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Short communication

Isotachophoretic determination of phosphate splitting from Amifostine and *p*-nitrophenyl phosphate in serum and neuroblastoma cells

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Abstract

Amifostine [WR-2721; H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-S-PO_3H_2] is used as a protecting agent in the chemotherapy of neuroblastoma. It is supposed that Amifostine will be transformed into its active form, the free thiol (WR-1065), easier by normal cells than by tumour cells. Analytical capillary isotachophoresis was used to determine the dephosphorylation of Amifostine in serum and on neuroblastoma cells and peripheral blood cells. Furthermore, the biological effects of Amifostine and its free thiol, on cell proliferation of neuroblastoma cells were measured in combination with Carboplatin. It was found that neuroblastoma cells did not split phosphate less efficiently than normal peripheral blood cells. Furthermore, neither Amifostine (as expected) nor the free thiol (not expected according to the theory) were able to inhibit the effects of Carboplatin. Therefore, the current hypothesis concerning the mode of action of Amifostine must be questioned.

Keywords: Pharmaceutical analysis; Amifostine; Nitrophenyl phosphate

1. Introduction

The pro-drug Amifostine {WR-2721; ethanethiol, 2-[(3-amino-propyl)amino]ethyldihydrogen-monothiophosphate}, is currently widely used in cancer therapy [1] e.g., in the therapy of neuroblastoma with Carboplatin. It is supposed that it protects selectively normal cells, but not cancer cells, from chemotherapy and radiotherapy [2,3], Fig. 1. Presumably only the active metabolite, the free thiol (WR-1065), will be incorporated into cells. It is assumed that phosphate (P) is split from Amifostine at the tissue side by membrane-bound alkaline phosphatase (AP) and that tumour cells have a reduced AP activity compared to normal cells [4]. The acidic pH found in many tumours may further reduce this effect [5]. The aim of the present study was to answer the following questions using analytical isotachophoresis: is Amifostine a good substrate for AP? Is it significantly activated by serum AP? And finally, is there a difference between AP activity on neuroblastoma cells and peripheral blood

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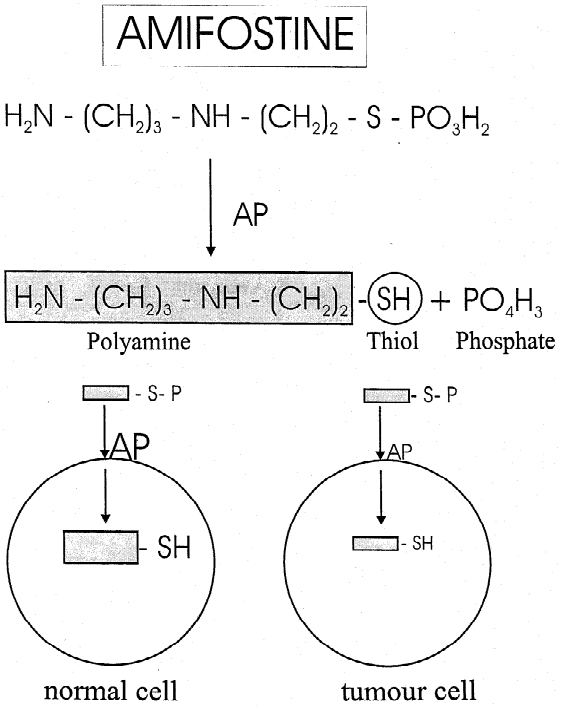

Fig. 1. Supposed mechanism for the selectivity of Amifostine. Only the active metabolite, the free thiol, is taken up by cells. The free thiol is formed at the tissue side by membrane-bound alkaline phosphatase; tumour cells have a reduced activity compared to normal cells.

cells? Furthermore, the influence of Amifostine and its free thiol on the proliferation of neuroblastoma cells in the absence and presence of Carboplatin was investigated.

