JPET Fast Forward. Published on October 20, 2003 as DOI:10.1124/jpet.103.057729

Title: Role of multidrug resistance protein 2 (MRP2, ABCC2) in alkylating agent detoxification: MRP2 potentiates glutathione *S*-transferase A1-1–mediated resistance to chlorambucil cytotoxicity*

Authors: Pamela K. Smitherman[1], Alan J. Townsend[1], Timothy E. Kute[2], and Charles S. Morrow[1]

From: Department of Biochemistry
Wake Forest University School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: MRP2 potentiates GSTA1-1–mediated resistance to chlorambucil

Corresponding author: Charles S. Morrow
Department of Biochemistry
Wake Forest University School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157
Telephone: 336-713-7218
FAX: 336-716-7671
Email: cmorrow@wfubmc.edu

Counts:
Text pages: 35
Tables: 3
Figures: 9
References: 40
Words: Abstract: 251
Introduction: 499
Discussion: 626

Abbreviations: ABC, ATP-binding cassette; BSA, bovine serum albumin; CHB and CHB-SG, chlorambucil and monoglutathionyl conjugate of chlorambucil; GSH, glutathione; GST, glutathione *S*-transferase, MRP, MRP1, and MRP2, multidrug resistance protein or multidrug resistance-associated protein, MRP isoform 1 (ABCC1), MRP isoform 2 (ABCC2); PBS, phosphate buffered saline

Section: Cellular and Molecular

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Our previous studies have shown that the glutathione *S*-transferases (GSTs) can operate in synergy with the efflux transporter, MRP1 (ABCC1), to confer resistance to the cyto- and genotoxicities of some anti-cancer drugs and carcinogens. The current study was designed to determine whether the alternative efflux transporter, MRP2 (ABCC2), can also potentiate GST-mediated detoxifications in HepG2 cells. HepG2 cells, which express high level MRP2 but not MRP1, were stably transduced with GST expression vectors under tetracycline-repressible transcriptional control. MRP2 was able to support GSTA1-1-mediated resistance to chlorambucil (CHB) cytotoxicity in HepG2 cells. Resistance was GST isozyme-specific in that GSTP1a-1a and GSTM1a-1a failed to confer protection from CHB toxicity. Moreover, inhibition of MRP2 with sulfinpyrazone completely reversed GSTA1-1-associated resistance indicating that MRP2-efflux function is required to potentiate GSTA1-1-mediated resistance. Relative transport by MRP1 versus MRP2 of monoglutathionyl-CHB (CHB-SG) was examined using inside-out plasma membrane vesicles derived from MCF7 cells transduced with MRP1 or MRP2 expression vectors. Both MRP1 and MRP2 transported CHB-SG efficiently, at the levels of protein expressed, with similar V_{max} and with K_m of 0.39 μM and 10 μM, respectively. We conclude that detoxification of CHB by GSTA1-1 requires the removal of the glutathione conjugate formed and that either MRP1 or MRP2 can serve this efflux function. These findings have implications for the role of MRP2 in detoxification of alkylating agents in the apical epithelium of liver and kidney where it is highly expressed as well as the role of MRP2 in the emergence of alkylating drug resistance in cancer cells.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Cellular mechanisms of xenobiotic detoxification are crucial elements involved in the emergence of drug resistance in cancer cells and in the successful elimination of reactive toxins from normal tissues. Recent work in our laboratory has focused on the interactions between the so-called phase II, or conjugating, with the phase III, or efflux, systems of detoxification (Ishikawa, 1992). In particular, we have examined the glutathione (GSH)/glutathione *S*-transferase (GST) conjugating and the ABC transporter efflux systems. We have shown that, for several of drugs, carcinogens, and other toxins, coordinated expression of these systems can effect cellular detoxification that is improved over that of either system alone (Morrow et al., 1998a; Morrow et al., 1998b; Morrow et al., 1998c; Morrow et al., 2000; Paumi et al., 2001).

While GSTs can catalyze the conjugation with GSH of a variety of electrophilic compounds rendering these compounds less reactive and generally less toxic, the over expression of GST alone is often insufficient to afford significant or consistent protection from the cytotoxicities of these electrophilic substrates (Fields et al., 1994; Hayes and Pulford, 1995; Leyland-Jones et al., 1991; Moscow et al., 1989; Townsend et al., 1992). Potential reasons for the failure of GSTs to confer complete protection include reversibility of some conjugation reactions (Witz, 1989), residual or, rarely, increased conjugate toxicities (Van Bladeren, 2000), or product inhibition of GST (Meyer, 1993; Meyer et al., 1992). Previously, our laboratory established that expression of GSTA1-1, which catalyzes the conjugation of GSH with chlorambucil (CHB), confers resistance to CHB in MCF7 cells but only when co-expressed with the ATP-dependent efflux transporter, MRP1 (ABCC1) (Morrow et al., 1998b). Subsequent studies suggested that the role for MRP1 in CHB detoxification is to remove the cellular monoglutathionyl conjugate of CHB, CHB-SG, thereby relieving the potent product inhibition of GSTA1-1 by CHB-SG (Paumi et al., 2001).

Work presented herein evaluates the ability of the alternative efflux transporter, MRP2 (ABCC2), to also potentiate GSTA1-1–mediated detoxification of CHB-SG. This is important because, while MRP1 and MRP2 have considerable substrate overlap, substrate specificies and transport kinetics of common substrates differ (Borst et al., 2000; Keppler et al., 1998; Konig et al., 1999). Moreover, the patterns of cellular and tissue distribution of MRP1 and MRP2 are distinct. MRP1 is ubiquitously expressed and

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

is often overexpressed in drug resistant tumor cells (Borst et al., 2000; Loe et al., 1996). MRP1 is localized in the plasma membrane and is targeted to the basolateral surface of polarized cells (Evers et al., 1996; Mayer et al., 1995). In contrast high-level expression of MRP2 is confined to limited tissues such as liver canalicular hepatocytes and renal tubule epithelium where MRP2 is localized on the apical surface of the plasma membrane (Buchler et al., 1996; Schaub et al., 1997). Although MRP2 may be overexpressed in some resistant tumor cells, it is likely to play a more important physiologic role in the excretory detoxification of xenobiotics.

In this study, HepG2 cells were used because they target significant levels of MRP2 to the plasma membrane under standard culture conditions yet express negligible MRP1 or cytosolic GST (Cantz et al., 2000; Jedlitschky et al., 1997; Morrow et al., 2000). To evaluate the ability of GSTs to confer alkylating agent resistance when coordinately expressed with MRP2, tetracycline/doxocycline regulatable vectors encoding GSTA1-1, GSTM1a-1a, and GSTP1a-1a were stably introduced into HepG2 cells. In order to assess the contribution of MRP2 to drug resistance, MRP2-mediated transport function was inhibited by sulfinpyrazone. The validity of this approach was supported by previous studies that showed MRP2 transport function is effectively inhibited by non-toxic doses of sulfinpyrazone (Morrow et al., 2000).

