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Protection of Normal Tissues from the Cytotoxic Effects of Chemotherapy and Radiation by Amifostine (WR-2721): Preclinical Aspects

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Amifostine is a radioprotective agent that prevents radiation- and chemotherapy-induced cellular injury through free-radical scavenging, hydrogen donation, and inhibition of DNA damage. Amifostine is metabolised and accumulated to a much greater extent in normal cells than in tumour cells. As a result, it exerts a protective effect from toxicity on normal tissues induced by chemo- or radiotherapy without reducing the antitumour effects of cancer therapy. Extensive preclinical studies have shown that amifostine protects against radiation damage and against the myelotoxic, nephrotoxic and neurotoxic effects of chemotherapeutic agents such as alkylating agents and platinum compounds. In some cases, the antitumour effects of these agents have been potentiated by amifostine. Amifostine has also been shown to protect against radiation- and chemotherapy-induced mutagenesis and, as a result, carcinogenesis. Use of amifostine allows for safer and more effective administration of radio- and anticancer therapy.

Key words: ethiofos, antineoplastic agents, radiation-protective agents, amifostine
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INTRODUCTION

THE COMPOUND amifostine, H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-S-PO_3H_2 (Figure 1), also known as WR-2721 and Ethyol^R, was developed originally as a radioprotectant as part of the Anti-Radiation Drug Development Program initiated by the United States Army at the Walter Reed Army Institute of Research during the 1950s. Amifostine was selected for clinical development from 4400 chemicals studied because it was shown to be one of the least toxic and most efficacious agents in protecting against lethal irradiation [1]. Amifostine is now being used in oncology as a protective agent against the toxic effects of platinum compounds, alkylating agents, and radiation therapy, allowing for safer and more effective administration of anticancer drugs and radiation, while at least maintaining destruction of cancer cells.

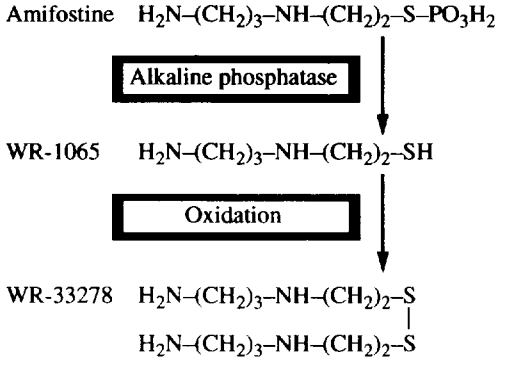

Figure 1. Structural formulae and activation of WR-2721.

MECHANISM OF ACTION

Amifostine does not or minimally exerts a protective activity by itself. The compound is activated through dephosphorylation to the free-thiol WR-1065, a process which is catalysed primarily by plasma membrane-bound alkaline phosphatase [2–4]. WR-1065 undergoes oxidation to WR-33278 (Figure 1).

Amifostine is thought to provide protection from radiation through free radical scavenging or hydrogen donation to repair DNA damage [5, 6]. Amifostine inhibits chemotherapy-induced DNA damage because the free-thiol WR-1065 serves as a site for nucleophilic attack for the charged carbonium of the activated alkylating agents, thus sparing the critical nucleic acids.

Amifostine can be expected to protect against chemotherapy-induced DNA damage in tissues that have a high capacity to dephosphorylate the compound to WR-1065, such as liver, kidney and small intestine [4], and then take up the thiol metabolite. Amifostine inhibits the toxic effects of chemotherapy to a far greater degree in normal tissue than in tumours, because normal tissues accumulate WR-1065 much more effectively than tumour cells. There are several reasons for this difference (reviewed by McCulloch and colleagues [5] and Grdina and Sigdestadt [6]). First, delivery of amifostine to normal tissue is more rapid because the vascularisation of normal tissue is superior to that of tumours. Second, conversion of amifostine to WR-1065 is faster in normal tissues because the alkaline phosphatase activity in the capillaries is greater than that in

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tumour tissue and because the relatively high pH of normal tissues results in a greater rate of WR-1065 formation and uptake than in tumour tissue. Finally, normal tissues have been shown to actively concentrate amifostine against a concentration gradient, whereas solid tumours fail to actively concentrate the compound, relying instead on passive absorption [7]. These differences between normal and cancerous tissues provide the biochemical and physiological basis for the selective protection of normal tissues by amifostine.

