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Effects of amifostine (WR-2721, ethyol) on tumor growth and pharmacology of cytotoxic drugs in human xenotransplanted neuroblastomas

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Amifostine was developed as a radio- and chemoprotective agent. It has shown protection against whole-body irradiation, and myelo- and nephrotoxicity of cytotoxic agents both in experimental and clinical studies. Some experimental trials revealed an influence of amifostine on tumor growth or the activity of cytotoxic drugs under certain circumstances. Therefore, it was the aim of our work to evaluate the pharmacological potential of amifostine in a preclinical *in vivo* situation with human xenotransplanted neuroblastomas. Human neuroblastoma cells (IMR5-75 and Kelly) were grown s.c. as xenografts in nude mice to palpable sizes (approximately 4 × 5 mm). Then the animals received 200 mg/kg amifostine i.p. and were treated 30 min later with one of the following cytotoxic drugs: cyclophosphamide, doxorubicin, cisplatin, ifosfamide, vincristine and etoposide. Amifostine as the only treatment did not influence the growth of the neuroblastomas IMR5-75 and Kelly. We observed no side effects of the compound itself. In no case did amifostine interact significantly with the antitumor effect of any cytostatic used in combination. However, amifostine mitigated the body weight loss induced by vicristine and the leukopenia induced by cyclophosphamide, cisplatin or ifosfamide, respectively. The side effects of the remaining cytostatics were—if observed at all—unchanged. We conclude that amifostine did not influence the tumor growth of xenotransplanted neuroblastomas and did not reduce the antineoplastic activity of the tested cytostatic drugs. Further investigation of amifostine as a protectant from side effects of chemotherapy in a clinical setting is warranted.

Key words: Amifostine, chemoprotection, cytostatics, neuroblastoma, side effects, xenografts, tumor growth.

Introduction

Amifostine was developed as a cytoprotective compound for normal tissues from side effects of chemo- or radiation therapy. It was selected from a panel of more than 4000 candidates by a program of the US Army. Amifostine (WR-2721) is a prodrug which is converted to a free thiol compound by membrane bound alkaline phosphatase. The active metabolite, WR-1065, is transported into the cells and exerts its effect as a scavenger of free radicals generated by the action of cytostatics, especially alkylating agents. The preferred selectivity of amifostine for normal compared to tumor cells is not fully enlightened, but to date the following facts are discussed as probable mechanisms:[1,2]

(1) Normal cells have a higher activity of alkaline phosphatase and therefore favor the formation of the active thiol.[3,4]
(2) The higher pH of normal cells intensifies both thiol formation and intracellular uptake.[5]
(3) WR-1065 is involved in an enzymatic DNA repair mechanism which leads to an accumulation of cells in the G_2/M phase;[6] it is suggested that differences in repair mechanisms between tumor and normal cells contribute to a different sensitivity towards thiol compounds.
(4) Amifostine promotes the growth of hematopoietic progenitors and in this way is able to counteract the bone marrow toxicity of cytostatic drugs.[7,8]
(5) The protective effect of amifostine is dependent on oxygen supply in the tissues; therefore, protection of hypoxic cells of solid tumors is reduced.[9]

Amifostine is eliminated from blood circulation after i.v. injection within 30 min; maximum concentrations in tissues are measured 15 min after administration.[10] The active thiol compound accumulates in experimental tumors at a lower rate than in other tissues. Therefore, it is proposed for amifostine to be administered 15–30 min prior to chemo- or radiation chemotherapy. The protection of normal tissues but not tumors has been proven in several preclinical and clinical studies.[11,12] In those investigations the bone marrow and nephrotoxicity of alkylating and platinum agents was especially mitigated by co-

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administration of amifostine. In most experimental systems tumor growth inhibition by cytostatic drugs was not reduced in the presence of amifostine.[13,14] Only a few studies reported a reduced antineoplastic effect of chemotherapy when the protector was co-administered, e.g. for Walker 256 carcinosarcoma in a rat model[15] and for some murine tumors.[16] Clinical randomized trials were conducted in ovarian, rectal, lung, head and neck carcinomas, and melanomas. No evidence of tumor protection was shown in these studies both concerning the negative effects on response rates to antineoplastic therapy or the survival of patients.[17]

Amifostine appears to be an interesting substance for neuroblastoma patients because of the considerable hemato- and nephrotoxic side effects of the current chemotherapeutic regimens. To exclude possible interactions of cytostatic with protective treatment we initiated preclinical investigations.

