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Effects of WR-2721 (amifostine) and its metabolite WR-1065 on the antiproliferative activity of chemotherapeutic agents on neuroblastoma cells *in vitro*

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Amifostine (WR-2721) is currently being investigated as a potential protector of normal tissues during chemotherapy in adult and pediatric cancer patients. The marked reduction of bone marrow and renal toxicity by amifostine is well documented, but data are lacking whether the anticancer activity of cytostatic drugs is also preserved in neuroblastoma as the second most common pediatric malignancy. We investigated the cytotoxic effect of six drugs on two neuroblastoma cell lines chosen for their presence or absence of N-*myc* amplification and PGY1 overexpression: IMR-5 (N-*myc* 25×, PGY1-negative), CHP-100 (N-*myc* 1×, PGY1-positive) *in vitro* in the presence and absence of WR-2721 and its active metabolite WR-1065 using the monolayer proliferation assay. Doxorubicin, vincristine, etoposide, cisplatin, 4-hydroperoxycyclophosphamide and 4-hydroperoxyifosfamide were equally cytotoxic with and without preincubation of WR-2721 (14 mM) or WR-1065 (40 μM) as shown by virtually identical dose–response curves and ID_{50} values. We conclude that WR-2721 and WR-1065 did not reduce the cytostatic activity of six commonly used drugs on neuroblastoma cell lines *in vitro*.

Key words: Alkylating agents, antibiotics, antineo-plastic, chemoprotection, cisplatin compounds, etoposide, neuroblastoma, tumor cells cultured, vinca alkaloids, WR-2721, WR-1065.

Introduction

Amifostine (Ethyol™) (Figure 1), formerly known as WR-2721, is a phosphorylated aminothiol that emerged out of a classified United States Army nuclear warfare research project begun in the 1960s. This program ultimately selected amifostine as the lead radioprotective compound based upon its ability to fully preserve a wide range of animal species from lethal doses of radiation.[1] The potential applications of amifostine were also explored in the medical field of oncology, particularly its role as a potential protector of normal tissues during chemotherapy.

$NH_2(CH_2)_3NH(CH)_2SPO_3H_2$

Amifostine

$NH_2(CH_2)_3NH(CH)_2SH$

WR-1065

Figure 1. Structures of amifostine (prodrug) and WR-1065, its active metabolite.

The basis for the selective cytoprotection of normal tissue is explained by the unique systemic and tissue distribution pharmacokinetics of amifostine. Following drug administration, the half-life of both the distribution and elimination phases are extremely rapid ($\alpha T_{1/2} < 1$ min; $\beta T_{1/2}$ 8 min). Ninety percent of the drug is cleared from the plasma within 6 min and the amount of the prodrug that is bioconverted to the free thiol in the systemic circulation is small.[2–4] Because of this rapid clearance, there is virtually no amifostine or free thiol in the systemic circulation 15–30 min later when the cytotoxic therapy is administered.[2,3] This strategy provides little opportunity for interactions within the systemic circulation between thiol and subsequent cytotoxic drugs.

Calabro-Jones *et al.*[5–7] have shown that normal tissues, especially at the capillary level, have a higher concentration of alkaline phosphatase, the membrane-bound enzyme responsible for dephosphory-

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lating amifostine to the free thiol, the active metabolite, thereby catalyzing the conversion of amifostine followed by rapid local uptake in normal tissues. In contrast, it has been suggested that the concentration of alkaline phosphatase in the endothelium of neoplastic capillaries is appreciably less, a feature that contributes to this apparent selective anabolism and uptake.[8] It was also demonstrated that the rate constant for the uptake of the free thiol across cell membranes is markedly accelerated with small differences in pH, favoring the pH of 7.4 which is found in normal tissues versus the relative acidity in some tumors. In addition to these local enzymatic mechanisms, differences in the transmembrane passage of the disulfide and intracellular activation of the free thiol between normal and neoplastic cells may also contribute to differential effects of amifostine in normal versus malignant tissue.[9–11]

Neuroblastoma is the second most frequent malignancy in childhood, which requires aggressive chemotherapy with the consequence of severe side effects to the patients. This study was initiated to investigate whether the anti-tumor activity of suitable chemotherapeutic agents against neuroblastoma cells is preserved or not with the use of amifostine.

