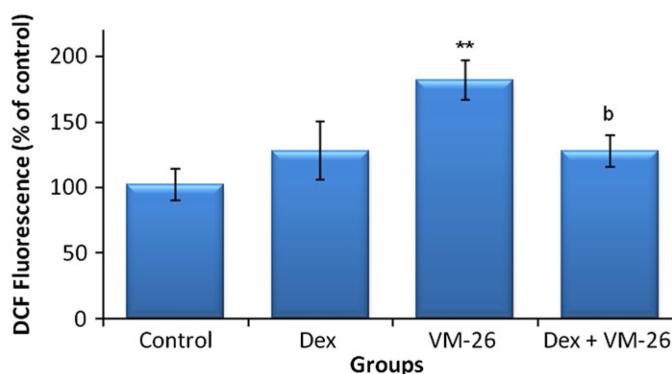


**Fig. 7.** Effects of dexrazoxane (Dex; 100 mg/kg) on teniposide (VM-26; 10 mg/kg)-induced generation of intracellular reactive oxygen species in the bone marrow cells of mice (mean ± SD). $^{**}P < 0.01$ versus control, $^{b}P < 0.01$ versus VM-26 alone (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test).

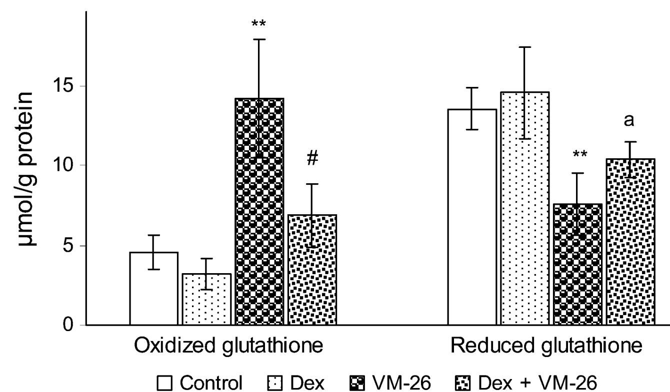


**Fig. 8.** Effects of dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg) on bone marrow oxidised and reduced glutathione levels in mice (mean ± SD). $^{**}P < 0.01$ versus control; $^{\#}P < 0.01$ versus VM-26 alone (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test). $^{a}P < 0.05$ versus VM-26 alone (unpaired *t*-test)

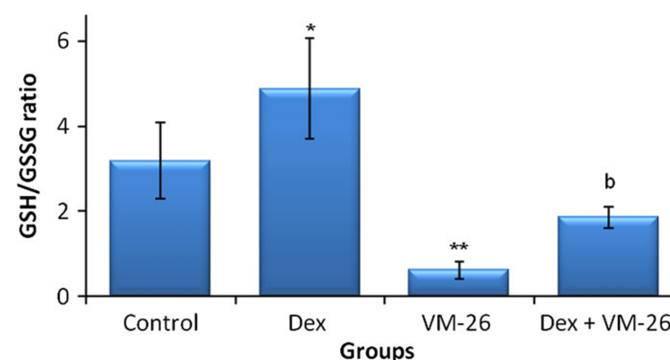


**Fig. 9.** Effects of dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg) on mouse bone marrow reduced and oxidised glutathione (GSH/GSSG) ratio (mean ± SD). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test). $^{b}P < 0.01$ versus VM-26 alone (unpaired *t*-test).

marrow cells against the VM-26-induced DNA damage, decline in the cell proliferation and apoptosis as observed by the use of standard techniques.

There is boundary of information on the *in vivo* metaphase damaging effects induced by VM-26, nevertheless, certain cytogenetic changes were measured in bone marrow and embryonic tissue from pregnant mice given a single intraperitoneal injection of 1.0 mg/kg VM-26 on Day 6, 7 or 8 of gestation and killed 48 h later. Treatment on Day 7 or 8 increased the frequency of embryonic cells with structural aberrations, one-fourth or more of which were stable, consisting of chromosomes with metacentric or submetacentric markers. VM-26 increased the percentage of embryonic cells with structural and numerical aberrations, but this was statistically significant only on Day 8 (36). On the other hand, in our study 0.05–2.5 mg/kg of VM-26 failed to induce chromosomal aberrations significantly at 24 h after treatment. Only the two highest doses tested induced a statistically significant increase in the total chromosomal aberrations. However, in contrast to our result, the induction of significant chromosomal aberrations with 1 mg/kg reported by Sieber *et al.* (36) might have been due to different animal age, species or the later harvesting time at 48-h post-treatment, whereas the harvesting of cells in the present study was at 24 h after treatment.

