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ORIGINAL ARTICLE

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In vitro antioxidant properties of amifostine (WR-2721, Ethyol)

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Abstract *Purpose*: Amifostine (WR-2721), a phosphorylated aminothiol pro-drug which is an analogue of cysteamine, is a selective cytoprotective agent for normal tissues from the toxicities associated with chemotherapy and irradiation. Despite a growing number of reports strongly supporting amifostine's clinical efficacy, few authors have focused on the biochemical basis of amifostine's antioxidant activity. *Methods*: We report on amifostine's free-radical scavenging activity against superoxide ($O_{\dot{2}}^{-}$), hydroxyl (OH^{-}) and lipoperoxyl radicals in an in vitro model, using pure chemical systems. Amifostine was dephosphorylated to its active metabolite, WR-1065, by adding 10% non-heat-inactivated serum; different amifostine concentrations (1, 10, 50, 100 μ*M* and 200 μ*M*) and pH conditions (pH 5, 7.4 and 9) were tested. *Results*: Independent of the concentration, amifostine exhibited no major activity against $O_{\dot{2}}^{-}$ ions, neither did any pH variations in the experimental model provide any scavenger effects of the drug against $O_{\dot{2}}^{-}$ radicals. On the other hand, the protective effect of amifostine against OH^{-} radicals was confirmed, yielding an EC_{50} of 255 μ*M* at pH 7.4 and 230 μ*M* at pH 5. Finally, amifostine exhibited scavenging activity against spontaneous lipoperoxidation, but no apparent antioxidant effect on iron ascorbate-induced lipoperoxidation. *Conclusions*: With this in vitro study, we are able to confirm the scavenging activity of the chemo- and radioprotector amifostine, whose activity seems to be particularly important from a biological point of view, since it is exerted mainly against highly reactive OH^{-}.

Key words Amifostine · Superoxide radicals · Hydroxyl radicals · Lipoperoxyl radicals · Antioxidant properties

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Introduction

Amifostine (WR-2721), a phosphorylated aminothiol pro-drug, which is an analogue of cysteamine, is a selective cytoprotective agent for normal tissues from the toxicities associated with chemotherapy and irradiation [4]. Indeed, WR-1065, the major active metabolite of amifostine, which is selectively produced by normal cells through dephosphorylation by membrane-bound alkaline phosphatase at a preferentially neutral pH [4], provides cytoprotection by at least three different mechanisms. First, it can bind directly to, and thus detoxify, the active species of alkylating [5] and platinum agents [22]. Second, it acts as a potent scavenger of drug- or radiation-induced oxygen free radicals [17]. Third, when administered after exposure to radiation and/or several chemicals (and not before, as in the above two cases), it can markedly reduce injury-induced apoptosis [18].

Many anticancer drugs, like alkylating and platinum agents, are highly reactive electrophilic compounds, while other compounds bind iron ions in the tissues and thus generate reactive oxygen species (ROS), e.g. doxorubicin-derived superoxide anions, which have been implicated in doxorubicin-induced cardiotoxicity [15, 2]. The exposure of normal tissues to such free radicals and oxidants causes damage to lipids, proteins and DNA,

resulting in several changes and ultimately leading to unwanted cell death. In the past few years, amifostine has been extensively studied in several clinical trials and has shown different cytoprotective activities. For example, it can prevent or ameliorate cisplatin-induced nephrotoxicity [1], anthracycline-induced cardiotoxicity (through its scavenging activity on doxorubicin-produced oxygen free radicals in a reducing environment) [6], chemotherapy-related thrombocytopenia [3], radiation-induced tissue damage [21], etc. Despite the fact that a growing number of reports strongly support amifostine's clinical efficacy, to our knowledge few authors have focused on the biochemical basis of its antioxidant activity [17, 20, 9]. In this study, we investigated amifostine's free-radical scavenging activity against superoxide ($O_{\dot{2}}^{-}$), hydroxyl (OH^{-}) and lipoperoxyl radicals in an in vitro system.

