Role of Multidrug Resistance Protein 1 (MRP1) and Glutathione S-Transferase A1-1 in Alkylating Agent Resistance

KINETICS OF GLUTATHIONE CONJUGATE FORMATION AND EFFLUX GOVERN DIFFERENTIAL CELLULAR SENSITIVITY TO CHLORAMBUCIL VERSUS MELPHALAN TOXICITY

Received for publication, October 16, 2000, and in revised form, December 1, 2000
Published, JBC Papers in Press, December 13, 2000, DOI 10.1074/jbc.M009400200

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We investigated the role of phase II (conjugation) and phase III (efflux) detoxification of the anticancer drugs melphalan (MLP) and chlorambucil (CHB). Although both drugs are substrates of Alpha-class glutathione S-transferases (GST) and the monoglutathionyl conjugates formed in these enzymatic reactions are transported by MRP1, we found that GSTA1-1 and MRP1 acted in synergy to confer resistance to CHB but not to MLP (Morrow, C. S., Smitherman, P. K., Diah, S. K., Schneider, E., and Townsend, A. J. (1998) J. Biol. Chem. 273, 20114–20120). To explain this selectivity of MRP1/GST-mediated resistance, we report results of side-by-side experiments comparing the kinetics of MLP- versus CHB-glutathione conjugate: formation, product inhibition of GSTA1-1 catalysis, and transport by MRP1. The monoglutathionyl conjugate of CHB, CHB-SG, is a very strong competitive inhibitor of GSTA1-1 (K_i 0.14 μM) that is >30-fold more potent than that of the corresponding conjugate of MLP, MLP-SG (K_i 4.7 μM). The efficiency of GSTA1-1-mediated monoglutathionyl conjugate formation is more than 4-fold higher for CHB than MLP. Lastly, both CHB-SG and MLP-SG are efficiently transported by MRP1 with similar V_max although the K_m for CHB-SG (0.37 μM) is significantly lower than for MLP-SG (1.1 μM). These results indicate that MRP1 is required for GSTA1-1-mediated resistance to CHB in order to relieve potent product inhibition of the enzyme by intracellular CHB-SG formed. The kinetic properties of MRP1 are well suited to eliminate CHB-SG at pharmacologically relevant concentrations. For MLP detoxification, where product inhibition of GSTA1-1 is less important, GSTA1-1 does not confer resistance because of the relatively poorer catalytic efficiency of MLP-SG formation. Similar analyses can be useful for predicting the pharmacological and toxicological consequences of MRP and GST expression on cellular sensitivity to various other electrophilic xenobiotics.

Our laboratory has studied the role in xenobiotic detoxification of coordinated phase II conjugation reactions and phase III efflux transport. Among the phase II enzymes, we have been particularly interested in the glutathione S-transferases (GST), a family of enzymes that catalyze the conjugation of glutathione with a variety of electrophilic toxins including cancer drugs, carcinogens, and other xenobiotics (1–4).

Although these conjugation reactions generally render the compound less chemically reactive and hence less toxic, the overexpression of the various isozymes of GST is frequently insufficient to confer significant protection from the cyto- and genotoxicities of these electrophiles. Indeed, we have shown that coexpression with GST of the glutathione conjugate efflux transporters, MRP1 or MRP2, is necessary to potentiate GST-mediated protection from the toxicities of the cancer drug chlorambucil (CHB) or the carcinogen 4-nitroquinoline 1-oxide (5–7). In these and other studies, high intracellular accumulation of the toxin-glutathione conjugate formed in the absence of MRP1 (or MRP2) is associated with increased toxicity whereas low intracellular toxin-conjugate accumulation resulting from MRP-dependent conjugate efflux is associated with cellular resistance (5, 7, 8). These results indicate that glutathione conjugates, especially when they accumulate to high intracellular levels, may themselves be directly or indirectly toxic.

