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Original Paper

Amifostine Protects Normal Tissues From Paclitaxel Toxicity While Cytotoxicity Against Tumour Cells is Maintained

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The objectives of this study were to evaluate the protective effects of amifostine against paclitaxel-induced toxicity to normal and malignant human tissues. Haematopoietic progenitor colony assays were used to establish the number of CFU-GEMM and BFU-E colonies after incubation with WR-1065 alone, Amifostine alone, paclitaxel (2.5 or 5 μM) +/- WR-1065 or amifostine. MTT and alkaline elution assays evaluated the *in vitro* growth inhibitory and DNA damaging effects, respectively, of paclitaxel with or without amifostine against normal human fibroblasts and human non-small cell lung cancer (NSCLC) cells. This combination was also evaluated *in vivo* using severe combined immune deficient (scid) mouse models of early (non-palpable tumours) and advanced (palpable tumours) human ovarian cancer. Human 2780 ovarian cancer cells were inoculated subcutaneously while paclitaxel and amifostine were administered intraperitoneally. A brief exposure (15 min) to amifostine not only protected human haematopoietic progenitor colonies from paclitaxel toxicity, but stimulated the growth of CFU-GEMM and BFU-E beyond control values. Amifostine protected normal human lung fibroblasts from paclitaxel-induced cytotoxicity and DNA single-strand breaks. However, paclitaxel cytotoxicity and DNA single-strand breaks were actually enhanced by pretreatment with amifostine in the NSCLC model. Importantly, amifostine did not interfere with paclitaxel antitumour activity even with prolonged exposure (24.5 h) of the lung cancer cells to high concentrations (1.2 mM) *in vitro* or following five repetitive high doses (200 mg/kg) given to scid mice with human ovarian cancer xenografts. Indeed, under certain circumstances, amifostine resulted in sensitisation of tumour cells to paclitaxel. Our results confirm previous reports of the ability of amifostine to protect normal tissues from the toxic effects of chemotherapy drugs and now extend these observations to paclitaxel. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

PACLITAXEL HAS broad antitumour activity in patients. Given as a single agent, an overall response rate of 56% has been noted in patients with metastatic breast cancer [2] and a nearly 25% response rate in patients with non-small cell lung cancer (NSCLC) [10]. These results have stimulated studies of paclitaxel in combination with doxorubicin in breast cancer [22] and cisplatin in NSCLC [10]. In a recent report by McGuire and associates, the combination of paclitaxel plus cisplatin produced superior progression-free and overall survival compared to the combination of cyclophosphamide plus cisplatin in patients with stage III and IV ovarian cancer [16].

Amifostine (WR-2721, Ethyol) is a cysteamine analogue initially developed by the United States Army as a radioprotective agent [3]. Amifostine is selectively dephosphorylated in normal tissues by membrane-bound alkaline phosphatase

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to a cytoprotective free thiol, WR-1065 [3]. Amifostine selectively protects against a spectrum of haematological and non-haematological toxicities induced by chemotherapy and radiation therapy without reducing antitumour activity from the same modalities [3, 4]. In a randomised comparison of cyclophosphamide and cisplatin with and without amifostine in advanced ovarian cancer patients, the amifostine treated group experienced significantly less haematological, renal neurotoxicity and less ototoxicity, but there was no difference in objective tumour response or survival [11]. In another randomised clinical trial, patients who received amifostine in conjunction with radiotherapy for advanced rectal cancer experienced fewer late radiation toxicities but similar response rates and survival compared to patients receiving radiotherapy alone [15]. *In vitro* investigations have shown that amifostine protects normal bone marrow progenitors from the cytotoxic effects of alkylating agents such as cyclophosphamide and its metabolites [5, 21].

The objectives of our studies were to evaluate the protective effects of amifostine against paclitaxel-induced toxicity in normal and malignant tissues. The normal tissues evaluated included normal human bone marrow and normal human lung fibroblasts. We were also interested in confirming that amifostine preserved paclitaxel cytotoxicity against tumour cells. For this purpose, we used an *in vitro* human non-small cell lung cancer model, as well as *in vivo*, minimal disease and advanced disease models of human ovarian cancer in the severe combined immune deficient (scid) mouse.