Selective uptake of amifostine by normal tissues has been demonstrated in several animal models. In one study, female Fischer 344 rats transplanted with 3M2N squamous cell carcinoma cells were injected intraperitoneally with ^{35}S-labelled amifostine at a dose of 200 mg/kg [7]. Tissue samples were harvested for determination of the tissue concentrations of radiolabelled amifostine and metabolites. As shown in Figure 2, uptake of labelled compounds in the tumour was markedly less than that in the serum, spleen, liver, heart, lung and kidney. The relatively large uptake by the kidney is especially important in view of the nephrotoxicity of cisplatin. Similar findings have been reported by Washburn and colleagues in mice bearing the P-1798 lymphosarcoma and CA-755 adenocarcinoma, and in rats bearing the RFT tumour [8], and by Millar and colleagues in mice bearing the Lewis lung tumour and human melanoma xenografts [9].

The greater cellular uptake of amifostine in normal cells relative to malignant tissue results in preferential protection of normal cells from the effects of cytotoxic compounds. Table 1 summarises the results of a number of studies in which amifostine was shown not to affect the antitumour activity of either a chemotherapeutic agent or radiation. Only in mice bearing syngeneic tumours, has tumour protection been postulated (Table 2). However, the effect was usually small, often not statistically significant, and highly variable between the various separate experiments. As discussed below, body temperature leading to for example a reduction of flow of blood and nutrients to the tumour, will cause an apparent protection of the tumour.

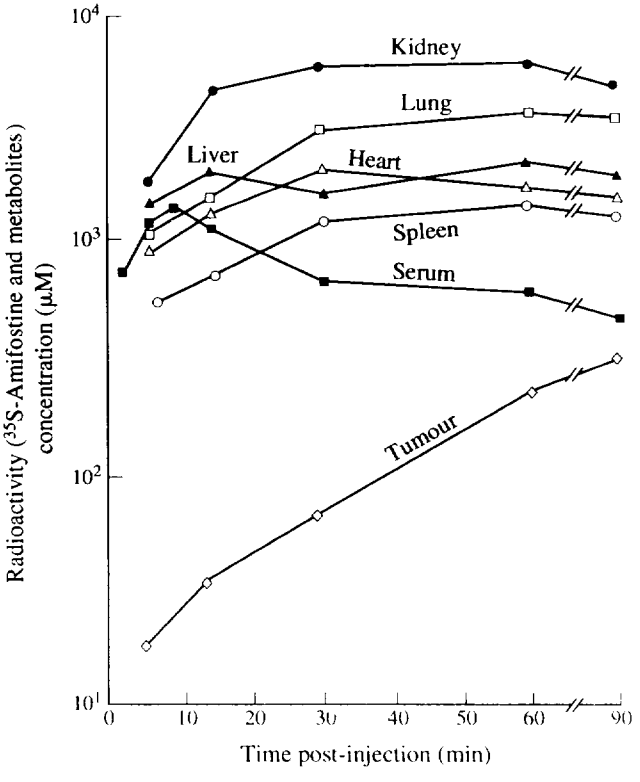

Figure 2. Concentration of amifostine in serum, tissue, and tumour of Fischer 344 rats as a function of time after intraperitoneal injection of 200 mg/kg of amifostine. Reprinted with permission from Yuhas *et al. Cancer Res* 1980, 40, 1519–1524 [7].

Table 1. Tumours shown not to be protected by amifostine from cytotoxic effects of drugs or radiation

Human tumour xenografts
Breast (MDA-MR-435)
Melanoma
Ovarian cancer (OVCAR-3)
Murine tumours
Colon tumours (colon 26, 38)
Leukaemia (AKR)
Leukaemia (P388)
Mammary tumour (EMT6)
Mammary adenocarcinoma (13762)
Mammary adenocarcinoma (R3230 AC)
Mammary adenocarcinoma (DMBA-1)
Mammary carcinoma (MCA-11)
Mammary squamous cell carcinoma (3M2N)
Sarcoma (C3HF)
Sarcoma (KHT)
Sarcoma (MDAH F)
Lung adenocarcinoma (line 1)
Lung adenoma (urethane-induced)
Drugs evaluated
Cisplatin
Carboplatin
Cyclophosphamide
Nitrogen mustard
Mytomycin-C
Melphalan
Doxorubicin
BCNU
5-FU
X-ray

These data have been compiled from a large number of references, including [5, 6, 9, 11, 12, 16, 25, 29, 31, 40].

