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Amifostine does not alter the antitumor activity of cisplatin in a pre-clinical model of testicular cancer

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Testicular germ cell tumors are so exquisitely sensitive to cisplatin that the majority of patients with this cancer are now cured with modern platinum-based chemotherapy. In contrast to some other tumor types, testicular germ cell tumors are known to express alkaline phosphatases (ALP). Amifostine is an aminothiol pro-drug which is rapidly dephosphorylated by ALP at the cell surface of healthy tissues and which exerts a clinically proven protective effect against chemotherapy associated toxicity. The aim of this pre-clinical study was to assess the potential of amifostine to protect platinum-sensitive non-seminomatous germ cell tumor (NSGCT) nude mouse xenografts established from an ALP-positive embryonal carcinoma (EC) cell line, from the cytotoxicity of cisplatin when both were administered at their individual maximally tolerated doses (MTD). The %T/C values calculated at day 30 for nude mice carrying H12.1 NSGCT xenografts treated with amifostine alone, amifostine plus cisplatin or cisplatin alone were, respectively, 93, 3 or 3%. Mean tumor volumes were not significantly different between mice treated with the combination versus cisplatin alone at day 14 or 30. The results of this study revealed no evidence of tumor protection by amifostine, confirming previous results in other tumor types.

Key words: Antitumor activity, amifostine, cisplatin, germ cell neoplasms, human xenografts, nude mice, WR-2721.

Introduction

Approximately 70–80% of patients with metastatic testicular cancer will become disease-free with platinum-based therapy and most of these patients will become long-term survivors. With this success, the reduction of acute and late toxicities such as nephro-, neuro-, oto- and vascular toxicity, fertility impairment, and the occurrence of secondary malignancies has gained increasing importance in the development of new treatment concepts.

Amifostine (WR-2721) is a phosphorylated, aminothiol pro-drug with clinically proven multiple organ chemoprotecting ability in cancer patients.[1] WR-2721 undergoes dephosphorylation to the active thiol WR-1065 by cell surface alkaline phosphatases (ALP).[2–4] The capacity of amifostine to specifically protect against chemotherapy-associated myelo-, nephro-, neuro- and ototoxicity is thought to be based upon its preferential activation to WR-1065 and up-take of the neutral form of WR-1065 by healthy tissues due to their high ALP activity and pH as compared to poorly vascularized, hypoxic tumor tissue.[5–8] This activation pathway by ALP might raise concern regarding a potential selective protection of human testicular germ cell tumors since they express ALP: detection of the placental form of ALP is used in the histological identification of carcinoma *in situ* of the testis, is a serum and tumor tissue marker in seminoma patients, and in contrast to several other tumor types, high level expression of the liver/bone/kidney ALP isoenzyme is a common feature of human embryonal carcinoma (EC) cells.[9–11] Before using amifostine in clinical trials in patients with germ cell cancer, a pre-clinical study to exclude the risk of a potential decrease of antitumor activity of cisplatin was desirable. We therefore evaluated the potential of amifostine to antagonize the antitumor activity of cisplatin in a pre-clinical xenograft model of human non-seminomatous germ cell tumor (NSGCT). H12.1 NSGCT xenografts in nude mice were previously shown to resemble tumors in patients in terms of histology, growth characteristics and tumor marker production.[12] In

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addition, NSGCT xenografts in nude mice treated with the maximally tolerated dose (MTD) of cytotoxic agents were able to predict the poor clinical activity of first line carboplatin-based therapy in testicular cancer patients[13,14] and the promising activity of taxol in cisplatin-resistant NSGCT.[15]

Materials and methods

Drugs and preparation of solutions for injection

Analytically pure amifostine (WR-2721) was supplied by US BioScience (West Coshohocken, PA) split into aliquots of 150 mg on arrival and stored at −20°C until required. On the day of the experiment, approximately 70 mg amifostine was weighed out, adjusted to a concentration of 40 mg/ml in sterile water and then immediately administered to mice by i.p. injection. Cisplatin was purchased from Medac GmbH (Hamburg, Germany) as a 20 mg/ml solution in 0.9% saline for clinical use. Control mice were injected with 200 μl of 0.9% saline.

