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The Preclinical Basis for Broad-spectrum Selective Cytoprotection of Normal Tissues From Cytotoxic Therapies by Amifostine (Ethyol®)

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Administered prior to cytotoxic chemotherapy or radiation, the aminothiol amifostine provides broad-spectrum cytoprotection of various normal tissues without attenuating antitumour response. The basis for the selectivity of action resides in the anabolism of amifostine at the normal tissue site by membrane-bound alkaline phosphatase. Dephosphorylation to the free thiol, WR-1065, is followed by rapid uptake into normal tissues by a carrier mediated, facilitated diffusion process; in contrast, uptake into tumour tissue is slow to negligible. Preclinical studies have shown that pretreatment with amifostine provides protection of normal tissues from the cytotoxic effects of alkylating agents, organoplatinums, anthracyclines, taxanes and radiation. Normal tissues protected include bone marrow, kidney, neural tissues, the heart, intestinal crypt cells and pulmonary tissues. Additionally, the mutagenic and carcinogenic effects of these modalities are also attenuated. With respect to bone marrow, preclinical studies have shown significant protection of progenitor cells that give rise to the red and white cells and platelets. Comparative *in vitro* and *in vivo* studies using murine and human tumour xenografts show no decrease of antitumour effects of these same therapies despite the protection of normal organs. The unique preclinical profile of amifostine serves as a model for the clinical development programme for this important new broad-spectrum cytoprotective agent.

Key words: cytoprotection, amifostine, chemotherapy, radiotherapy
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INTRODUCTION

THE TWO major obstacles to effective cancer therapy are drug resistance and toxicities to normal organs that prevent the use of optimal doses and schedules. For certain disease states, drug resistance may be overcome by dose-intensive therapy if the organ displaying the dose-limiting toxicity can be protected or rescued. This is reflected by the beneficial outcome of the extreme application of the concept of dose-intensive therapy followed by rescue with autologous marrow transplantation [1–4]. Other strategies that allow the delivery of moderately toxic doses of drugs include the use of bone marrow-stimulatory cytokines. The major limitation of these strategies is that marrow transplantation can usually be used only once, and the stimulatory cytokines have diminished efficacy following multiple cycles of therapy, reflecting a progressive depletion of the stem-cell pool by the impact of cumulative toxicity [5]. More limited strategies that enable the delivery of higher doses of chemotherapy are drug specific (e.g. mesna with ifosfamide or leucovorin rescue following high-dose methotrexate). Based on these examples, it becomes apparent that a broad-spectrum selective cytoprotective agent that improves patient tolerance to optimal doses and schedules, allows the delivery of higher cumulative doses of chemotherapy and improves quality of life would be a very useful adjunct in cancer medicine. A broad-spectrum selective cytoprotective agent can be defined as one that protects multiple normal organs from the toxicity of cytotoxic antineoplastic therapies without protecting the tumour.

The concept of cytoprotection antedates the modern era of cytotoxic cancer therapy. The original report by Patt and associates in 1949 showed that pretreatment with the sulphydryl amino acid cysteine could protect rats from lethal radiation [6]. These and similar observations stimulated extensive research on the role of thiol compounds as cytoprotectors. Amifostine (WR [Walter Reed]-2721, Ethyol®, for structure, see Bukowski, Figure 1, page S3), an analogue of cysteamine, is a phosphorylated aminothiol prodrug that is dephosphorylated at the tissue site by membrane-bound alkaline phosphatase to its active metabolite, the free thiol, WR-1065. WR-1065 is the form of the drug that is taken up into cells. Amifostine was originally developed during the height of the cold war by the Walter Reed Army Institute of Research as part of a United States Army classified research project to identify an agent that could be used to protect military personnel in the event of nuclear warfare. Of 4400 chemicals screened for this purpose, amifostine was selected as having the most effective radioprotective properties and a relative safety profile. Military experiments showed that amifostine had the ability to protect mice, dogs and monkeys from lethal doses of whole-body radiation [7]. Yuhas and coworkers subsequently showed that pretreatment with amifostine effectively protects normal tissues from the toxicities of therapeutic radiation without protecting

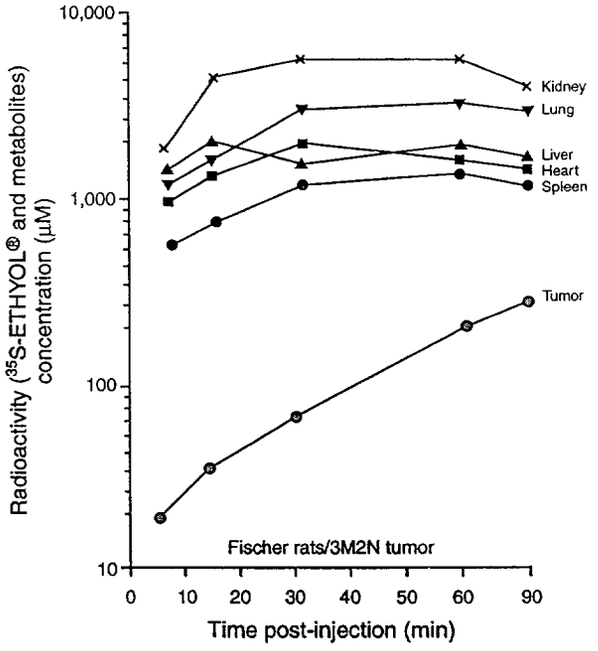

Figure 1. Concentration of ^{35}S-labelled amifostine and its metabolites in normal tissues, and tumour of Fischer 344 rats bearing 3M2N squamous cell carcinoma following a single intraperitoneal injection of amifostine at 200 mg/kg (1180 mg/m^2). Reproduced by permission of the American Association of Cancer Research, Inc., from Yukas JM, *Cancer Res* 1980, Vol. 40, pp. 1519–1524.

tumours [8]. Interest in the drug's potential value in cancer medicine was further increased when it was recognised that amifostine also protected normal tissues but not tumours against the toxic effects of alkylating agents and cisplatin [9–14]. Further laboratory studies have shown that amifostine possesses broad-spectrum selective cytoprotective properties and can protect against the toxicity of other classes of anticancer agents, such as anthracyclines [15, 16] and taxanes [17]. To date, it is the broad-spectrum cytoprotective agent with the largest preclinical and clinical database.

The basis for the selective cytoprotection of normal tissue by amifostine is explained by its unique systemic and tissue-distribution pharmacokinetics. Following drug administration, the half-lives of both the distribution and elimination phases in humans are extremely rapid ($T_{1/2\alpha} < 1$ min; $T_{1/2\beta} = 8.8$ min). Approximately 90% of the drug is cleared from the plasma within 6 min; thus the amount of the prodrug that is bioconverted to the free thiol in the systemic circulation relative to normal tissue is small [18–21]. Similar rapid clearance occurs in beagle dogs [22], rhesus monkeys [23] and mice [19]. The metabolites of amifostine include the free thiol, WR-1065; the symmetric disulphide, WR-33278; cysteamine and mixed disulphides containing L-cysteine; L-glutathione; and thiol-containing proteins [19]. Whereas the free thiol, WR-1065, is the major metabolite responsible for cytoprotection, the symmetric disulphide, WR-33278 [24] and cysteamine [25] have been shown to have cytoprotective properties, although to a lesser degree than WR-1065.

