Cancer Chemother Pharmacol (1999) 44: 498–504

ORIGINAL ARTICLE

Abdul-Kader Souid · Robert C. Fahey
Ronald L. Dubowy · Gerald L. Newton
Mark L. Bernstein

WR-2721 (amifostine) infusion in patients with Ewing's sarcoma receiving ifosfamide and cyclophosphamide with mesna: drug and thiol levels in plasma and blood cells, a Pediatric Oncology Group study

Received: 14 December 1998 / Accepted: 10 May 1999

Abstract *Purpose*: Previous WR-2721 human pharmacokinetic studies were limited to plasma levels in patients receiving platinum-based compounds, and none includes the effects of WR-2721 on endogenous thiols. In the present study (Pediatric Oncology Group study no. 9457), we measured the levels of WR-2721, its active metabolites, as well as cysteine and glutathione in whole blood, plasma, and blood cells in patients receiving high-dose alkylating agents with mesna. *Methods*: WR-2721 was administered (15 min intravenous infusion of 825 mg/m^2 per dose ×2) to five patients with metastatic Ewing's sarcoma receiving ifosfamide and cyclophosphamide with mesna. Intracellular and extracellular blood thiols were labeled with monobromobimane (mBBr) at the time of collection, and the low molecular weight (LMW) thiols were subsequently separated by HPLC and detected by fluorescence. *Results*: The active metabolite of the drug, WR-1065, peaked at 100 μ*M* in plasma and blood cells at the end of WR-2721 infusion and decayed with a rapid initial half-life. Detectable levels of WR-1065 and its LMW disulfides were present in plasma and blood cells at ∼1 h after the WR-2721 infusion. By the end of the first WR-2721 infusion (prior to mesna infusion), the mean cysteine level more than doubled and the mean Cys-SS-LMW (cystine and the mixed LMW disulfides) level decreased by ∼50% in both plasma and blood cells. In four of five patients, reduced glutathione levels in blood cells increased by the end of the first WR-2721 infusions, the average increment being ∼36%. *Conclusions*: (1) WR-1065 is rapidly formed from WR-2721 and equilibrates between plasma and blood cells; (2) WR-1065 decays in plasma and blood cells with similar rapid initial half-lives of ∼16 min; (3) WR-2721 treatment increases cysteine in plasma and blood cells, an effect similar to that of mesna; (4) WR-2721 treatment appears to increase glutathione levels in blood cells; (5) Mesna does not have a substantial effect on the fate of WR-2721 in patients.

Key words WR-2721 · Chemoprotection · Pediatric oncology · Thiols · Monobromobimane

A.-K. Souid (✉) · R.L. Dubowy
State University of New York,
Health Science Center at Syracuse,
Department of Pediatrics,
750 East Adams Street,
Syracuse, NY 13210, USA
e-mail: souida@hscsyr.edu
Tel.: +1-315-464-5294; Fax: +1-315-464-7238

R.C. Fahey · G.L. Newton
University of California, San Diego,
Department of Chemistry and Biochemistry,
9500 Gilman Drive, La Jolla,
CA 92093-0506, USA

M.L. Bernstein
McGill University – Montreal Children's Hospital,
Department of Hematology,
C 407, 2300 Tuper Street,
Montreal, QC H3H1P3, Canada

Introduction

WR-2721 (amifostine, Ethyol, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid) is an FDA-approved chemoprotective agent. Its clinical role in cancer therapy has been recently reviewed [2, 3]. WR-2721, a prodrug, is dephosphorylated by alkaline phosphatase to the active free thiol metabolite WR-1065 [*S*-2-(3-aminopropylamino)ethanethiol]. WR-1065, in turn, is oxidized to protein (WR-SS-protein) and low molecular weight (WR-SS-LMW) disulfides, including the symmetric disulfide, WR-33278.

Previous WR-2721 human pharmacokinetic studies have been limited to plasma levels in patients receiving cisplatin or carboplatin [9, 10, 16, 18]. However, WR-2721 is currently incorporated in many chemotherapeutic regimens containing high-dose alkylating agents with mesna (sodium-2-mercaptoethanesulfonate, $HS\text{-}CH_2\text{-}CH_2SO_3^-Na^+$ (M_r 164.18) [18], an extracellular uroprotective agent which prevents ifosfamide- and cyclophosphamide-induced hemorrhagic cystitis. Thus, it is

important to establish the fate of amifostine and its metabolites in patients receiving both amifostine and mesna. Furthermore, drug levels in blood cells and the effects of WR-2721 treatment on endogenous thiols have not been previously reported. Since blood cells comprise ~40% of the blood volume, it seemed important to establish the form and content of the drug in this compartment.

