Published in final edited form as:

J Nutr. 2004 November ; 134(11): 3205S–3206S. doi:10.1093/jn/134.11.3205S.

Glutathione and ABC Transporters as Determinants of Sensitivity to Oxidative and Nitrosative Stress[1]

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Keywords

Glutathione; GST (glutathione S-transferase), ABCA2; DNA-dependent protein kinase; JNK; nitric oxide

EXPANDED ABSTRACT

The development of novel drugs remains a high priority in the successful therapeutic management of cancer. We have applied our accumulated knowledge of the importance of glutathione (GSH), glutathione S-transferases (GSTs), and ATP-dependent binding cassette (ABC) transporters to the identification of novel targets and testing of new agents that are influenced by thiol homeostasis in tumors.

Glutathione S-transferase π (GSTπ) interacts with and suppresses the activity of c-Jun NH_2-terminal kinase (JNK) (1). GSTπ-deficient mice (GST$\pi^{-/-}$) have higher levels of circulating white blood cells than wild type mice. A peptidomimetic inhibitor of GSTπ disrupts the interaction between GSTπ and JNK and mimics in wild type mice the increased myeloproliferation observed in GST$\pi^{-/-}$ mice. In an in vitro hematopoiesis assay, interleukin-3, granulocyte-colony stimulating factor, and granulocyte-macrophage-colony stimulating factor were more effective at stimulating proliferation of hematopoietic cells in GST$\pi^{-/-}$ mice than in wild type mice. The GSTπ inhibitor increased myeloproliferation induced by these cytokines in wild type but not in GST$\pi^{-/-}$ mice, suggesting a key role for GSTπ in the effect of this drug. The JNK inhibitor, SP600125 which itself caused little inhibition of cytokine-induced myeloproliferation, prevented all myelostimulant effects of the GSTπ inhibitor. A more sustained phosphorylation of the STAT family of proteins was also observed in GST$\pi^{-/-}$ bone marrow derived mast cells exposed to IL-3. This was associated with an increased proliferation and a downregulation of expression of negative

[1]Presented as part of the conference “Free Radicals: The Pros and Cons of Antioxidants,” held June 26 –27 in Bethesda, MD. This conference was sponsored by the Division of Cancer Prevention (DCP) and the Division of Cancer Treatment and Diagnosis, National Cancer Institute, NIH, Department of Health and Human Services (DHHS); the National Center for Complementary and Alternative Medicine (NCCAM), NIH, DHHS; the Office of Dietary Supplements (ODS), NIH, DHHS; the American Society for Nutritional Science; and the American Institute for Cancer Research and supported by the DCP, NCCAM, and ODS. Guest editors for the supplement publication were Harold E. Seifried, National Cancer Institute, NIH; Barbara Sorkin, NCCAM, NIH; and Rebecca Costello, ODS, NIH.

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regulators of the JAK-STAT pathway. The increased activation of JNK and STATs in GST π-deficient mice provides a viable mechanism for the increased myeloproliferation in these animals. These data also confirm the important role that GSTπ plays in the regulation of cell signaling pathways in bone marrow (2).

TLK286 [γ-glutamyl-α-amino β (2-ethyl-N,N,N′,N′-tetrakis (2-chloroethyl) phosphorodiamidate)-sulfonyl-propionyl-(R)-(-) phenylglycine] is a novel GSTπ activated pro-drug (3). Because GSTπ is expressed at high levels in many solid tumors and in drug resistant tumor cells, the design rationale predicts that preferential release of the active cytotoxic species should occur in tumors, with the effective improvement of therapeutic index (4). Preclinical results support this contention. Interestingly, a potentially novel target for this drug has been identified. An IC_{50} inhibition constant of 1 μmol/L has been established for DNA-dependent protein kinase (DNA-PK). In addition, the drug has significant activity in Cisplatin resistant ovarian cancer cells (5), providing a rationale for its clinical use in ovarian cancer. Through the efforts of colleagues at Telik, the drug has completed successful phase I/phase II clinical testing and has now entered phase III trials.

Synthesis of PABA/NO (O^2-[2,4-dinitro-5-(*N*-methyl-*N*-carboxyphenylamino) phenyl] (1-*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate) provided a prodrug (6) that is metabolized to potentially cytolytic nitric oxide by the major cytosolic GST isozymes, including GSTπ. Mouse embryo fibroblasts null for GSTπ (GST$\pi^{-/-}$) showed a decreased sensitivity to PABA/NO. Cytotoxicity of PABA/NO was also examined in NIH3T3 cells that were stably transfected with GSTπ and/or various combinations of γ-glutamyl cysteine synthetase and the ABC transporter MRP1. Overexpression of MRP1 conferred the most significant degree of resistance and in vitro transport studies confirmed that a GSTπ-activated metabolite of PABA/NO was effluxed by MRP1 in a GSH-dependent manner. Additional studies showed that in the absence of MRP1, PABA/NO activated the extracellular-regulated and stress-activated protein kinases, ERK, JNK, and p38. Selective inhibitors showed that the activation of JNK and p38 were critical to the cytotoxicity of PABA/NO (7).