Table 2. Tumours postulated to be protected by amifostine from cytotoxic effects from drugs or radiation

Murine tumours
Fibrosarcoma (RIF-a)
Fibrosarcoma (FSA, NSFA)
Lewis lung carcinoma
Mammary tumour (EMT6)
Sarcoma (KHT)
(CAMT)
Drugs evaluated
Cyclophosphamide
Melphalan
CCNU
Cisplatin
X-ray

These data have been compiled from references [13, 18, 19, 25, 42–45]. It should be noted that several of the authors already postulated that the extent of protection is usually small and reproducibility between experiments is poor [45].

However, in several studies, amifostine even enhanced the antitumour activity of the therapy.

HYPOTHERMIA AND AMIFOSTINE TREATMENT IN MICE

One of the findings noted during initial studies of the effects of amifostine was a marked decrease in body temperature [10] following intraperitoneal administration of 525 mg/kg amifostine to female Balb/c mice [11, 12]. After 30 min, body temperature fell from about 37 to 32°C, and after 2.5 h, it had decreased to 27°C. As a result, the dose of amifostine was then reduced to 200 mg/kg. At this dose, a smaller decrease in body temperature of between 3 and 4°C was observed. A number of other investigators have used an amifostine dose of 525 mg/kg or even higher, but variable sensitivity to amifostine has been observed between the various strains and investigators [9, 13–17]. Several investigators have reported some tumour protection from cytotoxic therapy [13, 18, 19]. In these cases, tumour protection may have been a result of low body temperature. Hypothermia would lead to vasoconstriction which in turn would result in a reduction in the flow of blood and cytotoxic agent to the tumour. The severe hypothermia seen in Balb/c mice has also been reported in studies as early as 1965 [10], although in one study, 600 mg/kg amifostine was injected intraperitoneally in LAF_1 mice without reporting an effect on body temperature [14]. A dose of 525 mg/kg is higher than the dose that is currently given to patients [20–23].

AMIFOSTINE AND RADIOPROTECTION

The protective effects of amifostine against radiation damage have been studied extensively in rodents, dogs, and monkeys [15, 16, 24, 25]. In all of the tissues studied, a radioprotective effect was observed, although the degree of protection varied widely among the different tissues. Studies by Davidson and colleagues [24] in dogs and monkeys provided clear examples of the potent radioprotective activity of amifostine. In these studies, 5 dogs were pretreated with 200 mg/kg of amifostine 30 min before whole-body irradiation at 450 rads, and 5 additional dogs underwent whole-body irradiation without amifostine pretreatment and served as controls. After 30 days, none of the control dogs had survived, whereas all 5 of the dogs that had received amifostine were alive. In a similar trial, 6 monkeys were pretreated with 250 mg/kg of amifostine 10 min before whole-body irradiation at 1000 rads, and two additional monkeys underwent irradiation without amifostine pretreatment. After 30 days, neither of the untreated monkeys had survived, whereas all 6 of the amifostine-treated monkeys were alive.

AMIFOSTINE AND PROTECTION AGAINST THE TOXICITY OF CHEMOTHERAPEUTIC AGENTS

Amifostine has also been studied as a chemoprotector to inhibit the cytotoxic effects of chemotherapeutic agents such as cisplatin, carboplatin, melphalan, and nitrogen mustard. The results of these studies have shown that pretreatment with amifostine protects against haemato-, nephro-, oto-, or neurotoxicity induced by those agents and similar drugs.