Generation of the free thiol, WR-1065, occurs primarily at the tissue site [21]. Romanul and Bannister have shown that normal tissues, especially at the capillary level, have a high concentration of alkaline phosphatase [26], the enzyme responsible for dephosphorylating amifostine to the free thiol [27–29]. The localisation of alkaline phosphatase in the endothelium of capillaries [26] increases the conversion of amifostine to the free thiol for rapid local uptake in normal tissues. Additionally, recent studies have shown that the specific activity of membrane-bound alkaline phosphatase is 275-fold higher in normal lung cells compared with the activity in the cell membranes of human nonsmall-cell lung cancer cells [30]. Other local factors favour uptake in normal tissues. The neutral pH environment of normal tissues, relative to the acidic pH found in many tumours, favours the preferential enzymatic action of alkaline phosphatase in normal tissues [31]. This pH effect is also supported by data demonstrating that the rate constant for the uptake of the free thiol across cell membranes is markedly accelerated with small differences in pH, favouring the pH of 7.4 [26], which is found in normal tissues, versus the relative acidity noted in some tumours. Amifostine is not dephosphorylated by acid phosphatase [32].

These biochemical data are consistent with animal studies that have delineated the tissue distribution of amifostine and its metabolites. Using whole-animal autoradiography, Utley and coworkers have shown high uptake in various normal organs and absent uptake in EMT-6 tumour over a 60-min period [33]. Recent studies have shown that the organ: blood ratio for WR-1065 at 5–10 min following the intraperitoneal (i.p.) administration of 365 mg/kg (1095 mg/m^2) amifostine is as high as 6, consistent with tissue site anabolism and uptake [21]. *In vivo* and *in vitro* analyses of the uptake kinetics of radiolabelled amifostine in normal versus tumour tissues in mice, rats and rabbits have demonstrated that normal tissues concentrate the radiolabelled drug at a very fast rate [34, 35]. In contrast, uptake of the drug and its metabolites into tumour tissue is slow or negligible, even after multiple doses [34–36]. Figure 1 shows the selective uptake of ^{35}S-labelled amifostine and its metabolites by normal and tumour tissues in Fischer 344 rats bearing 3M2N squamous cell carcinoma. Within 10 min following an i.p. injection of 200 mg/kg (1180 mg/m^2) amifostine, high steady-state concentrations are achieved in normal tissues [35]. At 10–30 min after drug administration, the concentration of radiolabelled amifostine in normal tissues (i.e. kidney, lung, liver, heart and spleen) is as much as 50- to 100-fold greater than in tumour; and at least a 10-fold difference between normal tissues and tumour is maintained for at least 60 min. For the 3M2N carcinoma, the uptake of radiolabelled amifostine into the tumour increases gradually, approaching but not exceeding the declining serum levels. A study in mice bearing the RIF-1 sarcoma also showed avid uptake of radiolabelled amifostine in normal tissues and virtually no drug uptake in the tumour (Figure 2) [34]. Consistent with this observation, the daily administration of amifostine to mice for 21 days showed no accumulation of drug in tumour in contrast with a steady accumulation of the drug and its metabolites in normal tissues [36].

Studies examining the characteristics for cellular transport involved in the differential uptake of WR-1065 provide evidence for a carrier-mediated, temperature-dependent, non-adenosine triphosphate-dependent (e.g. determined by the classic inhibitors, potassium cyanide, sodium azide and ouabain) mechanism with no sodium dependence. These data are consistent with a facilitated diffusion transport process [11, 30, 35]. The differences in membrane-bound alkaline phosphatase activity and transport rates between normal and neoplastic cells suggest that dephosphorylation and transport may be linked processes. A consequence of the selective uptake and accumulation of the free thiol in normal tissues is the creation of a

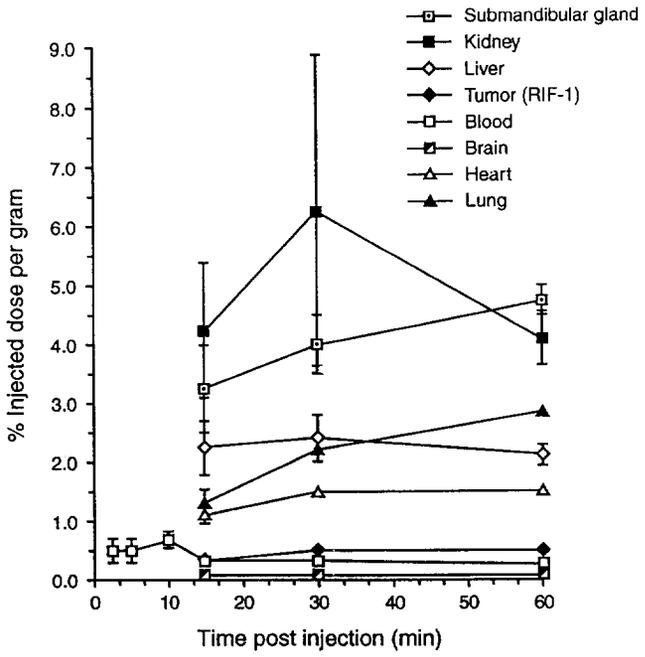

Figure 2. Concentration of ^{35}S-labelled amifostine and its metabolites in normal tissues and tumour of C3H mice bearing RIF-1 sarcoma following a single intraperitoneal injection of amifostine at 400 mg/kg (1200 mg/m^2). Reproduced by permission of Academic Press, Inc., from Rasey JS, *et al.*, *Radiat Res* 1985, Vol. 102, pp. 130–137.

temporary state of acquired resistance to the cytotoxic effects of cancer therapy in these tissues. In contrast, the low to absent concentration of the thiol in tumour tissues leaves them fully vulnerable to the cytotoxic effects of drugs or radiation.

Once inside normal cells, WR-1065 provides protection from cytotoxic therapies by several mechanisms. The free thiol can bind directly to, and thus detoxify, the active species of alkylating [37] or platinum [38, 39] agents. When amifostine or WR-1065 is administered prior to nitrogen mustard (HN_2) or cisplatin, the protective agent has been shown to reduce the formation of DNA–DNA interstrand crosslinks by the alkylating agent (Figure 3) [37] or platinum–DNA adducts (Figure 4a) [39]. It has also been shown that WR-1065 can also reverse performed platinum–DNA adducts when administered after cisplatin, albeit to a lesser extent compared with pretreatment with WR-1065 (Figure 4b) [39].

