RALBP1 IN STRESS RESISTANCE

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ABSTRACT

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Ralbp1[1] is a multi-specific transporter of glutathione conjugates as well as unconjugated amphiphilic toxins. Because glutathione conjugates are major metabolites of toxic lipid peroxidation products generated as a consequence of oxidant and radiant stress, the hypothesis is put forth that the function of Ralbp1 in-vivo and in vitro should be to protect organisms from chemical stress by removing toxic chemicals. Studies described in this dissertation were designed to prove this hypothesis. We tested the hypothesis by examining whether the inhibition or augmentation of Ralbp1 would sensitize or protect cells and whole animals from the toxic effects of chemicals and

[1] The human gene RALBP1 (18p 11.22) encodes a splice-variant designated RLIP76 when it was originally cloned through yeast two-hybrid method as the first known Ral effector by Julien-Flores et. al (Flores 2003). The corresponding animal genes from rat (Ralbp1, Ral-binding protein-1) and mouse (Rip1, Ral-interacting protein-1) encode a splice variant protein homologous to RLIP76, designated Ralbp1 (also written as RalBP1). For the purpose of this manuscript, we will use Ralbp1 to refer to the 76 kDa splice variant protein, and RALBP1 when referring to the gene.

radiation. These studies establish that Ralbp1 is a primary determinant of resistance to chemical stressors by the direct transport of these toxins.

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CHAPTER 1

INTRODUCTION

Cellular processes are tightly regulated to ensure that signals are properly relayed for the cell to maintain homeostasis. External stressors can cause instability of these tightly regulated systems and lead to diseases including metabolic disorders as well as cancer. Cancer is a leading cause of death in the developed world, with more than 1 million people diagnosed annually with various types of cancer in the United States alone (Howe 2007).

Cancer results from stress-induced chromosomal damage leading to mutations accumulating within the chromosomes (Arlt 2006). These mutations modify how the cell transcribes (DNA→RNA), translates (RNA→Protein) and/or processes mitosis/apoptosis, events that cause cells to exhibit autonomous growth along with characteristic morphology and behavior that identify it as cancerous. These characteristics include sequential appearance of: 1) over-expression of growth factors and/or resistance to growth-suppression factors, 2) increased rate of cell-proliferation, 3) immortality due to elongated telomerase, and suppression of programmed cell-death (apoptosis), 4) generation of new blood vessels (neovascularization), 5) ability to invade basement membranes and metastasize, 6) and disregard inhibitory or regulatory signals from immediate cellular environment with resultant crowding-out normal cells (Klaunig 2004). The culmination of these events leads to disseminated malignancy and death of

the host. Understanding how stress in its many facets alters proteins or genes involved in apoptosis and cellular regulation allows for the scientific community to design methodologies to inhibit or reverse the progression of stress related diseases including cancer.

Carcinogenesis

Exposure to stress in the form of radiant stress or chemical stress leads to carcinogenesis. Both exogenous and endogenous stressors results in carcinogenesis through cumulative damage to DNA directly, or indirectly through formation of reactive chemical species that arise from interaction of radiant energy with water as well as organic compounds including lipids, carbohydrates, proteins, nucleotides, and macromolecular complexes. It is estimated that ambient chemical and radiant stress inflicts about 10^5 lesions in cellular DNA each day (McKeown 2006). Carcinogenic lesions include adducts formed by reactions between nucleophilic sites on DNA-bases and electrophilic groups on endogenously generated or exogenous toxins. Such reactions can also occur between reactive toxins and cellular proteins and lipids. The products of these reactions can directly or indirectly inactivate tumor-suppressors, activate oncogenes, or disregulate mitotic check-points, resulting in unchecked cell-proliferation and other malignant phenotypes (Poklar 1996, Ioannou 1996, Chan 1993, Kastan 2004). These carcinogenesis mechanisms are briefly reviewed here.

Endogenous Carcinogens

Oxidative degradation of membrane and other lipids give rise to a large number of highly electrophilic and mutagenic species (Douki 2004). Lipid-derived reactive oxygen species (ROS) arising from poly unsaturated fatty acids (PUFA) are also known

to directly affect the balance between proliferation and apoptosis by interacting with and regulating key oncogenes including Ras, Ral, Myc, and tumor suppressors including P53 (Chang 2003). Oxidative degradation of membrane lipids occurs mainly through lipid peroxidation, a self sustaining chain reaction that results in formation of thousands of lipid-hydroperoxides starting with a single free-radical initiating reaction. Transition metal ions and other highly reactive chemical species with the tendency to participate in one-electron oxidation-reduction reactions (thus generating chemical species with unpaired electron, free-radicals), or high energy radiation are necessary to initiate the free-radical chain reaction known as lipid peroxidation. This process is markedly accelerated (propagated) by the presence of superoxide anion ($O^{\cdot}_2$) that is generated from mitochondrial electron leakage (Garrett 1999) as well as other oxidative processes in lysosomes and peroxisomes and endoplasmic reticulum. The CYP450 system which resides in the endoplasmic reticulum is a contributor to this process because of generation of reactive oxygen species through mono-oxygenation reactions and through formation of quinone containing compound that can undergo redox cycling to generate super oxide anions (figure 1.1).

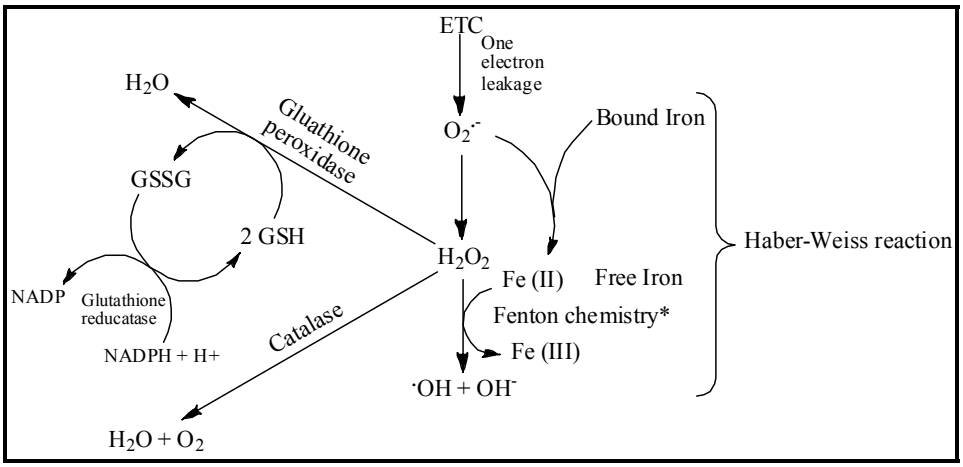

Figure 1.1 Oxygen Radical Formation and Quenching

Free radical reactions involving oxygen can be referred to as reactive oxygen species (ROS). The most common ROS include: the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($OH\cdot$), and hydrogen peroxide (H_2O_2). The electron transport chain (ETC) utilizes oxygen as the terminal electron acceptor as seen in Figure 1.1. When oxygen acquires an additional electron in the ETC the formation of superoxide anion occurs leaving the molecule with one unpaired electron. Literature suggests (Santago 2006) that anywhere from 2 to 5% of the oxygen present in the ETC forms the highly damaging superoxide anion radical. The toxicity of superoxide anion is the ability to liberate bound iron in the cell thus facilitating Fenton chemistry and as the primary step in the Haber-Weiss reaction forming the highly toxic ROS, the hydroxyl radical. Superoxide anions are also responsible in initiating lipid peroxidation of polyunsaturated fatty acids (PUFA's) forming lipid hydroperoxides Figure 1.3. The enzyme superoxide dismutase involves the dismutation of superoxide into oxygen and hydrogen peroxide. If H_2O_2 is not converted into water by the enzymes catalase and glutathione peroxidase the second step of the Haber-Weiss reaction proceeds leading to formation of the hydroxyl radical. Hydroxyl radicals are short-lived, but are the most damaging ROS. The formation of the hydroxyl radical via the Harber-Weiss reaction is slow but the interaction of iron (Fenton chemistry) acts as a catalyst for enhanced production of $OH\cdot$. *Fenton chemistry can occur with additional transition metals including copper, manganese, chromium, and nickel.

Superoxide anion O_2^{-}• are also generated in cytosol through a reduction of molecular oxygen during respiratory burst by NADPH oxidase (Afanas'ev 1989). Hydrogen peroxide is then formed through superoxide anions by enzymes possessing transition metal centers (Fe (II), Cu (I), Ni (II), Mn (II), and Cr (V)). Hydroperoxides contribute to the formation of the hydroxyl radical •OH (Halliwell 1984). The hydroxyl radical is the strongest oxidant in the cell with a 1-electron reduction potential of 2.8 V. It is responsible for reactions with biomolecules including nucleic acids (DNA and RNA), proteins (enzymes and regulatory proteins) and lipids (membrane) (figure 1.1) (Adler 1999).

Hydroxyl radicals are formed from interactions of high energy radiation with water molecules, or from reaction of hydrogen-peroxide and superoxide anions with transition metals. The hydroxyl radical is the only oxidant in biological systems capable of abstracting a hydrogen atom from membrane lipids leading to the formation of lipid alkyl radicals ($L^{\cdot}$). This event, known as 'initiation', leads to subsequent combination of the $L^{\cdot}$ with O_2 to form the highly reactive lipid hydroperoxy radical ($LOO^{\cdot}$). $LOO^{\cdot}$ is sufficiently reactive to cause double stranded DNA breaks. Unlike $OH^{\cdot}$ that has a very short $t_{1/2}$ in the order of 10^{-14} s, $LOO^{\cdot}$ are sufficiently stable ($t_{1/2}$ up to several seconds) and can diffuse considerable distances from the site of their formation (i.e. in or near membranes) to other macromolecular structures including DNA in the nucleus. The formation of $LOO^{\cdot}$ is critical for the propagation of the chain reaction because $LOO^{\cdot}$ can abstract alkyl hydrogen from surrounding lipids forming another lipid hydroperoxide (figure 1.2).

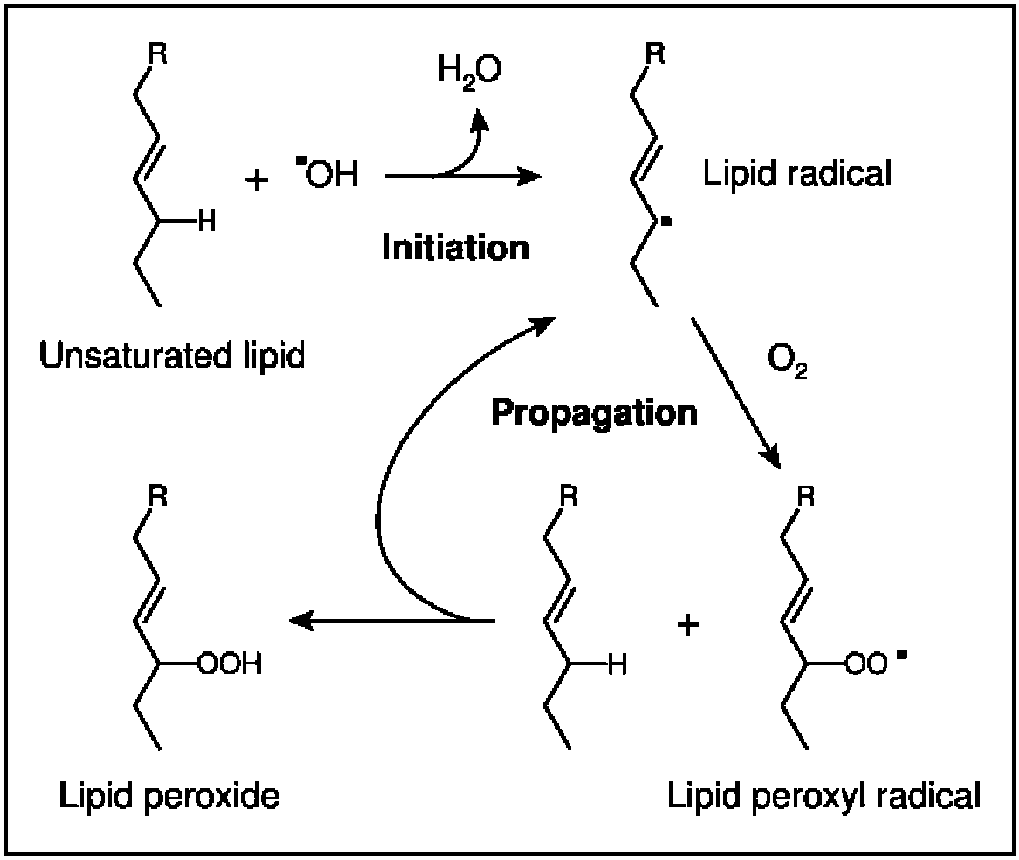

Figure 1.2 Lipid Hydroperoxide Formation

Polyunsaturated fatty acids (PUFAs) are fatty acids which contain more than one double bond. These lipids are abundant in cellular membranes and these PUFAs permit fluidity in membranes. ROS target the carbon-carbon double bond of PUFA. This is preferred because the double bond on the carbon weakens the carbon-hydrogen bond allowing for easy dissociation of the hydrogen by a free radical. A free radical (noted by the hydroxyl radical ·OH) prefers to steal electrons from the lipid membrane of a cell, initiating a free radical attack on the cell known as lipid peroxidation. A free radical will steal the single electron from the hydrogen associated with the carbon at the double bond satiating its electron deficiency. In turn this leaves a lipid radical. In an effort to stabilize the carbon-centered free radical molecular rearrangement occurs. The reaction with oxygen leads to the formation of a peroxy radical. The peroxy radical steals an electron from another lipid molecule and in the process propagates the lipid peroxidation process. This process continues in a chain reaction leading to increased formation of 4-HNE (figure 1.4).

regenerated. DNA damage by these ROS has been established through analysis of DNA repair; adduct formation and site directed mutagenesis studies on prokaryotic and eukaryotic cell lines (DeBont 2004, Nestmann 1996, Gupta 1999).

The unsaturated fatty acid hydroperoxides tend to undergo β–scission at their double-bonds to give rise to highly electrophilic alkenal moieties of various length. The dominant electrophilic alkenal is 4-hydroxy-trans-2-enal (4HNE) (figure 1.3) that is formed from the β–scission of hydroperoxides of n-6 polyunsaturated fatty acids , namely 15-hydroperoxyeicosatetraenoic acid (15-HpETE) and 13-hydroperoxy-linoleic acids (Bacot 2003, Guichardant 2006, Esterbauer 1998). 4HNE has three functional groups; the aldehyde moiety, the double bond and the hydroxyl group. Each of these reactive substituents can react and form adducts or even cross-links with cellular molecules and macromolecular structures.

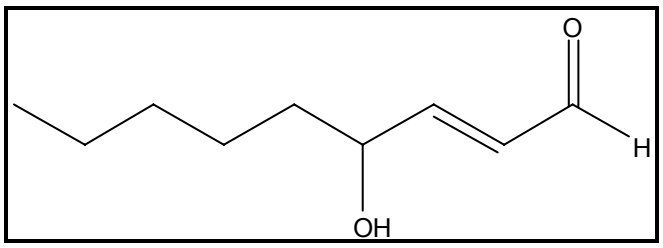

Figure 1.3 Structure of 4-hydroxy-trans-2-nonenal (4HNE)

4-hydroxy-trans-2-nonenal (HNE, 4-HNE) is a secondary oxidation chemical compound generated in the oxidation of lipids containing polyunsaturated omega-6 acyl groups, such as arachidonic or linoleic groups, and of the corresponding fatty acids.

Biological effects of 4HNE are very concentration-dependent. A vast body of literature accumulated demonstrates that 4HNE is directly involved in regulation of critical processes affecting cellular proliferation, differentiation, and apoptosis in a

concentration dependent manner (Barzilai 2002). Whereas low concentration of alkenals appear to be important for activation of signals that cause cell proliferation, very high concentration can lead to apoptosis through cross linking cellular macromolecules (Lamas 2006, Esterbauer 1991, O'Brien 2003 Abuja 1995,Young 2001, Esterbauer 1993, Esterbauer 1991). High concentrations (>20 μM) of 4HNE are acutely toxic (Esterbauer 1998) due to acute depletion of cellular thiols, and cross-linking of cellular macromolecules through electrophilic adducts and Schiff's base formation. Observable cellular changes associated with cell death through necrosis or apoptosis include lactate release, inhibition of glycolysis, inhibition of DNA and RNA synthesis, depletion of protein thiols, depletion of non-protein thiols (principally GSH), and enhancement of lipid peroxidation (Esterbauer 1993).

Moderate concentrations (1 to 20 μM) have been shown to cause inhibition of DNA, RNA and protein synthesis, inhibition of c-myc expression and stimulation of phospholipase-A2 (Esterbauer 1991). Inhibition of cell proliferation at concentrations between 1-20 μM appears to involve G2-phase arrest (Esterbauer 1991). These moderately high concentrations of 4HNE are also known to induce expression of stress-protective signaling mechanisms such as cellular Jun- kinase (JNK), transcription factors such as NFκB, and stress-protective proteins referred to as chaperones that function to keep cellular proteins in their proper functional conformation. Transcription factors such as HSF-1, that regulate the expression of chaperones, are known to be regulated by 4HNE (Kaarniranta 2005). If the stress is of sufficiently low magnitude and short duration to generate only enough 4HNE to turn on these stress-defense

mechanisms, their protective effects can predominate and prevent the onset of apoptosis; certainly, longer-duration or higher intensity stress that can overwhelm these defenses will lead to apoptotic cell death. In stark contrast to the effect of high or moderately high concentrations of 4HNE, low concentrations of 4HNE (up to 1 μM) appear to be required for physiological signaling including chemotaxis, adenylate cyclase modulation, guanylate-cyclase activity, and stimulation of phospholipase C (Siems 1992).

Exogenous Agents

In addition to endogenous mutagenic ROS and 4HNE, the cell is subjected to foreign toxic compounds (xenobiotic) which may have electrophilic or free radical behavior themselves or which may be metabolized by CYP450 into oxidant or free radical compounds. The formation of ROS through lipid peroxidation is also the primary mechanisms through which radiant stressor (heat, UV and X-irradiation) also exert their toxic effect. The toxicity of lipid-derived ROS is mediated through both direct toxic effects such as alkylation/scission of DNA and through regulation of signaling proteins known to be involved in defense of radiation toxicity. Ionizing radiation exposure stimulates signal transduction pathways. Xenobiotics are metabolized through enzymatic pathways that protect the cell. These pathways protect cells through the detoxification of xenobiotics, but in some instances result in the formation of activated metabolites that can react with bio-macromolecules (e.g. DNA, RNA, protein and lipids) (Katzung 2007). Xenobiotics can react with endogenous compounds enhancing their potency. Activation of xenobiotics occurs mainly in liver

and kidneys, since these organs are rich in CYP450 enzymes, also known as phase I biotransformation or phase I xenobiotic metabolism. Activated metabolites of xenobiotics generated in the liver can diffuse into the vascular system and distribute themselves throughout the body contributing to systemic toxicity. Through interaction with susceptible functional groups, xenobiotics exert their toxicity through the generation of electrophiles/nucleophiles, the evolution of free radicals, and reduction-oxidation active reactants (Klaassen 2001).

General Mechanisms of Xenobiotic Activation

Xenobiotics can display free radical mechanisms resembling those of endogenously formed free-radical toxins (Klaassen 2001). Initiation of a radical reaction by xenobiotics occurs via accepting or donating one electron, often resulting in homolytic fission of a covalent bond. Cytochrome P450 CYP450 activate many commonly used drugs through monooxgenation or homolytic bond fission, often called reductive fission. Redox cycling by quinone-containing compounds such as the anthracycline chemotherapy drug, doxorubicin (DOX; Adriamycin®) can lead to the generation of many superoxide anion radicals by redox cycling that generates $O^{\cdot}_2$ (Klaassen 2001). Bleomycin (BLM), another cytotoxic antineoplastic drug, can give rise to ROS formation directly, through participation in Fenton's transition metal catalysis (Hasler 1999).

Electrophiles are compounds exhibiting an electron deficiency. These molecules display partial or full positive charge characteristics and are often generated in the biotransformation or solubilization of lipophilic xenobiotics. Electrophiles are

often produced by insertion of an oxygen atom in the form of functional groups such aldehydes, ketones, and carboxylic acids which withdraw electrons from the neighboring carbon (Klaassen 2003). Unsaturated molecules containing functional groups can also display electrophilic characteristics as mentioned for α,β-unsaturated aldehydes and ketones. Formations of some electrophiles are catalyzed by CYP450, a detoxifying enzyme (Guengerich 2003).

Although many electrophilic products result from metabolism of xenobiotic compounds by CYP450, the majority of products of xenobiotic/drug metabolism are relatively nucleophilic compounds that are further metabolized by phase II biotransformation to glucuoronide or sulfate conjugates. Most nucleophilic products of CYP450 metabolism are relatively un-reactive and non-toxic, but some examples of toxic nucleophiles are known, and selenite is a good example (Tarze 2007).

Chemotherapy treatment of cancer cells frequently utilizes electrophilic compounds such as alkylating agents, which are subject to metabolism primarily by phase II biotransformation through conjugation with GSH catalyzed by GSH S-transferases (GSTs). Alkylating agents are so named for their ability to form DNA alkyl adducts leading to fragmentation of DNA by repair enzymes attempting to reverse the adduct. Crosslinking of DNA also occurs to prevent unwinding of DNA during transcription. Major classes for alkylating agents include; nitrogen mustards, ethylenimines, alkyl sulfonates, nitroureas, and triazenes (Dorr 1994).

Biotransformation of Xenobiotics

The biotransformation of xenobiotics, metabolism of xenobiotics, or xenobiotic chemistry are the terms synonymous for investigation into biomolecules namely proteins responsible for removal of xenobiotics and endobiotics. This process provides a pathway for converting toxic chemicals entering the cell into less toxic compounds that can be removed from the cell, thus reducing chemical stress.

Dr. R.T. Williams in 1963 published "Detoxication Mechanisms in Man" (Williams 1963). This manuscript laid the groundwork for modern toxicology when he proposed that xenobiotics were metabolized into two phases (figure 1.4), the first includes oxidations, reductions, and hydrolyses of the xenobiotic (phase I) and the next is comprised of conjugation reactions of the xenobiotics with cofactors (phase II) (Tredger 1991).

