KIN ETE ANALYSIS OF THE REACTIONS OF 4-HYDROPEROXYCYCLOPHOSPHAMIDE AND ACROLEIN WITH GLUTATHIONE, MESA, AND WR-1065

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(Received January 31, 2002; accepted April 26, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The kinetics of the reactions of glutathione (GSH) with 4-hydroperoxycyclophosphamide (4OOH-CP) and acrolein, a metabolite of 4OOH-CP, were investigated in a cell-free medium (pH 7.5) and peripheral blood mononuclear cells. The ability of the thiol drugs, sodium 2-mercaptoethane sulfonate (mesna) and WR-1065, to affect the reactions of cellular GSH with the alkylating agents was also studied. The amount of unreacted thiols in the various reactions was determined by derivatization with monobromobimane, followed by separation of fluorescent-labeled thioether adducts using high-pressure liquid chromatography. The second-order rate constants (k2) for reactions of GSH, mesna, and WR-1065 with 4OOH-CP in solution were 38 ± 5, 25 ± 5, and 880 ± 50 M⁻¹s⁻¹, respectively. The corresponding k2 for reactions of GSH, mesna, and WR-1065 with acrolein were 490 ± 100, 700 ± 150, and >2000 M⁻¹s⁻¹, respectively. The apparent rate constants for reactions of cellular GSH with acrolein and 4OOH-CP were smaller than those obtained in solution. Assuming that the k2 is the same inside and outside cells, we estimate the first-order rate constant (k1) for transfer of 4OOH-CP and acrolein across the cell membrane as ~0.01 and ~0.04 s⁻¹, respectively. WR-1065 was more effective than mesna in blocking depletion of cellular GSH (because it passes into the cell more quickly and has higher reaction rates with the alkylators than the latter compound). When WR-1065 and mesna were used together, the protection against cellular depletion of GSH was additive. Our results are relevant to the administration of thiol drugs with high-dose alkylating agents.

Cyclophosphamide [cis-(±)-2-(bis(2-chloroethyl)amino)tetrahydro-2-oxide-2H-1,3,2-oxazaphosphorine] is a leading anticancer agent. Activation of the drug requires hydroxylation by the hepatic microsomal cytochrome P-450 system in vivo. The resulting 4-hydroxylated metabolite spontaneously degrades via β-cleavage of its aldophosphamide tautomer to phosphoramide mustard (an active anticancer component) and acrolein (a highly electrophilic, α,β-unsaturated aldehyde) (Hohorst et al., 1976; Colvin, 1999; Kehrer and Biswal, 2000). Moreover, aldophosphamide is oxidized by aldehyde dehydrogenase to the inactive metabolite carboxyphosphamide (Bunting and Townsend, 1998; Sladek, 1999; Giorgianni et al., 2000). Other minor oxidations include dechloroethylation of a chloroethyl side chain to dechloroethylated metabolite and chloroacetaldehyde (Ludeman, 1999).

The reaction mechanism of phosphoramide mustard [N,N-bis-(2-chloroethyl)phosphorodiamicd, R-N(CH₂CH₂CH₂Cl)₂] involves generation of the intermediate phosphoramide aziridinium ion through an intramolecular nucleophilic attack (cyclization reaction) of the nitrogen on the β-carbon of a chloroethyl chain (Ludeman, 1999).

This work was submitted in partial fulfillment of the requirements for a Ph.D. degree for K. A. T. in the Biochemistry, Structural Biology and Biophysics Program at Syracuse University.

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Cellular thiols (e.g., GSH1) and other nucleophiles react rapidly with phosphoramide aziridinium ions, producing thioether products (Gamcsik et al., 1999). Moreover, reversible 4-(alkylthio)cyclophosphamide products have been observed (Hohorst et al., 1976; Niemeyer et al., 1984; Kwon et al., 1987; Lee, 1991a,b).

Reactions of thiols with acrolein (CH₂ = CH-CHO), on the other hand, are via nucleophilic addition at the β-carbon atom, forming stable thioether compounds (e.g., 3-oxopropyl glutathione) (Ramu et al., 1995, 1996). The 4-hydroxycyclophosphamide, aldophosphamide, and acrolein metabolites can readily cross the cell membrane. In contrast, phosphoramide mustard bears a negative charge at physiologic pH (pKₐ ~4.8) and, thus, is relatively membrane impermeable. Phosphoramide mustard is, therefore, primarily generated intracellularly (Boyd et al., 1986).