2. Experimental

2.1. Chemicals

Alkaline phosphatase from human placenta (15 U/mg) and *p*-nitrophenyl phosphate (p-NP) were from Sigma (Munich, Germany); Amifostine (WR-2721), the free thiol (WR-1065) and its disulfide form (WR-33278) were a gift from US Bioscience (West Conshohocken, PA, USA). Carboplatin was obtained from Ribosepharm (Munich, Germany).

2.2. Isotachophoresis (ITP)

ITP was carried out with a column coupling device equipment [ITA-CHROM EA 101 (J&M, Aalen, Germany)] according to Everaerts et al. [6]. A pre-column (capillary: 90 mm×0.8 mm I.D.×1.2 mm O.D.) and a separating column (160 mm×0.3 mm I.D.×0.7 mm O.D.) were used, both made of fluorinated ethylene–propylene polymer. Preseparation was carried out at 250 μA, the analysis at 50 μA (analysis of anions), and at 200 μA and 40 μA for the analysis of cations, respectively. For anionic analysis, 0.01 *M* HCl–histidine (pH 6) was used as leading electrolyte (L) and 0.005 *M* caproic acid–histidine (pH 6) as terminating electrolyte (T). For cationic analysis (WR-1065 and WR-33278), 0.01 *M* ammonium acetate (pH 5.2) was used as L and 0.01 *M* acetic acid (pH 3) as T (all from Merck, Darmstadt, Germany).

2.3. Incubation conditions

Splitting of phosphate from Amifostine and p-NP was carried out by incubation of both substances (5 m*M*) in diethanolamine buffer (pH 9.8) with 100 U/L alkaline phosphatase for 0 to 60 min. The mixture was subsequently diluted 1:10 with 0.01 *M* HCl; 30 μl were injected into the ITA-CHROM system.

Splitting of phosphate from 10 m*M* Amifostine in serum of healthy donors containing AP in the normal range (79–270 U/L; 37°C) was followed for 0 to 60 min. The reaction was stopped by addition of a 20-fold excess of 0.01 *M* HCl.

For determination of AP activity on peripheral blood cells and neuroblastoma cells, $40 \cdot 10^6$ cells/ml were incubated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline, containing Mg^{2+}, Ca^{2+} and glucose, with 10 m*M* p-NP for 30 min at 37°C. After centrifugation, the supernatant was diluted 1:20 with 0.01 *M* HCl prior to isotachophoretic analysis. Peripheral mononuclear blood cells (PMBCs) and granulocytes were obtained by standard gradient centrifugation procedures [7]. The human neuroblastoma cell line SK-N-LO was normally grown as a monolayer culture,

but was used as suspension culture for these experiments.

2.4. Cytotoxic assay

SK-N-LO cells were grown as monolayers in 96-well plates. They were incubated with 0.1–1 m*M* Amifostine (WR-2721), its free thiol (WR-1065) and the disulfide form [WR-33278: H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-S-S-$(CH_2)_2$-NH-$(CH_2)_3$-NH_2] for 30 min. After removal of the incubation medium, cells were incubated for further three days in the absence or presence of Carboplatin. Surviving cells were analyzed with the MTT test according to Mosman [8].

3. Results

3.1. Isotachophoretic characterization of amifostine

Amifostine could not be determined directly neither by the anionic nor by the cationic system used in this study, but indirectly by registration of the splitted phosphate (anionic system) and the formation of the thiol (cationic system). Purity and stability of the substance during storage and freezing procedures were determined in this way after establishment of the respective calibration curves. The amount of the free thiol during storage at 4°C for a time period of two months was below 5%. The free thiol is stable for some hours at room temperature, but was considerably converted to its disulfide form after freezing and after incubation with H_2O_2. Fig. 2 shows as an example the cationic isotachopherogram of the thiol (WR-1065) and its oxidized form, the disulfide (WR-33278). Thirty μl of a 250 μ*M* solution of the thiol was injected after a freezing period of two days. In the example given, about 35% of the free thiol was converted to its disulfide form.