The Pediatric Oncology Group (POG) is testing the clinical efficacy of WR-2721 in patients with metastatic Ewing's sarcoma (POG #9457) receiving high-dose ifosfamide and cyclophosphamide with mesna. In the present study, we measured WR-2721, WR-1065, and WR-SS-LMW in the patients' whole blood, plasma, and blood cells using methods recently developed for this purpose [5, 21]. In addition, the effects of WR-2721 treatment (prior to mesna infusion) on the cysteine and glutathione levels are reported.

Materials and methods

Chemicals

WR-2721, WR-1065 and WR-33278 were obtained from U.S. Bioscience (West Conshohocken, Pa.); monobromobimane (mBBr) from Molecular Probes (Eugene, Ore.); *E. coli* alkaline phosphatase (7.6 units/μl), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), cysteine, glutathione, tris(hydroxymethyl)-aminomethane (Tris) and high-performance liquid chromatography (HPLC) grade methanol, acetonitrile, methyl sulfoxide (DMSO) and dichloromethane from Sigma (St. Louis, Mo.); trifluoroacetic acid (TFA) and methanesulfonic acid (MSA) from Fluka BioChemika (Ronkonkoma, NY); highest purity dithiothreitol (DTT) from Calbiochem (San Diego, Calif.); and perchloric acid (PCA) from Aldrich (Milwaukee, Wis.).

WR-2721·3H_2O (M_r 268.26), WR-1065·2HCl (M_r 207.16), WR-33278·4HCl (M_r 412.31), mixed thiol standard, mBBr stock solutions, DTNB, DTT, and sodium methane sulfonate (NaMS) were prepared and stored as described previously [21]. The standard solutions were stored (up to 3 months) at −70 °C in small aliquots and thawed for single use. Each calibration level was validated (within-day and between-day validations) by assaying both freshly prepared and stored mixed thiol solutions. The bimane thiol derivatives were most stable when stored in the dark under mildly acidic (pH 5–6) conditions. The detection sensitivity was at the picomole level. The 2 μ*M* thiol standards were used to generate a calibration curve with each analytical run which was linear from 1.0 to 100 pmol. The concentrations of the standard thiols were measured using Ellman's reagent as described previously [4, 8, 21].

Patients

Patient #1 was an 11-year-old male, patient #2, a 24-year-old male patient #3, a 6-year-old female, patient #4, a 13-year-old female, and patient #5, a 16-year-old female. Because of advanced cancer, these patients may deviate from "normal status". The study was approved by the Institutional Review Board of each participating institution. Informed consent was obtained for each patient.

Drug administration and pharmacokinetic sampling

WR-2721 (825 mg/m^2) was administered intravenously (IV) over 15 min, beginning 30 min prior to ifosfamide and cyclophosphamide. WR-2721 was repeated 3 h after the first dose. Mesna was given as well, with the first dose just after the first WR-2721 dose. The cyclophosphamide course started 3 weeks after the ifosfamide course.

The ifosfamide course (days 1–5) was as follows: 0–0.75 h, etoposide 100 mg/m^2 over 0.75 h; 0.75–1 h, amifostine 825 mg/m^2 over 15 min; 1–1.25 h, mesna 400 mg/m^2 over 15 min; 1.25–3.25 h, ifosfamide 3.6 g/m^2 with mesna 400 mg/m^2 over 2 h; 3.25–6.25 h, mesna 400 mg/m^2 over 3 h; 3.75–4.0 h, amifostine 825 mg/m^2 over 15 min. During the ifosfamide course, blood samples were collected from each patient on day 1 at 0.75 h (i.e. just before first WR-2721 infusion), 1.0 h (i.e. immediately after the first WR-2721 infusion, but before mesna infusion), 1.25 h, 3.25 h, 3.75 h (i.e. just before the second WR-2721 infusion), 4.0 h (i.e. immediately after the second WR-2721 infusion), 4.25 h, and 4.5 h.

The cyclophosphamide course (days 1 and 2) was as follows: 0–0.25 h, amifostine 825/m^2 over 15 min; 0.25–0.5 h, mesna 400 mg/m^2 over 15 min; 0.5–1.0 h, cyclophosphamide 2.1 g/m^2 with mesna 400 mg/m^2 over 30 min; 1.0–4.0 h, mesna 400 mg/m^2 over 3 h; 3.0–3.25 h, amifostine 825/m^2 over 15 min; 4.0–4.25 h, mesna 400 mg/m^2 over 15 min repeated every 3 h ×2; 4.25–52.25 h, adriamycin 75 mg/m^2 over 2 days. During the cyclophosphamide course, blood samples were also collected from the same patients on day 1 at 0 h (i.e. just before the first WR-2721 infusion), 0.25 h (i.e. immediately after first WR-2721 infusion, but before mesna), 0.5 h, 1.0 h, 3.0 h (i.e. just before the second WR-2721 infusion), 3.25 h (i.e. immediately after the second WR-2721 infusion), 3.5 h, and 3.75 h.

