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**Glutathione Transferase P1 (GSTP1) Interacts Strongly with the Inner Leaflet of the
Plasma Membrane**

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Running title: Plasma Membrane Association of GSTP1

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ABBREVIATIONS: ABC, ATP-binding cassette; CT-B, cholera toxin subunit B; EDTA, ethylenediaminetetraacetic acid; GST, glutathione transferase; KI, potassium iodide; Na₂CO₃, sodium carbonate; pAb, polyclonal antibody; MRP, multidrug resistance protein

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ABSTRACT

Glutathione transferases (GSTs) are a superfamily of proteins best known for detoxifying harmful electrophilic compounds by catalyzing their conjugation with glutathione. GSTP1 is the most prevalent and widely distributed GST in human tissues, helping to detoxify a diverse array of carcinogens and drugs. In contrast with its protective role, overexpression of GSTP1 in a variety of malignancies is associated with a poor prognosis due to failure of chemotherapy. Although GSTP1 is classified as a cytosolic GST, we previously discovered that it is associated with the plasma membrane of the small cell lung cancer cell lines, H69 and H69AR. In the current study, endogenous and over-expressed GSTP1 in HEK293 and MCF-7 cell lines, respectively, were also found to associate with the plasma membrane, indicating that this interaction is not unique to H69 and H69AR cells. GSTP1 immunostaining in HEK293 and MCF7-GSTP1 cells only occurred under permeabilized conditions, suggesting that GSTP1 is associated with the intracellular surface of the plasma membrane. Cell surface biotinylation studies confirmed this. Immunogold electron microscopy revealed the presence of GSTP1 in close proximity to the plasma membrane. GSTP1 was not dissociated from plasma membrane sheets by high salt [potassium iodide (KI, 1 M) or KI / EDTA (1 M / 2 mM)] or alkaline sodium carbonate (Na_2CO_3 , 100 mM, pH 11.4), conditions known to strip peripherally associated membrane proteins. Thus, we report for the first time that GSTP1 is associated with the inner leaflet of the plasma membrane through a remarkably strong interaction.

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INTRODUCTION

Glutathione transferases (GSTs) are a superfamily of proteins best known for their role in detoxification. These enzymes catalyze the nucleophilic addition of reduced glutathione (GSH) to harmful electrophilic compounds, thus neutralizing reactivity. Human GSTs are classified into three major families: cytosolic, mitochondrial and microsomal (Hayes et al., 2005). The cytosolic proteins are further divided, based on amino acid sequences, into seven classes: Alpha (A), Mu, Omega, Pi (P), Sigma, Theta and Zeta. The human GSTP class contains a single member, GSTP1, the most prevalent and widely distributed GST in cells and tissues. GSTP1 is important in the detoxification of a diverse array of carcinogens, and variations in its genotype have been associated with differences in susceptibility to cancer (McIlwain et al., 2006). In contrast with this protective role, GSTP1 is over-expressed in a variety of malignancies, including lymphoma as well as ovarian, breast, colon and pancreatic cancers; this over-expression has been associated with the failure of chemotherapy and poor patient prognosis (McIlwain et al., 2006).

GSTP1 has multiple roles in addition to its GSH conjugation activity. These include the regulation of cell signalling pathways through *S*-glutathionylation, as well as the sequestration and negative regulation of the stress kinase c-jun N-terminal kinase (Tew and Townsend, 2011). Furthermore, GSTP1 can function non-enzymatically by binding non-substrate ligands and reactive compounds, leading to sequestration and intracellular transport (Hayes et al., 2005; Howie et al., 1989; Ralat and Colman, 2006). Monomers of GSTP1 are found in the media of cultured tumour cells and human platelets as well as in human plasma and bile (Howie et al., 1989; Kura et al., 1996; Ranganathan et al., 2005). The passage of GSTP1 across the cell membrane has been proposed as an excretory pathway for bound ligands, but the mechanism by which GSTP1 might traverse the membrane is not understood.