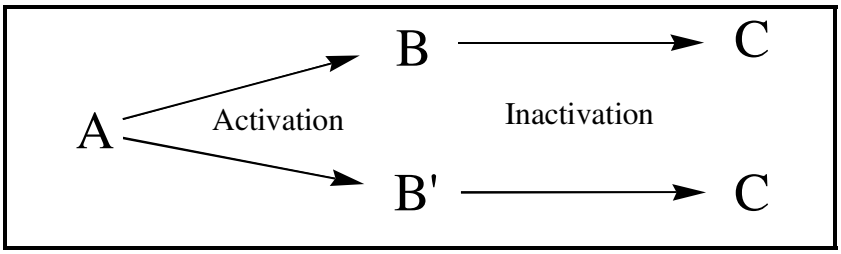

Figure 1.4 Detoxification Mechanisms (Williams 1963)

Recently, a third phase of biotransformation (phase III) describes proteins involved in transport of xenobiotics into the extracellular matrix by utilizing ATP (Ioannides 2002). Phase I enzymes include flavin monooxygenases, CYP450, aminoxidases, nitroreductases, azoreductases, and molybdenum hydroxylases (Klaassen 2003). Phase II enzymes responsible for the conjugating of xenobiotics include UDP

-Glucuronosyltransferases, sulfotransferases, arylamine acetyltransferases, methyltransferases, and of most important for electrophile detoxification, glutathione S-transferase GSTs. Phase III enzymes/mechanisms are as yet incompletely characterized and are the subject of intense interest at present. Current classification of this phase includes the ATP binding cassette (ABC) transporters and non-ABC members.

Phase I (Activating Enzymes)

The first step or phase of xenobiotic metabolism appears to be aimed at increasing the hydrophilicity of these compounds, most of which are lipophilic. Hydrolyzing, reducing, and/or oxidizing the foreign compound by members of phase I allows for an increase in polarity to solubilize the compound and creates functional groups (-OH, -SH, -NH, -COOH) such that phase II enzymes can subsequently react. Table 1.1 lists of the members of phase I along with their classification based on chemical reaction.

The phase I enzymes, the cytochrome P 450 (CYP450) monooxygenases, are best known for their ability to solubilize a diverse array of compounds (Guengerich 1988, Guengerich 1988). This family of enzymes is also known for activating intermediates into reactive species such as with benzo[a]pyrenediol epoxide (Waxmann 1999). The CYP450's name is based on the presence of a heme prosthetic group containing reduced state (Fe^{+2}) that complexes with carbon monoxide to yield a pigment that has a λ_{max} at 450 nm. CYP450's comprise a supergene family. There are 57 known CYP450s in at least 18 families in humans (Nelson 2003, Nelson 1998). Distribution of CYP450's is found in all tissues in microsomal fraction (endoplasmic reticulum) and

mitochondria (Yost 2001). CYP450 is a mono-oxygenase that inserts one atom of oxygen into a substrate.

CYP450 oxidation reactions include hydroxylation of compounds, epoxidation of a double bond, oxidative group transfer, cleavage of esters, dehydrogenation, and heteroatom (S, N, I) oxygenation/ N-hydroxylation/ (O, S, N, and Si) dealkylation (De Montellano 2005, Guengerich 2001).

Table 1.1 Enzymse Responsible for Phase I Xenobiotics Biotransformation (Willams 1971)

Reaction	Enzyme	Localization
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro- reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Flavin-monooxygenase	Microsomes

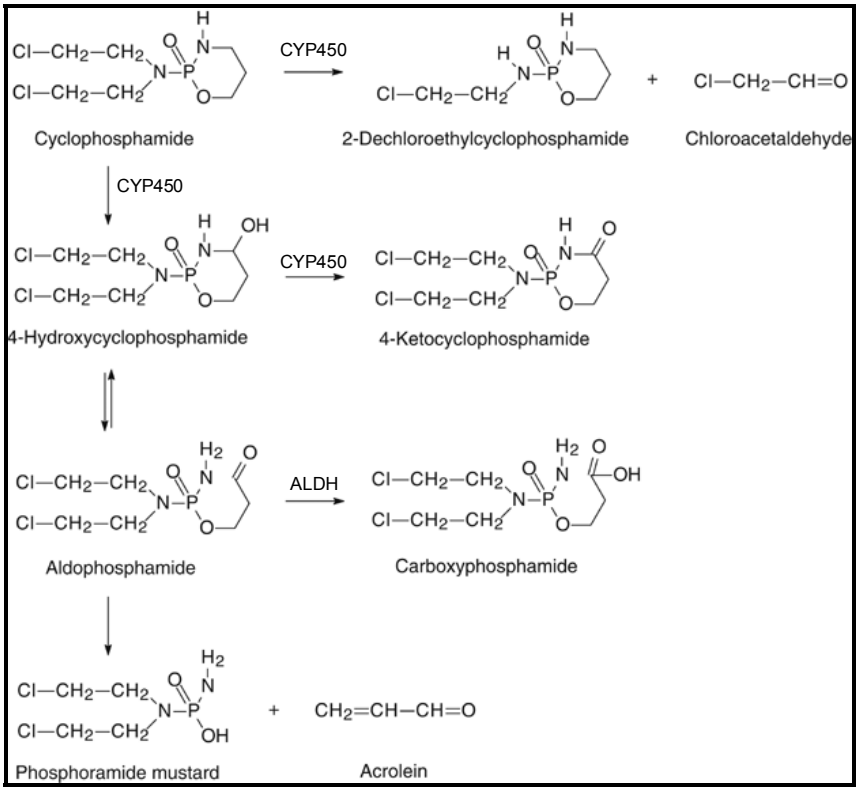

Figure 1.5 Pharmacology of cyclophosphamide through CYP450

Metabolism of cyclophosphamide, this intermediate of cyclophosphamide is in tautomeric equilibrium with aldophosphamide. 4-Hydroxyphosphamide is capable of spontaneously decomposing into the alkylating metabolite phosphoramide mustard and the nephrotoxic acrolein (Tim 2005). In a minor pathway, CYP450s result in release of the neurotoxic agent chloroacetaldehyde and 2-dechoroethylcyclophosphamide. The transformation of 4-hydroxyphosphamide into 4-ketocyclophosphamide occurs through CYP450 and carboxyphosphamide is formed by different isoforms of aldehyde dehydrogenase, ALDH.

The normal metabolic pathway for CYP450 is the addition of one atom of oxygen from the heme group is inserted into the substrate to produce the product (Dawson 1988, Schlichting 2000, Goeptar 1995). If the catalytic cycle of CYP450 is disrupted this disruption lead to production of superoxide anion, $O_2^{\cdot-}$ and H_2O_2 (De Montellano 2005). Activation of xenobiotics and endobiotics by CYP450's can enhance potency of toxins which can be genotoxic carcinogens, or even genotoxic antineoplastic agents (Bondy 1994). An excellent example is the metabolism of the nitrogen mustard cyclophosphamide; CYP450's activate cyclophosphamide into a powerful antineoplastic compound (Figure 1.5).

Hydroxylation of the α-carbon to the nitrogen ring leads to the rearrangement of cyclophosphamide to phosphoramide mustard releasing the toxic by-product acrolein (Sladek 1973). The specific example of cyclophosphamide activation thru CYP450 demonstrates one of the many positive examples of utilizing the enzymatic activity of CYP450. Introduction of compounds to CYP450 such as benzo[a]pyrene leads to a more potent carcinogen than the parent molecule (Fig. 1.6) (Parke 1987).

Flavin monooxygenase's (FMO) are enzymes that require flavin adenine dinucleotide (FAD) to oxidize a broad range of compounds (Lawton 1994). The predominant reactions for monooxygenases are nucleophilic heteroatoms (nitrogen, sulfur, and phosphorus) containing compounds to form their respective oxides (P-oxides, N-oxides, or S-oxides). Unlike CYP450 superfamily, the FMO family has only 6 isozymes (FMO1-FMO6) (Stehr 1998). FMO acts as an electrophilic oxygenating catalyst unlike CYP450 is reported not to oxidize carbon atoms. Other notable

differences in FMO biochemistry are the lack of induction of FMO by compounds known to induce CYP450, like phenytoin and ethanol (Klaassen 2001). FMO, like CYP450 requires NADPH and oxygen to catalyze the oxidation of a substrate (Cashman 1995). These activated compounds produced by phase I are toxic and can induce apoptosis. Fortunately phase II further metabolizes these xenobiotics to relatively un-reactive amphiphilic molecules that can be excreted safely.

Phase II (Conjugating Enzymes)

Phase II enzymes assists in the detoxification of reactive electrophiles and nucleophiles by the conjugation with biochemical cofactors facilitating their inactivation (Table 1.2).

Table 1.2 Enzymes Responsible For Phase II Biotransformation

Enzyme	Subcellular localization
Glucuronide conjugation	Microsomes
Sulfate conjugation	Cytosol
GSH conjugation	Cytosol, microsomes
Amino acid conjugation	Mitochondria, microsomes
Acylation	Mitochondria, cytosol
Methylation	Cytosol, microsomes, blood

Reactive electrophiles and nucleophiles that are substrates of phase II enzymes can come directly from extracellular sources, arise from intermediary metabolism, or by metabolism of xenobiotics by Phase I enzymes as described above. Products of phase II metabolism can function to inactivate toxins as well as to produce active metabolites or signaling molecules that exert important chemical and biological effects inside or outside cells (Liska 1998).

Glucuronidation is the major phase II xenobiotic path accounting for nearly 1/3 of drug conjugation. Glucuronidation UDP-glucuronosyltransferases, UGT's, (EC 2.4.1.17) are

a family of proteins consisting of 50-57 kDa enzymes that utilizes the cofactor uridine-5'-diphospho-α-D-glucuronic acid (UDP-GA) (Figure 1.8) to conjugate nucleophlic compounds (Iyanagi 2007).

These enzymes are distributed in endoplasmic reticulum of most tissues. There are approximately 30 isozymes of UGT divided into two families. UGT's prefer electron rich nucleophilic heteroatoms (oxygen, nitrogen, and sulfur) and target phenolic, alcohol, carboxylic acids, primary and secondary amines, and thiol functional groups as substrates. Molecules conjugated via UGT's produce polar products can be excreted in urine or bile (Hollinger 2003, Iyanagi 2007).

Sulfotransferase (SULT) (EC 2.8.2.2) another family of cytosolic phase II enzymes is capable of sulfonate conjugation utilizing the cofactor 3'-phosphoadenosine-5'phosphosulfate (PAPS).

44 SULT's have been identified in humans and 14 individual SULT isozymes are present (Bosch 2006, Nowell 2006). Substrate specificity is geared toward the large production of metabolites from phase I (aliphaltic alcohols and phenols) but is not limited to just compounds from phase I, some aliphatic/aromatic amines and aromatic hydroxylamine/amide can also be sulfonated (Raftogianis 2001). Unfortunately the sulfonate moiety being polar enhances the toxicity of these lipophilic molecules leading to tumorgenic metabolites. Redundancy in phase II limits sulfonation toxicity.

Acetyltransferases NAT's (EC 2.3.1.5) are enzymes that utilize Acetyl CoA to acetylate xenobiotics. This route is preferred for aromatic amines and hydrazines.

N-Acetylation is also important in mercapturic acid metabolism, where the GSH conjugates are acetylated to mercapturic acids. Distribution of NAT's are membrane

bound and their mechanism of action involves two sequential steps (Dupret 2005, King 1997); transfer of the acetyl group to a cysteine residue within NAT's followed by transfer of the acetyl group to the substrate. Expressions of NAT's are limited with only two isoforms existing in humans. NAT's can activate or deactivate molecules; activation can result through a CYP450 O-Acetyltransferase reaction as in the case of certain hydroxylated amines (Klaassen 2003, Hein 2002).

Methylation is a minor route of xenobiotic transformation in respect to UDP-glucocuronidation and sulfonation/GSH conjugation. Methionine adenosyltransferase (MAT) catalyze this reaction. These enzymes utilize the cofactor S-adenosylmethione (SAM) to methylate phenols, aliphatic and aromatic amines, N-heterocyclics, and sulfhydryl containing compounds (Loenen 2006).

Unlike other phase II enzymes the attachment of methyl moieties enhances lipophilicity. The decrease in toxicity is a greater benefit than the slight increase in lipophilicity. These enzymes are cytosolic and are further classified in humans based on the substrate specificity (Klaassen 2003, Fontecave 2004).

Amino acid conjugation can be considered the first discovered xenobiotic metabolism reaction when glycine reacted with benzoic acid to form hippuric acid in 1842 (Smith 1945). Glycine, taurine, and glutamine are samples of the amino acids that are utilized in these types of reaction.

Two types of reactions exist for amino acid conjugation; use of the amino functional group to conjugate carboxylic acids, or the use of the carboxylic functional groups to catalyze aromatic amines. Conjugation of hydroxylamine groups with amino acids conjugation can lead to the formation of nitrenium or carbonium ions from the reactive

N-ester product (Klaassen 2003).

GSH conjugation is of central importance to this manuscript because of its contribution to the discovery of Ral binding protein 1 (Ralbp1, RLIP76). Glutathione S-transferases, GST's (E.C 2.5.1.18) are enzymes capable of reacting the cofactor GSH to any electrophilic compound leading to the formation of a thioether and constitute upward of 10% of total cytosolic protein in some tissues (Nadkar 2006).

Functional groups targeted for GSH conjugation include epoxides, alkenes and electrophilic centers such as C, S, or N (Nebert 2004). The reaction mechanism can be an addition of GSH (such as to alkene epoxide) or the nucleophilic substitution using GSH as the nucleophile displacing the electrophile (Douglas 1987). The use of GSH as an antioxidant has been show to be important in mitigating the degradation of membrane lipids through quenching of lipid peroxidation by GSH peroxidase (Harman 1986, Ramakrishna 1990). Once GSH is conjugated to the xenobiotic, direct export of breakdown of the xenobiotics to a mercapturic acid or direct efflux from phase III allows GSH metabolite to be excreted from cells. GSTs are found in the cytosolic as well as microsomal fractions. Cytosolic GST's are considered a super family, possessing 7 gene families (α, μ, π, K, θ, ϕ, Z) with several sub-families within each family. Over 40 GST's or GST related proteins have been described in humans (Bjornestedt 1992). This large number of GST isozymes with different substrate specificity allows GST to conjugate a broad range of toxins. Although the products of GSH conjugates by GST's are generally less reactive, these enzymes can actually activate some compounds such as haloalkanes and haloalkenes (Dekant 1993, Monks 1990, Farber 1987). GST's also contribute to cell signaling through conjugation of

GSH and arachidonic acid leading to the formation of prostaglandins and leukotrienes (Penrose 1999, Hayes 1995, Shimizu 1990).

GST are particularly susceptible to product inhibition because of the realative high affinity of the active site for the product of glutathione conjugates. Thus, the activity of members of phase III enzymes in removing the glutathione conjugates is crucial to maintain the activity of GSTs.

Phase III (Transport Proteins)

Proteins of this class require energy in the form of ATP to transport metabolites of phase I and phase II, such as GSH conjugates, out of the cell. Two distinct classes of transporter proteins are currently known: ATP binding cassette transporters (ABC proteins) and non-ABC transporter proteins; the former being a superfamily of proteins. Structurally, ABC transporters posses at least two transmembrane domains and two ATP binding domains (Walker domain A and B) allowing them to actively transport drugs across membranes (Sarkadi 2006). They are grouped into superfamilies according to the characteristic of their 'C motif', present in the region following Walker domain B. The non-ABC transporter proteins are structurally quite dissimilar from ABC proteins, and include major vault protein (MVP) and Ral binding protein 1 (Ralbp1). Members of phase III are primarily responsible for stress resistance by decreased drug accumulation within the cell.

Problem: Stress Resistance-Drug Accumulation Defect

Presence of these three phases provides an opportunity for cells to survive oxidant exposure. However, the same mechanisms are also responsible for contributing to drug and radiation resistance in cancers.

The etiology of multidrug resistance by drug accumulation defect can be attributed to membrane, cytoplasmic, and nucleus alterations creating a situation negating the accumulation of the target compound. These alterations lead to cancerous tissues exhibiting resistance to a broad spectrum of structurally and chemically unrelated toxins. This term 'stress resistance' is more often attributed to the over expression of proteins contributing to phase III. Elevated expression of membrane-associated efflux proteins has been a defining cause through reduced accumulation of toxins.

P-glycoprotein (Pgp)

P-glycoprotein (Pgp) is a 170-180 kDa membrane-associated protein found in many tissues but is highly expressed in tissues with barrier functions such as liver, blood brain barrier, kidney, intestine, placenta and apical membranes. Introduction of Pgp as a multi-drug resistant protein was first recognized in CHO cells (Ficker 2004). In normal tissues Pgp posses a broad range of expression levels (Sarangi 1989). Its presence at the blood brain interface allows prevention of hydrophobic compounds to enter the central nervous system. Two molecules of ATP are required for this active efflux however the exact mechanisms of this action are debatable (Sauna 2000, Borges-Walmsley 2003, Hoffmann 2004). It has been implicated in energy-dependant transport of various neutral and cationic hydrophobic drugs out of the cellular membrane causing decreased drug accumulation. P-gp is demonstrated to be capable of transporting drug classes

anthracyclines, Vinca alkaloids and taxanes. Thus it has been widely studied as an indicator of multidrug resistance in various malignancies (Hamada 1986, Cornwell 1986, Horio 1985). Although P-gp is implicated in drug resistance, its expression and correlative stress resistance is inconsistent in clinical studies. Its over-expression was originally attributed to resistance (Beaulieu 1997) but under further review the conflict between P-gp expression levels, efflux activity and their corresponding effect warrant the exploration of other contributors to drug accumulation defect.

Multidrug Resistance Protein (MRP)

Biochemical investigation of Pgp-independent multi-drug resistance led to the identification of a family of drug resistance proteins termed multi-drug resistance associated protein (MRP) that were discovered following the cloning of MRP gene in 1992 (Cole 1992, Flens 1996, Taniguchi 1996, Kool 1999). MRP, a 190 kDa membrane-associated efflux protein, was subsequently shown to be a member of a large transporter superfamily refered to as the ATP binding cassette (ABC), to which P-gp also belongs (Borst 1999, Cole 1992).

MRP is ubiquitous and is expressed at a basal level in almost all normal tissues, while increased expression is common in tumor tissues (Choi 2005, Kruh 1995). Although structurally similar the notable difference of this protein from Pgp is the capability of transporting predominantly neutral or anionic organic compounds. MRP is capable of transporting organic anionic substrates with a noticeable activity towards these negatively charged compounds that have undergone phase II biotransformation (Loe 1996, Loe 1998, Kruh 1994, Breuninger 1995). In rare cases it has been demonstrated that positively charged chemotherapy drugs could be transported in conjunction with

the cofactor GSH (Tew 1994). Contribution of MRP1 to drug accumulation however mirrors that of Pgp and appears to conflict also among studies (Slovak 1993,Barrand 1994, Gekler 1995, Binaschi 1995, Eijdems 1995, Jain 1996, Withoff 1996, Vernhet 1999, Yoshida 2001). Similar inconsistency observed in biopsy samples, as only less than 1/3 of the samples studied had elevated MRP expression levels (Nooter 1996, Zhan 1997, Young 1999, Dingemans 1999). In MRP transfected HeLa cell lines resistance to doxorubicin, vincristine and etoposide was observed but not cisplatin (Grant 1994, Brock 1995, Campling 1997). The discrepancy is also observed in mitoxantrone-selected, taxol-selected and many other multidrug-resistance human tumor cells where MRP overexpression does not play a primary role (Futscher 1994, Binaschi 1995, Giaccone 1996, Laurencot 1997). In a drug accumulation study the decrease in drug level was not proportional to the expression of either the MRP mRNA or the protein in all of the non expressing P-gp resistant sub-lines (Versantvoort 1995). Analysis of the literature for MRP suggests that this second family member from ABC efflux family is not the predominant contributor to multidrug resistance.

Breast Cancer Related Protein (BCRP)

The observation of a ATP dependent transport in cell not over expressing Pgp or MRP suggested the existence of another transporter protein(s). This was confirmed by the finding of breast cancer resistance protein (BCRP) in MCF-7/AdrVp cell lines (Doyle 2005). BCRP a 72 kDa protein that is the third ABC transporter that has been implicated in multi drug resistance via drug accumulation defect. It is a half transporter in structural comparisons to its other family members Pgp/MRP, possessesing only one

ATP binding site. Its forced overexpression confirmed resistance to mitoxantrone, irinotecan, hydrocampothecin, mitomycin-C, doxorubicin in acute myloid leukemia (Raaijmakers 2005, Ciardiello 2000). The molecular targeting of BCRP in NSCLC patients with gefitinib, a BCRP substrate has shown to reduce drug resistance to chemotherapy (Nakamura 2005). However, BCRP is not significantly expressed in most human tissues including heart, lung, muscle, kidney, pancreas, spleen, thymus, or leukocytes (Krishnamurthy 2006) and by itself is not sufficient to explain the phenomenon of non-MRP, non-Pgp drug resistance, suggesting that other transporters are involved in mediating the accumulation defects observed in cells with multi-drug resistance.

Ralbp1

The ABC transporters were identified using genetic screening approaches that studied acquired drug-resistance due to repeated drug exposure. Another approach taken to identify the phase III transporters relied on a hypothesis that the xenobiotic/endobiotic transporter would be capable of binding numerous structurally unrelated toxins and that this would stimulate nucleotide hydrolysis, with resultant energy utilized for trans-membrane efflux.

In search for such transporters, an activity designated dinitrophenyl S-glutathione (DNP-SG) ATPase was identified in membrane fractions of human cells. This ATPase activity was stimulated in the presence of the GSH conjugate, DNP-SG (Sharma 2001, Awasthi 1994). Affinity purification, antibody preparation, and expression library screening with the antibodies repeatedly yielded the cloning of Ralbp1 a protein previously cloned from yeast 2 hybrid systems as a Ral-binding effector that was though to link the Ral-Ras pathway (Flores 2004). The identity of Ralbp1 with DNP-SG ATPase was established in a series of studies showing that 1) Recombinant Ralbp1 could be purified to apparent homogeneity using the same ligand affinity chromatography (DNP-SG-Sepharose) used to initially purify DNP-SG ATPase; 2) Recombinant Ralbp1 also is an ATPase which is stimulated by the same substrates and in a similar fashion to that previously established for DNP-SG ATPase.

Ralbp1 is a 76 KDa 655 amino acid splice variant encoded by the RALBP1 gene (18p11.22) (Awasthi 2000). It has been shown to be a Ral-binding protein, and a Rho-GTPase Activating Protein (GAP) protein that participates in clathrin coated

endocytosis (Rosse 2003, De Ruiter 2000). Expression of Ralbp1 is ubiquitous in all tissues including kidneys, liver, erythrocytes, leukocytes and present in most cancer cell lines (Awasthi 2002). Distribution of Ralbp1 in the cell is reported mainly in the membrane although recent studies, show significant presence in cytosol as well (Yadav 2004).

At the initiation of these studies that are the subject of this report, the transport activity of Ralbp1 had been demonstrated against a limited number of substrates, and preliminary studies had demonstrated that Ralbp1 could mediate resistance to some of these transported toxic substrates. Insufficent evidence was available at that time to generalize the findings to state that Ralbp1 could function as a multidrug transporter.