For each time-point, 1 ml of blood was collected and injected into a 3-ml foil-covered purple-topped (EDTA) Vacutainer tube containing a final concentration of 30 m*M* mBBr (from a 1.0 *M* stock in DMSO). Samples were mixed by inversions at room temperature (RT) for 2–3 min and shipped on ice. On arrival, the status of each sample was checked immediately, and only samples that arrived well-refrigerated and with no or minimum hemolysis were included in the results reported here.

Sample preparation

Plasma and blood cells were separated using a previously described method [21]. Four volumes of 2.5% perchloric acid, 2 *N* NaMS were added to whole blood, plasma, and blood cells. The samples were mixed by vortexing. The supernatants were recovered by centrifugation, extracted with H_2O-saturated dichloromethane, and stored at −70 °C until analysis.

Sample analysis

The acid-soluble supernatants of whole blood, plasma, and blood cells were processed in the dark with alkaline phosphatase and DTT (sample 1), DTT alone (sample 2), and without either (sample 3), for determination of phosphorothioate plus LMW disulfides plus thiols, thiols plus LMW disulfides, and thiols alone, respectively [21]. Sample 1 contained 100 μl supernatant, 40 μl 1.0 *M* Tris-base, and 20 μl alkaline phosphatase, final pH 8.0. The mixture was incubated at 37 °C for 30 min. Then, DTT was added to 3.0 m*M* from a freshly made 0.1 *M* ice-cold stock. The mixture was incubated at RT for 30 min. Additional DTT was added to achieve a total final concentration of 4.2 m*M*. The incubation was continued for another 30 min. Then, 10.7 m*M* mBBr was added from a 0.1 *M* stock solution in acetonitrile. The reaction was allowed to continue at RT for 30 min. Additional mBBr was added to achieve a total final concentration of 15.7 m*M*. The derivatization reaction was quenched by the additions of 5 μl 5.0 *M* MSA and 98 μl 10 m*M* MSA. The mixture was extracted once with H_2O-saturated dichloromethane, and 50 μl of the supernatant was injected onto the HPLC column and analyzed as described below. The dichloromethane extraction procedure did not reduce the recovery from control standards. Sample 2 was exactly as sample 1, except that the supernatant was incubated with 20 μl 0.5 *M* ammonium sulfate instead of alkaline phosphatase. Sample 3 was exactly as sample 2,

except that the supernatant was incubated with H_2O instead of DTT and acetonitrile instead of mBBr.

Final concentrations were calculated using the measured volume of whole blood, plasma and separated red cells. WR-2721 was calculated from the difference in WR-1065 value in samples 1 and 2, thiol-SS-LMW were calculated from the difference in thiol content between samples 2 and 3, and thiol values were established from the analysis of samples 3.

HPLC analysis

The HPLC analysis was performed on a Beckman (Fullerton, Calif.) HPLC system (model 125) gradient liquid chromatograph with autoinjector exactly as described previously [21]. The bimane derivatives were detected using a Model FL3000 fluorometer (Thermo Separation Products) equipped with a standard flow cell and operated with 390 nm excitation and 480 nm emission filters [15, 21]. A linear calibration curve, from 1.0 to 100 pmol of mixed thiol standard, was generated with each set of analytical runs. Levels of WR-1065 below 0.3 μM were not reliably measurable owing to background signals present in samples prior to the WR-2721 treatment. Final concentrations were calculated using the measured volume of whole blood, plasma, and separated blood cells. Values for the mixed disulfides were calculated as thiol equivalents, i.e. one equivalent of cystine equals two equivalents of Cys-SS-LMW.

Results

WR-2721 and its metabolites

Remarkable variability in WR-2721 concentrations in whole blood at the end of WR-2721 infusions existed among the patients (Table 1). However, on average, the remaining WR-2721 represented only ~5% of the administered dose, and this residual drug declined to below 10 μM (i.e. $<1\%$ of total dose) within minutes after the completion of the WR-2721 infusion.

At the end of WR-2721 infusion, the WR-1065 concentrations in plasma and blood cells were very similar, demonstrating that the plasma and blood cell pools equilibrate rapidly (Table 1 and Fig. 1). The data for the decline during the hour after the end of the infusion were fitted to a first-order decay to obtain a calculated maximum concentration (C_{max}) and a decay half-life ($t_{1/2}$), as shown in Table 2. Because of the large uncertainties, it was not immediately apparent that the C_{max} and $t_{1/2}$ values differed in a meaningful fashion between first and second infusions, between ifosfamide and cyclophosphamide courses, or between plasma and blood cells. Values of C_{max} (109 ± 34 μM, mean ± SD) and $t_{1/2}$ (16 ± 6 min) were obtained using all of the data in Table 2 (i.e. plasma and blood cell values for all WR-2721 infusions) and were used to generate the lines shown in Fig. 1. Most of the data points for the first infusion fell below the line while those for the second infusion fell above the line, for plasma as well as blood cells. We conclude that the pattern of the C_{max} for the second infusion exceeding the C_{max} for the first infusion in Table 2 reflects real differences. Low, but significant residual WR-1065 was measured in some patients at times just prior to the second infusion (Fig. 1), suggesting that the apparent first-order decay of WR-1065 does not continue beyond the first hour, but the data were too variable and too limited to fit a more complex model.

