**Leukemia (2000) 14**, 1642–1651

www.nature.com/leu

# Amifostine (WR-2721) selective protection against melphalan genotoxicity

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**Amifostine (WR-2721) is an aminothiol compound dephosphorylated at the tissue site by alkaline phosphatase to the active metabolite, which is able to inactivate electrophilic substances and scavenge free radicals. Amifostine effects against melphalan-induced DNA strand breaks were studied in normal human white blood cells (WBC) and K562 leukemic cells using the single cell gel electrophoresis (SCGE) or Comet assay, a reported method for measuring DNA damage in individual cells. Prior to treatment (1 h, 37°C) with increasing doses of melphalan, with or without S9, the cells were treated (15 min, 37°C) with a control medium or amifostine (3 mg/ml). Treatment of normal and leukemic cells with melphalan induced a dose-dependent 'comet formation'. Melphalan-induced DNA damage follows a normal distribution in WBC. On the other hand, in K562, a significant proportion of undamaged cells remains even with doses at which mean DNA damage is serious. Pretreatment with WR-2721 protects WBC, but not K562, against the genotoxic effect of melphalan. Amifostine might even strengthen the action of the antiblastic drug against K562 cells. S9 addition appears to enhance melphalan effectiveness. SCGE appears as a suitable primary screening method for *in vitro* and *in vivo* studies on drug–DNA interactions and their modulations by endogenous/exogenous factors.** *Leukemia* (2000) **14,** 1642–1651.

**Keywords:** Comet assay; antiblastic drugs; DNA damage; free radical scavenger

## Introduction

The aminothiol WR-2721 (Amifostine) and its active free thiol WR-1065, which is able to scavenge free radicals and conjugate to electrophilic substances, have been shown to reduce mutations from ionising radiation[1–4] and selectively protect normal but not neoplastic cells and tissues against the toxic effect of different antineoplastic drugs.[5–13] This selective protection is based on different membrane-bound alkaline phosphatase concentrations and different mechanisms of amifostine uptake.[14,15]

Melphalan, a phenylalanine derivative of nitrogen mustard, is an alkylating agent used as a therapeutic. Its reactivity with DNA can cause cell death. Side-effects of treatment include tissue toxicity and secondary malignancies, probably due to the genetic damage induced. Alkylating compounds mutagenicity is related to their ability to cross-link and/or produce monoadducts to DNA transferring an alkyl group. Melphalan reacts at $N^7$ of guanine, and also produces adenine–adenine intrastrand cross-links in DNA, which are proposed to be responsible for early termination of transcription.[16–18] The full mutagenic potential is realised in *Salmonella typhimurium* after metabolic activation, principally by cytochromes P-450.[19] The induction of micronuclei, sister chromatid exchanges, chromosome aberrations,[20] specific-locus mutations,[21] dominant lethal mutations and inheritable translocations in post-meiotic germ cells in mice[22] have been observed. *In vivo* data on melphalan toxicity modification by WR-2721[23–25] prompted us to evaluate the modulating effect of amifostine with different genetic endpoints, in various cellular systems.

The present study was undertaken to verify the selective protective effect *in vitro* of amifostine against melphalan-induced genotoxicity in normal white blood cells (WBC) *vs* the acute myelogenous leukemia cell line K562. Although, to our knowledge, melphalan has never been tested *in vitro* with exogenous metabolic activation, in this study its effectiveness on DNA was evaluated both with or without S9 mix since nitrogen mustards are normally activated by the hepatic cytochrome P-450 system *in vivo*.

We utilized K562 cells and normal WBC to better represent susceptibility to the drug *in vivo*. Indeed, from a clinical point of view, a cytoprotective agent must be able to give selective protection to normal *vs* tumor tissue from the actions of chemotherapy. Furthermore, our previous findings (unpublished) had shown a complete lack of alkaline phosphatase in the K562 cell line, while its presence is known in normal leukocytes.

After drug treatment, the cells were analyzed by alkaline single cell gel electrophoresis assay (SCGE or Comet test), a technique allowing DNA damage in a single cell to be shown in an epi-fluorescence microscope.[26,27] The Comet test is able to detect DNA strand breaks, alkali labile sites and incomplete excision repair events in individual cells.

DNA damage in the form of single or double strand breaks and alkali labile lesions may share dangerous mutagenic or carcinogenic properties of chemicals. Assays that measure DNA breaks generally require the unwinding of the double stranded DNA molecule for sensitive detection. A high pH is used to facilitate the denaturation, unwinding and expression of both single strand breaks and DNA breaks that only become apparent after exposure to alkali. Alkaline unwinding,[28] alkaline filter elution,[29] and alkaline DNA precipitation[30] assays require radiolabeling of DNA for sensitive detection of breaks and can only indicate the average number of breaks per cell. The Comet assay shows an exquisite sensitivity, without DNA radiolabelling, and is able to measure DNA damage in individual cells. The ability to score damage in a conspicuous number of individual cells gives it statistical strength. Furthermore, together with the assessment of *in vivo* genotoxicity of chemicals in multiple organs[31] or after environmental and occupational exposure,[32,33] it is an ideal tool for assessing variability in the response of different cell types *in vitro*.[34,35] The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay has also proved to be a sensitive and reliable method for measuring DNA strand breaks. However, the detection of highly damaged cells by the Comet assay occurred earlier than the detection of DNA modifications by the TUNEL assay[36] and more sensitively.[37] SCGE allowed a rapid analysis of genotoxic damage in individual nuclei such as flow cytometry, and a significant strong correlation was shown[38,39] with a higher sensitivity.[40] Furthermore, the Comet assay detected highly

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Received 6 October 1999; accepted 12 May 2000

damaged cells much earlier than did the flow cytometry method.[41]

SCGE, which requires only a small number of non-replicating cells and whose results can be obtained in a relatively short time, agrees with SCE induction[42] and appears to be more sensitive in revealing DNA damage.[43] The ratio between the amount of DNA breakage induced and the frequency of micronuclei expressed in the following interphase is unclear. With the development of SCGE it is possible to address this question at the cellular level.[44] An association between the determination of repair proficiency in the Comet assay and the mean frequency of micronuclei was determined in lymphocytes.[45] A relationship between the significantly increased frequency of chromosome aberrations (CA) and cancer risk was demonstrated in spite of CA unspecificity.[46] Studies proved the Comet assay to be more sensitive than CA[47] and a very strong association was shown between the two types of damage,[47,48] suggesting that the Comet assay responses may clearly predict cytogenetic damage.

## Materials and methods

### *Chemicals*

Amifostine (Ethyol) was provided by Schering-Plough (Milan, Italy), melphalan (Alkeran) was provided by Wellcome (Rome, Italy), and bleomycin (Bleomicina) by Rhône-Poulenc Rorer (Milan, Italy); S9 was purchased from MOLTOX (Molecular Toxicology Inc., Boone, NC, USA) whereas styrene and all other laboratory chemicals were purchased from Sigma-Aldrich (Milan, Italy).