In vitro systems have employed 4OOH-CP as an activated congener of cyclophosphamide, which spontaneously degrades to the reactive alkylating metabolites (Blomgren and Hallstrom, 1991). The 4OOH-

1 Abbreviations used are: GSH, glutathione; 4OOH-CP, 4-hydroperoxycyclophosphamide; mesna, sodium 2-mercaptoethanesulfonate; WR-2721, (amifostine), S-2-(3-aminopropylamino)ethyl phosphorothioic acid; WR-1065, S-2-(3-aminopropylamino)ethanethiol; PBMC, peripheral blood mononuclear cells; MSA, methanesulfonic acid; mBBR, monobromobimane; DNB, 5,5’-dithio-bis(2-nitrobenzoic acid); HPLC, high-pressure fluid chromatography; RSH, (GSH, mesna, or WR-1065); A, alkylator (4OOH-CP, acrolein, or mBBR); k₁, pseudo-first-order rate constant; k₂, second-order rate constant; RT, room temperature.
CP compound is used clinically to purge hematopoietic cells prior to autologous bone marrow transplantation. The sulphydryl compounds, mesna (HS-CH₂-CH₂SO₃Na), and WR-2721 [amifostine, S-2-(3-aminopropylamino)ethyl phosphorothioic acid, "H₂N-(CH₂),-NH₂-(CH₂),-S-PO₃H⁻"] are commonly used to ameliorate toxicities of cyclophosphamide and platinum-based compounds (Brock et al., 1982; Souid et al., 1999; Sadowitz et al., 2002). The protective mechanism of mesna and WR-1065 [WR-2721 active metabolite, "H₂N-(CH₂),-NH₂-(CH₂),-S-PO₃H⁻"] involves the thiol anions that participate in chemical reactions similar to those of GSH (Danehy and Noel, 1960). However, the reaction of mesna with the chloroethyl moieties of phosphoramide mustard is unfavorable (Seitz et al., 1989).

The distribution of WR-1065 and mesna differs markedly. WR-1065 distributes equally between the extra- and intracellular compartments, whereas mesna distributes mostly in the extracellular compartment (Newton et al., 1996; Souid et al., 2001).

The aim of the present study is to investigate the kinetics of reactions that are important for optimizing the clinical use of WR-1065 and mesna with high-dose alkylating agents. We estimated the rate constants for reactions of the protective thiols and GSH with 4OOH-CP and acrolein in a cell-free medium at physiologic pH (~7.5). We also investigated the kinetics of depletion of GSH inside PBMC by 4OOH-CP and acrolein in the presence and absence of mesna and WR-1065. The protective capacity of WR-1065 is compared with that of mesna, to provide a framework for drug thiol use during administration of high-dose alkylating agents.

Materials and Methods

Chemicals. 4OOH-CP (p-18864, MW 293.1) was obtained from ASTA Medica AG (Frankfurt, Germany); mesna (MW 164.18, purchased as 100 mg/ml solution) was obtained from Bristol-Myers Squibb Co. (Princeton, NJ); WR-1065 \( \cdot \) 2HCl (MW 207.16) was obtained from US Bioscience (West Conshohocken, PA); Ficoll-Paque from Amersham Biosciences AB (Uppsala, Sweden); methanesulfonic acid (MSA) and tris(hydroxymethyl)-aminomethane (Tris) was purchased from Fluka BioChemika (Ronkonkoma, NY); mBBr was purchased from Molecular Probes Inc. (Eugene, OR); acrolein (90%, or 0.45 M solution) was obtained from Sigma-Aldrich (St. Louis, MO); and RPMI 1640 medium (pH 7.15, without L-glutamine) were purchased from Mediatech (Herndon, VA).

The study was approved by the institutional review board (State University of New York, Upstate Medical University) for the protection of human subjects.

Solutions. Mesna and WR-1065 solutions were prepared in dH₂O and stored at \(-70^\circ\)C in small aliquots; their concentrations were determined by titration with DTNB immediately prior to each use (Souid et al., 1998).

Reactions of GSH, Mesna, and WR-1065 with 4OOH-CP and Acrolein. Reactions were carried out at 22 ± 2°C in flat-bottom glass vials with rapid stirring via a magnetic stirring bar in a total volume of 200 μl containing 20 mM Na₂HPO₄ and the thiol (final pH, ~7.5). Reactions were started by adding the alkylating agent (4OOH-CP or acrolein). At times indicated in the figures, reactions with thiol were terminated by adding 0.8 ml of 5 mM (final concentration) mBBr (diluted in 10 mM Na₂HPO₄ immediately prior to each addition), which reacts with the thiolate anion to form a fluorescent thioether derivative. Dilution of the medium by the addition of mBBr reduces the rate of reaction of thiol with alkylating agent by 5-fold. The samples were then incubated in the dark at room temperature (RT) for 15 min. Twenty microliters of 5 M MSA were added, mixed, and extracted with 1.0 ml H₂O-saturated CH₂Cl₂. Portions of the aqueous layers were loaded on high-pressure liquid chromatography (HPLC) and analyzed as described (Souid et al., 1999).

A linear calibration curve, from 0 to 100 pmol of mixed thiol standard was generated with each analytical run (r > 0.99). Peak identification was confirmed by retention time in comparison with authentic standards. Quantification was based on peak area against the appropriate reference standards.