Results from the determination of WR-SS-LMW are shown in Table 1 and Fig. 2, and exhibit an even wider variability than the values for WR-1065. This was not unexpected since WR-SS-LMW values were determined as the differences between the WR-1065 content and the total of the WR-SS-LMW plus WR-1065 content. Since both numbers were of the same magnitude, the error associated with the difference became large. The WR-SS-LMW values at the end of WR-2721 infusion were lower than those for WR-1065 (Table 1), and appeared to decline at a slower rate, in plasma as well as in blood cells (Fig. 2).

Table 1 Maximum levels of WR-2721, WR-1065, and WR-SS-LMW in whole blood, plasma, and blood cells. The values are mean ± SD C_{max} at the end of WR-2721 infusions and the numbers in parentheses are the number of WR-2721 infusions

Patient no.	Blood fraction	WR-2721 (μM)	WR-1065 (μM)	WR-SS-LMW (μM)
1	Whole blood	58 ± 14 (4)	105 ± 16 (4)	31 ± 12 (4)
	Plasma	–	101 ± 15 (4)	21 ± 19 (4)
	Blood cells	–	115 ± 37 (4)	15 ± 9 (4)
2	Whole blood	213 ± 182 (4)	75 ± 20 (4)	102 ± 77 (4)
	Plasma	–	98 ± 27 (4)	202 ± 167 (4)
	Blood cells	–	65 ± 4 (4)	42 ± 35 (3)
3	Whole blood	54	62	22
	Plasma	–	82	<0.3
	Blood cells	–	40	60
4	Whole blood	29	35	32
	Plasma	–	17	7
	Blood cells	–	78	–
5	Whole blood	9 ± 3 (4)	58 ± 14 (4)	14
	Plasma	–	74 ± 10 (4)	19 ± 8 (4)
	Blood cells	–	82 ± 47 (4)	25
Mean	Whole blood	86 ± 123 (14)	75 ± 26 (14)	46 ± 53 (14)
	Plasma	–	85 ± 27 (14)	75 ± 122 (14)
	Blood cells	–	83 ± 37 (14)	31 ± 26 (9)

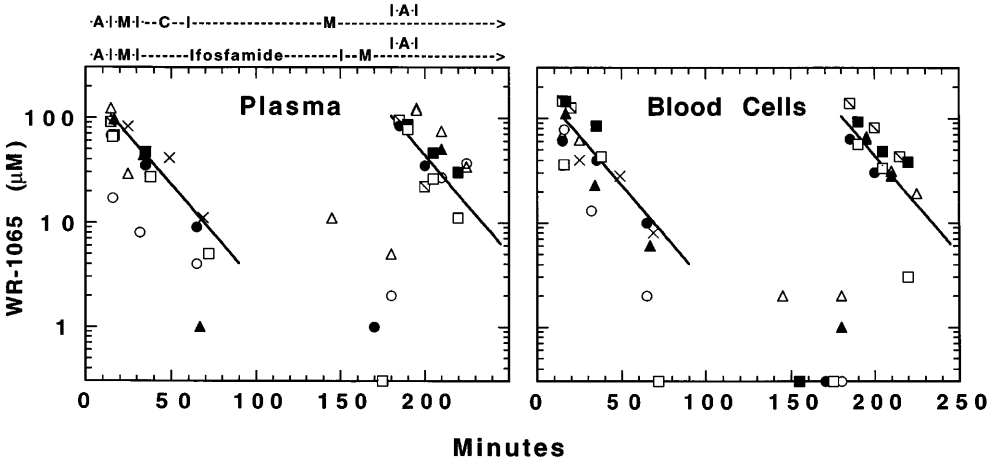

Fig. 1 WR-1065 concentrations in plasma and blood cells. Each set of concentrations depicts a patient's levels measured during one course of WR-2721, administered at times 0 and 180 min. The time-course of drug delivery is shown above the left panel (*A* amifostine, *M* mesna, *C* cyclophosfamide, *Ad* adriamycin). Solid lines were calculated for the first-order kinetic decay with $C_{max} = 109$ μ*M* and $t_{1/2} = 16$ min. The symbols represent patient 1 during the ifosfamide course, patient 1 during the cyclophosphamide course, patient 2 during the ifosfamide course, patient 2 during the cyclophosphamide course, patient 3 during the cyclophosphamide course, patient 4 during the ifosfamide course, patient 5 during the ifosfamide course, and patient 5 during the cyclophosphamide course