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Cell lines and tissue culture. Parental HepG2 and MCF7 (MCF7/WT) as well as their derivative cell lines were cultured in Dulbecco's modified Eagle medium plus 10% fetal bovine serum at 37^0C, 5 % CO_2. HepG2 cells stably expressing a doxocycline-repressible GSTP1a-1a vector, HepG2/π-3 have been described previously (Morrow et al., 2000).

All other transgenic cell lines were generated by stable transduction of retroviral vectors as described (Miller et al., 1993). Briefly, vectors were transiently transfected into PA317 amphotropic packaging cells and the resulting virus-containing supernatants were used to transduce HepG2 and MCF7 cells. Individual clones were selected in either G418 or hygromycin and analyzed separately for transgene expression.

HepG2 cell lines expressing doxocycline-repressible GSTA1-1 (HepG2/α-10 and HepG2/α-25) or GSTM1a-1a (HepG2/μ-2 and HepG2/μ-5) were constructed as follows. Parental HepG2 cells were transduced with pRevTet-Off (Clontech, Palo Alto, CA). Following selection in 0.5 mg/ml G418, one clone, HepG2/RTO-21, which expressed particularly high level doxocycline-repressible transactivator activity was chosen for subsequent transductions with GST expression vectors. *Eco*RI cDNA fragments encoding the human GSTA1-1 (839 bp, (Rhoads et al., 1987; Tu and Qian, 1986)) or GSTM1a-1a (1142 bp, (DeJong et al., 1988)) were blunt-end ligated into the *Hin*dIII site of pRevTRE (Clontech, Palo Alto, CA). These vectors were introduced into HepG2/RTO-21 cells, selected in 0.4 mg/ml hygromycin, and two clones expressing each GST vector were used.

MCF7 cells stably transduced with MRP1, MCF7/MRP1-10, have been described previously (Paumi et al., 2003). MCF7 cells expressing MRP2 were generated as follows. A 4689 bp cDNA fragment containing the complete coding sequence of MRP2 from the 5' Kozak consensus to the 3' stop codon was amplified from normal human liver RNA by RT-PCR using AMV reverse transcriptase and Expand High Fidelity DNA polymerase (Roche, Indianapolis, IN) (Ausubel et al., 1997). Using PCR-based site-directed mutagenesis, 9 bp encoding the C-terminal 3 amino acids were deleted. This modification was based upon a previous report suggesting that elimination of these amino acids, while not effecting the transport properties of MRP2, obviates the requirement of

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

cell polarization to target MRP2 to the plasma membrane (Harris et al., 2001). The veracity of the amplified MRP2 sequence was confirmed by DNA sequence analysis (DNA Synthesis Core Laboratory, Wake Forest University) and compared with published data base sequences (Genbank accession numbers U63970, X96395 and U49248). The modified MRP2 cDNA was inserted into pLNCX and transduced into MCF7 cells. Individual colonies were selected in 1 mg/ml G418 and one expressing high level membrane-associated MRP2, MCF7/MRP2-15, was used for transport studies.

Additionally, MCF7 cells expressing GSTA1-1 were generated. A 690 bp cDNA fragment encoding human GSTA1-1 from the Kozak consensus sequence through the stop codon was inserted into the *Cla*I site of pLHCX (Clontech). This vector was introduced by transduction into parental MCF7/WT and MCF7/MRP1-10 cells. Cells were selected in 0.4 mg/ml hygromycin. Colonies overexpressing GST activity were identified (Habig et al., 1974) and 2 colonies from each transduced cell line were chosen for cytoxicity experiments.

Analysis of transgenic cell lines. GST expression in HepG2 cells was examined by Western and Northern blotting as described previously (Morrow et al., 1998b) and by enzyme activity towards 1-chloro-2,4-dintrobenzene (Habig et al., 1974). GST levels were expressed as mU activity (nmol 2,4-dinitrophenyl glutathione formed/min•mg cellular protein). MRP1 and MRP2 expression was ascertained by Northern blot and Western blot analysis of membrane proteins as described (Hipfner et al., 1994) using mouse monoclonal antibodies QCRL1 ((Hipfner et al., 1994), MRP1) and M_2III-6 ((Kool et al., 1997), MRP2) purchased from Alexis Biochemicals (Carlsbad, CA).

Additionally, expression of MRP1 and MRP2 was examined by immunocytochemistry. Cells were fixed with 3.7 % formaldehyde in PBS (10 min, room temperature), washed with PBS, and blocked with 1 % BSA, 0.1 % saponin in PBS (10 min). After washing in PBS, cells were incubated for 30 min at room temperature with 1 % BSA, 0.1 % saponin in PBS containing the primary mouse monoclonal antibodies: QCRL3 (anti-MRP1, Alexis Biochemicals) at 1 µg/ml or M_2III-6 (anti-MRP2) at 10 µg/ml. PBS washed cells were then inclubated 30 minutes with secondary antibody, 25 µg/ml rhodamine-conjugated goat anti-mouse IgG (Immunotech, Hialeah, FL) in 1 %

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

BSA, 0.1 % saponin, PBS. Cells were post-fixed in 3.7 % formaldehyde in PBS. Phase contrast and fluorescent images were captured using an Axioplan 2 Zeiss microscope.

Cytotoxicity determinations. Cells were plated in 96 well plates at a density of 1200 cells per well (HepG2 and derivatives) or 300 cells per well (MCF7 and derivatives). Twenty-four hours later cells were treated with alkylating agents chlorambucil (Sigma, St. Louis, MO), melphalan (Sigma), or thiotepa (Drug Synthesis and Chemistry Branch, NCI, Bethesda, MD), or with vehicle control in serum free medium for one hour. Medium was replaced with DMEM plus 10 % fetal calf serum. Cells were fixed and stained using sulforhodamine B (Skehan et al., 1990). For some experiments, MRP2 activity was inhibited by the inclusion of 2 mM sulfinpyrazone (Sigma) in serum free medium added 15 min prior to alkylating drug exposure and continued for the 1 hour period of drug exposure as described previously (Morrow et al., 2000). In other experiments, GST activity was suppressed in transgenic HepG2 cells by prior growth in medium supplemented with 1 μg/ml doxocycline for 7-10 days. Doxocycline-suppressed cells were grown without doxocycline for 24 hours before plating and cytotoxicity experiments. This treatment completely suppressed GST expression (residual GST activity < 5 mU/min•mg cellular protein). Because the IC_{50} values towards the three alkylating agents were identical in the doxocycline-suppressed transduced cell lines and in parental HepG2 cells, doxocycline-suppressed and parental HepG2 cells were used interchangeably for minus GST controls.