Conclusion

Based on the evidence presented for Ralbp1 as a multi-specific transporter, I hypothesized that Ralbp1 is a major contributor to stress resistance of both endobiotics and xenobiotics. Ralbp1 has been demonstrated to show resistance to radiant stress along with chemical resistance of natural product drugs such as anthracyclines and vinca alkaloids. Overexpression of Ralbp1 should provide protection against stress agents both *in vitro* and *in vivo*. Conversely inhibition of Ralbp1 should decrease stress sensitivity. Because Ralbp1 was shown to be regulated by heat-shock, Heat shock transcription factor 1 was examined for the ability to regulate the transport activity of Ralbp1.

CHAPTER 2

METHODS FOR THE ROLE OF RALBP1

IN STRESS RESISTANCE

The present chapter describes the reagents, methods, and experiments for the present study.

Reagents

The following table 2.1 comprises the common reagents used in these studies.

Table 2.1 Reagents and Sources

Common name	Abbreviation	Formula weight	Chemical composition
Glutathione	GSH	307.1	$C_{10}H_{17}N_3O_6S$
Ethanolamine	MEA	61.1	C_2H_7NO
β-Mercaptoethanol	BME	78.13	C_2H_6OS
1-Chloro-2,4-dinitrobenzene	CDNB	202.5	$C_6H_3ClN_2O_4$
Adenosine Triphosphate	ATP	507.1	$C_{10}H_{16}N_5O_{13}P_3$
3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyletetrazolium bromide	MTT	414.3	$C_{18}H_{16}BrN_5S$
Dimethylsulfoxide	DMSO	78.1	C_2H_6OS
Doxorubicin (Adriamycin®)	DOX	543.5	$C_{27}H_{29}NO_{11}$
Daunorubicin (Daunomycin®)	DAU	527.5	$C_{27}H_{29}NO_{10}$

Table 2.1 continued

Common name	Abbreviation	Formula weight	Chemical composition
Melphalan	MEL	305.1	$C_{13}H_{18}Cl_2N_2O_2$
Cisplatin	CDDP	300.0	$Cl_2H_6N_2Pt$
Mitomycin-C	MMC	334.3	$C_{15}H_{18}N_4O_5$
Vincristine	VCR	824.9	$C_{46}H_{56}N_4O_{10}$
Vinblastine	VRL	810.9	$C_{46}H_{58}N_4O_9$
Navelbine	VLB	778.9	$C_{45}H_{54}N_4O_8$
Busulfan	BUS	246.3	$C_6H_{14}O_6S_2$
Cyclophosphamide	CYP	261.0	$C_7H_{15}Cl_2N_2O_2P$
Radiolabeled GSH	GSH	307.1	$C_{10}H_{17}N_3O_6S$
Radiolabeled DOX	DOX	543.5	$C_{27}H_{29}NO_{11}$
Radiolabeled VLB	VLB	778.9	$C_{45}H_{54}N_4O_8$
Ammonium acetate		77.1	$NH_4C_2H_3O_2$
Curcumin	CURC	368.3	$C_{21}H_{20}O_6$
SM2-bio beads			
Kanamycin	KAN	484.5	$C_{18}H_{36}N_4O_{11}$
Isopropyl β-D-1 thiogalactopyranoside	IPTG	238.3	$C_9H_{18}O_5S$
Phosphoric acid		98.0	H_3O_4P
Methanol	MeOH	32.0	CH_4O
Acetonitrile		41.0	C_2H_3N
Hydrochloric Acid		63.4	HCl
Sodium chloride		58.4	NaCl
Potassium chloride		74.5	KCl
Sodium acetate		82.0	CH_3COONa
Magnesium chloride		95.0	$MgCl_2$
Sucrose		342.3	$C_{12}H_{22}O_{11}$
Perchloric acid		100.4	$ClHO_4$
Trifluoroacetic acid	TFA	114.0	
Sodium borate		127.7	BNa_3O_3
BrCN-activated Sepharose 4B			
Epoxy activated Sepharose 6B			
1-Chloro 2,4-Dinitrobenzene	CDNB	202.5	$C_6H_3ClN_2O_4$
Boric acid		61.8	$H_2B_4O_7$
Sodium dodecyl sulfate	SDS	288.4	$C_{12}H_{25}NaO_4S$
Butylhydroxytoluene	BHT	220.3	$C_{15}H_{24}O$

Table 2.1 continued

Common name	Abbreviation	Formula weight	Chemical composition
Glutathione	GSH	307.1	$C_{10}H_{17}N_3O_6S$
Ethanolamine	MEA	61.1	C_2H_7NO
β-Mercaptoethanol	BME	78.13	C_2H_6OS
1-Chloro-2,4-dinitrobenzene	CDNB	202.5	$C_6H_3ClN_2O_4$
Adenosine Triphosphate	ATP	507.1	$C_{10}H_{16}N_5O_{13}P_3$
Potassium phosphate (monobasic)		136.0	H_2KO_4P
Fetal bovine serum	FBS		
Trypan blue	TN		
Scintillation fluid			
Geimsa-May- Grunwald stain			
Phosphate buffered saline	PBS		
Penicillin/ streptomycin solution	P/S		
N,N,N',N'-Tetramethylethylenediamine	TEMED	116.2	$C_6H_{16}N_2$
Acrylamide		71.1	C_3H_5NO
Polidocanol			
Tris (hydroxy methyl) aminomethane	TRIS	121.1	$C_4H_{11}NO_3$
3', 3'', 5', 5''-Tetrabromophenolsulphonephthalein	Bromophenol Blue (BPB)	699.9	$C_{19}H_{10}Br_4O_5S$
Butanol		74.1	$C_4H_{10}O$
Ammonium Acetate		77.1	CH_3COONH_4
Phenylmethylsulfonyl fluoride	PMSF	174.1	$C_7H_7FO_2S$
4-Hydroxynonenal	HNE	156.2	$C_9H_{16}O_2$
Sodium hydrogen carbonate		84.0	$CHNaO_3$
[Ethylenebis (oxyethylenenitrilo)] tetraacetic acid	EGTA	380.351	$C_{14}H_{24}N_2O_{10}$
N,N'-Methylenebisacrylamide	Bisacrylamide	154.2	$C_7H_{10}N_2O_2$

Cell lines and cultures

CML K562 was studied. All cells were cultured at 37 ^{o}C in a humidified atmosphere of 5 % CO_2 (Napco 5300 CO_2 incubator) in RPMI-1640 medium supplemented with 10 % (v/v) heat-inactivated FBS, and 1% (v/v) P/S solution.

Animals

As per institutional regulations, animal safety training was completed, and use of animals was according to IACUC approved protocols. C57B wild-type mice 10-12 weeks of age were obtained from Harlan Labs and quarantined for 14 d prior to studies. Ralbp1 heterozygous knockout mice were obtained originally from Lexicon Genetics, Woodlands, TX. Colonies of wild-type, heterozygous knockout, and homozygous knockout mice were established and genotyped using tail-tip tissue for restriction fragment length polymorphism analysis to definitively identify genotype as wild type ($Ralbp1^{+/+}$), heterozygous ($Ralbp1^{+/-}$), and homozygous ($Ralbp1^{-/-}$) knockout mice. For animal toxicity studies to compare the genotypic groups with respect to chemotherapeutic agent cytotoxicity, the drugs were given intraperitoneally in a 0.1 mL volume of diluent (0.9 % NaCl) using a 26 gauge needle and a 1 cc syringe. The animals were monitored three times daily with the endpoint of the study being survival time. Animals exhibiting moribund status were subjected to CO_2 euthanasia as per approved protocol. Graphpad Prism software was used to analyze data for survival.

Buffers

1. Potassium phosphate buffer (1 M monobasic and dibasic potassium phosphate buffer pH 7.2)

2. GST assay buffer (100 mM potassium phosphate buffer pH 6.5)
3. GSH elution buffer (50 mM Tris-HCl pH 9.6 containing 1.4 mM BME, 10 mM GSH final pH 7.2)
4. GSH Linking buffer (44 mM sodium hydrogen phosphate and potassium phosphate buffer pH 7.0)
5. GSH Affinity buffer (22 mM phosphate buffer containing 1.4 mM BME pH 7.0)
6. GSH Coupling buffer (1 M sodium hydrogen carbonate containing 5 M sodium chloride pH 7.9)
7. Buffer A (10 mM potassium phosphate buffer pH 7.0)
8. Buffer B (22 mM potassium phosphate buffer pH 7.0)
9. Ralbp1 Washing buffer (Ralbp1 lysis buffer containing 0.01 % SDS pH 7.4)
10. Ralbp1 Lysis buffer (10 mM Tris-HCl pH 7.4 containing 50 µM BHT, 100 µM PMSF, and 1.4 mM BME)
11. Ralbp1 Elution buffer (Ralbp1 lysis buffer containing 10 mM $MgCl_2$, 10 mM ATP, 0.025 % Polidocanol, 0.2 mM DNP-SG)
12. Reconstitution buffer (10 mM Tris-HCl pH 7.4 containing 2 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, 40 mM sucrose, 2.8 mM BME, 0.05 mM BHT, 0.025 % Polidocanol)
13. Transport buffer (1 M Tris-HCl pH 7.4, containing 1 M $MgCl_2$, 1 mM EGTA, 25 mM KCl, 1 mM sucrose)
14. Ralbp1 regeneration buffer 1 (0.5 M potassium chloride in 0.1 M sodium acetate pH 4.0)

15. Ralbp1 regeneration buffer 2 (0.5 M potassium chloride in 0.1 M boric acid pH 8.0)
16. TAE buffer (Tris-HCl pH 9.6 containing 40 mM EDTA, 1 M acetic acid)
17. RNA loading buffer (1 M MOPS containing 64 mM EDTA pH 7.0)
18. Sigma DNA loading buffer (25 % bromophenyl blue, 30 % glycerol, and 45 % water)
19. SDS-PAGE Protein loading buffer (approximately 80 % water, 12 % glycerol, 5 % SDS 1%, Tris pH 6.8)
20. Sigma Bradford reagent (85 % phosphoric acid, Coomassie brilliant blue G-250, 95 % methanol, water)

Preparation of GSH-sepharose affinity resin for GST purification

Epoxy-activated Sepharose 6B is a 6 % agraose bead coupled to a 12 atom spacer (1, 4-bis (2, 3-epoxypropoxy-) butane) that upon addition of the ligand GSH forms a stable covalent bond allowing us to purify GST. We first need to couple GSH to the Sepharose resin. 1.2 g of epoxy-activated Sepharose 6B resin (1 g freeze-dried powder gives about 3.5 ml final volume of medium) was placed into a 15 mL centrifuge tube and washed with de-ionized water. After washing resin was centrifuged at 1,400 x g to remove the water. Once the water was removed linking buffer was added to the resin and the resin was deoxygenated with nitrogen. GSH was added to a final concentration of 0.3 M pH, 7.2 and the solution was again deoxygenated with nitrogen. The mixture was transferred to an orbital shaker at 4 °C and allowed to react overnight. The resin was centrifuged down at 1,400 x g and excess GSH removed followed by washing with

deionized water. After the removal of water the un-reacted epoxy groups were blocked by the addition of 1 M ethanolamine, pH 8.0, for four hours at room temperature on an orbital shaker. The resin was centrifuged at 1,400 x g and the ethanolamine removed and again washed with deionized water. The resin was then washed, centrifuged sequentially with sodium acetate pH 4.0, sodium borate pH 8.0, and affinity buffer*.

*The last three buffers are used for the regeneration of the GSH-Sepharose resin.

Purification of GST

GST consists of a super-family of cytosolic, microsomal, and mitochondrial dimeric isoenzymes varying between 45-55 kDa in size (Wilce 1994). GSTs catalyze the rate limiting step of mercapturic acid biosynthesis, and play a central role in phase II metabolism of electrophilic xenobiotics and endobiotics (Ketterer 1998). Recombinant human GST α 1-1 was selected for use in catalytic synthesis of DNP-SG because it was ready availability. GST purification was achieved by GSH-affinity chromatography modified for use with recombinant human GST expressed in E. coli.

E. coli BL21 (DE2) containing the recombinant GST A 1-1 gene inserted into a pET30a (+) prokaryotic expression vector was obtained from Dr. Yogesh C. Awasthi's laboratory, UTMB, Galveston. A 3 mL starter culture was prepared initially, followed by inoculation of a larger-scale 200 mL culture in LB medium, pH 7.4. The culture medium was incubated overnight at 37 °C with gentle shaking. Growth was monitored using a UV-Vis spectrophotometer at 600 nm and when the culture reached ideal optical density (0.6) 0.4 mM IPTG was added, and the culture stored overnight at 37 °C with gentle shaking. The culture was removed from the incubator transferred to sterile

centrifuge tubes and centrifuged at 3,000 x g for 5 min. The centrifuged pellet was re-suspended in 10 mL Buffer A and 1.4 mM BME. After sonication at 50 W for 30 s x 3, the centrifuge tube was transferred to an orbital shaker at 4 °C for 3-4 hours. After mixing, the tube was centrifuged at 12000 x g for 45 min and the supernatant fraction was collected. A sample of 100 μL was removed and placed in a 1.5 mL microcentrifuge tube for GST activity and protein content as determined. The remaining supernatant fraction was dialyzed overnight against 100x volumes of Buffer A. An affinity chromatography column was packed with GSH-Sepharose affinity resin and allowed to equilibrate overnight with Buffer B. The dialyzed sample was removed and centrifuged at 12000 x g and an additional aliquot of 100 μL was removed for GST activity assay and protein determination. The remaining sample was applied to the column at a flow rate of 8 mL/hour and loaded onto the column. The unabsorbed fraction was collected and a sample of this fraction was collected for GST assay and protein determination. The column was washed overnight with buffer B at a flow rate of 8 mL/hour. The next day the bound GST α 1-1 was eluted with elution buffer containing 10 mM GSH in 50 mM Tris-HCL, pH 9.6 containing 1.4 mM β-mercaptoethanol, a sample of this elution was removed for GST assay and protein determination. This elution was dialyzed against 100 x buffer A overnight. The next day the sample was removed from dialysis another aliquot was removed for GST assay and protein determination and the remaining sample was stored in a 15 mL centrifuge tube for preparation of DNP-SG. Protein was lyophilized and SDS-PAGE and Western

blot were performed to confirm purification of protein. Bradford assay was performed to assess protein quantity.

GST Activity Determination

GST catalyzes the formation of DNPSG from CDNB and GSH. Since the reaction product, DNPSG, is highly colored, GST activity can be measured by monitoring the formation of DNPSG spectrophotometrically according to the method of Jakoby (Pabst, 1974).

Table 2.2 Assay of GST activity towards CDNB

Assay Components	(Blank)	Experimental
GST Assay Buffer	850 µL	830 µL
10mM GSH	100 µL	100 µL
GST homogenate enzyme	-	20 µL
20 mM CDNB	50 µL	50 µL

Solutions of CDNB, GST assay, GSH, and the GST homogenate were prepared. 20.26 mg of CDNB in 5 mL of absolute ethanol was prepared. GST assay buffer was prepared by mixing a 1 M solution of K_2HPO_4 with 1 M solution of KH_2PO_4 according to a calculated ratio obtained from the Henderson-Hasselbach equation to obtain a pH of 6.5. GSH solution (GSH) was prepared by dissolving 15.4 mg of GSH in 5 mL of assay buffer. Male mouse wild type liver was dissected and weighed using a Sartorius analytical balance. The tissue was washed with phosphate buffered saline (PBS) to remove blood and placed in a glass test tube containing 2.5mL Buffer A. The volume

was adjusted to achieve a 10 % (w/v) homogenate. The tissue was homogenized using a tissumizer. Reaction mixtures with a final volume 1 mL were prepared by sequential addition of GST assay buffer, GSH, GST as shown (Table 2.2) into 1.5 mL quartz cuvettes. The controls included no GST or no GSH added. Absorbance for the formation of DNPSG was monitored for 4 min at 340 nm using a Varian cary bio 300 UV Vis spectrophotometer. Specific activity was calculated for the slope of increase in DNPSG using the extinction coefficient 340 nm for DNPSG (9.6) and the protein concentration was determined by Bradford's method.

Protein Determination

The Bradford dye-binding assay is a colorimetric assay for measuring total protein concentration. It is based the binding of Coomassie brilliant blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues. The anionic (bound form) has absorbance maximum at 595 nm whereas the cationic form (unbound form) has an absorbance maximum at 470 nm. The assay is monitored at 595 nm in a spectrophotometer, and thus measures CBBG complexed with the protein (Bradford 1976). After mixing the components, the reagent was stored in a dark bottle at room temperature overnight, followed by filtration through Whatman #1 filter paper. The final reagent was stored in the dark at room temperature, and standardized against BSA. The protocol for the additions to control and experimental cuvettes is shown in Table 2.3. The linear concentration range was 1.23 mg/ml of protein, using BSA (bovine serum albumin) as the standard protein.

Table 2.3 Bradford's protein assay protocol

Experimental (µL)		Blank (µL)	
Protein	10	Protein	-
H_2O	90	H_2O	100
Bradford Reagent	1000	Bradford Reagent	1000
Total	1100		1100

Preparation of DNP-SG

1-chloro-2, 4-dinitrobenzene (CDNB) is an alkylating agent class chemotherapy drug used for local therapy of human melanoma (Jungnelius 1994). It is the best model-substrate for GSTs, and the product DNP-SG is formed in high purity and yield, without significant formation of by-products seen when GSH and CDNB are reacted in the absence of enzyme. Thus, the enzymatically synthesized, purified DNP-SG is most ideally suited for purification of Ralbp1 via affinity chromatography (Zou 2002).

For the coupling reaction to prepare the affinity resin all buffers and reagents were purged and stored under a nitrogen atmosphere. The 50 mL reaction mixture contained 50 mM GSH, 40 mM CDNB, 5 % ethanol, 95 % 100 mM phosphate buffer pH 6.5, and 5 units purified GST α 1-1 activity. The CDNB stock solution of 400 mM in ethanol was added last in a dropwise fashion to the reaction mixture. After overnight incubation, the mixture was lypholized and washed with pure ethanol 2-3 times and centrifuged at 3000 x g. On the final centrifugation the pellet was collected and re-

suspended in 2-3 mL of deionized water. To purify the DNP-SG the aqueous mixture was subjected to TLC with a solvent system consisting of 7:2 acetonitrile and water. The top yellow band was removed from the TLC and the DNP-SG containing silica was then placed into a test tube re-suspended with water and allowed to shake for one hour at 4 °C. This was followed by centrifugation at 1000 x g for 10 min. The supernatant fluid was removed and diluted 100 fold. Confirmation of DNP-SG was done by UV-VIS (Varian-Cary Bio300 spectrophotometer).

Preparation of DNP-SG resin

Cyanogen bromides react with the hydroxyl groups on Sepharose to form cyanate ester groups, which are highly reactive. Proteins, peptides, amino acids or nucleic acids can be coupled to BrCN-activated Sepharose, under mild conditions, via primary amino groups or similar nucleophilic groups. The activated groups react with primary amino groups on the ligand to form isourea linkages. The coupling reaction is spontaneous and requires no special chemicals or equipment. The resulting multi-point attachment ensures that the ligand does not hydrolyze from the matrix. The activation procedure also cross-links Sepharose and thus enhances its chemical stability, offering considerable flexibility in the choice of elution conditions.

DNP-SG affinity resin was prepared by addition of ~1-4 g of cyanogen bromide activated sepharose 4B in acidified solution with 1 mM HCl to 3 mM DNP-SG at 4 °C overnight. The resin was then centrifuged down 1,400 x g and excess uncoupled DNP-SG was washed with 100 mL coupling buffer. The resin was then placed in 1 M ethanolamine, pH 8.0, followed by centrifuged and washed in the sequence addition of

0.1 M sodium acetate buffer pH 4.0, 0.1 M sodium borate buffer pH 8.0, and coupling buffer respectively. Resin was stored in the dark at 4 °C.

Purification of RalBP1/Ralbp1

Bacterial pellet fraction of E. coli strain BL21 (DE3)-RLIP76 induced by 0.4 mM IPTG. All purification steps were carried out at 4 °C unless otherwise specified. The supernatant was collected and centrifuged again at 100,000 x g for 60 min on a Sorvall RC-5. Supernatant was then discarded and the membrane pellet thus obtained was solubilized in Ralbp1 lysis buffer. The mixture was sonicated 3 times for 30 seconds at 50 W and incubated for 4 h at 4 °C with occasional shaking. After incubation, the mixture was centrifuged at 100,000 x g for 1 h at 4 °C and the pellet was discarded. The supernatants were applied to affinity chromatography over a column of DNP-SG linked to BrCN-activated Sepharose 4B, equilibrated with lysis buffer. RLIP76 was purified by binding the resulting membranes to DNP-SG Sepharose 4B affinity resin followed by removal of contaminating proteins and elution with 10 mM ATP, 10 mM $MgCl_2$, 0.2 mM DNP-SG and 0.025 % $C_{12}E_9$ in lysis buffer.

Preparation of Protein Liposomes

Liposomes are commonly used for drug or protein delivery because of their innate ability to absorb into membrane. Our experiments utilize purified recombinant Ralbp1 reconstituted into proteoliposomes to study the effects of Ralbp1 transport.

In 4 °C conditions, the purified Ralbp1 was dialyzed against reconstitution buffer. A homogeneous emulsion of soybean asolectin (40 mg/mL) and cholesterol (10 mg/mL) was prepared in reconstitution buffer by sonication. 100 µL of mixture was

removed and 900 µL of reconstitution buffer containing 20 µg of purified dialyzed Ralbp1 was added. This protein/liposome mixture was then sonicated at 50 W for 30 s. Formation of vesicles was precipitated by the addition of 200 mg of SM-2 Bio beads pre-equilibrated in reconstitution buffer without polidocanol. Vesicles were allowed to form for 4 h after which the SM-2 Bio beads were removed using centrifugation and liposomes were stored at 4 °C.

Transport Studies using Crude Membrane Vesicles

Transport studies that allowed measurement of [14-^{14}C]-DOX (sp. act. 8.9 x 10^4 cpm/nmol) in IOVs were performed by a method developed and published by our lab. (Awasthi 1994; Awasthi 2000). Stock solutions of 40 mM $MgCl_2$ and 40 mM of ATP were prepared in phosphate buffer containing 250 mM sucrose and 10 mM Tris-HCl, pH 7.4.The reaction mixture (120 ml) consisted of IOVs protein (80 mg), 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 4 mM $MgCl_2$ and either 4 mM ATP or an equimolar concentration of NaCl (6 mM). Addition of radiolabeled compound was added and the reaction was allowed to incubate at 37 °C for 10 min. The transport was stopped by rapid filtration of a fixed aliquot (30 ml) of the reaction mixture through 96-well nitrocellulose plates (0.45 mm pore size) using a 96-well vacuum filtration device. After filtration, the bottoms of the nitrocellulose membranes were blotted dry with filter paper, removed and the associated radioactivity was measured by placing in liquid scintillation counting vials containing 10 ml of scintillation fluid. Scintillation vials were mixed, and allowed to rest for 1 h at room temperature. The material was analyzed in a liquid scintillation counter (Beckman LS 230).