WR-1065, the active metabolite of amifostine, enters cells via passive diffusion which *per se* does not allow a selective targeting of normal versus malignant cells. Recent data, however, suggests that WR-1065 requires intracellular protein-dependent mechanisms to become active.[12–16] Although the conversion from WR-2721 to WR-1065 happens rapidly and uptake of WR-1065 occurs immediately thereafter, there remains the theoretical possibility of small amounts of WR-1065 being circulating for a limited period of time. An important objective of our work was to explore if direct exposure of tumor cells to WR-1065 impacts on their growth inhibition achieved by cytotoxic and cytostatic agents. The experiments reported here have therefore tested the anti-tumor activity of several chemotherapeutic agents (doxorubicin, vincristine, etoposide, cisplatin, cyclophosphamide and ifosfamide) on neuroblastoma cell lines in the presence of both WR-2721 or WR-1065.

Materials and methods

Drugs

WR-2721 (amifostine, Ethyol®) and its active metabolite WR-1065 was kindly provided by US Bioscience (West Conshohocken). The following drugs were tested: 4-hydroperoxycyclophospha- mide and 4-hydroperoxyifosfamide (Asta Medica, Frankfurt, Germany, kindly provided by Dr Pohl), cisplatin and etoposide (Bristol Arzneimittel, Munich, Germany), vincristine (Rhone-Poulenc, Cologne, Germany), and doxorubicin (Farmitalia, Freiburg, Germany).

Cell lines

The two neuroblastoma cell lines were chosen for their presence or absence of N-*myc* amplification and PGY1 overexpression:[17] IMR-5 (N-*myc* 25×, PGY1-negative), CHP-100 (N-*myc* 1×, PGY1-positive). Cells were maintained in monolayer culture in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (Gibco, Eggenstein, Germany), 2 mM L-glutamine (Boehringer, Mannheim, Germany), 100 μg/ml gentamicin (Boehringer) and were incubated at 37°C in 95% air/5% CO_2.

Monolayer proliferation assay

The monolayer proliferation assay was performed as described by Dietel *et al.*[18] In general, viable cells remain adherent to tissue culture plates, whereas non-viable cells detach from the underlying surface. Aliquots of 1 ml of 0.05% trypsin and 0.02% EDTA without Ca^{2+} and Mg^{2+} (Boehringer) were added to the cultures for 3–5 min to obtain a single-cell suspension and cells were separated by careful mechanical aspiration. Cells were counted using a standard hemocytometer. Between 30 and 40 000 cells in 750 μl of medium were seeded in each well of a 12-well tissue culture plate (Costar, Bielefeld, Germany). To assess the amount of DNA prior to drug incubation, one plate was rinsed with Hank's balanced salt solution (Seromed) after 72 h preincubation and stored at −20°C until determination of DNA levels. The cells on the remaining plates were incubated for 15 min at 37°C with 14 mM WR-2721 and 40 μM WR-1065, respectively. The concentrations of WR-2721 and of WR-1065 were chosen equal or in excess of peak plasma levels measured in man, thus reflecting a relevant and possible dose for interference with the cytostatic drugs.[32] The plates were washed once with Hank's balanced salt solution to remove WR-2721 or WR-1065 prior to drug incubation. Cells were treated with six different concentrations of cytostatic agents for 2 h at 37°C.

After an additional 72 h incubation in fresh medium without drugs, cell growth was assessed microscopically. The medium was then removed, the non-adherent dead cells were washed off with Hank's balanced salt solution and the amount of DNA in the remaining adherent cells was determined. Cell growth was calculated by the difference in DNA content before and after drug incubation. The cell growth of the drug exposed cells was defined as relative cell growth according to the formula:

$$\text{relative growth (\%)} = \frac{\text{cell growth of exposed cells}}{\text{cell growth of control cells}}$$

$$= \frac{DNA_{exposed} - DNA_{pre}}{DNA_{control} - DNA_{pre}}$$

For simplicity, negative growth (destruction of cells) was set to 0%.