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During the course of investigating the cellular metabolism and efflux of arsenic, a high-priority environmental carcinogen, we detected GSTP1 in plasma membrane fractions of the H69 and H69AR small-cell lung cancer cell lines (Leslie et al., 2004). Imaging experiments showed a co-localization of GSTP1 with the H69AR plasma membrane (Leslie et al., 2004). The association of GSTP1 with the plasma membrane was unexpected, because GSTP1 is classified as a cytosolic GST. The close association of GSTP1 with the plasma membrane could be important for numerous cell functions. For example, non-substrate ligands bound to GSTP1 could be brought closer to the plasma membrane for excretion. Alternatively, GSTP1 is known to confer resistance to the toxic effects of electrophilic drugs and carcinogens by acting synergistically with several ATP-binding cassette (ABC) transport proteins (Depeille et al., 2004; Leslie et al., 2004; Peklak-Scott et al., 2005; Smitherman et al., 2004), including plasma membrane efflux transporters such as the multidrug resistance proteins MRP1 (*ABCC1*) and MRP2 (*ABCC2*). We and others have proposed that the conjugating enzymes and the plasma membrane must be in close proximity for efficient conjugation and efflux of either labile conjugates or hydrophobic toxicants (Bauer et al., 2008; Leslie et al., 2004; Singh et al., 2002).

It was the purpose of the current study to determine if the GSTP1 plasma membrane association was an anomaly of the H69 and H69AR cell lines and to biochemically characterize the plasma membrane association. Endogenous and over-expressed GSTP1 in HEK293 and MCF7 cell lines, respectively, were found to associate with the plasma membrane. Cell surface biotinylation assays and immunoelectron microscopy suggested that GSTP1 is localized to the inner leaflet of the plasma membrane. Lastly, the association of GSTP1 with the plasma membrane was found to be remarkably strong and was not dissociated under harsh membrane stripping conditions.

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METHODS

Materials- The GSTP1 GS72 rabbit polyclonal antibody (pAb) was from Oxford Biomedical Research (Oxford, MI). The GAPDH (6C5) mouse monoclonal and Na⁺/K⁺ATPase (H-300) rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The MRP1 rat monoclonal antibody (MAb) MRPr1 was from Novus Biologicals (Littleton, CO). The anti-calnexin and anti-Mn superoxide dismutase rabbit pAbs were from Stressgen (Ann Arbor, MI) and the anti-histone H1 mouse MAb was from US Biological (Swampscott, MA). Alexa Fluor 555 conjugated cholera toxin subunit B (CT-B), and DAPI were from Molecular Probes (Eugene, OR). G418 (Geneticin) was from Invitrogen by life technologies. Mini CompleteTM protease inhibitor tablets and FuGENETM6 were from Roche Diagnostics (Indianapolis, IN). All other reagents were purchased from Sigma.

Construction of vector encoding GSTP1 protein- pBluescriptSK(-)GSTP1 was used as a template in PCR using a 5' primer with an engineered *Bam*HI site (5'...GAGGATCCGCCATGC CGCCGTATAACCGTGGTC...3') and a 3' primer with an engineered *Xho*I site (5'...GTCTCGAG TCACTGTTTCCCGTTGCCATTGATG....3'). The PCR product was digested with *Bam*HI and *Xho*I and ligated into pcDNA3.1(+).

Cell lines- The SV40-transformed human embryonic kidney (HEK293) cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine and 10% fetal bovine serum. The MCF7-GSTP1 and MCF7-vector stable cell lines were created as described (Ito et al., 2001), except levels of GSTP1 expression in G418 selected populations were determined by immunoblot analysis with the GSTP1-specific antibody GS72, as described (Leslie et al., 2004). GSTP1-expressing populations were cloned by limiting dilution to obtain populations of >90% as assessed by GSTP1 immunostaining and fluorescence microscopy.

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Cells were maintained in DMEM with 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS and 1000 µg/mL G418.

Confocal microscopy. Triple staining experiments for nuclei, plasma membrane and GSTP1 were performed using the HEK293 and MCF7-GSTP1 cell lines, as described previously (Leslie et al., 2004), with minor modifications. HEK293 cells were transiently transfected with pcDNA3.1(-)MRP1 (as a plasma membrane marker) (Ito et al., 2001) using FuGENE™6, according to the manufacturer's instructions. Alexa Fluor 555 conjugated cholera toxin subunit B (CT-B) was used as a plasma membrane stain for the MCF7-GSTP1 cell line. Seventy-two hrs post-seeding, cells were fixed with paraformaldehyde for 10 min and MCF7-GSTP1 cells incubated with CT-B (1:1000) for 10 min. The rest of the staining procedure was identical to that described in Leslie et al., 2004 except that the anti-GSTP1 antibody GS72 was used. Cells were also stained under non-permeabilizing conditions using the conditions described above, but in the absence of Triton X-100. Cells were viewed with a Zeiss LSM510 confocal microscope and photographed.