Studies in two neuroblastoma cell lines *in vitro* revealed that both amifostine and its active metabolite WR-1065 did not reduce the cytostatic potential of six commonly used drugs.[18]

The aim of the present investigations was to study the pharmacological effects of amifostine on human neuroblastomas xenotransplanted to nude mice. We were especially interested in facts concerning the influence of the protectant on tumor growth and its possible interaction with cytostatic treatments both concerning antitumor and side effects.

Materials and methods

Mice and tumor models

Two neuroblastomas were selected from a panel of several cell lines for *in vivo* investigations because of a 100% take and a sufficient growth rate as xenotransplants in nude mice. The tumors IMR5-75 and Kelly are characterized by a 25- or 100–120-fold N-*myc* amplification, respectively.[19] Since the two tumors grew in a sex-dependent manner we used female Ncr: *nu/nu* for IMR5-75 and male NMRI: *nu/nu* mice for Kelly.

Tumor fragments of 1–2 mm in diameter were transplanted s.c. to adult nude mice. The mice were held under sterile conditions at 24–26°C room temperature, 50% relative humidity and a 12 h light–dark rhythm in laminar flow shelves. They received autoclaved food (Sniff, Soest, Germany) and bedding. The drinking water was filtered and acidified (pH 4.0). Tumor size was measured twice weekly with a calliper-like instrument in two dimensions. Individual tumor volumes (V) were calculated from these values by the formula $V = (\text{length} + [\text{width}]^2)/2$ and related to the values of treatment day (relative tumor volume, RTV). At each measurement day treated/control values (T/C) were calculated as percentage; the optimum values obtained within 4 weeks after treatment were used for evaluation of the efficacy and are presented in the corresponding tables. The body weight of mice was determined twice weekly and related to the weight of treatment day (body weight change). Between 3 and 4 days after cytostatic treatment blood was taken from the retro-orbital venous plexus and blood parameters [white blood cell counts (WBC), erythrocytes, platelets, hemoglobin] were determined with a Coulter Counter.

Drugs and drug treatment

We selected for our studies cytostatics, which are also common for the clinical treatment of neuroblastomas (Table 1). Amifostine was a kind gift of Dr Oster (USB Pharma, UK). Cytostatics were purchased from the mentioned companies. Drugs were freshly solved as prescribed for clinical use and

Table 1. Drugs used, sources and application procedures

Drug	Supplier	Dose (mg/kg)	Route
Cyclophosphamide	Asta Medica AG, Frankfurt/M	150	i.p.
Ifosfamide	Asta Medica AG, Frankfurt/M	400	i.p.
Cisplatin	Medac GmbH, Hamburg	8	i.p.
Adriablastin	Farmitalia	10	i.v.
Vincristine	Lilly Deutschland GmbH, Giessen	1	i.p.
Etoposide	Bristol Arzneimittel GmbH, München	40	i.p.
Amifostine[a]	USB Pharma	200	i.p.

[a]Amifostine was administered 30 min before the cytostatic drug.

administered in a volume of 0.2 ml/20 g body weight.

Doses and schedules were chosen according to our experience in animal experiments and corresponded roughly with the maximum tolerated doses (LD_{10}). Amifostine was administered 30 min before the cytostatic drug.

Treatment of tumor-bearing mice started when the tumors were at a palpable size (4–5 mm diameter). Each treatment group consisted of six to eight mice; one control group within each experiment received saline only.

