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Determination of the cytoprotective agent WR-2721 (Amifostine, Ethyol®) and its metabolites in human blood using monobromobimane fluorescent labeling and high-performance liquid chromatography

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Abstract *Purpose*: WR-2721 [*S*-2-(3-aminopropylamino)ethylphosphorothioic acid] is a chemoprotective agent that is currently in pediatric clinical trials. It is a prodrug that is dephosphorylated by alkaline phosphatase to the active free thiol form, WR-1065 [*S*-2-(3-aminopropylamino)ethanethiol]. It is likely that adequate and sustained cellular levels of the drug are necessary for optimum cytoprotection. To date, a method to measure both plasma and cellular levels of WR-2721 and its metabolites in clinical samples has not been available. *Methods*: In the study reported here the monobromobimane (mBBr) fluorescent labeling method was used to measure these levels when drug was added in vitro to blood samples from normal volunteers. In addition, we present pharmacokinetic data from a pediatric patient receiving WR-2721 (825 mg/m^2 × 2). *Results*: The results can be summarized as follows: (1) WR-2721 was detected in the patient's plasma with a half-life of about 10 min; (2) the WR-1065 concentration in the blood cellular fraction was similar to that of plasma; (3) both WR-1065 and WR-SS-low molecular weight (WR-SS-LMW) metabolites disappeared from plasma and the cellular fraction by 3.6 h after WR-2721 infusion; (4) a large proportion of WR-1065 was oxidized in plasma to WR-SS protein and WR-SS-LMW; (5) a large proportion of WR-1065 in the cellular fraction was oxidized to WR-SS-protein; (6) the WR-SS-LMW concentration in the cellular fraction was low; and (7) saturation of plasma and cellular protein binding sites was possible. *Conclusions*: The pharmacokinetic data that were generated with this technique could guide clinical trials using WR-2721.

Key words WR-2721 · Monobromobimane · Chemoprotection · Cancer therapy · Pediatric oncology

Abbreviations *ACN* acetonitrile · *DMSO* methyl sulfoxide · *DTNB* 5, 5-dithio-bis(2-nitrobenzoic acid) · *DTT* dithiothreitol · *EDTA* ethylenediaminetetraacetic acid · *HPLC* high-performance liquid chromatography · *IV* intravenous · *LMW* low molecular weight · *mBBr* monobromobimane · *MSA* methanesulfonic acid · *Na MS* sodium methane sulfonate · *PCA* perchloric acid · *RT* room temperature · *TFA* trifluroacetic acid · *Tris* tris(hydroxymethyl)aminomethane · *WR-SH* WR-1065 · *WR-SP* WR-2721 · *WR-SS-WR* WR-33278

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Introduction

WR-2721 (Amifostine, Ethyol®) is a chemoprotective agent [28–31] approved for use with cisplatin in adults. It is currently in pediatric clinical trials. A major obstacle in the development of optimal administration regimens is the lack of a suitable method to measure its cellular levels. To date, only limited plasma levels from adults have been reported [12, 21, 23, 24].

In this study, we adapted a method for the analysis of biological thiols, using fluorescent labeling with monobromobimane (mBBr), to the problem of measuring WR-2721, WR-1065 and the disulfide forms in whole blood, plasma and blood cellular fraction

[7, 8, 13, 14, 18]. The formulae of these compounds are as follows:

WR-2721 (WR-SP): $^{+}H_3N-(CH_2)_3-NH_2^{+}-(CH_2)_2-S-PO_3H^{-}$

WR-1065 (WR-SH): $^{+}H_3N-(CH_2)_3-NH_2^{+}-(CH_2)_2-SH$

WR-33278 (WR-SS-WR): $^{+}H_3N-(CH_2)_3-NH_2^{+}-(CH_2)_2-S-\bar{S}(CH_2)_2-NH_2^{+}-(CH_2)_3-NH_3^{+}$

Our results indicate that clearance rates of WR-2721 and WR-1065 in a pediatric patient were comparable to rates reported for adults. A large proportion of the drug was recovered in oxidized forms. Low molecular weight WR-SS (WR-SS-LMW) is a utilizable metabolite of the drug. Drug levels in the blood cellular fraction are the first to be reported.

