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Enhanced glutathione depletion, protein adduct formation, and cytotoxicity following exposure to 4-hydroxy-2-nonenal (HNE) in cells expressing human multidrug resistance protein-1 (MRP1) together with human glutathione S-transferase-M1 (GSTM1)

Lisa P. Rudd, Sandra L. Kabler, Charles S. Morrow, and Alan J. Townsend

Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC

Abstract

4-hydroxy-2-nonenal (HNE) is one of the most reactive products of lipid peroxidation and has both cytotoxic and genotoxic effects in cells. Several enzymatic pathways have been reported to detoxify HNE, including conjugation by glutathione-S-transferases (GSTs). Removal of the resulting HNE-glutathione conjugate (HNE-SG) by an efflux transporter may be required for complete detoxification. We investigated the effect of expression of GSTM1 and/or the ABC efflux transporter protein, multidrug-resistance protein-1 (MRP1), on HNE-induced cellular toxicity. Stably transfected MCF7 cell lines were used to examine the effect of GSTM1 and/or MRP1 expression on HNE-induced cytotoxicity, GSH depletion, and HNE-protein adduct formation. Co-expression in the MCF7 cell line of GSTM1 with MRP1 resulted in a 2.3-fold sensitization to HNE cytotoxicity (0.44-fold IC_{50} value relative to control) rather than the expected protection. Expression of either GSTM1 or MRP1 alone also resulted in slight sensitization to HNE cytotoxicity (0.79-fold and 0.71-fold decreases in IC_{50} values, respectively). Co-expression of GSTM1 and MRP1 strongly enhanced the formation of HNE-protein adducts relative to the non-expressing control cell line, whereas expression of either MRP1 alone or GSTM1 alone yielded similarly low levels of HNE-protein adducts to that of the control cell line. Glutathione (GSH) levels were reduced by 10–20% in either the control cell line or the MCF7/GSTM1 cell line with the same HNE exposure for 60 minutes. However, HNE induced > 80% depletion of GSH in cells expressing MRP1 alone. Co-expression of both MRP1 and GSTM1 caused slightly greater GSH depletion, consistent with the greater protein adduct formation and cytotoxicity in this cell line. Since expression of GSTM1 or MRP1 alone did not strongly sensitize cells to HNE, or result in greater HNE-protein adducts than in the control cell line, these results indicate that MRP1 and GSTM1 collaborate to enhance HNE-protein adduct formation and HNE cytotoxicity, facilitated by GSH depletion mediated by both MRP1 and GSTM1.

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Corresponding author: Dr. Alan J. Townsend, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, N.C. 27157, Tel: (336) 713-7215; Fax: (336) 716-7671, atown@wfubmc.edu.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

glutathione S-transferase; multidrug resistance protein; glutathione depletion; 4-hydroxynonenal; protein adducts; cytotoxicity; lipid peroxidation

1. INTRODUCTION

Oxidative damage to cellular macromolecules is known to constitute a major component of a wide range of disease processes, such as inflammation, atherosclerosis, cardiac and neural ischemia-reperfusion injury, and neurodegenerative diseases [1]. Lipid peroxidation (LPO) is a free radical-mediated chain reaction whereby polyunsaturated fatty acids are degraded via oxidation in solution or in biological membranes to form reactive and toxic saturated and α,β -unsaturated aldehydes [2–4]. One of the most prevalent, highly reactive and cytotoxic aldehydes produced from this process is 4-hydroxy-2-nonenal (HNE) [5]. The C2=C3 double bond of HNE forms an electrophilic Michael addition site that readily undergoes nucleophilic attack by nitrogen in nucleic acids or by nitrogen or sulfur in proteins to form covalent adducts. The reaction with nucleic acids can result in mutagenic etheno-purine exocyclic adducts, while the most common type of HNE-protein adduct formed is through the addition of cysteine sulfhydryl groups, and to a lesser extent lysine and histidine nitrogen residues to the 3-carbon of HNE, resulting in a Michael addition product that may undergo additional reactions. The terminal (C1) aldehyde can also react with amino groups, resulting in Schiff base products, and thus HNE can induce peptide cross-linking in addition to the Michael adduct [6, 7].