Rarely, the glutathione conjugate may be more reactive than the parent compound resulting in increased conjugate toxicity (9–11). However, in most cases, the conjugate is less reactive than the parent compound. Thus for the majority of glutathione conjugates, the basis for any apparent conjugate toxicity is unknown but may involve residual conjugate reactivity, product inhibition of GST by the conjugate (12, 13), or novel toxicities of the conjugate when present at high intracellular concentrations.

Previously we showed that expression of both GSTA1-1 and MRP1 are required to confer resistance to CHB; expression of GSTA1-1 or MRP1 alone afforded no protection from CHB cytotoxicity (6). However, combined expression of GSTA1-1 and MRP1 failed to confer protection from the cytotoxicity of the related drug, melphalan (MLP). This was surprising because both of these structurally similar drugs (Fig. 1) are reportedly substrates of Alpha-class GST (12, 14–17), and because their monoglutathionyl conjugates are transported by MRP1 (18). To

*This work was supported by National Institutes of Health Grants CA70338 and CA64579. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: GST, GSTA1, and GSTA1-1, glutathione S-transferase, human GST isoform A1 monomer, and A1-1 dimer; CHB and CHB-SG, chlorambucil and monoglutathionyl conjugate of chlorambucil; CDBN, 1-chloro-2,4-dinitrobenzene; MLP and MLP-SG, melphalan and monoglutathionyl melphalan; MRP, MRP1, and MRP2, multidrug resistance protein or multidrug resistance-associated protein; MRP isoform 1, MRP isoform 2; HPLC, high performance liquid chromatography; WT, wild-type.
explain the basis for this selectivity of MRP/GST-mediated resistance, here we report the results of side-by-side experiments designed to compare the kinetics of CHB- versus MLP-glutathione conjugate: formation, inhibition of GSTA1-1, and transport by MRP1. Other investigators have shown that MLP and CHB are substrates of Alpha-class GST, but to our knowledge no direct comparisons of the kinetic constants of purified GSTA1-1 toward the two substrates have been reported. Whereas, the monoglutathionyl conjugate of CHB, CHB-SG, is known to inhibit Alpha-class GST (12), again no direct comparison between inhibition by CHB-SG and the corresponding conjugate of MLP, MLP-SG, has been reported. Finally, previous studies on MRP1-mediated MLP-SG and CHB-SG transport did not report kinetic constants for the transport of these two compounds (18).

Our results show that the kinetic parameters for CHB-SG and MLP-SG formation, efflux, and GSTA1-1 inhibition can explain the selectivity of GST/MRP1-mediated resistance synergy toward CHB versus MLP. The data indicate that the most important toxicity of CHB-SG is indirect and involves the inhibition of GSTA1-1 catalysis, which limits further drug detoxification.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture, and Cytotoxicity Determinations—All cell lines were derived from parental MCF7/WT human breast carcinoma cells. MCF7/WT cells have low GST activity, no detectable GSTA1-1, and extremely low level MRP1 expression. The multidrug resistant variant, MCF7/VP, expresses high level MRP1 but again has low GST activity. The multidrug resistant variant, MCF7/WT, expresses extremely low level MRP1 expression. The multidrug resistant variant, MCF7/WT or MRP1

Preparation of Glutathione Conjugates of MLP and CHB—Monoglutathionyl derivatives of CHB and MLP were prepared in 1–5 ml reactions containing 0.13 mM CHB or MLP, 10 mM glutathione (Sigma), 0.1 mM sodium phosphate, pH 7.5, and 0.2 mM NaCl. Mixtures were incubated for 30 min to 1 h at 37 °C, and the reactions were terminated by the addition of perchloric acid to 6.4% (v/v). Samples were loaded onto Waters (Milford, MA) Oasis HLB 3-cc extraction cartridges and washed with 2 ml each of 0.05% (v/v) trifluoroacetic acid and successively higher concentrations of methanol in 0.05% trifluoroacetic acid. MLP-SG eluted in 40% methanol and CHB-SG in 80% methanol. The purity and identity of each conjugate was verified by analytical HPLC (below) and electrospray mass spectrometry (MLP-SG: m/z [M+H]+ 575.12). Concentrations of conjugate preparations were estimated by analytical HPLC using integrated absorbance peak areas (254 nm) of conjugates compared with CHB and MLP standards of known concentrations.