Material and methods

Chemicals

WR-2721, WR-1065 and WR-33278 were obtained from US Bioscience (West Conshohocken, Pa.); 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 (ACN), methyl sulfoxide (DMSO), dichloromethane and chloroform from Sigma (St. Louis, Mo.); trifluroacetic acid (TFA) and methanesulfonic acid (MSA) from Fluka BioChemika (Ronkonkoma, N.Y.); highest purity dithiothreitol (DTT) from Calbiochem (San Diego, Calif.); and perchloric acid (PCA) from Aldrich (Milwaukee, Wis.).

WR-1065 · 2HCl (M_r 207.16), WR-2721 · $3H_2O$ (M_r 268.26) and WR-33278 · 4HCl) (M_r 412.31) 100 m*M* stock solutions were prepared in water and stored at −70 °C. The final concentrations of WR-1065 and WR-2721 were determined by DTNB titration, as described below. The WR-2721 and WR-33278 stocks contained <2% thiol by DTNB titration. Full recovery of WR-2721 was obtained with DTNB titration in the presence of alkaline phosphatase. Sodium methane sulfonate (Na MS) was prepared from 15.4 *M* MSA by addition of one equivalent of sodium hydroxide and dilution to 4.0 *M*. Two mBBr stock solutions, 1.0 *M* in DMSO (dissolved at 37 °C to force into solution) and 0.1 *M* in ACN, were stored at −20 °C and protected from light. Fresh 0.1 *M* DTT was made in water and placed on ice. DTNB 10 m*M* stock was made in 100 m*M* Tris-Cl, pH 8.0, and stored at 4 °C.

DTNB titration of free thiols

Thiol concentrations were measured using Ellman's reagent [6]. Diluted thiol samples were added to a 1.5 ml, 1 cm cuvette containing 0.2 m*M* DTNB in 100 m*M* Tris-Cl, pH 8.0. The absorbance at 412 nm ($\varepsilon_m = 13.6 \times 10^3$) was determined after the reduction of the DTNB by the free thiols was complete (5–15 min) [11].

Preparation of thiol standards

Cysteine, WR-1065 and glutathione were suspended to 10 m*M* in water and their final concentrations were determined by DTNB titration. Each bimane-thiol derivative was prepared by mixing to give final concentrations of 1 m*M* thiol, 3 m*M* mBBr and 20 m*M* Tris-sulfate, pH 8.0. The mixture was incubated in the dark at room temperature (RT, 25 °C) for 15 min. Then, MSA was added to a final concentration of 25 m*M* from a 5.0 *M* stock solution. Mixed thiol standards, 2 µ*M*, were made from the 1.0 m*M* bimane-thiol derivatives in 10 m*M* MSA and stored in small aliquots at −70 °C for up to 3 months. The 2 µ*M* thiol standards were used to generate a calibration curve which was linear from 1.0 to 100 pmol of standard injected on the HPLC column. The bimane thiol derivatives were most stable when stored in the dark in a mildly acidic (pH 5–6) condition [19]. The standard stock solutions were stored 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 detection sensitivity was at the picomole level.

HPLC analysis

Analysis was performed on a Beckman (Fullerton, Calif.) HPLC system, model 125 gradient liquid chromatograph with autoinjector. 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. Solvent A was 0.1% (v/v) TFA-water and solvent B was HPLC-grade methanol. The column, 4.6 × 250 mm Beckman Ultrasphere IP column, (cat. no. 235335), was operated at RT at a flow rate of 1.2 ml/min. The chromatography protocol employed linear gradients as follows: 0 min, 10% B; 18 min, 18% B; 19 min, 100% B; 28 min, 10% B; 40 min, reinject [8, 13,18]. At least three standard samples were included with each set of analytical runs. Peak identification was confirmed by retention time in comparison with authentic standards. Quantification was based on peak area against the appropriate reference standards. The minimum quantifiable level, with a signal-to-noise ratio of >3:1, was approximately 1 pmol and the minimum quantifiable concentration in the blood samples was 0.3 µ*M*.