Preparation of Melphalan S-GSH

Melphalan exhibits its cytotoxicity via DNA intrastrand crosslinks by bifunctional alkylation of 5'-GGC sequences (Bauer 1997). Melphalan is known to cyclize into an unstable intermediate and is shown to react with the nucleophile GSH to form glutathione melphalan conjugates. Melphalan GSH conjugate synthesis was performed by an established method (Paumi 2001). 0.1 M potassium phosphate buffer, pH 7.3 was added followed by the addition of 0.2 M NaCl, 10 mM GSH, and 1 mM melphalan and allowed to react at 37 °C for 45 min. The reaction was terminated by the addition of 6.4 % perchloric acid (v/v). Solid phase extraction was performed by Waters Oasis® HLB 3 cc extraction columns pre equilibrate with 1mL of methanol followed by 1 mL of water. Solid phase extraction was performed as recommended by the manufacturer using 1mL portion of 10 % incremental MeOH w/ 0.05 % TFA to 100 % MeOH. The eluted fractions were collected and 100 µL samples were subjected to HPLC and MALDI-MS analysis for identification of conjugate.

HPLC Analysis

HPLC analysis was used to separate melphalan glutathione conujgates. Two HPLC columns were utilized depending on the study an Aligent 1100 using a 15 cm C_{18} column and a Spectra Physics 2200 using a 17 cm C_{18} column. The following describes the solvent systems used in HPLC analysis in different mobile phases.

MeOH/H_2O mobile phase

Stock reaction mixtures of 50 mM GSH and 10 mM melphalan were prepared as according to method above. A sample of the reaction mixture was removed and

injected into the HPLC pre-equilibrated with H_2O with 0.01 % TFA (A1) and MeOH with 0.01 % TFA (A2). The HPLC system ran a gradient beginning at 2 min till 14 min from 100 % A1 to 100 % A2

Ammonium acetate/ MeOH mobile phase

Ammonium acetate for the mobile phase was selected based on literature (Zhang 2005) for the isolation of Melphalan conjugates as opposed to the use of TFA.

Stock reaction mixtures of 50 mM GSH and 10 mM melphalan were prepared as according to the method above. A sample of the reaction mixture was removed and was injected into the HPLC that was pre equilibrated with ammonium acetate (B1) and MeOH (B2). The HPLC system ran a gradient beginning at 0 min till 35 min from 100 % B1 to 100 % B2. 20 µL samples were removed and applied onto the HPLC. Once conjugates were identified a 2 mL loop was obtained along with a larger C18 column and upscale was performed to increase yield.

MS Analysis

MS analysis using MALDI-TOF consisted of a Bruker autoflex MALDI-TOF utilizing a nitrogen laser at 337 nm laser with matrix consisted of α-cyano-4 hydroxy-cinniminic acid. Following MALDI-TOF MS analysis the MS was performed by LC/MS. 1 ml of melphalan glutathione conjugate sample was submitted for analysis using a Thermo Finnigan LXQ LC/MS.

Preparation of Ralbp1/pcDNA 3.1 gene for transfection

Blank pet30a(+) prokaryotic expression vector was extracted from E. Coli BL21(DE3) using a Qiaprep® Miniprep Kit (Qiagen Cat. # 27104) this kit allows lysis of bacterial

cells using alkaline solution while adsorption of DNA onto silica followed by elution in the presence of a high salt solution. Upon elution of DNA into a 1.5 mL microcentrifuge tube (total volume 50 µL). We prepare the DNA according to manufacturers instructions. 3 µL was removed from and subjected to 1 % agarose gel electrophoresis to confirm presence of intact plasmid. The remaining DNA elution was subjected to digestion by BamHI and XhoI restriction endonucleases.

DNA Agarose Gel Electrophoresis

To prepare the agarose gel 300 mg of agarose powder was mixed with 30 mL of 1x TAE buffer. This mixture was warmed allowing the agarose to dissolve. The agarose/TAE solution was allowed to cool to 55 °C followed by the addition of 3 µL of ethidium bromide. The agarose ethidium bromide was mixed and poured into the casting mold and allowed to cool solidify into a slab. Samples were prepared by the addition of 5:1 sample buffer: sample. The gel was then submersed in 1x TAE and the gel was ran at 80 v for 1 hour.

Amplification of Ralbp1 gene

Components from were added according to table 2.4 and placed in a applied biosystems geneamp pcr 2700 pcr instrument and amplification occurred according to figure 2.1.

Table 2.4 Components for PCR amplification of Ralbp1

Components for PCR amplification of Ralbp1	
Components	Volume (µL)
H_2O	82
Bovine Serum Albumin (100x)	1
Thermopal Buffer (10x)	10
Forward Primer	1.5
Reverse Primer	1.5
DNA Template (Ralbp1)	1
dNTP	2
Vent Polymerase	1
Total	100

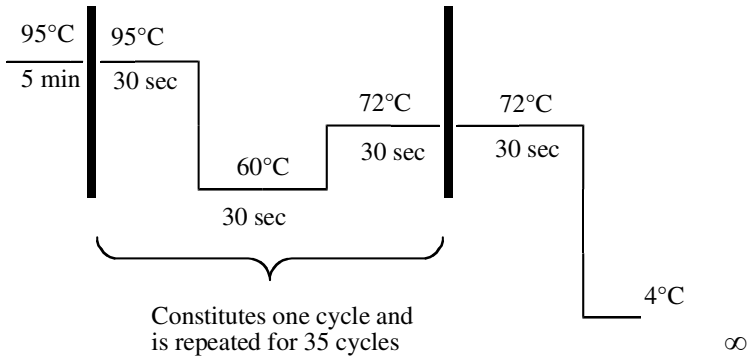

Figure 2.1 PCR condition for amplification cycle of Ralbp1 gene

The cycling of PCR involves 3 main steps indicated between the solid black lines. First the deanturation step, is a high temperature step allowing the DNA to melt for 30 seconds disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA. The second step, the annealing step, lowers the temperature to 60 °C for 30 seconds allowing annealing of the primers to the single-stranded DNA template. The last step in the cycle, extension/elongation step, allows for the polymerase to synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's that are complementary to the template in 5' to 3' direction. As shown this cycle was repeated for 35 cycles leading to the amplification of Rablp1 gene.

The samples were removed from PCR and purified with electrophoresis using a low melting agarose gel. The band was visualized, excised, and extracted from gel using Qiagen gel extraction kit. Digestion of Ralbp1 using BamH1 and Xho 1 endonucleases was performed in a similar to above but total volume was increased to 40 µL. The digested sample was removed from 37 °C water bath, placed in 60 °C water bath to inhibit further digestion of the gene and purified again by running low melting agarose gel and extracting with gel extraction kit eluting with a volume of 20 µL.

Ligation of Ralbp1 gene and pcDNA 3.1 vector

The ligation was performed and as shown below the following components were added and allowed to ligate at room temperature overnight.

Table 2.5 Components ligation of Ralbp1 gene and pcDNA 3.1 vector

Components for ligation of Ralbp1 gene and pcDNA 3.1 vector	
Components	Volume (μL)
H_2O	12
Buffer ligase (10x)	2
pcDNA (+) vector	1
Ralbp1	4
T_4 DNA ligase	1
Total	20

Transformation of ligation mixture into DH5α

Preparation of SOB and SOC media were made accordingly. 200 μL of E. coli DH5α was removed and 10 μL sample of ligation mixture was added to the bacteria. This mixture was cooled on ice for 30 min. followed by heat shock for 1 min. After heat shock the mixture was placed on ice for additional 5 min followed by bacterial growth in 600 μL of SOC medium for one hour. Post incubation the culture was on LB agar plates and allowed to grow overnight. The plates were removed and colonies were selected and grown in LB medium.

Purification of pcDNA 3.1/ (Ralbp1) plasmid using the QIAprep® and transformation into E.coli

According to Qiagen this protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from 1–5 mL overnight cultures of E. coli in LB (Luria-Bertani) medium. Purification was performed according to the manufacturers instruction and transformation of the plasmid into E.coli was performed in a similar manner as to DH5α.

Cell Transfection

In cellular transfection certain conditions may facilitate the need to transiently express the gene. The DNA in transient expression is introduced in the same manner as stable expression however the foreign material is removed when the cell undergoes mitosis. As in stable transfection the Invitrogen Lipofectamine 2000® transfection reagent kit utilized in a similar manner to stable transfection. To ensure successful transfection DNA to lipofectamine ratio should be maintained to 1:2 - 1:3.

Before transfection 4-8 million K562 cells were placed in 500 μL of growth medium without antibiotics P/S. 4 μg of the Ralbp1 gene was diluted in 250 μL of DMEM serum and gently mixed. In a separate tube 10 μL of Lipofectamine 2000® was diluted in 250 μL of DMEM medium. Both tubes were incubated for 5 mins. at room temperature, post incubation both tubes were mixed and incubated for an additional 20 min. at room temperature. Mixture was divided into 6 aliquots in a 6-well plate. The cells were incubated at 37 °C in a CO_2 incubator for 18 to 48 hours prior to testing for transgenic expression was checked by RT-PCR.

Stable Transfection

The stable transfection was performed in a similar manner as the transient transfection. Following transfection 600 μL of G418 was used to select for successful transfection.

Isolation of DNA and RNA by Trizol method (Invitrogen)

Description and method provided by Invitrogen are cited from Chomczynski (1987). TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Extraction was performed according to manufacturers protocol. DNA, RNA, and protein fractions were confirmed by Agarose and Acrylamide gels.

RT-PCR of cell genomic extract

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to ensure the level of expression for our gene in the cell transfect was in fact overexpressed. PCR was performed in an Applied Biosystems, Gene Amp, PCR system 2700 thermocycler (Figure 2.2). Samples were purified using low melting agarose gel electrophoresis and Qiagen gel extraction kit as previously stated.

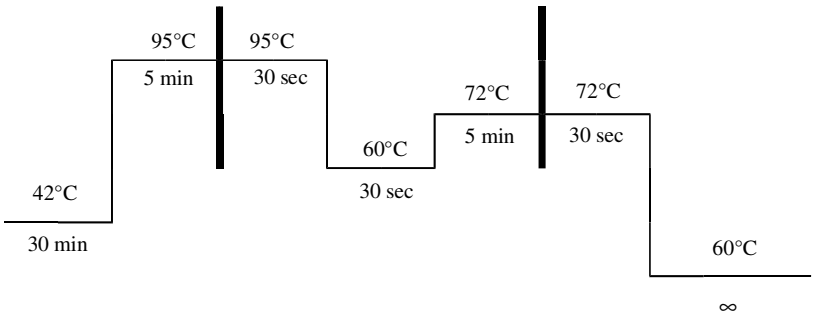

Figure 2.2 PCR condition for RT-PCR of Ralbp1 in cells

In the first step of RT-PCR, called the "first strand reaction," complementary DNA is made from a messenger RNA template using dNTPs and an RNA-dependent DNA polymerase (reverse transcriptase, RT) through the process of reverse transcription for 30 min at 42 °C. This was followed by the "second strand reaction" or standard PCR reaction as described in figure 2.3.

Cell Viability assay

The MTT assay determines drug sensitivity of selected antineoplastic agents.

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is yellow in color and is reduced by mitochondrial reductases into formazan crystals. This formazan crystal, purple in color, is solubilized in DMSO and absorbance determined using a spectrophotometer at 540 nm (figure 2.3) (Mossmann 1983).

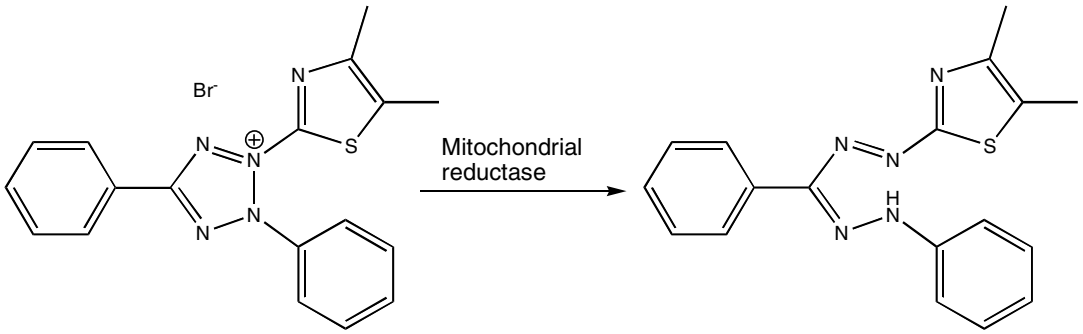

Figure 2.3 Reaction of (MTT)

MTT assay is a laboratory test for measuring IC_{50} cytotoxicity of administred toxic compounds. It can also be used to determine cellular proliferation (cell growth). Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) [shown left] is reduced to purple formazan [shown right] in the mitochondria of living cells. A solubilization solution DMSO is added to dissolve the insoluble purple formazan product leading to formation of a purple colored solution. The absorbance of this colored solution can be quantified by measuring with a UV-VIS spectrophotometer.

K562 cells (Control), pcDNA 3.1 transfected K562, and Ralbp1 transfected were counted using trypan blue and twenty thousand cells per well were seeded into a 96 well plate. Plates were placed in 37 °C for 24 hrs, post incubation aliquots of drug in concentrations ranging from 1 nm to 2,000 nm for DOX, DAU, VCR, VBL, VRL, MTC, MTX and drug concentrations ranging from 1 μm to 2,000 μm for CIS and MEL were added to their respective plate.

Following 72 h incubation, 20 μL MTT (5 mg/mL in PBS) was added to each well and plates were incubated for 2 hrs at 37 ºC. The plates were centrifuged at 1200 x g for 10 min. media remove and cells lysed with 100 μL DMSO. Absorbance was determined using an el311 Biotek Instruments ELISA plate reader. Survival fraction

(IC_{50}) was tabulated. This data was treated to a non-linear regression and fitted to a modified form of the Hill equation for dose response curve analysis.

Cell Uptake Studies

For tritium labeled uptake K562 control, K562 pcDNA 3.1 vector transfected and Ralbp1 transfected cells were collected and rinsed with 1 mL PBS. After counting, cell aliquots containing 5 x 10^6 cells were placed in 80 µL fresh RPMI medium followed by centrifugation at 300 x g. These cells were pelleted, and re-suspended in 90 µL medium. 10µL of 1 µM [^{3}H]-NAV was added to the medium (Final concentration 100 nM). This was allowed to incubate for a predetermined time period (5, 10, 20, and 30 minutes) at 37 ºC. Drug uptake was stopped by rapid cooling on ice. Cells were centrifuged at 2000 x g for 5-10 min. at 4 ºC and supernatant was decanted. Radioactivity was determined in the cell pellet after rinsing twice with ice-cold PBS and addition of scintillation fluid. Radioactive counts were performed using a Beckman LS-230 scintillation counter.

For [14-^{14}C] DOX uptake the similar procedure was followed with exception of 10 µL of 1 µM [14-^{14}C]-DOX [32,000cpm] was then added to the medium (Final concentration 100 nM) in place of tritium labeled drug.

Cell efflux studies

K562 control, K562 vector transfected (pcDNA 3.1), and Ralbp1 transfected cells were collected and rinsed with PBS. Samples containing 5 x 10^6 cells were suspended in fresh medium and centrifuged at 300 x g, pelleted, and re-suspended in 90 µL medium. 10 µL of [^{3}H]-drug was added to the medium and allowed to incubate for 60 min at 37

ºC. Cells were centrifuged at 300 x g for 5 minutes and cell pellet washed twice with PBS. Pellet was suspended in 1 mL of PBS. 50 µL samples were removed every minute for 15 minutes and added to 5mL of scintillation fluid and counted using a Beckman LS-230 scintillation counter. Back-addition for the cellular drug versus time were constructed and reported.

IHC studies on H358 (NSCLC) and K562 using monochlorobimane and DOX

K562 cells were grow in suspension, 0.5 x 10^6 cells were placed into 12 well plates and allowed to grow for 24 h into medium and transferred to histological slides using Cytopro Cytocentrifuge at 200 x g (Wescor, Inc., UT). Cells were treated with either 50 µM monochlorobimane or 10 µM DOX for 20 min at 37 ^{0}C. Subsequently, cells were washed with PBS 4-5 times, followed by fixation with 4 % paraformaldehyde in PBS. Finally cover-slips were mounted on slides with Vectashield mounting medium for fluorescence (Vector Laboratories, CA). Fluorescence photomicrographs were taken using a LEICA DMLB (Germany) fluorescence microscope at 400-x magnification. The excitation wavelength of MCB and DOX were MCB 495 nm and 380 nm and emission was 550 nm and 470 nm respectively.

Mae Grunwald Geimsa Stain

Using K562 control, pcDNA 3.1 vector transfected K562, and RLIP transfected K562 cells were grown to a density of 500,000 cells/mL and pelleted using centrifugation at 1,100 x g. Cells were washed with Hank's PBS and suspended in RPMI 1640 medium containing HNE with a final concentration of 20 µM. After 2 hours of exposure, cells were washed with PBS and fixed to glass slides using a Cytospin at 200 x g for 5 min at

low acceleration. After drying slides they were stained with May-Grumwald-Giemsa stain and visualized on a light microscope.

Hemoglobin Estimation

Cells were treated with HNE in a similar manner above. After HNE treatment cells were washed and place in 1mL of PBS and sonicated at 50 W for 30 sec. Samples were treated to Drabkin's reagent, incubated for 3 min. prior to analysis using a Cary win bio 300 UV-Vis spectrophotometer at 340 nm.

Purification of recombinant Hsf1

Rec-Hsf1 was purified by metal affinity chromatography over Ni-NTA superflow resin (QIAGEN) with slight modifications as described below. E. coli BL21(DE3) expressing Hsf1 was lysed in 20 mM Tris-HCl containing 250 mM NaCl; 100 μM PMSF and 5 mM imidazole; pH 7.9, sonicated and incubated for 4 hours at 4 ^{o}C with gentle shaking followed by centrifugation at 11,000 x g for 30 min. The supernatant was mixed with Ni-NTA Superflow resin pre-equilibrated with the same buffer. The resin was stored overnight at 4 ^{o}C with gentle shaking and washed with wash buffer (20 mM Tris-HCl, 300 mM NaCl; 20 mM imidazole and 100 μM PMSF; pH 7.9) until OD_{280} was zero. The bound protein from the resin was eluted with elution buffer (20 mM Tris; 500 mM NaCl; 400 mM imidazole and 100 μM PMSF pH 7.9) and was dialyzed against 10 mM Tris-HCl (pH 7.4), 100 μM EDTA and 100 μM PMSF.

Functional reconstitution of purified Ralbp1 into artificial liposomes and transport studies

Purified Ralbp1 was dialyzed against reconstitution buffer (10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA, 100 mM KCl, 40 mM sucrose, 2.8 mM BME, 0.05 mM BHT, and 0.025 % polidocanol). An aqueous emulsion of soybean asolectin (40 mg/ml) and cholesterol (10 mg/ml) was prepared in the reconstitution buffer by sonication and 0.1 ml of this mixture will be added to 0.9 ml aliquot of dialyzed purified rec-Ralbp1 containing 20 μg proteins. The reaction mixture was sonicated at 50 W for 30 sec. Vesiculation was initiated by addition of 200 mg SM-2 Bio-beads pre-equilibrated in the reconstitution buffer without polidocanol. Vesiculation was carried out for 4 h at 4 ^{o}C, followed by removal of SM-2 Bio-beads by centrifugation and the vesicles (Ralbp1-liposomes) were collected. Control vesicles (control-liposomes) were prepared using an equal amount of crude protein from E. coli not expressing Ralbp1. ATP-dependent transport of [^{14}C]-DOX in the rec-Ralbp1 reconstituted proteoliposomes was performed by rapid filtration technique (Awasthi 2005).

Optimization of transport assay

To state if the apparent transport seen for Ralbp1 is optimized we performed as series of experiment to optimize the transport assay. Four variables were monitored to determine the best condition for transport, these variable are time, temperature, osmolarity, protein concentration. Enzyme kinetics for both the substrate and the energy currency ATP were also determined as established for both HNE-SG and DNP-SG.

Protein determination was the first variable examined. Five different protein concentrations were selected ranging from 25 ng to 250 ng and as seen on the figure A the protein concentration for transport is linear indicating that increased protein leads to greater efflux. The second variable examined was time; the alteration of time followed a general logarithmic curve with the optimal range appearing between 2.5-7.5 min. For temperature the ideal temperature for transport activity is 37 °C.

Determination of transport activity

Once establishing the ideal conditions and ensuring they correlate with previous published studies we could then check the activity of RLIP efflux against the substrates DNP-SG and HNE-SG by correlating it to classical enzyme kinetics. By varying the substrate concentration and ATP concentration to their respective substrate the data were using the Lineweaver Burk method.

Transport of [14-^{14}C]-DOX by Ralbp1 and its inhibition by POB1 and HSF1

For these experiments, fixed amount of purified rec-Ralbp1 (20 μg) was reconstituted in proteoliposomes along with varying amounts (0-64 μg) of POB1 and HSF1 and transport of [14-^{14}C]-DOX was measured in these proteoliposomes. In control, equivalent amount of BSA was reconstituted in Ralbp1 proteoliposomes in place of POB1 or HSF1.

[14-^{14}C]-DOX efflux and uptake studies

[14-^{14}C]-DOX efflux and uptake studies were performed in a similar manner to uptake and efflux listed above with slight modification. Purified recombinant HSF1 up to 300 ng was added in the reconstitution of liposome.

CHAPTER 3
ROLE OF GST, GSE, AND GSH IN Ralbp1 CHEMISTRY

Introduction

Mechanistic studies of Ralbp1 require an understanding of GSH chemistry and the biochemistry of enzymes which utilize reduced or oxidized GSH (GSSG) as substrate, particularly GST.

This sulfhydrl cofactor GSH (γ-glutatmyl-cysteinyl-glycine) is remarkable in its contribution to cellular survival. Cellular concentration of GSH, approximately 2-10 mM, is, remarkably high in comparison to other small biomolecules inside the cell (Wilhelm 1997). The γ-glutamyl linkage is remarkable for its ability to resist proteolytic degradation caused by peptidases (Orlowski 1970). In addition to being a substrate for glutathione S-transferase and GSH peroxidase, GSH participates in several reactions including as a cofactor for formaldehyde dehydrogenase, as a cosubstrate for glyoxalase I, and as a part of the structure for glyoxalase II and γ–glutamyltranspeptidase. (Awasthi YC 2006). GSH also participates in inflammation-signaling through the formation of leukotrienes. GSH and also contributes to basic cellular survival by functioning to maintain reduced thiol cysteines in proteins, and by detoxification of xenobiotics and free radicals.

