Haematologica 2000; 85:820-825
original paper

Hemostatis & Thrombosis

Amifostine, a reactive oxygen species scavenger with radiation- and chemo-protective properties, inhibits *in vitro* platelet activation induced by ADP, collagen or PAF

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ABSTRACT

Background and Objectives. **Reactive oxygen species (ROS) generation has been suggested to represent an important regulatory mechanism of platelet reactivity in both physiologic and pathologic conditions; consistent with this hypothesis is the observation that free-radical scavengers may inhibit platelet activation, thus contributing to the regulation of their reactivity. The purpose of the present study is to study the *in vitro* effects of amifostine (WR-2721, ethyol™), a selective cytoprotective agent for normal tissues against the toxicities of chemotherapy and radiation, on platelet activation induced by the physiologic agonists ADP, collagen and PAF.**

Design and Methods. **The effect of amifostine, added to the experimental system at final concentrations ranging from 10^{-7} M to 10^{-5} M, was studied on platelet aggregation induced by the following physiologic agonists at the given concentrations: ADP (1 µM), collagen (2 µg/mL), and PAF (0.1 µg/mL). Platelet aggregation was investigated using a platelet ionized calcium aggregometer and was expressed as the percentage change in light transmission. Furthermore, thromboxane B_2 (TxB_2) levels and nitric oxide (NO) production were determined by radioimmunoassay and by evaluating the total nitrite/nitrate concentration using a commercially available colorimetric kit, respectively, both in the control system and after the addition of amifostine.**

Results. **Amifostine inhibited both platelet aggregation and TxB_2 production induced by ADP, collagen and PAF, in a dose-dependent manner. Amifostine proved to be an effective inhibitor of platelet function and the effect was more pronounced if platelets were stimulated with ADP, intermediate when collagen was the chosen agonist, and less evident, though present, when PAF was used. Platelets stimulated with ADP, collagen or PAF produced significant amounts of NO over the baseline. When amifostine was added at a final concentration of 5 µM, it significantly increased ADP, collagen and PAF-induced NO production, which suggests that NO release by activated platelets was involved in the inhibitory effect of amifostine.**

Interpretation and Conclusions. **Amifostine proved to be an effective inhibitor of platelet activation induced *in vitro* by physiologic inducers. This previously unrecognized effect was more evident with the weak agonist ADP and was related to reduced NO consumption by free radicals generated during platelet activation. Amifostine proved to be not only a powerful cytoprotectant, but, more generally, a therapeutic agent endowed with several relevant, though largely unknown, biological effects. Finally, our data once again support the concept that oxidative balance is of crucial importance in regulating platelet reactivity in both health and disease.**

Key words: amifostine, reactive oxygen species, platelet function, nitric oxide

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Reactive oxygen species (ROS), which include the superoxide anion (O_2^-) and the hydroxyl radicals (OH^-), together with hydrogen peroxide (H_2O_2) and the singlet oxygen ($^1\Delta gO_2$), are highly reactive substances that react with lipids, proteins and DNA, causing irreversible changes in their biomolecular structure. ROS also play an essential role in controlling various cell functions; indeed, they are intermediate metabolites in several enzyme reactions, are involved in post-translational protein turnover, and play a key role in the control of signal transduction.

Finally, ROS are also known to be able to activate platelet function[1-5] and thus play an important role in many vascular and thrombotic disorders, including atherosclerosis.[6-8] Conversely, many components of the vascular system, including leukocytes, monocytes, endothelial cells, and also platelets, especially when stimulated by different agonists, are sources of H_2O_2, O_2^- and OH^-.[9-11]

Therefore, ROS generation has been suggested to represent an important regulatory mechanism of platelet reactivity in both physiologic and pathologic conditions;[2,4] consistent with this hypothesis is the observation that free-radical scavengers may inhibit platelet activation, thus contributing to regulating their reactivity.[4,12-14]

Amifostine [NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-S-PO_3-H_2] is an organic thiophosphate pro-drug, an analog of cysteamine, originally developed during the cold war by the Walter-Reed Army Institute of Research as part of a US Army classified research project to identify potential agents to protect troops from radiation in the event of nuclear warfare.