Sample preparation

The use of plasma from a volunteer to develop the assay technique was approved by the local Institutional Review Board (IRB) for the Protection of Human Subjects. Blood was freshly drawn from a volunteer into a 3.0 ml purple-top (EDTA) Vacutainer tube containing a final concentration of 30 m*M* mBBr (from 1.0 *M* stock in DMSO) and various concentrations of WR-2721, WR-1065 or WR-33278. The sample was mixed by inversion at RT for 2 min and then placed in the dark at 4 °C until analysis. Plasma was separated by centrifugation in an International Clinical Centrifuge (IEC, Model CL) for 15 min at 2800 rpm and stored at −70 °C until analysis. The surface of the cellular fraction was rinsed twice with normal saline. Two volumes of 2.5% PCA, 2 *N* Na MS (or ACN) were added to whole blood, plasma and the cellular fraction, and mixed by vigorous vortexing. The supernatants were recovered by centrifugation, extracted twice with water-saturated dichloromethane and stored at −70 °C until analysis. The acid-precipitated pellets were washed twice with normal saline, resuspended in 0.1 *M* Tris-sulfate, pH 8.0, and stored at −70 °C until analysis.

Sample analysis

The protocol shown in Fig. 1 was used to measure WR-2721 and its thiol-disulfide forms. The samples were analyzed in the dark with alkaline phosphatase and DTT, DTT alone, and without either. **Reaction 1** contained 100 µl sample, 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 before addition of 3.0 m*M* DTT. The mixture was incubated at RT for 30 min. Additional DTT was added to achieve a total final concentration of 4.2 m*M*, and the incubation was continued for another 30 min. Then, 10.7 m*M* mBBr was

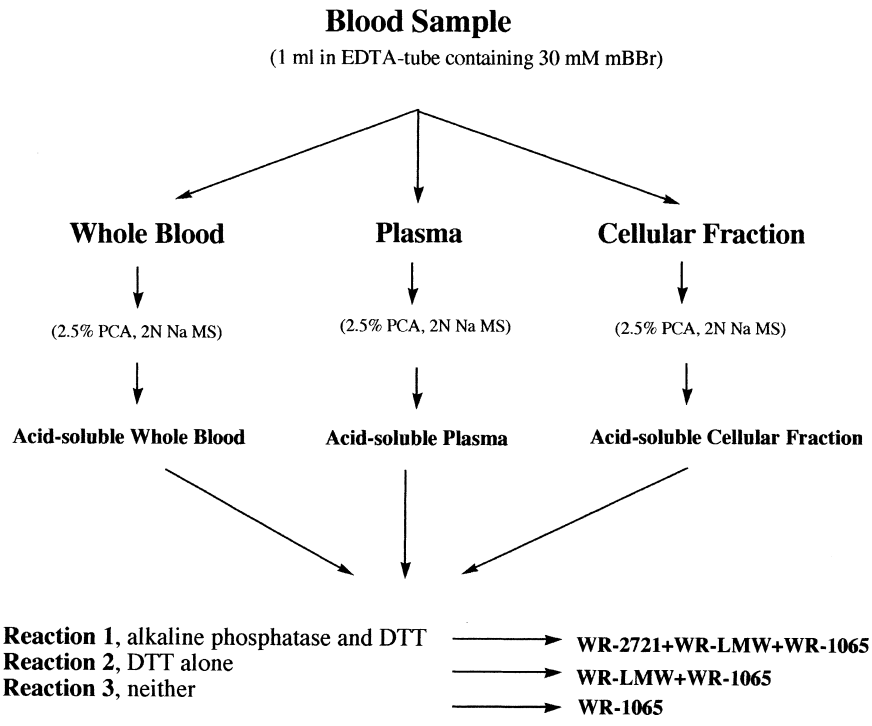

Fig. 1 Protocol for measuring WR-2721 and its various thiol forms in blood samples

added from a 0.1 m*M* stock in ACN. The reaction was allowed to continue at RT for 30 min and repeated with 5.0 m*M* mBBr (total mBBr 15.7 m*M*). The derivatization reaction was followed by additions of 5 µl 5.0 *M* MSA and 98 µl 10 m*M* MSA. The mixture was extracted once with water-saturated dichloromethane and 50 µl was injected onto the HPLC column and analyzed as described above. The dichloromethane extraction procedure did not reduce the recovery from control standards. **Reaction 2** was exactly as reaction 1 except that samples were incubated with 20 µl 0.5 *M* ammonium sulfate instead of alkaline phosphatase. **Reaction 3** was exactly as reaction 2 except that samples were incubated with water instead of DTT and ACN instead of mBBr. Plasma and the acid-insoluble pellets were analyzed as described above, except that 40 µl 1.0 *M* Tris-sulfate, pH 8.0, was used instead of Tris base and 50 µl of their acid-soluble supernatant was injected onto the HPLC column. Final concentrations were calculated using the measured volume of whole blood, plasma and separated red cells. Each set of experiments was repeated at least twice.