### *Cells*

EDTA-anticoagulated peripheral blood was obtained by venipuncture from consenting healthy non-smoker donors. In order to isolate leukocytes, the blood was centrifuged twice in a lysis buffer (155 mM $NH_4Cl$, 5 mM $KHCO_3$, 0.005 mM $Na_2EDTA$, pH 7.4), washed with phosphate-buffered saline (PBS) and resuspended ($1 \times 10^6$ cells/ml) in RPMI-1640 medium (Gibco, Grand Island, NY, USA).

The K562 acute myelogenous leukemia cell line was maintained in the suspension culture in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, Canada) and L-glutamine (2 mM). Exponentially growing K562 cells, washed with PBS and resuspended ($1 \times 10^6$ cells/ml) in RPMI-1640 medium, were used throughout this study.

### *Melphalan and amifostine treatment*

Appropriate volumes of melphalan and 0.1 M phosphate buffer (pH 7.4) or S9-mix were added to an Eppendorf tube containing 1 ml cell suspension. S9 was prepared as described by Maron and Ames.[49] It was diluted 1:10 in 0.1 M phosphate buffer (pH 7.4) containing NADP (4 mM), glucose-6-phosphate (5 mM), $MgCl_2$ (8 mM), and KCl (33 mM) to constitute S9-mix, 250 $\mu$l of which was added to each tube to make a total of 1 ml cell suspension. The cells were treated at 37°C with or without WR-2721 (3 mg/ml) 15 min prior to treatment for 1 h at 37°C at different doses of melphalan, and then washed twice in PBS. Cell viability and apoptotic cells were checked and the Comet assay performed only with a viability $\geqslant$95% (Trypan blue exclusion method) and apoptotic cells $\leqslant$4%. The apoptosis percentage was determined by TUNEL assay.[50]

### *Alkaline SCGE assay*

SCGE was performed basically according to Singh *et al*.[26] Degreased slides were previously dipped in 1% normal melting agarose for the first layer. The cells (~$2 \times 10^5$ cells) were then mixed with 85 $\mu$l of 0.7% low melting agarose (LMA) and placed on the first layer. Lastly 85 $\mu$l of LMA were added as the top layer. The cells were lysed at 4°C in the dark, for at least 1 h, in an ice-cold freshly prepared solution of 2.5 M NaCl, 10 mM $Na_2EDTA$, 10 mM Tris-HCl, 1% Triton X-100 and 10% DMSO, pH 10. The slides were then placed on a horizontal gel electrophoresis unit. The DNA was allowed to unwind for 20 min in an electrophoretic alkaline buffer (1 mM $Na_2EDTA$, 300 mM NaOH, pH 13) and subjected to electrophoresis for 20 min at 0.78 V/cm and 300 mA. Alkali and electrophoresis treatments were performed in an ice bath. All the steps described above were performed under a yellow light to prevent additional DNA damage. Once electrophoresis had been carried out, the slides were washed in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove alkali and detergent. Subsequently, the slides were dried and fixed by immersion in absolute methanol for at least 60 s. The slides were prepared in duplicate for each sample. Immediately before the examination, the DNA was stained with 100 $\mu$l ethidium bromide (2 $\mu$l/ml). The samples were examined at 400× magnification under a fluorescent microscope (Leitz Dialux 20; Leitz, Milan, Italy) equipped with an excitation filter BP 515–560 nm and a barrier filter LP 580 nm, using an automatic image analysis system (Cometa Release 2,1, Sarin, Florence, Italy). The image analysis system gives a quantitative description of comets by various parameters such as migration distance toward anode of DNA fragments, between the edge of the comet head and end of the tail, head diameter, and percentage DNA fluorescence intensity of total DNA in the head and tail of the comet. The tail moment is an integrated value considering both the distance and amount of migrated DNA, ie tail length × tail %DNA fluorescence intensity. The comet parameter tail moment was chosen to represent the data on genotoxic effects. One hundred cells (50 cells in two slides) per sample, selected at random, were analyzed under constant sensitivity.

The results are presented as frequency distributions of single cell DNA damage or as box and whisker plots. In this case measured values at the tested concentrations are shown as boxes that include 50% of the data. The top and bottom of the boxes mark the 25th and 75th centiles and the inner line marks the median value; 25% of data above the 75th centile and 25% below the 25th centile are marked as 'whiskers' limited by the maximum or minimum values. Outliers are displayed as points.

A negative control (0.1% DMSO) and positive controls (100 $\mu$g/ml bleomycin and 50 $\mu$M styrene without and with S9 mix, respectively) were performed.

The samples were coded and evaluated blind. All tests were generally performed three times.

### *Cell survival assay*

The cells were pre-treated with 0 or 3 mg/l of amifostine for 15 min, and then, as previously described, treated with various doses of melphalan, w/o S9-mix. The cells were washed, then resuspended in RPMI-1640 and maintained in a humidified incubator at 37°C with 5% $CO_2$. Cell survival was detected by Trypan blue exclusion 24 h after treatment. The tests were performed in triplicate.

### *Statistical analysis*

The data were analyzed using the statistic and graphic functions of SigmaPlot 5.0 and SigmaStat 2.0. A one-way analysis of the variance test was performed. If a significant $F$ value ($P \leqslant 0.05$) was obtained, the comparison between the grade of DNA damages was analyzed by using Student's $t$-test.

## Results

Normal WBC and K562 leukemic cells, pre-incubated at 37°C with or without WR-2721, were analyzed for the increase in electrophoretic mobility of nuclear DNA (comet formation) after exposure to increasing concentrations of melphalan, with or without S9 mix. A range of drug concentrations (0.25, 0.5, 1, 2.5, 5, 7.5, 10, 12.5 and 15 $\mu$g/ml were previously tested for cell toxicity. The chosen doses (0–10 $\mu$g/ml) were within the range showing, immediately after treatment, a cell survival $\geqslant$95%. Concentrations (12.5 and 15 $\mu$g/ml) showing a higher toxicity (70 and 55% in WBC, and 67 and 56% in K562, respectively) were rejected. Amifostine dose and time pre-treatment were chosen from previous studies.[10,51,52] Three independent experiments for each condition were performed (Tables 1 and 2). The average mean and the average median tail moments are reported together with the average 95th percentiles. The dispersion of the tail moment, ie variance/mean, is also shown.