MATERIALS AND METHODS

Materials

[Methyl-3 H]thymidine (specific radioactivity 60 Ci/mmol) was purchased from Morvek Biochemicals Inc., Brea, California, U.S.A. MTT, DNase-free RNase A and proteinase K were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Amifostine was obtained from US Bioscience, Inc., West Conshohocken, Pennsylvania, U.S.A. PBS solution consisted of 2.7 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM $MgCl_2$, 138 mM NaCl and 8.1 mM Na_2HPO_4 at a final pH of 7.4. Tissue culture medium and serum were obtained from Gibco BRL Inc., Gaithersburg, Maryland, U.S.A.

Cell lines

Human diploid lung fibroblasts (MRC-5) and A427 human NSCLC cells were purchased from the American Type Culture Collection, Rockville, Maryland, U.S.A. Human ovarian carcinoma cells (2780) were grown in RPMI 1640 media (Sigma, St. Louis, Missouri, U.S.A.) supplemented with 10% fetal bovine serum (Gibco, Grand Island, New York, U.S.A.), 1% L-glutamine (Fisher, Phoenix, Arizona, U.S.A.) and 1% penicillin streptomycin (Sigma) and maintained in humidified 5% CO_2–95% air atmosphere at 37°C. Cell lines were grown to 95–100% confluence. Trypsin (1 ml/5 ml of Hank's balanced salt solution, HBSS) was added to detach adherent cells. Cells for scid mouse inoculation (90–100% viability) were counted and resuspended in a volume of 10×10^6 cells/200 µl of sterile saline.

Cell viability assay

Human lung fibroblasts and non-small cell lung cancer cells were grown as monolayers in 96-well microtitre plates. The cells were pretreated with either 0 or 1.2 mM amifostine for 30 min, then paclitaxel (0.05 µM) was added. The cells were then incubated with both drugs for an additional 24 h Cell viability was determined using the MTT colorimetric method as previously described [6].

Scid mouse colony and procedures

Female scid mice 7–9 weeks of age (Balb/cByJSmn-scid/J) were housed in microisolator cages (Allentown Caging Equipment Company, Allentown, New Jersey, U.S.A.) under specific pathogen-free conditions. The mice were fed LM45 5% fat, irradiated pellets (Tekland Premier, Madison, Wisconsin, U.S.A.) and autoclaved water. Every 3 months the mice were screened for mycoplasma, mouse hepatitis virus and Sendai virus. Only mice with ≤ 20 µg/ml immunoglobulin were used for the experiments. Mice were weighed once weekly. Tumour cells (10×10^6 in 200 µl) were injected subcutaneously (s.c.) in the right flank. Amifostine was administered by intraperitoneal injection (200 µl) in the left side of the abdomen, and paclitaxel (200 µl) was given 30 min later in the right side of the abdomen. For the minimal tumour burden ovarian cancer model, tumour cells were injected on day 0 and drugs were administered on days 1, 3, 5, 7 and 9. In the advanced ovarian cancer model, tumour cells were injected on day 17, and mice were evaluated for tumour growth by estimating tumour volume (cm^3) in accordance with the formula (tumour width^2 × tumour length/2) [23]. Mice with tumours measuring 0.2 cm × 0.2 cm were randomly assigned to treatment groups and received drug injections on days 0, 2, 4, 6 and 8. Mice with excessive tumour burden were sacrificed by cervical dislocation after anaesthesia with ketamine HCl 60–70 mg/kg (Aveco Co., Inc., Fort Dodge, Iowa, U.S.A.) and xylazine 5–7.5 mg/kg (Lloyd Laboratories, Shenandoah, Iowa, U.S.A.) using standard procedures approved by the University of Arizona Institutional Animal Care and Use Committee.

Human normal bone marrow progenitors

Bone marrow samples were obtained from normal bone marrow donors according to approved University of Arizona Human Subjects Committee procedures. Heparinised bone marrow samples were separated by Ficoll–Hypaque density centrifugation.