DNA determination

The amount of DNA as a function of number of adherent, living cells was determined by the method of Giles and associates using diphenylamine.[19] For standardization, salmon sperm DNA (Boehringer) was used in triplicates (0.5–60 μg DNA). Then 10% perchloric acid (Merck, Darmstadt, Germany) was added to each well for cell lysis and the plates were heated at 90°C for 30 min. After cooling to room temperature, 1 ml of 4% diphenylamine (Sigma, Munich, Germany) dissolved in glacial acid (Merck) and 0.016% acetaldehyde (Merck) was added for the color reaction. After a 24 h incubation at room temperature, the optical density of the solution was measured at 595 nm using a Beckmann spectrophotometer model Acta CIII.

Statistics

Each drug concentration was tested in triplicate. The mean, standard deviation and variation coefficient were calculated. Regression analysis was used for the correlation of cell number and level of DNA. Dose–response curves were plotted on a semilogarithmic scale with relative growth against drug concentration. The dose effective at inhibiting 50% cell growth, i.e. the ED_{50}, was obtained by interpolation from dose–response curves.

Results

A linear relationship and close correlation ($r > 0.99$) between cell number ($0.5-8 \times 10^5$ cells) and level of DNA for the two neuroblastoma cell lines had previously been reported.[20] Reduced cell growth by the monolayer proliferation assay was uniformly associated with a corresponding number of dead cells by microscopic evaluation (data not shown). S-shape dose–response curves were obtained in all instances.

Figure 2 shows the dose–response curves of IMR-5 and CHP-100 cells after treatment with a broad range of cytotoxic agents (including doxorubicin, vincristine, VP-16, cisplatin, cyclophosphamide and ifosfamide) with or without WR-2721 preincubation, at concentrations achieved *in vivo*. In both cell lines, dose–response curves with WR-2721 pretreatment were almost identical when compared to those without WR-2721 pretreatment.

Lack of any protective effect of the prodrug WR-2721 in neuroblastoma cell cultures against cytotoxic drugs might partly be due to the fact that WR-2721 is not converted to its active metabolite WR-1065, e.g. because of low or absent biological activity of alkaline phosphatase on the surface of neuroblastoma cells. In contrast to the prodrug WR-2721, the active metabolite WR-1065 (free thiol) enters cells via passive diffusion, thereby assuring intracellular accumulation. Therefore and for reasons elucidated in the Introduction we further investigated the effect of WR-1065 in the experimental setting. Both cell lines, IMR-5 and CHP-100, were exposed to the same list of cytotoxic agents with or without WR-1065. The dose of WR-1065 was chosen to mimic the *in vivo* situation where most of the WR-2721 is immediately transformed and enters the cell as WR-1065. However, minor concentrations of WR-1065 circulating for short periods of time cannot be excluded. Again none of the experiments displayed in Figure 3 revealed any evidence for interference of WR-1065 with the antitumor agent.

As a next step IC_{50} concentrations were assessed for each cell line and each cytotoxic agent with or without WR-2721 and WR-1065. In IMR-5 cells, a concentration of 0.02 nmol/ml doxorubicin was necessary to produce a 50% inhibition of growth in the absence or presence of WR-2721. In CHP-100 cells, concentrations of 0.09 and 0.08 nmol/ml doxorubicin were required for comparable growth inhibition with and without pre-exposure to WR-2721, respectively.

ED_{50} values of all tested drugs are listed in Table 1. Addition of WR-2721 or WR-1065 did not signifi-