Subsequently, it has been developed as a selective cytoprotective agent for normal tissues against the toxicities of chemotherapy and radiation.[15]

Amifostine provides cytoprotection by several different mechanisms, through the direct action of WR-1065, its major active metabolite which is selectively produced by normal cells through dephosphorylation by membrane-bound alkaline phosphatase at a preferentially neutral pH.[15]

Amifostine can bind directly to, and thus detoxify, the active species of alkylating[16] and platinum agents;[17] furthermore, when administered after exposure to radiation and/or several chemical agents, it can markedly reduce injury-induced apoptosis.[18] Finally, amifostine acts as a potent free-radical scavenger, targeting preferentially highly dangerous OH^- radicals, with a calculated EC_{50} of 255 µM at pH 7.4 and 230 µM at pH 5.[19] This latter activity seems the key to the pharmacologic action of the drug, since OH^- radicals are extremely aggressive against cell structures and may both initiate and then self-propagate cell damage.[19]

The purpose of the present study is to investigate the *in vitro* effects of amifostine on platelet activation induced by the physiologic agonists ADP, collagen and PAF.

Design and Methods

Platelet aggregation studies

Blood was obtained from apparently healthy subjects aged 20-35 years by puncture of the antecubital vein and gently mixed with trisodium citrate (9 volumes of blood and 1 volume of 3.8% trisodium citrate) in plastic tubes. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained as previously described elsewhere;[20] when necessary, PRP was diluted with PPP to give a final platelet concentration ranging from 2.5 to 3.0x10^8/mL.

Platelet aggregation was investigated by Born's method[21] using a platelet ionized calcium aggregometer (Chrono-Log, Havertown, PA, USA). PRP (0.5 mL) was pipetted in a siliconized glass cuvette and placed in the appropriate compartment of the aggregometer at 37°C. After 3 minutes the agonist was added and aggregation recorded; the aggregation response was expressed as the maximum of the first aggregation wave. Platelet aggregation inducers were used at the following final concentrations: ADP (Sigma, St. Louis, MO, USA) 1 µM, collagen (Mascia Brunelli, Milan, Italy) 2 µg/mL, and PAF (Sigma, St. Louis, MO, USA) 0.1 µg/mL.

In other experiments, platelets were preincubated for 3 minutes in the aggregometer cuvette with amifostine at final concentrations ranging from 10^{-7} M to 10^{-5} M before addition of the agonist. This preincubation allowed amifostine to be converted into its active metabolite WR-1065,[15] by platelet membrane-bound alkaline phosphatase.

Platelet aggregation was expressed as the percentage change in light transmission.

Thromboxane B_2 and nitric oxide assay

Thromboxane B_2 (TxB_2) levels were determined by radioimmunoassay using commercially available reagents (Amersham Italia, Milan, Italy), while nitric oxide (NO) production was measured by evaluating the total nitrite/nitrate concentration using a commercially available colorimetric assay (Cayman Chemicals, Ann Arbor, MI, USA).

PRP samples collected 6 minutes after addition of the agonists, when aggregation was complete, were centrifuged at 11,000 g for two minutes in an Eppendorf centrifuge and the levels of TxB_2 and NO were assessed in the supernatants. From these values we subtracted the levels of TxB_2 or NO in the supernatants of PRP treated in exactly the same way, but after addition of buffer alone without the agonists.

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) for repeated measures. A probability value of less than 0.05 was considered significant. Amifostine concentrations able to produce half-maximum inhibition of platelet aggregation and TxB_2 production (IC_{50}) were calculated by log-probit analysis.

Results

Effect of amifostine on platelet aggregation and TxB_2 production induced by different agonists

Amifostine inhibited both platelet aggregation and TxB_2 production induced by ADP, collagen and PAF, in a dose-dependant manner.

Indeed, as far as ADP-induced platelet aggregation and TxB_2 production are concerned, amifostine at lowest doses did not significantly modify platelet aggregation and TxB_2 production, while at higher concentrations, i.e., 10^{-5} M for platelet aggregation and 10^{-6} and 10^{-5} M for TxB_2 production, it significantly inhibited the two events (Figures 1 and 2).