Reaction of the thiolate ions with the alkylating metabolites are as shown in eq. 1:

\[
RSH \leftrightarrow RS^- + H^+ \quad K
\]

\[
RS^- + A \rightarrow RS-A \quad k_2
\]

where RSH = GSH, mesna or WR-1065 and A = 4OOH-CP or acrolein. Equation 1 is the ionization of the thiol, characterized by its pK. If the kinetics to reach equilibrium for eq. 1 are rapid, the concentration of RS⁻ is proportional to the concentration of RSH, and the equilibrium can be ignored. The alkylation reaction then becomes

\[
RSH + A \rightarrow RS-A \quad k_2
\]

These chemical reactions are illustrated in Schemes 1 and 2.

Reaction (eq. 2), with the effective rate constant \( k_2 \), is thus a mixed second-order reaction, and the rate equation is easily integrated. The reaction of a thiolate ion with mBBr to form the bimane (thioether) adduct RS-B (Scheme 3) is also a mixed second-order reaction with the rate constant \( k_2' \).

The kinetics of alkylation reactions were analyzed using the second-order rate equation:

In eq. 3, [RSH]₀ and [A]₀ are the initial concentrations of the thiol and alkylating agent, respectively. The concentration of 4OOH-CH or acrolein that has reacted with the thiol at time \( t \), [RS-A], is calculated as the difference between [RSH]₀ and [RS-A] as determined by titration using DTNB (Souid et al., 1998). The initial concentration of acrolein in water was determined spectrophotometrically at 310 nm, using an extinction coefficient of 11.8 M⁻¹ cm⁻¹ (r > 0.99). The initial concentration of 4OOH-CP was determined by weight. The value of \( k_2 \) was determined by constructing linear plots (least-squares) of In Q versus time; \( t_{1/2} \) was the slope of the line divided by \( [A]_0 - [RSH]_0 \). The rate of the reaction was calculated from the second-order rate equation:

\[
\ln \left( \frac{1}{2} [RSH]_0 \right) = \ln \left( \frac{[A]_0 - [RSH]_0}{2[A]_0} \right) = \left( [A]_0 - [RSH]_0 \right) k_2 t_{1/2}
\]
The $t_{1/2}$ (s) for the thiol was calculated from

$$
\ln \left( \frac{[A]_0 - \frac{1}{2} [RSH]_0}{\frac{1}{2} [A]_0} \right) = [A]_0 - [RSH]_0 k_2 t_{1/2}
$$

The pseudo-first-order rate equation (eq. 6) was used when the concentration of alkylator was in large (>25-fold) excess, so $[A]$ always remained close to $[A]_0$.

$$
\ln \frac{[RSH]_0}{[RSH]_0 - [RSH] - [A]} = [A]_0 k_2 t
$$

The pseudo-first-order rate constant ($k_2$, s$^{-1}$) in this case is

$$
k_1 = k_2 [A]_0
$$

The $t_{1/2}$ (s) for depletion of thiol for pseudo-first-order reactions is calculated as

$$
t_{1/2} = \ln 2 / k_1
$$

The stability of acrolein in Na$_2$HPO$_4$ at RT was determined using HPLC (Beckman Coulter Inc.) as follows. Acrolein was detected at 310 nm using a spectrophotometer equipped with a standard flow cell. The column, 4.6 x 250 mm Beckman Ultrasphere IP (Beckman Coulter Inc.) was operated at RT at 1.0 ml/min. Separa-

The reactions contained 20 mM Na$_2$HPO$_4$ (pH 7.5), either 100 µM GSH, 100 µM mesna or 100 µM WR-1065, and 500 µM mBBr (final volume 200 µl). The mixtures were incubated at RT for indicated time. At the end of the incubation periods, 5 µl of 5 M MSA were added. The samples were then diluted to 1.0 ml with 10 mM MSA and extracted with 1.0 ml H$_2$O-saturated CH$_2$Cl$_2$. The thiol-bimane derivatives were quantitated on HPLC as described under Materials and Methods. Spontaneous oxidation of the thiols during the 120-s period was <1%. Shown are values of ln Q (see eq. 3) calculated from experimental data for GSH (circles), mesna (squares), and WR-1065 (diamonds). Linear least-square fits to ln Q versus $t$ are shown (solid, long-dashed, and short-dashed lines, respectively); $k_2$ is determined from the slope in each case.

Results

Kinetics of Thiol Reactions with 4OOH-CP and Acrolein in a
Cell-Free Solution. We first explored the rates of reactions of mBBr (500 µM) with GSH, mesna, and WR-1065 (100 µM) in 20 mM Na$_2$HPO$_4$, pH 7.5 (final volume, 200 µl). The mixtures were incubated at RT for 15 to 120 s and quenched by lowering the pH with 5 µl of 5 M MSA. The results are given in Fig. 1, where the quantity

$$
Q = \frac{[A][RSH]}{[A][RSH]}
$$

(see eq. 3) is plotted. The value of $k_2$, for the reaction of GSH with mBBr was $7.3 \pm 0.5$ M$^{-1}$s$^{-1}$ ($t_{1/2}$, 3.4 min), mesna $6.1 \pm 0.3$ M$^{-1}$s$^{-1}$ ($t_{1/2}$, 4.0 min), and WR-1065 9.4 $\pm 0.9$ M$^{-1}$s$^{-1}$ ($t_{1/2}$, 2.6 min), Fig. 1 ($r > 0.98$ for all three).