Bleomycin (100 $\mu$g/ml), used as a genotoxic agent able to produce DNA lesions just after treatment,[53] displayed an increase in DNA damage (mean tail moments were $27.23 \pm 1.68$ $\mu$m in WBC and $30.27 \pm 2.06$ $\mu$m in K562, respectively). Styrene (50 $\mu$M), a compound which is metabolized by cytochrome P450 isozymes to reactive epoxides,[54] was used both with and without S9 to show the effectiveness of the S9 mix. Styrene strongly induced DNA damage in the presence of exogenous metabolic activation (mean tail moments were $40.78 \pm 3.28$ and $44.51 \pm 3.88$ $\mu$m in WBC and K562, respectively). However, DNA damage was also observed without S9 ($10.29 \pm 0.37$ *vs* $6.47 \pm 0.44$ $\mu$m in WBC and $12.41 \pm 1.16$ *vs* $7.42 \pm 0.26$ $\mu$m in K562).

### *Normal white blood cells (WBC)*

When the amifostine-untreated WBC were exposed to increasing doses of melphalan, a clear dose–response effect was

**Table 1** Genotoxic effects of different doses of melphalan in normal white blood cells (WBC)

| *Dose ($\mu$g/ml)* | *Amifostine* | *Average mean ($\mu$m ± s.d.)* | *Average median ($\mu$m ± s.d.)* | *Average 95th perc ($\mu$m ± s.d.)* | *Dispersion ± s.d. (Variance/Mean)* |
|---|---|---|---|---|---|
| *–S9* | | | | | |
| 0 | – | 6.47 ± 0.44 | 6.95 ± 0.41 | 9.62 ± 0.08 | 1.58 ± 0.46 |
| | + | 7.31 ± 0.84 | 7.05 ± 0.27 | 12.65 ± 4.69 | 1.08 ± 0.66 |
| 0.25 | – | 7.38 ± 0.41 | 7.05 ± 0.09 | 14.69 ± 0.33 | 1.42 ± 0.37 |
| | + | 7.73 ± 0.33 | 7.32 ± 0.12 | 10.69 ± 3.06 | 0.85 ± 0.64 |
| 0.50 | – | 7.52 ± 0.04 | 6.91 ± 0.41 | 17.41 ± 1.42 | 2.46 ± 0.39 |
| | + | 7.02 ± 0.45 | 7.03 ± 0.23 | 11.37 ± 2.88 | 0.83 ± 0.31 |
| 1.00 | – | 9.23 ± 1.68 | 7.04 ± 0.26 | 24.27 ± 4.01 | 2.71 ± 0.55 |
| | + | 7.46 ± 0.18 | 7.31 ± 0.13 | 12.77 ± 1.05 | 0.87 ± 0.25 |
| 2.50 | – | 12.25 ± 0.43 | 9.87 ± 0.38 | 31.42 ± 2.57 | 2.57 ± 0.73 |
| | + | 8.82 ± 0.22 | 8.35 ± 0.29 | 15.22 ± 3.13 | 1.14 ± 0.87 |
| 5.00 | – | 16.78 ± 3.32 | 14.13 ± 1.31 | 34.62 ± 6.63 | 2.88 ± 1.27 |
| | + | 9.02 ± 0.90 | 8.14 ± 0.66 | 19.25 ± 2.05 | 2.24 ± 0.44 |
| 10.00 | – | 18.92 ± 0.54 | 18.54 ± 0.59 | 36.43 ± 0.81 | 2.86 ± 0.83 |
| | + | 10.55 ± 0.62 | 9.35 ± 0.19 | 20.85 ± 6.92 | 1.49 ± 0.40 |
| *+S9* | | | | | |
| 0 | – | 7.04 ± 0.12 | 7.39 ± 0.31 | 10.56 ± 0.21 | 1.16 ± 0.45 |
| | + | 6.72 ± 0.39 | 7.50 ± 0.27 | 11.04 ± 0.80 | 1.20 ± 0.17 |
| 0.25 | – | 8.07 ± 1.68 | 8.29 ± 1.58 | 16.00 ± 3.86 | 2.20 ± 0.04 |
| | + | 7.21 ± 0.47 | 7.23 ± 0.57 | 12.38 ± 0.13 | 1.19 ± 0.42 |
| 0.50 | – | 12.20 ± 0.07 | 11.48 ± 0.95 | 19.83 ± 2.90 | 2.23 ± 0.20 |
| | + | 6.30 ± 1.36 | 6.22 ± 1.50 | 10.64 ± 1.38 | 1.20 ± 0.53 |
| 1.00 | – | 13.80 ± 1.33 | 12.89 ± 0.81 | 23.43 ± 3.10 | 1.96 ± 0.13 |
| | + | 6.41 ± 1.70 | 6.58 ± 1.80 | 11.97 ± 2.93 | 1.43 ± 0.27 |
| 2.50 | – | 15.64 ± 2.04 | 14.95 ± 2.85 | 26.94 ± 2.03 | 2.82 ± 0.01 |
| | + | 8.15 ± 0.76 | 7.52 ± 0.11 | 16.79 ± 1.05 | 3.33 ± 0.60 |
| 5.00 | – | 21.24 ± 0.22 | 20.67 ± 0.59 | 36.10 ± 3.15 | 2.44 ± 0.03 |
| | + | 9.51 ± 0.71 | 10.01 ± 0.66 | 15.57 ± 2.18 | 1.63 ± 0.74 |
| 10.00 | – | 26.56 ± 0.63 | 26.26 ± 1.11 | 38.86 ± 1.25 | 1.39 ± 0.09 |
| | + | 9.17 ± 0.11 | 8.75 ± 0.18 | 17.70 ± 4.20 | 1.79 ± 0.76 |

Average mean, median, 95th percentile and dispersion values of the comet parameter tail moment are reported.
s.d., standard deviation of the average summary statistic (ie averaged mean values or the averaged median values).