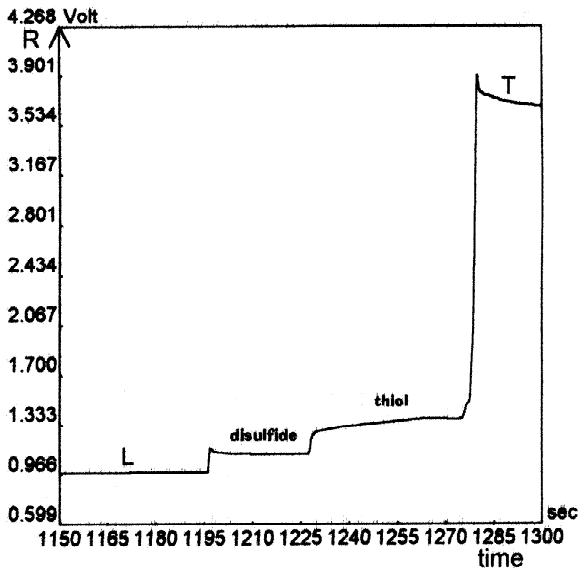

Fig. 2. Isotachopherogram (cationic system) of the free thiol (WR-1065) and its disulfide form (WR-33278).

3.2. Splitting of phosphate from p-NP and Amifostine by AP

p-NP is a widely used substrate in clinical chemistry for registration of AP activity. AP activity of the pure enzyme, enzyme present in serum and on cell surfaces was determined by registration of phosphate formation or the determination of remaining p-NP (Fig. 3). Phosphate cleavage from Amifostine was found to be reduced compared to p-NP (Fig. 4).

For clinical application, it is important to know in which proportions cleavage of P takes place in blood after intravenous (i.v.) injection of Amifostine. Using sera with AP activities in the normal clinical range (200 U/L), P is only moderately splitted from Amifostine (about 6% after 60 min).

3.2.1. AP activity on neuroblastoma cells compared to blood cells

It is supposed that Amifostine can enter cells only after cleavage of phosphate, and that this splitting takes place to a greater extend on normal cells compared to tumour cells, explaining the selectivity of protection. Since Amifostine is used in our clinic in the high dose chemotherapy of neuroblastoma [9], we tested AP activity on a human neuroblastoma cell line compared to blood cells. In both cell systems only a small splitting of phosphate (using p-NP as substrate) was observed (Fig. 5). Furthermore, an acidic pH environment that may be present in the tumour area, did not further reduce this process