Results

Tumor growth

The results with neuroblastoma tumors obtained from IMR5-75 and Kelly cells are listed in Tables 2 and 3, respectively. In each experiment, a direct comparison of cytostatic treatment with or without amifostine was performed. Tumor growth curves of the different treatment and control groups are presented in Figures 1 and 2.

The tumor IMR5-75 can be considered as a very sensitive model, in which all tested cytostatics showed a different but significant growth inhibition. Ifosfamide, adriablastin, cisplatin and etoposide revealed the most impressive efficacy. On contrary, in the neuroblastoma Kelly only three of six of the tested cytostatics (ifosfamide, adriablastin and etoposide) led to a significant tumor size inhibition, vincristine was only marginally active, cyclophosphamide and cisplatin lacked activity at the chosen dose intensity.

Amifostine as a single treatment of 200 mg/kg once i.p. neither inhibited nor accelerated tumor growth. In no case, could a significant influence of amifostine on the cytostatic-mediated antitumor effect be registered.

Body weight

All cytostatic treatments induced a more or less pronounced body weight loss in the treated mice with a nadir at about 3–4 days after therapy. Amifostine, oppositely, had no decisive influence on this parameter, neither when used alone nor in combination with the antineoplastic drugs. Differences observed between the two neuroblastoma models are probably due to the use of nude mice with different sexes and genetic backgrounds showing a

Table 2. Pharmacological effects of cytostatics with and without amifostine pretreatment in neuroblastoma IMR5-75 xenografts

Drug	Amifostine[a]	Optimum T/C[b] (%)	BWC[c] (%)	WBC[d] (% of controls)	Platelets (% of controls)
Cyclophosphamide	–	54*	−1	28*,**	115
	+	45*	−2	55	102
Ifosfamide	–	32*	−15*	23*	78
	+	12*	−15*	57*,**	63*
Cisplatin	–	36*	−9*	70	75
	+	27*	−9*	126	74
Adriablastin	–	15*	−6*	91	94
	+	9*	0	82	77
Vincristine	–	58*	−12*	70	73
	+	48*	−6*	88	65*
Etoposide	–	49*	4	112	60*
	+	40*	6	110	82
Amifostine	+	77	7	101	94

Six to eight mice per group were treated with the corresponding cytostatic ± amifostine, starting when tumors had reached a size of 4–5 mm diameter.
*Significant compared to controls ($p \leq 0.05$).
**Significant compared to cytostatic alone ($p \leq 0.05$).
[a]Amifostine (200 mg/kg, i.p.) was administered 30 min before the cytostatic.
[b]Treated/control value of relative tumor volumes registered within 4 weeks after treatment.
[c]Nadir of body weight change in relation to day of treatment.
[d]White blood cell counts determined 3 or 4 days after treatment.

Table 3. Pharmacological effects of cytostatics with and without amifostine pretreatment in neuroblastoma Kelly xenografts

Drug	Amifostine[a]	Optimum T/C[b] (%)	BWC[c] (%)	WBC[d] (% of controls)	Platelets (% of controls)
Cyclophosphamide	–	76	−8*	53*	75
	+	94	−6*	46*	88
Ifosfamide	–	40*	−16*	39*	60*
	+	42*	−22*	33*	58*
Cisplatin	–	77	−9*	117	87
	+	74	−8*	108	94
Adriablastin	–	43*	−15*	82	97
	+	49*	−14*	79	82
Vincristine	–	54	−5*	116	75
	+	60	−9*	87	78
Etoposide	–	48*	−6*	73*	102
	+	49*	−4*	70*	117
Amifostine	+	106	0	117	92

Six to eight mice per group were treated with the corresponding cytostatic ± amifostine, starting when tumors had reached a size of 4–5 mm diameter.
*Significant compared to controls ($p \leq 0.05$).
**Significant compared to cytostatic alone ($p \leq 0.05$).
[a]Amifostine (200 mg/kg, i.p.) was administered 30 min before the cytostatic.
[b]Treated/control value of relative tumor volumes registered within 4 weeks after treatment.
[c]Nadir of body weight change in relation to day of treatment.
[d]White blood cell counts determined 3 or 4 days after treatment.