Total and cell surface expression of GSTP1. HEK293 and MCF7-GSTP1 cells were seeded at 1×10^6 cells in a T-75 flask and 72 hrs later washed with ice-cold borate buffer (0.62 mg/ml boric acid, pH 9.0). Biotinylation reactions and sample preparation for SDS-PAGE were performed as described previously (Leslie et al., 2007). GSTP1 protein was detected using the anti-GSTP1 antibody GS72 (1:1000). To ensure that the cell surface biotinylation worked correctly blots were stripped and probed for the Na⁺/K⁺ATPase (integral plasma membrane protein) using the rabbit anti-Na⁺/K⁺ATPase pAb (1:500). To ensure the biotinylation was specific to cell surface proteins, the blots were also probed for the cytosolic protein GAPDH using the rabbit anti-GAPDH pAb (1:1000).

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Immunoelectron microscopy. The subcellular localization of GSTP1 in MCF7-GSTP1 cells was examined using immunogold electron microscopy. Cells were seeded at 1×10^6 cells in a T-75 flask and 72 hrs later pelleted and washed with PBS. Cells were fixed with 0.1% glutaraldehyde, 3% paraformaldehyde, 3% sucrose, 1.5 mM CaCl_2 in 0.1M sodium cacodylate buffer, pH 7.2 for 1 hr at 4°C . Sample preparation, immunogold labelling [using primary antibody anti-GSTP1 GS72 (1:50) for 2 hr and a 12 nm colloidal gold-donkey anti-rabbit IgG secondary antibody (1:10) for 1 h], and viewing followed previously described procedures (Ilkow et al., 2010).

Plasma membrane sheet preparation, stripping and immunoblot analysis. Plasma membrane sheets were prepared from MCF7-GSTP1 cells collected from ten 150 mm^2 Petri-plates, as described previously (Hubbard et al., 1983). The purity (of the plasma membrane sheets) was assessed by immunoblot using antibodies against calnexin (endoplasmic reticulum marker), nuclear histone H1 (nuclear protein marker), GAPDH (cytosolic marker), and manganese superoxide dismutase (mitochondrial marker). Plasma membrane sheets were then subjected to various chemical treatments known to strip peripherally associated proteins. Thus, plasma membrane sheets (10 μg) were incubated with ten volumes of 1M Tris HCl (pH 7.4), 1 M potassium iodide (KI), 1 M KI with 2 mM EDTA, or 100 mM sodium carbonate (Na_2CO_3 , pH 11.4) for 30 min at 4°C . To increase the stringency of stripping, the Na_2CO_3 condition was also done using 50- and 100-volumes. Treated plasma membrane sheets were then pelleted by centrifugation at $100,000 \times g$ for 1 hr, resuspended in Laemmli buffer, resolved by 11% SDS-PAGE, transferred and probed with the anti-GSTP1 GS72 antibody (1:1000) as described previously (Leslie et al., 2004), the blot was then stripped and probed with the anti- Na^+/K^+ ATPase pAb, and finally stripped and probed with the anti-GAPDH antibody.

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RESULTS AND DISCUSSION

The purpose of the current study was to extend our investigations of the plasma membrane association of GSTP1 (Leslie et al., 2004). Thus, in an investigation of MRP1-mediated transport of arsenic triglutathione, GSTP1 was found to be in close proximity with the plasma membrane of H69 and H69AR cells (Leslie et al., 2004). To determine if the GSTP1 plasma membrane association was a characteristic unique to the H69 and H69AR cell lines the localization of GSTP1 was investigated in HEK293 and MCF7-GSTP1 cell lines. HEK293 cells contain endogenous GSTP1 while the MCF7-GSTP1 cell line are stable transfectants. Cell lines were immunostained with the anti-GSTP1 GS72 antibody and a significant fraction of GSTP1 was localized to a region resembling the plasma membrane of HEK293 and MCF7-GSTP1 cells (Fig. 1A, first column). To define the plasma membrane region of the HEK293 cells they were immunostained with the anti-MRP1 antibody MRPr1 after transient transfection with the plasma membrane protein MRP1 (Fig. 1A, column 2, top). To define the plasma membrane region of the MCF7-GSTP1 cells the plasma membrane marker CT-B was used (Fig. 1A, column 2, bottom). Merged images of GSTP1 and the respective plasma membrane marker are shown (Fig. 1A, column 3) and for cellular orientation the nuclei were stained (Fig. 1A, column 4).