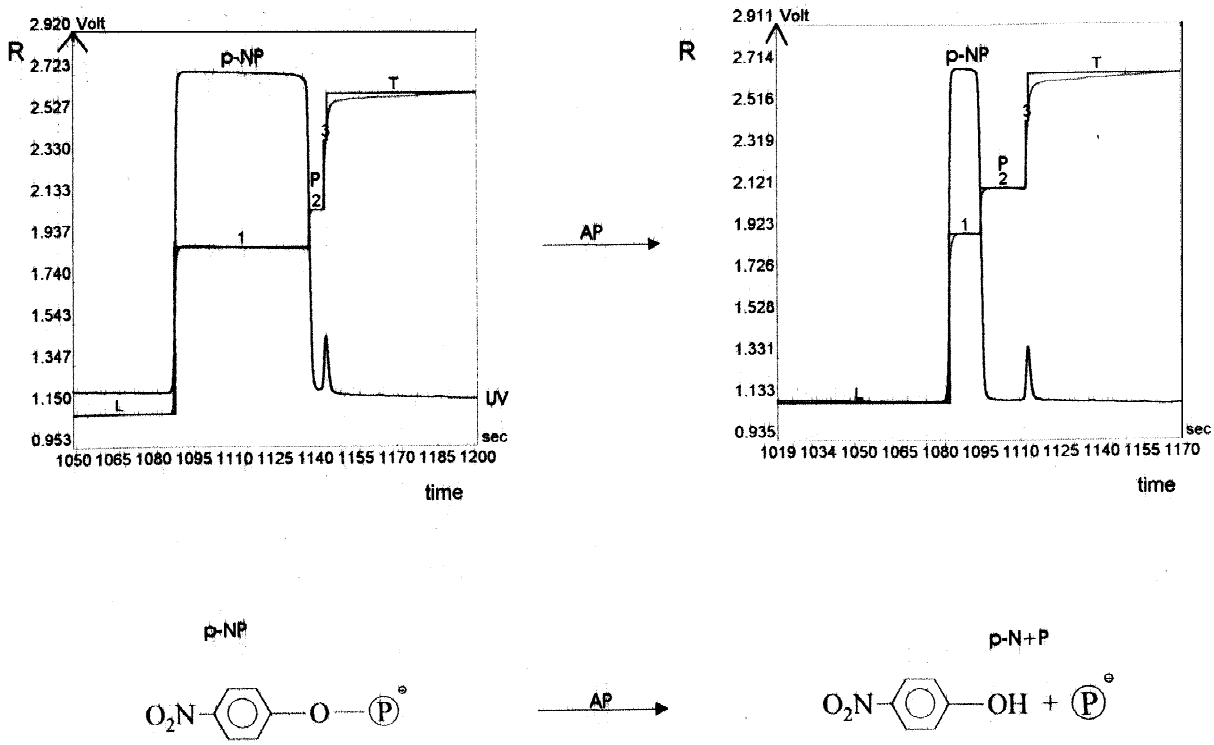

Fig. 3. Decrease of p-NP and increase of phosphate (P) after a 30-min incubation time with alkaline phosphatase in diethanolamine buffer. The phosphate zone present at t_0 represents impurities of the commercially available p-NP.

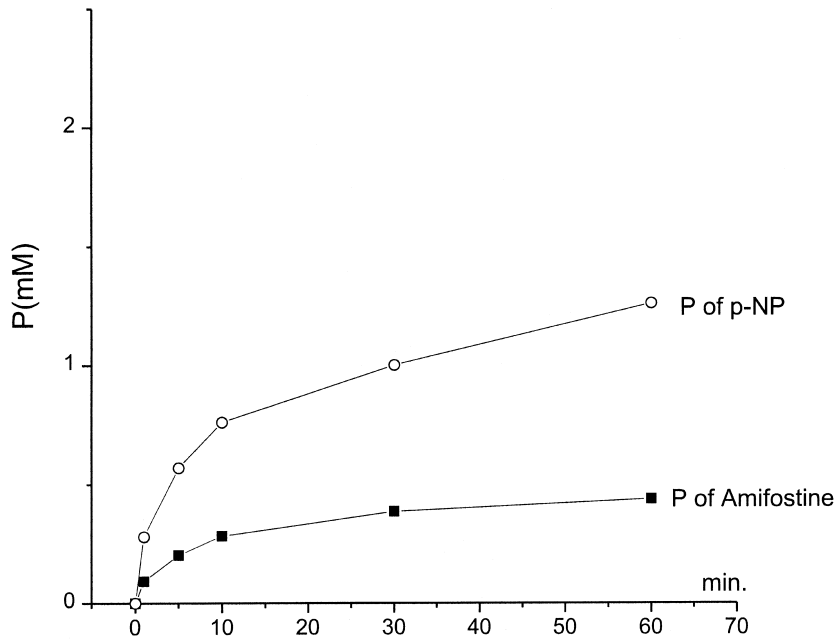

Fig. 4. Comparison of the phosphate cleavage from p-NP and Amifostine during incubation with 100 U/L alkaline phosphatase in diethanolamine buffer, pH 9.8