MRP1- and MRP2-dependent transport of monoglutathionyl-chlorambucil (CHB-SG) in isolated membrane vesicles. Inside-out membrane vesicles were prepared from HepG2, MCF7/MRP1-10, and MCF7/MRP2-15 cells as described previously (Paumi et al., 2001). Uptake of ^{3}H-CHB-SG was determined and kinetic parameters calculated as described (Paumi et al., 2001). ATP-dependent transport was calculated by subtracting uptake observed in minus ATP controls containing 4 mM of the non-hydrolyzable analog, β,γ-methyleneadenosine 5'-triphosphate, from uptake observed in the presence of 4 mM ATP as described.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Cell lines. Expression of the three GST isozymes, A1-1, M1a-1a and P1a-1a, in HepG2 cells was examined by Northern (Fig. 1) and Western blots (Fig. 2). These cytosolic GST isozymes, undetectable in parental HepG2 cells, were observed only in the corresponding transgenic HepG2 derivative cell line. Northern blot and Western blot analysis of membrane-associated proteins (Fig. 3) showed that, as reported previously (Cantz et al., 2000; Morrow et al., 2000), HepG2 cells do not express significant levels of MRP1 (Fig. 3A) but do express high levels of MRP2 (Fig. 3B). Moreover, co-expression with GSTA1-1, GSTM1a-1a, or GSTP1a-1a had no effect on MRP2 expression (Fig. 3B). Culture of HepG2 cells in 1 μg/ml doxocycline to suppress transgenic GST activity had no effect on MRP2 expression (not shown).

Western blot analysis showed that high level expression of membrane-associated MRP1 or MRP2 was achieved in the transduced MCF7 derivatives, MCF7/MRP1-10 (Fig. 3A) or MCF7/MRP2-15 (Fig. 3C), respectively. The summary in table 1 describes all of the cell lines used in the following studies.

Verification that MRP1, expressed as a transgene, potentiates GSTA1-1-mediated resistance to CHB. Previous studies using etoposide-selected MCF7 cells overexpressing MRP1 (MCF7/VP cells) showed that co-expression of MRP1 and GSTA1-1, but not MRP1 or GST alone, conferred significant levels of resistance to CHB cytotoxicity (Morrow et al., 1998b). To eliminate any potential confounding, unrecognized alterations in gene expression that may have occurred during cytotoxic drug selection, we examined the role of MRP1 expressed as a transgene in GSTA1-1-associated resistance to CHB. As shown in table 2, expression of GSTA1-1 alone (MCF7/α7 and MCF7/α8) resulted in very little resistance to CHB (1.5-2.3 fold relative resistance). In contrast, co-expression of MRP1 and GSTA1-1 (MRP1-10/α2 and MRP1-10/α13) conferred considerable resistance to CHB cytotoxicity (5.8-7.1 fold relative resistance). These results confirm the importance of MRP1 in potentiation and augmentation of GSTA1-1-mediated resistance to CHB.

MRP2 supports CHB-SG transport and GSTA1-1-mediated resistance to CHB in HepG2 cells. MRP2 is the dominant MRP-family transporter expressed on the plasma membrane of HepG2 cells (Jedlitschky et al., 1997; Morrow et al., 2000). To determine if

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

MRP2 could support CHB-SG efflux, uptake of radiolabeled CHB-SG was examined in inside-out membrane vesicles prepared from HepG2 cells. Vesicles derived from HepG2 cells showed substantial ATP-dependent uptake of CHB-SG whereas MRP-minus control vesicles derived from MCF7/WT cells did not (Fig. 4). To evaluate whether this capacity of MRP2-mediated CHB-SG export present in HepG2 cells was sufficient to support GSTA1-1-dependent resistance to CHB, CHB cytotoxicity was examined in two independent HepG2 clones overexpressing GSTA1-1, HepG2/α-10 (70-110 mU GST/mg) and HepG2/α-25 (110-153 mU GST/mg). Compared with parental HepG2 cells (< 5 mU GST/mg), expression of GSTA1-1 in HepG2/α-10 and HepG2/α-25 cells conferred 3.5 and 4.5 fold resistance, respectively, to CHB cytotoxicity (Fig. 5 and table 3). That this resistance is dependent upon GSTA1-1 was confirmed by additional experiments in which GSTA1-1 expression was completely suppressed by culture in 1 μg/ml doxocycline. Suppression of GST expression completely reversed CHB resistance resulting in cytotoxicity profiles indistinguishable from those of parental HepG2 cells (not shown). Moreover, GST-mediated resistance was GSTA1-1 isozyme specific in that overexpression of GSTP1a-1a (HepG2/π-3) or GSTM1a-1a (HepG2/μ-2 and HepG2/μ-5) did not confer resistance to CHB (table 3).

The contribution of MRP2 to GSTA1-1-associated CHB resistance was evaluated by the use of sulfinpyrazone to inhibit MRP2. Previous studies showed that 2 mM sullfinpyrazone can inhibit MRP2 transport of glutathione conjugates and does so without significant toxicity (Morrow et al., 2000). As shown in Fig. 6, 2 mM sulfinpyrazone also inhibits MPR2-dependent transport of CHB-SG in isolated membrane vesicles. The inclusion of sulfinpyrazone just prior to and during the period of exposure to CHB had no effect on the sensitivity of parental HepG2 cells (Fig. 7A). However, sulfinpyrazone strongly sensitized GSTA1-1-expressing HepG2/α-10 and HepG2/α-25 cells yielding cytotoxicity profiles that were nearly identical to parental HepG2 control cells (Fig. 7B and C). These results indicate that CHB resistance in this system is dependent upon the simultaneous expression of both GST and MRP2 activities.

The ability of GST and MRP2 to influence sensitivities to other alkylating agents was examined. As shown in table 3, MRP2 expressed in HepG2 cells was unable to support cytoprotection from melphalan or thiotepa toxicities in cells expressing any of the

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

three GST isozymes tested. Thus resistance conferred by combined GST and MRP2 expression is also specific for the alkylating drug used.

Kinetics of MRP1- versus MRP2-dependent transport of CHB-SG. The relative efficacy of MRP1 and MRP2 transport of CHB-SG was compared in MCF7 cells transduced with MRP1 (MCF7/MRP1-10) and MRP2 (MCF7/MRP2-15). Immunocytochemistry revealed that the majority of MRP1 was correctly targeted to the plasma membrane (Fig. 8A, lower panel). Neither MRP1 nor MRP2 was detected in parental MCF7/WT cells (Fig. 8A and B, upper panels). MCF7/MRP2-15 cells were generated using an expression vector containing a C-terminal 3 amino acid truncation in an effort to promote plasma membrane targeting in these non-polarized cells (Harris et al., 2001). While much of the MRP2 was indeed found associated with the plasma membrane, considerable protein remained in cytoplasmic compartments despite this modification (Fig. 8B, lower panel). Nevertheless, inside-out vesicles derived from MCF7/MRP2-15 cells supported robust transport of CHB-SG as did vesicles from MCF7/MRP1-10, but not MCF7/WT, cells (Fig. 9). At the levels of protein expressed, MRP1 and MRP2 transported CHB-SG with similar V_{max} (82 and 99 nmol/min•mg, respectively) but with a significantly lower K_m for MRP1 (0.39 μM, MRP1; 10 μM, MRP2). This K_m for MRP1 is very close to the value previously determined (0.37 μM) for MRP1 expressed in the drug-selected MCF7/VP cell line (Paumi et al., 2001).

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The present study establishes that MRP2, like MRP1, can support GST-mediated detoxification of CHB. Either absence (or suppression) of transgenic GSTA1-1 or inhibition of MRP2 activity abolished the observed resistance to CHB (Figs. 5 and 7 and table 3). Thus, coordinated expression of GSTA1-1 and MRP2 activities are required to confer resistance to CHB in the model HepG2 cells used. The role of MRP2 in resistance is the consequence of robust ATP-dependent CHB-SG efflux activity (Figs. 4 and 9)—activity that is required to relieve the potent product inhibition of GSTA1-1 by CHB-SG (Paumi et al., 2001).