Reduced GSH is required for many catalytic functions. The concentration of reduced GSH in cells is maintained by the activity of reductases and transhydrogenases which reduce oxidized GSH (GSSG) back to GSH. Reduced GSH is utilized by glutathione-peroxidases to reduce hydroperoxides to alcohols thus ameliorating formation of free radical from peroxides. Reduced GSH is also utilized by glutathione S-transferases in phase II conjugation reactions with electrophilic xenobiotics (E), leading to the formation of glutathione conjugate (GS-E). Once the electrophile is conjugated it is metabolized ultimately to a mercapturic acid.

The discovery of Ralbp1 originated from studies that isolated and characterized the product of reaction for a model substrate of GST, 1-chloro-2, 4-dinitrobenzene. This GS-E, designated dinitrophenyl-S-glutathione (DNP-SG) was used as an affinity chromatography ligand to purify mechanisms involved in active transport of GS-E from cells. A DNP-SG stimulated ATPase protein was purified by this affinity method, and designated DNP-SG ATPase. In subsequent studies, this protein was shown to be identical with RLIP76, a splice variant protein encoded by the human RALBP1 gene. The purification of this protein both from eukaryotic and recombinant prokaryotic sources still utilizes the same DNP-SG affinity purification procedure originally used to purify DNP-SG ATPase.

Because optimal preparation of DNP-SG for the affinity resin is through an enzymatic procedure, purification of GST's was necessary for preparation of the DNP-SG affinity resin used in present studies to purify Ralbp1. The activity of the DNP-SG affinity purified recombinant as well as cell and tissue Ralbp1 was assessed through transport

assays that use purified Ralbp1 reconstituted into artificial liposomes. A major advantage of this assay is that it has been shown to be devoid of any other known transporter proteins, thus the activity measured is wholly that of Ralbp1. This, along with a number of other functional assays were used to confirm that Ralbp1 is a highly active and dominant transporter not only of glutathione conjugate, but also of many unconjugated amphiphilic cationic xenobiotics.

Results

The synthesis of the glutathione conjugate DNP-SG is required for the purification of Ralbp1. By utilizing an established technique linking the cofactor GSH to epoxy-activated Sepharose 6B, we coupled GSH for use in purification of GST's from homogenate mouse tissues and recombinant protein. Purification was followed by measuring activities and protein concentrations for each respective fraction along purification process (Table 3.1). The purification table for GST from mouse liver shows 71.6 % and a final specific activity of 44.95 U/mg.

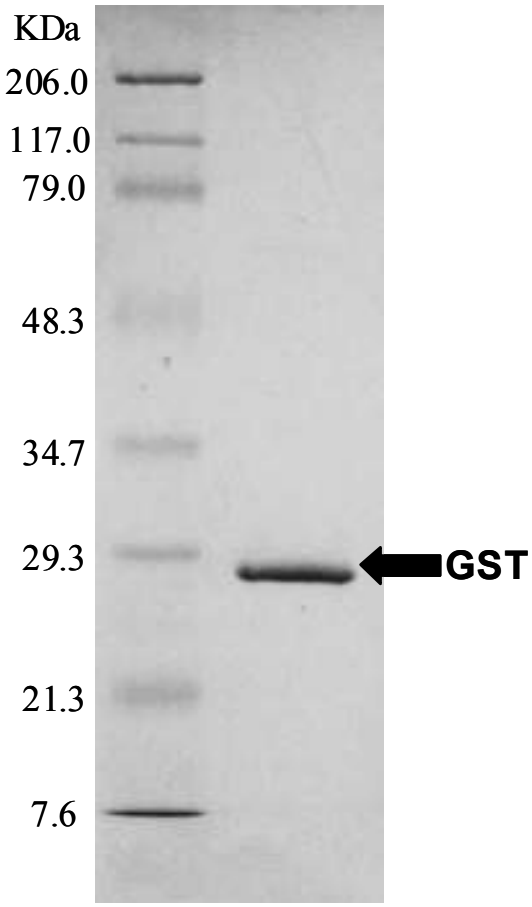

Figure 3.1 Western Blot of GST A 1-1

Western Blot of GST A 1-1 using rabbit anti-GST antibodies as the primary antibody.

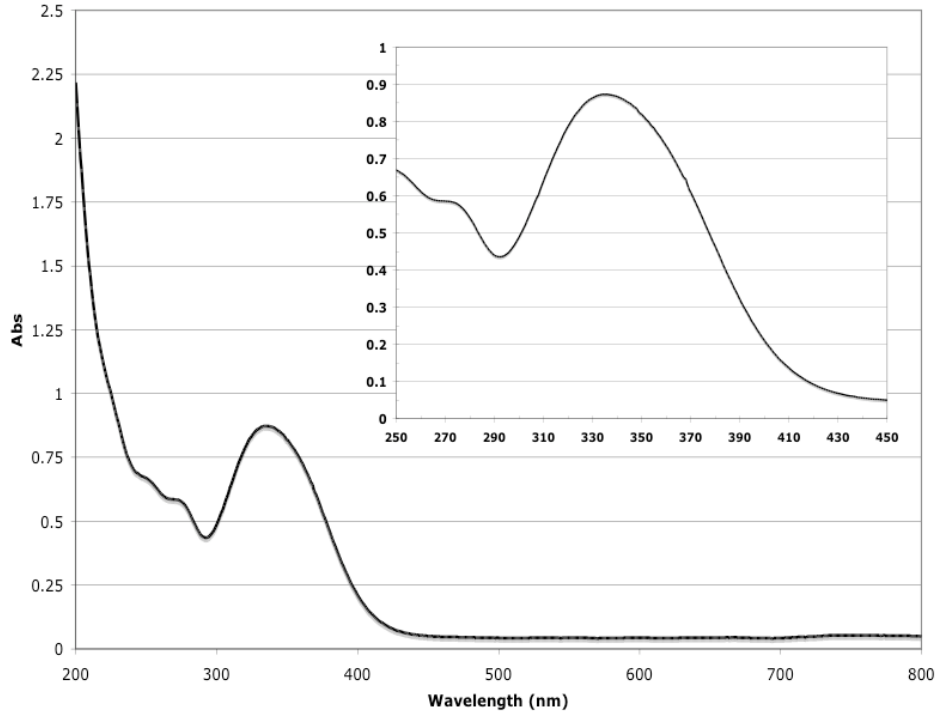

Figure 3.2 UV-VIS λ_{max} determination for DNP-SG.

Presence of pure DNP-SG, was confirmed by a sample of DNP-SG diluted 1:250 and scanned on a Varian Cary Bio 300 UV-Vis spectrophotometer. λ_{max} for DNP-SG was recorded at 339 nm. Samples were analyzed in triplicate and concentration tabulated using Beer Lambert's law.

After confirming protein activity protein was confirmed by western blot. Polycolonal anti-GST 1-1 as the primary antibody followed by anti-rabbit goat secondary antibody confirmed the protein to be GST 1-1.

DNP-SG synthesis

Upon successful purification of GST, DNP-SG was used as the affinity-ligand for purification of Ralbp1.

Table 3.1 Tabulation of DNP-SG concentrations.
The final concentration of DNP-SG was calculated by the division of the absorbance time dilution by the extinction coefficient.

	Absorbance
First Sample	0.98
Duplicate Sample	0.92
Triplicate Sample	0.96
Average Absorbance	0.95
Standard Deviation	0.03
Dilution of sample	1:250
Extinction Coefficient	9.6
Final Concentration (mM)	24.8

To confirm the presence of pure DNP-SG, the product was scanned on a Varian Cary Bio 300 UV-Vis spectrophotometer. λ_{max} for DNP-SG was determined to be 339 nm. Samples were analyzed at λ_{max} in triplicate and concentration tabulated using Beer Lambert's law (Table 3.3). Final concentration was determined to be 24.8 mM.

Mel-SG synthesis

DNP-SG was used in these studies not only as the affinity ligand for Ralbp1 purifcation, but also as a substrate for measuring stimutalted ATPase activity, as well as, substrate stimulated transport assay. Because the glutathione conjugates of another electrophilic

chemotherapy drug, melphalan, have been extensively characterized we wanted to synthesize the GSH conjugate of melphalan to use as an alternate substrate for transport assays. We thus undertook the task to synthesize melphalan glutathione conjugates. Melphalan undergoes a ring cyclization that leaves the intermediate, the aziridine ring, susceptible to reaction to nucleophiles including amino, carboxyl, imidazole sulfhydryl and hydroxyl groups (Dulik 1986, Tipnis 1999). A known reaction of melphalan biotransformation is through GST's to form a melpahlan glutathione conjugate MEL-SG (Zhang 2005). We attempted to isolate the melphalan monoglutathionyl conjugates possessing original chloro substituent for use in future studies.

We have replicated the past methodology of Morrow et. al. (Paumi 2001) with slight modification. Our attempt to synthesized MEL-SG by this method proved unsuccessful as our MS data generated a m/z value 577 whereas MEL-SG mass is 551 g/mol. We attempted another method for synthesis of MEL-SG (Awasthi 1996). As indicated this method requires a decrease in pH by the addition of HCl in 20 µL increments to a total percentage of HCl is 10 % of the total volume. By using HCl this allowed the melphalan to dissolve quite readily as opposed to 30 min. to 45 min.

GSH conjugate was carried out at 37 °C after the addition of GSH for 1 hour yielding a white floating precipitate-fraction. The solution was centrifuged at 1,400 x g to allow the non-dissolvable precipitate-fraction to collect toward the bottom. The supernatant-fraction was applied to the HPLC using Aligent 1100 HPLC solvent delivery system and separated on 15 cm C18 Waters Atlantis column. Mobile phase consisted of 90/10 of 50 mM ammonium acetate/MeOH at a flow rate of 1 mL/min. Wavelength of detection was set at 254 nm. After these conditions were chosen the standard melphalan

was injected and the HPLC spectrum was obtained. The standard was confirmed and reaction mixture consisting of MEL-SG was injection in 20 μL samples (figure 3.3).

It was clearly apparent an isocratic separation would not be feasible due to the long retention time for melphalan and the corresponding derivatives (45 min. for monohydroxy and 231 min. for melphalan). Using a Thermo Finnigan LXQ LC/MS to detect conjugate spectra we infused a fresh MEL-SG sample after determination of standards for melphalan and GSH (figure 3.4). We obtained a value of 576 m/z in positive mode corresponding to a mass of 577 g/mol which in the expected mass for monoglutathionyl monochloro melphalan. In addition to product we also determined the mass for the monochloro monohydroxy melphalan 558 g/mol and the diglutathionyl melphalan with a mass of 847 g/mol..

Attempts at scaling up the synthesis of MEL-SG were not successful. It appeared that the hydrochloric acid in larger quantities deactivates the basicity of the amine in the N, N-bis(2-chloroethyl)amine moiety. This tertiary amine from the reactions with hydrochloric acid forms quaternary amine. Attempts to synthesis the conjugate in an aprotic environment (to keep the melphalan from degradation) were also unsucessful. This result was likely due to the fact that aprotic solvents such as dimethlylsulfoxide (DMSO) and tetrahydrofuran (THF) cannot abstract the hydrogen off of cysteine in GSH, therefore GSH cannot be ionized to react with melphalan.

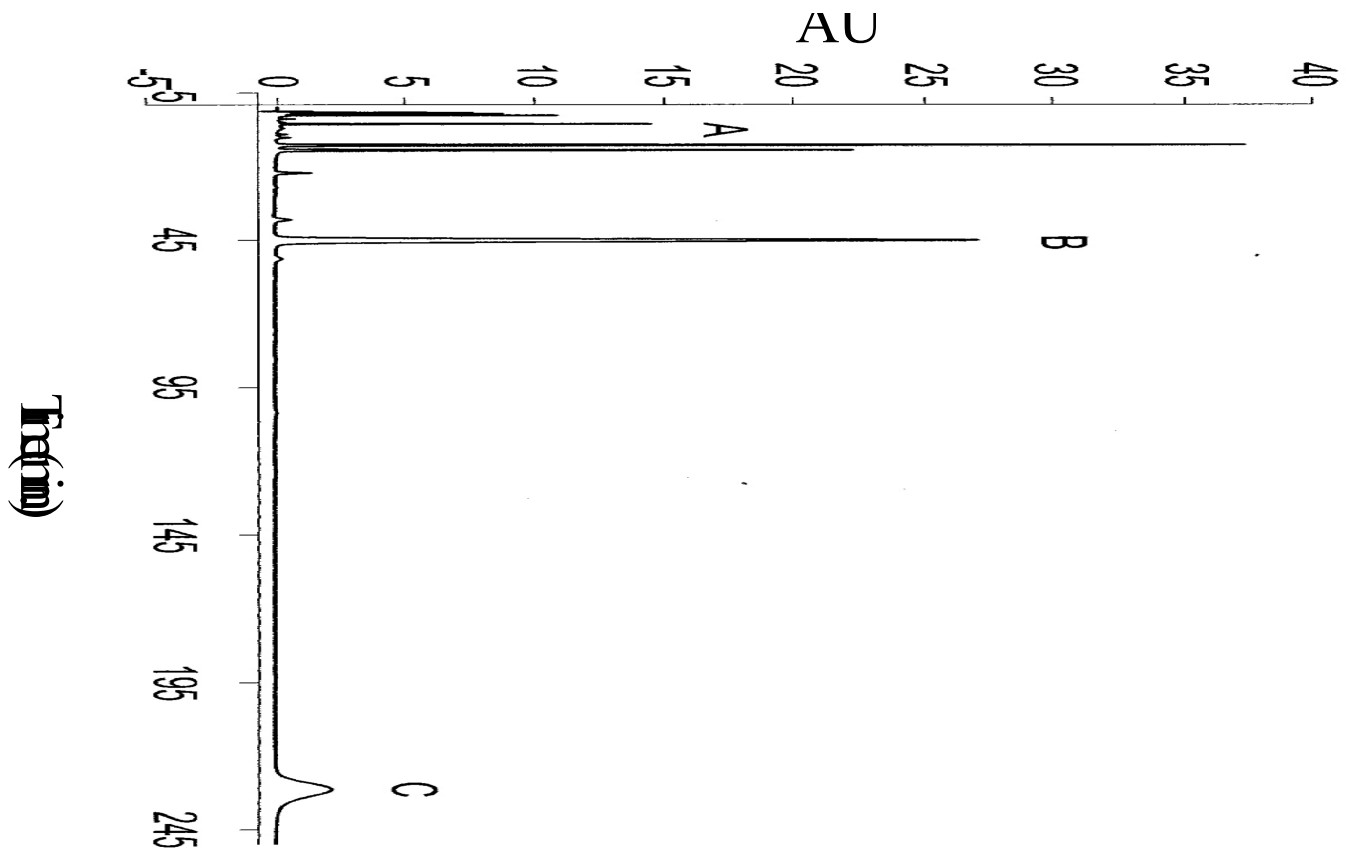

Figure 3.3 MEL and GSH standards on HPLC

This chromatogram displays the relative retention times for GSH (A) and melphalan (B) and the dihydroxy melphalan (C). 20 µL was injected into a aligent 1100 HPLC. HPLC solvent system consisted of an isocratic mobile phase of 90/10 of 50mM ammonium acetate/MeOH at a flow rate of 1mL/min.

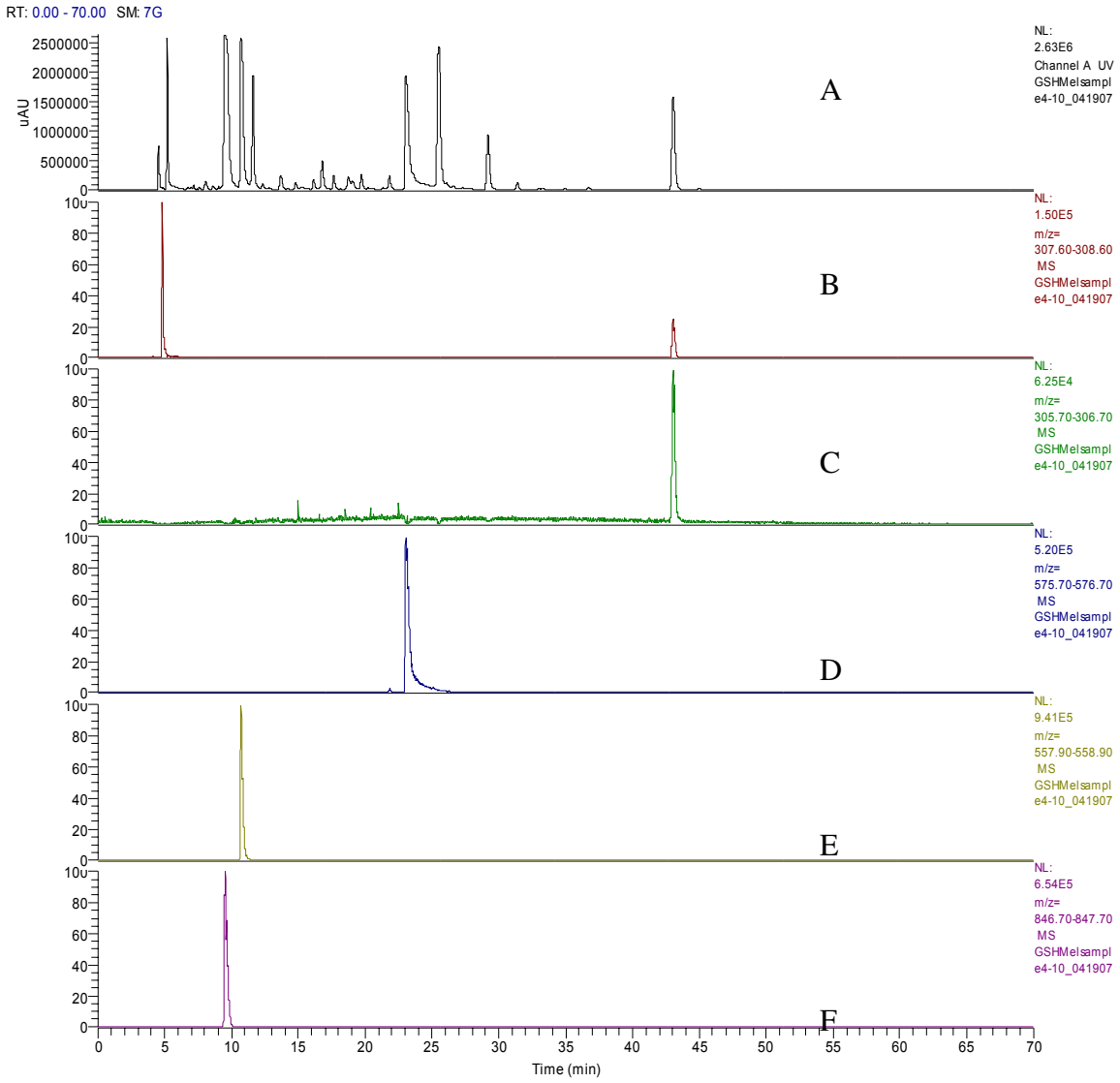

Figure 3.4 LC-MS of melphalan GSH conjugates

Characterization of the supernatant fraction from melphalan GSE synthesis was performed. Spectrum A is the entire FPLC run from the LC/MS, individual peaks were selected for MS analysis to see if any of the FPLC peaks corresponded to expected masses. Spectrum B the representative peak for the melphalan standard corresponding to a mass of 306 in positive mode the smaller peak is contamination with glutathione. Spectrum C is representative standard glutathione with a mass of 306 in positive mode. Spectrum D is the monochloro monoglutathione melphalan conjugate with a mass of 576. Spectrum E is the monohydroxy monoglutathione melphalan conjugate with a mass of 551 spectrum F is the di-glutathionyl melphalan conjugate with a mass of 847.

Conclusion

The buildup of the conjugate through readily synthesized has been shown to be partially electrophilic and toxic. The GSE's DNP-SG and HNE-SG shall be demonstrated capable of being transported via Ralbp1. The enzymatic synthesis and purification of DNP-SG was carried out successfully using purified mouse liver GST. The yield and purity of DNP-SG was consistent with prior studies (Singhal 2003). Preparation of MEL-SG was more difficult. Though the formation of the proposed conjugate was confirmed, sufficient amounts of the conjugate were not obtained to be used either in affinity chromatography or transport activity.

CHAPTER 4

ROLE OF Ralbp1 IN STRESS RESISTANCE

Introduction

Stress resistance in the form of multidrug-resistance (MDR), is a phenotype exhibited by many cancers that is typified by resistance to the cytotoxic effects of structurally unrelated cytotoxic agents. MDR remains a major obstacle to the eradication of malignancy with chemotherapy. Numerous biochemical mechanisms have been identified as contributors to MDR, though the MDR phenotype is most frequently associated with a decrease in cellular accumulation of drug due to active energy-dependent efflux of drugs or metabolites (Nooter 1994, Leith 1998, Sharma 2003, Takara 2006). MDR is mediated by a diverse array of transporter proteins, some of them are well-characterized as transporters and others for which there is indirect evidence for transporter function (Veuger 2003, Sargent 2001, Doyle 1995, Olson 2005, Pajic 2005, Kostrzewa-Nowak 2005, Qadir 2005, Kuwazuru 1990, Wuchter 2000, Sugimoto 1987, Deffie 1998, Gelkler 1998, Niethammer 1989, Assaraf 2007, Ma 1987, Paietta 1997, Ueda 1987, Chauncey 2001). Pgp (mdr-1 gene product, ABCB1) was the first identified drug-efflux pump shown to mediate resistance to a broad variety of weakly basic amphiphilic xenobiotic compounds through their ATP-dependent efflux (Gottesman 1993, Szakacs 2006). MRP (ABCC1) was subsequently identified as another related transporter protein also capable of mediating MDR through drug-efflux (Borst 1999, Szakacs 2006, Haimeur 2004). Subsequently, at least 46 other ABC family proteins have been identified, some with demonstrated transport activities comparable with Pgp or

MRP, and others with presumed transport function on the basis of homology (Sharma 2003, Szakacs 2006, Haimeur 2004). The difficulty in determining the sole contribution of ABC transporters Pgp and MRP to MDR is that expression is not correlative to resistance (Nadkar 2006). A recently discovered non-ABC transporter Ralbp1 was demonstrated to transport doxorubicin and *Vinca* alkaloids. Additional parallel studies have focused on the role of Ralbp1 as an anti-apoptotic GS-E and drug-transporter (Singhal 2006). These observations along with others place Ralbp1 as a central anti-apoptotic effector mechanism, an assertion validated by studies of others who have independently identified the function of Ralbp1 as being directly linked as an effector in multiple signaling pathways critical in malignancy (Ikeda 1998, Rosse 2003, Hu 2003, Goldfinger 2006, Yang 2003). Since Ralbp1 is also expressed in cell lines derived from hemato-logical malignancies (Singhal 2003), we have directly addressed the question of whether Ralbp1 can play a role in mediating multidrug resistance in K562 human erythroleukemia cells. The results presented here indicate that Ralbp1 can function as a multidrug resistance mediating protein in K562 cells.