Patient

A 10-year-old girl with a primary suprasellar germinoma and diabetes insipidus experienced severe nephrotoxicity (serum creatinine rising from 0.7 to 1.6 mg% and salt-wasting) following her first course of cisplatin-containing combination chemotherapy. In the second course, she received WR-2721, 825 mg/m^2 IV over 15 min prior to the start of the cisplatin dose (100 mg/m^2 IV over 6 h). The WR-2721 treatment was repeated 218 min later. Her body weight was 35.5 kg, body surface area 1.15 m^2 and calculated blood volume approximately 2.5 l. Her serum creatinine rose from 0.9 to 1.1 mg% with this course. She experienced transient vomiting and hypotension during WR-2721 infusions.

Pharmacokinetic sampling and analysis

A 1 ml blood sample was collected in a foil-covered purple-top Vacutainer tube containing a final concentration of 30 m*M* mBBr before the WR-2721 infusion and 2, 10, 28, 218, 258 min and 20 h after the completion of the first WR-2721 infusion. Samples were mixed by inversion at RT for 2 min and placed on ice for 6 h or less. Plasma and blood cells were separated as described above. Two volumes of 2.5% PCA, 2 *N* Na MS were added to whole blood, plasma and cellular fractions and mixed by vigorous vortexing. The supernatants were recovered by centrifugation, extracted twice with water-saturated dichloromethane and stored at −70 °C until analysis. The acid-soluble supernatants of whole blood, plasma and blood cellular fraction were analyzed as described above. Each set of experiments was repeated at least twice.

Results

Recovery and fate of WR-1065 in volunteer's plasma

Blood was freshly drawn from a volunteer into a purple-top Vacutainer tube and the plasma was separated by centrifugation. WR-1065 was added to the plasma to a final concentration of 50 µ*M* or 500 µ*M*. The mixture was allowed to incubate at 37 °C for 10 min and analyzed as described in Methods and the legend to Table 1. As can be seen in Table 1, the bulk of WR-1065 was in the disulfide forms. Only 2.4% of the 50 µ*M* WR-1065 and 21% of the 500 µ*M* WR-1065 was in the WR-SH form. A 10-fold increase in WR-1065 concentration was associated with a 90-fold increase in measured WR-1065 (0.73 µ*M* versus 66 µ*M*). This increment was mostly due to a lower proportion of WR-SS-protein at the higher amount (38% versus 61%), while the WR-SS-LMW percentage remained almost the same (41% versus 36%; Table 1).

The WR-1065 recovery in these experiments was only 60% and 62%. In replicate experiments, recoveries were 68% to 100%. The wide range of recoveries was due to

Table 1 Fate and recovery of WR-1065 from volunteer's plasma. Blood was freshly drawn from a volunteer into a purple-top Vacutainer tube and plasma was separated by centrifugation. WR-1065 was added to the plasma to a final concentration of 50 μ*M* or 500 μ*M* and incubated at 37 °C for 10 min. An equal volume of ACN was added to the samples and the supernatants were recovered by centrifugation. The ACN-soluble plasma was incubated in the dark with 3.0 m*M* mBBr at RT for 30 min, followed by additions of 5 μl 5.0 *M* MSA and 98 μl 10 m*M* MSA. The mixture was extracted with water-saturated dichloromethane and 50 μl was injected onto the HPLC column and analyzed as described Methods (HPLC analysis). In parallel reactions, the plasma and the ACN-soluble plasma were processed in the presence of DTT as described in Methods, Sample analysis (reaction 2). An equal volume of ACN was added to the plasma reaction before the dichloromethane extraction, and 50 μl of the supernatant was injected onto the HPLC column and analyzed as described above. WR-SH was set equal to the WR-S-bimane derivative in the ACN-soluble plasma in the absence of DTT. WR-SS-LMW was set equal to the WR-S-bimane derivative in the ACN-soluble plasma in the presence of DTT minus WR-SH. WR-SS-protein was set equal to the WR-S-bimane derivative in the plasma in the presence of DTT minus WR-LMW

WR-1065 added to plasma	Thiol recovered	Concentration (μ*M*)
50 μ*M*	WR-1065	0.73
	WR-SS-protein	18.4
	WR-SS-LMW	10.9
500 μ*M*	WR-1065	66
	WR-SS-protein	120
	WR-SS-LMW	127

variable detections of the WR-SS-protein form of the drug.