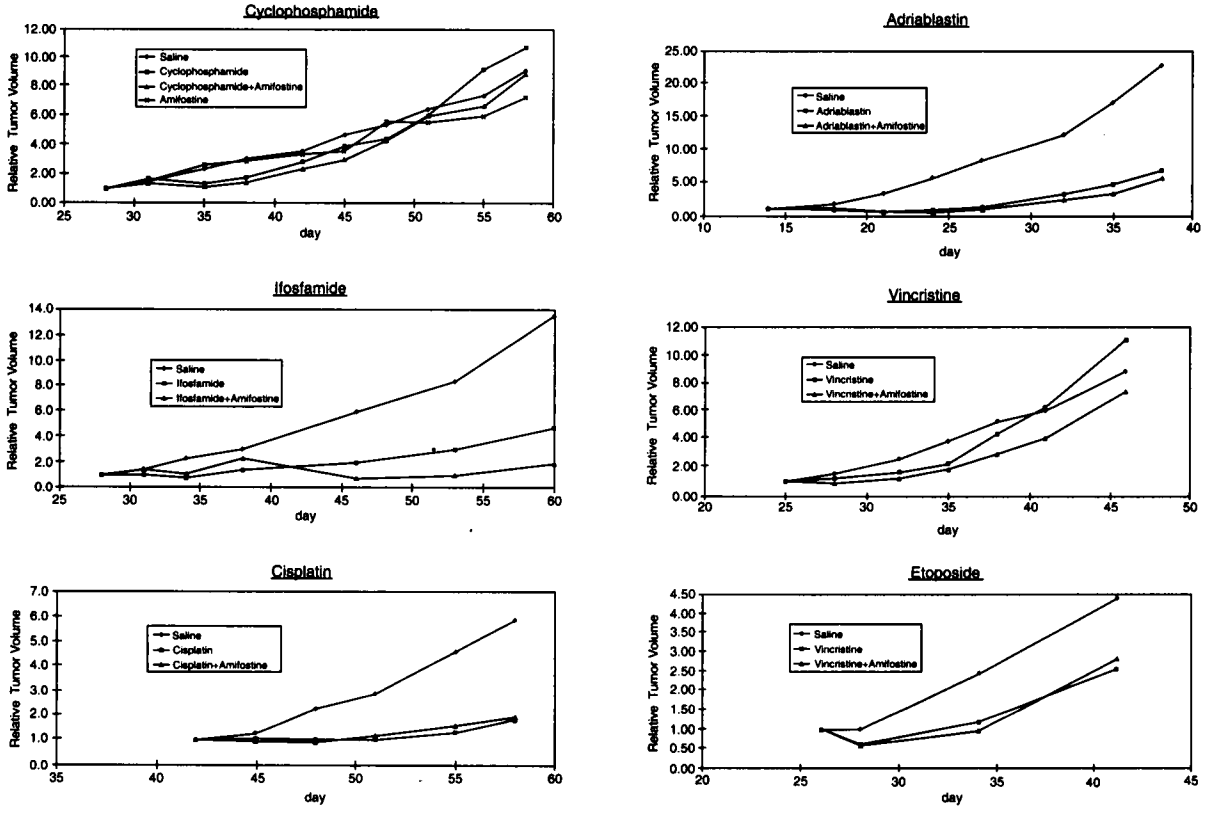

Figure 1. Tumor growth curves of neuroblastoma IMR5-75. Tumors were transplanted s.c.. At palpable sizes groups of six to eight mice each were treated with a cytostatic drug alone or in combination with amifostine (doses and schedules according to Table 1). One control group was treated with saline and another group with amifostine alone.