Preliminary dose finding/toxicity study of amifostine

Amifostine has been previously demonstrated to induce rapid, murine-specific hypothermia with unique i.p. doses in the region of 100–200 mg/kg.[16] Since the routine MTD dose of cisplatin used in our laboratory was 3 mg/kg/day injected i.p. on days 1–5, we investigated the MTD of amifostine when injected on days 1–5, so that the two drugs could be investigated in combination. The MTD of amifostine was defined as the dose inducing a maximum of 3°C fall in body temperature, in accordance with other studies of this type previously reported in the literature.[16] Groups of five non-tumor carrying nude mice were injected i.p. over days 1–5 with either 100 or 200 mg/kg/day amifostine every 24 h. Body temperature was measured from days 1 to 8 using a specially designed anal probe with a digital read-out (measurement error ± 0.2°C; Technical Department, Hannover Medical University). Body temperature was measured before injection (0 h/24 h), and then 2 and 5 h after each injection. Mouse weight was also measured daily before each amifostine injection. Mortality was recorded over days 1–30.

Human NSGCT cell line and heterotransplantation into nude mice

H12.1 was established from an orchiectomy specimen of a 19-year-old previously untreated patient.[12] The histology of the original tumor, cell line and nude mouse xenograft is shown in Table 1. H12.1 xenografts for mouse to mouse transplantation were obtained as previously described:[12,13] briefly, 0.2 ml RPMI 1640 culture medium containing 1×10^7 viable, trypsinized H12.1 cells was injected s.c. into the right flank of 10 athymic male NMRI-*nu* mice (Bommice, Denmark). Approximately 10 mm^3 non-necrotic tumor fragments were then transplanted into the left flank of the appropriate number of nude mice for the antitumor activity studies.

Antitumor activity studies

Nude mice carrying with H12.1 xenografts were stratified by tumor size into groups of eight mice on day 0 and treated after weighing on days 1–5 by i.p. injection with either 200 mg/kg amifostine (group 1), 200 mg/kg amifostine injected 15 min before 3 mg/kg cisplatin (group 2), 3 mg/kg cisplatin (group 3) or 200 μl 0.9% saline (vehicle only control; group 4). The dose of cisplatin used was the previously determined MTD[13,14] and is routinely used in our laboratory. Mice were weighed and the tumor volume ($a \times b^2 \times 0.5$), where a is the longest diameter and b is the diameter perpendicular to a, measured every 2–3 days until day 30. The relative tumor volumes (rTV), the TV on a given day divided by the TV on day 1 for individual mice, were plotted graphically over days 1–30. The %T/C (mean tumor volume of treated mice divided by the mean tumor volume of control mice × 100) were calculated and the Mann–Whitney *U*-test/Wilcoxon rank sum *W*-test performed to evaluate significant differences between tumor volumes in the different treatment groups at day 14 and 30. Differences with $p > 0.05$ were considered insignificant. Experiments

Table 1. Origin and histology of the NSGCT cell line used for heterotransplantation into nude mice

Histology of original tumor	Cell line characterized as	Histology of nude mouse tumor
EC, CC, T, S	EC	EC, STGC, T

EC, embryonal carcinoma; CC, choriocarcinoma; T, teratoma; STGC, syncitiotrophoblastic giant cell.

were performed once only with the individual MTD of each drug alone or in combination.

Results

An initial dose-finding toxicity study was first performed to evaluate the MTD of amifostine for use in antitumor activity studies in combination with cisplatin. The hypothermia induced was rapid and dependent on the dose of amifostine injected (Table 2). The fall in temperature at 2 h following injection of 200 mg/kg/day amifostine on days 1–5 was 2.53 ± 0.21°C. This was similar to that reported in BALB/c mice (approximately 3°C at 2 h following injection of 200 mg/kg amifostine) treated by one i.p. injection of amifostine.[16] A dose of 200 mg/kg/day was chosen as the optimal dose to combine with cisplatin and 5-fluorouracil in the latter study, and was the dose chosen in our study since it induced a fall in body temperature not exceeding 3°C, which was not cumulative or otherwise toxic when injected on five consecutive days.