The toxicity of HNE and other LPO products has resulted in adaptive evolution of metabolic routes for the biotransformation of LPO products to less reactive and less toxic species [7–9]. One important pathway of detoxification is through conjugation with glutathione (GSH), catalyzed by glutathione-S-transferases (GSTs) [10]. These ubiquitous conjugases can detoxify a wide range of electrophilic compounds, including cytotoxic drugs, carcinogens, and α,β -unsaturated lipid aldehydes, via conjugation with the thiol group of GSH. However, expression of GST alone may not be sufficient to provide protection against cytotoxicity of some of these compounds [11, 12]. Reasons for this limitation may include product inhibition of the GST by the conjugated product [13], possible reversibility of conjugation [14], and less commonly, increased toxicity of the conjugate relative to the parent compound [15]. Further, the conjugated HNE-SG product undergoes cyclization to form a thiohemiacetal ring structure (95%) that is in tautomeric equilibrium via reversible ring opening to yield an open-chain conjugate (5%), which regenerates the free aldehyde functional group that can react with cellular protein amines via Schiff base chemistry [16, 17]. Accumulation of HNE-SG conjugates has been reported to cause product inhibition of GSTs which would limit further detoxification of HNE [18]. In addition to potential conjugate toxicity, excessive formation of HNE-SG may cause toxicity due to depletion of the cellular glutathione pool and attenuation of GSH functions as a cellular conjugating and reducing agent to convert reactive electrophiles to less toxic species. Hence, while removal of HNE-SG by an efflux transporter may be necessary to enable efficient detoxification of HNE via conjugation by GST, this cooperative interaction could also enhance the rate of GSH depletion, potentially resulting in toxicity by oxidative or other mechanisms.

The multidrug resistance-associated proteins (MRPs) are a family of ATP-binding cassette (ABC)-containing cellular transporters that function in the ATP-dependent transmembrane efflux of organic anions and a variety of endo- and xenobiotics, alone or as glutathione, glucuronate, or sulfate conjugates [19]. The MRPs play a critical role in detoxification via export of toxic or inhibitory compounds from the cell. A synergistic interaction between

GST enzymes and MRP1 or MRP2 was reported by our group that demonstrated resistance to the chemotherapeutic agent chlorambucil due to detoxification by heterologously expressed GSTA1, but only if MRP1 or MRP2 was also co-expressed [20, 21]. In another study, a requirement for MRP1 co-expression to enable protection by GSTP1 against 4-nitroquinoline-1-oxide (NQO) toxicity in MCF7 cells was reported. There was no protection against NQO cytotoxicity in the cell line expressing only GSTP1, and 4-fold resistance with only MRP1 expression, while the cell line expressing both MRP1 and GSTP1 $\pm$ MRP1 exhibited a 20-fold resistance to NQO [11, 12].

Evidence for a significant role of MRP1 and MRP2 as transmembrane efflux transporters for HNE-SG conjugates has been reported [22, 23]. However, while expression of MRP1 was associated with protection against HNE toxicity in a human small cell lung cancer cell line, GLC₄ [23], expression of stably transfected MRP2 in MDCK cells unexpectedly exacerbated HNE toxicity [24]. In contrast, expression via stable transfection of hGSTA4, which has high activity for HNE conjugation, conferred protection against HNE in human HepG2 hepatoma cells [25], which express MRP2 constitutively. The combined role of GSTs with the MRP1 transporter has not been directly addressed with lipid aldehydes, such as HNE, as substrates. Since HNE is effectively conjugated with GSH by GST isozymes of the alpha and mu classes [26], we used a cell line model derived by stable transfection of MCF7 human breast carcinoma cells to investigate the effect of expression of human GSTM1 and MRP1, either alone or combined, in the detoxification of HNE. Although most cell lines and tissues express one or more GST isozymes [27], there are some cell lines that express little or no GST activity or protein, including MCF7, T47D, and HepG2 [21, 28–30]. The MCF7 cell line was chosen since this cell line expresses no detectable endogenous activity of either GSTM1 or MRP1 [12, 20, 31]. This also mimics the polymorphic expression demographics in humans, in which GSTM1 is deleted in about half of the population [32]. Sensitivity to HNE-induced cytotoxicity was assessed, and GSH depletion and HNE-protein adduct formation were examined as potential components of the molecular mechanisms underlying the differential effects of exposure to HNE.

2. MATERIALS AND METHODS

2.1 Reagents

All chemicals were of analytical grade and were obtained from Fisher (Atlanta, GA) or Sigma (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) and Advanced Dulbecco's Modified Eagle's Medium (Advanced DMEM) were purchased from Gibco/BRL, Grand Island, NY. 4-hydroxy-2-nonenal (HNE) was purchased from Cayman (Ann Arbor, MI). Rabbit antibody directed against HNE-protein Michael adducts was purchased from Calbiochem (San Diego, CA). Mouse β -actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit HRP conjugate secondary antibody was purchased from Bio-Rad (Hercules, CA). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL).