Several reports have documented that GSTP1 is found in the culture media of human tumour cells and human platelets as well as in human plasma and bile (Howie et al., 1989; Kura et al., 1996; Ranganathan et al., 2005). In addition, GSTP1 has been found to associate with the external surface of cells (Hemachand et al., 2002). To determine if GSTP1 was interacting with the internal surface of the plasma membrane or binding to the external surface after exiting the cell, immunostaining using non-permeabilized conditions and cell surface biotinylation studies were undertaken.

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HEK293, MCF7-GSTP1 and H69AR cells were immunostained with an anti-GSTP1 antibody under non-permeabilized conditions, and then examined by confocal microscopy. In contrast with permeabilized conditions [Fig. 1A first column and previously reported for H69AR (Leslie et al., 2004)], GSTP1 was not detected (data not shown). The location of the anti-GSTP1 antibody epitope is not known, making it difficult to conclude that GSTP1 is localized entirely to the inner leaflet of the plasma membrane; however, this data suggests GSTP1 is not entirely on the extracellular surface.

To determine if any portion of GSTP1 was located on the outer surface of the cell, cell surface expression of GSTP1 in MCF7-GSTP1 and HEK293 cells was measured using biotinylation and compared to total expression (Fig. 1B). GSTP1 was detected in the total protein for both cell lines, but was not present in the cell surface fractions (Fig. 1B). The cytosolic protein GAPDH was also detected in the total protein, but not the cell surface fraction, while the Na⁺/K⁺ ATPase (a plasma membrane protein) was detected in the total protein and the cell surface fraction. The GAPDH and Na⁺/K⁺ ATPase controls indicate that the biotinylation method successfully enriched the fraction for proteins expressed at the cell surface. Overall, the data suggests that no portion of GSTP1 is located at the extracellular surface of HEK293 or MCF7-GSTP1 cells.

To confirm the cellular localization of GSTP1, MCF7-GSTP1 cells were analyzed using electron microscopy immunogold labelling. Consistent with previous reports of other cell lines, GSTP1 was distributed throughout the cytoplasm, mitochondria and nucleus of the MCF7-GSTP1 cells (Fig. 2) (Goto et al., 2009). GSTP1 was not evenly distributed throughout the cytoplasm, but clustered, suggesting that GSTP1 could be associated with organelles or other intracellular structures. In addition, plasma membrane staining for GSTP1 was observed.

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To determine the biochemical nature of the association between GSTP1 and the plasma membrane, plasma membrane sheets were prepared from MCF7-GSTP1 cells and treated under stringent membrane stripping conditions known to remove peripherally associated membrane proteins. Immunoblot analysis of plasma membrane sheets revealed they were very pure with little contamination by endoplasmic reticular, mitochondrial or nuclear proteins (data not shown). Plasma membrane sheets treated with 10-volumes of sodium carbonate (Na_2CO_3 , 100 mM, pH 11.4) or potassium iodide (KI, 1 M) \pm EDTA (2 mM) retained virtually all of the GSTP1 and the integral membrane protein Na^+/K^+ ATPase (Figure 3). A substantial amount of GSTP1 was retained in the membrane after treatment with 50- and 100-volumes of Na_2CO_3 . Interestingly, even the integral membrane protein Na^+/K^+ ATPase was beginning to be stripped from the membrane at 100-volumes of Na_2CO_3 , emphasizing how harsh this stripping condition was. In contrast, the cytosolic protein GAPDH was almost completely removed from the membrane by a wash with Tris (1 M, pH 7.4) as well as all of the harsh stripping conditions. Thus, these results unexpectedly showed that the association between the plasma membrane and GSTP1 was very strong, and comparable to the integral membrane protein Na^+/K^+ ATPase.