Protection against nephrotoxicity

Cisplatin is an effective antineoplastic agent whose use is limited primarily by nephrotoxicity. Yuhas and associates showed that resistance to cisplatin-induced nephrotoxicity was increased by a factor of 1.7 when rats were injected intraperitoneally with 200 mg/kg of amifostine 30 min prior to cisplatin administration [17]. In a subsequent experiment, Treskes and colleagues studied the effect of amifostine on cisplatin-induced nephrotoxicity in BALB/c mice (8 per group) and the dependence on the relative times of administration of amifostine and cisplatin [26]. Specifically, these authors wished to determine whether the protective potential of 200 mg/kg amifostine administered intraperitoneally 30 min prior to cisplatin could be increased by giving the same dose 5 min before or 30 min after cisplatin administration. Figure 3 shows the modulation of cisplatin-induced nephrotoxicity on day 4 in terms of plasma urea concentrations. Plasma urea increased with cisplatin dose among the mice administered cisplatin alone (control). Administration of amifostine 30 min after cisplatin provided no protection against nephrotoxicity. In contrast, when amifostine was given either 30 or 5 min before cisplatin administration, there was clear protection against cisplatin-induced nephrotoxicity.

Protection against myelotoxicity

Carboplatin is a second-generation analogue of cisplatin which is less nephrotoxic than cisplatin but is associated with dose-limiting myelotoxicity. In a study of the effect of amifostine on carboplatin-induced myelotoxicity, one group of mice received carboplatin alone, a second group received 200 mg/kg amifostine followed after 5 min by carboplatin, in a third group the interval was 30 min before carboplatin, while in the fourth group amifostine was given 30 min after carboplatin [27]. Figure 4 shows the effect of carboplatin with and without amifostine on the proliferation of bone marrow cells in the presence of M-CSF and IL-3 as a percentage of control. Amifostine alone did not affect the proliferation of the bone marrow cells. When carboplatin was administered with amifostine, myelotoxicity was less severe which allowed the administration of higher doses of the cytotoxic agent [27].

Green and Schein [28] have reported similar findings in studies in which assays of myelotoxicity included determinations of white blood cell counts. In these studies, the nadir for white blood cells was higher when cytotoxic therapy was combined with amifostine than when given alone. Van Laar and colleagues combined carboplatin with 5-fluorouracil. The chemotherapy-induced fall in thrombocytes could be partially prevented by administration of 200 mg/kg amifostine 5 min before carboplatin [29]. In this study, the dose of carboplatin could be increased

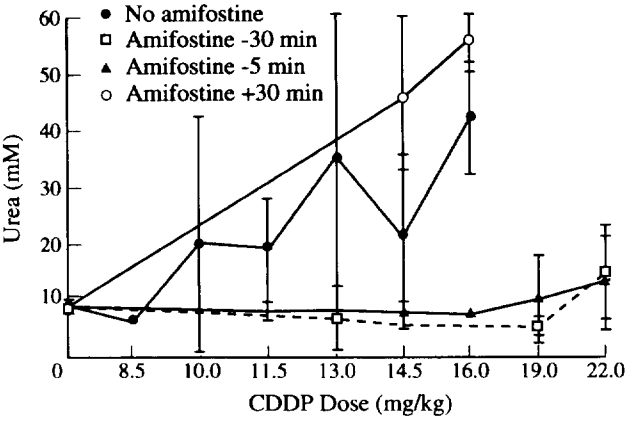

Figure 3. Plasma urea levels (mM) of BALB/c mice given cisplatin alone or cisplatin plus an intraperitoneal injection of 200 mg/kg amifostine 30 min before (−30 min), 5 min before (−5 min), or 30 min after (+ 30 min) cisplatin administration. Adapted with permission from Treskes *et al. Cancer Res* 1992, 52, 2257–2260 [26].

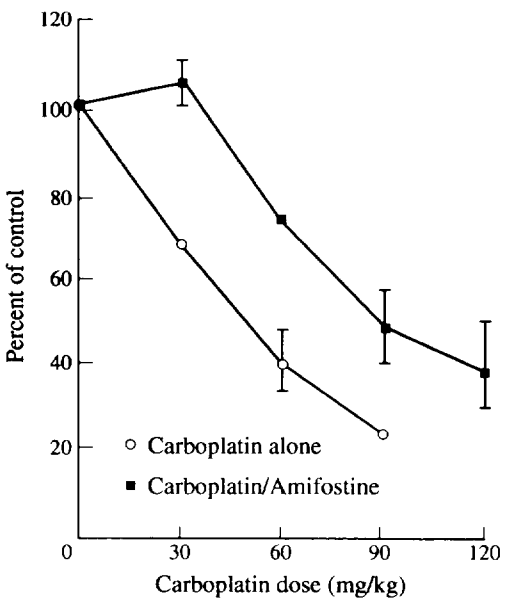

Figure 4. Effect of amifostine on carboplatin induced bone marrow toxicity was evaluated as proliferation of isolated bone marrow cells in the presence of M-CSF and IL-3. Amifostine (at 200 mg/kg) was administered 5 min before carboplatin. Adapted with permission from Treskes *et al. Eur J Cancer* 1994, 30A, 183–187 [27].