Another mechanism of cytoprotection involves the ability of the free thiol to act as a potent scavenger of oxygen free radicals, such as those derived from radiation therapy or from specific drugs—e.g. doxorubicin-derived superoxide anions, which have been implicated in the production of doxorubicin-induced cardiac toxicity [40]. In addition to the cytoprotective effects of WR-1065, two additional metabolites, cysteamine and the symmetric disulphide, WR-33278, have cytoprotective properties [25, 41]. The symmetric disulphide has structural similarities to the polyamine, spermine. WR-33278 binds more avidly to DNA than spermine does and enhances relaxation of DNA supercoils mediated by topoisomerase type I [42]. WR-33278 has also been shown to protect cells from radiation-induced cytotoxicity and mutations [24]. The attenuating effects of amifostine and its metabolites or alkylating agent/DNA interactions and free radical damage of DNA not only have implications regarding the acute and chronic manifestations of cytotoxic cancer therapy, but also affect the potential genotoxic effects of cytotoxic therapies manifested as secondary malignancies. In contrast with the cellular protection provided by pretreatment with amifostine or WR-1065, post-treatment

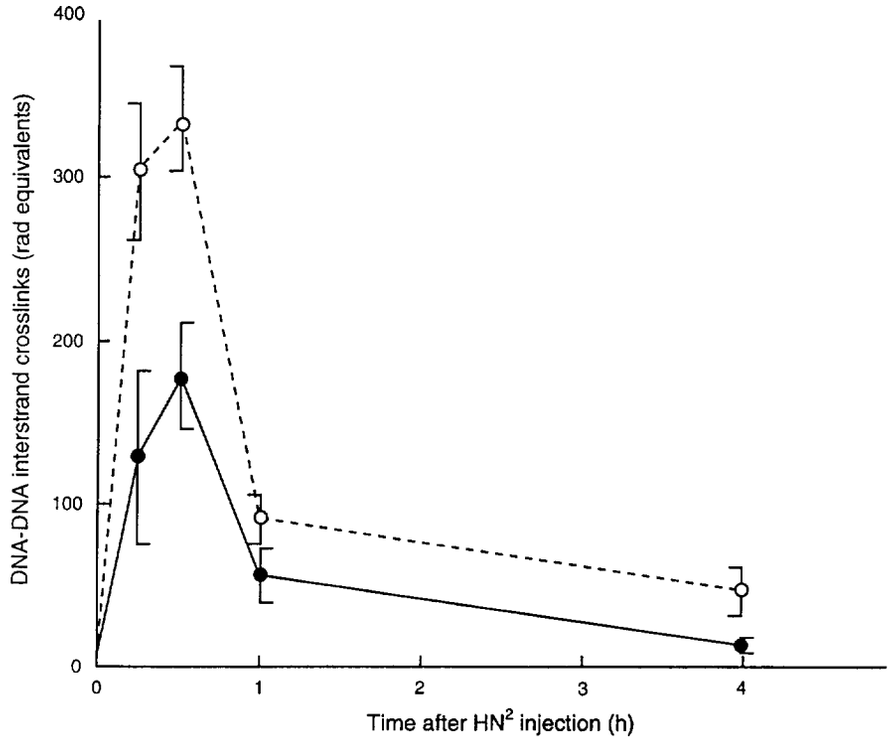

Figure 3. Time course of the formation and disappearance of DNA–DNA interstrand crosslinks in normal bone marrow cells of AKR mice treated intraperitoneally with amifostine (●) (15 mg/mouse), followed 15 min later by nitrogen mustard (HN_2) [0.1 mg/mouse] or by HN_2 (○) [0.1 mg/mouse] alone. Values are means ± SEM of three independent experiments. Reproduced by permission of the American Association of Cancer Research, Inc., from DeNeve WJ, *et al.*, *Cancer Res* 1988, Vol. 48, pp. 6002–6005.

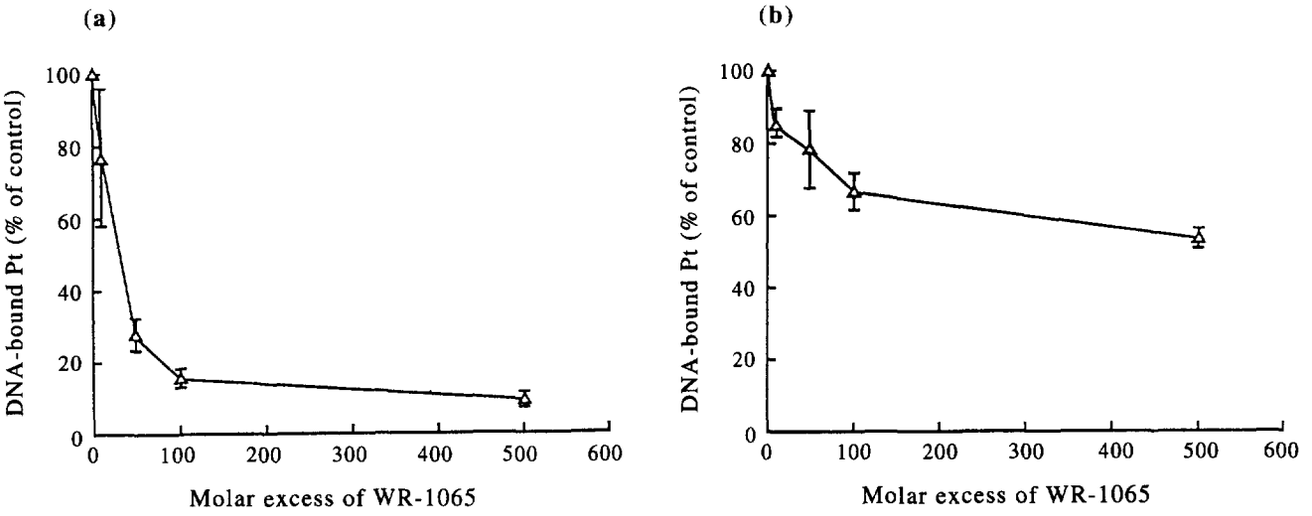

Figure 4. (a) Effect of pretreatment with molar excess of WR-1065 on the platination of salmon sperm DNA. (b) The reversal of DNA-bound platinum (salmon sperm DNA) by WR-1065 at several molar excess. Pt, platinum. Reproduced by permission of Elsevier Science Inc., from Treskes M, *et al.*, *Biochem Pharmacol* 1992, Vol. 43, pp. 1013–1019.

of irradiated cells with WR-1065 has been shown to attenuate markedly radiation-induced apoptosis. WR-1065 has also reduced apoptosis caused by several chemicals [43]. Possible mechanisms of this effect include the binding of WR-1065 to DNA and nuclear proteins, thereby altering the structure of the internucleosomal region of chromatin and rendering it less vulnerable to degradation.

Based on the uptake and tissue-retention characteristics described above, the laboratory and clinical protocols that describe amifostine cytoprotection have recommended administration of amifostine 5–30 min prior to the cytotoxic therapy. Whereas most studies have utilised only one dose of amifostine, expanding laboratory [44] and clinical experience [45–47] utilises multiple doses of amifostine to achieve maximal cytoprotection. The multiple-dose practice is based on considerations of the systemic and tissue-distribution pharmacokinetics of amifostine and its metabolites relative to the pharmacokinetics of certain anticancer drugs such as carboplatin. The laboratory and clinical experience, to date, with two or three doses of amifostine indicates that the dosing schedule is safe and does not interfere with antitumour activity. Other laboratory studies have shown that protracted concurrent exposure to amifostine or WR-1065 for 24–72 h and either cisplatin [48], 5-fluorouracil (5-FU) [48] or paclitaxel [49] does not affect antitumour activity.