We then explored the rates of reactions of 4OOH-CP and acrolein with the thiols, using 5 mM mBBr to quench the reactions. With this large excess of mBBr, the labeling reaction is pseudo-first-order, with $k_1$ between 0.03 and 0.05 s$^{-1}$ and $t_{1/2}$ between 15 and 23 s (calculated using eqs. 7 and 8, respectively).

Unfortunately, the $k_2$ values for the reactions of thiols with acrolein and 4OOH-CP (Table 1) are much larger than the $k_2$ values given above. Thus, even though the mBBr concentration is much larger than those of acrolein and 4OOH-CP, the thiol-alkylation reaction competes with the thiol-labeling reaction, and not all of the thiol is derivatized with mBBr. One should really consider simultaneous second-order equations:

$$
RS^- + A \rightarrow RS-A
$$

$k_2$
shown), but only results to estimate for infinitely fast. The numerical calculations show, however, that a good line is far from going through the origin; if we impose a zero initial concentration of mBBr is much larger than [A].

Since there is no closed-form solution to these rate equations, we assume that the final values of [RS-B] are equal to the values of [RSH] before addition of mBBr and calculate [A] from the values of [RSH]. If the results for [RS-B] from numerical calculations are treated in this way,

$$\ln Q = \ln \left( \frac{[A]}{[A]_0} \right) [RSH]$$

is not proportional to $t$, as it would be if the labeling reactions were infinitely fast. The numerical calculations show, however, that a good estimate for $k_2$ can be obtained from the initial slope, $(dQ/dt)_{t=0}$. The results of the experiments are plotted in Figs. 2 (reactions of thiols with 4OOH-CP) and 3 (reactions of thiols with acrolein). Table 1 summarizes values of $k_2$ for the reactions shown in these figures. Since the scatter in the data (Figs. 2–3) is too large for the curvature in the plots to be determined, we have used the slope of each least-square linear fit to determine $k_2$.

The GSH reaction with 4OOH-CP (Fig. 2, circles) contained 100 μM GSH and 50 μM 4OOH-CP. Data were taken to $t = 900$ s (not shown), but only results to $t = 300$ s (14 points) were used to obtain $k_2$, giving $25 \pm 5 \text{ M}^{-1}\text{s}^{-1}$ ($r = 0.87$) and $t_{1/2} = 320 \pm 60$ s. The best-fit line is far from going through the origin; if we impose a zero $y$-intercept, the best-fit line ($r = 0.57$) gives $k = 38 \pm 5 \text{ M}^{-1}\text{s}^{-1}$. The mesna reaction with 4-OOH-CP (Fig. 2, squares) contained 200 μM mesna and 100 μM 4OOH-CP. Eight data points were taken to $t = 120$ s; $k_2$ was calculated as $25 \pm 5 \text{ M}^{-1}\text{s}^{-1}$ ($r = 0.91$), giving $t_{1/2} = 164 \pm 31$ s. The WR-1065 reaction with 4-OOH-CP (Fig. 2, triangles) contained 100 μM WR-1065 and 75 μM 4OOH-CP. Ten data points were taken to $t = 60$ s; $k_2$ was calculated as $880 \pm 50 \text{ M}^{-1}\text{s}^{-1}$ ($r = 0.98$), giving $t_{1/2} = 10.2 \pm 0.6$ s.

The GSH reaction with acrolein (Fig. 3, circles) contained 100 μM GSH and 50 μM acrolein; $k_2$ was $490 \pm 100 \text{ M}^{-1}\text{s}^{-1}$, and $t_{1/2}$ was $16 \pm 3$ s. The mesna reaction with acrolein (Fig. 3, squares) contained 200 μM mesna and 100 μM acrolein; $k_2$ was $700 \pm 150 \text{ M}^{-1}\text{s}^{-1}$, and $t_{1/2}$ was $5.6 \pm 1.2$ s. The WR-1065 reaction with acrolein was too fast for accurate measurement of $k_2$. From the data, it appears that it is greater than 2000 $\text{M}^{-1}\text{s}^{-1}$.

Rates of the Reversed Chemical Reactions. The rate for removal of thiolates from products formed in the reaction of alkylating agents with thiols (that is, the reverse rate) was investigated by adding 5 mM mBBr to reactions containing 100 to 200 μM thiol (GSH, mesna, or WR-1065) and 50 to 100 μM alkylating agent (acrolein or 4OOH-CP). The mixtures were incubated for 1, 5, or 15 min before the
addition of MSA. For all incubation times, there was no change in the amount of free thiol detected by mBBr, implying that, during the longest time interval, the reaction product does not decompose to give free thiol.