**Table 2** Genotoxic effects of different doses of melphalan in acute myelogenous leukemia cell line (K562)

| *Dose (μg/ml)* | *Amifostine* | *Average mean (μm ± s.d.)* | *Average median (μm ± s.d.)* | *Average 95th perc (μm ± s.d.)* | *Dispersion ± s.d. (Variance/Mean)* |
|---|---|---|---|---|---|
| *–S9* | | | | | |
| 0 | – | 7.42 ± 0.26 | 6.90 ± 0.18 | 12.00 ± 1.27 | 0.88 ± 0.46 |
| | + | 7.40 ± 0.63 | 6.80 ± 0.75 | 13.52 ± 1.29 | 0.96 ± 0.14 |
| 0.25 | – | 9.39 ± 0.04 | 8.67 ± 0.17 | 15.95 ± 0.74 | 1.11 ± 0.19 |
| | + | 8.62 ± 0.97 | 8.00 ± 1.40 | 14.37 ± 0.86 | 0.92 ± 0.05 |
| 0.50 | – | 16.16 ± 1.90 | 13.46 ± 1.34 | 35.24 ± 4.10 | 4.81 ± 0.73 |
| | + | 17.72 ± 2.77 | 16.30 ± 3.79 | 34.43 ± 1.60 | 3.94 ± 0.53 |
| 1.00 | – | 18.49 ± 0.52 | 17.52 ± 1.39 | 36.71 ± 2.35 | 4.37 ± 1.10 |
| | + | 21.71 ± 0.33 | 17.73 ± 0.08 | 38.65 ± 0.89 | 4.28 ± 0.35 |
| 2.50 | – | 19.66 ± 0.01 | 17.18 ± 1.99 | 40.82 ± 0.50 | 5.95 ± 0.12 |
| | + | 22.78 ± 0.24 | 22.65 ± 0.45 | 42.69 ± 3.80 | 4.16 ± 1.39 |
| 5.00 | – | 21.61 ± 1.80 | 18.70 ± 0.63 | 46.55 ± 3.95 | 7.42 ± 1.10 |
| | + | 23.28 ± 1.65 | 22.49 ± 1.56 | 38.33 ± 0.86 | 4.01 ± 0.23 |
| 10.00 | – | 24.09 ± 2.62 | 19.31 ± 1.39 | 43.00 ± 2.94 | 5.81 ± 1.20 |
| | + | 33.26 ± 0.28 | 32.32 ± 0.33 | 56.74 ± 1.73 | 4.73 ± 0.10 |
| *+S9* | | | | | |
| 0 | – | 9.40 ± 0.45 | 8.67 ± 1.40 | 12.25 ± 1.30 | 0.58 ± 0.16 |
| | + | 9.91 ± 0.16 | 9.71 ± 0.67 | 12.57 ± 3.24 | 0.41 ± 0.26 |
| 0.25 | – | 12.41 ± 0.01 | 6.56 ± 0.59 | 36.83 ± 0.06 | 10.15 ± 1.59 |
| | + | 15.44 ± 1.42 | 10.03 ± 1.27 | 44.31 ± 5.82 | 9.70 ± 1.77 |
| 0.50 | – | 15.28 ± 0.10 | 8.85 ± 0.37 | 38.86 ± 0.42 | 9.01 ± 0.76 |
| | + | 19.26 ± 1.51 | 12.81 ± 0.69 | 51.94 ± 3.32 | 11.05 ± 0.74 |
| 1.00 | – | 16.64 ± 1.72 | 10.41 ± 2.76 | 38.39 ± 1.45 | 10.49 ± 1.58 |
| | + | 21.13 ± 0.04 | 13.10 ± 2.47 | 45.09 ± 1.19 | 11.29 ± 1.09 |
| 2.50 | – | 19.93 ± 1.80 | 12.69 ± 1.52 | 44.58 ± 1.61 | 10.71 ± 0.60 |
| | + | 22.96 ± 1.57 | 20.81 ± 4.18 | 48.25 ± 5.93 | 9.76 ± 2.16 |
| 5.00 | – | 25.65 ± 0.45 | 26.54 ± 2.03 | 45.09 ± 0.87 | 6.67 ± 0.31 |
| | + | 29.92 ± 1.83 | 30.26 ± 1.73 | 51.66 ± 2.88 | 6.77 ± 0.11 |
| 10.00 | – | 30.07 ± 0.99 | 31.10 ± 0.23 | 41.26 ± 2.52 | 2.15 ± 0.17 |
| | + | 34.08 ± 0.47 | 34.71 ± 2.94 | 49.53 ± 0.60 | 2.97 ± 0.07 |

Average mean, median, 95th percentile and dispersion values of the comet parameter tail moment are reported.
s.d., standard deviation of the average summary statistic (ie averaged mean values or the averaged median values).

shown, both with and without S9 (Table 1). With S9 addition, mean tail moments for control WBC and WBC treated with melphalan at 10 μg/ml were 7.04 and 26.56 μm (Student's *t*-test $P < 0.001$), respectively. In the absence of S9, the mean tail moment for WBC treated with melphalan at 10 μg/ml was increased three times as compared to the control WBC (Student's *t*-test $P < 0.001$). The addition of microsomial extract increased cell sensitivity; the first effective dose was 0.5 μg/ml (*t*-test $P < 0.001$) in WBC + S9 and 1 μg/ml (*t*-test $P < 0.01$) in WBC – S9 (Table 1). The presence of amifostine determined considerable protection against the genotoxic effectiveness of melphalan: at 10 μg/ml, a significant effect was evident (–S9: mean tail moment reduced to 10.55 μm *vs* 18.92 μm, *t*-test $P < 0.001$; +S9: 9.17 μm *vs* 26.56 μm, *t*-test $P < 0.001$). Furthermore, the first effective dose became 5 μg/ml both with (*t*-test $P < 0.001$) and without (*t*-test $P < 0.01$) S9.

## *Acute myelogenous leukemia cell line K562*

Melphalan genotoxicity increases with larger doses of the drug, both with and without exogenous metabolic activation and irrespective of amifostine pre-treatment (Table 2). When K562 cell treatment was performed with no microsomial extract, the first active doses were 0.5 μg/ml (*t*-test $P < 0.001$) and 0.25 μg/ml (*t*-test $P < 0.01$) for WR-2721-treated and untreated cells, respectively. In the presence of S9 mix, the first dose with genotoxic effectiveness was 0.25 μg/ml both with (*t*-test $P < 0.05$) and without (*t*-test $P < 0.01$) amifostine. Pre-treatment with amifostine did not protect K562 cells from the genotoxic action of melphalan.

The median and 95th percentile values, reported in Tables 1 and 2, confirmed the protective effectiveness of the pro-drug WR-2721 against DNA damage induced by melphalan in WBC and its inefficacy in K562 cells. Another difference between WBC and K562 cells was the frequency distribution of the amount of DNA damage as shown by dispersion values.

The results of one (Exp. No. 1) out of three independent experiments for each condition are reported in Figure 1 as box and whisker plot. The different behavior of WBC and K562 cells in response to WR-2721 pre-treatment was evident both in terms of cell population as well as individual cell response.