NMRI *nu/nu* mice heterotransplanted with H12.1 were treated on days 1–5 with amifostine combined with cisplatin versus each drug alone. No deaths were observed in the groups treated with cisplatin, which is in accordance with previous results using this schedule (mortality ≤ 20%). Tumor volume was followed over days 1–30 and the mean rTV over days 1–30 calculated for each mouse relative to its tumor volume on day 1 in each treatment group (Figure 1). Cisplatin showed good antitumor activity in this tumor model, as observed previously,[13,14] and amifostine did not itself show any tumor-inhibiting or tumor-stimulatory activity over days 1–30 (Figure 1). The reductions in tumor volume achieved with cisplatin alone or in combination with amifostine were, respectively, 21 or 30% at day 14 and were equivalent at day 30 (T/C values 3%) (Table 3). The differences in tumor volumes in the latter groups were not significant at day 14 ($p = 0.92$) or at day 30 ($p = 0.89$).

Table 2. Mean temperature loss (°C) per group of nude mice over days 1–5 at 2, 5 and 24 h following injection of 100 or 200 mg/kg amifostine

Dose of amifostine injected (mg/kg/day)	Mean temperature loss (°C) per group of mice over days 1–5 at 2, 5 or 24 h following injection of amifostine:		
	2 h	5 h	24 h
100	0.98 ± 0.53	1.16 ± 0.23	0.27 ± 0.39
200	2.53 ± 0.21	1.58 ± 0.11	0.24 ± 0.11

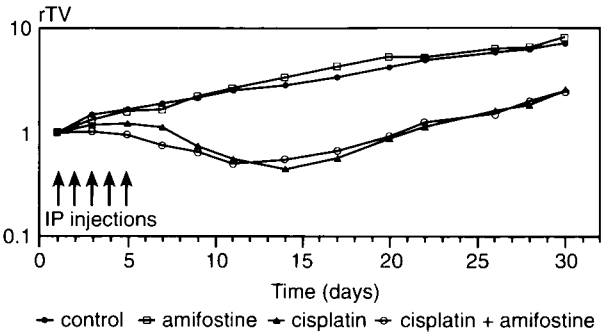

Figure 1. Mean rTV of H12.1 NSGCT xenografts in nude mice treated with the individual MTD of amifostine combined with the previously determined, individual MTD of cisplatin versus each drug alone following i.p. injection on days 1–5.

Table 3. Mean %T/C values for H12.1 xenografts on day 30 in treatment groups 1–4

Group	Treatment	%T/C:[a] day 14	%T/C: day 30
1	amifostine	112	93
2	amifostine + cisplatin	30	3
3	cisplatin	21	3
4	vehicle only control	100	100

[a]%T/C = mean tumor volume per group of treated mice/mean tumor volume per group of control mice at day 14 or day 30.

Discussion

Before using amifostine in clinical trials in patients with testicular germ cell cancer, it was desirable to test the potential of amifostine to antagonise the antitumor activity of cisplatin in a cisplatin-sensitive xenograft model of human NSGCT in nude mice. Protection of the antitumor efficacy of cisplatin was not observed in our xenograft model treated with the individual MTD of each drug. One possible explanation for this result is that H12.1 xenografts possess low levels of ALP activity, and therefore cannot dephosphorylate the pro-drug and internalize it. However, human EC cell lines cultures, including H12.1, possess very high levels of ALP activity (W Muller and J Casper, Hannover Medical University, unpublished data;[9–11,17]) and previous studies have shown that H12.1 xenografts *in vivo* still contain EC elements following heterotransplantation.[12] Amifostine has previously been reported to increase the antitumor activity of platinum compounds,[18–20] and

the alkylating agents melphalan, nitrogen mustard and mafosfamide.[21–24] A pharmacokinetic interaction between amifostine and carboplatin has been demonstrated in nude mice carrying ovarian cancer xenografts, releasing increased platinum levels in tumor and plasma ultrafiltrate.[24] Additional mechanisms are not clear at the present time but could possibly involve down-regulation of glutathione or production of toxic metabolites such as acrolein in tumor tissue.[25] In addition, WR-2721 and WR-1065 bear a striking resemblance to spermidine, and the disulfide moiety of WR-2721, WR-33278, is reported to be structurally and functionally very similar to spermine.[25,26] These polyamines are known to bind strongly to DNA and to mediate effects on DNA confirmation and DNA–protein binding.[27–29] It is intriguing to speculate as to whether amifostine metabolites which resemble polyamines could serve to modify the amount of DNA damage or the repair of DNA damage induced by cisplatin or alkylating agents. Further studies are required in this and other NSGCT models with a wider range of doses to investigate the potential cytotoxic interaction of amifostine with cisplatin, and also with ifosfamide, bleomycin, etoposide and vinblastine, included in combination therapy protocols for treatment of testicular cancer patients. An important issue for future studies which has not previously been adequately investigated remains the quantification of ALP isoenzyme and activity levels present in the xenografts at the time of drug treatment.