Barring few fragments, rings and polyploidy, the VM-26-induced chromosomal aberrations recorded in our study were mostly gaps and breaks (data not shown). The total numbers of structural and numerical chromosomal aberrations were noticed to be maximum after treatment with 10 mg/kg of VM-26. The treatment of mice with Dex before exposure to VM-26 resulted in significant reduction in the total number of chromosomal aberrations induced by VM-26. The genotoxic protection was also directly correlated with mitotic activity as an obvious protection was noted with Dex pretreated animals when bone marrow suppression was examined at metaphase stage, which is in harmony with our previous study (22) and the study by Hofland *et al.* (21). Where, reductions in etoposide-induced myelosuppression in mice pretreated with Dex were observed.

Using an MN test, VM-26 caused significant increases in MN induction at all doses tested. However, an inverse dose response was found between 0.5 and 10 mg/kg. These results are similar to the one obtained by conventional staining of VM-26-induced MN in mouse bone marrow cells (13). In the mouse

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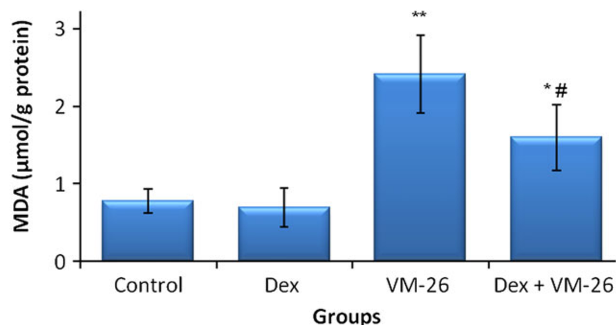


**Fig. 10.** Effects of dexrazoxane (Dex; 100 mg/kg) and/or teniposide (VM-26; 10 mg/kg) on bone marrow lipid peroxidation level (MDA) in mice (mean ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.01$ versus VM-26 alone (one-way ANOVA and post hoc Tukey–Kramer multiple comparison test).

bone marrow MN test, VM-26 caused a dose-dependent increase in MN induction up to 0.3 mg/kg. Twenty-four hours following intraperitonial injection and in the tested dose range of 0.01–10 mg/kg, the lowest genotoxic dose was 0.01 mg/kg. An inverse dose response was found between 0.3 and 10 mg/kg (13). 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. Jagetia and Aruna (13) found that the decline in PCE frequencies was dose dependent up to a dose of 0.3 mg/kg. With a further increase in drug dose, the decline in PCE frequencies was arrested. The PCE frequencies registered an elevation after administration of 0.6 mg/kg VM-26 in comparison with 0.3 mg/kg drug dose. This trend was dose dependent up to a dose of 10 mg/kg, where the highest PCE frequency was observed among drug-treated groups. On the other hand, our results demonstrated that VM-26 caused a dose-dependent suppression of erythroblast proliferation; the highest suppressive dose was 10 mg/kg and significantly decreased the frequency of PCE from 48.8 to 33.2%. The differences between these results can be attributed to different animal species and the technical features of the test procedures. The current data showed significant inhibitory effects of Dex on the MN produced by VM-26 and this protection was also directly correlated with mitodepression amelioration when mitotic activity was examined at interphase stage.

In mice metaphase chromosomal analysis and MN assay, which need DNA replication, 24-, 48- and 72-h sampling times are usually recommended to detect chromosome aberrations resulting from early DNA damage. For the comet assay, Sasaki *et al.* (37) have shown that DNA damages were generally detected 1–4 h after drug treatment. According to the pharmacokinetic of the drugs used in this study, a short sampling time 1 h and a longer one 24 h were chosen. In our study, at the 1-h sampling time, both topoisomerase-II inhibitors produced significant DNA damage as observed by increases in the bone marrow olive tail moment, tail length and tail intensity as compared to the control group. Moreover, pretreatment with Dex was found to significantly decrease the DNA damage relative to the values obtained after treatment with VM-26 alone. However, this protection was still statistically significant in comparison to the solvent control group. With 24-h sampling time, conversely, the level of DNA damaged cells in Dex-treated group decreased to close to that in the control animals. Additionally, animals pretreated with Dex produced a clear significant inhibitory effect on the level of DNA damage induced by VM-26 and statistically significant in comparison to the VM-26 alone. This indicates that DNA damage observed by Dex alone at 1-h sampling time was weak and easily repaired. An additional general observation was that the levels of DNA damage at 24-h sampling time seems to be higher than the value observed at 1-h sampling time for the controls. The difference between these values might be attributed to the conditions of animal accommodation or the technical features of the comet assay procedure.