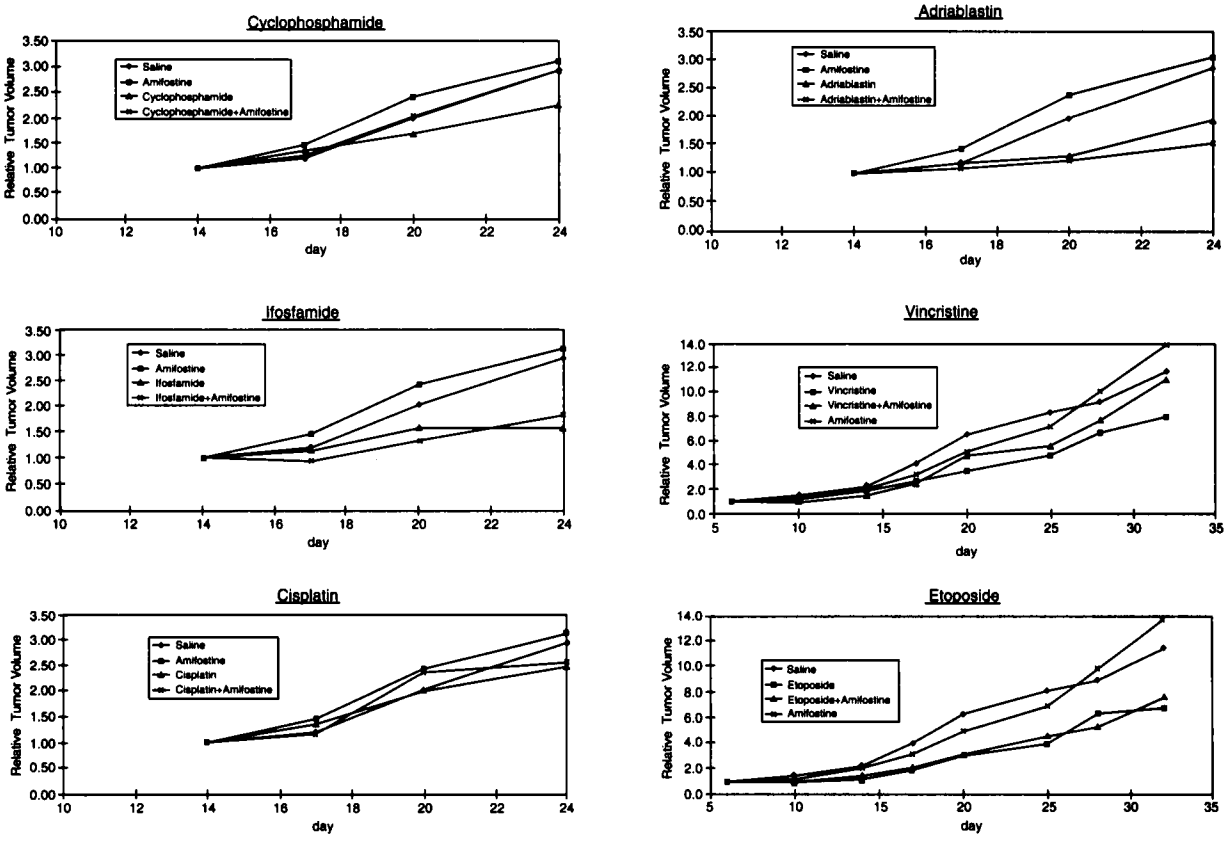

Figure 2. Tumor growth curves of neuroblastoma Kelly. Procedure as described for Figure 1.

different sensitivity to the compounds used. One representative example of body weight course in the neuroblastoma IMR5-75 is shown in Figure 3; the vincristine-induced body weight loss is marginally reduced when amifostine is given in combination.

WBC

As in the clinical use of cytostatics, some agents also induced a depression of leukocyte counts in our animal experiments. This phenomenon was noticed in the neuroblastoma IMR5-75-bearing mice after treatment with cyclophosphamide and ifosfamide (Table 2), while in the Kelly-bearing mice etoposide additionally led to a leukopenia (Table 3). Amifostine itself had no influence on this parameter, but mitigated the leukopenia induced by cyclophosphamide (Figure 4) or ifosfamide in the neuroblastoma IMR5-75 in a significant way. Also the cisplatin-mediated leukopenia was relieved in this model, but the differences were not statistically significant (Table 2). In the mice used for the transplantation of the neuroblastoma Kelly no influence of amifostine on the cytostatic-induced leukopenia could be registered (Table 3).