Materials and methods

Chemicals

Amifostine (Ethyol) was donated by Schering-Plough, Milan, Italy. In the different experiments performed, amifostine was used immediately after vial opening to prevent drug degradation. All the other chemicals and reagents were purchased from Sigma Chemicals, St. Louis, Mo., USA.

Amifostine activation

Amifostine was dephosphorylated to its active metabolite WR-1065 by adding 10% non-heat-inactivated serum, which provided alkaline phosphatase, which is known to be intrinsic to the serum, as previously described by our own group as well as by other authors [4, 19, 14]. This dephosphorylation procedure was the same as the one we had previously used to document the selective cytoprotective effect of WR-1065 from cisplatin-induced cytotoxicity on human microvascular endothelial cells in vitro [14]. Different pH conditions (pH 5, 7.4 and 9) were also investigated, in order to assess the role of pH in the antioxidant activity of amifostine. Finally, the scavenging activity of amifostine was investigated when 1–200 μM of the drug reacted with ROS in different experimental conditions.

Superoxide radical scavenging activity

The degree to which amifostine quenched $O_{\dot{2}}^{-}$ was measured, as described by Halliwell [10]. Briefly, $O_{\dot{2}}^{-}$ were generated in vitro by the 500 μM hypoxanthine/0.01 U/ml xanthine oxidase system in a reaction mixture containing 20 μM cytochrome *c* at pH 7.4; amifostine's antioxidant activity was measured on per cent inhibition of the reduction of cytochrome *c* measured spectrophotometrically at 550 nm. Different amifostine concentrations (1, 10, 50, 100 μM and 200 μM) and pH conditions (pH 5, 7.4 and 9) were tested. Xanthine oxidase activity in the absence of cytochrome *c* was also evaluated by measuring the increase in absorbance at 290 nm due to ureate formation. This assay was included to ascertain that the tested compound did not interfere with xanthine oxidase activity.

Hydroxyl radical scavenging activity

Amifostine's scavenging activity against OH^{-} radicals was tested using the deoxyribose degradation method, originally described by Halliwell et al. [13]. Briefly, in this experimental model deoxyribose (3 mM) is degraded by the OH^{-} generated according to Fenton's reaction, by a mixture of ascorbic acid (100 μM), H_2O_2 (5 mM) and $FeCl_3$ (10 mM). Deoxyribose degradation products, reacting with thiobarbituric acid, develop thiobarbituric acid reactive substances (TBARs). This reaction is easy to quantify with the colorimetric test at 532 nm, according to Ohkawa et al. [16]. Different concentrations (1, 10, 50, 100 μM and 200 μM) of amifostine in phosphate buffer (20 mM) were then added to the reaction mixture to evaluate to what extent, if any, amifostine inhibited deoxyribose degradation by OH^{-}. Once again, different pH conditions (pH 5, 7.4 and 9) were tested.

Lipid peroxidation assay/Statistics

Lipid peroxidation was evaluated spectrophotometrically by measuring the production of TBARs at 532 nm according to Ohkawa et al. [16]; in this case, TBARs resulted from the lipoperoxidation of cell membranes by ROS. Briefly, rat brain homogenate diluted 1:2 in 0.9% NaCl was used in this assay as the source of lipid membrane bilayers. Brain homogenate was incubated at 37 °C for 1 h with or without 1, 10, 50, 100 μM and 200 μM amifostine (basal conditions); the reaction was stopped by adding 20% acetic acid. Peroxidation was then stimulated by adding 200 μM Fe^{2+}/250 μM ascorbate to the reaction mixture (stimulated conditions). The anti-lipoperoxidative action of amifostine was tested by measuring the drug's capability to decrease the formation of lipoperoxidation end products, particularly malonyldialdehyde (MDA). Lipid peroxidation was also evaluated colorimetrically by detecting the production of MDA and 4-hydroxynonenal (4-HNE) at 586 nm by means of the Esterbauer and Cheeseman method [7]. Values are expressed as the means ± standard error of the mean (SEM) of at least five experiments. Differences were statistically evaluated using the Student's *t*-test.