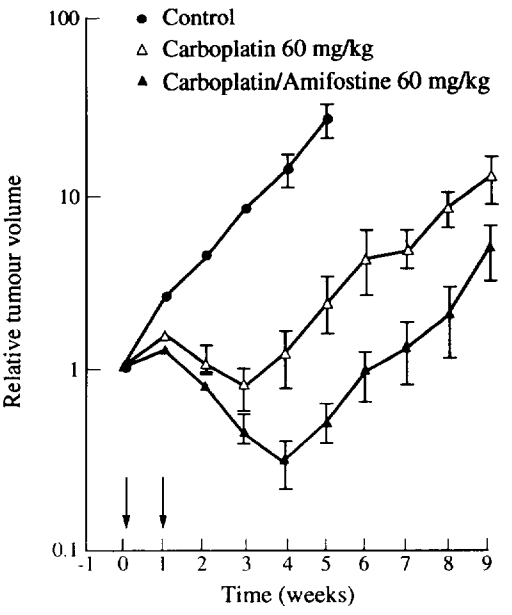

Figure 5. Antitumour activity over time in OvCar3 XG-bearing nude mice that received no treatment (control), 60 mg/kg carboplatin alone, or 60 mg/kg carboplatin with amifostine (at 200 mg/kg, given 5 min before carboplatin). Treatment was given twice with a weekly interval (indicated by arrows). Adapted with permission from Treskes *et al. Eur J Cancer* 1994, 30A, 183–187 [27].

from 45 to 60 mg/kg when amifostine was administered 5 min before carboplatin. In another study, amifostine was given before ^{131}I-labelled antibodies or total body irradiation [30]. Partial protection against total body irradiation was observed, but amifostine failed to prolong survival or delay myelosuppression from the ^{131}I-labelled antibody.

Although the ability of protective agents to inhibit the deleterious effects of cytotoxic therapy is important, the selectivity of these agents is equally important. Compounds that extend equal protection to normal and neoplastic tissues will be of little therapeutic value. The effect of amifostine on the antitumour activity of carboplatin was studied in groups of OvCar3 XG-bearing nude mice over time [27]. One group of 6 animals received 60 mg/kg carboplatin alone, the second group received 60 mg/kg carboplatin plus amifostine at 200 mg/kg 5 min before carboplatin, and the third group received no treatment and served as controls. As shown in Figure 5, tumour growth was reduced in mice that received carboplatin alone. However, when the same dose of carboplatin was administered in combination with amifostine, further reduction in tumour volume was observed, indicating that amifostine potentiated the antitumour activity of carboplatin [27].

Valeriote and Tolen conducted a study investigating the effect of amifostine on the cytotoxic and antitumour activity of nitrogen mustard in the AKR mouse [31]. The effect of treatment on cytotoxicity was assessed on the basis of the spleen colony assay for both normal haematopoietic stem cells and AKR leukaemia cells. Amifostine treatment resulted in greater survival of the CFU-S fraction and decreased survival of the LCFU fraction. These findings indicate that amifostine potentiated the antileukaemic activity of nitrogen mustard while inhibiting its myelotoxic effects.

Enhancement of antitumour activity following administration of amifostine has also been reported by others. Millar and colleagues administered 400 mg/kg amifostine to groups of 10 thymectomised mice 30 min prior to treatment with 20 mg/kg melphalan [9]. An additional group of 10 thymectomised mice received melphalan alone and served as controls. In this study, all 10 control mice died, whereas all 10 of the amifostine-treated mice survived. Survival of gut microcolonies of cryptogenic cells was 61% in mice given melphalan plus amifostine intraperitoneally, compared with 12% in mice given doses of melphalan alone. The protective effect of amifostine was slightly less when both agents were given intravenously. In this case, survival was 11% in mice treated with melphalan alone compared with 30% in those that also received amifostine. Finally, amifostine also exerted a protective effect on bone marrow, increasing survival of bone marrow CFU-S by a dose modification factor of 1.6. In this model, amifostine potentiated the antitumour effect of melphalan against the human melanoma xenograft. A dose of 12 mg/kg melphalan alone led to a tumour growth delay of 22 days, while combination of this dose with amifostine produced a delay of 27 days.