Results

Stable overexpression of Ralbp1 was confirmed in K562 cells by RT-PCR and Western blot (Figure 4.1). RT-PCR using the forward (nt 1496-1515) and reverse (nt 1948-1968) primers demonstrated a ~2.7-fold greater expression of Ralbp1 mRNA content in comparison to control and empty vector transfection. Ralbp1 mRNA as well as protein level was unaffected by empty vector transfection. Quantifying Ralbp1 protein by densiometry showed a 3.8-fold increase in Ralbp1 protein in transfected cells as compared with wild-type or empty vector transfected cells. Ralbp1 overexpression did

not cause significant morphological alteration, but the growth rate of cells was increased as evidenced by a reduction in doubling time by 20 % (results not shown).

Cellular efflux/uptake

The uptake and efflux of radiolabeled vinerolbine [^{3}H]-VRL and doxorubicin [^{14}C]-DOX was compared between wild-type, empty-vector, or Ralbp1 transfected K562 cells. A marked decrease in total uptake of both drugs was observed in cells overexpressing Ralbp1 (Figure 4.2 panels A and B). The efflux of both drugs from cells loaded with either drug was greater with Ralbp1-overexpression (Figure 4.2 panels C and D) while the empty-vector was unaffected. The greater cellular accumulation of DOX was also confirmed by fluorescence microscopy. The effect of Ralbp1 on GS-E accumulation was also examined using monochlorobimane (MCB) which yields a fluorescent glutathione conjugate (Briviba 1993, Millis 1997, Sarkadi 2004). As with DOX, MCB accumulation in cells was reduced significantly in Ralbp1 overexpressing cells. Fluorescence micrographs taken of cells incubated with MCB and DOX independently as well as both together and examined with dual-excitation at 380 and 470 nm and emission at 495 and 550 nm for MCB and DOX respectively. A relatively greater accumulation of DOX was found in the nuclear compartment. Ralbp1 overexpression reduced the overall uptake of both drugs and this effect was particularly apparent for DOX in the membrane compartment.

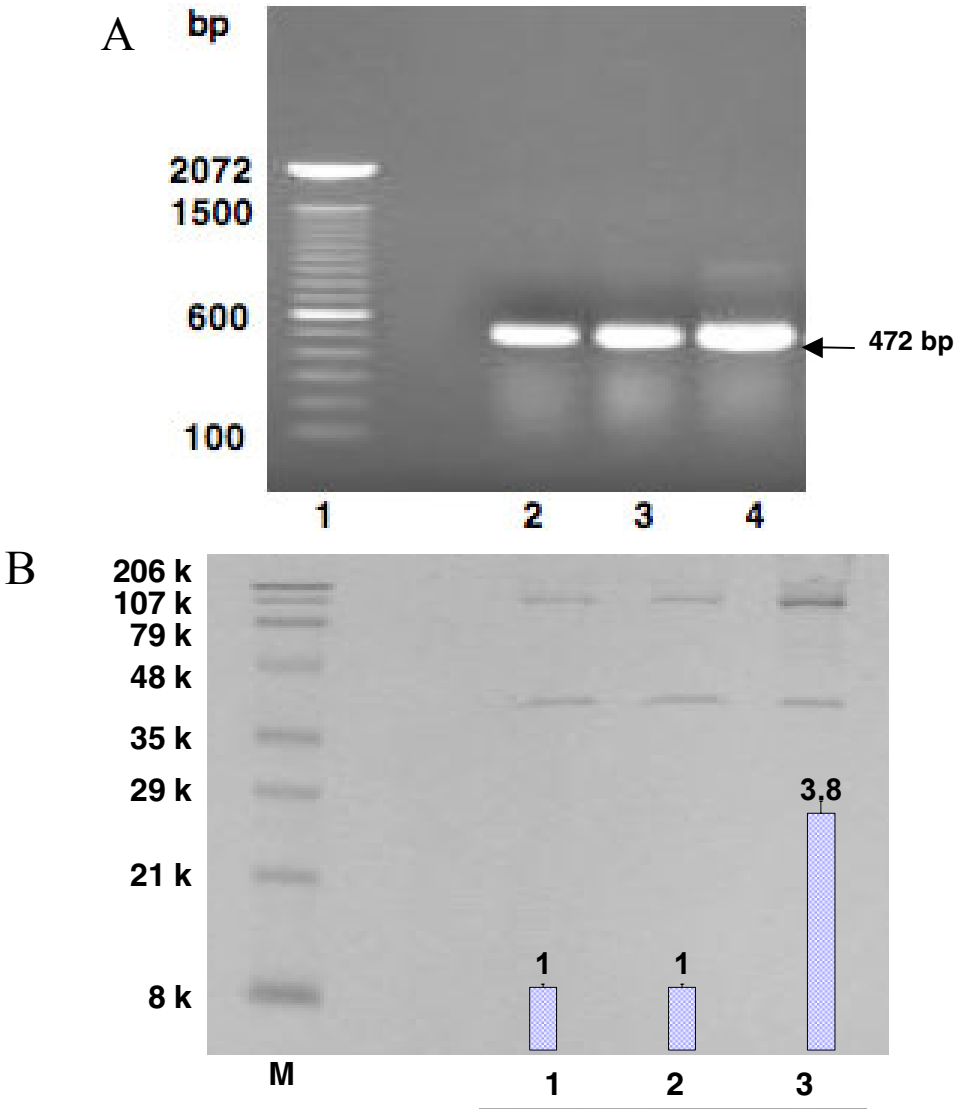

Figure 4.1 RT-PCR and Western blot for the transfection of Ralbp1 in K562 cells

K562 cells were transfected using eukaryotic expression vector alone (pcDNA3.1) or with pcDNA3.1-Ralbp1 using Lipofectamine 2000 Transfection Reagent kit (Invitrogen). A) RT-PCR was performed using Ralbp1 gene-specific primers [nt 1496-1515 (upstream primer) and nt 1948-1968 (downstream primer)]. Lane 1, 100 bp DNA marker; Lanes 2-4 contained DNA, a product of RT-PCR from control, vector transfected and Ralbp1-transfected K562 cells, respectively. B) Visualization of protein overexpression of Ralbp1 in K562 cells was evaluated by applying 200 µg aliquots of crude extracts applied to SDS-PAGE, and Western blotting against Ralbp1 IgG. Lane 1-3 shows the total crude protein from control, vector transfected and Ralbp1 transfected cells respectively while lane M shows the protein marker. Fold induction of Ralbp1 was quantified scanning densitometry.

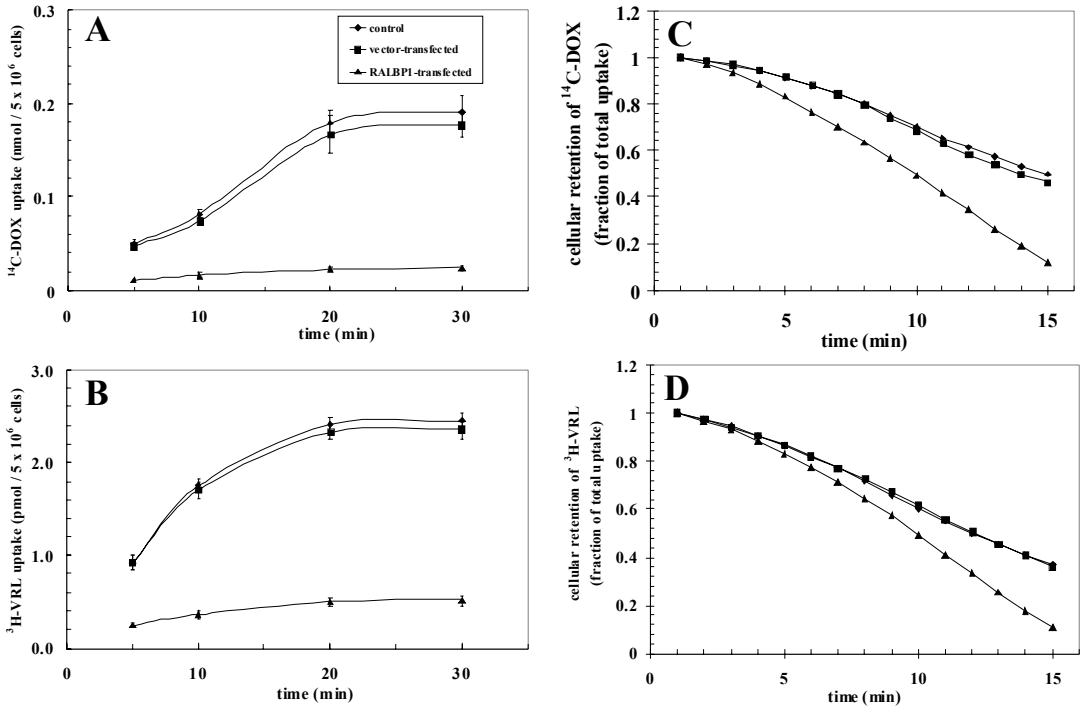

Figure 4.2 Effect of Ralbp1 overexpression on DOX and VRL uptake and efflux in K562 erythroleukemia

The rates of cellular accumulation of [^{14}C]-DOX and [^{3}H]-VRL were evaluated using established methods (Awasthi 2000). Results for cellular accumulation of VRL and DOX are shown (panels A & B) for wild-type (●), vector (■) and Ralbp1-transfected cells (▲). The efflux of DOX was determined by integration of measurements of DOX or VRL appearing in the medium as well as the residual at the end of the experiment to obtain cellular drug-level and efflux rates as per results of previously published studies (Stuckler 2005) (panels C & D).

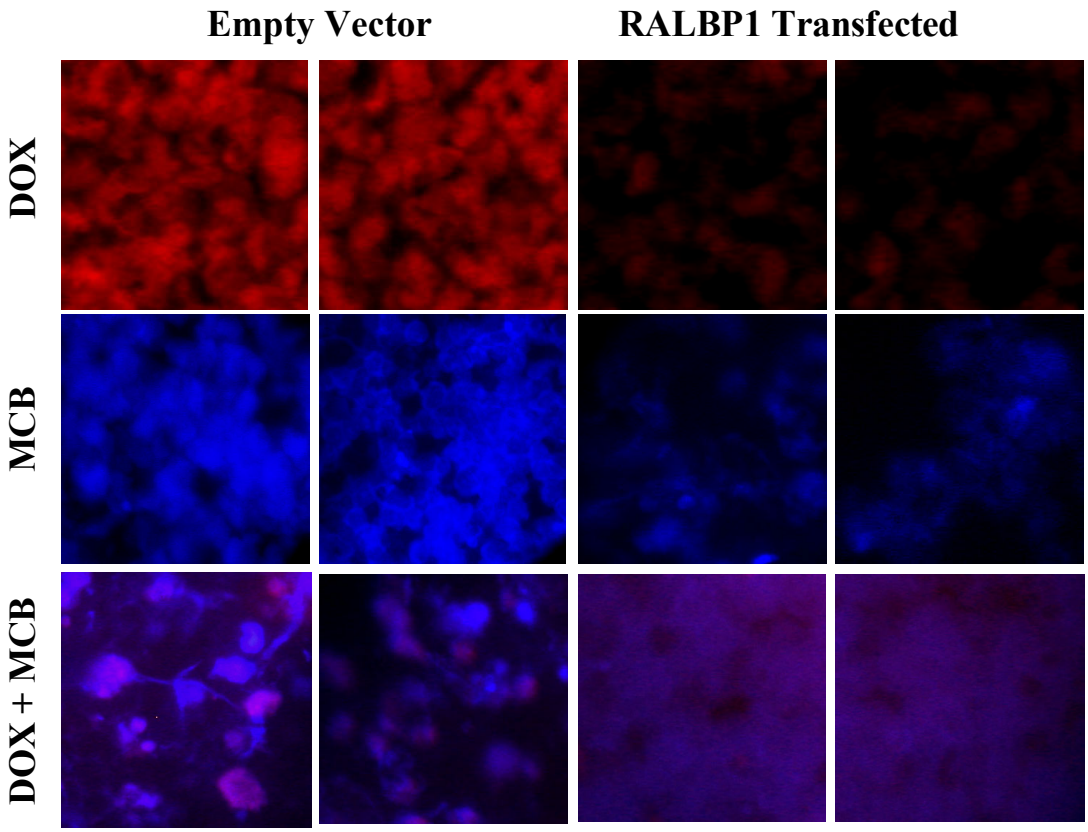

Figure 4.3 Effect of Ralbp1 overexpression on DOX and MCB uptake

Cells growing in a suspension culture at a density of 5 x 10^5 cells / mL were treated with either 50 µM MCB, 10 µM DOX, or both for 20 min at 37 ^oC, rinsed with PBS, and cytospun on to glass slides at 400 x g. The excitation and emission filters used were 380 nm and 470 nm respectively for MCB, and 495 and 550 nm respectively for DOX. Representative fluorescence micrographs at 400 x magnification are presented.

Table 4.1 Effect of Ralbp1 overexpression and anti-Ralbp1 IgG on sensitivity of selected chemicals in K562 cells

The IC_{50} values in nanomolar concentrations are presented as mean $\pm$ SD from three separate determinations with eight replicates each (n = 24). Antibody concentration (either pre-immune or anti-Ralbp1 IgG) is 37 μg / ml.

Compounds	Pre-immune IgG				Anti-Ralbp11 IgG				Ratio IC_{50} Ralbp1/Pre-immune IgG		
	Control	Vector	Ralbp1	Ratio IC_{50} (Ralbp1/ Vector)	Control	Vector	Ralbp1	Ratio IC_{50} (Ralbp1/ Vector)	Control	Vector	Ralbp1
DOX	90+8	80+11	410+38	5.1	38+6	36+4	47+4	1.3	0.4	0.5	0.1
DAU	220+28	280+26	550+56	2.0	80+14	90+11	120+9	1.3	0.4	0.3	0.2
VCR	40+5	38+3	97+6	2.6	17+2	18+1	22+3	1.2	0.4	0.5	0.2
VLB	10+1	10+1	24+3	2.4	4+1	5+1	7+1	1.4	0.4	0.5	0.3
VRL	4+1	4+1	11+1	3.1	2+0.2	2+0.2	3+0.3	1.3	0.4	0.4	0.2
MMC	25+3	28+3	68+7	2.4	14+1	14+1	19+2	1.4	0.6	0.5	0.3
MEL	6,000 +770	5,000 +330	22,000 +1950	4.4	2,500 +220	2,500 +220	3,500 +510	1.4	0.4	0.5	0.2
CDDP	3,500 +560	4,000 +530	9,000 +825	2.3	2,000 +420	2,500 +340	3,500 +470	1.4	0.6	0.6	0.4

Multi drug resistance conferred by Ralbp1 overexpression

Cytotoxicity assays were performed to examine the effects of Ralbp1 transfection on anthracyclines (doxorubicin DOX, daunorubicin DAU), *Vinca* alkaloids (vincristine VCR, vinblastine VBL, and vinerolbine VRL), alkylating agents (melphalan MEL, mitomycin-C MMC) and platinum coordinate (cisplatinum CDDP). The IC_{50} was determined in K562 erythroleukemia cells transfected with empty-vector, Ralbp1 as well as the control untransfected cells growing in log phase 24 h after addition of pre-immune IgG or anti-Ralbp1 IgG (Table 4.1).Whereas empty vector transfection had no detectable effect, Ralbp1 overexpression yielded 2-5-fold resistance to the cytotoxic effects for all drugs tested. Anti-Ralbp1 IgG reduced the IC_{50} of all cell lines, and caused reversion of resistance to the drugs in the Ralbp1-overexpressing cells to near that seen in the wild-type or empty-vector transfected. For confirming that Ralbp1 overexpression is responsible for this effect, anti-Ralbp1 antibodies were used to demonstrate complete reversal of fold increase from 2-5 folds to 1.2-1.4 fold.

Ralbp1 reconstituted efflux

After establishing that Ralbp1 is capable of mediating resistance to a broad range of amphiphillic molecules we proceeded to show whether transport activity is responsible for this resistance by reconstituting purified recombinant Ralbp1 into artificial proteoliposomes composed of asolectin and cholesterol. These proteoliposomes were characterized and optimized for transport studies as described in previous studies for doxorubicin transport (Awasthi 1998). Substrates selected were the classic glutathione conjugates [^{3}H]-DNP-SG and the endogenous glutathione conjugate [^{3}H]-HNE-SG. Uptake of DNP-SG or HNE-SG by proteoliposomes was measured with respect to time

(figure 4.4). Five minutes appears to be the optimal time due to linearlity with respect to substrate uptake. Temperature dependence was determined to ensure optimal transport activity with respect to temperature. For these studies (figure 4.5), ATP-dependent transport was found to be optimal near physiological temperature, 37 °C for both [^{3}H] DNP-SG and [^{3}H] HNE-SG. The decrease in transport activity at slightly higher temperature suggests this protein is prone to heat inactiviation. At low temperature transport is markedly decreased. These results indicate that at physiologicial temperature, Ralbp1 posseses maximal transport activity for both [^{3}H] DNP-SG and [^{3}H] HNE-SG across membranes. It is reported that the amphiphilic nature of glutathione conjugates would permit a significant amount of it to be membrane associated (Awasthi 1998). To account for this membrane association, estimation of the membrane-bound fraction was performed by measuring ATP-dependent uptake varying osmolarity. As shown (Figure 4.6). the amount for [^{3}H] DNP-SG and [^{3}H] HNE-SG vesicular uptake is comparable to published studies (Awasthi 1998, Sharma 2003). Conditions for transport were establish by these studies allowing for the quantification of the kinetic properties for Ralbp1 to be determine with respect to the glutathione conjugate substrates (figure 4.7). From these optimization studies the effect of Ralbp1 transport was determined.

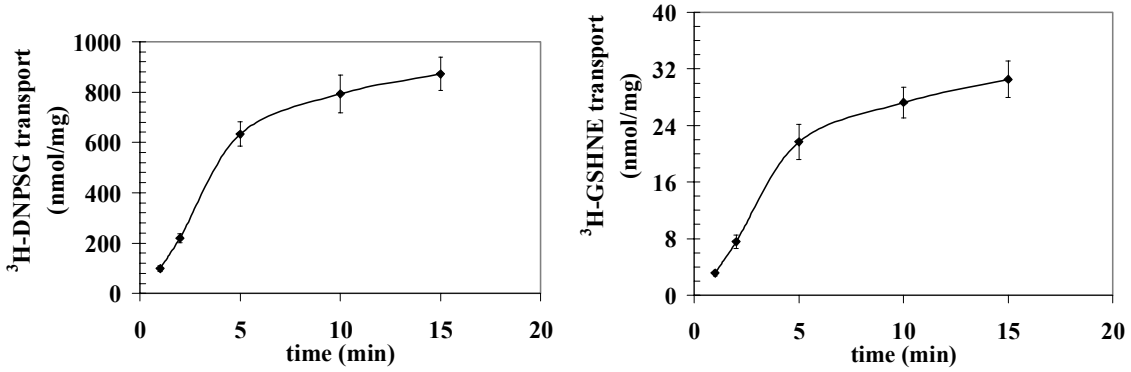

Figure 4.4 Time dependence of Ralbp1 transport

The time dependence of [^{3}H] DNP-SG and [^{3}H] HNE-SG uptake in proteoliposomes was measured. Proteoliposomes reconstituted with 250 ng protein were diluted in transport buffer containing 100 μM [^{3}H] DNP-SG (panel A) or 10 μM [^{3}H] HNE-SG (panel B). Aliquots of transport buffer (50 μL) were filtered in triplicate after varying incubation times (1-15 min.) and radioactivity remaining was quantified. Points are average of three determinations each performed in triplicate.

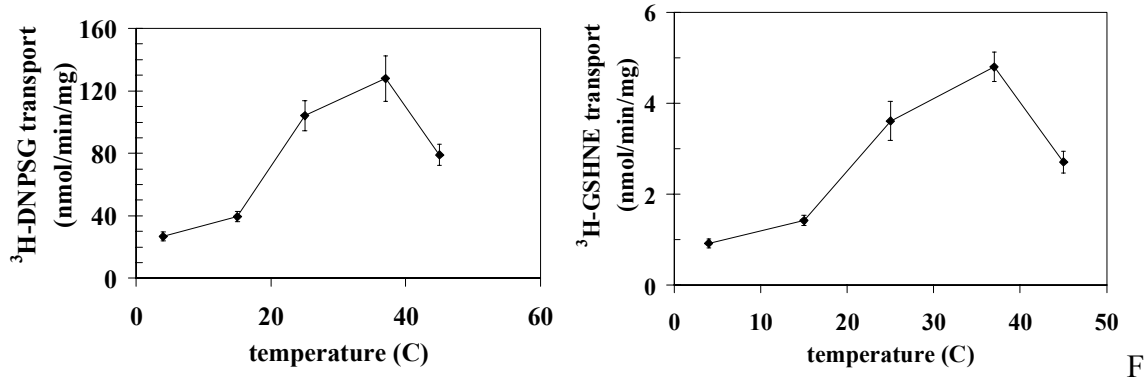

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Figure 4.5 Temperature dependence of Ralbp1 transport

The temperature dependence of DNP-SG and HNE-SG uptake in proteoliposomes was measured. Proteoliposomes reconstituted with 250 ng protein were diluted in transport buffer containing 100 μM [^{3}H] DNP-SG (panel A) or 10 μM [^{3}H] HNE-SG (panel B). After equilibration at temperatures ranging from 4 to 45 °C for 10 min. Samples of transport buffer (50 μL) were filtered in triplicate after 5 min, incubation at the respective temperature was followed by determination of radioactivity. The points represent means of triplicate measurements in a single experiment at respective temperature.

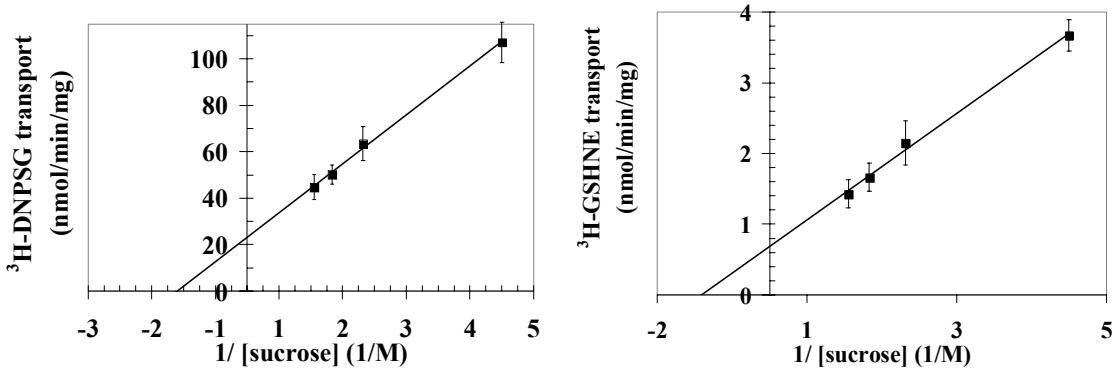

Figure 4.6 Osmolar sensitivity of Ralbp1 transport

Osmolar sensitivity of ATP-dependent DNP-SG and HNE-SG uptake was measured. The temperature dependence of DNP-SG and HNE-SG uptake in proteoliposomes was measured. Proteoliposomes reconstituted with 250 ng protein were diluted in transport buffer containing 100 μM [^{3}H]DNP-SG (panel A) or 10 μM [^{3}H] HNE-SG (panel B) along with varying concentrations of sucrose (between 0.25 and 1 M). After equilibration at 37 °C for 10 min, samples of transport buffer (50 μL) were filtered in triplicate after 5 min, and radioactivity remaining was quantified. Points represent averages of three determinations performed in duplicate.