Radialed conjugates were prepared as described above with the following modifications. Total reaction volumes were reduced to 100 μl; MLP or CHB was added to 1 mM or 3 mM, respectively; and glysine-2-[3H]glutathione (PerkinElmer Life Sciences) was added to 0.51 mM (specific activity: 0.98 nCi/mole). Monoglutathionyl conjugates were purified by HPLC using a C18 reverse phase column (Beckman) and a solvent system consisting of a 90-min linear gradient of 5–90% (v/v) methanol in 0.05% trifluoroacetic acid run at 0.5 ml/min. Chromatography was monitored spectrophotometrically at 254 nm with CHB-SG eluting at 57 min and MLP-SG at 41 min. Drug conjugates were dried under nitrogen and dissolved in water just prior to use.

Enzyme Kinetics—Recombinant human GSTA1-1 was prepared as described previously (6). For the analysis of monoglutathionyl conjugate inhibition of GSTA1-1 activity, an adaptation of the CDNB assay described by Habig et al. (21) was used. Reactions contained 0.1 mM potassium phosphate, pH 6.8, nonlimiting concentration (2 mM) glutathione, 0.5–2 mM CDNB (Sigma), as the variable substrate, ± 0.2 μg/ml purified GSTA1-1, and variable concentrations of MLP-SG or CHB-SG. Reactions were initiated at 25 °C by the addition of CDNB, and formation of its conjugate was monitored spectrophotometrically at 340 nm (21). Enzyme-dependent catalysis was determined by subtracting conjugate formed in the absence of GSTA1-1.

GSTA1-1 catalysis of MLP-SG and CHB-SG formation was determined as follows. Reactions contained 0.1 mM sodium phosphate, pH 6.5, 140 mM KCl, 2 mM glutathione, ± 0.27 μg/ml recombinant GSTA1-1, and either 50 or 500 μM CHB or 50 or 1000 μM MLP as the variable substrate. Reactions were initiated at 25 °C by the addition of CHB or MLP. At 1, 3, 5, 10, 20 min, aliquots were removed, and reactions were terminated by the addition of perchloric acid to 5% (v/v). Conjugate formation was quantified by analytic HPLC as described above. Enzyme-dependent catalysis was determined by subtracting conjugate formed in the absence of GSTA1-1 from conjugate formed in the presence of GSTA1-1. Kinetic constants were calculated from the initial velocities of enzyme-dependent conjugate formation fitted to the Michaelis-Menten equation using Synergy KaleidaGraph 3.0 software for the Macintosh.

Preparation of Inside-Out Plasma Membrane Vesicles and Determination of MRP1-dependent Conjugate Uptake—Membrane vesicles were prepared by modification of a method described by Loe and coworkers (22, 23). Frozen cell pellets from ~4 × 10⁸ cells (MRP1-MCF7/WT or MRP1-MCF7/VP cells) were thawed in 7 ml of the homogenization mixture (including fresh protease inhibitors) as described previously (22, 23). Cells were disrupted at 4 °C by nitrogen cavitation at 1250 psi with constant stirring for 20 min. The homogenate was centrifuged at 1700 rpm in a Sorvall RT6000 centrifuge at 4 °C for 15 min. The supernatant was overlaid on a 3 ml sucrose cushion (35% (w/v) sucrose, 1 ml EDTA). Following centrifugation at 24,000 rpm for 2 h at 4 °C (Beckman SW41 rotor), the opaque interface was collected, diluted into 5 parts TS (10 mM Tris, pH 7.5, 250 mM sucrose), and centrifuged at 35,000 rpm for 40 min at 4 °C (Beckman 60 Ti rotor). The pellet was suspended in 1 ml of 50 mM Tris, pH 7.5, 250 mM sucrose, gently dispersed by 4 passages through a 27-gauge needle, and stored in aliquots at −80 °C.