MRP2/GST detoxification is GST isozyme specific. GSTA1-1, but not GSTP1a-1a or GSTM1a-1a, can confer resistance to CHB in MRP2 expressing HepG2 cells (table 3). In contrast, no GST isozyme/MRP2 pair tested conferred resistance to thiotepa or melphalan—alkylating agents that can also form conjugates with glutathione (Dirven et al., 1995; Dulik and Fenselau, 1987; Dulik et al., 1986; Srivastava et al., 1999). Therefore, MRP2/GST resistance is drug selective. Differences in MRP2-mediated transport of thiotepa- or melphalan-glutathione conjugates may have contributed to the drug selectivity but were not examined in the present study. Alternatively, or additionally, the selectivity of drug resistance may be attributable to differences in the catalytic efficiency of GST-dependent conjugate formation or in the potency of conjugate product inhibition of GST—differences previously observed for GSTA1-1 and its substrates melphalan and CHB (Paumi et al., 2001).

In transgenic MCF7 cells, the apparent efficiency of CHB-SG transport (V_{max}/K_m) is ~25 fold greater for MRP1 than MRP2—a difference due to the higher K_m of MRP2 for CHB-SG (Fig. 9). Nevertheless, MRP2 effectively potentiates GSTA1-1 resistance in HepG2 cells. This suggests that the level of MRP2 in cells that naturally express the protein is more than sufficient to relieve CHB-SG product inhibition despite the relatively higher K_m of transport.

Harris et al. reported that the C-terminal 3 amino acids of MRP2 are required for proper targeting of MRP2 to the apical plasma membrane of polarized cells (Harris et al., 2001). While trafficking of MRP2 to the plasma membrane is generally poor in non-polarized cells, these investigators found that deletion of the C-terminal 3 amino acids

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

resulted in efficient expression of MRP2 on the plasma membrane of murine L1210 leukemia cells. We attempted to take advantage of this observation to target MRP2 to the plasma membrane of non-polarized MCF7 cells. Although significant MRP2 associated with the plasma membrane, considerable protein remained in the cytosolic compartments (Fig. 8). The failure to more efficiently target MRP2 to the plasma membrane may represent inherent differences in membrane protein trafficking between MCF7 and L1210 cells. Additionally, more recent findings by Mateus et al. indicate that the N-terminal transmembrane domain of MRP2, a region not modified in the MRP2 protein expressed in MCF7/MRP2-15 cells, is crucial for correct and stable targeting of MRP2 (Mateus Fernandez et al., 2002). It may be that modification in this N-terminal region would be required to efficiently target MRP2 to the plasma membrane of non-polarized cells.

In summary, the GSH conjugate efflux activity of MRP2 can support resistance to CHB mediated by GSTA1-1. Although the resistance observed was drug and GST isozyme specific, it is likely that a variety of drugs and other xenobiotics are similarly detoxified by various GST isozyme/MRP2 pairs (Morrow et al., 2000). Overexpression of MRP2 has been observed in some cancer cells (Konig et al., 1999) and may therefore play a role in the emergence of resistance to chemotherapeutic drugs including some alkylating agents. Moreover, high level MRP2 expression is observed on the apical, excretory surfaces of tissues such as the tubular epithelium of kidney and the canalicular hepatocytes of liver (Buchler et al., 1996; Schaub et al., 1997)—the latter representing a particularly rich source of several cytosolic isozymes of GST (Hayes and Pulford, 1995). Using the model cell lines developed in our laboratory, we have shown how the interplay between MRP2 and the GSH/GST conjugating systems can effect detoxification of reactive, electrophilic toxins including some alkylating agents and other xenobiotics.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors appreciate the expert assistance of Stephanie Jilcott in the construction of transgenic cell lines.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (1997) Current Protocols in Molecular Biology. John Wiley and Sons, New York.

Borst, P., R. Evers, M. Kool, and J. Wijnholds (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst*. 92:1295-1302.

Buchler, M., J. Konig, M. Brom, J. Kartenbeck, H. Spring, T. Horie, and D. Keppler (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J Biol Chem*. 271:15091-15098.

Cantz, T., A.T. Nies, M. Brom, A.F. Hofmann, and D. Keppler (2000) MRP2, a human conjugate export pump, is present and transports fluo 3 into apical vacuoles of Hep G2 cells. *Am.J.Physiol.Gastrointest.Liver Physiol.* 278:G522-G531.

DeJong, J.L., C.-M. Chang, J. Whang-Peng, T. Knudsen, and C.-P.D. Tu (1988) The human glutathione *S*-transferase gene superfamily: expression and chromosome mapping of an Hb subunit cDNA. *Nucl. Acids Res.* 16:8541-8554.

Dirven, H., E. Dictus, N. Broeders, O.B. Van, and B.P. Van (1995) The role of human glutathione S-transferase isoenzymes in the formation of glutathione conjugates of the alkylating cytostatic drug thiotepa. *Cancer Res*. 55:1701-1706.

Dulik, D.M., and C. Fenselau (1987) Conversion of Melphalan to 4-(glutathio-nyl)phenylalanine. A novel mechanism for conjugation by glutathione-S-transferases. *Drug Metab Dispos*. 15:195-198.

Dulik, D.M., C. Fenselau, and J. Hilton (1986) Characterization of melphalan-glutathione adducts whose formation is catalyzed by glutathione transferases. *Biochem. Pharmacol.* 35:3405-3409.

Evers, R., G.J.R. Zaman, L. van Deemter, H. Jansen, J. Calafat, L.C.J.M. Oomen, R.P.J.O. Elferink, P. Borst, and A.H. Schinkel (1996) Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *J. Clin. Invest.* 97:1211-1218.

Fields, W.R., Y. Li, and A.J. Townsend (1994) Protection by transfected glutathione *S*-transferase isozymes against carcinogen-induced alkylation of cellular macromolecules in MCF-7 cells. *Carcinogenesis*. 15:1155-1160.

Habig, W., M. Pabst, and W. Jakoby (1974) Glutathione S-transferase: the first enzymatic step in mercapturic acid formation. *J. Biol Chem*. 249:7130-7139.

Harris, M.J., M. Kuwano, M. Webb, and P.G. Board (2001) Identification of the Apical Membrane-targeting Signal of the Multidrug Resistance-associated Protein 2 (MRP2/cMOAT). *J Biol Chem.* 276:20876-20881.

Hayes, J.D., and D.J. Pulford (1995) The glutathione *S*-transferase supergene family: Regulation of GST and the contribution of the isozymes to cancer chemoprevention and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* 30:445-600.

Hipfner, D.R., S.D. Gauldie, R.G. Deeley, and S.P.C. Cole (1994) Detection of the Mr 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. *Cancer Res.* 54:5788-5792.

Ishikawa, T. (1992) The ATP-dependent glutathione S-conjugate export pump. *TIBS.* 17:463-468.