2.2 Cell lines and Cell Culture

Cell lines were grown in DMEM with 10% serum in a 5% CO₂ atmosphere at 37° C. Transduced MCF7 human breast carcinoma cell lines genetically modified to express human MRP1, or hGSTM1, or both were derived by stable transduction, using retroviral expression vectors as previously described [30]. The parental MCF7 and empty vector-transduced control cell lines have been characterized by activity assays and western blot to have no detectable endogenous expression of MRP1 and GST activity [30]. This low background expression is ideal for comparing toxicity endpoints in the presence or absence of MRP1 and/or GSTM1. The MCF7/GSTM1 and MCF7/GSTM1/MRP1 were previously

characterized by western blot and activity assay and shown to express similar levels of GSTM1, without or with MRP1, respectively [30]. Expression of MRP1 was previously shown to be equal in the MCF7/MRP1 and the MCF7/GTM1/MRP1 cell lines as compared by western blot analysis [30]. The MCF7/pLNCX and MCF7/MRP1 lines were selected with 1.5 mg/ml G418. The MCF7/GSTM1 line was selected with 0.2 mg/mL hygromycin and MCF7/GSTM1/MRP1 was selected with 1.5 mg/ml G418 and 0.2 mg/ml hygromycin. Cells were maintained continuously under G418 and/or hygromycin selection, except prior to plating experiments they were removed from selection for one passage (~2 days).

2.3 GST enzyme assay

GST activity in cell lysates was measured with minor modifications as described previously [33, 34]. Cell pellets were collected by scraping into cold phosphate buffered saline (PBS) + 5 mM EDTA and centrifuged at $1,000 \times g$ for 5 min at 4 °C. The cell pellet was lysed in 50 mM Tris + 5 mM EDTA by sonication (Fisher sonic dismembrator) at a setting of 35 % power, for 10 seconds. The lysate was centrifuged at $12,000 \times g$ for 5 min. GST activity was measured at room temperature using 10–20 μ L cell lysate in a reaction buffer composed of 1 mM glutathione (GSH) in 0.1 mM K_2PO_4 , pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in absorbance at 340 nm was monitored for a total of 90 sec at 15 sec intervals. Activity was calculated based on the $\Delta A/\text{min}$ and extinction coefficient $[9.6 \text{ (mmol/L)}^{-1}\text{cm}^{-1}]$, and background activity was corrected for by subtraction of a reagent blank. Total protein concentration in the cell lysate was determined using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Enzyme activities were expressed as nmol/min/mg protein.

2.4 Cytotoxicity determination

MCF7-derived cell lines were plated in 96-well plates at a density of 400 cells/well in DMEM with 10 % FBS. After attachment for 18 – 24 hours, cells were exposed to various concentrations of HNE or solvent (ethanol, 0.1 % final concentration) in controls for 2 hours in serum-free medium. After dosing, the serum-free medium was removed and replaced with medium containing 10% serum. Cells were allowed to incubate for 5 days, then stained with 0.4 % sulforhodamine B in 1 % glacial acetic acid, washed, and solubilized in 10 mM Tris base solution (no pH adjustment) as previously described [35]. The IC_{50} value (concentration of HNE that reduced cell density by 50%) was determined from the measured absorbance of triplicate wells at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The cytotoxicity values are expressed as fold-difference relative to the control cell line [$\text{transfectant cell line } IC_{50} \text{ value} / \text{control cell line } IC_{50} \text{ value}$].

2.5 Immunoblot determination of HNE-protein adduct

HNE modification of cellular proteins was determined by immunoblot. Briefly, cells were plated at a density of $1.5 \times 10^6/100 \text{ mm dish}$ 18 – 24 hours prior to treatment with HNE. Following exposure to HNE for 2 hours in serum-free medium, cells were rinsed with serum-free medium and scraped into cold phosphate-buffered saline (PBS) + 5 mM EDTA and centrifuged at $1,000 \times g$ for 5 min. The cell pellet was lysed in 150 μ L of 50 mM HEPES/150 mM NaCl, 1 % Igepal pH 7.5 with 1 $\times$ Halt Protease Cocktail (Thermo Scientific, Rockford, IL), by sonication with a Fisher sonic dismembrator at 35 % power setting for 10 seconds. The lysate was centrifuged at $12,000 \times g$ for 5 min and an aliquot of the supernatant was removed to be assayed for total protein concentration using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. The remaining supernatant was immediately chemically reduced by addition of sodium borohydride (Sigma) to 100 mM and incubation for 30 min at room temperature, in order to stabilize Michael protein adducts formed as a result of HNE exposure. The HNE-adducted proteins were separated on a 10 % SDS/PAGE gel, transferred to PVDF membrane