Depletion of Cellular GSH by 4OOH-CP and Acrolein. The filled circles in Fig. 4 show $[S]/[S]_0$, the fraction of PBMC GSH as a function of 4OOH-CP (left panel) and acrolein (right panel) concentrations in the incubation medium. The incubation time was 60 min. Concentrations of 4OOH-CP $\leq$10 $\mu$M hardly affected cellular GSH. Fitting the results in the left panel to an exponential (dashed curve), we find that 50% cellular GSH depletion occurs for 39 $\mu$M 4OOH-CP, 90% for 123 $\mu$M, and 95% for 159 $\mu$M. Acrolein was more effective than 4OOH-CP at depleting cellular GSH. Fitting the results in the right panel to an exponential (dashed curve) shows that, with acrolein, 50% cellular GSH depletion occurs for 6 $\mu$M acrolein, 90% for 20 $\mu$M, and 95% for 25 $\mu$M. Exponential fits are appropriate if the rate of transfer of alkylating agent across the cell membrane is much faster than the rate of reaction.

Kinetics of Cellular GSH Depletion by 4OOH-CP and Acrolein. Figure 5, filled circles, shows PBMC GSH depletion as a function of time with 100 $\mu$M 4OOH-CP (left panel) and acrolein (right panel) in the incubation medium. If the rate of transfer of alkylating agent across the cell membrane is much faster than the rate of reaction inside the cell, the kinetics becomes pseudo-first-order, and fitting the data to exponentials (dashed curves) is justified. This gives, for 4OOH-CP, a pseudo-first-order rate constant ($k_1$) of 0.099 $\pm$ 0.009 min$^{-1}$ and $t_{1/2}$ = 7.0 $\pm$ 0.6 min ($r = 0.98$). Since [4OOH-CP] = 100 $\mu$M, the $k_2$ for reaction of 4OOH-CP with cellular GSH is 16.5 M$^{-1}$s$^{-1}$ (calculated using eq. 7). This is lower than the $k_2$ for reaction of 4OOH-CP with extracellular GSH, 38 M$^{-1}$s$^{-1}$ (Table 1). Acrolein depletes cellular GSH much more quickly than 4OOH-CP; fitting the data for acrolein to exponential decay gives 0.26 $\pm$ 0.10 min$^{-1}$ for the $k_1$ and $t_{1/2}$ = 2.7 $\pm$ 1.0 min ($r = 0.79$). The $k_2$, assuming [acrolein] = 20 $\mu$M, is 220 $\pm$ 80 M$^{-1}$s$^{-1}$, lower than the value for extracellular GSH, 490 $\pm$ 100 M$^{-1}$s$^{-1}$ (Table 1).

Estimation of Transport Rate Across Cell Membrane. The reason that the $k_2$ values for reactions of 4OOH-CP and acrolein with cellular GSH are smaller than the $k_2$ values for their reactions with GSH in solution (Table 1) may be that the transport of the alkylating agents across the cell membrane is not extremely fast, or that $k_2$ is slower inside the cell than in the cell-free medium. However, since the reactants are small molecules that collide by diffusion through solvent, the overall rates leading to product inside and outside the cell should be comparable. If one assumes that $k_2$ is the same inside and outside the cell, one can estimate the transport rate coefficient. Nevertheless, since the experimental conditions are not identical, these coefficient values should be considered approximates.

Let $[A]$ be the concentration of alkylating agent in the intracellular space and $A_e$ the concentration in the extracellular medium. Assume that $A_e$ is held constant in time and that the rate of transport into the intracellular space is proportional to $A_e - [A]$. Then

$$\frac{d[A]}{dt} = k_1(A_e - [A]) - k_2[A][S]$$

(9)

where $[S]$ is the thiol concentration, and $d[S]/dt = -k_2[A][S]$. If each of the two processes is fast compared with $d[A]/dt$ (steady-state assumption), we can set $d[A]/dt$ equal to 0 and find

$$[A] = \frac{k_1A_e}{k_1 + k_2[S]}$$

Then

$$\frac{d[S]}{dt} = -\frac{k_1k_2[A][S]}{k_1 + k_2[S]}$$

which integrates to

$$k_1k_2\frac{[S]}{[S]_0} + k_2[S]_0\left(\frac{[S]}{[S]_0} - 1\right) = -k_1k_2A_e t$$

(10)
If $k_1 \gg k_2([S] - [S]_0)$, so that $[A]$ is always close to $A_c$ and the second-order reaction is the rate-limiting step, this reduces to pseudo-first-order kinetics, $\ln([S]/[S]_0) \approx -k_2 A_c t$. If, on the other hand, $k_2([S] - [S]_0) \gg k_1$, the transport across the cell membrane is the rate-limiting step, and $[S] - [S]_0 = -k_1 A_c t$. Plots of $[S]/[S]_0$ versus $A_c$ with $r$ fixed (Fig. 4), or versus $r$ with $A_c$ fixed (Fig. 5), resemble exponential decay more than linear decay, indicating that we are closer to the first case (i.e., that the transport is faster than the reaction with GSH).