A completely different aspect of thiol protection was the blockage by WR-1065 of drug- and radiation-induced programmed cell death or apoptosis in thymocytes [32]. WR-1065 inhibited the Ca^{++} and Mg^{++} dependent internucleosomal DNA fragmentation, characteristic for apoptosis [32]. WR-1065 also protected against dexamethasone and calcium ionophore A23187-induced apoptosis.

The studies reviewed above indicate that amifostine selectively protects normal tissues from the cytotoxic effects of cancer therapy, but does not interfere with its antitumour activity. In these studies, amifostine potentiated the antitumour activity of carboplatin, nitrogen mustard, and melphalan. Other studies have shown that amifostine also increases the antineoplastic effects of the combinations of 5-fluorouracil plus carboplatin [29] or cisplatin [11].

Protection against neurotoxicity

The findings of some studies have suggested that amifostine may also provide protection to neurological tissue against chemo-

therapy-induced toxicity. Muller and colleagues conducted studies of the effect of amifostine on cisplatin-induced neurotoxicity using neurons of the snail, *Lymnaea stagnalis*, in an *in vitro* system [33]. Toxic effects were assessed on the basis of condensation of nuclear and nucleolar chromatin and changes in cytoplasmic organelles. When amifostine was administered with cisplatin, the induction of lysosomes and the presence of intercellular spaces and swollen axons that were seen with cisplatin alone were prevented. These findings, and others in the pig and in humans [23], suggest that amifostine protects nervous tissue against cisplatin-induced toxicity.

PREVENTION OF MUTAGENESIS AND CARCINOGENESIS

Although ionising radiation is carcinogenic, the occurrence of radiation-induced tumours as a side effect of radiation therapy is rare. Nevertheless, every possible step must be taken to reduce this risk. As a result, amifostine has been investigated as a protective agent against radiation-induced carcinogenesis and mutagenesis. In a study by Milas and colleagues, 47 C3Hf/Kam mice were injected intraperitoneally with 400 mg/kg amifostine 30 min before exposure of their right hind legs to single doses of gamma rays ranging from 3400 to 5700 rads [34]. 40 additional mice that did not receive amifostine and were irradiated similarly served as controls. The incidence of tumour in these mice is shown in Figure 6. By the end of the 786-day observation period, 38 of the 40 (95%) control mice had died, compared with only 19 of the 47 (40%) amifostine-treated mice. The overall actuarial tumour incidence was 87% in the control group compared with only 26% in the amifostine-treated group. The results of this study indicate that protection against radiation-induced tumours is an additional therapeutic benefit of amifostine. This was also observed in other studies in which B6CF1 mice were injected intraperitoneally with 400 mg/kg amifostine prior to irradiation with 10 cGy neutrons. Survival of thiol-protected mice was enhanced compared with saline-injected mice [35].

The effect of WR-1065, the dephosphorylated metabolite of amifostine, on cisplatin-induced mutagenesis was explored by Nagy and colleagues at the hypoxanthineguanine phosphoribosyl transferase locus in V79 Chinese hamster cells [36]. WR-1065 was added to cells before, during, or after treatment with cisplatin; additional cells were treated with cisplatin alone for comparison. Under all conditions, the induction of mutants was linear and a function of cisplatin concentration (Figure 7). The frequency of mutation per microgram of cisplatin was as follows: 25×10^{-7} for cells exposed to cisplatin alone, 1×10^{-7} for cells treated with WR-1065 prior to exposure to cisplatin, 5×10^{-7} for cells treated with WR-1065 during exposure to cisplatin, and 11×10^{-7} for cells treated with WR-1065 after exposure to cisplatin. These findings indicate that amifostine provides protection against the mutagenic effects of cisplatin and that protection is greatest when amifostine is administered before cisplatin.

Protection against radiation-induced mutagenesis was also observed by Grdina and associates [37, 38] using a similar model. The mutation frequency at the hypoxanthine–guanine phosphoribosyltransferase locus was decreased 2- to 5-fold.