The present results confirm the literatures that have described the catalytic inhibitors, which produce low levels of topoisomerase-II-mediated DNA cleavage as having only modest or even no mutagenic activity (38,39). Certain *in vitro* studies have shown that VM-26 induce DNA strand breaks essentially in dividing cells. DNA strand breaks were observed in HeLa cells exposed to the VM-26 for a short period 40 min (39). These authors found with the comet assay that most of the cells treated with VM-26 showed a comet-like pattern of DNA staining, which represented significant DNA damage in those cells. Of considerable interest, cells treated with the catalytic topoisomerase-II inhibitor merbarone for 40 min exhibited few DNA breaks. Our *in vivo* data at 1-h sampling time were concordant with this *in vitro* data. However, at 24-h sampling time, no significant DNA damage was observed with the catalytic inhibitor Dex-treated animals. A possible explanation for the differences in the DNA damaging effects is that the DNA damage caused by Dex is easier to repair than that caused by VM-26. Moreover, our *in vivo* antigenotoxic results confirm the findings of previous *in vitro* studies of the inhibition of topoisomerase-II poisons-induced DNA damage by Dex. Using alkaline elution assays, Dex in a dose-dependent manner inhibited the formation of DNA single-strand breaks as well as DNA-protein cross links induced by topoisomerase-II poisons etoposide, amsacrine, daunorubicin and doxorubicin which are known to stimulate DNA–topoisomerase-II cleavable complex formation (40).

The intriguing question was whether Dex has influence on apoptosis induced by VM-26 in mouse bone marrow cells. VM-26-induced apoptosis has been demonstrated in various cell types including oral squamous cell carcinoma (41) and human FLC and HL-60 cells (42). Data presented here indicate that VM-26 induce death in bone marrow cells with morphological and biochemical characteristics typical of apoptosis. Dex did not significantly change the frequency of apoptotic cells in animals not post-treated with VM-26, whereas it significantly decreased the degree of apoptosis in VM-26-treated animals, which correlated well with the level of fragmented cells detected by comet assay. In the current study, Dex decreased the fragmentation of nuclear DNA induced by VM-26 as detected by the appearance of nuclei with hypodiploid DNA content, moreover, caspase-3 activation, an event that has been shown to play a critical role in apoptosis signalling transduction becomes less in animals pretreated with Dex. It is worthwhile to note that VM-26-induced apoptosis is dependent on caspase-3 as shown by abrogation of apoptosis after inhibition with the pan-caspase inhibitor z-VAD-fmk. Information on the modulatory effects of catalytic inhibitors 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 (43). Moreover, the results of Hasinoff *et al.* (44) had shown that

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Dex reduced doxorubicin-induced apoptosis, which are consistent with its ability to prevent daunorubicin-induced apoptosis of myocytes (45,46).

The exact mechanism(s) by which the catalytic inhibitor Dex protected against VM-26-induced DNA damage is not well known and the detailed mechanism(s) of the Dex antigenotoxic action remain to be investigated in future. However, the mechanism of protection could be the result of reduction in the amount of cleavable complex formation (47) or simultaneous treatment with Dex that would allow interception of free radicals generated by VM-26 before they reach DNA and induce damages. Like etoposide, VM-26 has been reported to generate phenoxyl radical or quinone intermediates in the redox reaction (48,49). Phenoxyl radicals can oxidise intracellular thiols to reactive thiyl radicals. These thiyl radicals can further react to generate disulfide anion radicals, which can donate an electron to oxygen. Superoxide anion radical thus produced can form, in the presence of transition metal complexes, the extremely reactive hydroxyl radical that damages DNA (50,51). Moreover, it is believed that accumulation of lipid peroxyl radicals induced by podophyllotoxins during their oxidation may cause damage to cell membrane leading to lipid peroxidation (52,53). Conversion of etoposide and VM-26 to the *O*-demethylated metabolites (catechol and quinone) has also been reported. These metabolites are highly redox active molecules, which can redox cycle with their semiquinone radicals leading to formation of reactive oxygen species (48,54,55). Accumulation of these reactive oxygen species may cause damage to cellular genome and other critical biomolecules, ultimately inducing genotoxicity and leukaemia (56–58). 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 VM-26, oxidative stress markers such as generation of reactive oxygen species, lipid peroxidation, GSH and GSSG were determined after the animals were treated with VM-26, compared with the pretreatment with Dex and the solvent control groups.