Since $A_c$ and $t$ appear in eq. 10 only as product, we use eq. 10 to fit the data for cellular GSH depletion as a function of $A_c$ ($t$ fixed) and the data for cellular GSH depletion as a function of $t$ ($A_c$ fixed). We set $k_2$ for each alkylating agent equal to the value determined for cell-free solution and find $k_1$ by minimizing the sum, for all $[S]$, of the squared deviations of experimental $A_c$ values from calculated $A_c$. We assume $[S]_0 = 2$ mM, an approximate concentration of cellular GSH.

The precision of the data does not justify varying all three parameters ($k_1$, $k_2$, and $[S]_0$) to obtain the best fits to eq. 10.

Treating the concentration-dependent data for 4OOH-CP and acrolein (Fig. 4) in this way gives $k_1 = 0.006$ s$^{-1}$ for 4OOH-CP and 0.037 s$^{-1}$ for acrolein. The resulting plots are shown as solid curves in Fig. 4. The sum of the squared deviations of $A_c$ (fitted) from $A_c$ (measured) is $9 \times 10^{-10}$ M$^2$ for 4OOH-CP and $2.1 \times 10^{-11}$ M$^2$ for acrolein. The dashed curves are the fits to $\ln([S]/[S]_0) = -k_2 A_c t$, with the best value of $k_2$. The sum of the squared deviations of $A_c$ (fitted) from $A_c$ (measured) is $6 \times 10^{-11}$ M$^2$ for 4OOH-CP and $6 \times 10^{-11}$ M$^2$ for acrolein. The model of eq. 10 is clearly superior for acrolein, and both models are equally good for 4OOH-CP.

The time-dependent data of Fig. 5 is treated the same way. Assuming $[S]_0 = 2$ mM and $k_2$ = cell-free solution value, we find the value of $k_1$ that minimizes the sum of the squared deviations of $t$ (fitted) from $t$ (measured). The results are shown in Fig. 5 as solid curves, the sum of the squared deviations being 60 min$^2$ for 4OOH-CP, and $157$ min$^2$ for acrolein. The best fits to the logarithmic functions are shown as dashed curves. For 4OOH-CP, the sum of the squared deviations is 157 min$^2$, so the model of eq. 10 is clearly superior.

For acrolein (right panel of Fig. 5), both models are equally good. The best logarithmic fit gives $k_1 = 427$ s$^{-1}$ (sum of squared deviations = 13.6) and the best fit to (10) gives $k_1 = 4.0$ s$^{-1}$ (sum of squared deviations = 14.2). The data point for $t = 1$ min is clearly wrong, since $([S]/[S]_0)$ must be significantly larger for 1 min than for 2 min. Although this point makes both fits look poor, it was left in and used in the fits because there was no specific reason to eliminate it.

**Cellular GSH Protection by Mesna and WR-1065.** Figure 6, left panel, shows PBMC GSH protection by mesna (squares) and WR-1065 (circles) in the presence of 100 mM 4OOH-CP. Figure 6, right panel, shows PBMC GSH protection by mesna (squares) and WR-1065 (circles) in the presence of 20 mM acrolein. The protection ($P$) is calculated according to

$$P = [\text{GSH recovered from cells after exposure to alkylator}] / [\text{GSH recovered from cells not exposed to alkylator}]$$

Measurement of the quantity in the denominator was performed four times, giving 378, 347, 450, and 387 pmol per 50 μl (volume injected into HPLC), i.e., 390 ± 37.5 (mean and root mean square deviation from the mean). If the same 9.6% uncertainty obtains for all measurements of GSH recovered, the uncertainty in each value of $P$ is 13.6%.

Modeling the results of these experiments would require taking into account the following processes: 1) extracellular reaction of alkylating agent with drug thiol; 2) transport of the alkylating agent and the drug thiol across the cell membrane; 3) reaction of the alkylating agent with the drug thiol in the intracellular medium; and 4) intracellular reaction of the alkylating agent with GSH. In addition to a rate constant associated with each of these processes, a value for intracellular GSH would have to be assumed. It does not seem advisable to try to fit the experimental results by adjusting so many parameters, although values for some of these are obtainable from the experiments discussed previously.

We have performed numerical calculations, integrating all the rate equations. The results show that a plot of $P$ versus drug thiol concentration [D] should be S-shaped.

For the four sets of data in Fig. 6, we have fit $P - P_0$, where $P_0$ is the protection with no drug thiol, as a function of $c$, concentration of drug thiol, to the S-shaped two-parameter function

$$P - P_0 = \frac{1}{2} 100e^{\frac{c - C}{B}}, \ c \ll C; \ P - P_0 = 100(1 - \frac{1}{2}e^{\frac{c - C}{B}}), \ c \gg C$$

Here $P_0$ is the measured protection, as defined by eq. 11, for a drug thiol concentration of zero, and $B$ and $C$ are fitting parameters. The quantity, $P_0$, would be zero if the incubation time were infinite. The resulting plots are shown in Fig. 6, solid for WR-1065 and dashed for mesna. Agreement between the fitting function and experiment is within the stated 13.6% of experimental error. The fit for mesna and 4OOH-CP is not shown, because it is largely determined by a single point ($P = 30\%$, [D] = 1 mM).