As shown in Table 1, a broad spectrum of normal tissues has been protected by amifostine. This list is consistent with the tissue distribution of amifostine and its metabolites as described above. Amifostine-mediated protection of the marrow progenitor cells from the cytotoxic effects of drugs and radiation has been the most extensively studied system. Pretreatment with amifostine has shown significant protection of bone marrow toxicities induced by a broad range of antineoplastic agents, including cisplatin, carboplatin, cyclophosphamide, nitrogen mustard, melphalan, mitomycin-C, carmustine and 5-FU, as well as ionising radiation in both normal and tumour-bearing animals [10, 44, 50–53]. Detailed studies of the effect of amifostine on the cytotoxicity of various drugs to human marrow progenitor cells have also been conducted. Pretreatment with amifostine protected normal marrow progenitor cells (colony-forming units-granulocyte macrophage and -granulocyte, erythrocyte, macrophage, megakaryocyte and/or burst-forming units-erythrocyte) from the cytotoxic effects of carboplatin [54], various cyclophosphamide derivatives including 4-hydroperoxycyclophosphamide and mafosfamide [55–57], paclitaxel [17] and photodynamic therapy [58].

In initial studies using tumour-bearing animals, Yuhas [10] showed that the administration of amifostine 15 min before nitrogen mustard administration doubled its LD_{50} dose (e.g. death from a haematopoietic failure) without altering the effect of nitrogen mustard on line 1 lung carcinoma (Figure 5). Valeriote and Tolen [50] further explored the comparative effects of amifostine against the toxic effects of nitrogen mustard on AKR leukaemia and on colony-forming units-spleen (CFU-S) pluripotential stem cells in AKR mice. When mice were pretreated with amifostine prior to nitrogen mustard administration, a 2-log increase occurred in the survival of CFU-S compared with the cytotoxic effects of nitrogen mustard alone (Figure 6a). Paradoxically, pretreatment with amifostine resulted in a 3-log synergistic enhancement of the cytotoxic effects of nitrogen mustard on colony-forming units-leukaemia compared with the effects of nitrogen mustard alone (Figure 6b) [50]. These data indicate that amifostine pretreatment of tumour-bearing animals has the capacity to increase the therapeutic index (TI) of anticancer drugs.

Similar enhancement of the TI has been noted in other studies. Comparative toxicological and antitumour studies have been conducted in immunodeficient mice bearing human melanoma xenografts treated with melphalan with or without amifostine. Pretreatment of mice with amifostine provided significant protection of CFU-S pluripotential haematopoietic

Table 1. Normal tissue protection by amifostine (Ethyol®)

Protected		Not protected
Bone marrow	Oesophagus	Brain
Immune system	Kidney	Spinal cord
Skin	Liver	
Small intestine	Salivary gland	
Colon	Oral mucosa	
Lung	Testes	

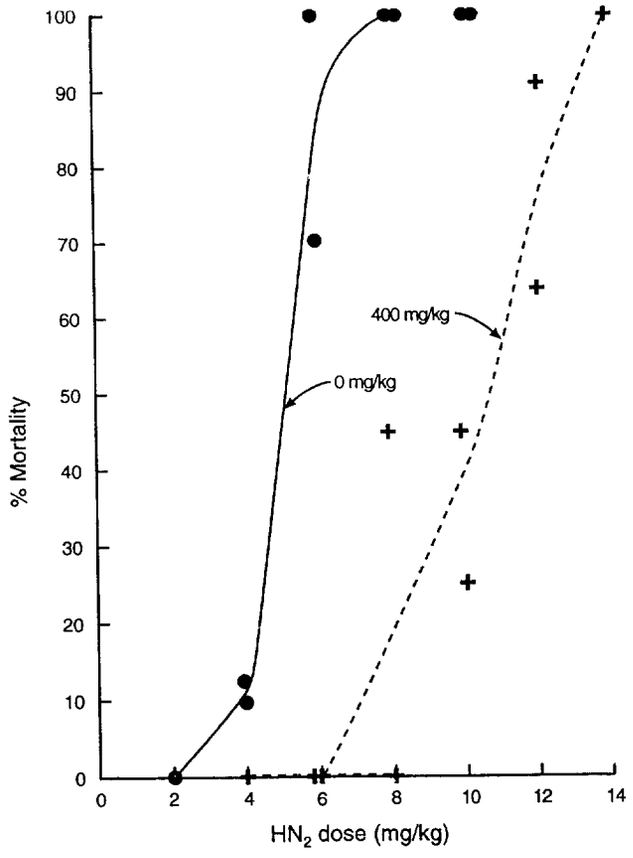

Figure 5. Per cent mortality of C57BL/6J mice at 30 days after nitrogen mustard (HN_2) injection without (——) or with (---) amifostine injection (400 mg/kg i.p.) administered 15 min earlier. Reproduced by permission of the *Journal of the National Cancer Institute*, from Yuhas JM, *Cancer Treat Rep* 1979, Vol. 63, pp. 971–976.

stem cells through a 4-fold dose escalation range of melphalan. In contrast, the combined therapy with amifostine and melphalan resulted in a longer growth delay of the melanoma than that achieved following treatment with either agent alone [52]. Also noted has been amifostine sensitisation of tumours to the cytotoxic effects of other agents such as mafosfamide [57], carboplatin [53], doxorubicin [15], photodynamic therapy [58] and paclitaxel [17]. The pharmacological basis for this apparent tumour sensitisation with concurrent protection of normal organs remains to be explored. Two reports illustrate the potential haematoprotective effect of amifostine from 5-FU toxicity [48, 51].

Preclinical studies have shown that the sequential use of the cytoprotector amifostine is complemented when followed by bone marrow-stimulatory cytokines. As noted above, repetitive use of bone marrow-stimulatory cytokines meets with diminished efficacy through multiple cycles of chemotherapy [5], probably because of progressive depletion of the progenitor-cell pool. Consequently, preservation of the progenitor-cell pool by a cytoprotector administered before cytotoxic therapy would preserve the capacity of the cytokine to accelerate bone marrow recovery. This hypothesis has been tested in mice and dogs treated with whole-body radiation [59–61]. As noted in Figure 7, pretreatment of mice with amifostine before the administration of whole-body radiation exceeded the survival-enhancing potential of granulocyte colony-stimulating factors (G-CSF). The administration of amifostine prior to radiation followed by administration of G-CSF after radiation provides further enhancement of survival reflecting the complementary role of combining a cytoprotector with a bone marrow-stimulatory cytokine.