A peripheral association of P and Mu GST isoforms with the plasma membrane of goat sperm has been described (Hemachand et al., 2002). Furthermore, murine GSTA4-4 and several human GSTA isoforms were found to associate with the plasma membrane and outer nuclear membrane, respectively, through electrostatic interactions (Singh et al., 2002; Stella et al., 2007). We have found that GSTP1 is still associated with the plasma membrane after high salt treatment and alkaline carbonate extraction, ruling out the possibilities of electrostatic and/or peripheral interaction of GSTP1 with the plasma membrane. This represents the first report of a strong, non-peripheral association of a “cytosolic” GST with the plasma membrane. Although the

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strength of the GSTP1 plasma membrane interaction is characteristic of an integral membrane protein, nothing about the reported structure of GSTP1 suggests that this would be the case (Oakley et al., 1997). Intriguingly, members of the chloride intracellular channel (CLIC) family, which are also GST fold superfamily members, exist primarily as soluble proteins, but can also auto-insert into cellular membranes to form ion channels, through a poorly understood mechanism (Littler et al., 2010). Whether or not GSTP1 could also interact with the plasma membrane through a similar mechanism is currently unknown. The observation that GSTP1 is simultaneously present in the cytosol and the plasma membrane of individual cells (Fig. 1A), also raises the possibility that GSTP1 associates with the plasma membrane after a reversible post-translational modification. Investigations are currently underway to explore this possibility.

In conclusion, GSTP1 is associated with the inner leaflet of the plasma membrane through a remarkably strong interaction. The localization of GSTP1 to the plasma membrane could have important implications for the role of GSTP1 in detoxification, anticancer drug resistance as well as the many other functions of GSTP1.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Qazi, Osoria Pérez, and Leslie

Conducted experiments: Qazi, Osoria Pérez, and Sam

Performed data analysis: Qazi, Osoria Pérez, and Leslie

Wrote the manuscript: Qazi and Leslie

Other: Leslie acquired funding for the research

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REFERENCES

- Bauer B, Hartz AM, Lucking JR, Yang X, Pollack GM and Miller DS (2008) Coordinated nuclear receptor regulation of the efflux transporter, Mrp2, and the phase-II metabolizing enzyme, GSTpi, at the blood-brain barrier. *J Cereb Blood Flow Metab* **28**(6):1222-1234.
- Depeille P, Cuq P, Mary S, Passagne I, Evrard A, Cupissol D and Vian L (2004) Glutathione S-transferase M1 and multidrug resistance protein 1 act in synergy to protect melanoma cells from vincristine effects. *Mol Pharmacol* **65** 897-905.
- Goto S, Kawakatsu M, Izumi S, Urata Y, Kageyama K, Ihara Y, Koji T and Kondo T (2009) Glutathione S-transferase pi localizes in mitochondria and protects against oxidative stress. *Free Radic Biol Med* **46**(10):1392-1403.
- Hayes JD, Flanagan JU and Jowsey IR (2005) Glutathione transferases. *Annu Rev Pharmacol Toxicol* **45**:51-88.
- Hemachand T, Gopalakrishnan B, Salunke DM, Totey SM and Shaha C (2002) Sperm plasma-membrane-associated glutathione S-transferases as gamete recognition molecules. *J Cell Sci* **115**(Pt 10):2053-2065.
- Howie AF, Hayes PC, Bouchier IA, Hayes JD and Beckett GJ (1989) Glutathione S-transferase in human bile. *Clin Chim Acta* **184**(3):269-278.
- Hubbard AL, Wall DA and Ma A (1983) Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J Cell Biol* **96**(1):217-229.
- Ilkow CS, Weckbecker D, Cho WJ, Meier S, Beatch MD, Goping IS, Herrmann JM and Hobman TC (2010) The rubella virus capsid protein inhibits mitochondrial import. *J Virol* **84**(1):119-130.