Haematopoietic progenitor colony assays

Mononuclear cell fractions (2×10^6 cells) were incubated with WR-1065 or amifostine (U.S. Bioscience) at concentrations of 0.01 or 0.1 mM, respectively for 15 min. The cells then were centrifuged and the cell pellets washed twice with IMDM (Iscove's Modified Dulbecco Medium) (Life Technologies, Grand Island, New York, U.S.A.), and resuspended at 2×10^5 cells/ml plus paclitaxel (2.5 or 5 µM) (MeadJohnson, Princeton, New Jersey, U.S.A.) for 60 min at 37°C.

Granulocyte, erythroid, macrophage and megakaryocyte colony forming units (CFU-GEMM) and erythroid burst forming units (BFU-E) were determined using a clonogenic assay [17]. The cells were washed twice with IMDM. One ml of mononuclear cells (2×10^5 cells) in IMDM plus 0.8% methylcellulose (Stem Cell Technologies, Vancouver, British Columbia), 30% fetal bovine serum (HyClone, Logan, UT), 3 U/ml erythropoietin (Amgen, Thousand Oaks, California, U.S.A.) and 5% phytohemagglutinin-stimulated leucocyte-conditioned media (Stem Cell Technologies) were plated in 35 ml petri dishes (VWR, Phoenix, Arizona, U.S.A.) and

incubated in humidified air with 5% CO_2 at 37°C to assess progenitor growth. Using an Olympus CK2 inverted microscope (Scientific Instruments, Tempe, Arizona, U.S.A.), CFU-GEMM and BFU-E colonies (containing 40 cells) and clusters (3–40 cells) were scored after 14 days.

Alkaline elution analysis

Alkaline elution analysis was performed as described by Kohn [12]. Cells ($0.5–1 \times 10^6$) were labelled with [^{3}H]thymidine (0.1 mCi/ml) for 20 h, washed twice to remove excess radioactivity, and then grown for 24 h in fresh medium. The cells were treated with paclitaxel (0.1 μM) with and without amifostine (0.4 mM) for the same time periods described for the cell viability assay. The cells were collected on polyvinyl filters, lysed with a solution of 2% SDS–25 mM EDTA and then digested with proteinase K at a final concentration of 0.5 mg/ml. Alkaline elution was carried out with a solution of 10% tetrapropylammonium hydroxide, 20 mM EDTA and 0.1% SDS at a pH of 12.1. Six fractions were collected at 3 h intervals with a total elution time of 18 h. Radioactivity was measured using a scintillation counter and the numbers of paclitaxel-induced DNA strand breaks were expressed as x-ray equivalents [12]. Retention of [^{3}H]DNA correlated with the dose (100–3000 rad) of x-irradiation administered to the cells ($r = 0.92$).

Chemotherapy drugs and diluents

Paclitaxel (18 or 27 mg/kg in sterile saline), amifostine (100 or 200 mg/kg in phosphate buffer solution) and diluents were freshly prepared. Amifostine and diluents were heated to mouse body temperature (approximately 35°C) prior to injection.

Statistical analysis

The growth of CFU-GEMM and BFU-E colonies were analysed by two-way analysis of variance. The outcome variable, cell count, was adjusted by dividing the actual cell count by the cell count with no pretreatment and no paclitaxel. Since the adjusted cell count was not normally distributed, the $\log_{10}$ transformation was used. The two factors were pretreatment drug (WR-1065 versus amifostine) and paclitaxel dose. The interaction term between pretreatment drug and paclitaxel dose was also tested. When the interaction term was significant at the 0.10 level of significance, two Student's *t*-tests were done at each paclitaxel dose: one *t*-test to determine the significance of WR-1065 versus no pretreatment, and the other to determine the significance of amifostine versus no pretreatment. The Bonferroni adjustment for multiple comparisons was used in calculating the *P* values. A proportional hazards regression survival analysis assessed the effect of amifostine and paclitaxel on time to death or tumour volume greater than 3 cm^3. The amifostine by paclitaxel interaction effect was also tested.