Platelets

In the IMR5-75-bearing mice ifosfamide, vincristine and etoposide induced a thrombopenia. Amifostine had a very different influence on this parameter. It strengthened the thrombopenic effect of ifosfamide and vincristine but mitigated the etoposide-mediated thrombopenia (Table 2), but in a non-significant way. In the mice bearing the neuroblastoma Kelly only ifosfamide induced a significant decrease in platelet numbers; amifostine had no influence on this parameter, neither alone nor in combination with any cytostatic drug.

Discussion

Neuroblastomas are the most frequent solid tumors of early childhood.[20] One unique feature of this tumor is the possibility for spontaneous regression

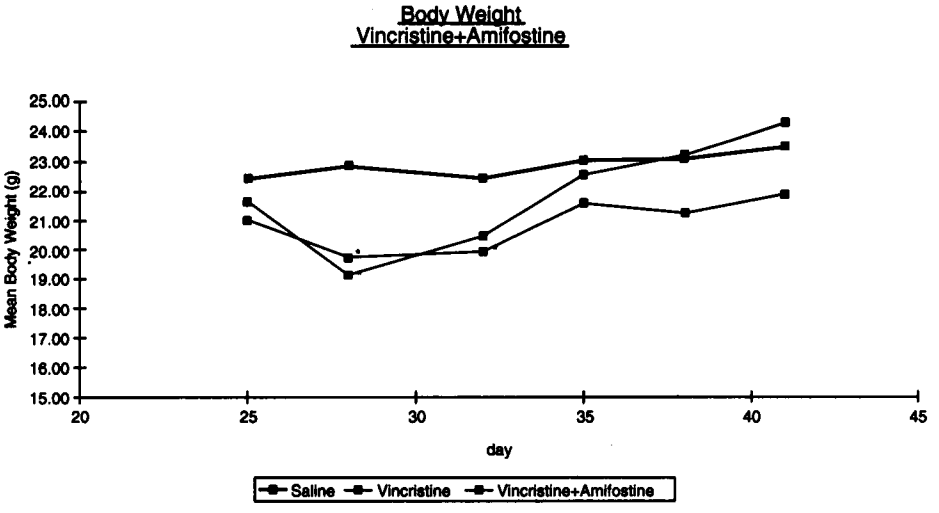

Figure 3. Body weight course of IMR5-75-bearing mice following treatment with vincristine alone or in combination with amifostine. Treatment: day 25, i.p. *Significant.