Again, when collagen was considered, amifostine proved to be able to inhibit both platelet aggregation and TxB_2 production significantly when added at high concentrations, i.e., 10^{-5} and 5x10^{-5} M (Figures 3 and 4). Finally, the same effect was recorded for PAF-induced platelet aggregation and TxB_2 production, amifostine being able to inhibit these events significantly at concentrations of 10^{-5} and 5x10^{-5} M (Figures 5 and 6).

These results clearly show that amifostine is an effective inhibitor of platelet function. This effect is more pronounced if platelets are stimulated with ADP, intermediate when collagen is the chosen agonist, and less evident, though present, when PAF is used. Furthermore, TxB_2 production appears to be inhibited slightly more efficiently than aggregation. IC_{50} for inhibition of both parameters after platelet activation with the above different agonists are reported in Table 1.

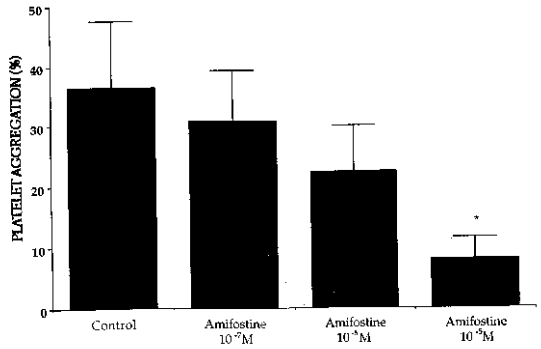

Figure 1. Effect of amifostine at different concentrations on platelet aggregation induced by 1 µM ADP (means ± S.E. of 5 independent experiments). *$p < 0.02$ versus control.

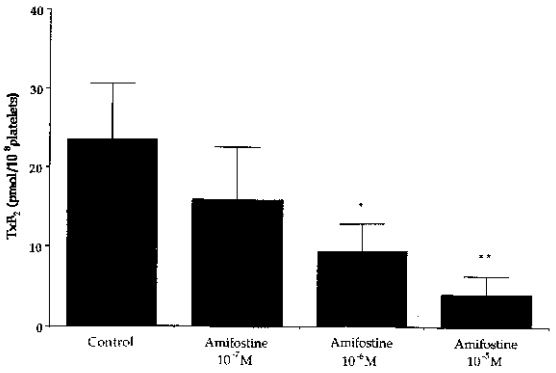

Figure 2. Effect of amifostine at different concentrations on TxB_2 production induced by 1 µM ADP (means ± S.E. of 5 independent experiments). *$p < 0.05$ versus control; **$p < 0.01$ versus control.

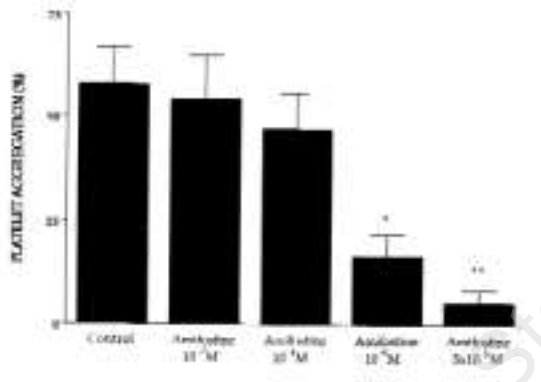

Figure 3. Effect of amifostine at different concentrations on platelet aggregation induced by 2 µg/mL collagen (means ± S.E. of 5 independent experiments). *$p < 0.02$ versus control; **$p < 0.01$ versus control.

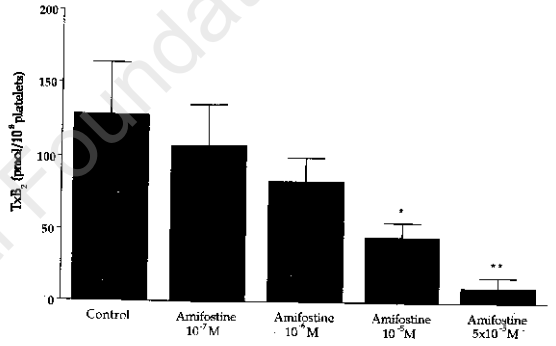

Figure 4. Effect of amifostine at different concentrations on TxB_2 production induced by 2 µg/mL collagen (means ± S.E. of 5 independent experiments). *$p < 0.05$ versus control; **$p < 0.01$ versus control.