The present study demonstrates that Dex pretreatment reduced the VM-26-induced reactive oxygen species generation, lipid peroxidation, GSSG accumulation and prevented the depletion in GSH significantly. The increased GSH and GSH/GSSG levels suggest that protection by Dex may be mediated through the modulation of cellular antioxidant levels. These observations confirm earlier studies in which Dex was reported to elevate reduced glutathione, glutathione peroxidase, superoxide dismutase and to reduce lipid peroxidation (22,59). It has been reported that Dex is a prodrug that is hydrolysed to its ring-opened metal-ion-binding metabolite (ADR-925) with a structure similar to EDTA. ADR-925 could remove iron from the iron–drug complex or bind free iron to decrease reactive oxygen species formation (60). More recently, the results of Junjing *et al.* (61) demonstrated that Dex was an antioxidant that could effectively scavenge hydroxyl, superoxide, lipid, diphenylpicrylhydrazyl and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) free radicals *in vitro* solution systems and the scavenging effects of Dex did not require the enzymatic hydrolysis to its ring-opened forms. In addition, Dex has potent intrinsic scavenging activity not only against hydroxyl radicals, the typical free radical product of the redox reaction of iron complexes but also against peroxyl radicals and the peroxynitrite radicals as compared to some classic antioxidants as glutathione, trolox and uric acid, indicating that the antioxidant properties of Dex were not solely dependent on iron chelation, although iron chelation may be a factor contributing to the its decrease on iron-based free radical generation (61,62). Thus, scavenging of free radicals by Dex seems to be an important mechanism against the VM-26-induced DNA damage and apoptosis.

In fact, when Dex given in combination with anthracyclines (e.g. doxorubicin or epirubicin), the pharmacokinetics of anthracyclines are unchanged (63). In addition, escalating doses of epirubicin did not significantly alter the pharmacokinetics of Dex (64). Interaction studies of Dex and etoposide have also been described. The pharmacokinetics of Dex are not altered by etoposide treatment (65). Moreover, the pharmacokinetics of etoposide were also unaffected by Dex rescue (66). Therefore, pharmacokinetics cannot account for the protective effects described for Dex against these anticancer drugs. A crucial consideration of coadministration of topoisomerase-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 anti-tumour effects. Dex reduced myelosuppression (21), ameliorated intestinal cell damage in mice (67) and prevented skin ulceration following experimental extravasation (68) from several different anthracyclines treatment, without compromisinG the anti-tumor efficacy of the anthracyclines. The mechanism of action on these preventions is unclear, whereas the current understanding is that Dex protects the heart from iron-mediated oxidative damage through the iron-chelating properties of the ring-opened molecule rather than through its topoisomerase-II inhibitory properties (69,70).

In conclusion, a critical point of this study is the possibility that there may be a therapeutic window for the use of VM-26 in combination with Dex, so that its DNA damaging and apoptogenic effects in non-tumour cells are minimised. The DNA damaging effects of VM-26 might be, at least in part, mediated by an oxidative stress mechanism that may be prevented or reduced by radical scavengers. VM-26 has a direct inhibitory effect on topoisomerase-II, an important component of its anti-tumour activity, and this will be unchanged by any manipulations that alter the redox reaction. Finally, this study provides for the first time that Dex has a protective role in the abatement of VM-26-induced DNA damage and apoptosis in the bone marrow cells of mice that resides, at least in part, in its radical scavenger activity. Therefore, Dex can be a good candidate to decrease the adverse effects of VM-26 in the non-tumour cells of cancer patients treated with this drug.

## Funding

Research Center of Pharmacy, King Saud University, Riyadh, Saudi Arabia (CPRC 245, 080146).

## Acknowledgements

The authors are grateful to Dr White S. L. for providing the teniposide and dexrazoxane samples and Dr Ilse-Dore Adler for stimulating discussions and her assistance with this and related studies.

Conflict of interest statement: None declared.

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