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- Ito K, Olsen SL, Qiu W, Deeley RG and Cole SP (2001) Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport. *J Biol Chem* **276**(19):15616-15624.
- Kura T, Takahashi Y, Takayama T, Ban N, Saito T, Kuga T and Niitsu Y (1996) Glutathione S-transferase-pi is secreted as a monomer into human plasma by platelets and tumor cells. *Biochim Biophys Acta* **1292**(2):317-323.
- Leslie EM, Haimeur A and Waalkes MP (2004) Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. *J Biol Chem* **279**(31):32700-32708.
- Leslie EM, Watkins PB, Kim RB and Brouwer KL (2007) Differential Inhibition of Rat and Human Na⁺-Dependent Taurocholate Cotransporting Polypeptide (NTCP/SLC10A1) by Bosentan: A Mechanism for Species Differences in Hepatotoxicity. *J Pharmacol Exp Ther* **321**(3):1170-1178.
- Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, Valenzuela SM, Mazzanti M, Brown LJ, Breit SN and Curmi PM (2010) The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? *FEBS Lett* **584**(10):2093-2101.
- McIlwain CC, Townsend DM and Tew KD (2006) Glutathione S-transferase polymorphisms: cancer incidence and therapy. *Oncogene* **25**(11):1639-1648.
- Oakley AJ, Lo Bello M, Battistoni A, Ricci G, Rossjohn J, Villar HO and Parker MW (1997) The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. *J Mol Biol* **274**(1):84-100.

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- Peklak-Scott C, Townsend AJ and Morrow CS (2005) Dynamics of glutathione conjugation and conjugate efflux in detoxification of the carcinogen, 4-nitroquinoline 1-oxide: contributions of glutathione, glutathione S-transferase, and MRP1. *Biochemistry* **44**(11):4426-4433.
- Ralat LA and Colman RF (2006) Identification of tyrosine 79 in the tocopherol binding site of glutathione S-transferase pi. *Biochemistry* **45**(41):12491-12499.
- Ranganathan PN, Whalen R and Boyer TD (2005) Characterization of the molecular forms of glutathione S-transferase P1 in human gastric cancer cells (Kato III) and in normal human erythrocytes. *Biochem J* **386**(Pt 3):525-533.
- Singh SP, Janecki AJ, Srivastava SK, Awasthi S, Awasthi YC, Xia SJ and Zimniak P (2002) Membrane association of glutathione S-transferase mGSTA4-4, an enzyme that metabolizes lipid peroxidation products. *J Biol Chem* **277**(6):4232-4239.
- Smitherman PK, Townsend AJ, Kute TE and Morrow CS (2004) Role of multidrug resistance protein 2 (MRP2, ABCC2) in alkylating agent detoxification: MRP2 potentiates glutathione S-transferase A1-1-mediated resistance to chlorambucil cytotoxicity. *J Pharmacol Exp Ther* **308**(1):260-267.
- Stella L, Pallottini V, Moreno S, Leoni S, De Maria F, Turella P, Federici G, Fabrini R, Dawood KF, Lo Bello M, Pedersen JZ and Ricci G (2007) Electrostatic association of glutathione transferase to the nuclear membrane: Evidence of a defense barrier at the nuclear envelope. *J Biol Chem* **282**:6372-6379.
- Tew KD and Townsend DM (2011) Regulatory functions of glutathione S-transferase P1-1 unrelated to detoxification. *Drug Metab Rev.* DOI: 10.3109/03602532.2011.552912.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: GSTP1 is associated with the HEK293 and MCF7-GSTP1 plasma membrane but is not cell surface biotinylated. *A*, Confocal microscopy of the HEK293 (*top panels*) and MCF7-GSTP1 (*bottom panels*) cell lines was done using the GSTP1 pAb GS72 visualized with Alexa Fluor 488- conjugated goat anti-rabbit IgG (H+L) secondary antibody, and is shown in green (first column). The plasma membrane of the HEK293 and MCF7-GSTP1 is shown in red (second column). Nuclei were stained with DAPI and are shown in blue (fourth column). The scale bars are 10 μm . Identical experiments were done under non-permeabilized conditions and no staining was observed. MCF7-vector control cell lines were also stained under the same conditions (permeabilized and non-permeabilized) and GSTP1 was not detected. *B*, Total and cell surface biotinylation of GSTP1 in HEK293 (left panel) and MCF7-GSTP1 cells (right panel). Total (12.5 μl , 1.25 μg) (left) and cell surface (25 μl , 50% of protein captured on streptavidin beads) (right) proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. Blots were probed with anti-GSTP1 antibody, stripped, probed with anti- Na^+/K^+ -ATPase antibody, stripped and probed with anti-GAPDH antibody.