Recovery and fate of WR-1065 in volunteer's blood

Blood wash freshly drawn from a volunteer into a purple-top Vacutainer tube containing a final concentration of 0.5 m*M* or 5.0 m*M* WR-1065, mixed thoroughly and incubated at 37 °C for 15 min. Then, 5.0 m*M* mBBr was added and mixed by inversion at RT for 2 min. The samples were prepared and analyzed as described in Methods and the legend to Table 2. At the 0.5 m*M* drug concentration, WR-1065 accounted for only 8% of the measured drug, whereas, at 5 m*M*, WR-1065 comprised 47% of the total. The cellular level of WR-1065 was comparable to the plasma level at the 5 m*M* drug concentration, but severalfold greater than that at the lower drug concentration. The intracellular WR-SS-LMW concentrations were quite low, presumably reflecting the ability of the cell to reduce LMW disulfides.

Reduction and recovery of WR-33278 in volunteer's blood

Blood was freshly drawn from a volunteer into a purple-top Vacutainer tube containing final concentrations of 100 μ*M* WR-33278 and 30 m*M* mBBr, mixed by inversion at RT for 2 min and analyzed immediately or after 5 days at 4 °C. The plasma was collected by centrifugation and analyzed using reactions 2 and 3 as described in Methods, Sample analysis. Of the WR-33278, 13% was recovered as the WR-S-bimane derivative in the plasma in the absence of DTT and 100% in the presence of DTT. The same values were obtained after 5 days at 4 °C.

Table 2 Fate and recovery of WR-1065 from volunteer's blood. Blood was drawn from a volunteer into a purple-top Vacutainer tube containing 0.5 m*M* or 5 m*M* WR-1065. The mixture was incubated for 15 min at 37 °C and then 30 m*M* mBBr was added and mixed by inversion for 2 min at RT. Plasma was separated by centrifugation. Two volumes of 2.5% PCA, 2 *N* Na MS were added to the plasma and the cellular fraction and mixed vigorously. The supernatants were recovered by centrifugation, extracted twice with water-saturated dichloromethane and analyzed as described in Methods (Sample analysis). The acid-insoluble pellets were also washed twice with normal saline, resuspended in 10 m*M* Tris-SO_4, pH 8.0, and analyzed as described in Methods. WR-1065 was set equal to the WR-S-bimane derivative in the acid-soluble samples in the absence of DTT. WR-SS-LMW was set equal to the WR-S-bimane derivative in the acid-soluble samples in the presence of DTT minus WR-1065. WR-SS-protein was set equal to the WR-S-bimane derivative in the acid-insoluble pellets

WR-1065 added to blood	Thiol recovered	Plasma (μ*M*)	Cellular fraction (μ*M*)
0.5 m*M*	WR-1065	23	81
	WR-SS-protein	120	140
	WR-SS-LMW	150	< 0.3
5.0 m*M*	WR-1065	1600	1600
	WR-SS-protein	600	2000
	WR-SS-LMW	1200	27

Hydrolysis and recovery of WR-2721 in volunteer's blood

Blood was freshly drawn from a volunteer into a purple-top Vacutainer tube containing final concentrations of 100 μ*M* WR-2721 and 30 m*M* mBBr, mixed by inversion at RT for 2 min and analyzed immediately or after 5 days at 4 °C. The plasma was separated by centrifugation and analyzed using reactions 1–3 as described in Methods, Sample analysis. Of the WR-2721, 12% was detected in the plasma in the presence of DTT alone and 63% in the presence of DTT plus alkaline phosphatase. Similar values, 8% and 62%, respectively, were obtained after 5 days at 4 °C. When WR-2721 was directly added to plasma, 11% was hydrolyzed in the presence of DTT alone and 80% with DTT plus alkaline phosphatase.