The kinetics of 3H-labeled conjugate uptake by vesicles was determined using an adaptation of the membrane rapid filtration method (27). Briefly, 25–50 μl reaction mixtures contained 50 mM Tris, pH 7.5, 10 mM MgCl₂, 250 mM sucrose, either 4 mM ATP or 4 mM β,γ-methyleneadenosine 5′-triphosphate (nonhydrolyzable ATP control), and varying concentrations of [3H]MLP or [3H]CHB-SG. Mixtures were warmed to 37 °C, and reactions were initiated by addition of membrane vesicles (32 μg/50 μl reaction). At 30-s intervals, 10-μl aliquots were removed, and reactions were terminated in 1 ml of ice-cold TS. Samples were immediately filtered with vacuum through 25 mm hydrophilic membrane filters (GVWP, Millipore), and the retained vesicles were washed twice with 1 ml of ice-cold TS prior to liquid scintillation counting.

RESULTS

Previous studies demonstrated that coexpression of MRP1 and GSTA1-1 confers resistance to CHB (6). Additional experiments showed that both MRP1 and GSTA1-1 are required for...
resistance; expression of GSTA1-1 or MRP1 alone was ineffective for cytoprotection. The cytotoxicities of CHB versus MLP were compared in the MCF7 derivative cell lines expressing (i) neither MRP1 or GSTA1-1 (MCF7/WT), (ii) MRP1 alone (MCF7/VP), or (iii) MRP1 and GSTA1-1 in combination (MCF7/VPa). These data, shown in Fig. 2, revealed that whereas combined expression of MRP1 and GSTA1-1 conferred resistance to CHB, combined expression had no effect on cellular sensitivity to MLP. We hypothesized that this selectivity of MRP1/GSTA1-1 cytoprotection may result from quantitative differences in (i) GSTA1-1-mediated MLP-SG versus CHB-SG conjugate formation, (ii) product inhibition of GSTA1-1 by MLP-SG versus CHB-SG, or (iii) MLP-SG versus CHB-SG efflux by MRP1.

Using purified recombinant GSTA1-1 and CDNB as the variable substrate, experiments shown in Fig. 3, A and B) demonstrated that both CHB-SG and MLP-SG are competitive inhibitors of GSTA1-1. CHB-SG is an exceptionally potent inhibitor with a \( K_i \) of 0.14 \( \mu \)M whereas MLP-SG is considerably less potent with a \( K_i \) of 4.7 \( \mu \)M (Fig. 3C). These data indicate that MRP1 may be required to potentiate GSTA1-1-mediated resistance to CHB in order to relieve product inhibition of the enzyme by CHB-SG.

For MLP, product inhibition of GSTA1-1 by MLP-SG is relatively less important. Thus, it is unclear from these data why GSTA1-1 alone or in combination with MRP1 did not confer resistance to MLP. To address this issue, the kinetics of GSTA1-1-mediated formation of the monoglutathionyl conjugates of MLP versus CHB were examined (Fig. 4). Whereas both compounds are substrates of GSTA1-1, the \( V_{max} \) is significantly higher and the \( K_m \) lower for CHB than for MLP resulting in a catalytic efficiency (\( V_{max}/K_m \)) that is more than 4-fold superior for CHB (Table I). These data predict that GSTA1-1 should be less efficient in the conjugation and detoxification of MLP, a prediction consistent with the cytotoxicity data (Fig. 2).

A comparison of CHB-SG and MLP-SG uptake by isolated inside-out membrane vesicles revealed that both conjugates are efficiently transported by MRP1 in an ATP-dependent manner (Fig. 5). Initial velocities of MRP1-mediated transport were determined for CHB-SG and MLP-SG inhibition experiments are represented by closed circles and squares, respectively. These data indicate that MRP1 is considerably less effective for CHB-SG transport, 0.37 \( \mu \)M (Fig. 6). The relatively low \( K_m \) for CHB-SG transport indicates that it is one of the better substrates so far reported for MRP1 and that MRP1-mediated efflux of CHB-SG operates efficiently at low, GSTA1-1 inhibitory concentrations of CHB-SG.