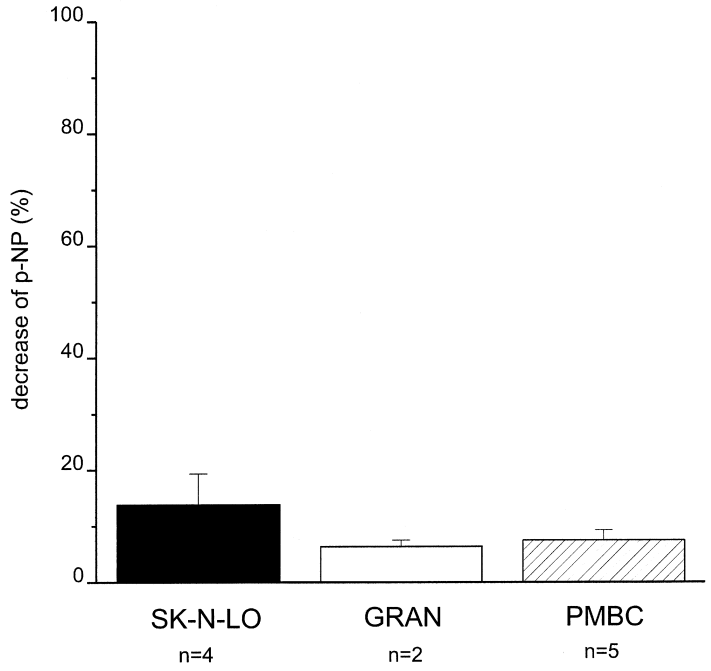

Fig. 5. Splitting of phosphate from p-NP after a 30-min incubation time in the presence of SK-N-LO cells, granulocytes (GRANs) and peripheral mononuclear blood cells (PMBCs)

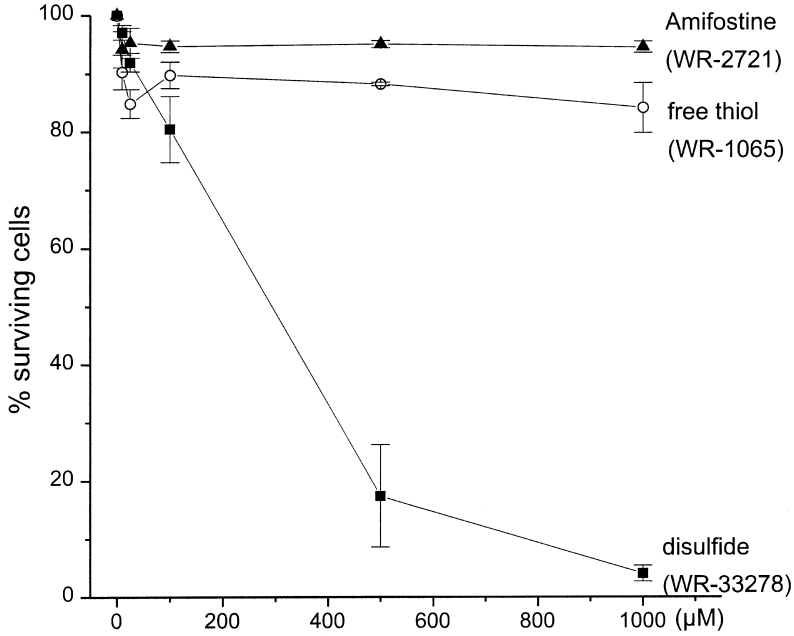

Fig. 6. Concentration-dependent cytotoxicity of Amifostine (WR-2721), its free thiol (WR-1065) and the disulfide form (WR-33278) on the neuroblastoma cell line SK-N-LO (MTT test; mean±SD; $n=3$).