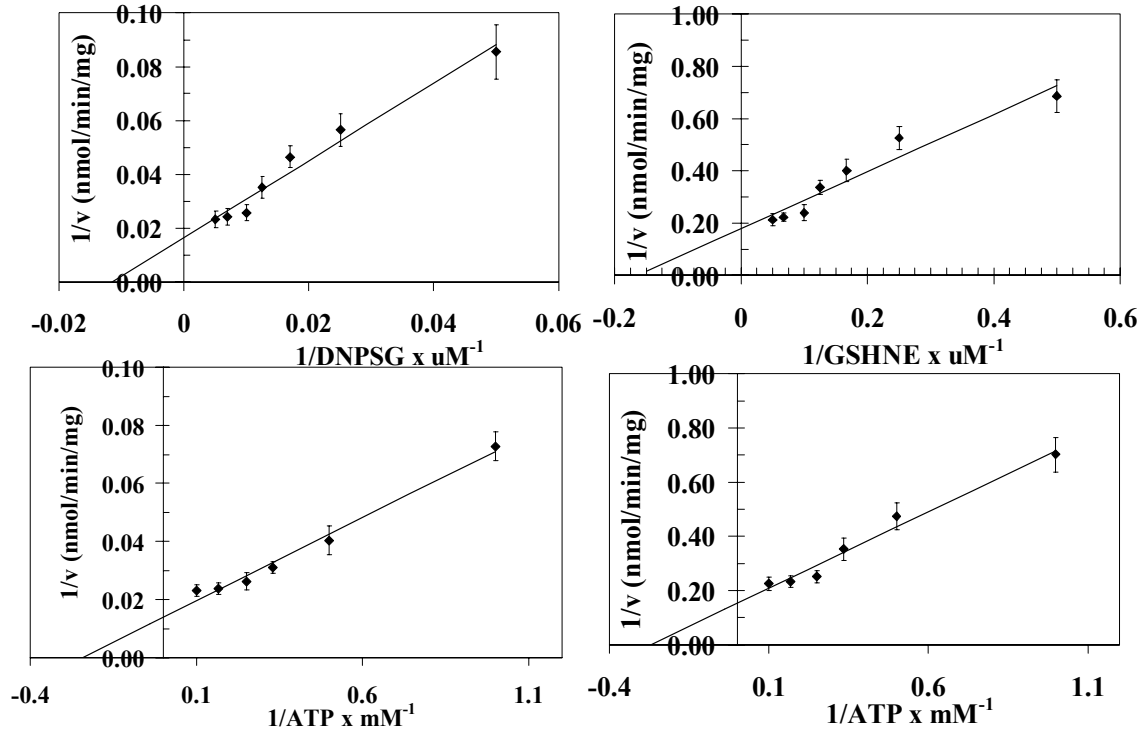

Figure 4.7 Kinetics of ATP-dependent DNP-SG and HNE-SG transport with respect to varying DNP-SG or HNE-SG and ATP

Proteoliposomes reconstituted with 250 ng of protien were diluted in transport buffer containing varying concentraton of substrate (12-170) μM [^{3}H]DNP-SG (panel A) or (1-10) μM [^{3}H] DNP-SG (panel B). Final ATP concentrations in the transport buffers were (1-10) mM. After equilibration at 37 °C for 10 min, samples of transport buffer (50 μL) were filtered in triplicate after 5 min radioactivity remaining was quantified. Data were regressed using Graphpad's Prism enzymatic regression program.

Proteoliposomes were prepared with a fixed protein/lipid ratio of 3 μg of protein/5 mg of lipid (figure 4.8). Transport of [^{3}H] DNP-SG or [^{3}H] HNE-SG by liposomes was not increased by control (-A –P), ATP (+A -P), or protein (-A +P) whereas uptake of [^{3}H] HNE-SG or [^{3}H] DNP-SG in proteoliposomes after 5 min in the presence of both protein and ATP uptake for both substrates was more than 2 fold for [^{3}H] HNE-SG and 3 fold [^{3}H] DNP-SG in comparison to the other three groups. The increase in fold for [^{3}H] DNP-SG can be attributed to a 10 fold increase in concentration (100 μM) versus [^{3}H] HNE-SG (10 μM).

To confirm if the transport in a model system devoid of the contribution by other transporters which are present in the membranes of live cells, the transport function was verified by using plasma membrane vesicles (inside out vesicles) prepared from K562 cells. These cells as described above were stably transfected with the gene RALBP1 leading to increase production of Ralbp1 protein. ATP dependent transport activity was increased in inside out vesicles prepared from the Ralbp1-tarnsfected cells as compared to those prepared from control or blank vector transfected cells (figure 4.9). This clearly indicates that the acquired resistance seen in the cytotoxicity studies was due to enhanced Ralbp1 mediated transport. In addition, the results demonstrate that Ralbp1 expressed in K562 cells are capable of transporting [^{3}H] DNP-SG and [^{3}H] HNE-SG.

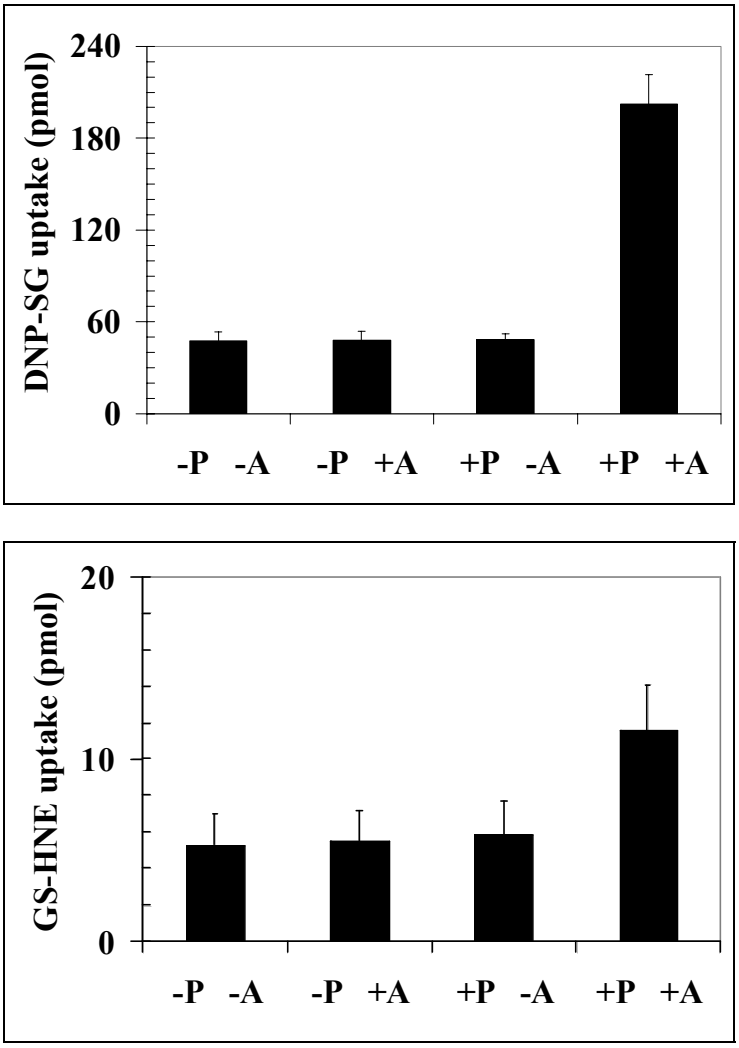

Figure 4.8 [^{3}H]-DNP-SG and [^{3}H]-HNE-SG transport

Uptake by control liposomes or proteoliposomes of [^{3}H] DNP-SG (sp. act. 1.87×10^4 cpm/nmol) and [^{3}H] HNE-SG (sp. act. 3.51×10^4 cpm/nmol) was compared in the presence of ATP (A) or Protein (P). Protein was omitted for reconstitution of control liposomes and examined in the presence of 6M NaCl (-P –A) or 6M ATP (-P +A). Proteoliposomes used for these studies were reconstituted with 3 µg of protein and 5 mg of liposomes. Liposomes or proteoliposomes were diluted 2-fold in transport buffer containing 100 µM [^{3}H] DNP-SG or 10 µM [^{3}H] HNE-SG. After equilibration at 37 °C for 10 min, transport was initiated in the proteoliposome by addition of either 6 mM NaCl (-A +P) or an equiosmolar amount (6 mM) of ATP (+A +P). Samples of transport buffer (30 µL) were removed and proteoliposomes filtered onto a nitrocellulose membrane and radioactivity remaining on the nitrocellulose filter was quantified by a scintillation counter. Data are presented in terms of uptake pmol/5 min (n = 3).

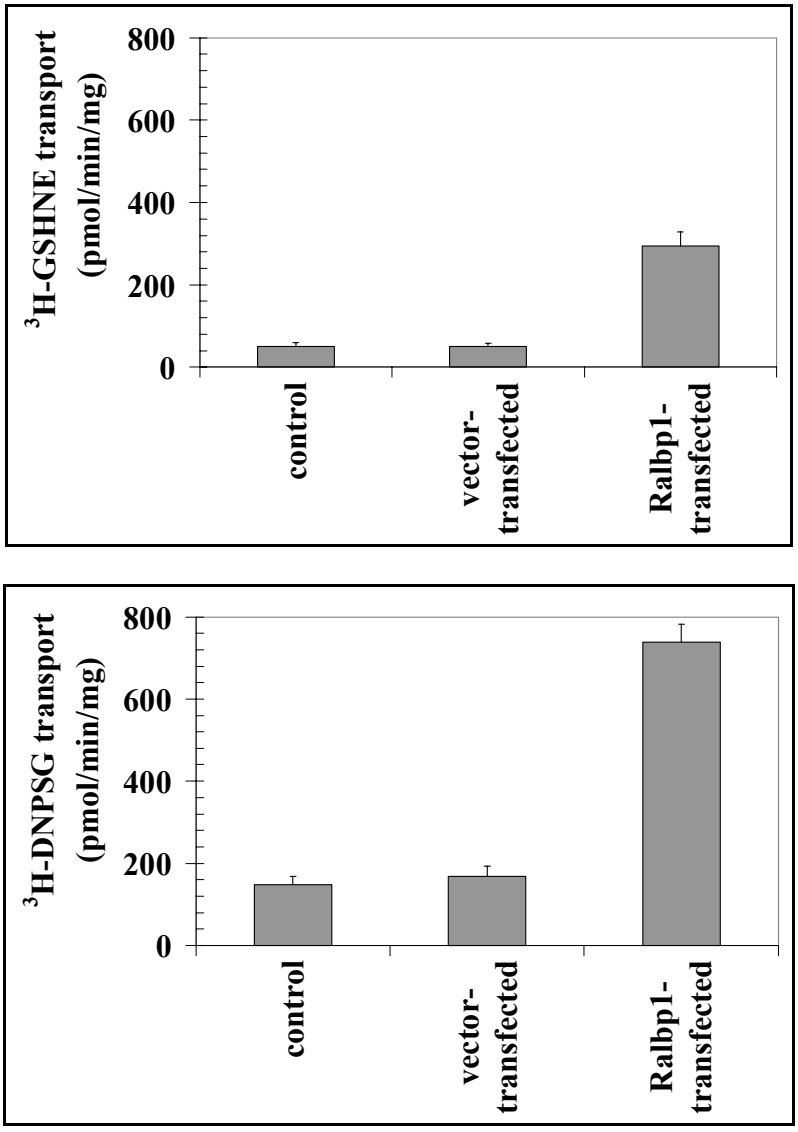

Figure 4.9 [^{3}H]-DNP-SG and [^{3}H]-GS-HNE transport in in-side out vesicles prepared from K562 cells

K562 cells were diluted 40-fold in 0.5 mM sodium phosphate buffer pH 8.0, incubated overnight at 4 °C, and centrifuged at 28000 x g for 1 h. The pellet was suspended in the same buffer and passed repeatedly through a 25-gauge needle. Inside-out vesicles were purified by passing mixed vesicles over a wheat germ agglutinin-Sepharose column which selectively retained right-side-out vesicles (Awasthi 2000). Transport studies in IOVs were done by the same method as described above for the transport using rec-Ralbp1 proteoliposomes with the noted exception of the no protein control. This negative control was done by heat-inactivated IOVs.

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Differentiation of K-562 into RBC's using 4HNE

K-562 is a human erythroleukemia cell line, which characteristically differentiates into hemoglobin-containing erythrocyte precursors upon exposure to a variety of differentiating agents including but not limited to Ara-C, cytosinearabinoside, DMSO, dimethyl sulfoxide; HMBA, hexamethylene bisacetamide, radiation, HNE, 6-mercaptopurine (6MP), methotrexate (MTX), trans-retinoic acid, ATRA (vitamin A), Flavonoids (including apigenin, luteolin, quercetin, genistein) and 1,25-D3 (vitamin D3) 1 ,25-dihydroxyvitamin D3 as examples (Ichikawa 1969, Gann 1977, Collins 1978, Weiss 1978, Breitmann 1981, Lin 2007). Since differentiation therapy has been shown to be very beneficial in another human leukemia, acute promyelocytic leukemia (Savickiene 2006), examination of the mechanisms which trigger differentiation could help to yield novel differentiation therapies for other malignancies. It is notable, that the lists of agents known to trigger partial differentiation of K562 cells are all known to increase lipid-peroxidation. HNE, a known toxic endobiotic in concentration greater than 1 μM, has been reported to conjugate with GSH and form HNE-SG (Esterbauer 1999). This differentiation could be blocked by overexpressing GST isoenzyme A4-4, which displays highest specific activity towards the conjugation of HNE to HNE-SG. GSTA4-4 transfection resulted in lowering the intracellular concentration of HNE to about 1/5 of normal, increased rate of proliferation in transfected cells, and blockade of HNE-mediated terminal differentiation. Because HNE, an obligate byproduct of lipid-peroxidation, can itself trigger nearly complete differentiation to the enucleate erythrocyte, a proposal was put forth that HNE formation was a final common pathway for triggering apoptosis through these diverse agents (Cheng 1999).

Recently, a series of studies has demonstrated that HNE-SG and its reduced metabolite DHN-SG (produced through aldose reductase-mediated catalysis) play a key role in regulation of cell-proliferation pathways in malignant cells (Awasthi 2005). Since reducing total HNE reduced differentiation capacity and increased rate of proliferation, the conclusion drawn was that HNE is necessary for these events. Alternatively, a down-stream metabolite of HNE may be more directly responsible for the signaling effects. Recent studies have demonstrated in a rather dramatic fashion that inhibiting the conversion of HNE-SG to DHN-SG by depleting aldose reductase with antisense technology causes a complete cessation of growth of colon cancer xenografts (Singhal 2007). These findings indicate that the glutathione-conjugates of HNE and other reactive alkenals are the key signaling molecules. If this were the case, depleting these conjugates by augmenting cellular Ralbp1 level should antagonize the differentiating effects of HNE.

Though the differentiating effects of several other weaker differentiating agents require more sophisticated approaches (Awasthi 2006), the differentiation mediated by HNE can be easily followed by measuring total hemoglobin in a pellet of cells exposed to HNE, using Drabkin's reagent. Results of these experiments (Fig. 4.10) were quite clear in showing that addition of HNE caused a significant increase in cellular hemoglobin in control or vector transfected cells, but over-expresion of Ralbp1 cause a complete abrogation of HNE-mediated differentiation to hemoglobin-producing cells. These results were also evident from Wright-Giemsa stained cytospin preparations (Fig. 4.10). The appearance of enucleate 'hemoglobin-lakes' and condensed nuclei caused by HNE is attenuated by the over-expression of Ralbp1. Analysis of cellular growth rate confirmed that Ralbp1 transfected cells also demonstrated increased rate of proliferation (Fig. 4.11),

as was also seen previously with GST A4 transfected cells.

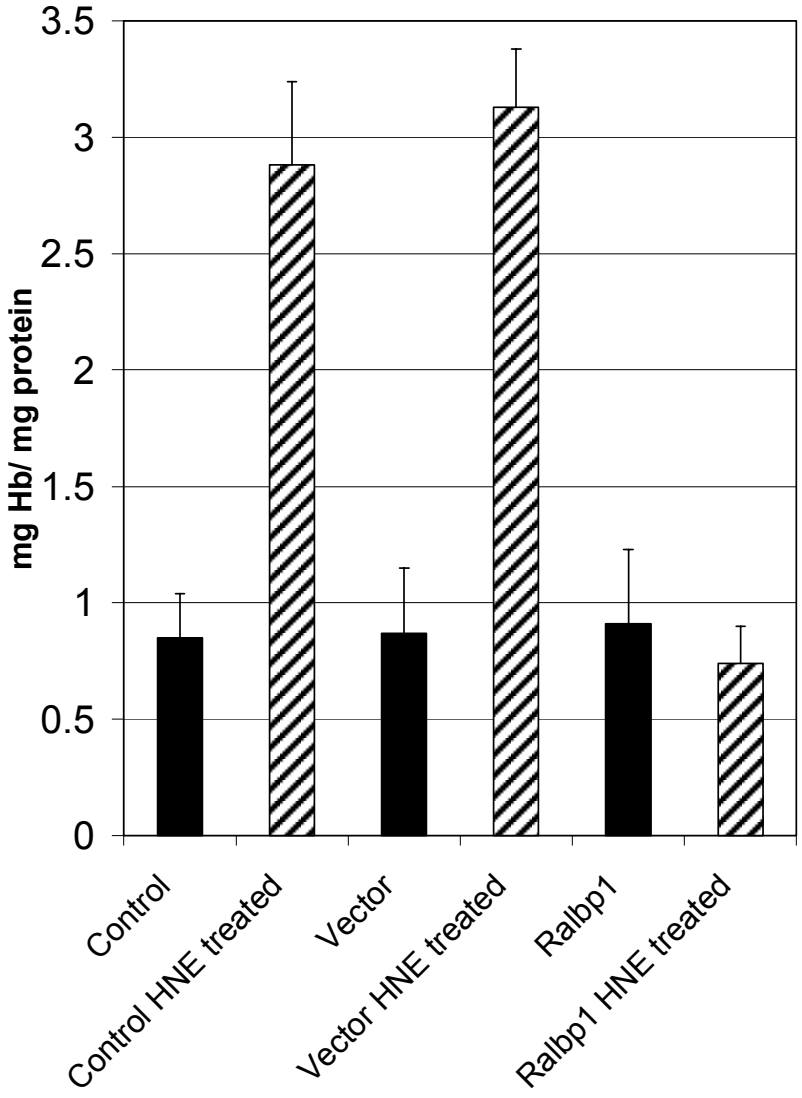

Figure 4.10 Differentiation K-562 into hemoglobin-producing cells by HNE
Cells were grown over a 24 hour period in the presence or absence of 20 μM HNE. Post HNE treatment cell were washed with PBS and lysed. Cell extracts were treated with Drabkin's reagent and quantified in a UV-Vis spectrophotometer at 540 nm.

In vivo toxicity determination

It has been clearly demonstrated in this study that in vitro overexpression of Ralbp1 correlates to a marked increase in cellular survival. However for this observation to be correct in a clinical aspect, alteration of Ralbp1 expression should correlate with a change in survival *in vivo*. C57B mice, both wild type and genetically knocked out for Ralbp1 ($Ralbp1^{-/-}$) were utilized to examine this effect. Injections correlating to the respective median lethal dose LD_{50} of DOX, MEL, BUS, and CYP were administered intraperitoneally, i.p., followed by monitoring subjects from time till death. Health and survival of the animal was recorded and their corresponding survival per population is presented (Figure 4.11).

The ensuing loss of Ralbp1 in the $Ralbp1^{-/-}$ mice conferred an increase in sensitivity to all selected chemotherapy compounds. Each chemotherapy compound decreased survival for the $Ralbp1^{-/-}$ in comparison to wild type by at least 5-13 days. In the wild type, the alkylating agents melphalan and busulfan demonstrated that Ralbp1 is effective in mediating resistance associated with compounds known to be processed by glutathione conjugation. CYP also known to be conjugated by glutathione is more toxic leading to all wild type mice succumbing to the effect of the drug. As seen in figure 1.6 CYP exerts its toxicity through other mechanisms besides the alkylation using the bischloroethyl amine moieties. This *in vivo* study complements and completes this chapter by demonstrating the expression of Ralbp1 correlates to the survival through the removal of toxic stress molecules in both the *in vitro* and *in vivo* model.

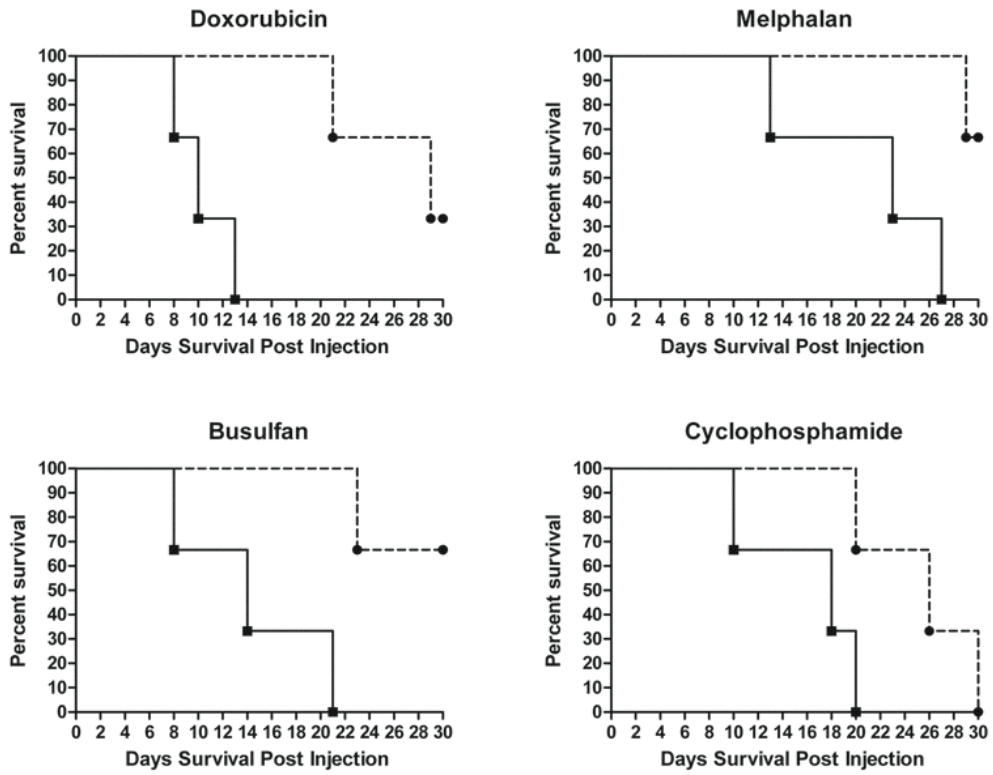

Figure 4.11 Survival curve for DOX, MEL, BUS, and CYP

Survival curves are derived from three C57B wild-type mice (----) and three C57B mice knocked out for the gene Ralbp1 (Ralbp1$^{-/-}$) (¯). Mice were inoculated i.p. with the median lethal dose, LD_{50}, for DOX (11.16 mg/kg), MEL(6 mg/kg), BUS(86 mg/kg), CYP (159 mg/kg) followed by observation for one month till time to death.