This latter point is better shown in Figure 2, which reports the frequency distribution of tail moment values for different melphalan concentrations, with (Figure 2a) and without (Figure 2b) S9 mix, in one of the experiments. The data of their positive controls are also reported (Figure 2c). WBC are a mixture of cell types, including monocytes, lymphocytes and granulocytes, whereas K562 is an established cell line. This might have suggested a distinct response from various cell types for WBC,[55] and a more regular response for K562. Unexpectedly, sensitivity appeared to be quite similar in each of the WBC cell types (frequency distributions maintain a similar normal distribution at the increasing melphalan concentrations). On the other hand, K562 cells showed a high

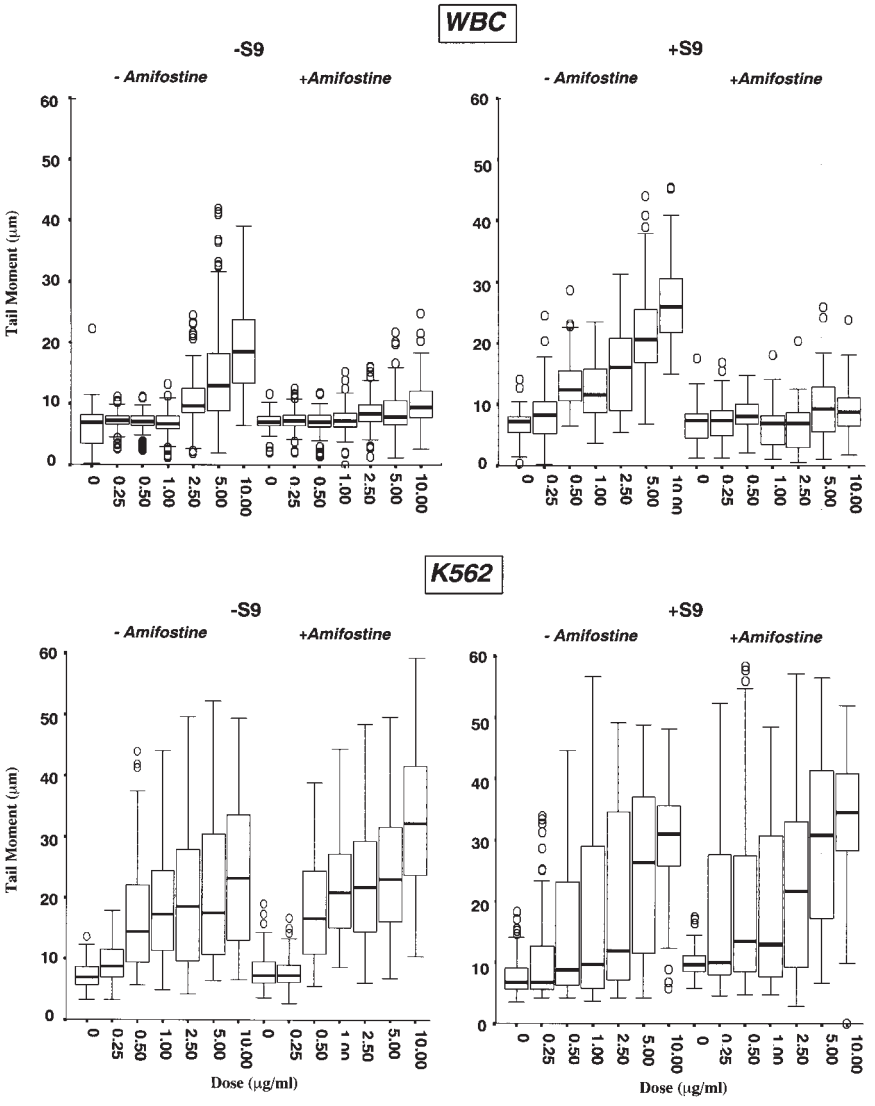


**Figure 1** Genotoxic effects of different doses of melphalan: the results of one (Exp. No. 1) out of three independent experiments are presented by the comet parameter tail moment. Results from 100 comet images per treatment are presented in box and whisker plots (see Materials and methods). WBC, normal white blood cells; K562, acute myelogenous leukemia cell line.

response variability with high drug resistance in subpopulations and a consequent onset of undesirable behavior against drug effectiveness.

The mechanisms of DNA damage induction appeared different in WBC and K562 cells.

A good linear correlation between DNA damage and melphalan doses was evident in WBC. The S9 mix significantly increased DNA damage in WBC which were not pre-treated with amifostine (one-way ANOVA, $P < 0.001$, and *t*-test, $P \leqslant 0.001$ for melphalan concentrations $\geqslant 0.5$ $\mu$g/ml, the first effective dose in WBC + S9).

In K562 cells, a significant proportion of undamaged cells remains even with doses at which DNA damage is strong. The addition of microsomal extract to K562 cells increased the mean tail moment in the controls (± amifostine) whereas it does not appear to influence the 95th percentile values (Table 2). However, a significant difference between cells treated w/o S9 was shown both without (one-way ANOVA, $P = 0.003$) and with (one-way ANOVA, $P < 0.001$) amifostine. Pre-treatment with WR-2721 did not protect K562 cells. Although in some cases amifostine appeared to strengthen melphalan effectiveness on DNA, scientific justification to support the data should be provided.

Even if melphalan is able to produce high DNA damage, 'ghost cells', in which DNA is extensively degraded allowing most of the comet head to migrate under electrophoresis, were not observed in either WBC or K562.

Apoptosis data ($\leqslant$4%), detected by TUNEL assay immediately after treatment, confirm the lack of hedgehog cells such as cell viability which, immediately after treatment, is always $\geqslant$95%.

This high survival percentage does not preclude the later appearance of cytotoxicity. Therefore cell survival was measured after 24 h (Table 3). In actual fact, the data do not show any clear relationship with drug doses, cellular types or treatment (w/o amifostine, w/o S9 mix). At the doses of melphalan used, efficient mechanisms can probably repair DNA damage. However, further investigations will have to be performed to clarify these issues.