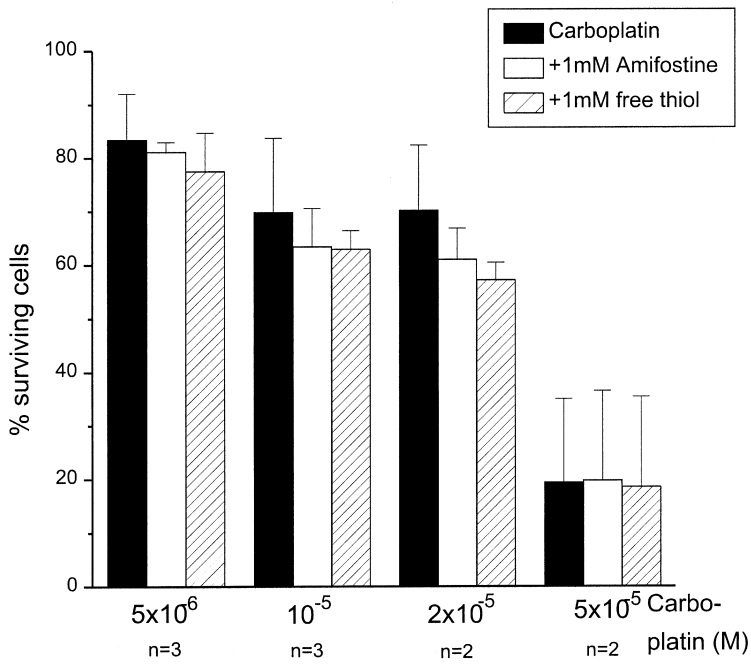

Fig. 7. Influence of Amifostine and its free thiol on the cytotoxicity of Carboplatin on the neuroblastoma cell line SK-N-LO (MTT test; mean±SD).

(SK-N-LO cells were incubated at pH 6.2 compared to pH 7.2, data not shown).

3.2.2. Biological effects of Amifostine and its metabolites on neuroblastoma cells in the absence and presence of Carboplatin

In contrast to the disulfide form WR-33278, neither Amifostine nor the free thiol influenced the proliferation of neuroblastoma cell line SK-N-LO (Fig. 6). Since Carboplatin is used in the high dose treatment of neuroblastoma [9] in combination with Amifostine, we tested the effects of Amifostine and its active thiol in combination with Carboplatin on the neuroblastoma cell line SK-N-LO. According to the theory (Fig. 1), it could be expected that the free thiol, but not Amifostine, is able to protect neuroblastoma cells from the toxic effects of Carboplatin. However, both Amifostine and its thiol did not influence the growth of SK-N-LO cells (Fig. 7). The same results were obtained with two other neuroblastoma cell lines, SK-N-SH and Sima.

4. Discussion

According to the literature [1,2,3] Amifostine protects normal cells better than tumour cells from chemotherapy and radiotherapy by increased formation of the protecting free thiol. However, in our studies presented in this paper, no evidence for this hypothesis could be obtained. Compared to the standard substrate p-NP, Amifostine is only a weak substrate for AP, and it is only slightly activated by AP present in serum. Indeed, phosphate splitting activity on neuroblastoma cell membrane proved to be rather small, but was not reduced compared to peripheral blood cells, and no further decrease at acidic pH could be observed. Furthermore, according to the literature, it could be expected that the free thiol WR-1065, but not Amifostine, should reduce the cytotoxicity of Carboplatin. However, neither Amifostine (expected) nor the free thiol (not expected according to the current hypothesis) reduced the toxicity of Carboplatin against neuroblastoma cells. Therefore, this proposed mechanism for the

selective action of Amifostine on normal cells compared to tumour cells must be questioned.

Acknowledgements

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References

[1] R. Kath, H.D. Peters, K. Höffken, Onkologe 2 (1997) 191.
[2] R.L. Capizzi, Seminars Oncol. 23 (1996) 2.
[3] C.M. Spencer, K.L. Goa, Drugs 50 (1995) 1001.
[4] P.M. Calabro-Jones, J.A. Aguilera, J.F. Ward, Cancer Res. 48 (1988) 3634.
[5] J.L. Yang, D.J. Fernandes, L. Speicher, Proc. Am. Ass. Cancer Res. 36 (1995) 290.
[6] F.M. Everaerts, Th.P.F.M. Verheggen, F.E.P. Mikkers, J. Chromatogr. 169 (1979) 21.
[7] C.E. Gerber, S. Kuci, M. Zipfel, D. Niethammer, G. Bruchelt, Eur. J. Chem. Clin. Biochem. 34 (1996) 901.
[8] T. Mosman, J. Immunol. Methods 65 (1983) 55.
[9] Bone Marrow Transplantation Protocol for Neuroblastoma, Children's Hospital, University of Tübingen, Tübingen, 1998.