Cysteine and glutathione components

The cysteine content of plasma increased significantly during the 15-min WR-2721 infusion (Table 3 and Fig. 3, left panel), whereas the Cys-SS-LMW value declined sharply in most patients during this interval (Fig. 3, right panel). The cysteine values remained elevated and the Cys-SS-LMW content continued to decline (or remained low) during the subsequent 15-min mesna infusion which followed. Qualitatively similar, but substantially reduced changes occurred during the second infusion (Fig. 3). The total cyst(e)ine (i.e. cysteine + Cys-SS-LMW) levels in plasma did not increase following the WR-2721 infusions (Table 3). Thus, the cysteine increments appeared to reflect rapid reductions of Cys-SS-LMW to cysteine (Fig. 3). Cellular cysteine was also elevated during the first WR-2721 infusion, and in some patients during the second infusion (Fig. 4, left panel).

Remarkable variability in cellular GSH levels existed among the patients (Fig. 4, right panel), possibly reflecting different clinical situations, as well as individual variations. In four of the five patients cellular reduced glutathione (GSH) exhibited a modest or marked increase during the first WR-2721 infusion, but in one patient it declined slightly (Fig. 4, right panel). The cellular GSH level prior to the first WR-2721 infusions was 609 ± 291 μ*M* (mean ± SD, $n = 6$) and at the end of the first infusions (i.e. prior to the first mesna) 831 ± 277 μ*M* ($n = 6$), that is, an average increment of 36%. The increment between GSH and total glutathione did not yield reliable values for glutathione disulfides (data not given) because the increment was comparable to the uncertainties in the values.

Discussion

Recently, we adapted the method of fluorescent labeling of biologic thiols with mBBr to determine WR-2721, WR-1065, and WR-SS-LMW in whole blood, plasma, and blood cells [21]. Thiols react rapidly with mBBr to form fluorescent bimane derivatives, which are readily separated by HPLC and detected with good sensitivity [6, 11, 15]. Since WR-1065 and other thiols oxidize within minutes in plasma [5, 14, 16], it is important to rapidly trap them at the time of blood sample collection to avoid losses leading to low values for thiols and elevated values for disulfides. Collection of blood in Vacutainer tubes containing mBBr has been shown to give reliable results for endogenous thiols [14], and in our protocol blood samples were injected into Vacutainer tubes containing mBBr immediately after collection [21].

Table 2 Kinetic parameters for decay of WR-1065 in plasma and blood cells. The numbers in parentheses are the number of WR-2721 infusions

Blood fraction	Chemotherapy course	End of first WR-2721 infusion		End of second WR-2721 infusion	
		C_{max} (μ*M*)	$t_{1/2}$ (min)	C_{max} (μ*M*)	$t_{1/2}$ (min)
Plasma	Ifosfamide	58 ± 22 (8)	13 ± 5 (8)	97 ± 36 (10)	26 ± 15 (10)
	Cyclophosphamide	100 ± 35 (12)	13 ± 3 (12)	148 ± 60 (7)	12 ± 4 (7)
Blood cells	Ifosfamide	124 ± 35 (9)	9 ± 3 (9)	134 ± 33 (8)	18 ± 4 (8)
	Cyclophosphamide	69 ± 13 (11)	18 ± 3 (11)	145 ± 68 (7)	9 ± 2 (7)

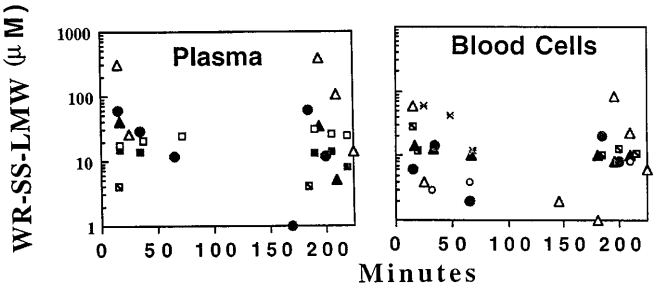

Fig. 2 WR-SS-LMW concentrations in plasma and blood cells. Each set of concentrations depicts a patient's levels measured during one course of WR-2721, administered at times 0 and 180 min. The time course of drug delivery is shown above Fig. 1, left panel. Symbols are as described for Fig. 1