Clearly, WR-1065 gives greater protection than mesna against both 100 mM 4OOH-CP and 20 mM acrolein. For 4OOH-CP, $P$ increased only slightly for WR-1065 concentrations less than 400 μM, but complete protection ($P = 100$) was observed at concentrations ≥800 μM (Fig. 6, left panel, circles). In contrast, $P$ never exceeded 32% for mesna.

Table 2 shows the results of protection experiments using 50 μM extracellular mesna, 50 μM extracellular WR-1065, and both. The alkylating agent was acrolein, also extracellular. It is clear that the combination of drug thiols gives more cytoprotection than either separately. The PBMC GSH level is reduced to 3% by the presence of...
20 μM acrolein. With addition of 50 μM mesna, the PBMC GSH level is raised to 29%, with addition of 50 μM WR-1065 to 34%, and with addition of both thiols it becomes 48%.

**Cellular GSH Protection by Intracellular Mesna and WR-1065.**

The contribution of intracellular drug thiols to cellular GSH protection was also investigated. PBMC (~10^7 cells per condition) were first incubated (37°C for 30 min) in RPMI medium without or with 300 μM mesna or WR-1065. Cells were then collected by centrifugation, suspended in fresh medium without or with 20 μM acrolein, and re-incubated at 37°C for 60 min. At the end of the incubation period, cells were collected, labeled with mBBr, and analyzed for glutathionebimane-bimane derivatives as described under Materials and Methods.

The percentage of GSH was calculated as 100 times the ratio of amount of GSH found to amount of GSH in the control sample (that is, cells incubated without any addition). The percentage of GSH in the absence of drug thiols was ~6%, whereas the presence of either mesna or WR-1065 raised this to ~40%. Although the k_2 values for WR-1065 and mesna with acrolein are different (Table 1), the long period of the reaction, 60 min, insures that all of the acrolein has been consumed in the alkylation process.

### Discussion

Rate constants were measured for reactions of thiols (GSH, mesna, and WR-1065) with mBBr (Fig. 1). It was concluded that with 5 mM mBBr, the rate of thiol reaction with mBBr is faster than the rate of thiol reaction with 4OOH-CP and acrolein (at the concentrations used in most of our experiments). This means that mBBr at this concentration can be used to quench most thiol reactions with 4OOH-CP and acrolein. If the reaction with mBBr is not fast enough, the term “ln Q” in eq. 3 will not be a linear function of time, as it should be for second-order reactions. Numerical calculations (shown under Results) explained how to treat experimental data when this is the case.

We then examined reactions of GSH, mesna, and WR-1065 with 4OOH-CP and acrolein in Na_2HPO_4 buffer at physiologic pH (~7.5) and 22 ± 2°C (Fig. 2–3). The rate constants obtained from these measurements are given in Table 1. These values are “effective rate constants” since they are calculated using total thiol concentration, whereas thiols react mainly by nucleophilic attack of thiolate anions on the reactive moieties of 4OOH-CP and acrolein. Assuming that thiol ionization is rapid enough so the equilibrium in eq. 1 is maintained, the concentration of thiolate is proportional to thiol concentration. Concentration of available nucleophile is determined by the thiol’s pK_a, which is ~8.7 for GSH, ~9.1 for mesna and ~7.7 for WR-1065 (Daney and Noel, 1960; Whitesides et al., 1977; Shaked et al., 1980; Szajewski and Whitesides, 1980; Newton et al., 1992).

Calculating using the equation, pH = pK_a + log (RS^-/RSH), we find that, at pH 7.5, thiolate anion represents ~6% total GSH, ~2% total mesna, and ~39% total WR-1065. Thus, at pH 7.5, the effective rate constant for WR-1065 is expected to be ~6.5 times greater than that for GSH and ~20 times greater than that for mesna. The results in Table 1 show that the k_2 for WR-1065 reaction with 4OOH-CP is more than 20-fold higher than that for GSH with 4OOH-CP (880 M^-1s^-1 versus 38 M^-1s^-1) and about 35-fold higher than that for mesna with 4OOH-CP (880 M^-1s^-1 versus 25 M^-1s^-1). The k_2 for WR-1065 reaction with acrolein was too high to measure, but the k_2 for GSH-acrolein (490 M^-1s^-1) is 0.7 times the k_2 for mesna-acrolein (700 M^-1s^-1) (Table 1), whereas, from the percentage of thiolates calculated above, the ratio should be 6.2. This implies that factors other than pK_a also determine relative rate constants.