FIG. 2. Coexpression of MRP1 and GSTA1-1 confer resistance to chlorambucil (CHB) but not melphalan (MLP) cytotoxicity. Chlorambucil (A) or melphalan (B) cytotoxicities were determined for the various cell lines as described under “Experimental Procedures.” Represented are cytotoxicity profiles for MCF7/WT cells (MRP1-, GSTA1-1, open squares), MCF7/VP cells (MRP1+, GSTA1-1, open circles), and MCF7/VPa cells (MRP1+, GSTA1-1, closed circles). Points represent the mean values of eight determinations \( \pm 1 \) S.D.

FIG. 3. Differential sensitivity of GSTA1-1 to inhibition by monogluthathionyl conjugates of chlorambucil (CHB) versus melphalan (MLP). Enzyme kinetics were performed as described under “Experimental Procedures” using CDNB as the variable substrate and monoglutathionyl conjugates of CHB (CHB-SG) or MLP (MLP-SG) as inhibitors. Shown are Hanes plots for CHB-SG inhibition (A) or MLP-SG inhibition (B) and plots of \( K_m/V_{max} \) versus inhibitor concentrations (C). A, CHB-SG concentrations used were 0 (closed circles), 0.05 \( \mu \)M (open circles), 0.1 \( \mu \)M (closed squares), and 0.2 \( \mu \)M (open squares). B, MLP-SG concentrations used were 0 (closed circles), 1.5 \( \mu \)M, 2 \( \mu \)M, and 3 \( \mu \)M (plots using intermediate inhibitor concentrations were omitted from B for clarity); and 4 \( \mu \)M (open circles). C, data derived from CHB-SG and MLP-SG inhibition experiments are represented by closed circles and squares, respectively.

DISCUSSION

This report characterizes the roles of phase II (conjugation) and phase III (efflux) in CHB and MLP detoxification in model MCF7 cell lines. Both drugs share detoxification pathways that
involve GSTA1-1 catalysis of monoglutathionyl conjugate formation and MRP1-dependent conjugate efflux. Despite these shared processes, differential protection from drug toxicities is observed in cells expressing both GSTA1-1 and MRP1. In these MCF7/VPα cells, significant resistance is observed only toward CHB, not MLP (Fig. 2). The preferential detoxification of CHB over MLP can be primarily attributed to the superior catalytic efficiency of GSTA1-2 toward CHB (Fig. 4, Table I). The greater efficiency of MRP1-mediated transport of CHB-SG may make some additional contribution to the differential protection conferred by GSTA1-1 expressing cells (Fig. 6, Table II).

The role of MRP1, or other glutathione conjugate efflux transporters, is an important issue in GST-mediated detoxification. GSTs catalyze the conjugation with glutathione of reactive electrophilic centers of several toxic xenobiotics, including MLP and CHB (2). These reactions generally decrease the reactivity and presumably the toxicity of the electrophile. However, as we have also noted for the cyto- and genotoxic compound 4-nitroquinoline 1-oxide, expression of GST alone is insufficient to afford measurable protection from CHB cytotoxicity (5–7). In these studies, GST-mediated resistance required the coexpression of a glutathione conjugate efflux transporter such as MRP1 (CHB and 4-nitroquinoline 1-oxide) or MRP2 (4-nitroquinoline 1-oxide). These results suggest that the glutathione conjugates, which accumulate within the cell in the absence of MRP, have some residual or novel toxicities. Indeed, in the absence of MRP1, GST-expressing MCF7 cell derivatives treated with only micromolar concentrations of CDNB or 4-nitroquinoline 1-oxide can rapidly accumulate millimolar concentrations of the respective glutathione conjugates, S-(2, 4-dinitrophenyl)glutathione or 4-(glutathione-S-yl)-quinoline 1-oxide (5, 7, 8). Under similar conditions, MRP1-expressing cells are able to maintain very low intracellular levels of these conjugates.