Results

Superoxide radical scavenging activity

Amifostine exhibited no major activity against superoxide ions, independent of the concentration, neither did any pH variations in the experimental model provide any scavenger effects of the drug against $O_{\dot{2}}^{-}$ radicals. Amifostine did not significantly decrease the rate of cytochrome *c* reduction relative to control conditions (no amifostine) in any of the experimental settings (Table 1).

Table 1 Inhibition of the reduction of cytochrome *c* by 1, 10, 50, 100 mM and 200 mM amifostine at pH 5, 7.4 and 9. Per cent values are plotted against control condition in absence of amifostine (100%). Values are the mean of at least five separate experiments with SEM ± 15%. *SEM* standard error of the mean

	Control	1 μM	10 μM	50 μM	100 μM	200 μM
pH 5% inhibition	100	89.61 ± 8.91	91.92 ± 12.34	105.51 ± 8.50	82.11 ± 9.23	92.61 ± 3.22
pH 7.4% inhibition	100	103.27 ± 3.42	93.81 ± 5.33	97.25 ± 5.11	92.90 ± 3.63	96.74 ± 4.89
pH 9% inhibition	100	105.89 ± 6.11	107.9 ± 7.51	102.84 ± 7.73	98.76 ± 10.02	94.32 ± 11.10

Hydroxyl radical scavenging activity

A progressive increase in the inhibition of TBAR formation is observed at pH 7.4 (Fig. 1a); the antioxidant effect of amifostine against OH^- radicals is even stronger at an acid pH (pH 5) where TBAR formation was significantly and progressively reduced by all the drug concentrations (Fig. 1b). The amifostine concentrations necessary to inhibit TBAR formation by 50% under the above experimental conditions (EC_{50}) were calculated by extrapolation and were 255 μM at pH 7.4 and 230 μM at pH 5. Finally, at pH 9 amifostine decreased TBAR production much less significantly; indeed, the resulting EC_{50} was so high that this figure clearly shows that the latter effect has little – if any – biological significance.