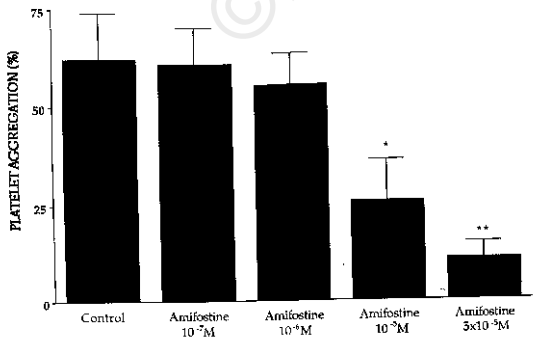

Figure 5. Effect of amifostine at different concentrations on platelet aggregation induced by 0.1 µg/mL PAF (means ± S.E. of 5 independent experiments). *$p < 0.05$ versus control; **$p < 0.01$ versus control.

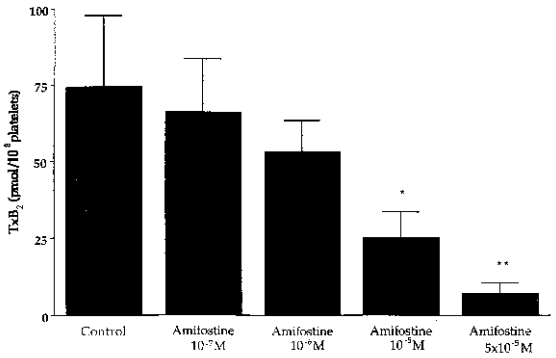

Figure 6. Effect of amifostine at different concentrations on TxB_2 production induced by 0.1 µg/mL PAF (means ± S.E. of 5 independent experiments). *$p < 0.05$ versus control; **$p < 0.01$ versus control.

Table 1. Concentrations of amifostine able to produce 50% inhibition (IC_{50}) of platelet activation induced by ADP, collagen or PAF.

Agonist	*Platelet function*	*IC_{50} (mM)*
ADP 1 µM	Aggregation	1.57
	TxB_2 production	0.83
Collagen 2 µg/mL	Aggregation	2.58
	TxB_2 production	1.98
PAF 0.1 µg/mL	Aggregation	4.15
	TxB_2 production	3.61

Table 2. Effect of amifostine (5 µM final concentration) on agonist-induced NO production by platelets (means ± SE of 5 independent experiments).

	NO (pmol/10^8 platelets)		
Agonist	*Control*	*Amifostine*	*p*
ADP 1 µM	51.0±6.6	85.3±11.7	< 0.05
Collagen 2 µg/mL	84.0±13.8	147.4±18.2	< 0.02
PAF 0.1 µg/mL	47.3±7.8	91.4±16.9	< 0.02

Effect of amifostine on NO production by platelets stimulated with different agonists

Platelets stimulated with ADP, collagen or PAF produced significantly increased amounts of NO compared to baseline values. When amifostine is added at a final concentration of 5 µM, it significantly increased ADP, collagen and PAF-induced NO production, with resulting mean concentrations of 85.3, 147.4 and 91.4 pmol/10^8 platelets, respectively (vs. 51.0, 84.6 and 47.3, respectively, of controls), as shown in Table 2. This effect suggests that NO release by activated platelets is involved in the inhibitory effect of amifostine.

Discussion

Vast experimental evidence indicates that platelets are activated upon exposure to ROS-generating systems, including those derived from polymorphonuclear leukocytes and red blood cells.[22]

Indeed, the effects of ROS on platelet function have been extensively studied in the past years by means of chemical- and cellular-generated fluxes of free radicals. Back in 1977, Handin *et al.* demonstrated that $O2^-$ causes platelet aggregation and induces serotonin release, while superoxide dismutase (SOD) prevents superoxide-induced platelet activation;[23] more recent experimental evidence indicates that H_2O_2 potently favors the activation of platelets primed with arachidonic acid or collagen;[24] furthermore, the activation of platelets exposed to H_2O_2 was demonstrated to be mediated by OH^- formed in an extracellular Fenton-like reaction.[5]