Jedlitschky, G., I. Leier, U. Buchholz, J. Hummel-Eisenbeiss, B. Burchell, and D. Keppler (1997) ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochem. J.* 327:305-310.

Keppler, D., I. Leier, G. Jedlitschky, and J. Konig (1998) ATP-dependent transport of glutathione S-conjugates by the multidrug resistance protein MRP1 and its apical isoform MRP2. *Chem Biol Interact*. 111-112:153-161.

Konig, J., A.T. Nies, Y. Cui, I. Leier, and D. Keppler (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta*. 6:377-394.

Kool, M., M. de Haas, G.L. Scheffer, R.J. Scheper, M.J. van Eijk, J.A. Juijn, F. Baas, and P. Borst (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res*. 57:3537-3547.

Leyland-Jones, B.R., A.J. Townsend, Tu, C.D., K.H. Cowan, and M.E. Goldsmith (1991) Antineoplastic drug sensitivity of human MCF-7 breast cancer cells stably transfected with a human alpha class glutathione S-transferase gene. *Cancer Res.* 51:587-594.

Loe, D.W., R.G. Deeley, and S.P. Cole (1996) Biology of the multidrug resistance-associated protein, MRP. *Eur J Cancer*. 32A:945-957.

Mateus Fernandez, S.B., Z. Hollo, A. Kern, E. Bakos, P.A. Fischer, P. Borst, and R. Evers (2002) Role of the N-terminal transmembrane region of the multidrug resistance protein MRP2 in routing to the apical membrane in MDCKII cells. *J. Biol. Chem.*:M204267200.

Mayer, R., J. Kartenbeck, M. Buchler, G. Jedlitschky, I. Leier, and D. Keppler (1995) Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. *J Cell Biol*. 131:137-150.

Meyer, D.J. (1993) Significance of an unusually low Km for glutathione in glutathione transferases of the a, m, and π classes. *Xenobiotica*. 23:823-834.

Meyer, D.J., K.S. Gilmore, J.M. Harris, J.A. Hartley, and B. Ketterer (1992) Chlorambucil-monoglutathionyl conjugate is sequestered by human alpha class glutathione *S*-transferases. *Br. J. Cancer*. 66:433-438.

Miller, A.D., D.G. Miller, J.V. Garcia, and C.M. Lynch (1993) Use of retroviral vectors for gene transfer and expression. *Meth. Enz.* 217:581-599.

Morrow, C.S., S. Diah, P.K. Smitherman, E. Schneider, and A.J. Townsend (1998a) Multidrug resistance protein and glutathione S-transferase P1-1 act in synergy to confer protection from 4-nitroquinoline 1-oxide toxicity. *Carcinogenesis*. 19:109-115.

Morrow, C.S., P.K. Smitherman, S.K. Diah, E. Schneider, and A.J. Townsend (1998b) Coordinated action of glutathione S-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1- and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells. *J Biol Chem.* 273:20114-20120.

Morrow, C.S., P.K. Smitherman, and A.J. Townsend (1998c) Combined expression of multidrug resistance protein (MRP) and glutathione S-transferase P1-1 (GSTP1-1) in MCF7 cells and high level resistance to the cytotoxicities of ethacrynic acid but not oxazaphosphorines or cisplatin. *Biochem Pharmacol*. 56:1013-1021.

Morrow, C.S., P.K. Smitherman, and A.J. Townsend (2000) Role of multidrug-resistance protein 2 in glutathione S-transferase P1-1-mediated resistance to 4-nitroquinoline 1-oxide toxicities in HepG2 cells. *Mol Carcinog*. 29:170-178.

Moscow, J.A., A.J. Townsend, and K.H. Cowan (1989) Elevation of the π class glutathione S-transferase activity in human breast cancer cells by transfection of the GSTπ gene and its effect on sensitivity to toxins. *Mol. Pharmacol.* 36:22.

Paumi, C.M., B.G. Ledford, P.K. Smitherman, A.J. Townsend, and C.S. Morrow (2001) 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. *J. Biol. Chem.* 276:7952-7956.

Paumi, C.M., M. Wright, A.J. Townsend, and C.S. Morrow (2003) Multidrug Resistance Protein (MRP) 1 and MRP3 Attenuate Cytotoxic and Transactivating Effects of the Cyclopentenone Prostaglandin, 15-Deoxy-Delta(12,14)Prostaglandin J(2) in MCF7 Breast Cancer Cells. *Biochemistry*. 42:5429-5437.

Rhoads, D., R.P. Zarlengo, and C.D. Tu (1987) The basic glutathione *S*-transferases from human livers are products of separate genes. *Biochem. Biophys. Res. Commun.* 145:474-481.

Schaub, T.P., J. Kartenbeck, J. Konig, O. Vogel, R. Witzgall, W. Kriz, and D. Keppler (1997) Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules. *J Am Soc Nephrol*. 8:1213-1221.

Skehan, P., R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, and M.R. Boyd (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82:1107-1112.

Srivastava, S.K., S.S. Singhal, X. Hu, Y.C. Awasthi, P. Zimniak, and S.V. Singh (1999) Differential catalytic efficiency of allelic variants of human glutathione *S*-transferase Pi in catalyzing the glutathione conjugation of thiotepa. *Arch.Biochem.Biophys.* 366:89-94.

Townsend, A.J., C.P. Tu, and K.H. Cowan (1992) Expression of human mu or alpha class glutathione S-transferases in stably transfected human MCF-7 breast cancer cells: effect on cellular sensitivity to cytotoxic agents. *Mol. Pharmacol.* 41:230-236.

Tu, C.-P.D., and B. Qian (1986) Human liver glutathione *S*-transferases: complete primary sequence of a Ha subunit. *Biochem. Biophys. Res. Commun.* 141:229-237.

Van Bladeren, P.J. (2000) Glutathione conjugation as a bioactivation reaction. *Chem.Biol.Interact.* 129:61-76.

Witz, G. (1989) Biological interactions of alpha,beta-unsaturated aldehydes. *Free Radic Biol Med*. 7:333-349.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

[*] This work was supported by NIH grant CA70338.

Address all correspondence and reprint requests to: Charles S. Morrow, Department of Biochemistry, Medical Center Boulevard, Wake Forest University School of Medicine, Winston-Salem, NC 27157

[1] Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC.

[2] Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Fig. 1. Northern blot analysis of GST isozymes expressed in transgenic HepG2 cells.

Whole cellular RNA (10 μg) was examined by Northern blotting. The blot was hybridized, stripped and re-hybridized to cDNA fragments specific for the human GSTA1 (top panel), GSTM1 (second panel), or GSTP1 (third panel) mRNAs. The bottom panel is an ethidium bromide stain of the electrophoretically separated RNA samples prior to transfer. RNA samples are derived from the following parental and transgenic HepG2 cell lines: lane 1, parental HepG2; lane 2, HepG2/α-10; lane 3, HepG2/α-25; lane 4, HepG2/μ-2; lane 5, HepG2/μ-5; lane 6, HepG2/RTO-21; lane 7, HepG2/π-3.

Fig. 2. Western blot analysis of GST isozymes expressed in HepG2 derivatives.