RESULTS

A 1 h exposure to paclitaxel alone decreased CFU-GEMM and BFU-E progenitor cell growth from normal human bone marrow cells to 72% (Figure 1(a)) and 63% (Figure 1(b)) of control, respectively. However, a 15 min pre-exposure to amifostine or WR-1065 before paclitaxel not only prevented the paclitaxel-induced inhibition, but resulted in significant stimulation of progenitor cell growth to greater than control for both CFU-GEMM (Figure 1(a)) and BFU-E (Figure 1(b)). Pretreatment with amifostine followed by paclitaxel at 2.5 μM stimulated CFU-GEMM colony growth by 70% over control ($P = 0.039$) and BFU-E colony growth by 56% over control ($P = 0.013$). Stimulation of progenitor growth was attenuated at the higher paclitaxel concentration (5 μM) for both amifostine and WR-1065. Treatment with 0.01 mM WR-1065 or 0.1 mM amifostine alone increased CFU-GEMM growth by 1.59-fold and 1.56-fold of control levels, respectively and BFU-E growth by 2.13-fold and 1.73-fold of control, respectively.

Using the MTT *in vitro* assay of cell growth, paclitaxel alone (24 h exposure) decreased the growth of MRC-5 human lung fibroblasts to 60% of control (Figure 2). However, 30 min pre-exposure to amifostine followed by 24 h exposure to both amifostine and paclitaxel resulted in significantly less growth inhibition ($P < 0.01$) compared to paclitaxel alone (Figure 2). Amifostine alone was not cytotoxic. Paclitaxel alone also decreased the growth of A427 non-small cell lung cancer cells (by 20%), although to a lesser degree than seen with MRC-5 cells (Figure 2). However, amifostine exposure of the lung cancer cells did not result in protection from paclitaxel cytotoxicity, with A427 viability significantly ($P < 0.01$) decreased for the combination of amifostine and paclitaxel compared to paclitaxel alone (Figure 2).

Consistent with the viability assays, alkaline elution analysis showed significantly ($P = 0.023$) fewer DNA single-strand breaks in MRC-5 cells following 30 min pre-exposure to amifostine than 24 h of exposure to both amifostine and

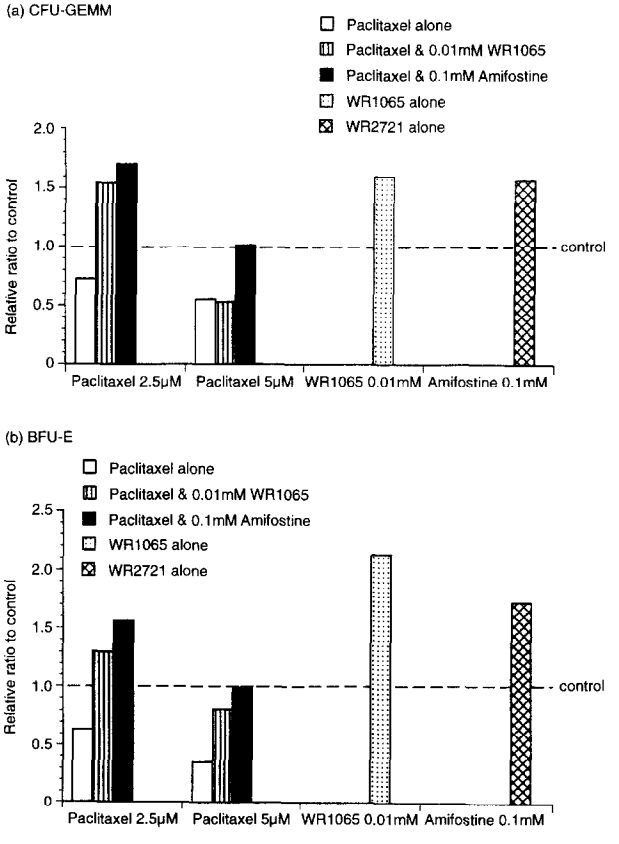

Figure 1. CFU-GEMM (a) and BFU-E (b) progenitor growth from normal human bone marrow specimens following exposure to paclitaxel and amifostine. Data are shown as a relative ratio of control (no drug exposure) growth (shown as a broken line).