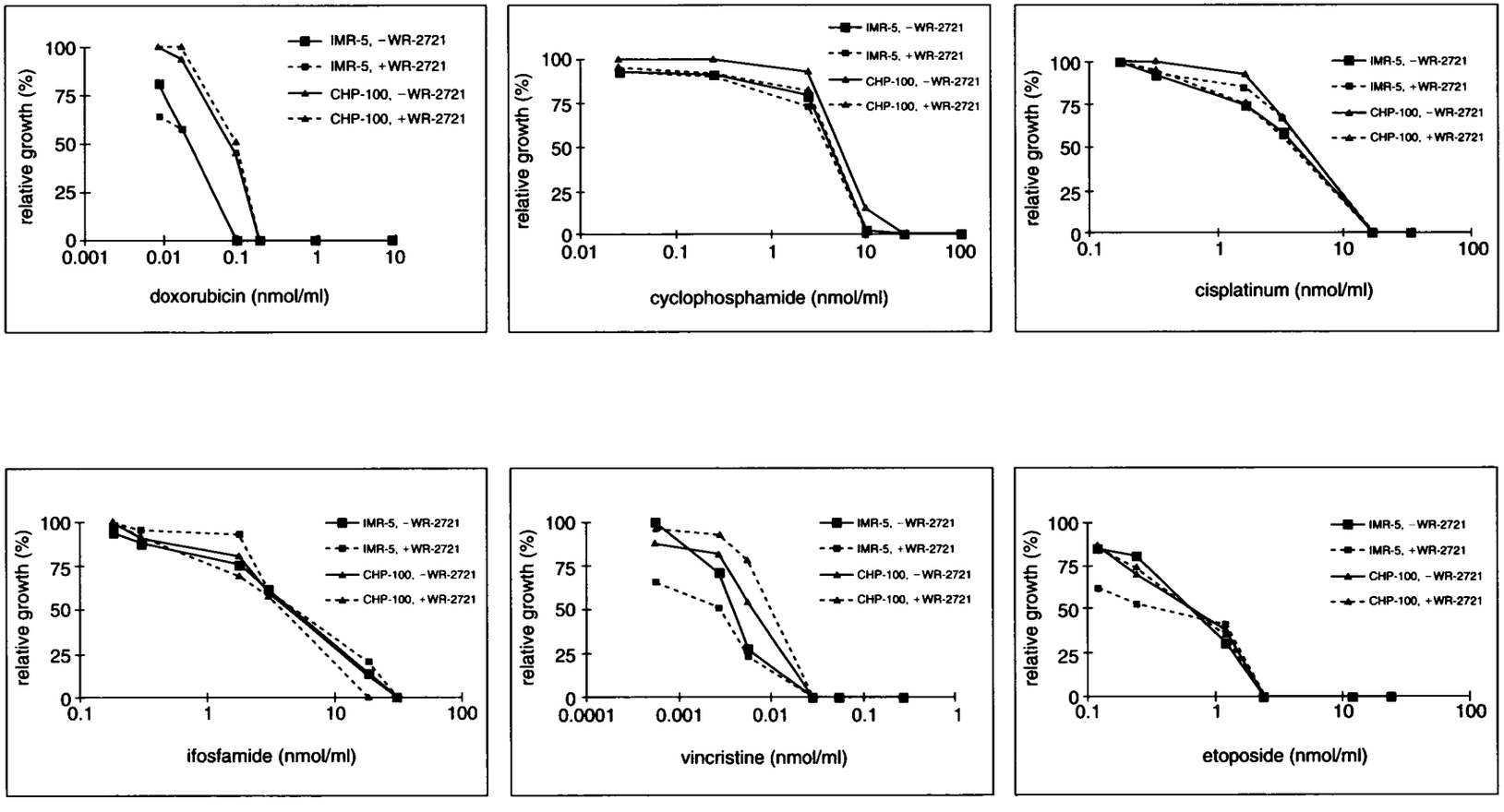

Figure 2. Dose–response curves of two neuroblastoma cell lines after 2 h exposure to doxorubicin, vincristine, VP-16, cisplatin, cyclophosphamide and ifosfamide as determined by monolayer proliferation assay: without WR-2721 pretreatment (solid lines), with WR-2721 pretreatment (broken lines).