Total cellular protein (50 μg) was analyzed by Western blot. Following SDS polyacrylamide gel electrophoresis and transfer, replicate samples were examined using polyclonal antibodies specific for alpha (top panel), mu (middle panel), and pi (lower panel) class GST. Lanes contained protein from HepG2 derivatives as follows: lane 1, parental HepG2; lane 2, HepG2/α-10; lane 3, HepG2/α-25; lane 4, HepG2/μ-2; lane 5, HepG2/μ-5; lane 6, HepG2/π-3. Bars on the left correspond to 29kDa (upper) and 21.5 kDa (lower) molecular weight standards.

Fig. 3. Expression of MRP1 and MRP2 in parental and transgenic MCF7 and HepG2 cells.

Shown are the expression levels of MRP1 (A) or MRP2 (B and C) in the indicated cell lines. Western blot analyses using 50 μg membrane-associated protein are shown in the upper panels. The position of the 217 kDa marker is shown. Results shown are representative of Western blots done using at least two independent protein preparations from each cell line. Northern blot analyses using 10 μg whole cell RNA are shown in the lower panels. The positions of 28S and 18S RNA are indicated. Ethidium bromide staining of the gel prior to transfer revealed similar loading in each of the lanes (not shown).

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4. MRP2-mediated ATP-dependent uptake of CHB-SG by inside-out vesicles derived from HepG2 cells.

Shown is the ATP-dependent transport of 10 μM CHB-SG by inside-out vesicles derived from HepG2 (MRP2 plus) and MCF7WT (MRP minus) cells. Points are the means of at least three determinations and error bars represent ± 1 SD.

Fig. 5. Expression of GSTA1-1 confers resistance to CHB cytotoxicity in MRP2-containing HepG2 cells.

Shown are cytotoxicity profiles of parental HepG2 cells (open circles) and transgenic derivatives expressing GSTA1-1 (open squares, HepG2/α-10; open diamonds, HepG2/α-25). Indicated are mean values from 8 replicate determinations ± 1 SD.

Fig 6. Sulfinpyrazone inhibits MRP2-dependent transport of CHB-SG.

Shown is ATP-dependent transport of 0.5 μM CHB-SG by inside-out vesicles derived from MCF7 cells stably transduced with MRP2 (MCF7/MRP2-15) in the absence (open circles) or presence (closed circles) of 2 mM sulfinpyrazone. Vesicles were pretreated with 2 mM sulfinpyrazone or vehicle control at 37^{o}C for 15 min prior to the addition of ^{3}H-CHB-SG substrate. Points are the means of triplicate determinations ± 1 SD.

Fig. 7. Inhibition of MRP2 by sulfinpyrazone reverses GSTA1-1-associated resistance to CHB.

CHB cytotoxicity was determined in cells treated with 2 mM sulfinpyrazone (closed symbols) or vehicle control (open symbols) as described in Methods. Panel A shows the effect of sulfinpyrazone on CHB cytotoxicity in parental HepG2 cells (GST minus). Panels B and C show the sensitization of HepG2 derivatives expressing GSTA1-1 by sulfinpyrazone: panel B, HepG2/α-10 cells (squares); panel C, HepG2/α-25 cells (diamonds). For comparison, the cytotoxicity profile of parental HepG2 cells minus sulfinpyrazone is reproduced in A, B and C (open circles). Points are the mean values from 8 determinations ± 1 SD.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

These experiments were done simultaneously with those shown in Fig. 5. Data represented by open symbols (minus sulfinpyrazone controls) are identical to data shown in Fig. 5 and are reproduced in these separate panels for comparison and clarity.

Fig. 8. Immunocytochemistry of transgenic MCF7 cells expressing MRP1 and MRP2.

Parental (MCF7/WT) and cells transduced with expression vectors encoding MRP1 (MCF7/MRP1-10) or MRP2 (MCF7/MRP2-15) were prepared for immunocytochemistry as described in Methods. Cells were incubated with mouse anti-MRP1 (panel A) or anti-MRP2 (panel B) monoclonal antibodies. Fluorescent labeling was accomplished using a rhodamine-conjugated goat anti-mouse IgG. Phase contrast images are shown on the left and fluorescent images on the right.

Fig. 9. Kinetics of CHB-SG transport by MRP1 versus MRP2.

Shown are initial velocities of ATP-dependent CHB-SG uptake by inside-out membrane vesicles prepared from parental MCF7/WT cells (closed triangles) and MCF7 cells transduced with MRP1 (MCF7/MRP1-10, closed circles) or MRP2 (MCF7/MRP2-15, open circles) expression vectors. Represented are the mean values from ≥ 3 determinations and one standard deviation from the mean.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Cell lines.

Cell Line	Description	MRP	GST
HepG2	parental	MRP2 (endogenous)	–
HepG2/RTO-21	transduced doxocycline-repressible transactivator	MRP2	–
HepG2/α-10 HepG2/α-25	transduced GST (doxocycline-repressible)	MRP2	GSTA1-1
HepG2/μ-2 HepG2/μ-5	transduced GST (doxocycline-repressible)	MRP2	GSTM1a-1a
HepG2/π-3	transfected GST (doxocycline-repressible)	MRP2	GSTP1a-1a
MCF7/WT	parental	–	–
MCF7/α7 MCF7/α8	transduced GST	–	GSTA1-1
MCF7/MRP1-10	transduced MRP1	MRP1	–
MRP1-10/α2 MRP1-10/α13	MCF7/MRP1-10 transduced with GST	MRP1	GSTA1-1
MCF7/2-15	transduced MRP2	MRP2	–

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. MRP1 expressed as a transgene potentiates and augments GSTA1-1-mediated resistance to CHB.

Cell Line	GST[a]	Relative Resistance[b]
MCF7/WT	< 5	1.0
MCF7/α7	204 ± 17	2.3 ± 0.3
MCF7/α8	124 ± 13	1.5 ± 0.2
MCF7/MRP1-10	< 5	1.0
MRP1-10/α2	276 ±23	5.8 ± 1.0
MRP1-10/α13	310 ± 38	7.1 ± 0.9

[a] GST activity is expressed in mU/mg cellular protein (nmol 1-chloro-2,4-dinitrobenzene conjugated/min•mg). Shown are mean values from ≥ 5 determinations ± 1 SEM.

[b] Relative resistance to CHB cytotoxicity was defined and determined as follows. For MCF7/WT, MCF7/α7, and MCF7/α8 cells, relative resistance is defined as IC_{50} ÷ IC_{50} MCF7/WT. For MCF7/MRP1-10, MRP1-10/α2, and MRP1-10/α13, relative resistance is calculated as IC_{50} ÷ IC_{50} MCF7/MRP1-10. Values shown are means from ≥ 5 determinations ± 1 SEM.

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.