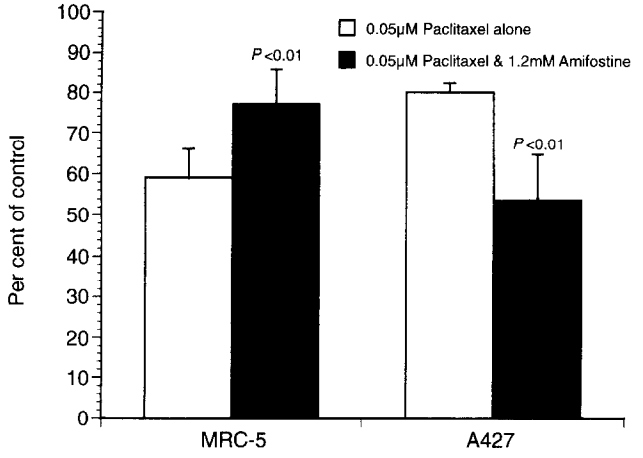

Figure 2. MTT assay results for paclitaxel alone and paclitaxel plus amifostine. The data are shown as percentage of control (no drug) growth. MRC-5: normal lung fibroblasts; A427: non-small cell lung cancer cells. Amifostine alone was not cytotoxic. The data are expressed as the means of 12 determinations ± S.E.M. The *P* values were obtained from a comparison of paclitaxel + amifostine versus paclitaxel alone.

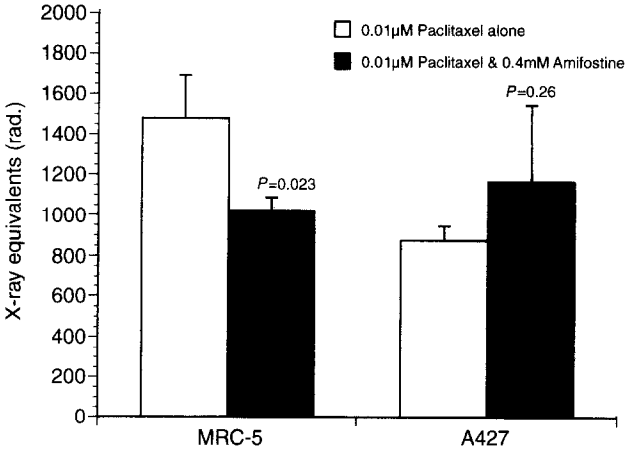

Figure 3. Alkaline elution assay results for paclitaxel alone and paclitaxel plus amifostine. The results are expressed in x-ray equivalents, indicative of DNA single-strand breaks. MRC-5: normal lung fibroblasts; A427: non-small cell lung cancer cells. The data are expressed as the means of six determinations ± S.E.M.

paclitaxel compared to paclitaxel alone (Figure 3). However, similar analysis showed no protection from paclitaxel-induced DNA single-strand breaks in A427 non-small cell lung cancer cells (Figure 3).

In a scid mouse model of minimal tumour burden human ovarian cancer, mice were injected with tumour cells SC on day 0 and received drug treatment i.p. on days 1, 3, 5, 7 and 9. Mice were treated with paclitaxel (0 or 27 mg/kg) in combination with amifostine (0, 100 or 200 mg/kg) (6 mice per treatment group, 6 treatment groups). The combinations of paclitaxel at 27 mg/kg plus amifostine at either 100 or 200 mg/kg resulted in 100% survival up to day 76 when the experiment was terminated (data not shown). Paclitaxel alone at 27 mg/kg resulted in 83% survival at 76 days. Mice receiving drug diluents only or amifostine alone at 100 or 200 mg/kg on day 76 had survival rates of 17%, 17% and 33%, respectively. Thus, in this model of minimal tumour burden, 5 repetitive doses of amifostine as high as 200 mg/kg did not attenuate the antitumour effect of paclitaxel.