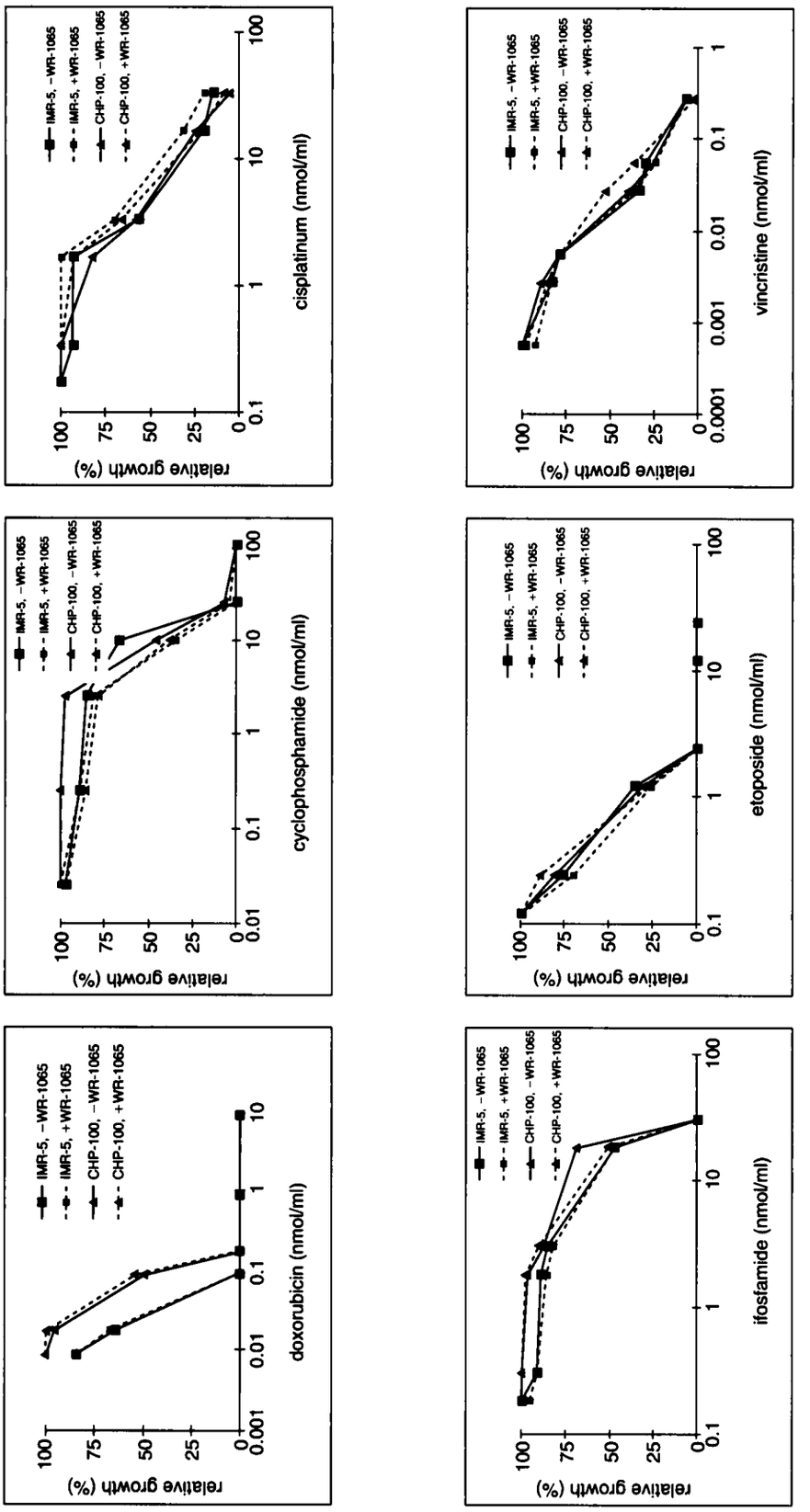

Figure 3. Dose–response curves of two neuroblastoma cell lines after 2 h exposure to doxorubicin, vincristine, VP-16, cisplatin, cyclophosphamide and ifosfamide as determined by monolayer proliferation assay: without WR-1065 pretreatment (solid lines), with WR-1065 pretreatment (broken lines).

Table 1. ED_{50} values (nmol/ml) of two neuroblastoma cell lines after 2 h drug exposure as measured by monolayer proliferation assay

	IMR-5 (N-*myc* 25×, PGY$^-$)				CHP-100 (N-*myc* 1×, PGY$^+$)			
Drugs	−WR-2721	+WR-2721	−WR-1065	+WR-1065	−WR-2721	+WR-2721	−WR-1065	+WR-1065
ADR	0.02	0.02	0.026	0.026	0.08	0.09	0.085	0.09
VCR	0.004	0.0027	0.014	0.015	0.006	0.01	0.018	0.03
VP-16	0.6	0.4	0.65	0.5	0.6	0.6	0.65	0.65
DDP	4	4	5	8	5	5	5.5	6
CP	4	4	12	6.5	5.5	4	9	6.5
IFO	4.5	4.5	15	15	4.5	4	20	18