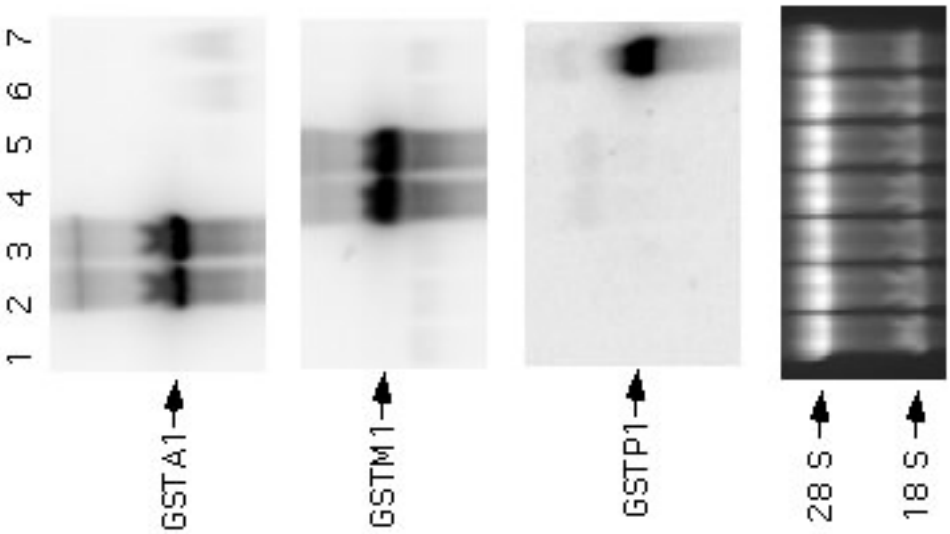
Table 3. MRP2-associated resistance to alkylating agents is drug and GST isozyme specific.

	GST[a]		Relative resistance[b]		
Cell Line	- dox	+dox	chlorambucil	melphalan	thiotepa
HepG2	<5	<5	1.0	1.0	1.0
HepG2/α-10	70-110	<5	**3.5**	1.0	1.0
HepG2/α-25	110-153	<5	**4.5**	1.0	1.0
HepG2/π-3	220-250	<5	1.1	0.95	0.93
HepG2/μ-2	303	<5	0.76	0.93	0.71
HepG2/μ-5	229	<5	0.94	0.89	0.71

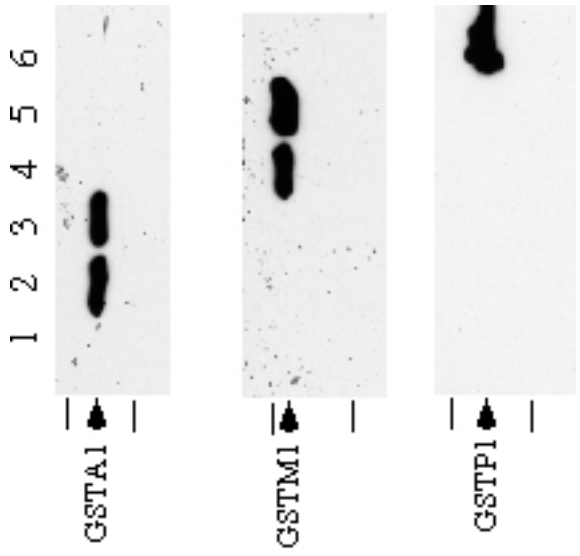
[a] GST activity is expressed in mU/mg cellular protein (nmol 1-chloro-2,4-dinitrobenzene conjugated/min•mg). GST activity was measured in cytosolic extracts obtained from cells grown in the absence (- dox) or presence (+ dox) of 1 μg/ml doxocycline.

[b] Relative resistance is defined as IC_{50} of the derivative cell line ÷ IC_{50} of parental HepG2 cells.

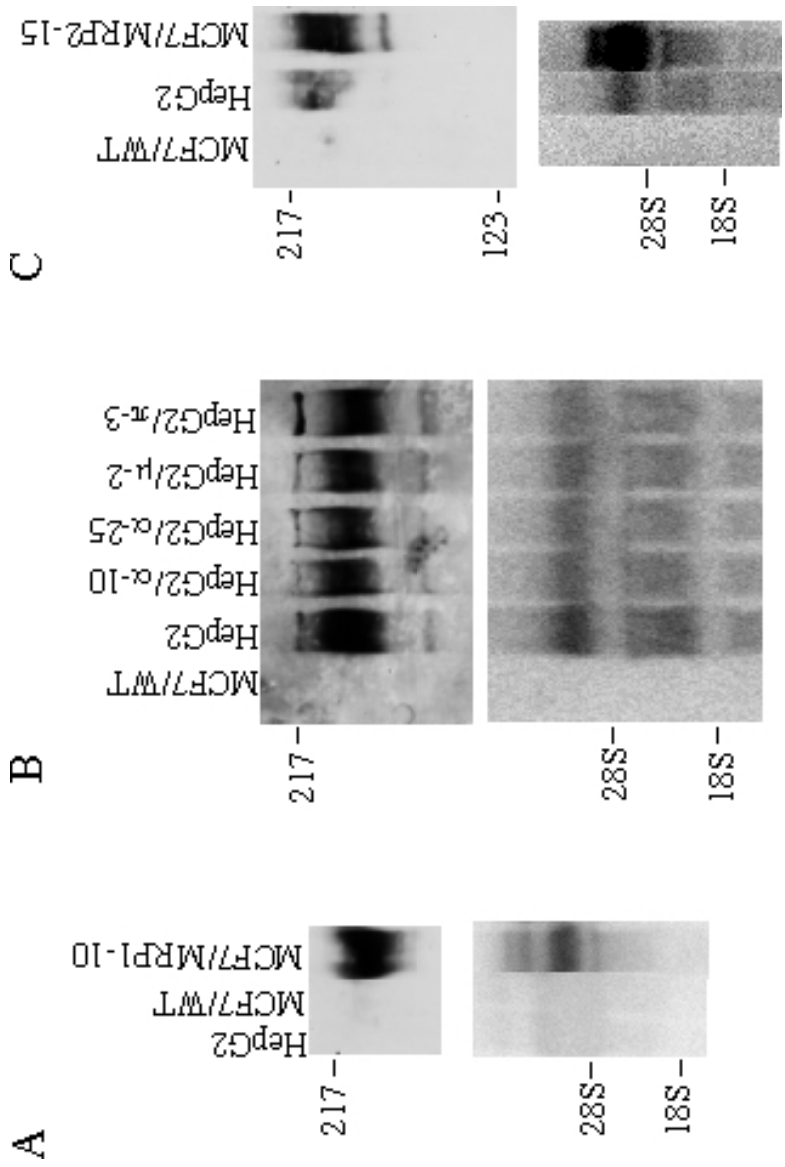
JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729