For the advanced ovarian cancer scid mouse model, drug treatment was delayed until SC tumours were palpable (0.2×0.2 cm). Figure 4 shows Kaplan–Meier survival curves for groups of scid mice in the advanced ovarian cancer model treated with paclitaxel (27 mg/kg) in combination with amifostine (100 or 200 mg/kg every other day for 5 doses). Control mice received PBS, the drug diluent for paclitaxel and amifostine. Median time to death or tumour volume $> 3\,cm^3$ for mice receiving paclitaxel alone, paclitaxel plus amifostine at 100 mg/kg or 200 mg/kg were 57, 64 and > 89 days, respectively. Compared to control or amifostine alone, survival was improved for mice treated with paclitaxel with and without amifostine and the paclitaxel effect was significant ($P = 0.0001$). While the median time to death for mice treated with 200 mg/kg amifostine plus paclitaxel was the longest (> 89 days), there was no paclitaxel–amifostine interaction effect ($P = 0.5135$), indicating that the paclitaxel effect was the same regardless of the presence or absence of amifostine.

DISCUSSION

Amifostine protected normal human haematopoietic progenitor cells and normal human lung fibroblasts from the cytotoxic effects of paclitaxel, yet paclitaxel antitumour activity was preserved *in vitro* against human non-small cell lung cancer cells and *in vivo* using minimal and extensive tumour burden models of human ovarian cancer. Of significance in the observed lack of tumour protection was the use of protracted *in vitro* exposure of the lung cancer cells (24.5 h) to high concentrations of amifostine (1.2 mM) and the repetitive high-dose regimen (amifostine 200 mg/kg) used in the mouse models with low and high tumour burden. Additionally, brief amifostine exposure resulted in significant stimulation of both CFU-GEMM and BFU-E and abrogated the expected *in vitro* cytotoxic effects of paclitaxel on normal human bone marrow. This marrow stimulatory property of amifostine has been described in both human normal and myelodysplastic marrows [13, 14].

Prior studies of amifostine cytoprotection of normal or tumour cells utilised a brief pretreatment period with amifostine followed by a washout, prior to treatment with the cytotoxic agent [3]. This approach was intended to mimic the rapid clearance of amifostine from the plasma as described in its pharmacokinetic profile [19]. The rapid removal of extracellular amifostine or WR-1065 by systemic clearance *in vivo* or wash out *in vitro* was also intended to avoid any potential intracellular drug–drug interaction and any theoretical potential for extracellular drug–drug interaction and any theoretical potential for extracellular interaction of the cytotoxic agent and the thiol [3]. This sequential method of thiol–cytotoxic exposure has been extensively explored *in vivo* and *in vitro*. Two consistent observations emerge from this body of literature: protection of normal tissues and lack of protection of diverse human and murine tumours [3]. Our experiments placed more stringent conditions on the phenomenon of selective cytoprotection mandated by the schedule of paclitaxel use. Laboratory experiments with paclitaxel describe increased cytotoxic effect related to protracted exposure periods for 24–72 h compared to shorter periods of drug exposure [1]. Accordingly, we conducted a comparative

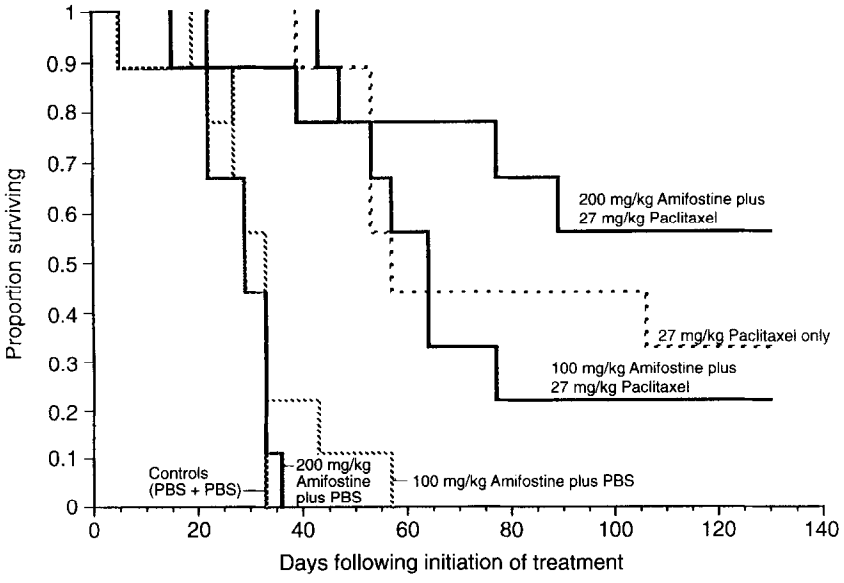

Figure 4. Survival of scid mice inoculated with human ovarian tumour cells and treated with paclitaxel and amifostine.