Several studies illustrate selective cytoprotection by amifostine against cisplatin-induced nephrotoxicity without interfering with cisplatin's antitumour response. Yuhas and coworkers [9, 12] demonstrated that pretreatment of mice or rats with

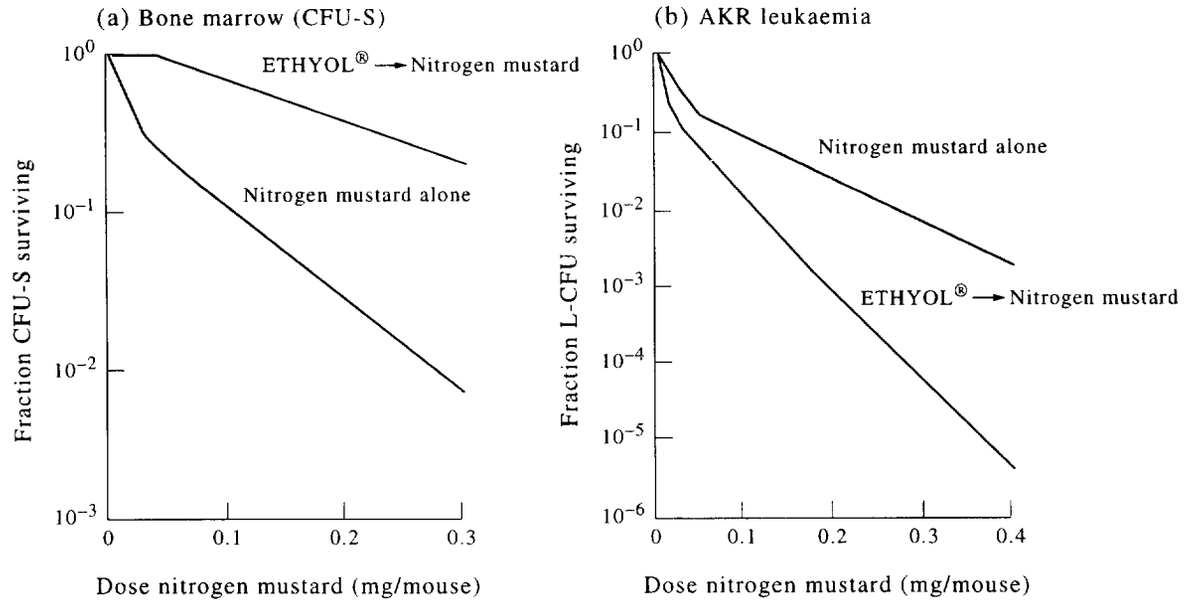

Figure 6. (a) Survival of colony-forming units-spleen (CFU-S) and (b) survival of leukaemia colony-forming units (L-CFU) exposed *in vivo* either to nitrogen mustard (HN_2) alone or to amifostine administered 15 min before HN_2. Following treatment *in vivo*, marrow or leukaemia stem cells were harvested and viability was assayed as CFU-S or L-CFU in recipient mice. Reproduced by permission of the American Association of Cancer Research, Inc., from Valeriote F and Tolen S, *Cancer Res* 1982, Vol. 42, pp. 4330–4331.

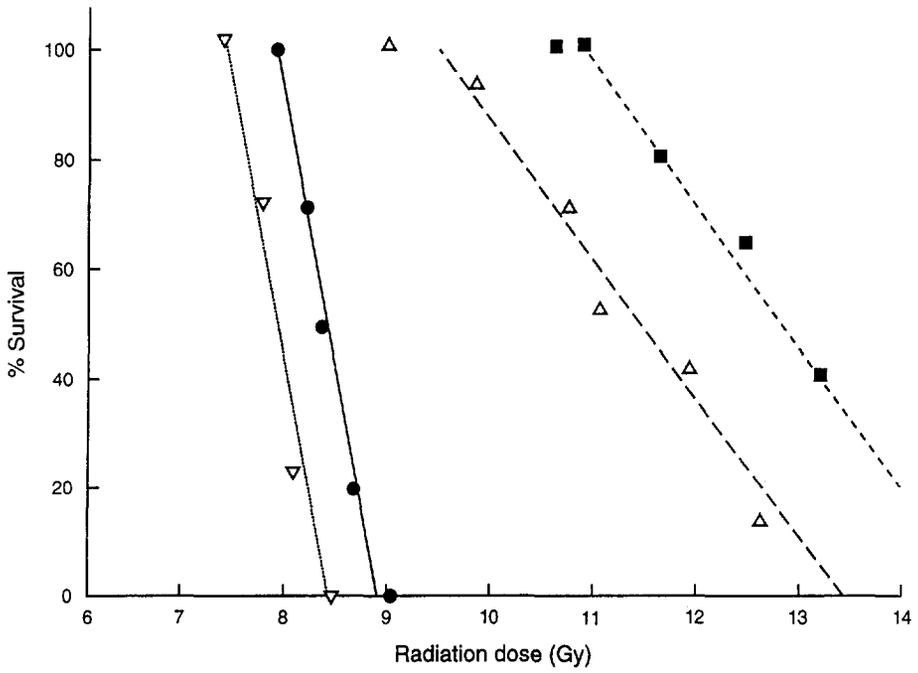

Figure 7. Effects of saline (▽), granulocyte colony-stimulating factor (G-CSF) (●), amifostine (△) and amifostine plus G-CSF (■) on survival of irradiated mice. C3H/HeN mice were administered amifostine (200 mg/kg i.p.) 30 min before ^{60}Co irradiation and G-CSF (125 μg/kg/d subcutaneously) on days 1–16 post-irradiation. Each data point represents results obtained from 30 mice.

amifostine protected against nephrotoxicity induced by both single and repeated doses of cisplatin without affecting the antitumour effects of cisplatin. As shown in Table 2, pretreatment with single doses of amifostine at either 100 or 200 mg/kg i.p. 30 min before cisplatin administration, increased the resistance of BALB/c mice and Fischer 344 rats to cisplatin-induced nephrotoxicity by a dose-modification factor ranging from 1.2 to 1.7. In these studies, the dose-modification factor, as defined in nephrotoxicity studies, is the ratio of cisplatin doses required to produce a peak blood urea nitrogen (BUN) measurement of 40 mg/dl in pretreated versus control animals. The observed protection was related to the dose of amifostine in both species. Histological examination of the kidney from these rats one month later was performed to examine the extent of the protective effect of amifostine. Far less renal tubular injury was observed in the amifostine-treated rats than in the control rats receiving cisplatin alone [12].

Similar results are illustrated in Figure 8a: pretreatment of Fischer 344 rats with 200 mg/kg amifostine allowed the safe administration of 7.5 mg/kg cisplatin, a dose that was otherwise fatal to 60% of the rats [9]. Figure 8b demonstrates that pretreatment with 200 mg/kg amifostine 30 min before cisplatin can increase the resistance of rats to nephrotoxicity as evidenced by higher peak BUN levels. In this same study, amifostine did not impair the cisplatin dose response on each of three transplantable tumours (Table 3) [9]. Similar cytoprotection from cisplatin nephrotoxicity was evident in rats and mice treated with five daily doses of cisplatin ± amifostine (Figure 9) [12]. In these same experiments, amifostine did not alter the therapeutic effect from cisplatin on two rat (3M2N and DMBA-14) and one mouse (MCa-11) mammary carcinomas.

Table 2. Amifostine-induced dose-modification factors (DMFs) against cisplatin nephrotoxicity in rats and mice

Species	Amifostine dose (mg/kg)	DMG
Rat	100	1.3
	200	1.7
Mouse	100	1.2
	200	1.5

Adapted from Yuhas JM *et al. Br J Cancer* 1980, **42**, 574–585.