(Millipore, Bedford, CA) and probed with antibody directed against reduced HNE-protein Michael adducts (Calbiochem, San Diego, CA). Following blocking in 5% nonfat milk, the primary antibody was used at a dilution of (1:4,000) in 1 % nonfat milk and incubated overnight at 4° C. After several washes in PBS, blots were probed with goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) at a dilution of (1:10,000) in 1 % non-fat milk and incubated for 2 hrs at room temperature. Immunoreactive proteins were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) with either film exposure or imaging on a FujiFilm LAS-3000 Imaging/Gel documentation system. Protein loading equivalency was determined by stripping the blot with 0.2 M glycine pH 2.0 and reprobing with β -actin antibody. For protein-adduct quantification, MultiGauge Version 3.0 (FujiFilm Corp.) was used.

2.6 GSH depletion

Cells were plated at a density of 1×10^6 /100 mm dish 48 hours prior to treatment with HNE. A time course study of GSH depletion was performed at 30 μ M and 60 μ M HNE for exposure times up to 1 hour. HPLC analysis of GSH levels in treated cells was performed as previously described [36]. Following HNE treatment, cells were harvested by scraping into 5 % perchloric acid (wt/vol), containing 0.2 M boric acid and an internal standard, 10 μ M γ -glutamyl-glutamate. The samples were then centrifuged at $14,000 \times g$ for 2 min to separate precipitated proteins from the acid-soluble sample supernatant. The supernatant was transferred and treated with iodoacetic acid and subsequently derivatized with dansyl chloride. The derivatized samples were separated by HPLC on a Supelcosil LC-NH2 column, 4.6×25 cm, (Supelco, Bellefonte, PA) using a Shimadzu modular HPLC system and a Shimadzu RF-10A fluorescence detector, set to an excitation wavelength of 335 nm and an emission wavelength of 550 nm. GSH levels were quantified based on the ratio of integrated peak area to internal standard area relative to a standard curve, and expressed as nmol GSH/mg protein. Cells were plated in a separate plate for analysis of cellular protein using the BCA protein assay (Pierce, Rockford, IL) with BSA as a standard. Alternatively, the protein pellet from each sample was resuspended in 500 μ L of 1 M NaOH, neutralized and analyzed for protein content by the Bradford method with BSA as a standard.

2.7 Statistical Analysis

Data analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Cytotoxicity and GSH depletion data are expressed as the mean $\pm$ standard deviation (SD) of three or more independent experiments. Statistical differences were evaluated by ANOVA and Tukey's post-hoc test to evaluate statistical significance. P values < 0.05 were considered significant.

3. RESULTS

3.1 GST enzyme activity in transfected cell lines

GST enzyme activities in transfected cell lines were measured to ensure that the GSTM1 transfected cell line had adequate GST expression and that background GST levels were low in non-expressing cell lines. The MCF7 empty vector-transfected control cell line, as well as the MCF7/MRP1 cell line, had low GST activities (< 5 nmol/min/mg). The two GST expressing cell lines both expressed moderate levels of GST activity. There was no significant difference ($p > 0.05$) in activities between the MCF7/GSTM1 (216 nmol/min/mg) and MCF7/GSTM1/MRP1 (230 nmol/min/mg) cell lines (Table 1). Thus, stable and closely matched GST expression was confirmed with the cell lines transfected with GSTM1 alone or together with MRP1, and background activity was low in the control cell lines.

3.2 Cytotoxicity of HNE

The HNE cytotoxicity dose-response was compared in the MCF7 control and GSTM1 and/or MRP1 transfected cell lines. Moderate sensitization was observed for HNE cytotoxicity in the GST-only expressing cell line (MCF7/GSTM1), which had a mean IC_{50} value of $15.9 \pm 1.4 \mu\text{M}$, a decrease of 0.79-fold in the IC_{50} value relative to the control cell line, which had an IC_{50} value of $20.0 \pm 1.5 \mu\text{M}$ (Table 2; $p < 0.01$). A similarly moderate sensitization was observed for the cell line expressing only MRP1 (MCF7/MRP1) with a mean IC_{50} value of $14.4 \pm 1.4 \mu\text{M}$, a 0.71-fold decrease in IC_{50} value relative to control (Figure 1 and Table 2; $p < 0.01$). An even greater sensitization was observed in the cell line expressing both GSTM1 and MRP1 (MCF7/MRP1/GSTM1), with mean IC_{50} value for this cell line of $8.9 \pm 1.2 \mu\text{M}$, a 0.44-fold decrease relative to the empty vector control ($20.0 \pm 1.5 \mu\text{M}$) ($p < 0.0001$). The difference in sensitization between the dual-transfected line and either the MCF7/GSTM1 or the MCF7/MRP1 single-transfected cell lines was also highly significant ($p < 0.001$; Table 2). These data suggest that there is an additive effect of GST and MRP1 in the sensitization of MCF7 cells to HNE cytotoxicity since the greatest sensitization was in the MCF7/MRP1/GSTM1 cell line.