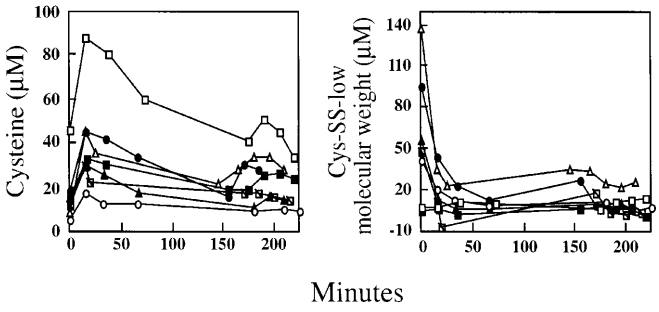

Fig. 3 Plasma levels of cysteine and Cys-SS-LMW following WR-2721 infusions. Each curve depicts a patient's levels measured during one course of WR-2721 administered at times 0 and 180 min. The time course of drug delivery is shown above Fig. 1, left panel. Symbols are as described for Fig. 1

The C_{max} values determined here for WR-2721 at the end of the infusion exhibited marked variation (Table 1), in part because they were calculated as the difference between two comparable numbers. Nevertheless, they showed that, on average, ~95% of the administered dose was cleared from the plasma by the end of the WR-2721 infusion. This is in general accord with other studies in humans involving WR-2721 delivery as a bolus dose [16], or 15-min infusion [9, 10].

The C_{max} values for plasma WR-1065 (Tables 1 and 2) were as much as twofold greater than those determined in other studies for infusion of comparable doses [10, 18]. This difference cannot be ascribed to an effect of mesna preventing or reversing the loss of WR-1065 by oxidation, because the WR-1065 values of Table 1 were determined at the end of the first WR-2721 infusion and prior to the first mesna infusion. The difference more probably derives from the different methods used to quench the oxidation of WR-1065. The earlier studies utilized ice-cold perchloric acid to quench oxidation immediately after collection and manipulation of samples. In the present work, WR-1065 reacted with mBBr immediately after the blood was collected. The latter protocol may capture WR-1065 more efficiently, accounting for the higher values found here.

It has been reported previously that the second and third infusions of WR-2721 produce C_{max} values for plasma WR-1065 that are, respectively, 66% and 79% higher than for the first infusion [10]. A somewhat smaller increase (58% with ifosfamide and 33% with cyclophosphamide, Table 2) was found for the second infusion in the present study.

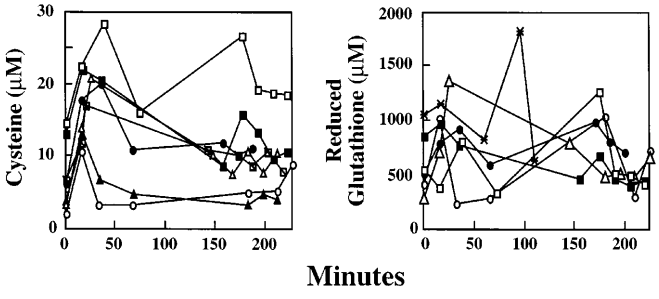

Fig. 4 Blood cell levels of cysteine and GSH following WR-2721 infusions. Each curve depicts a patient's levels measured during one course of WR-2721, administered at times 0 and 180 min. The time course of drug delivery is shown above Fig. 1, left panel. Symbols are as described for Fig. 1

The rate of WR-1065 decay measured in the present study is also in accord with the earlier work [10]. The values of $t_{1/2}$ for initial decay of WR-1065 in plasma (Table 2) yield an average of 12.7 min, which is in reasonable agreement with the value of $t_{1/2\alpha}$ of 10.8 min reported earlier [10]. These kinetics are determined during and immediately following the infusion of mesna, so this agreement indicates that mesna does not markedly influence the decay of WR-1065 in plasma.

The results obtained for WR-SS-LMW did not prove very useful, in part because of the large error associated with the method of determination (as a difference between measured values of comparable magnitude). More precise data would have resulted had a separate blood

Table 3 Levels of cysteine and total cyst(e)ine in whole blood, plasma, and blood cells before and immediately after the first WR-2721 infusion (i.e. prior to any mesna infusion). The values are means ± SD, and the numbers in parentheses are the number of WR-2721 infusions. Total cyst(e)ine represents (cysteine + Cys-SS-LMW)

Thiol	First WR-2721 infusion	Whole blood	Plasma	Blood cells
Cysteine (μ*M*)	Before	12 ± 6 (14)	16 ± 13 (7)	7 ± 5 (7)
	After	26 ± 12 (14)	40 ± 24 (7)	17 ± 5 (7)
Total cyst(e)ine (μ*M*)	Before	60 ± 30 (14)	72 ± 43 (7)	34 ± 29 (7)
	After	57 ± 31 (14)	63 ± 30 (7)	32 ± 21 (7)

sample been taken in which WR-1065 and other thiols were quenched by reaction with *N*-ethylmaleimide prior to determination of disulfides [10]. However, this would have doubled the number of blood samples required.