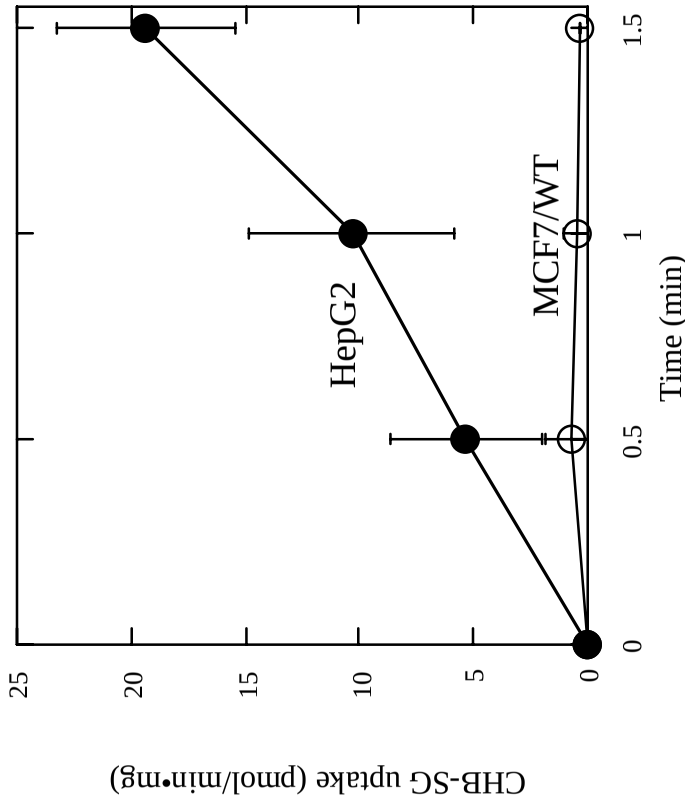
JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729



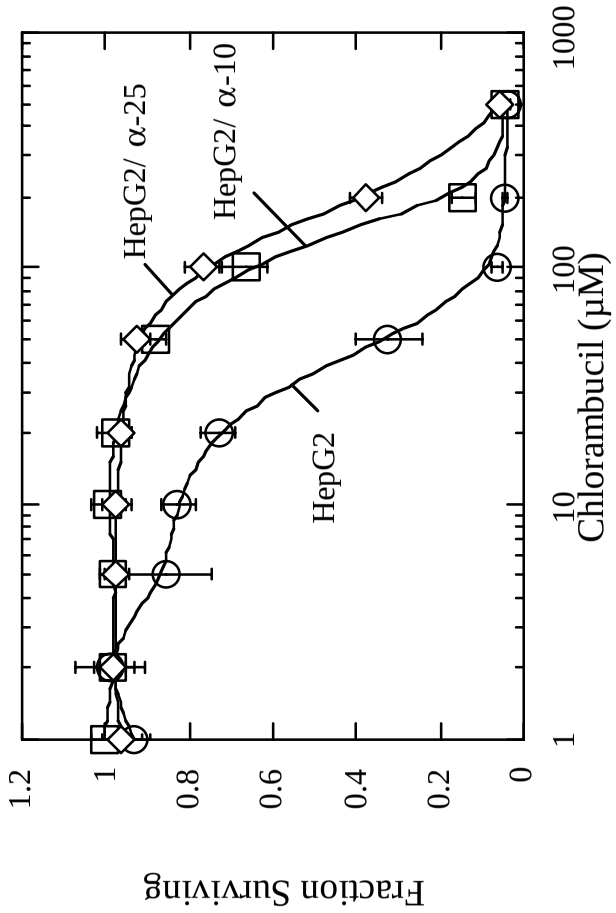
JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729



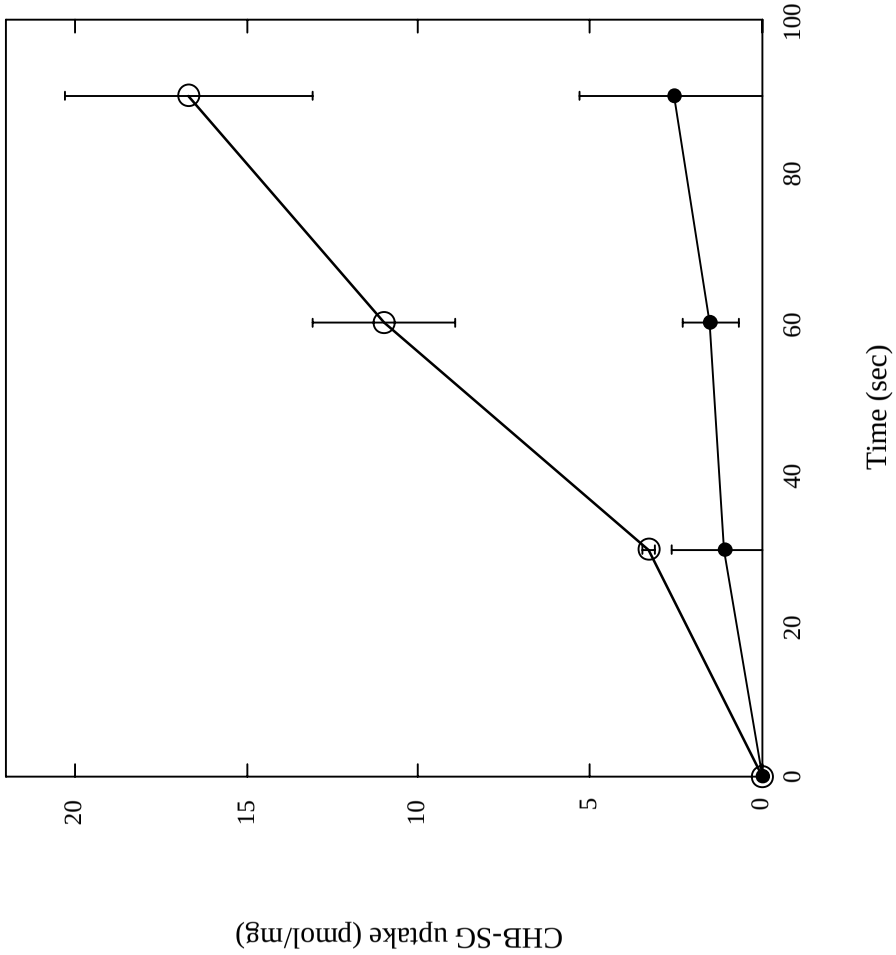
JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.



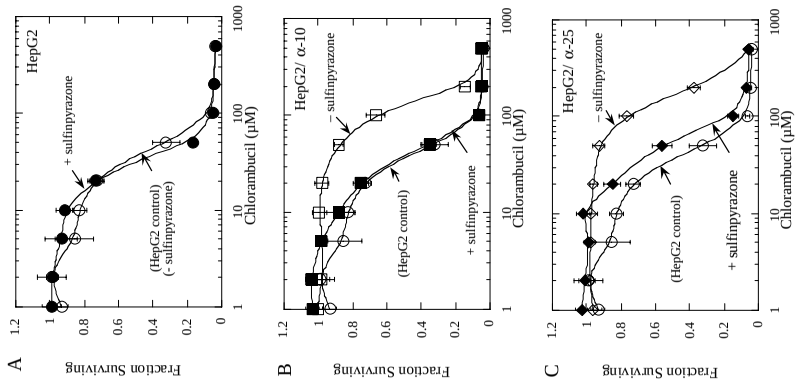
JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.



JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.



JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.



A

MCF7/WT

MCF7/MRP1-10

B

MCF7/WT

MCF7/MRP2-15

JPET Fast Forward. Published on October 20, 2003 as DOI: 10.1124/jpet.103.057729
This article has not been copyedited and formatted. The final version may differ from this version.