Fate of WR-2721 in patient's blood

WR-2721 (825 mg/m^2 or 26 mg/kg, i.e. 910 mg or 3.4 mmol) was administered by a 15-min IV infusion, and repeated after 218 min. Timed blood samples were collected and analyzed as described in Methods.

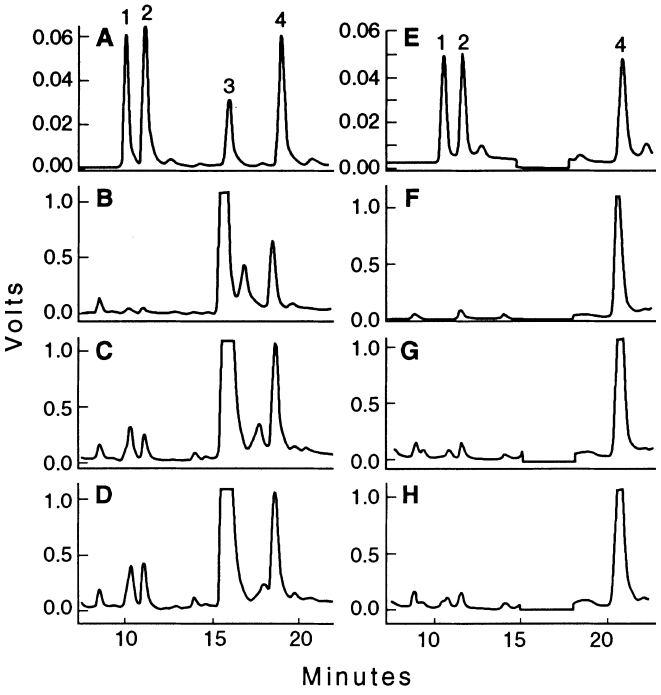

Fig. 2 Representative HPLC chromatograms. A 1-ml blood sample was collected from the patient and prepared and analyzed as described in Methods (Pharmacokinetic sampling and analysis). Chromatograms of the whole blood (**B–D**) and the cellular fraction (**F–H**) samples are shown. Chromatograms **A** and **E** are analytical runs of 50 µl of the 2 µ*M* standards. *Peak 1* is the cysteine-bimane derivative, *peak 2* the WR-S-bimane derivative, *peak 3* the reagent, and *peak 4* the glutathione-bimane derivative. Chromatograms **B** and **F** are the products of reaction 3, chromatograms **C** and **G** reaction 2, and chromatograms **D** and **H** reaction 1, as described in Methods, Sample Analysis. In runs **E–H**, the excitation wavelength was changed to 550 nm and the emission wavelength to 600 nm from 15 min to 18 min to avoid overloading the photomultiplier tube by the reagent peak

Representative chromatograms are shown in Fig. 2 and the results in Table 3. WR-2721 was detected in plasma with a half-life of about 10 min. WR-1065 was also rapidly cleared from plasma with a similar half-life. The predominant metabolite in plasma was WR-SS-LMW (in these experiments WR-SS-protein was not measured). After 218 min, i.e. before the second WR-2721 infusion, both WR-1065 and WR-SS-LMW metabolites had disappeared from the plasma and the cellular fraction.

The WR-1065 level in the cellular fraction was maintained for at least 30 min following the infusion. Similar to the findings when WR-1065 was added to volunteer's blood, the WR-SS-LMW concentration in the cellular fraction was only 10–20% of that in the plasma (Table 3). The whole blood, plasma and the cellular fraction concentrations immediately following the second WR-2721 infusion were very similar to the values shown in Table 3, at 2 min. As expected, 20 h following the infusion, WR-1065 and WR-SS-LMW were not detected.