3.3 GSH depletion

The initial characterization of these cell lines indicated that resting basal levels of GSH in the MCF7/pLNCX vector control and MCF7/GSTM1 cell lines (76 and 65 nmol/mg, respectively) were not significantly different (Table 1; $p > 0.05$). Basal GSH levels were also quite similar in the cell lines expressing MRP1 (MCF7/MRP1 and MCF7/GSTM1/MRP1: 46 nmol/mg and 44 nmol/mg, respectively; $p > 0.05$). However, the basal GSH levels in the two MRP1-expressing cell lines were significantly decreased by 30–40% compared to the two cell lines without MRP1 expression (Table 1; $p < 0.01$).

A time-course comparison of the GSH depletion in cells exposed to $60 \mu\text{M}$ HNE was conducted with the four MCF7-derived cell lines. The MCF7/GSTM1 line exhibited a similar degree of GSH depletion as that of the control MCF7/pLNCX cell line throughout the 60 minute exposure. The GSH levels decreased by up to 20% over the initial 20 minutes in these cell lines, and then did not decrease further up to 60 minutes; GSH levels were not significantly different for MCF7/pLNCX and MCF7/GSTM1 at all time points up to 60 minutes (Figure 2; $p > 0.05$). The two MRP1-expressing cell lines, with or without co-expression of GSTM1, also exhibited a similar, albeit much greater degree of GSH depletion relative to the control cell line at all time points (Figure 2). Both cell lines had nearly identical initial (44–46 nmol/mg) and final levels (7–8 nmol/mg) of GSH remaining after 60 min of a $60 \mu\text{M}$ HNE exposure. However, the MCF7/MRP1/GSTM1 cell line appeared to exhibit up to 2-fold greater depletion at the 10, 20, and 40 minute time intervals compared to the MCF7/MRP1 line, although the differences did not achieve statistical significance (Figure 2; $p > 0.05$).

3.4 HNE-protein adducts

The formation of HNE-protein adducts was compared at several HNE concentrations in the MCF7 control and GSTM1- and/or MRP1-expressing cell lines, by western blotting using a primary antibody specific for HNE-protein Michael adducts. The cell lines expressing either MRP1 alone or GSTM1 alone yielded similar protein adduct profiles relative to the control MCF7 cell line at all concentrations studied (Figure 3). In contrast, MCF7/GSTM1/MRP1 (expressing both GST and MRP1) had substantially more HNE-protein adducts compared to the MCF7/pLNCX control cell line at all concentrations studied, and this difference was particularly pronounced at $60 \mu\text{M}$ HNE. Based on these observations, neither MRP1 alone nor GSTM1 alone appear to have much effect on HNE-protein adduct formation, while expression of both MRP1 $\pm$ GSTM1 greatly enhances HNE-protein adducts.

4. DISCUSSION

Numerous reports have documented the role of members of the MRP gene family of lipophilic anion efflux transporters in cellular resistance to a range of cytotoxic and genotoxic compounds, including both conjugated and unconjugated drugs, signal transduction molecules, carcinogens, and oxidized and reduced glutathione [37, 38]. Generally the resistance is attributable to ATP-dependent active extrusion of the toxic compound or a metabolite, a process that may depend on the presence of GSH for efflux of some MRP substrates [39, 40]. The MRP1 transporter also exports the oxidized form of GSH, glutathione disulfide (GSSG) [41], and even reduced GSH, albeit to a lesser extent [39, 42]. Thus, there is a logical basis and precedent for the lower basal GSH levels in MCF7 cells expressing MRP1 via transfection, as compared to the empty vector-transfected isogenic parental line. This effect has been observed previously in cells expressing MRP1 [43], and the same rationale has been invoked to explain higher GSH levels in the tissues of mice rendered deficient in MRP1 by targeted deletion, in comparison with the same tissues of wild type mice [44]. Nevertheless, a report that cells expressing stably transfected MRP2 were in fact rendered more sensitive than the control parental cells to the toxic effects of the lipid aldehyde HNE [24] was unexpected and surprising, especially since an earlier study had reported protection against HNE toxicity in cells expressing stably transfected MRP1 [23]. The seemingly paradoxical sensitization to HNE merits further investigation to determine whether it is universal in cells expressing MRP2, and whether and why expression of MRP1 via stable transfection results in protection or sensitization to HNE in other cell types.