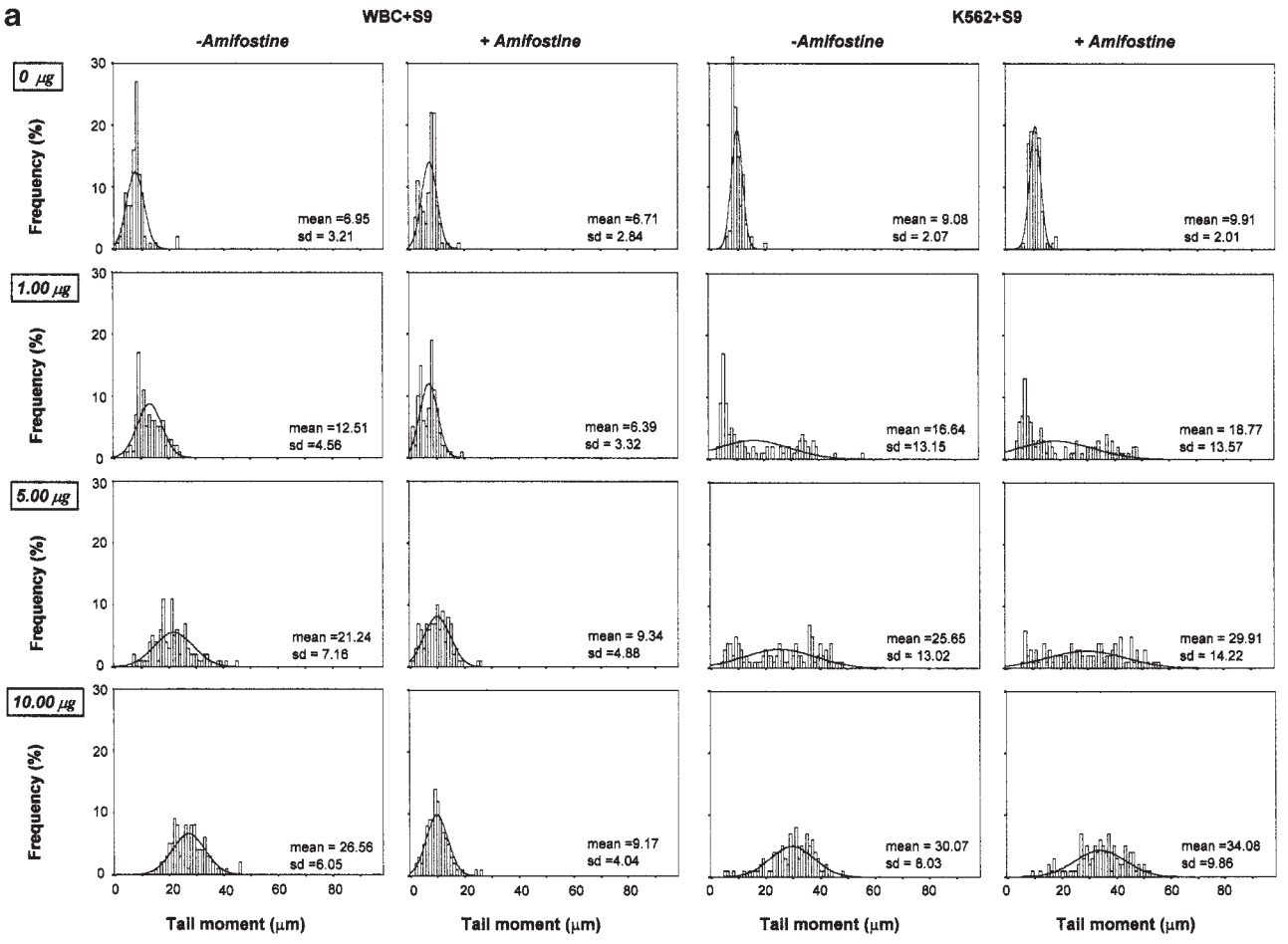


**Figure 2** Genotoxic effects of different concentrations of Melphalan on normal white blood cells (WBC) and acute myelogenous leukemia cell line (K562), with (+) and without (–) amifostine. The cells were treated in the presence (a) or in the absence (b) of S9. Positive controls are reported (c). The data are displayed as frequency distribution of tail moment values. SD, standard deviation; S9, exogenous metabolic activation.

## Discussion

Antiblastic drugs are characterized by a reduced therapeutic index, ie difference between toxic and effective dose. Side-effects of treatment include tissue toxicity and secondary malignancies, probably due to the genetic damage induced. Therefore, a selective protection of normal *vs* tumor tissues by cytoprotective agents seems to be an important goal. Amifostine has been reported to be a selective cytoprotective compound.[5–13] The pro-drug WR 2721 is an organic thiophosphate compound dephosphorylated into the active metabolite by membrane alkaline phosphatase at the target tissue.

In our study, the possible protective effects of WR 2721 against melphalan-induced genotoxicity in human WBC from healthy donors as well as in the K562 leukemic cell line was detected by the SCGE assay. WR-2721 appears to selectively protect healthy leukocytes but not K562 tumoral cells. This selective action is mainly based on the efficient dephosphorylation of aminothiol by alkaline phosphatase in WBC, whereas K562 cells are unable to activate amifostine for the complete lack of membrane-bound enzyme. On the other hand, amifostine strengthens melphalan genotoxic effects in tumoral cells. Further investigations are required to provide scientific rationale to clarify this effect.

The reaction of a nitrogen mustard such as melphalan with a DNA base can be summarized. One 2-chloroethyl side chain undergoes an intramolecular cyclization with release of $Cl^-$ and formation of a highly reactive intermediate that can resemble a nucleophilic substitution reaction. For example, alkylation of the $N^7$ of guanine residues in DNA may have various effects of considerable biological importance, such as abnormal base pair, labilization of imidazole ring with a possible ring cleavage or depurination by excision of base residues, cross-linking with a second base or another nucleophilic moiety. Any of these effects could adequately explain both the mutagenic and the cytotoxic effects of the drug. In this study we applied the alkaline SCGE assay to detect mainly alkali labile sites. DNA repair events might also be detected. To be able to evaluate the contribution of repair, the treatment should be performed at 4°C, that is, at a temperature that slows down the enzymatic systems such as excision repair processes.[56] A detailed analysis of the molecular pharmacology of cross-linking drugs cannot be provided by this type of assay. To identify the presence of cross-links, cells should be exposed to DNA-damaging agents such as ionizing radiation: the degree of migration inhibition can be used as a measure of DNA cross-linking.[57] The Comet assay is a sensitive test for the detection of a variety of DNA lesions, but it does not readily detect cross-linking agents in the test conditions we adopted. However, our main goal was to evaluate whether pre-treatment with amifostine could protect WBC, and not K562 cells, against the effects of melphalan. On the other