The mean maximum value for WR-SS-LMW of 75 μM (Table 1) was intermediate between the value of 7–11 μM reported for the symmetrical disulfide WR-33278 after a bolus dose of WR-2721 [16] and the mean value of 184 μM found for all disulfide forms, including WR-SS-protein, after an initial 15-min infusion of WR-2721 [10]. The WR-SS-protein content, not measured in the present study, appears to make a large contribution to the total drug in plasma, and we expect to include this component in future studies.

The most aberrant value for WR-SS-LMW was found in the plasma of patient 3, in which no disulfides could be detected (Table 1). This does not appear to be the result of a defective determination, since the values for WR-SS-LMW measured in blood cells and whole blood verify the low plasma content. Thus, the blood cell value of 60 μM contributes approximately 20 μM to the whole blood content in this patient with a hematocrit of 30%. The measured whole blood content was only 22 μM, leaving little or no contribution from the plasma. Detectable levels of WR-1065 and WR-SS-LMW were present in both plasma and blood cells $\sim$1 h after the infusion (Figs. 1 and 2). The cationic character of WR-1065 allows it to concentrate near the DNA (in a similar manner to biologic polyamines) [19, 20], where its protective capacity is likely to be enhanced [22]. A second dose, perhaps 2 h after the first dose may be necessary for alkylating agents that are long-acting or infused over a long time (e.g. ifosfamide). It is unknown whether or not patient blood levels correlate with tissue levels. Marked variability in WR-1065 uptake exists among different tissues in animal models [17, 23]. However, during the interval 5–30 min after a single intraperitoneal injection of WR-2721 in mice, the pattern of variation in WR-1065 levels for several tissues is generally similar to that in whole blood, although the values for the tissue levels are mostly larger than those in blood [17].

The production of WR-1065 in plasma leads to the reduction and release of disulfide-bound cysteine residues, increasing the plasma concentration of cysteine (Fig. 3, left panel). The plasma cysteine levels drop shortly after the first infusions, and the increments following the second infusions are less than those following the first (Fig. 3, left panel). Since mesna has a similar effect on plasma cysteine (see below), cysteine levels after the first mesna infusion reflect combined effects of WR-2721 and mesna. The cysteine levels increased in the plasma 2–7-fold and in blood cells 2–12-fold by the end of the first WR-2721 infusion (i.e. prior to any mesna infusion; mean increment 2.5-fold for each; Table 3). The cysteine increments in blood cells (Fig. 4, left panel) presumably reflect increased uptake of cysteine from plasma. This was accompanied by an increase in GSH levels in four of the five patients studied. Addition of thiols to the culture medium leads to release of bound cysteine which is taken up by cultured cells and used to enhance the cellular GSH content [1, 7]. This effect has not been seen in WR-2721 treatment of experimental animals [17, 23]. It remains to be seen whether or not the observed variation in glutathione levels correlates with the toxicity to alkylating agents.

Mesna has an effect on plasma cysteine similar to that described above for WR-2721. In healthy volunteers, 5 min after mesna administration (7.3 mmol, $\sim$1.2 g), plasma cysteine levels increased from 8.2 μM to 54 μM and total cyst(e)ine (i.e. cysteine, cystine, and all mixed disulfides) levels decreased from 276 μM to 102 μM. The authors concluded that the rise in the cysteine was due to a reduction in circulating cystine as a result of the presence of mesna, and the decrease in total plasma cysteine was due to increased cysteine uptake into cells and increased urinary excretion of cysteine [12]. Our findings show that mesna does not further increase cysteine over the post-WR-2721 value, but it may contribute to maintaining plasma Cys-SS-LMW at a low value so that the second WR-2721 treatment produces a smaller increase in plasma cysteine than that seen following the first treatment (Fig. 3, left panel), with no increase in cellular cysteine (Fig. 4, left panel). In three children with bone marrow failure syndromes who received WR-2721 (200 mg/m^2 IV push) without mesna or chemotherapy, the cysteine levels returned to the baseline 1 h after the dose. In these patients, the plasma cysteine level prior to WR-2721 administration was 7 ± 2 μM, immediately after 24 ± 2 μM, and 1 h after 7 ± 2 μM (unpublished data). Cys-SS-protein was not determined in these studies and may contribute significantly to total cyst(e)ine content.

The high doses of ifosfamide and cyclophosphamide cannot be given without mesna, and WR-2721 soon may become an important component of this combination. The plasma levels of WR-1065 and WR-SS-LMW shown in Tables 1 and 2 and Figs. 1 and 2 (left panels) do not substantially deviate from levels reported without mesna [9, 10, 13, 16–18, 21, 23]. Therefore, the same WR-2721 dosages can be used for mesna-containing regimens.