The major route of HNE detoxification and elimination in liver is via GST-catalyzed conjugation with GSH [45]. Among the commonly expressed alpha, mu, and pi classes of soluble GST isozymes, GSTM1 is one of the most abundantly expressed, and has significant activity for HNE conjugation [46]. Expression of GSTM1 in humans is polymorphic, with the GSTM1 gene deleted in approximately half of individuals, and this deletion of GSTM1 in humans is associated with increased risk of lung and bladder cancer in smokers [32, 47]. We have previously shown that human GSTM1 confers protection against cytotoxicity and mutagenicity of the procarcinogen benzo[a]pyrene (B[a]P) when co-expressed with human CYP1A1, which activates B[a]P to the potent alkylating diol-epoxide metabolite [35]. As mentioned previously, co-expression of MRP1 or MRP2 was required to fully enable protection against cytotoxicity of the chemotherapeutic alkylating agent chlorambucil or the carcinogen NQO by alpha- and pi-class GSTs, respectively [12, 21, 48, 49]. Transgenic expression of human GSTA4, which has high activity for conjugation of HNE, conferred protection against HNE at low (< 15 μ M), but not high concentrations in HepG2 cells, which constitutively express MRP2 [25]. Given the preponderance of evidence for protective functions of MRPs, we investigated whether MRP1 would protect or sensitize cells to HNE in the presence of GSTM1 catalysis of conjugation.

Stably transfected cell lines derived from the MCF7 parent cell line, which lacks any endogenous expression of GSTs, MRP1, or MRP2 [49] were used to examine the role of GSTM1 and MRP1, expressed alone or together, on HNE sensitivity. The expression of either GSTM1 or MRP1 alone resulted in slight sensitization to HNE (0.79-fold or 0.71-fold lower IC₅₀, respectively; $p < .01$). When both GSTM1 and MRP1 were expressed together in the same cell line, the IC₅₀ was 0.44-fold lower ($p < .0001$), indicating more than 2-fold sensitization. The effect of MRP2 expression on HNE cytotoxicity was also examined, using HepG2 cells, a human liver epithelial line. The parental HepG2 cell line, which expresses MRP2 constitutively, had an average IC₅₀ value of 39.5 μ M, while the HepG2/ μ 2 cell line (expressing both human GSTM1 and MRP2) had an average IC₅₀ value of 33.1 μ M, a 0.84-

fold sensitization relative to the HepG2 parental cell line (data not shown). These results are consistent with the results of the MCF7 cytotoxicity data.

MRP1-expressing cell lines, both with and without GSTM1 expression, had lower basal resting levels of GSH (44–46 nmol/mg protein compared to 65–76 nmol/mg protein in the MRP1 non-expressing cell lines) (Figure 2). This has been reported previously in MRP-transfected cell lines [24], and may be due to efflux of either free GSH, or GSH conjugates of endogenous substrates, or as oxidized glutathione disulfide (GSSG) [50, 51]. Since the MCF7/GSTM1 line did not exhibit significantly greater HNE-induced GSH depletion compared to the MCF7 control, this indicates that conjugation of HNE by GSTM1 by itself was not sufficient to deplete cellular GSH pools. This may be due to inhibition of HNE conjugation as a result of intracellular accumulation of the HNE-SG conjugate, which has been shown to inhibit human GSTA4-4, an isozyme with high specific activity for HNE conjugation [18]. Conjugate accumulation might also explain the slight sensitization to HNE by GSTM1 expression alone. The HNE-SG conjugate is present mostly (> 90%) as a cyclic GSH thiohemiacetal Michael addition product, but the remaining fraction (< 10%) exists in tautomeric equilibrium as a ring-opened linear glutathionyl alkanal that can form toxic Schiff-base adducts with cellular proteins [17].

Although HNE is generally considered to arise as a result of radical-mediated lipid peroxidation, it can also cause ROS-mediated oxidative stress and GSH depletion, creating a positive reinforcement loop that exacerbates toxicity [52, 53]. We examined GSH levels in cells exposed to HNE and found that it was significantly depleted in MRP1-expressing MCF7 cell lines (MCF7/MRP1 and MCF7/GSTM1/MRP1), with only 7–8 nmol/mg GSH remaining after exposure to 60 μ M HNE for 60 minutes, compared to 58–61 nmol/mg GSH remaining in the MRP1 non-expressing cell lines (MCF7 and MCF7/GSTM1) under the same condition (Figure 2). The GSH depletion was more rapid in the MCF7/GSTM1/MRP1 than in the MCF7/MRP1 line, reaching a minimum of approximately 10% residual GSH at 20 minutes, whereas the cells expressing MRP1 reached the 10% nadir only at the end of the 60 minute exposure. Thus the cells expressing both MRP1 and GSTM1 were subjected to the lowest GSH levels reached for 40 minutes longer than the cells expressing MRP1 alone.