Conclusion

The results of these studies show that overexpression of Ralbp1 results in resistance in K562 leukemia cells to the cytotoxic effects of alkylating-agent, transition metal, and natural product class toxins, as well as resistance to the differentiating effects of the natural alkylating-agent, HNE. Resistance encompassed by the classical Pgp substrates (*Vinca* alkaloids and anthracyclines), as well as GS-E forming drugs (MMC, CDDP, MEL) known to be transported by Pgp, MRP1, and other MRP-related transporters (Sarkadi 2003). The resistance was due to decreased overall cellular drug-accumulation caused by greater efflux. The specific nature of resistance was demonstrated by reversal using anti-Ralbp1 IgG which has been shown previously to bind to a cell-surface epitope of Ralbp1 (aa 171-185) and inhibit the transport activity of Ralbp1 (Yadav 2004). The differential ability of Ralbp1 to mediate greater resistance to DOX than DAU suggests a possible reason for the greater efficacy of DAU in leukemia as compared with DOX. The ability of Ralbp1 overexpressing cells to preferentially lower nuclear accumulation of DOX suggests that this transporter is operational also at the nuclear membrane. In a similar manner depletion or absence of Ralbp1 *in vivo* clearly demonstrates the importance of Ralbp1 transport in mediate additional forms of stress.

The predictions of cell-culture studies with respect to the ability of Ralbp1 to protect against the toxins were entirely confirmed in animal studies that clearly demonstrated that loss of Ralbp1 translates into significantly earlier death after poisoning by alkylating agents, metabolically activated alkylating agent (cyclophosphamide), platinum coordinate, or anthracycline.

The HNE-mediated differentiation studies have interesting implications when taken in context with previous studies evaluating the effect of HNE-depeletion using

GST-transfectants. In GST-transfectants, though HNE was decreased, if Ralbp1 remined unchanged, HNE-SG (or DHN-SG) should have increased. Thus, the observed effect of resistance to differentiation and increased proliferation caused by HNE-depletion could have been due to either a decrease in HNE, or relative increase in the glutatathionylated metabolites (due to GST-mediated catalysis). Present studies dispel the latter prediction, because they show that increased expression of a protein that directly lowers glutathionylated metabolite levels also causes resistance to differentiation, and increase in proliferation. Because the Michael addition reaction that gives rise to HNE-SG from reaction of GSH and HNE is near equilibrium under cellular conditions (in which a high GSH concentration and plenty of GST enzyme activity are present), it can be assumed that increased removal of HNE-SG or DHN-SG will result in lowering or HNE levels as well. Indeed, this has been confirmed by previous studies showing that Ralbp1 over-expression results in decreased cellular total HNE content (Dwivedi 2007). Thus, we conclude that processes that act to deplete HNE will function to inhibit differentiation and promote cell-proliferation, and as a logical consequence, conditions that increase cellular HNE should inhibit cell-proliferation, promote differentiation, and (as clearly demonstrated for a number of other cancer cells), promote apoptosis. Identification of each of the particular protein-HNE interactions responsible most directly for the differentiating, proliferation, or apoptosis effects of modulating cellular concentration of HNE remain to be fully elucidated, but studies (Awasthi 2006) indicate that the list of protein targets of HNE include glucose-6-phosphatase, adenylate cyclase, DNA polymerase , DNA polymerase , adenine nucleotide translocator, Na^+K^+-ATPase, ADP ribosyl transferase, and NADPH oxidase (Siems 2003).

CHAPTER 5
MODULATION OF RALBP1 STRESS RESISTANCE
THROUGH HEAT STRESS PATHWAY INTERACTIONS

Introduction

Ralbp1 has been demonstrated to posses an array of functions in the cell in addition to transmembrane efflux including mitosis and clathrin-dependent receptor-ligand endocytosis (Awasthi 2003, Flores 2005). In order to perform this broad array of functions, Ralbp1 must be capable of protein-protein interactions in concert with transmembrane transport. A majority of protein-protein interaction for Ralbp1 occurs through the C-terminal region. The C-terminal region of Ralbp1 allows Ralbp1 to interact with Ral as an effector protein and provides downstream signaling of endocytosis through partner of Ralbp1 (POB1). This interaction between Ralbp1 and POB1 has been shown to regulate the transport activity of Ralbp1 (Yadav 2005). Recently Ralbp1 has been shown to bind to heat shock transcription factor 1 (HSF1) and heat shock protein 90 kDa (HSP90) in a trimeric complex, which affects expression of heat shock protein 70 kDa (HSP70) (Hu 2003, Yamamoto 2007). Since HSF1 is also known to interact with the C-terminal of Ralbp1 we expect HSF1 to regulate transport activity of Ralbp1 in a similar manner as POB1 (Figure 5.1).

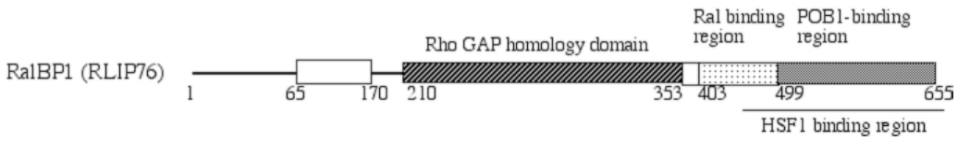

Figure 5.1 Topology of Ralbp1 (Mivechi 2003)

The ability of a heat shock transcription factor to possess protein interactions with Ralbp1 adds the possibility of a novel role for a GSH conjugate transporter protein to be linked with heat stress pathways.

Heat shock transcription factor 1 is a well characterized 57.3 kDa transcription factor that is responsible for activation of heat shock response genes that lead to the production of heat shock proteins, chaperones. This transcription factor forms a homotrimer when activated by phosphorylation (figure 5.2, Xia 1997).

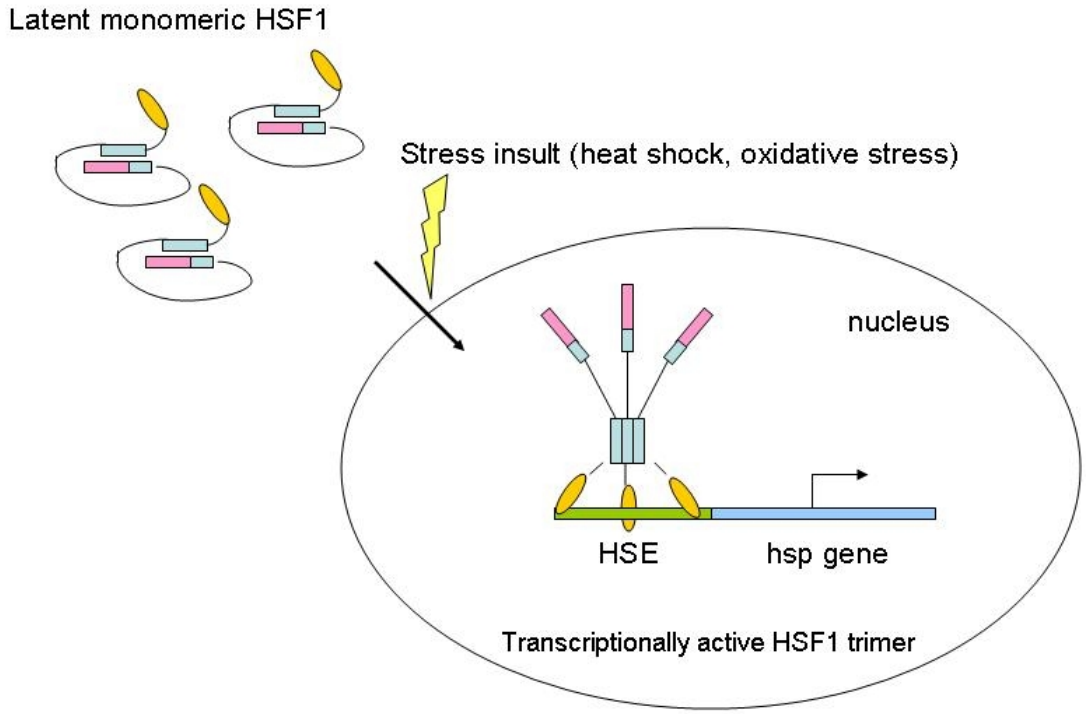

Figure 5.2 Trimerization of HSF1

Monomeric HSF1 under the induction of stress translocates into the nucleus to which it trimerizes to form transcriptionally active HSF1 which transcripts mRNA for heat shock proteins.

The DNA binding domain recognizes the sequences nGAAn and begins transcription of the RNA for chaperone proteins. This transcription response was discovered by

experimentation with elevated temperatures and later found to be induced by chemical, radiant, and physiological stressors which lead to the discovery that the heat shock response is regulated by oxidative stress in addition to thermal stress (Ahn 2003). The heat shock response element is present in organisms from prokaryotes to eukaryotes (Holzer 2007, Arrigo 1999, Westerheide 2005, Voellmy 2006, Anckar 2007, Voellmy 2007).

This chapter addresses the hypothesis that increase HSF1 regulates the transport activity of Ralbp1. This hypothesis was tested by examining Ralbp1 transport activity in the presence of purified rec-HSF1. These studies intend to establish that HSF1 regulates the transport activity in a similar fashion as POB1 (Singhal 2008).

Effect of protein inhibitors on RALBP1 uptake and efflux

Crude membrane vesicles from cells were prepared and the subsequent uptake and efflux of DOX was determined in the presence and absence of POB1, HSF1 or an adjuvant administration of both HSF1 and POB1. As demonstrated by figure 5.3 both uptake and efflux were affected by the presence of either HSF1 or POB1 with POB1 having a great effect of inhibition than HSF1. Remarkably POB1 and HSF1 together led to a mildly synergistic effect.

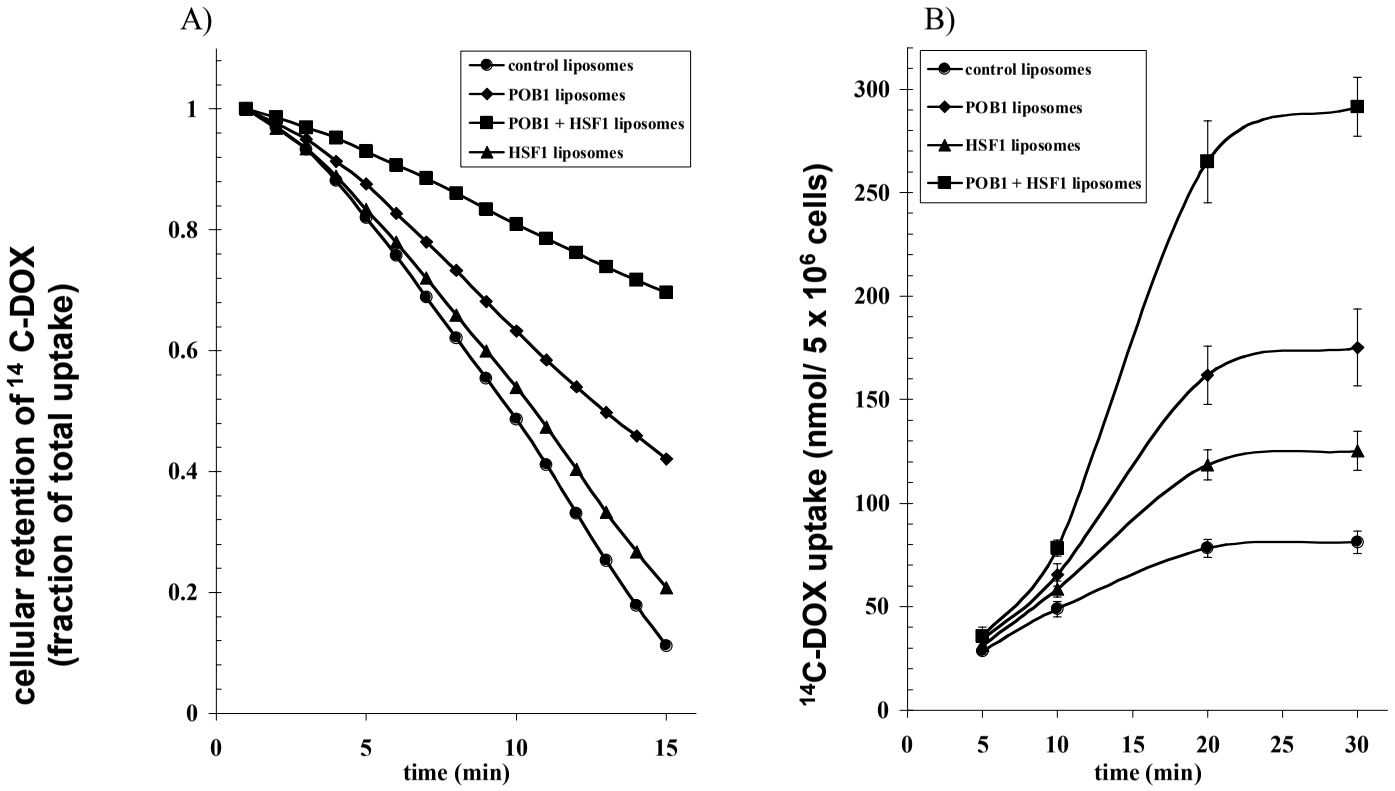

Figure 5.3 Effect of RALBP1 uptake and efflux in the prescence of HSF1, POB1 and HSF1 and POB1
The rate of cellular accumulation of [^{14}C]-DOX was evaluated using established methods (Awasthi 2000). Results for cellular efflux (panel A) and cellular accumulation of DOX are shown (panels B) for control (●), POB1 liposomes (◆), POB1 and HSF1 liposomes (■) and HSF1 liposome (▲).

Effect of POB1, HSF1, and POB1+HSF1 on[^{14}C]-Dox transport

Results of our transport studies examining the effect seen in uptake and efflux corroborated with previous studies indicating that increasing HSF1 concentration decreased transport activity. The transport activity was further inhibited in the presence of POB1 nearly 55 % compared to HSF1 inhibition of approximately 40 %. Similarly to the efflux/uptake studies, the adjuvant administration of both POB1 and HSF1 to Ralbp1 liposomes shows an inhibition of nearly 80 % transport activity when compared to control (figure 5.4). This inhibition of transport activity by HSF1 can serve as a regulation for Ralbp1 on the efflux of HNE since low levels of HNE are known to enhance cellular proliferation. By regulating the amount of HNE transported by Ralbp1 the cell can regulate signaling and apoptotic pathways depended upon HNE signaling.

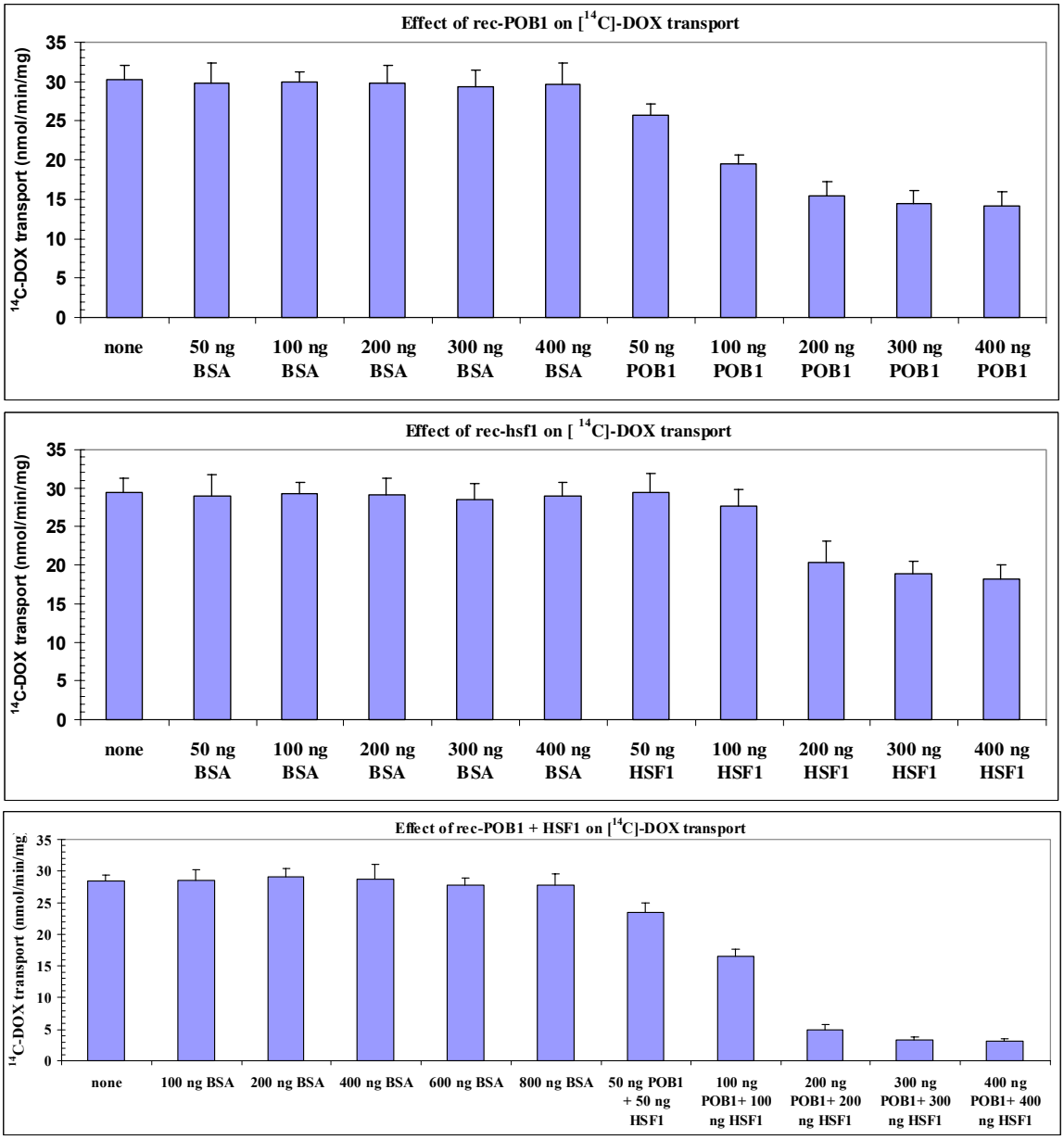

Figure 5.4 Effect of POB1, HSF1, and POB1+HSF1 on [^{14}C]-Dox transport Proteoliposomes containing 3 µg of protein per 5 mg of liposome were utilized to monitor the effect of increased protein of either BSA (control), POB1, HSF1, or POB1+HSF1 synergy. Concentrations of protein are in nanograms delivered using proteoliposomes.

CHAPTER 6

CONCLUSIONS

The significance of showing that Ralbp1 is a major contributor to stress resistance through a drug accumulation defect mechanism rather than ABC transporters is, ABC transporters lack correlative evidence of their expression relative to their drug resistant phenotype. This lack of evidence suggests the presence of other energy-dependent transmembrane transporter proteins to be contributors to resistance through drug accumulation defect. Knockout mice for Ralbp1 were shown not to lose their GSH conjugate transport capacity whereas, the MRP1 knockout mouse lost 80 % of its DNP-SG transport capacity (Awasthi 2003).

Ralbp1 over-expression assists in stress resistance by conferring resistance to endogenous and exogenous stressors by contributing to the efflux of either the parent molecule itself or its associated GSH metabolite. Contrasting that statement, inhibition of Ralbp1 allows a build up of GSH conjugates that inhibit other proteins responsible for the prevention of oxidative damage leading to a cell with increased stressors that lead to apoptosis or enhance mutagenesis. The hypothesis examined within this manuscript states that Ralbp1 is a major contributor to stress resistance of both endobiotics and xenobiotics, and modulation can occur through protein interactions of heat stress pathways. The material presented represents a novel finding that overexpression of Ralbp1 results in an extensive stress resistant phenotype. This drug-accumulation defect has been clearly demonstrated

by transport of GSE in a model system devoid of other efflux proteins. Within the cell line K562 we tested a broad range of substrates to confirm the hypothesis that Ralbp1 transport is indeed capable of mediating the stress associated with the introduction of xenobiotics. Substrates tested were chemotherapy agents ranging from the classical ABC transporter family of substrates anthracyclines DOX and DAU to compounds from the *Vinca* alkaloids, alkylating agents, platinum coordinates, and mitomycin C. It has been clearly established that Ralbp1 is capable of mediating the stress associated with these agents. Reversal of this accumulation defect has been established by inhibition of Ralbp1 using IgG against Ralbp1. Ralbp1 contribution to stress resistance has been demonstrated in vivo, through the studies involving KO animals. Loss of Ralbp1 has been clearly demonstrated to decrease time to death upon the administration of xenobiotic stress. In addition to clearly demonstrating Ralbp1's role in stress resistance the interaction with the C-terminal of Ralbp1 to HSF1 demonstrates that transport activity can be regulated in a similar fashion as previously reported by a known downstream partner to Ralbp1 termed partner of Ralbp1 (POB1) (Yadav 2005). The evidence presented calls for the importance of investigating other proteins associated with stress resistance thereby gaining a better understanding of how biological systems remediate stress.

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BIOGRAPHICAL INFORMATION

Kenneth Drake received his baccalaureate degree from Cameron University with a major in chemistry and a minor in biology. During his time at Cameron University Kenneth was part of several organizations helping him prepare for a venture into graduate study most notably the Ronald E. McNair scholars program and the Louis Stokes Alliance for Minority Participation. From these two opportunities he has pursued a doctorate at the University of Texas at Arlington with the hope of being the first in his family to earn a doctorate degree.