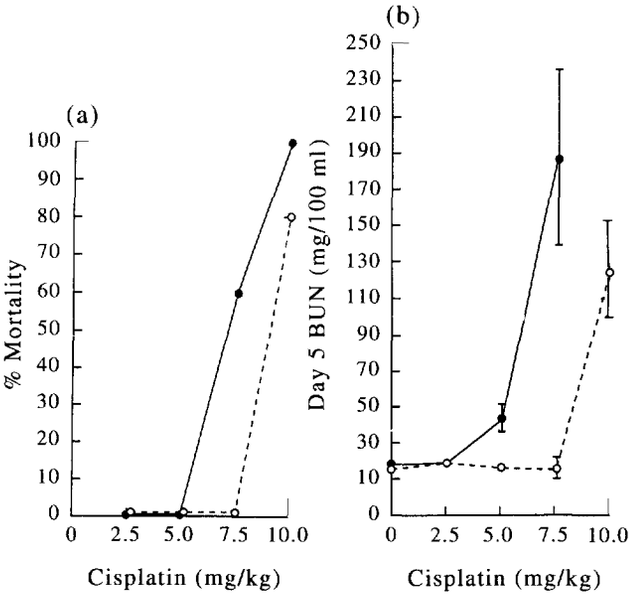

Figure 8. (a) Per cent mortality at 30 days after injection of graded doses of cisplatin in Fischer 344 rats given saline (●) or 200 mg/kg of amifostine (○) 30 min prior to cisplatin. (b) Blood urea nitrogen (BUN) levels on day 5 as a function of cisplatin dose for rats pre-treated with saline (●) or 200 mg/kg of amifostine (○) 30 min prior to cisplatin. Reproduced by permission of the *Journal of the National Cancer Institute*, from Yuhas JM and Culo F, *Cancer Treat Rep* 1980, Vol. 64, pp. 57–64.

Table 3. Delay in tumour growth from 10 mm *to* 14 mm: *days ± SE*

Tumours	Control (saline) plus 5 mg/kg cisplatin	Amifostine 200 mg/kg i.p. plus 5 mg/kg cisplatin	Amifostine 200 mg/kg i.p. plus 7.5 mg/kg cisplatin
3M2N	6.0 ± 0.52	6.1 ± 0.81	10.0 ± 1.10
13762	8.3 ± 0.37	7.9 ± 0.66	12.6 ± 0.91
R3230AC	2.4 ± 0.36	3.2 ± 0.71	5.0 ± 0.18

Adapted from Yuhas JM, Culo F. *Cancer Treat Rep* 1980, **64**, 57–64.

Experiments conducted at the Free University Hospital in Amsterdam confirmed the above data. The investigators reported a time dependence for the protection from cisplatin-induced nephrotoxicity by amifostine in mice [62]. Amifostine was equally effective in protecting mice from cisplatin-induced nephrotoxicity (dose modification factor of 2.2) and lethality when administered either 30 or 5 min before cisplatin treatment (Figure 10a); however, no protection was observed when amifostine was administered 30 min after cisplatin. The authors concluded that amifostine protects against cisplatin-induced toxicities by preventing rather than reversing cellular damage [62, 63]. As a result of the protection from cisplatin-induced nephrotoxicity, mice were able to tolerate larger doses of cisplatin, which in turn resulted in improved antitumour effects against OVCAR-3 (human ovarian) carcinoma implanted in nude mice (Figure 10b) [62].

Amifostine was also effective in reducing nephrotoxicity in Fischer 344 rats treated with tetraplatin, a second-generation platinum compound [64]. Rats treated with tetraplatin alone exhibited significant increases in BUN and creatinine. When amifostine (e.g. 200 mg/kg i.p.) was administered 30 min before tetraplatin, levels of BUN and creatinine were significantly lower in comparison with rats receiving tetraplatin alone. Moreover, none of the rats receiving amifostine prior to tetraplatin exhibited any significant pathological changes in the kidney.

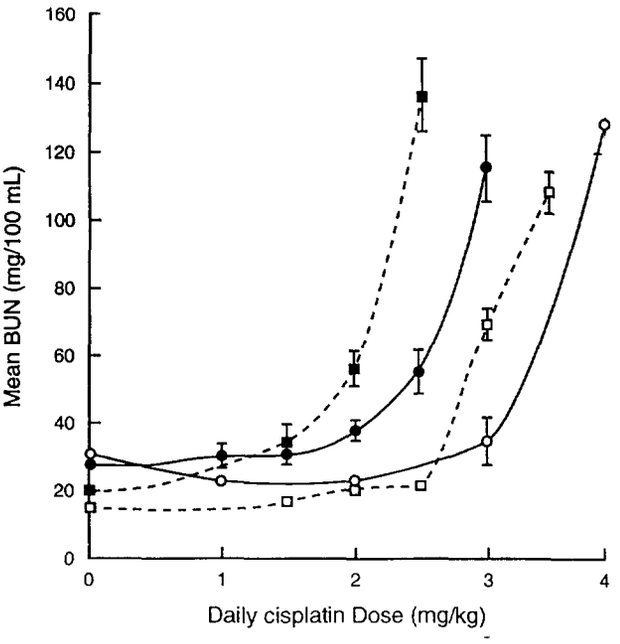

Figure 9. Peak blood urea nitrogen (BUN) levels observed in mice and rats as a function of cisplatin dose administered daily for five days. ■, Control; □, rats pretreated with 100 mg/kg of amifostine 30 min prior to each cisplatin injection; ●, control mice, ○, mice treated with 100 mg/kg of amifostine 30 min prior to each cisplatin injection. Reproduced by permission of The Macmillan Press Ltd, from Yuhas JM, *et al.*, *Br J Cancer* 1980, Vol. 42, pp. 574–585.

This cytoprotective effect on cisplatin nephrotoxicity is consistent with the tissue-distribution pharmacokinetics of amifostine and its active metabolite, the free thiol, WR-1065. Following administration to mice and rats, the concentration of amifostine and its metabolites reaches steady-state levels within 10 min (see Figures 2 and 3). These high levels in the kidney