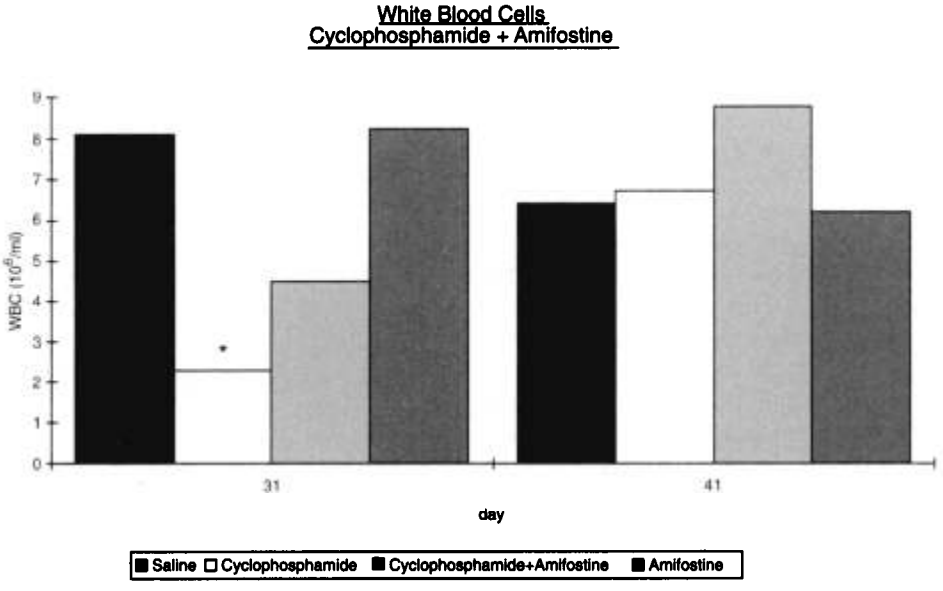

Figure 4. WBC counts of IMR5-75-bearing mice on days 31 and 41 following treatment with cyclophosphamide alone or in combination with amifostine. Treatment: day 28, i.p. *Significant versus control.

even observed with widely spread stages. Besides age at diagnosis and stage, N-*myc* amplification and deletions in chromosome 1 have also been suggested as parameters for an assessment of prognosis.

Resection of locally growing tumor mass is used as a therapeutic modality for early stages of the disease. More advanced and disseminated neuroblastomas are treated by polychemotherapy including cisplatin, etoposide, vinca alkaloids, ifosfamide and adriablastin. In recent years, bone marrow transplantation and peripheral stem cell rescue modalities offered the possibility for dose intensification and escalation leading to a substantial increase in disease-free survival. Therefore, the search for chemoprotectants allowing even higher dosages seems worthwhile in this tumor localization. Amifostine could be one candidate for combined use with high-dose chemotherapy because of its protective effects, especially concerning bone marrow and nephrotoxicity of alkylating and platinum drugs.

Despite a lot of preclinical and clinical studies concerning the protective influence of amifostine

both on tumor and normal tissues,[11,12] its special pharmacological behavior was to be tested in experimental neuroblastomas before considering it for clinical use.

A comprehensive study was already performed in order to elucidate the effectiveness of cytostatic drugs in neuroblastoma cell lines[21] and, further, to assess the influence of amifostine on cytostatic-induced growth inhibition *in vitro*.[19] Investigations described in the present paper dealt with the question if amifostine had any influence on the growth of xenotransplanted neuroblastomas. For this purpose, two cell lines with N-*myc* amplification, IMR5-75 and Kelly, were established as xenotransplants in T cell-deficient nude mice. In these models six different cytostatics, which are common for the clinical treatment of neuroblastomas, were tested according to their antitumor and side effects. IMR5-75 was a very chemosensitive tumor in which all compounds revealed a significant growth inhibition, whereas the model Kelly has to be considered as a more resistant neuroblastoma. It could be demonstrated that ifosfamide, adriablastin and etoposide showed a comparatively high effectiveness in both models.

A further question to be considered in this study was the influence of amifostine on the growth of the xenotransplants. It can be concluded from our results in two neuroblastomas that amifostine neither influenced the tumor development as a single treatment nor interfered significantly with the cytostatic-mediated growth inhibition.

Although it was not the primary objective of our studies to look at the protection of normal tissues exerted by amifostine, we concomitantly registered parameters of gastrointestinal (body weight) and bone marrow (peripheral blood cells) toxicity in the therapeutically oriented experiments.

The cytostatic-induced body weight loss was mainly uninfluenced by amifostine, pointing to the fact that the protectant probably does not decisively mitigate the gastrointestinal toxicity of chemotherapeutic drugs. On the other hand, leukopenia induced by cyclophosphamide, ifosfamide or cisplatin could be relieved by a concomitant application of amifostine in mice bearing the neuroblastoma IMR5-75.

The effect of amifostine on platelet nadirs induced by cytostatics in therapeutic doses as used in our experiments was different and did not show a clear tendency for protection.

In the light of our preclinical *in vivo* studies in two xenotransplanted neuroblastomas we conclude that amifostine had no influence on tumor growth and did not protect the tumors from a cytostatic effect. Side effects of chemotherapeutic agents were mainly uninfluenced, bone marrow toxicity of alkylants was relieved in some cases. Therefore, amifostine can be considered as possible protective agent for high-dose chemotherapy of neuroblastomas.

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