In conclusion, the data presented here indicate that amifostine may not compromise the clinical activity of cisplatin in NSGCT patients although further pre-clinical studies are necessary to establish this result conclusively using xenograft models with documented ALP activity. Studies in testicular cancer patients are desirable with amifostine since acute and chronic side-effects, including nephro- and ototoxicity and peripheral neuropathy, could possibly be avoided by inclusion of amifostine into chemotherapy protocols.[30–32] In addition, neuro- and nephrotoxicity, which may become dose limiting with high-dose chemotherapy protocols with hematopoeitic factors and/or peripheral blood stem cell rescue to ameliorate myelotoxicity,[33] could potentially be reduced.

References

1. Capizzi RL. Protection of normal tissues from the cytotoxic effects of chemotherapy by amifostine (Ethyol). Clinical experiences. *Semin Oncol* 1994; **21**(5) suppl 11: 8–15.
2. Calabro-Jones P, Fahey R, Smoluk G, *et al.* Alkaline phosphatase promotes radioprotection and accumulation of WR-1065 in V79-171 cells incubated in medium containing WR-2721. *Int J Radiat Biol* 1985; **47**: 23–7.
3. Nakamura J, Shaw LM, Brown DQ. Hydrolysis of WR-2721 by mouse liver cell fractions. *Radiat Res* 1987; **109**: 143–52.
4. Smoluk GD, Fahey RC, Calabro-Jones PM, *et al.* Radioprotection of cells in culture by WR-2721 and derivatives: form of the drug responsible for protection. *Cancer Res* 1988; **48**: 3641–7.
5. Utley JF, Seaver N, Newton GL, *et al.* Pharmacokinetics of WR-1065 in mouse tissue following treatment with WR-2721. *Int J Radiat Oncol Biol Phys* 1984; **10**: 1525–8.
6. Calabro-Jones P, Aguilera J, Ward J, *et al.* Uptake of WR-2721 derivatives by cells in culture: identification of the transported form of the drug. *Cancer Res* 1988; **48**: 3634–40.
7. Shaw LM, Glover D, Turrisi A, *et al.* Pharmacokinetics of WR-2721. *Pharmac Ther* 1988; **39**: 195–201.
8. Treskes M, Boven E, Holwerda U. Time dependence of the selective modulation of cisplatin-induced nephrotoxicity by WR2721 in the mouse. *Cancer Res* 1992; **52**: 2257–60.
9. Hustin J, Collette J, Franchimont P. Immunohistochemical demonstration of placental alkaline phosphatase in various states of testicular development and in germ cell tumours. *Int J Androl* 1987; **10**: 29–35.
10. Andrews PW, Bronson DL, Wiles MV, *et al.* The expression of MHC antigens by human teratocarcinoma derived cell lines. *Tissue Antigens* 1981; **17**: 493–500.
11. Benham FJ, Andrews PW, Knowles BB, *et al.* Alkaline phosphatase isoenzymes as possible markers of differentiation in human testicular teratocarcinoma cell lines. *Dev Biol* 1981; **88**: 279–87.
12. Casper J, Schmoll H-J, Schnaidt U, *et al.* Cell lines of human germinal cancer. *Int J Androl* 1987; **10**: 105–13.
13. Harstrick A, Casper J, Schmoll HJ. Comparative antitumor activity of cisplatin and two new cisplatin-analogues JM8 and JM9 in human testicular carcinoma xenografts. *Int J Androl* 1987; **10**: 139–45.
14. Harstrick A, Casper J, Guba R, Wilke H, Poliwoda H, Schmoll HJ. Comparison of the antitumor activity of cisplatin, carboplatin, and iproplatin against established human testicular cancer cell lines *in vivo* and *in vitro*. *Cancer* 1989; **63**: 1079–83.
15. Dunn TA, Schmoll HJ, Grunwald V, Casper J. Preclinical activity of taxol in non-seminomatous germ cell tumor (NSGCT) cell lines and nude mouse xenografts. Paper submitted.
16. van der Wilt CL, van Laar JAM, Gyergyay FS, Smid K, Peters GJ. Biochemical modification of toxicity and the anti-tumor effect of 5-fluorouracil and *cis*-platinum by WR-2721 in mice. *Eur J Cancer* 1992; **28A**: 2017–24.
17. Andrews PW, Banting G, Damjanov I, *et al.* Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embry-