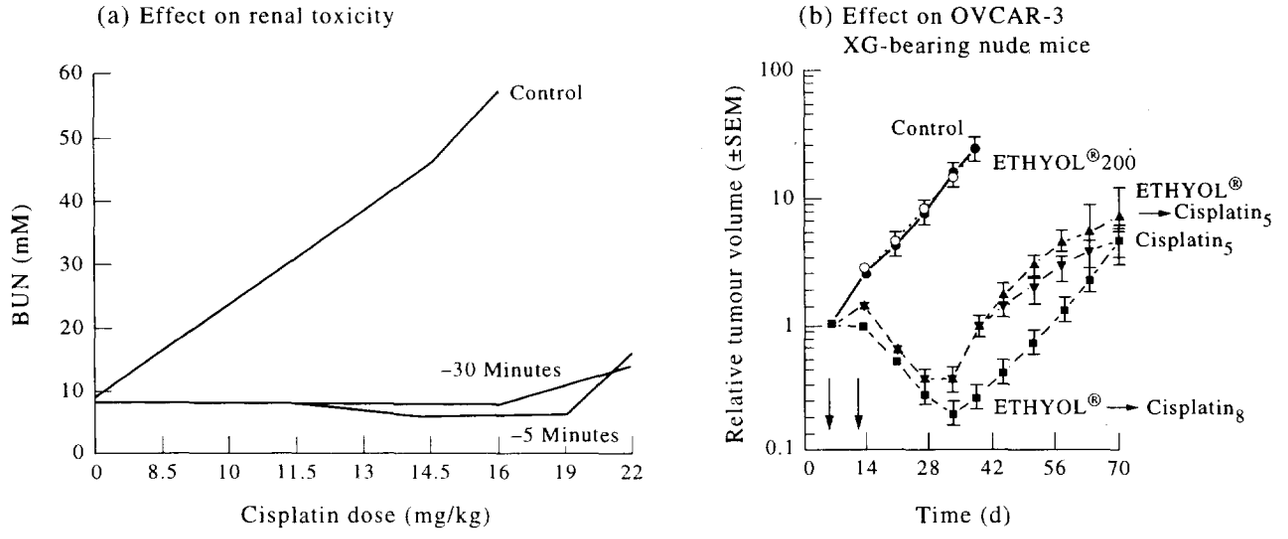

Figure 10. (a) The effect of amifostine (200 mg/kg i.v.) on cisplatin-induced nephrotoxicity in BALB/c mice (n = 8) when administered 30 or 5 min before cisplatin as measured by blood urea nitrogen levels (BUN) at day 4. (b) The effect of amifostine on tumour growth as measured by relative tumour volume of OVCAR-3 (ovarian cancer) xenografts grown in nude mice. Symbols represent untreated (○) and amifostine-treated (●) mice, 5 mg/kg (days 0 and 7) of cisplatin alone (▼) and in combination with amifostine 5 min prior to cisplatin (▲) or amifostine 5 min prior to an equitoxic dose of 8 mg/kg of cisplatin (■). Reproduced by permission of the American Association of Cancer Research, Inc., from Treskes M, *et al.*, *Cancer Res* 1992, Vol. 52, pp. 2257–2260.

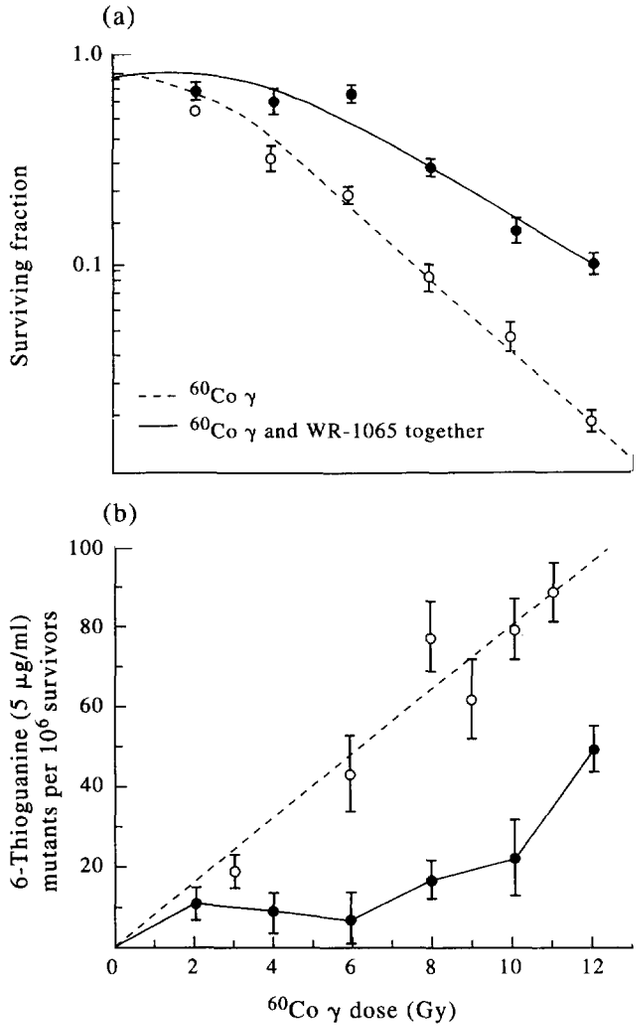

Figure 11. The response of V79 cells exposed to ^{60}Co γ-rays in the absence or presence of 4mM WR-1065. (a) Cell survival. (b) Mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus. WR-1065 was added to cells 30 min prior to and removed 3 h following irradiation. Reproduced by permission of Oxford University Press, from Grdina DJ, *et al.*, *Carcinogenesis* 1985, Vol. 6, pp. 929–931.

and other organs persist (e.g. tissue retention) for at least 90 min.

The primary cisplatin nephrotoxic effect is on the renal tubules [65]. Alkaline phosphatase, the enzyme that converts amifostine to WR-1065, is primarily localised in the brush border of the proximal tubules [66]. This histochemical feature correlates with the tissue-distribution pharmacokinetics and the pharmacodynamics of amifostine nephroprotection observed in rodents and humans.

Amifostine has cytoprotective effects on other organs. Amifostine pretreatment has been shown to protect intestinal crypt cells from melphalan toxicity, while not affecting the cytotoxic effect on human melanoma xenografts [52]. Pretreatment with amifostine has been shown to protect intestinal crypt cells from radiation damage [67–69]. Mollman and coworkers [70] and Müller and coworkers [71] have shown that pretreatment with amifostine resulted in a significant decrease in cisplatin-induced toxicity to neural tissues. Pierson and Moller [72] showed that pretreatment with amifostine reduced aminoglycoside-induced hearing loss, the mechanism of which is similar to that caused by cisplatin. Pulmonary toxicity in mice treated with high-dose cyclophosphamide [73] or radiation [74] was abrogated by pretreatment of the mice with amifostine. Anthracycline toxicity to murine fetal cardiac myocytes was attenuated by pretreatment with amifostine or WR-1065 [75]. In contrast, there was no loss of antitumour activity from doxorubicin when mice bearing human breast cancer xenografts were pretreated with amifostine compared with the effect of doxorubicin alone [15]. In addition to the reduction of acute reversible radiation toxicities, amifostine may significantly attenuate radiation-induced permanent destruction of certain tissues such as salivary glands [76, 77] and soft tissues of the limb, causing limb contractures [78], issues that, in the clinical setting, have substantial impact on quality of life.

One of the more devastating delayed complications of cytotoxic therapy is the occurrence of drug- or radiation-induced secondary neoplasms. Laboratory data indicate that amifostine can significantly reduce the carcinogenic [79], mutagenic [41, 80–82] and clastogenic [83, 84] effects of cytotoxic cancer therapy. Extensive *in vivo* and *in vitro* evaluations of the protective effects of amifostine and WR-1065 against radiation- and chemotherapy-induced at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in V79 Chinese hamster lung fibroblasts [85–87] and radiation-induced carcinogenesis [79] have been carried out. As shown in Figure 11, pretreatment with WR-1065 (4 mM) provided protection against radiation-induced cell lethality as well as radiation-induced mutations at the HGPRT locus in V79 cells, as assessed by 6-thioguanine resistance [85, 86]. Similar protection has been demonstrated against cisplatin-, bleomycin- and nitrogen mustard-induced mutations at the HGPRT locus [86, 87]. The data in Figure 12 are representative of these effects. Pretreatment of V79 cells with WR-1065 provided maximal protection; however, antimutagenic effects were also obtained with concurrent treatment and even post-treatment with amifostine [88]. The latter effect is consistent with biochemical data showing removal of platinum bound to DNA by post-treatment with WR-1065 (Figure 4b). Amifostine also afforded protection against high- and low-energy radiation-induced carcinogenesis.