Glutathionylation is emerging as an important post-translational modification that influences the function and stability of a variety of proteins. Glutathionylation occurs through a thiol disulfide exchange reaction with oxidized glutathione or the conjugate of glutathione and nitric oxide, GSNO, and available cysteine residues in target proteins. In response to oxidative and nitrative stress, such modifications can alter the characteristics of a spectrum of proteins. Using two dimensional gel electrophoresis with monoclonal antibodies directed against glutathionylated cysteine residues, PABA-NO produced a time- and dose-dependent glutathionylation of >50 proteins within 5 min of treatment. In NIH3T3 cells overexpressing MRP1, glutathionylation was significantly reduced. Similarly, in HL60 cells resistant to adriamycin (HL60-ADR), overexpression of MRP1 also reduced total protein glutathionylation in a manner consistent with the observed collateral resistance to PABA-NO. Once NO is released, it can combine with GSH to form GSNO, and this is known to be a potent stimulator of protein glutathionylation and a plausible substrate for MRP1, explaining the reduced effects in MRP1 overexpressing cells.

The human ABCA2 transporter is a member of a large family of ATP-binding proteins that transport a variety of macromolecules across biological membranes. Its overexpression has been implicated in the acquired resistance of cells to the chemotherapeutic drug estramustine (8). We have identified a novel first exon, which we designate 1A, that is located 699 bp upstream of the previously characterized first exon, which we designate 1B (9). These first exons are alternatively spliced to the second exon of the ABCA2 transcript resulting in a protein that has a unique amino terminus. For exon 1A, the new amino terminus encoded by the first exon is 52 amino acids and for exon 1B, 22 amino acids. We observed that among adult tissues examined, the highest expression of the 1A isoform was in peripheral blood leukocytes. Laser scanning confocal microscopy revealed that the 1A isoform, as well as the 1B isoform colocalizes with the lysosome-associated membrane proteins-1 and −2 (LAMP1 and 2). Since both isoforms of the ABCA2 transporter have similar subcellular localization, we propose that they are also functionally redundant. It is likely that expression of ABCA2 by 2 independent promoters constitutes locus of regulation controlling levels of the protein to meet requirements in different tissues.

We now have evidence that the expression of the ABCA2 gene is correlated with changes in cellular cholesterol levels. The metabolism of LDL-derived cholesterol has been extensively studied and several diseases that manifest defective cholesterol trafficking have been described, including Niemann-Pick type C (NPC). Few genes have been identified that have a functional role in the trafficking of LDL-derived cholesterol, among them NPC1 and NPC2. Expression of ABCA2 increases when cells are grown in lipoprotein-deficient medium and declines when 25-hydroxycholesterol is exogenously supplied. Similar to the NPC1 protein, ABCA2 colocalizes with LAMP2 containing vesicles (10). Agents that cause lysosomal accumulation of LDL-derived cholesterol, the steroid hormone progesterone, and the hydrophobic amine U18666A elevate ABCA2 expression. We also observe an increase in ABCA2 expression in NPC and Familial Hypercholesterolemia fibroblasts. These results represent the first characterization of a functional role for the ABCA2 transporter in uptake of LDL in the maintenance of cellular cholesterol homeostasis (11).

Transporter function may be influenced by interactions with other proteins within the local membrane environment. Amplified Differential Gene Expression (ADGE) and ADGE microarray were developed in this laboratory to detect small changes of gene expression with little starting material (12). Application of ADGE microarray to an ABCA2 transfected cell line revealed altered expression of gene clusters with functions related either to *1*) transport or *2*) oxidative stress response and **β**-amyloid metabolism. These included: *1*) fatty acid binding protein, phospholipid binding protein, phospholipid synthesis protein, and transporter cofactors; *2*) seladin-1, amyloid **β** precursor protein, vimentin, and low-density lioprotein receptor-related protein. The ABCA2 transfected cell line was resistant to the free radical initiator AAPH. This response together with the altered gene expression patterns may suggest a possible role for the transporter in diseases such as Alzheimer's (13).

Some novel research questions can be derived from these results. For example, GSTs have important ligand binding activities that are distinct from their catalysis functions. How do they influence kinase mediated signaling pathways? Glutathionylation of proteins can alter the structure and function of proteins. How important and widespread is this post-

translational modification? Determining substrate specificity for ABC transporters can be a difficult proposition. With what disease states can these transporters be associated? How does the expression of certain transporters influence the ability of a cell to deal with oxidative stresses? What links exist between thiol mediated detoxification pathways and transporter functions? There are plenty of opportunities to answer such questions through empiric experimentation.

Acknowledgments

Supported by grants NCI RO1 CA083778 and CA085660.

Abbreviations used:

ABC	ATP-dependent binding cassette
ADGE	Amplified Differential Gene Expression
GSH	importance of glutathione
GST	glutathione S-transferase
GSTπ	glutathione S-transferase π
GST$\pi^{-/-}$	GSTπ deficient mice
JNK	c-Jun NH_2-terminal kinase
LAMP1 and 2	lysosome-associated membrane proteins-1 and -2
NPC	Niemann-Pick type C
PABA/NO	O^2-[2,4-dinitro-5-(*N*-methyl-*N*-4-carboxyphenylamino) phenyl] 1-*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate

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