To investigate the mechanism by which amifostine inhibits cisplatin-induced mutagenesis, Treskes and colleagues studied the effect of amifostine and its metabolites WR-1065 and WR-33278 on the formation and stability of cisplatin-DNA adducts *in vitro* [39]. In these studies, salmon sperm DNA was incubated with 25 μg/ml (83 μM) cisplatin in the presence or absence of the modulating agents. Platination in DNA exposed to cisplatin was inhibited by all three modulators. DNA platination was 26% that of controls in the presence of WR-1065, 37% that of controls in the presence of WR-33278, and 49% that of controls in the presence of amifostine. Amifostine and its metabolite WR-1065 exert their protective effects through inhibition of DNA platination in those tissues able to metabolise and accumulate them. Because amifostine is metabolised and accumulated more effectively in normal than in neoplastic cells, amifostine effects protection against cisplatin-induced DNA changes in normal cells without interfering with these changes in the tumour.

CONCLUSIONS

Amifostine selectively protects normal cells from early and late radiation injury and from the toxicities of various chemotherapeutic agents. Because amifostine is metabolised and accumulated in normal tissues to a much greater extent than in tumour

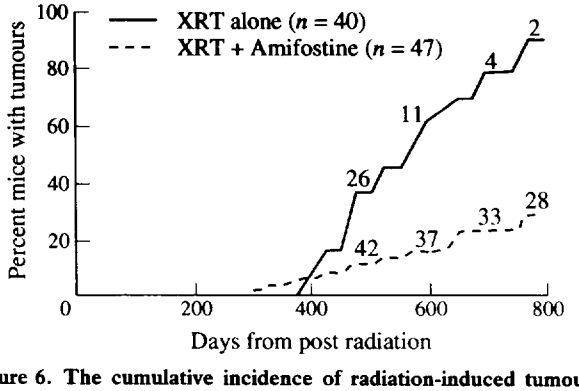

Figure 6. The cumulative incidence of radiation-induced tumours following irradiation (XRT) of the right hind legs of mice injected intraperitoneally with 400 mg/kg amifostine 30 min before irradiation and mice that did not receive amifostine (control). Numbers in the figure are the remaining mice without tumours. Reprinted with permission from Milas *et al. Cancer Res* 1984, 44, 5567–5569 [34].

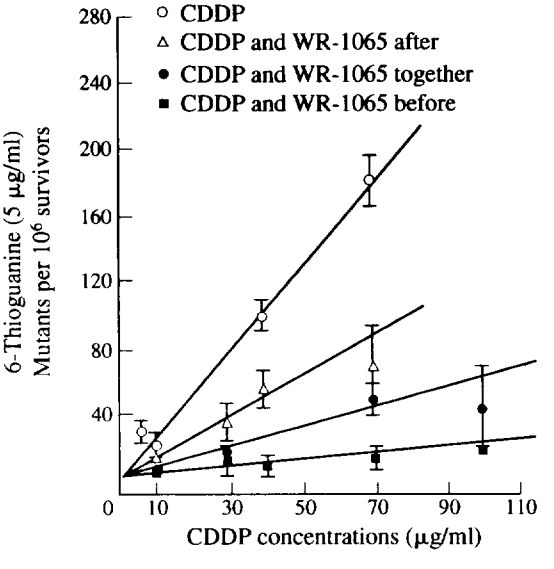

Figure 7. Mutagenesis as a function of cisplatin dose in V79 Chinese hamster cells treated with cisplatin alone or with cisplatin with amifostine added before, during, or after cisplatin treatment. Reprinted with permission from Nagy B *et al. Cancer Res* 1986, 46, 1132–1135 [36].

tissue, this agent provides protection from cytotoxicity to normal tissues, but not to the tumour. Amifostine is also effective in preventing radiation and chemotherapy-induced mutagenesis and carcinogenesis. It is of special interest that amifostine not only protects against radiation- and chemotherapy-induced myelotoxicity, but also against other dose-limiting toxicities such as nephro- and gastro-intestinal toxicity. The mechanism of myeloprotection seems to be different from that of various growth factors [5, 40]. This makes the combination of both growth factors and amifostine as protecting agents in conjunction with chemotherapy and radiotherapy very attractive. The safety and efficacy of such cancer treatments is expected to be enhanced [27, 41].

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