Table 3 Fate and detection of WR-2721 from patient's blood. WR-2721 was administered to the patient intravenously over 15 min. A 1-ml blood sample was collected in a foil-covered purple-top Vacutainer tube containing a final concentration of 30 m*M* mBBr at the indicated minutes following the completion of the first WR-2721 infusion. Samples were prepared and analyzed as described Methods (Pharmacokinetic sampling and analysis). WR-1065 was set equal to the WR-S-bimane derivative in reaction 3. WR-SS-LMW in reaction 2 minus WR-1065, and WR-2721 in reaction 1 minus WR-SS-LMW (see Methods, Sample analysis)

Minutes	WR-2721 (µ*M*)	WR-1065 (µ*M*)	WR-SS-LMW (µ*M*)
Whole blood			
2	50	21	61
10	–	23	84
28	< 0.3	16	42
218	< 0.3	< 0.3	< 0.3
Plasma			
2	100	25	143
10	57	16	152
28	20	< 0.3	82
218	< 0.3	< 0.3	< 0.3
Cellular fraction			
2	6	38	28
10	< 0.3	31	16
28	< 0.3	20	14
218	< 0.3	< 0.3	< 0.3

Discussion

WR-2721 is a chemoprotective agent recently approved by the FDA. It requires activation by dephosphorylation to produce the free thiol metabolite WR-1065 [4, 20, 25]. The activation step is presumably catalyzed by the capillary, membrane-bound alkaline phosphatase. WR-1065 is taken up by cells by passive diffusion which is the dominant import mechanism in the millimolar concentration range [5]. However, the symmetric disulfide, WR-33278, can be taken up by the polyamine transport systems and reduced intracellularly [16, 19].

The protective capacity of WR-1065 is related to the nucleophilic activity of the thiol anion group. The thiolate ion reacts with electrophilic agents such as cisplatin and other alkylating agents to produce inactive products. In addition, the cationic character of WR-1065 and WR-33278 allows them to be concentrated near DNA where their protection capacity is likely to be enhanced [26, 32].

The clinical utilization of WR-2721 in cancer treatment has been extensively reviewed [2, 3]. In several adult trials WR-2721 has been shown to reduce the bone marrow suppression induced by alkylating agents and platinum-based compounds [9, 10 ,17]. A pediatric maximum tolerated dose for WR-2721 of 1650 mg/m^2 in a single dose has been suggested [1]. A dose of 825 mg/m^2 × 2 is currently being implemented in a Pediatric Oncology Group clinical trial, and was used in our patient. It is likely, however, that WR-2721 administration schedules will need to be tailored to particular

chemotherapeutic agents in order to achieve optimal protection. The effective design of such schedules will depend on the availability of data pertaining to its intracellular pharmacokinetics. At present, only limited plasma levels from adults have been reported [12, 21, 23, 24].

We adapted the method for analysis of biological thiols, based upon fluorescent labeling with mBBr, to determine WR-2721, WR-1065 and its disulfides in plasma and the cellular blood fraction. The mBBr fluorescent label is a derivative of *syn*-9,10-dioxabimane: 1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione [7, 8, 13, 14, 18]. It rapidly penetrates cells and reacts with both intracellular and extracellular free thiols. The bimane thioether derivatives are stable, highly fluorescent and trapped inside cells, as are the derivatives of other charged thiols. Therefore, both extracellular and intracellular thiols can be rapidly stabilized and kept separate by labeling with mBBr. The hydrophobic bimane group facilitates their separation by HPLC on a silica-based reverse-phase column, and they are detected with great sensitivity at the picomole level [18]. In view of the rapid oxidation of WR-1065, we used a high concentration of mBBr (i.e. 30 m*M*) to ensure the derivatization of all thiols in a few seconds [18].

The mBBr and its solvent cause partial hemolysis. The use of a concentrated stock solution of 1.0 *M* mBBr in DMSO minimizes, but do not eliminate, the problem of hemolysis. Hemolysis in the sample was estimated at 15% based upon the measured glutathione level in plasma above the normal plasma level ($\leq 20\,\mu M$). This level of hemolysis would not appreciably change the values reported in Tables 2 and 3.

The symmetric disulfide metabolite, WR-33278, cannot be directly measured by our analytical method . The measured values for WR-SS-LMW include WR-SS-WR, as well as other low molecular weight mixed disulfides. The WR-SS-LMW forms disappeared from both plasma and the cellular fraction by 3.6 h after the administration of the WR-2721 dose. Our results show that, in blood, the extracellular and intracellular concentrations of WR-1065 are very similar. However, marked variability in WR-1065 uptake exists among different tissues in animal models [27]. Immediately after infusion 80–90% of WR-2721 was gone from the plasma, in accord with other results on adults.

It is not known whether the drug levels found in the cellular fraction in this study are comparable to those in other tissues. It is possible that the blood cell values represent mean tissue values. The analytical method described in this report can be applied to measure drug levels in the marrow mononuclear fraction, a major target tissue for protection.