The slight sensitization of the cells expressing MRP1 alone may be caused in part by the lower basal level of GSH in these cells prior to HNE challenge. However, while GSH depletion may contribute to sensitization of the two cell lines expressing MRP1, it does not explain the slight sensitization to HNE of cells expressing GSTM1 alone. Together with the more than additive effect of combined expression of GSTM1 and MRP1, this suggests a role for GST in sensitizing cells to HNE cytotoxicity other than enhancement of GSH depletion, at least in the absence of an efflux pathway. The ring-opened tautomer of the HNE-SG conjugate formed by GSTs is likely still toxic to the cell [17], and both forms would accumulate in cells due to impermeability of the charged glutathionyl moiety. The presence of GSTM1 accelerates the rate of conjugate formation and accumulation until it becomes inhibited by high levels of HNE-SG product, likely resulting in greater exposure to the presumably toxic conjugate. Thus, removal of the HNE-SG conjugate may be required for complete HNE detoxification.

The formation of HNE-protein Michael adducts (Figure 3) was consistent with the HNE cytotoxicity data (Table 2), with substantially increased adduct formation only in the MCF7/GSTM1/MRP1 cell line. The large increase in protein adduct formation in this line was coincident with a more rapid depletion of GSH that persisted over a longer portion of the exposure interval. Since HNE has been shown to partially inhibit MRP1 via alkylation [54], the role of the GSTM1 could be to prevent MRP1 inhibition by conjugating HNE with GSH, facilitating greater GSH depletion by MRP1 efflux of HNE-SG and GSSG, as well as via

conjugation by GSTM1. The low protein adduct formation in the MCF7 empty vector control and MCF7/GSTM1 lines correlated with weak GSH depletion, and the strong protein adduct formation in the MCF7/GSTM1/MRP1 line correlated with strong GSH depletion and sensitization to HNE cytotoxicity. However, the weak adduct formation in the MCF7/MRP1 line was consistent with the slight sensitization to HNE but not with the strong GSH depletion in this line. The better correlation of cytotoxicity with protein adducts than with GSH depletion suggests that the latter may be a necessary condition conferred by MRP1 expression that enables increased HNE-protein adduct formation via a GST-dependent mechanism. The HNE-protein adducts may exert cytotoxic effects via the cumulative deleterious effects on the functionality of a broad range of proteins, as suggested by the broad size distribution of HNE-adducted proteins shown in the western blot (Figure 3). Alternatively, there may be select protein targets that mediate the most lethal effects of HNE, such as protein kinases and other signal transduction proteins that have a role in critical cellular functions including cell cycle control and apoptosis [55, 56].

Overall, these results indicate that MRP1 and GSTM1 collaborate to enhance HNE-protein adduct formation and HNE cytotoxicity, via depletion of GSH in cells exposed to HNE by MRP1, facilitated by GSTM1. In addition to GSTM1 conjugation of HNE and MRP1 efflux of HNE-SG, another possible explanation for the strong GSH depletion in both of the MRP1-expressing cell lines could be enhanced HNE-induced formation of GSSG, resulting in GSH depletion via efflux of GSSG by MRP1 [51]. Glutathione disulfide (GSSG) reductase was shown to be inhibited by 10 μ M HNE [57], providing an additional mechanistic basis for this rationale. Further studies will be required to distinguish among these possibilities.

Highlights

- We asked whether MRP1 + GSTM1 protect against 4-hydroxy-2-nonenal toxicity (HNE)
- MRP1 + GSTM1 sensitized 2.3-fold, not protected, MCF7 cells against HNE
- Glutathione was depleted in cells expressing MRP1 $\pm$ GSTM1 (faster with GSTM1)
- HNE-protein adducts were strongly enhanced when both MRP1 and GSTM1 are expressed

Abbreviations

GSH	glutathione
GSSG	oxidized glutathione disulfide
GSTM1	human mu class glutathione S-transferase M1a-M1a
HNE	4-hydroxy-2-nonenal
HNE-SG	glutathione S-conjugate of HNE
LPO	lipid peroxidation
MRP1	human multidrug resistance protein 1 (ABCC1)
MRP2	human multidrug resistance protein 2 (cMOAT; ABCC2)
MCF7/pLNCX	empty vector-transfected control MCF7 cell line