onal carcinoma cells. *Hybridoma* 1984; **3**: 347–361.
18. Glover D, Grabelsky S, Fox K. Clinical trials of WR-2721 and *cis*-platinum. *Int J Radiat Oncol Biol Phys* 1989; **16**: 1201–04.
19. Ghiorghis A, Talebian A, Schein PS, *et al.* Effect of anticancer drugs against PA-1 human ovarian cancer cells pretreated with the chemoprotective agent WR2721. *Proc Am Ass Cancer Res* 1992.
20. Treskes M, Boven E, van de Loosdrecht AA. Effects of the modulating agent WR2721 myelotoxicity and antitumor activity in carboplatin-treated mice. *Eur J Cancer* 1994; **30A**: 183–7.
21. Douay L, Hu C, Giarratana M, *et al.* Amifostine improves the antileukemic therapeutic index of mafosfamide: implications for bone marrow purging. *Blood* 1995; **86**: 2849–33.
22. Valeriate F, Tolen S. Protection and potentiation of nitrogen mustard cytotoxicity by WR-2721. *Cancer Res* 1982; **42**: 4330–1.
23. Millar JL, McElwain TJ, Clutterbuck RD, *et al.* The modification of melphalan toxicity in tumor bearing mice by *S*-2(3-aminopropylamino)-ethylphosphorothoic acid (WR 2721). *Am J Clin Oncol* 1982; **5**: 321–8.
24. Korst AEC, Boven E, van der Vijgh WJF. Influence of amifostine on the platinum concentration in plasma and tumor tissue of nude mice treated with carboplatin. *Ann Oncol* 1994; **5** (suppl 5): S205.
25. Meier T, Issels RD. Degradation of 2-(3-aminopropylamino)-ethanethiol (WR-1065) by Cu-dependent amine oxidases and influence on glutathione status of chinese hamster ovary cells. *Biochem Pharmacol* 1995; **50**: 489–96.
26. Shigematsu N, Schwartz JL, Gardina DJ. Protection against radiation-induced mutagenesis at the hprt locus by spermine and *N,N''*-(dithiode-2,1-ethanediyl)bis-1,3-propanediamine (WR33278). *Mutagenesis* 1994; **9**: 355–60.
27. Hasan R, Alam MK, Ali R. Polyamine induced *Z*-conformation of native calf thymus DNA. *FEBS Lett* 1995; **368**: 27–30.
28. Van-den-Broeck D, Van-der-Straeten D, Van-Montagu M, *et al.* A group of chromosomal proteins is specifically released by spermine and loses DNA-binding activity upon phosphorylation. *Plant Physiol* 1994; **106**: 559–66.
29. Thomas T, Gallo MA, Klinge CM, *et al.* Polyamine-mediated conformation perturbations in DNA alter the binfing of estrogen receptor to poly(dG–m5dC)·poly(dG–m5dC) and a plasmid containing the estrogen response element. *J Steroid Biochem Mol Biol* 1995; **54**: 89–99.
30. Cornelison TL, Reed E. Nephrotoxicity and hydration management for cisplatin, carboplatin and ormaplatin. *Gynecol Oncol* 1993; **50**: 147–58.
31. Bokemeyer C, Frank B, van Rhee J, *et al.* Peripheral neuropathy following cancer chemotherapy. Long term results in patients with curable neoplasms. *Tumordiagn Ther* 1993; **14**: 232–7.
32. Bokemeyer C, Berger C, Kynast B, *et al.* Ototoxicity following therapy for testicular cancer. *Eur J Cancer* 1993; **29A** (suppl 6): 1357.
33. Siegert W, Beyer J, Strohscheer I, *et al.* High dose treatment with carboplatin, etoposide and ifosfamide followed by autologous stem cell transplantation in relapsed or refractory germ cell tumors—a phase I/II study. *J Clin Oncol* 1994; **12**: 1223–31.

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