The molecular mechanisms by which ROS interact with platelet function have also been extensively investigated. Briefly, oxidative stress activates tyrosine kinases associated with the plasma membrane, thus inducing tyrosine phosphorylation which in turn phosphorylates MAP-kinase. This kinase subsequently phosphorylates and activates cytosolic phospholipase A_2 (PLA_2) and produces an increase in substrate, i.e., arachidonic acid, for the enzyme prostaglandin endoperoxide synthase.[22]

Alternatively, ROS may affect platelet function by reacting with NO, a natural vasodilating substance released by endothelium, in response to stimulation by a variety of endogenous substances, and also by platelets, where it inhibits aggregation by increasing cGMP.[25-27] The biological activity of NO is limited by the concurrent presence of $O2^-$, which reacts rapidly with NO in acqueous solution to form the peroxinitrite anion ($OONO^-$), which in turn decomposes to HO^- at neutral pH.[28,29]

Taken together, these findings suggest that ROS behave as stimulators of platelet aggregation by acting at different levels of the signal transduction pathways, i.e., by inhibiting NO and by stimulating arachidonic acid metabolism, Ca^{++} influx and tyrosine kinases.[22]

Within this complex context, free-radical scavengers might interrupt ROS-induced platelet activation, leading to decreased platelet aggregation. In fact, a number of antioxidants exhibited such an effect both *in vitro* and *in vivo*, including natural substances such as vitamin E[30] and ascorbic acid,[31] synthetic antioxidants such as butylated hydroxyanisole[32] and butylated hydroxytoluene,[33] and therapeutically active substances such as the antiplatelet and vasodilator drug dipyridamole;[34] glutathione (GSH) had already proved to be an inhibitor of both platelet activation and TxB_2 production induced by the physiologic agonists ADP, collagen and PAF.[14]

In this study, we demonstrated that amifostine, a broad-spectrum cytoprotectant which has recently been added to our armamentarium of anticancer agents to minimize the toxixity of both chemotherapy and radiation on normal tissues,[35] not only acts as an anti-apoptotic, pro-differentiating drug,[36,37] but could act as an effective inhibitor of platelet activation induced by physiologic stimuli.

Indeed, amifostine proved to be a stronger inhibitor of platelet aggregation than GSH, at least *in vitro*, having IC_{50} values for the different agonists tested in the micromolar range, instead of the millimolar range previously evidenced for GSH.[14]

The platelet antiaggregating effect of amifostine is more evident with the weak agonist ADP and proved to be related to reduced NO consumption by free radicals generated during platelet activation.

Thus, once again amifostine proved to be not only a powerful, though still little studied, cytoprotectant, but, more generally, a therapeutic agent exhibiting a number of relevant, though largely unknown, biological effects. Its antiaggregating properties, for example, deserve further studies, especially *in vivo*. Furthermore, since platelet activation plays a key role in the process of tumor invasion and metastasis[38] and a number of anticoagulants and antiaggregating

agents have consequently been investigated for a possible antineoplastic activity,[39,40] it should be investigated whether amifostine has potential effects in cancer patients different from those already well acknowledged.

Finally, the data reported here once again support the concept that oxidative balance is of crucial importance in the regulation of platelet reactivity, and that its manipulation could thus represent a reasonable target of pharmacologic intervention to treat conditions related to platelet hyperaggregation.

Contributions and Acknowledgments

CP and GG had the original idea for the study, AM and AT performed all experiments; CP wrote the paper, while GG supervised it and, as Senior Author, is cited last. We gratefully thank Dr. Alfonso Ferrarini and the late Dr.ssa Stefania Monselice of Schering-Plough, Italy, who provided us with the amifostine.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received March 3, 2000; accepted June 19, 2000.

Potential implications for clinical practice

- The data here reported support the concept that oxidative balance is of crucial importance in the regulation of platelet reactivity, and that its manipulation could thus represent a target of pharmacologic intervention to treat conditions related to platelet hyperaggregation.
- Amifostine proved to be not only a powerful, though still little studied, cytoprotectant, but, more generally, a therapeutic agent exhibiting a number of relevant biological effects.
- Since platelet activation plays a key role in the process of tumor invasion and metastasis, the role of amifostine in cancer patients should be investigated for potential effects different from those already well acknowledged.

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