In conclusion, the most important finding in this study was that the WR-2721 protective metabolite, WR-1065, rapidly appears in blood cells at $\sim$100 μM and decays rapidly; however, measurable levels are still present 1 h after the infusion. In addition, the WR-2721 treatment modulates endogenous thiols, an effect that may contribute to the cytoprotective capacity of the drug. Mesna does not appear to substantially modify WR-1065 levels.

Acknowledgements The authors are in debt to Dr. Annelies E.C. Korst for careful review of the manuscript. The study was supported in part by grants from the National Cancer Institute (CA-28439, CA-33587, CA-30969, CA-35982), a grant from U.S. Bioscience to the Pediatric Oncology Group, and a generous gift from Alfred T. Murphy.

References

1. Aguilera JA, Newton GL, Fahey RC, Ward JF (1992) Thiol uptake by Chinese V79 cells and aerobic radioprotection as a function of the net charge on the thiol. Radiat Res 130: 194
2. Amifostine (Ethyol®): current and future applications in cytoprotection (1996). Eur J Cancer 32A [Suppl. 4]: S1–S49
3. Applications of amifostine in cancer treatment (1996). Semin Oncol 23 [Suppl. 8]: 1–99
4. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82: 70
5. Fahey RC, Newton GL (1985) Measurement of WR-2721, WR-1065, and WR-33278 in plasma. Int J Radiat Oncol Biol Phys 11: 1193
6. Fahey RC, Newton GL (1987) Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. Methods Enzymol 143: 85
7. Issels RD, Nagele A (1989) Promotion of cysteine uptake, increase of glutathione biosynthesis, and modulation of glutathione status by S-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR-2721) in Chinese hamster cells. Cancer Res 49: 2082
8. Jocelyn PC (1987) Spectrophotometric assay of thiols. Methods Enzymol 143: 44
9. Korst AEC, Gall HE, Vermorken JB, van der Vijgh WJF (1996) Pharmacokinetics of amifostine and its metabolites in the plasma and ascites of a cancer patient. Cancer Chemother Pharmacol 39: 162
10. Korst AEC, Eeltink CM, Vermorken JB, van der Vijgh WJF (1997) Pharmacokinetics of amifostine and its metabolites in patients. Eur J Cancer 33: 1425
11. Kosower NS, Kosower EM (1987) Thiol labeling with bromobimanes. Methods Enzymol 143: 76
12. Lauterburg BH, Nguyen T, Hartmann B, Junker E, Kupfer A, Cerny T (1994) Depletion of total cysteine, glutathione, and homocysteine in plasma by ifosfamide/mesna therapy. Cancer Chemother Pharmacol 34: 132
13. Mangold DJ, Huelle BK, Miller MA, Geary RS, Sanchez-Barona DOT, Swynnerton NF, Fleckenstein L, Ludden TM (1990) Pharmacokinetics and disposition of WR-1065 in the rhesus monkey. Drug Metab Dispos 18: 281
14. Mansoor MA, Svardal AM, Ueland PM (1992) Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. Anal Biochem 200: 218
15. Newton GL, Fahey RC (1995) Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. Methods Enzymol 251: 148
16. Shaw LM, Turrisi AT, Glover DJ, Bonner HS, Norfleet AL, Weiler C, Kligerman MM (1986) Human pharmacokinetics of WR-2721. Int J Radiat Oncol Biol Phys 12: 1501
17. Shaw LM, Bonner HS, Brown DQ (1994) Metabolic pathway of WR-2721 (ethyol, amifostine) in the BALB/c mouse. Drug Metab Dispos 22: 895
18. Shaw LM, Bonner HS, Schuchter L, Schiller JH, Nakashi MA, Lieberman R (1996) Population pharmacokinetics of amifostine in cancer patients (abstract 1515). Proc Am Soc Clin Oncol 15: 478
19. Smoluk GD, Fahey RC, Ward JF (1986) Equilibrium dialysis studies of the binding of radioprotector compounds to DNA. Radiat Res 107: 194
20. Smoluk GD, Fahey RC, Ward JF (1988) Interaction of glutathione and other low-molecular-weight thiols with DNA: evidence for counterion condensation and coion depletion near DNA. Radiat Res 114: 3
21. Souid AK, Newton GL, Dubowy RL, Fahey RC, Bernstein ML (1998) Determination of the cytoprotective agent WR-2721 (amifostine, Ethyol®) and its metabolites in human blood using monobromobimane fluorescent labeling and high-performance liquid chromatography. Cancer Chemother Pharmacol 42: 400
22. Treskes M, van der Vijgh WJF (1993) WR-2721 as a modulator of cisplatin- and carboplatin-induced side effects in comparison with other chemoprotective agents: a molecular approach. Cancer Chemother Pharmacol 33: 93 (and references therein)
23. Utley JF, Seaver N, Newton GL, Fahey RC (1984) Pharmacokinetics of WR-1065 in mouse tissue following treatment with WR-2721. Int J Radiat Oncol Biol Phys 10: 1525