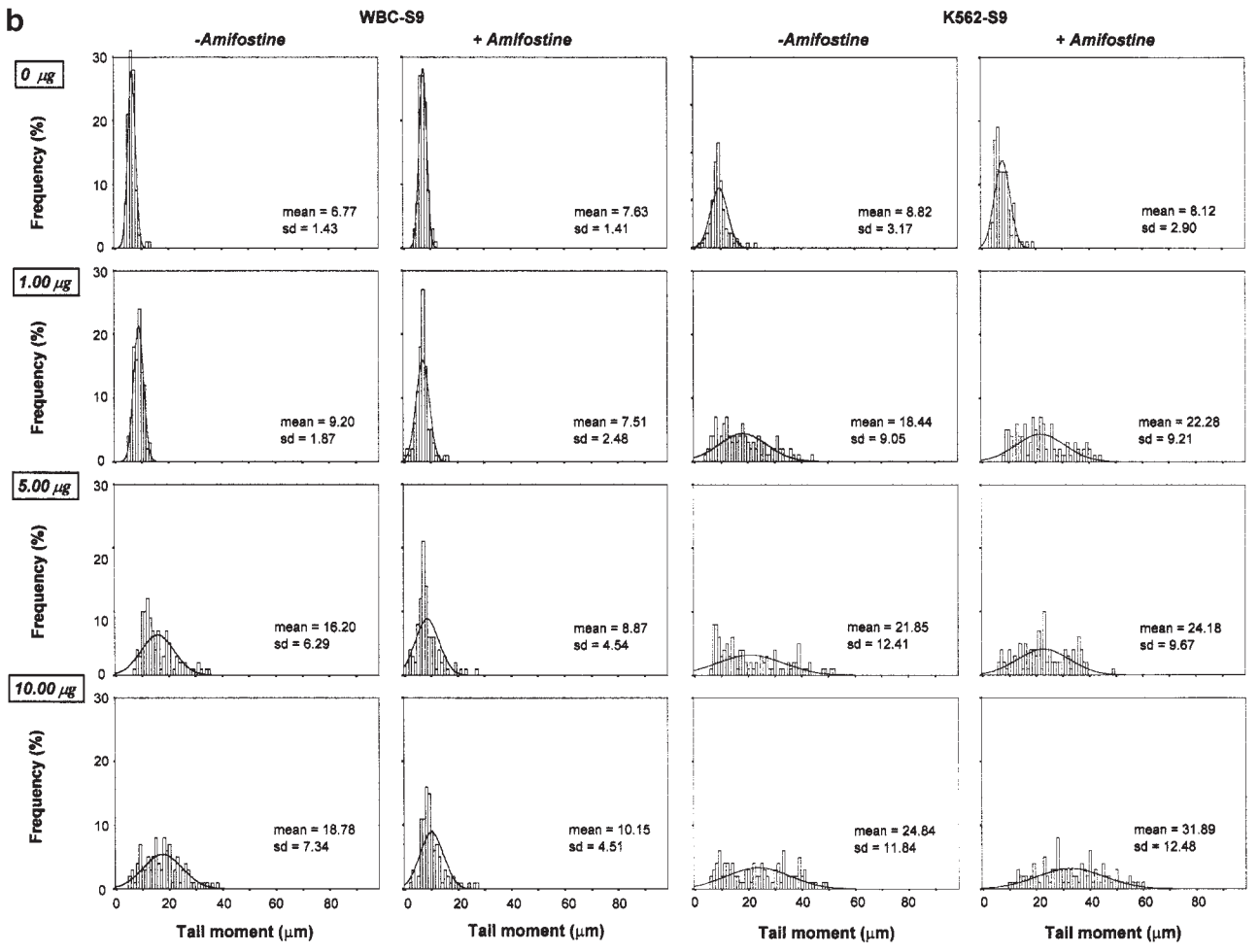


**Figure 2** *Continued.*

hand, we tried to relate the overall effects in the comet assay to cytotoxic ones. In contrast to the clear concentration-related genotoxic effects seen for both WBC and K562 cells, cell survival, determined 24 h after treatment, did not show any clear relationship with drug doses.

All nitrogen mustards are chemically unstable but vary greatly in their degree of instability. For example, cyclophosphamide requires biochemical activation by cytochrome P-450 complex before its cytotoxicity becomes evident. In this context, exogenous metabolic activation was included in the assays to try to enhance the effects of melphalan.

S9 really enhances (about 1.5-fold) the effectiveness of this phenylalanine derivative of nitrogen mustard, and at the same time magnifies the amifostine protective action by reducing the tail moment values at the same level detected in the absence of S9 in WBC. This suggests the formation of a more electrophilic compound than the original drug, which shows, at the same time, a higher affinity both to DNA and amifostine: the increase of melphalan-induced DNA damage could be due to the formation of a more reactive derivative. This also appears more efficient than parental compound in conjugating with WR-2721 derivative thiol that consequently shows higher cytoprotective effects. In actual fact, melphalan is a substituted aniline, and aniline is a selective substrate towards various cytochrome P450s.[58–60] Furthermore, ring hydroxylation can increase aniline genotoxicity, with the formation of DNA adducts.[59]

On the other hand, melphalan is also able to induce dose-related effects in WBC tested without S9 mix. Our knowledge concerning the xenobiotic-metabolizing capacity of fresh human blood cells is rather limited, however, monocytes were shown to constitutively express some P-450 enzymes.[61]

The K562 cells present a complex behavior in relationship to S9 addition which increases tail moment values in the control group and at the same time protects against the genotoxic effects of melphalan, specially at low doses (mean tail moment increase/dose unit: 10.72 *vs* 15.48 $\mu$m and 7.09 *vs* 12.35 $\mu$m in amifostine-treated and untreated cells, respectively). Culture conditions could influence the cell response, however, further investigations need to be performed to clarify this point.

Resistance to chemotherapeutic drugs and radiation represents a major obstacle in human cancer therapy. Most of the methods currently used to quantify DNA damage are capable of providing measurements of average response, and, in heterogeneous populations, averages are not sufficient to describe the effects of physical and chemical agents. One of the potential uses of the Comet assay is to predict the response of human tumor cells to therapy.[62,63] The ability of this method of detecting resistant and sensitive subpopulations of tumour cells, is particularly valuable.[35] In this study, the K562 cells showed a higher heterogeneity in DNA damage than WBC against melphalan treatment. It can derive both from genetic differences, such as cell subpopulations with various