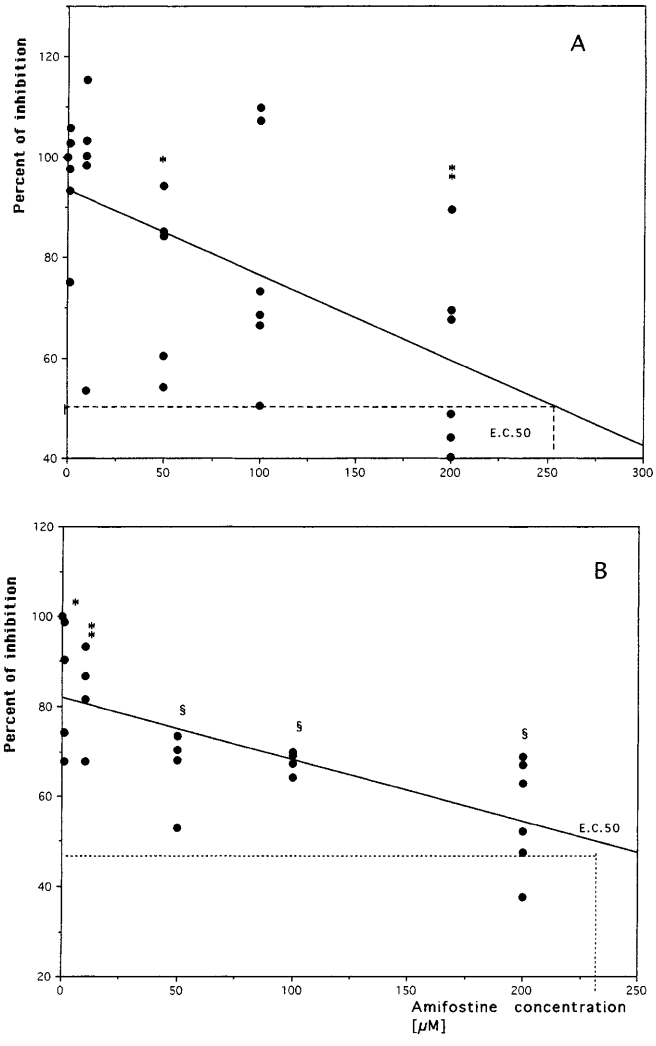

Fig. 1 **a** Rate of TBAR inhibition from deoxyribose degradation at different amifostine concentrations at pH 7.4 and calculation of the relative EC_{50}. Percent values are plotted against control condition in absence of amifostine (100%). Points are the mean of at least five separate experiments with SEM = 15%. Statistical analysis: $*P = 0.05$, $**P = 0.02$ versus control condition. **b** Rate of TBAR inhibition from deoxyribose degradation at different amifostine concentrations at pH 5 and calculation of the relative EC_{50}. Per cent values are plotted against control condition in the absence of amifostine (100%). Points are the mean of at least five separate experiments with SEM = 15%. *TBAR* thiobarbituric acid reactive substance, EC_{50} amifostine concentration necessary to inhibit TBAR formation by 50%, *SEM* standard error of the mean. $*P = 0.05$; $**P = 0.02$; $^{\S}P = 0.001$ versus control condition

Lipid peroxidation assay

As for amifostine's antioxidant action against cell membrane lipoperoxidation, TBAR production was progressively more inhibited under spontaneous conditions for amifostine doses ranging from 1 to 100 μM. It seems to decrease at 200 μM (Fig. 2), which confirms the efficacy of amifostine against spontaneous lipoperoxidation, but not any antioxidant effect on lipoperoxidation stimulated by adding an iron ascorbate mixture to the experimental system (Fig. 3).

Discussion

Despite the fact that amifostine, a chemo- and radioprotective agent of the thiol family, is increasingly used in clinical practice to minimize treatment-related toxicities [4], the biochemical bases of its antioxidant function have not been extensively studied. In an in vitro study, using a pure chemical system, Ohnishi et al.

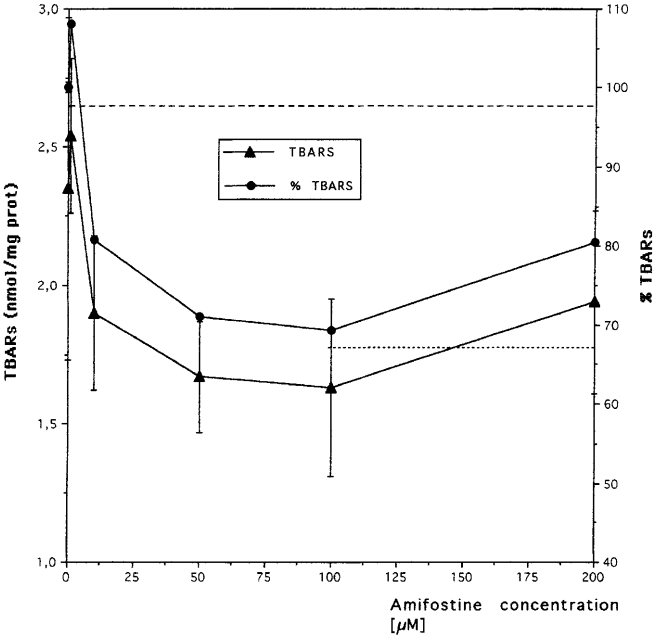

Fig. 2 Basal TBAR levels measured in rat brain homogenate in the presence of 1–200 μM amifostine. Basal TBAR values are expressed as nmol/mg protein and as the rate of inhibition of chromogenous substances relative to control. Values are the mean ± SEM of at least five separate experiments, and per cent values are plotted against control condition in the absence of amifostine (100%). *TBAR* thiobarbituric acid reactive substance, *SEM* standard error of the mean