ED_{50}, *in vitro* concentration for 50% growth inhibition; ADR, doxorubicin; VCR, vincristine; VP-16, etoposide; DDP, cisplatin; CP, cyclophosphamide; IFO, ifosfamide; N-*myc*, N-*myc* amplification; PGY$^+$, PGY1 overexpression; PGY$^-$, no PGY1 overexpression.

cantly reduce cytotoxicity of doxorubicin, vincristine, etoposide, cisplatin, cyclophosphamide and ifosfamide on the two neuroblastoma cell lines.

Discussion

Amifostine, an analog of cysteamine, is a phosphorylated aminothiol prodrug that is dephosphorylated at the tissue site by membrane-bound alkaline phosphatase to its active metabolite, the free thiol, WR-1065. WR-1065 is the form of the drug that is taken up into cells via passive diffusion. The disulfide form is taken up actively via a polyamine transporter system.[11]

Preferential uptake and differential concentration of the free thiol in normal versus malignant tissue allows a temporary state of acquired resistance to the effects of chemotherapy. Amifostine has been reported and clinically developed as an agent which protects normal hematopoietic cells against the toxic effects of alkylating and platinum agents, and prevents renal damage from cisplatin. Neuroblastoma is the most common extracranial solid tumor in childhood.[21] The last 25 years have only slightly improved the chances of cure for the 75% of children with neuroblastoma who present with advanced disease (stages 3 and 4). Currently aggressive chemotherapy with increased dose intensity is being explored.[20] The experience with amifostine in children is very preliminary. Adamson *et al.*[33] reported on a phase I study in 19 patients with refractory cancer (no child had neuroblastoma). The data demonstrated a good tolerability of amifostine without reaching the maximum tolerated dose at 2700 mg/m^2. Borsi *et al.*[34] showed a reduced grade II–IV leuco- and thrombocytopenia after high-dose carboplatin in a child with medulloblastoma.

The data presented here shows that amifostine does not reduce the anti-tumor activity of the chemotherapy agents commonly applied for this tumor. Neither WR-2721, the inactive prodrug, has shown interaction with the series of drugs tested (i.e. doxorubicin, vincristine, etoposide, cisplatin, cyclophosphamide and ifosfamide) nor did WR-1065 negatively impact on growth inhibition of neuroblastoma cells. Of particular importance is the integrity of anti-tumor activity along the full stretch of dose–response curves, as presented for doxorubicin in the presence or absence of WR-2721 or WR-1065. This excludes the clinically relevant aspects of reduced activity, e.g. in the high dose range of doxorubicin.

The latter observation is also important in view of the mechanisms underlying the selective protective effects of amifostine which have not been fully revealed. Whereas the discrepant alkaline phosphatase activity on normal versus malignant cells may be an important factor allowing rapid uptake and protective concentrations in normal tissue, the lack of protection in malignant cells after exposure to WR-1065 requires a different mechanism. Murley and Grdina recently described an intracellular protein-dependent mechanism which is initiated by WR-1065 resulting in a shift of cell cycles to the late S-phase which is typically providing protection against toxic agents.[12] These newly defined intracellular mechanisms of WR-1065 appear to facilitate the efficacy and fidelity of intracellular repair systems. It may well be that malignant cells lack or exhibit different kinetics of this mechanism.

Further studies are warranted to explore the differential intracellular mechanisms of WR-1065 between normal and malignant tissue. Several reports are available on the lack of evidence of reduced anti-tumor effects when chemotherapy is given adjacent to amifostine.[10,22–31] Interaction stud-

ies in neuroblastoma, however, have not yet been published. Our data are reassuring that amifostine, which has unequivocal protective efficacy on hematopoietic and non-hematopoietic normal cells, does fully preserve the anti-tumor efficacy of potent chemotherapy agents which are commonly used for the treatment of neuroblastoma. We thus have also established the rationale to explore amifostine in animal models and phase I clinical trials in this disease.

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