Figure 2: Cellular localization of GSTP1 by immunogold electron microscopy. MCF7-GSTP1 cells were fixed and processed as described in the methods section. Cells were stained with primary antibody anti-GSTP1 GS72 (1:50) for 2 hrs and a 12 nm colloidal gold-donkey anti-rabbit IgG secondary antibody (1:10) for 1 h. Samples were viewed with a Philips 410 transmission electron microscope. Nu, nucleus; Mt, mitochondria; PM, plasma membrane. Arrows indicate plasma membrane regions with GSTP1.

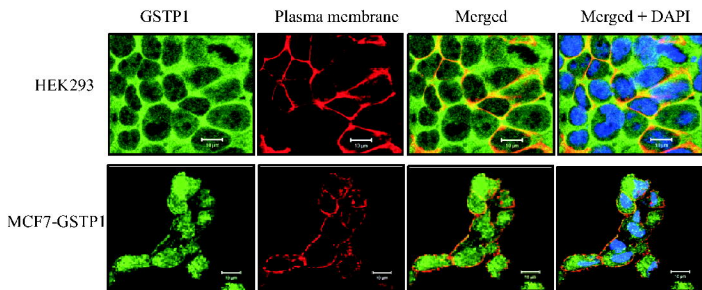
Figure 3: GSTP1 is strongly associated with the plasma membrane. Plasma membrane sheets isolated from the MCF7-GSTP1 cell line (10 μg) were incubated with ten volumes of Tris (1 M,

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pH 7.4), Na₂CO₃ (100 mM, pH 11.4), KI (1 M), and KI + EDTA (1 M + 2mM) at 4°C for 30 min. The Na₂CO₃ treatment was also done under identical conditions using 50 and 100 volumes (indicated by 50x and 100x). Treated plasma membrane sheets were then pelleted by centrifugation, resolved by SDS-PAGE, transferred and probed with the anti-GSTP1 pAb GS72 (*top panel*). The same blot was stripped and re-probed with the anti-Na⁺/K⁺ ATPase (*middle panel*) and anti-GAPDH (*bottom panel*) pAbs, as cytosolic and plasma membrane protein controls, respectively.

Figure 1

A



B

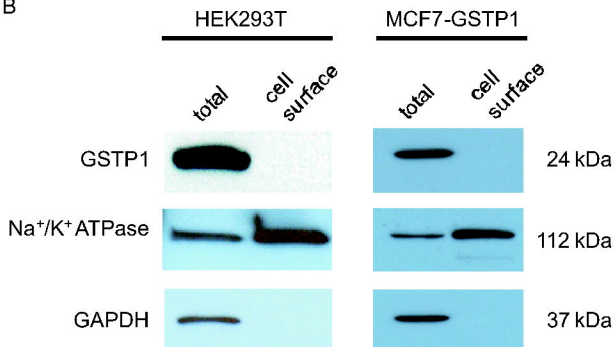


Figure 2

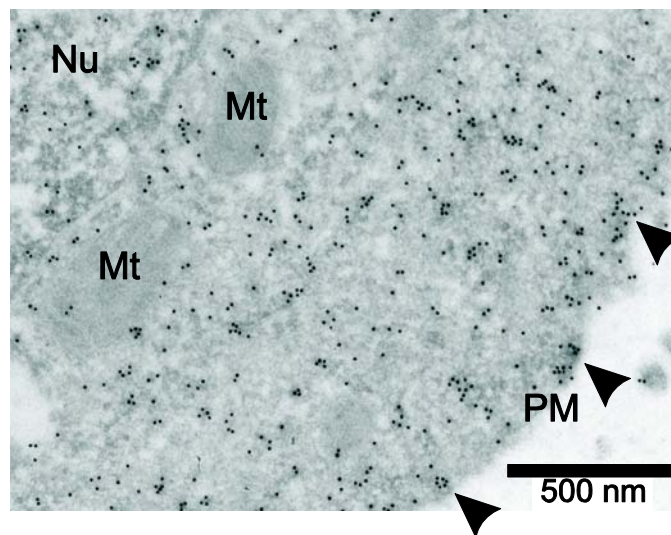


Figure 3