There are at least two plausible mechanisms for the apparent toxicity of CHB-SG in cells lacking MRP1. The first is the

![Fig. 4. Differential GSTA1-1 catalysis of monoglutathionyl-chlorambucil (CHB-SG) versus -melphalan (MLP-SG) formation.](image)

Shown are the initial velocities of GSTA1-1-mediated CHB-SG (closed circles) versus MLP-SG (open circles) formation at varying substrate concentrations. Data were obtained as described under “Experimental Procedures” with each point representing the mean initial velocities determined from two independent experiments. Error bars show the range of values measured.

| Substrate        | $K_m$  | $V_{max}$ | $V_{max}/K_m$ |
|------------------|--------|-----------|---------------|
| chlorambucil     | 111    | 712       | 6.4           |
| melphalan        | 170    | 251       | 1.5           |

![Fig. 5. MRP1- and ATP-dependent uptake of monoglutathionyl conjugates of CHB and MLP by inside-out membrane vesicles.](image)

Shown are ATP-dependent uptake of CHB-SG (A) and MLP-SG (B) into inside-out membrane vesicles derived from MRP1– MCF7/WT cells (closed circles) or MRP1– MCF7/VP cells (open circles). Transport studies were accomplished as described under “Experimental Procedures.” Points are the means of four determinations, and the error bars represent 1 S.D.

| Substrate        | $K_m$  | $V_{max}$ | $V_{max}/K_m$ |
|------------------|--------|-----------|---------------|
| CHB-SG           | 0.37   | 130       | 0.35          |
| MLP-SG           | 1.14   | 130       | 0.11          |
potential direct toxicity of this monoglutathionyl conjugate of CHB. GSTA1-1 catalyzes the formation of CHB-SG but leaves the second reactive chloroethyl group intact. Whereas CHB-SG can no longer form DNA strand cross-linkages, it retains the ability to form monovalent adducts with cellular nucleophiles including proteins and nucleic acids. Therefore, it would not be surprising to find that the monoglutathionyl derivative of CHB retained significant cytotoxicity at high intracellular concentrations. Several findings argue that this mechanism contributes relatively little to the CHB toxicity overcome by combined expression of GSTA1-1 and MRP1. First, it is known that finite levels of CHB-SG are formed nonenzymatically, in the absence of GSTA1-1. MRP1 will support efflux of this conjugate (Figs. 5 and 6), yet MRP1 alone does not confer resistance (MCF7/VP, Fig. 2). Indeed, expression of MRP1 alone consistently results in either no change or a modest sensitization to CHB toxicity (6, 19).

The data suggest that a second potential mechanism of CHB-SG toxicity is more important. This mechanism is indirect and involves product inhibition of GSTA1-1. As originally described by Meyer et al. (12) and shown here (Fig. 3, Table I), CHB-SG is a potent inhibitor of GSTA1-1 ($K_i \approx 0.14$ $\mu M$). In contrast, MLP-SG is a much less effective inhibitor of GSTA1-1 ($K_i \approx 4.7$ $\mu M$). Hence, removal of intracellular CHB-SG is particularly critical to maintain GSTA1-1 activity and continued detoxifying conjugation of CHB. MRP1 efficiently supports CHB-SG efflux (Figs. 5 and 6 and Table II). The $K_m$ of transport is remarkably low ($0.37$ $\mu M$), which indicates that MRP1-mediated transport is kinetically suited to remove CHB-SG at low, GSTA1-1-inhibitory concentrations of the conjugate.

In summary, MRP1 is essential to potentiate GSTA1-1 detoxification of CHB in order to relieve product inhibition of this conjugating enzyme. In contrast to its effect on CHB toxicity, GSTA1-1 is unable to confer measurable protection from MLP toxicity because of its relatively poorer efficiency toward this substrate. These studies indicate that the determination of quantitative differences in the kinetics of phase II conjugation and phase III efflux processes can be used to accurately predict the sensitivities of cells and tissues to the toxicities of some drugs and other xenobiotics.

Acknowledgments—We thank Emmanuelle Rocchi and Erasmus Schneider for helpful suggestions on vesicle preparation.

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