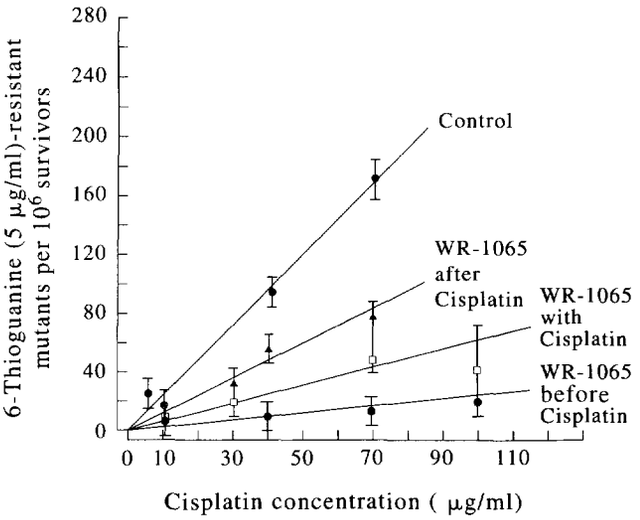

Figure 12. The effect of WR-1065 (4 mM) on mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase locus in V79 cells as a function of cisplatin concentration. Reproduced by permission of the American Association of Cancer Research, Inc., from Nagy B, *et al.*, *Cancer Res* 1986, Vol. 46, pp. 1132–1135.

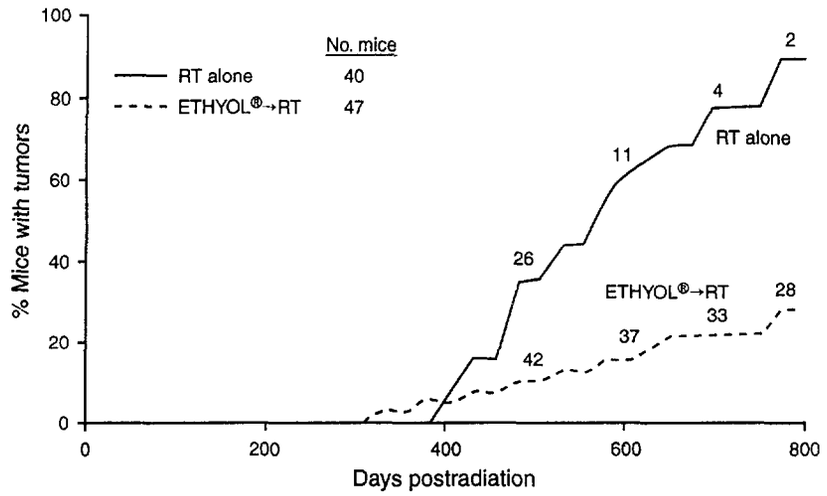

Figure 13. Cumulative incidence of radiation-induced tumours (fibrosarcoma) as a function of time following irradiation (3400- to 5700-rad single dose γ-radiation) of the hind leg of C_3Hf/Kam mice with or without pretreatment with amifostine (400 mg/kg i.p.). RT, radiotherapy. Reproduced by permission of the American Association of Cancer Research, Inc., from Milas L, *et al.*, *Cancer Res* 1984, Vol. 44, pp. 5567–5569.

As shown in Figure 13, there was a significant ($P > 0.001$) reduction in the incidence of radiation-induced tumours in mice pretreated with amifostine (400 mg/kg i.p.) 30 min before single doses of gamma radiation [79].

In summary, the preclinical spectrum of amifostine-mediated cytoprotection is broad, as indicated by data from numerous laboratories around the world. Many of the above citations discussed comparative studies of the protection of normal organs in tumour-bearing animals. The results of these studies show that amifostine does not attenuate and, in specific instances, may significantly enhance the cytotoxic effect on tumour despite the reduction of normal organ toxicity. A catalogue of the murine and human tumours that have been studied to determine the potential interaction between amifostine and/or WR-1065 and various cytotoxic therapies is shown in Tables 4 and 5. These represent a broad range of histologies, including carcinomas, sacromas and leukaemias. The *in vitro* studies used concentrations of amifostine or WR-1065 that are 50- to 100-fold higher than those achieved in the systemic circulation following the administration of therapeutic doses. Even with direct contact between the tumour cells and these high concentrations of drugs, there was no evidence of attenuation of the antitumour effects in any of these experiments. The unique preclinical profile of amifostine described above serves as the basis for the clinical development programme for this important new broad-spectrum selective cytoprotective agent.

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Table 4. Catalogue of murine tumours for the assessment of amifostine pretreatment on the effect of various cytotoxic therapies: amifostine pretreatment does not attenuate cytotoxic activity

Murine tumours	Antitumour treatment
Colon 26 tumour	
Colon 38 tumour	Carboplatin
Leukaemia (AKR)	Carmustine
Leukaemia (P388)	Cisplatin
Lung adenocarcinoma (line 1)	Cyclophosphamide
Lung adenoma (urethane induced)	Doxorubicin
Mammary tumour (EMT6)	5-Fluorouracil
Mammary adenocarcinoma (13762)	4-Hydroperoxycyclophosphamide
Mammary adenocarcinoma (R3230 AC)	Mitomycin-C
Mammary adenocarcinoma (DMBA-1)	Nitrogen mustard
Mammary adenocarcinoma (spontaneous C57)	Radiation
Mammary carcinoma (MCA-11)	
Mammary squamous cell carcinoma (3M2N)	
Morris hepatoma (FTO)	
Morris hepatoma (FAO)	
Sarcoma (C3HF)	
Sarcoma (KHT)	
Sarcoma (MDAH F)	

Table 5. Catalogue of the preclinical assessment of amifostine pretreatment on the effect of various cytotoxic therapies: amifostine pretreatment does not attenuate cytotoxic activity

Human tumour cell lines (*in vitro*)	Treatment
Ovarian (PA-1; BG-1; A2780; 2780/S; 2780-CP70; OVCAR 3; OVCAR 10)	Bleomycin Carboplatin Cisplatin
Acute lymphoblastic leukaemia (REH CEM-CCRF/MOLT-4)	Cytosine arabinoside Doxorubicin
Acute myelocytic leukaemia (human)	Etoposide
Breast cancer (CAMA)	5-Fluorouracil
Fibrosarcoma Glioma Melanoma (SK-MEL, PA01)	4-Hydroperoxycyclophosphamide (active metabolite of cyclophosphamide) Haematoprophyrin derivative photo treatment
Nonsmall-cell lung cancer (A549, CALU-6, A427)	Idarubicin
Small-cell lung cancer (ATCC HTB-110; HTB 120)	Light-activated merocyanine 540 photo treatment Mafosfamide
Human tumour xenografts	Melphalan Methylprednisolone + etoposide + ilmofosine
Breast (MDA-MR-435) Lung Melanoma Ovary	Mitomycin C Mitoxantrone Nitrogen mustard Radiation therapy
Human tumour stem-cell assay	Paclitaxel Docetaxed
Bladder Breast Large-cell lung cancer Pancreas	Vinblastine Vincristine

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