MCF7/GSTM1	human GSTM1a-transfected MCF7 cell line
MCF7/MRP1	human MRP1-transfected MCF7 cell line
MCF7/GSTM1/MRP1	MCF7 cell line dual-transfected with GSTM1 and MRP1
NQO	4-nitroquinoline-1-oxide

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Figure 1. Dose-response plot of HNE cytotoxicity in MCF7-derived cell lines
Cells were plated in 96-well plates and exposed to 0 – 40 μM HNE in serum-free medium for 2 hours, then medium was changed and cells were allowed to grow for 5 days and processed as described in Materials and Methods. Results are the average of 4 independent determinations (error bars omitted for clarity; see Table 2 for statistics).

Figure 2. GSH depletion by HNE in MCF7 derived cell lines

Cells were exposed to 60 μ M HNE for 10, 20, 40, or 60 min in serum-free DMEM, then harvested and GSH content was analyzed as described in Materials and Methods. Legend: solid bars, MCF7/pLNCX; gray bars, MCF7/GSTM1; open bars, MCF7/MRP1; cross-hatched bars, MCF7/MRP1/GSTM1. * Significantly different ($p < 0.001$) from the MCF7/pLNCX control cell line. The difference in GSH levels between MCF7/pLNCX and MCF7/GSTM1 at each time point measured was not statistically different ($p > 0.05$). The difference in GSH levels between MCF7/MRP1 and MCF7/MRP1/GSTM1 at each time point measured also was not statistically different ($p > 0.05$). The results represent a mean $\pm$ SD for 3 independent experiments.

Figure 3. HNE-protein adduct formation in MCF7 derived cell lines

Cells were exposed to HNE concentrations of 0, 30 μ M, 60 μ M, or 90 μ M HNE for a 2 hour time interval in serum-free media. Cells were harvested, lysed, and borohydride-reduced cytosolic protein (40 μ g/lane) was resolved on a 12 % SDS-PAGE gel and transferred to a PVDF membrane. Following blocking in 5% nonfat milk, protein adducts were detected using a polyclonal antibody (1:4000 dilution) directed against chemically reduced HNE-protein Michael adducts as described in Materials and Methods. Sample loading order: 1) MCF7/pLNCX; 2) MCF7/MRP1/GSTM1; 3) MCF7/MRP1; 4) MCF7/GSTM1. Bands at the bottom are from the stripped blot re-probed with β -actin as an index of equal protein loading.

Table 1
Characterization of GST activities and GSH levels in transgenic cell lines

Results for GST activities and basal (unchallenged) GSH levels are the mean $\pm$ SD of three or more experiments.

Cell line	Description	GST activity (nmol/min/mg)	Basal GSH levels (nmol/mg)
MCF7/pLNCX	Empty vector control	< 5	76 $\pm$ 6
MCF7/MRP1	MRP1	< 5	46 $\pm$ 4 ^b
MCF7/GSTM1	GST M1-1	216 $\pm$ 21 ^a	65 $\pm$ 6 ^c
MCF7/MRP1/GSTM1	MRP1 and GST M1-1	230 $\pm$ 18 ^a	44 $\pm$ 3 ^{b,d}

^a GST activities for the GSTM1-expressing MCF7 cell lines were not significantly different ($p > 0.05$)

^b Significantly different ($p < 0.005$) from the mean for the MCF7/pLNCX control

^c Not significantly different ($p > 0.05$) from the mean for the MCF7/pLNCX control

^d Significantly different ($p < 0.01$) from the mean for the MCF7/GSTM1 line

Table 2
Cytotoxicity of HNE in MCF7 derived cell lines expressing MRP1 ± GSTM1 or GSTM1 alone, compared to the empty vector control line

Cytotoxicity of HNE was measured as the IC₅₀ using the sulforhodamine B dye method as described in Materials and Methods. Values represent the mean of the IC₅₀ ± 1 SD for four independent experiments.

Cell line	IC ₅₀ values (μM HNE)	Fold-Difference Relative to control
MCF7/pLNCX	20.0 ± 1.5	(N/A)
MCF7/MRP1	14.4 ± 1.4 ^a	0.71
MCF7/GSTM1	15.9 ± 1.4 ^a	0.79
MCF7/MRP1/GSTM1	8.9 ± 1.2 ^{b,c}	0.44

^aSignificantly different (p < 0.01) from the mean for MCF7/pLNCX control

^bSignificantly different (p ≤ 0.001) from the mean for MCF7/MRP1 or MCF7/GSTM1

^cSignificantly different (p < 0.0001) from the mean for MCF7/pLNCX control