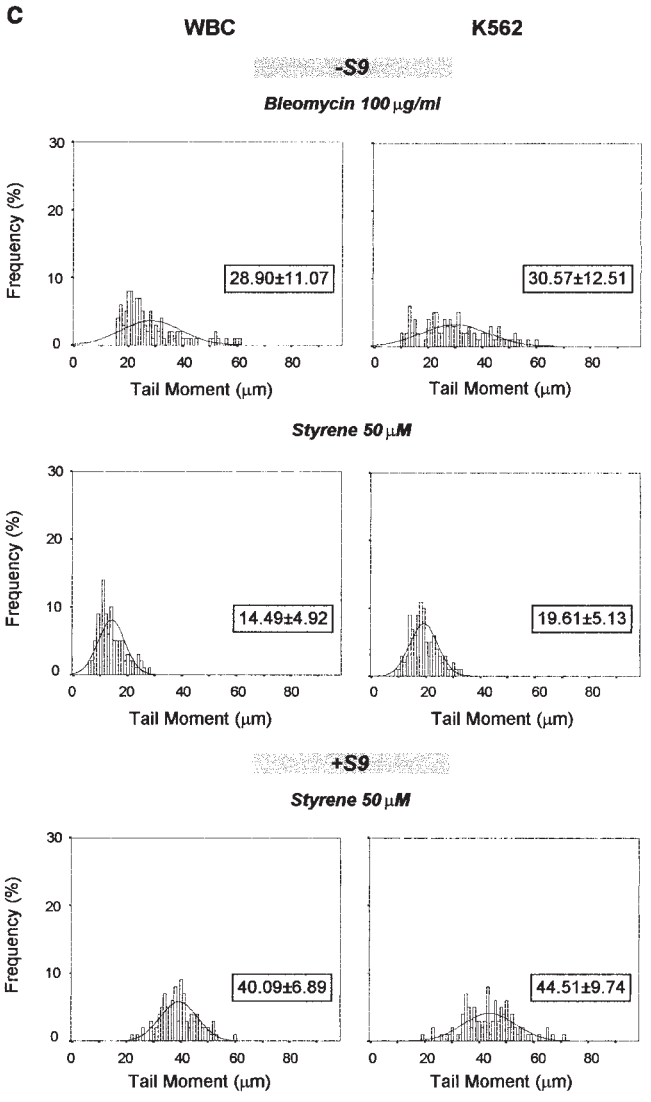


**Figure 2** *Continued.*

resistance/sensitivity to xenobiotics, and phenotypic differences related to cell cycle steps. Cell cycle differences, which are critical to cell survival, do not appear to influence tail moment values in radiation-induced DNA damage.[62] The same authors[64] established that drugs such as etoposide, selectively acting in proliferating cells, showed different tail moment values in relation to the cell cycle differences. Paclitaxel, an inhibitor of tubuline depolymerization, induced single strand breaks on proliferating lymphocytes but not in resting cells:[56] DNA damage could be linked to a direct DNA damaging effect on replicating cells but also to a high frequency of cells in the S-phase cycle. In another study,[65,66] untreated exponentially growing cells in $G_1$ phase showed a lower tail moment than S and $G_2$/M cells. The same cell cycle dependence was evidenced in cells treated with low doses of hydrogen peroxide, while, at the highest doses, all cells showed a similar level of damage.[66] Melphalan is not cell cycle specific and may act on cells at any stage of the cycle, thus suggesting the probable presence of genetically determined melphalan-resistant subpopulations. However, the analysis of a cell population, with prolonged survival, that is able to renew itself and provide an adequate model for hematoprotection studies,[13] could provide additional results to those observed for WBC and also a better comparison with K562 cells (ie cell cycle with a similar S/$G_2$/M cumulative phase percent). Furthermore, the influence of cell cycle status on melphalan genotoxicity and DNA protection by amifostine might be clarified.[66]

When the aim is to cause the tumoral cells to became sterile, the critical factor is not whether they all resist an appropriate level of DNA damage but rather if some do escape sufficient damage. Our data (Table 3) show that DNA damage assessment proved inadequate in predicting survival following melphalan treatment. This may depend on the doses used, which were selected for their low cytotoxicity. However, the significant proportion of undamaged cells which remains even at doses that determine a high mean DNA damage is more likely itself to repopulate the tumor. Furthermore, the normal level of apoptosis, detected by TUNEL assay and the lack of 'ghost cells' in the Comet assay can confirm the specificity of melphalan-induced genotoxicity in treated cells.

Our results appear to be in line with previous reports show-

**Table 3** Survival percentage in WBC and K562 cells with (+) or without (–) amifostine pre-treatment, in the absence or presence of S9 mix (±S9), 24 h after melphalan treatment

| *Dose (μg/ml)* | *Amifostine* | *WBC* | | *K562* | |
|---|---|---|---|---|---|
| | | *–S9 (cell %)* | *+S9 (cell %)* | *–S9 (cell %)* | *+S9 (cell %)* |
| 0 | – | 85.3 ± 4.1 | 90.2 ± 1.9 | 92.0 ± 2.2 | 87.2 ± 3.2 |
| | + | 89.8 ± 3.3 | 91.6 ± 3.4 | 92.3 ± 4.1 | 88.4 ± 2.9 |
| 0.25 | – | 92.2 ± 3.5 | 84.9 ± 2.7 | 93.5 ± 3.3 | 78.9 ± 3.5 |
| | + | 86.5 ± 2.8 | 85.2 ± 3.9 | 92.7 ± 3.6 | 77.1 ± 2.5 |
| 0.50 | – | 82.0 ± 4.3 | 79.8 ± 3.2 | 79.6 ± 3.1 | 77.2 ± 2.7 |
| | + | 84.7 ± 3.0 | 80.7 ± 2.8 | 90.7 ± 2.8 | 79.3 ± 3.6 |
| 1.00 | – | 78.3 ± 1.8 | 74.7 ± 4.2 | 86.1 ± 4.9 | 80.4 ± 3.3 |
| | + | 86.4 ± 3.7 | 90.2 ± 3.5 | 93.0 ± 2.6 | 76.7 ± 2.8 |
| 2.50 | – | 80.6 ± 3.1 | 74.5 ± 3.5 | 92.6 ± 4.0 | 75.9 ± 3.3 |
| | + | 90.1 ± 2.2 | 85.9 ± 2.9 | 94.9 ± 2.3 | 78.3 ± 3.7 |
| 5.00 | – | 73.7 ± 3.3 | 82.1 ± 3.2 | 87.2 ± 5.1 | 80.5 ± 2.9 |
| | + | 81.9 ± 1.2 | 77.0 ± 3.8 | 92.9 ± 3.3 | 76.7 ± 3.6 |
| 10.00 | – | 79.7 ± 3.6 | 73.2 ± 2.1 | 80.8 ± 3.2 | 74.8 ± 2.9 |
| | + | 86.3 ± 3.1 | 85.6 ± 3.4 | 91.1 ± 5.3 | 72.8 ± 3.3 |

ing hematoprotection in normal and enhanced drug-induced toxicity in leukemic cells by amifostine. In particular, the dual effect of WR-2721 protection of normal WBC coupled with amifostine-induced sensitization of the acute myelogenous leukemia cell line K562 towards melphalan can suggest its potential application for removing contaminating tumoral cells in bone marrow purging.[52]

In conclusion, amifostine selective protection against melphalan-induced DNA damage in normal and tumoral cells was stated by using the Comet assay. The relevance of the protective action exerted by amifostine on normal hematopoietic cells should be tested in appropriate clinical settings, eg, patients undergoing high-dose chemotherapy who are exposed to a significant chemotherapy-related toxicity on normal hematopoietic cells. Such toxicity together with the associated genetic damage might in turn represent the first hit triggering secondary neoplastic events.

The selective amifostine-mediated protection in normal blood cells from melphalan genotoxicity and the contemporary improvement of drug action on K562 cells confirm[52] the potential application of this aminothiol also for bone marrow purging.

While many methods are population-based assays which assume that all cells within a population respond in a similar manner to a DNA-damaging drug, the Comet assay confirms that it provides adequate resolution in detecting the presence of cell subpopulations with different sensitivity. Specific information regarding direct genotoxicity of drugs and DNA repair can be obtained in cells which are not in apoptosis and also by modifying the assay.[56,57,67,68] The findings indicate the suitability of this technique as a primary screening method for *in vitro* and *in vivo* studies on drug–DNA interactions and their modulations by endogenous/exogenous factors.

## Acknowledgements

This work was supported in part by grants from 'Ministero dell'Università e della Ricerca Scientifica e Tecnologica' (MURST – 40% & 60%), and 'Associazione Italiana per la Ricerca sul Cancro' (AIRC, Milano). ER is a recipient of an AIRC fellowship.

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