Although the chemical reactions of thiols with alkylating agents are reversible, the rates of the reverse reactions are so slow that they have no effect on the measurements (see Results). This observation (that is, a slow reverse rate) is in agreement with the literature (Esterbauer et al., 1975; Wlodek, 1988; Ramu et al., 1995, 1996).

The alkylating agents are known to produce cellular GSH depletion in vitro as well as in vivo (Gurtoo et al., 1981; Crook et al., 1986; Souid et al., 2001). In the present study, the rates of cellular GSH depletion by acrolein and 4OOH-CP were measured, and the effective rate constants were determined (Fig. 4–5). The measured k_2 for cellular GSH depletion by acrolein was ~13 times larger than that by 4OOH-CP (~220 M^-1s^-1 versus ~16.5 M^-1s^-1, see Results), about the same as the ratio of the k_2 for GSH reactions with acrolein and 4OOH-CP in solution (~490 M^-1s^-1 versus ~38 M^-1s^-1, Table 1). This suggests that (a) the rate constants for intracellular reaction with GSH are the same as for extracellular reaction and (b) the rate of transport across the cell membrane is fast for both agents.

The concentration of acrolein that depletes cellular GSH by 50% is ~5-fold less than that of 4OOH-CP (~7 μM versus ~35 μM; Fig. 4, right and left panels, respectively). These results are in accord with the previous report in Chinese hamster lung fibroblast cells, showing a total cellular GSH depletion within 30 min incubation at 37°C with either 10 μM acrolein or 100 μM 4OOH-CP (Bunting and Townsend, 1998).

Assuming the rate constants for reactions of thiols with alkylating agents were the same inside as outside cells, we were able to estimate the rate constants (k_1) for the transport across the cell membrane from the data of Figs. 4 and 5. Writing the rate of increase of the alkylating agent concentration inside the cell, d[A]/dt, as k_1[A]_e – k_2[A]__i [eq. 2], we estimated that k_1 for 4OOH-CP was ~0.15 s^-1 and k_1 for acrolein was ~0.04 s^-1. To completely neglect transport across the cell membrane, the transport rate must be much higher than the reaction rate. To compare these two rates, suppose [A]_e – [A] = 100 μM and k_1 = 0.02 s^-1, so the rate of transport is 2 × 10^-3 M s^-1. Then, if k_2 = 100 M^-1s^-1 and [A] = [thiol] = ~50 μM, the rate of reaction is 2.5 × 10^-7 M s^-1, less than an order of magnitude slower than the transport rate.

That GSH reacts faster with acrolein than with 4OOH-CP (Table 1 and Fig. 5) is due partly to faster transport of acrolein across the cell membrane and partly to larger k_2. The rapidity of the reaction explains the failure to obtain a complete cellular GSH protection even with 25-fold molar excess (i.e., 500 μM) of WR-1065 or mesna (Fig. 6, right panel), whereas a complete cellular GSH protection over 4OOH-CP is obtained with only a 10-fold molar excess of WR-1065 (i.e., 1 mM) (Fig. 6, left panel, circles). Mesna (Fig. 6, left panel, squares) has a more limited cytoprotective capacity, since, in the presence of a 10-fold molar excess of mesna (i.e., 1 mM), cellular GSH was below 50%.

The results in Fig. 6 (left panel) are in accord with the previous
study showing that mesna, GSH, and N-acetylcysteine protect Chinese hamster lung fibroblast cells from the toxic effects of 4-OH-CP (Bunting and Townsend, 1998). In another report, a 100-fold molar excess of mesna ameliorated $<50\%$ of the growth inhibitory activity of either 100 $\mu$M 4-OH-CP or 100 $\mu$M acrolein in various cell types (Blomgren and Hallstrom, 1991). Moreover, binding of cyclophosphamide metabolites to intact cells or cellular components was decreased by only $\sim50\%$ in the presence of an equal molar concentration of mesna or GSH (Wildenauer and Oehlmann, 1982). The fact that a complete cytoprotection requires significant molar excess of WR-1065 over the alkylating agent (Fig. 6, left panel, circles) may account for the lack of cytoprotection by WR-2721 during very intensive chemotherapy (Shapiro et al., 1998).

Studies of cellular uptake of mesna and WR-1065 demonstrate that significant uptake of mesna does occur but at a level much less than that of WR-1065 (Souid et al., 2001). For both agents, the predominant intracellular form is the free thiol (Souid et al., 2001). The present study shows that cytoprotection by WR-1065 and mesna is additive (Table 2), supporting their clinical use in combination (Souid et al., 1999, 2001).

The cytoprotective capacity of a thiol drug is influenced by its $pK_a$, its rate of reaction with the alkylating agent, and the rate of its uptake by the cell. The data show a superior protection of cellular GSH by WR-1065. This observation is interpreted in terms of the rate constants derived from kinetic analysis of the measured depletion data. A kinetic model to interpret the protection data is also possible but would require assumed values for several parameters in addition to the measured rate constants.

Acknowledgments. We appreciate the technical support on the kinetics by professor Robert C. Fahey.

References