study of two cell types derived from human lung, the normal lung fibroblast (MRC-5) and the non-small cell lung cancer cell line (A427). Both were exposed to paclitaxel for 24 h ± pretreatment with amifostine for 30 min, followed by concurrent exposure to amifostine and paclitaxel for the entire treatment period. The results of cytotoxicity assessment (MTT assay) and biochemical measure of paclitaxel effect (DNA single-strand breaks) showed consistent selective cytoprotection of non-neoplastic cells. Amifostine significantly attenuated the effect of paclitaxel on the normal lung cells. In contrast, similar pretreatment and protracted concurrent exposure of the non-small cell lung cancer cells significantly enhanced tumour cytotoxicity. At the concentrations employed, amifostine alone was not cytotoxic. Similar tumour sensitisation has been noted for alkylating agents, doxorubicin, organoplatinums and photodynamic therapy [3]. The biochemical basis for this tumour sensitization remains to be defined. Repetitive doses of amifostine over 9 days did not attenuate the therapeutic effect of paclitaxel on small- or large-volume ovarian cancer. Indeed, tumour growth delay, as assessed by time to reach 3 cm^3 or median survival time, was increased by combined treatment with 200 mg/kg amifostine and paclitaxel. This sensitisation effect on ovarian cancer is consistent with similar observations by Treskes and associates who showed that amifostine pretreatment enhanced the carboplatin effect on human ovarian cancer xenografts in nude mice [25].

The mechanism of action most commonly attributed to paclitaxel is abnormal stabilisation of microtubules resulting in disordered cell division [18]. However, paclitaxel has also recently been shown to have genetic toxicity and potential for DNA damage in mouse bone marrow micronucleus assays [24]. Of note, amifostine appears to have a number of DNA protective effects including inhibition of platinum–DNA adduct formation [26] and decreased nitrogen mustard-induced DNA–DNA interstrand cross-links in normal bone marrow but not in tumour tissue [7]. The symmetrical disulphide of WR-1065 (WR-33278) stimulates topoisomerase I unwinding of negatively supercoiled DNA, possibly conferring DNA protection [9]. Also, WR-33278 has polyamine-like effects that could stabilise DNA [20]. The results of our alkaline elution studies suggest that paclitaxel also induces DNA single-strand breaks *in vitro* in human non-malignant and malignant lung cell models. We found that pretreatment with amifostine decreased paclitaxel-induced DNA single-strand breaks in normal lung fibroblasts without significantly affecting the formation of single-strand breaks in non-small cell lung cancer cells.

In summary, our results confirm the broad-spectrum normal tissue protective effects of amifostine and confirm the lack of protection of neoplastic cells. It is also reasonable to suggest, based on our data, that amifostine can sensitise tumour cells to cytotoxicity by paclitaxel. Thus, amifostine may improve the therapeutic index for paclitaxel by decreasing toxicity to normal tissues and enhancing tumour-specific cytotoxicity. These results have substantial clinical relevance for patients with breast or ovarian cancer where clinical studies of dose-intensive paclitaxel therapy are in progress. The availability of a broad-spectrum cytoprotective agent with the ability to the decrease toxicity of paclitaxel to normal tissues and preserve, if not enhance, cytotoxicity to tumour cells could potentially improve the therapeutic index for paclitaxel and result in improved clinical outcome. In an ongoing Phase I dose escalation trial in patients with advanced malignancies, the highest dose of paclitaxel (3 h infusion) administered thus far with amifostine pretreatment has been 270 mg/m^2; dose-limiting neuropathy or myalgia/arthralgia have not been reached [8]. These data suggest that further clinical trials of the combination of amifostine and paclitaxel are warranted.

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