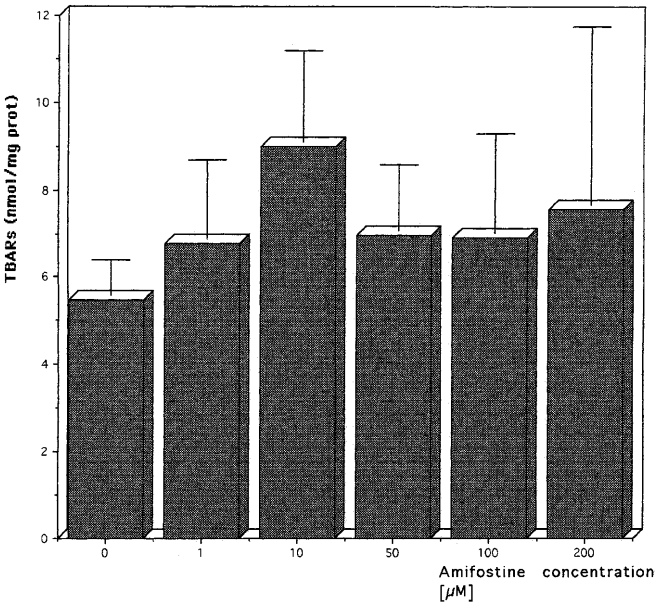

Fig. 3 Stimulated TBAR levels measured in rat brain homogenate in the absence or presence of 1–200 μ*M* amifostine. Stimulated TBAR values are expressed as nmol/mg protein and the rate of inhibition of chromogenous substances relative to control. Values are the mean ± SEM of at least five separate experiments. *TBAR* thiobarbituric acid reactive substance, *SEM* standard error of the mean

demonstrated that WR-1065, the active metabolite of amifostine, was able to scavenge hydroxyl radicals, superoxide anions and doxorubicin-derived superoxide anions [17]. The results of our study only partially confirm these characteristics of amifostine.

In fact, in none of the experiments performed did amifostine show major $O_{\dot{2}}^{-}$ scavenging action, while the protective effect of amifostine against OH^{-} radicals was confirmed, yielding an EC_{50} of 255 μ*M* at pH 7.4 and 230 μ*M* at pH 5. This different antioxidant behaviour may be explained by the different reaction capabilities of $O_{\dot{2}}^{-}$ and OH^{-}. $O_{\dot{2}}^{-}$ does indeed react poorly with intracellular structures and substances, such as lipids and antioxidants, the rate constant of $O_{\dot{2}}^{-}$ with glutathione being $< 7.7 \times 10^{5}\ M^{-1}\ s^{-1}$ [23].

In contrast, OH^{-} is an aggressive species, reacting with extremely high rate constants ($10^{9}\ M^{-1}\ s^{-1}$) with nearly every type of cell molecule, and also with disulfide compounds such as amifostine [12]. The observed decrease in the amount of TBARs from deoxyribose degradation in the presence of amifostine, suggests that OH^{-} radicals react first of all with the drug's thiol group, converting it into a relatively innocuous thiol radical. The known preferential activity of amifostine as a selective scavenger of OH^{-} appears to be the key element in the drug's efficacy, since OH^{-} radicals are surely the most dangerous ROS from a biological viewpoint. Indeed, they are extremely aggressive against cell structures and may both initiate and then self-propagate cell damage, while $O_{\dot{2}}^{-}$ seem to play a secondary role [11, 8].

Finally, amifostine exhibited only moderate antioxidant action on membrane lipids. Thus, the normal production of peroxide radicals in rat brain homogenate was 30% lower when amifostine was added, while no major effects were evidenced when lipid peroxidation was stimulated in vitro, suggesting limited antioxidant activity on lipoperoxidation. Regarding the different pH conditions, the antioxidant action of amifostine seems to increase at pH 5 and decrease at pH 9. This underlines the importance of careful checking of pH conditions during in vitro oxidation experiments. In conclusion, our study confirms the scavenging activity of the chemo- and radio-protector amifostine. This activity seems to be particularly important from a biological point of view, since it is exerted mainly against highly reactive OH^{-}.

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