In a pharmacokinetic study of WR-1065 in the Rhesus monkey model, a 10-min infusion of WR-1065, 60 mg/kg, was followed by peak plasma levels of WR-1065 of $452\,\mu M$, and of total recoverable WR-1065 (i.e. free and disulfide forms) of $645\,\mu M$. Twice that dose caused a disproportionate increase in the measured levels. The WR-1065 level had declined 1 h after infusion by about two orders of magnitude [15]. In a pharmacokinetic study of WR-2721 in the BALB/c mouse model, WR-2721 was administered IV at 500 mg/kg. Maximal WR-1065 tissue levels occurred 5–15 min after the injection, with levels in liver, kidney, lungs and salivary gland exceeding 1000 μmol/kg. The decline in levels varied markedly among the tissues: in lungs a six-fold drop was observed by 30 min and in salivary glands only 15% by 3 h. In a mouse treated with ^{14}C WR-2721, 15 min after the IV injection, WR-1065 accounted for over half of the total drug recovered from all normal tissues [27]. In another Balb/c mouse study, 365 mg/kg of WR-2721 was injected intraperitoneally. Blood concentrations of WR-2721 peaked at 5 min and had declined tenfold at 30 min. Free WR-1065 was detected in tissues, with the highest concentrations in the liver (965 μmol/kg) and kidneys (2195 μmol/kg) [22].

In a pharmacokinetic study of WR-2721 in humans a single 150 mg/m^2 (i.e. 20% of our patient's dose) bolus dose of WR-2721 was administered IV to 13 adult patients. Less than 10% of the WR-2721 remained in the plasma 6 min after administration. The average plasma clearance of WR-2721 was 2.17 l/min. The volume of distribution was small (6.4 l) and the average urinary excretion was low ($< 1\%$). Plasma and cellular levels of WR-2721 metabolites were not measured [21]. In a subsequent study, 24 adult patients were treated with WR-2721, either 740 or 910 mg/m^2 IV over 15 min. At the end of the infusion, WR-2721 levels were $93 \pm 38\,\mu M$ and $238 \pm 68\,\mu M$, respectively. The peak concentration of WR-1065, measured in 7 patients, was $50 \pm 33.6\,\mu M$, and had decreased to $6.6 \pm 5.7\,\mu M$ by 30 min after the completion of the infusion [24].

In a recent study, WR-2721 (740 mg/m^2) was administered as a 15-min IV infusion to an adult patient before and at 2 and 4 h after administration of carboplatin (500 mg/m^2). The plasma concentrations of WR-2721, WR-1065 and mixed disulfides (WR-SS-LMW plus WR-SS-protein) were measured. Peak levels for all three compounds were observed at completion of the WR-2721 infusion. Average peak concentration of WR-2721 was $225\,\mu M$, WR-1065 $65\,\mu M$, and mixed disulfides $190\,\mu M$. At 15 min after each infusion, the plasma concentration of WR-2721 was only 2–5%, WR-1065 42–50% and mixed disulfides 95–56% of the peak values. WR-2721 and WR-1065, had disappeared from the plasma within 2 h, but mixed disulfides (probably all protein bound) remained for at least 24 h [12].

The effective clinical utilization of WR-2721, aiming to provide selective protection of normal tissues, depends on the achievement of adequate intracellular drug levels; that is, a time-concentration profile of the cytoprotectant that matches, in a molar sense, the intracellular levels of the cytoxic drugs. It is likely that different WR-2721 doses and schedules will be necessary for use with different chemotherapeutic agents. The assay methods reported in this study provide a tool to study intracellular (e.g. peripheral blood or bone marrow cells)

levels of WR-1065 and metabolites in patients receiving WR-2721 in various doses and schedules. By correlating the cellular levels achieved with the degree of clinical cytoprotection produced (for any combination of chemotherapy and WR-2721 regimens), it may be possible to more intelligently design WR-2721 regimens.

In conclusion, our results show that a large proportion of WR-2721 was oxidized to WR-SS-protein (Tables 1 and 2). WR-1065 and WR-SS-LMW disappeared from blood by 3.6 h after the infusion (Table 3), suggesting that both forms can be utilized by tissues.

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