COMPARISON OF REDOX-ACTIVE AND CONJUGATIVE ENZYMES WITH AND WITHOUT EFFLUX TRANSPORTERS AND THEIR COMPARATIVE EFFECTIVENESS IN PROTECTION AGAINST CELLULAR TOXICITY OF 4-HYDROXY-2-NONENAL (HNE), AN ALDEHYDE LIPID PEROXIDATION PRODUCT

BY

Lisa P. Rudd

A Thesis Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

In Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Molecular Biology

August 2009

Winston-Salem, North Carolina

Approved by:

Alan Townsend, Ph.D., Thesis Advisor

Examining Committee:

Mark Miller, Ph.D., Committee Chair

Charles Morrow, Ph.D.

Robert Wykle, Ph.D.
ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Alan Townsend, my committee chair, Dr. Mark Miller, and committee members Dr. Larry Daniel, Dr. Charlie Morrow, and Dr. Bob Wykle for their time, expertise, guidance, and suggestions during the course of my Master’s studies at Wake Forest University.

I would also like to acknowledge my colleagues in Dr. Townsend’s lab: Ms. Sandra Kabler, Ms. Melissa Fleming, Ms. Patricia Durant and Dr. Sarfaraz Ahmad. I would like to especially thank Ms. Sandra Kabler for her technical support and Dr. Sarfaraz Ahmad for his assistance with experimental design and data analysis.

I would like to thank Dr. Trevor Penning and Dr. Thomas Kensler for providing the cDNAs for the AKR1B1, AKR1C1, and hAOR respectively. I would like to thank Dr. Charlie Morrow for providing the MCF7 and HepG2 derived cell lines and Ms. Pam Smitherman for her assistance and technical support.

I would like to acknowledge support from the faculty and students in Department of Biochemistry during my studies at Wake Forest University.

I would like to give special thanks to my friends for their trust and kindness and my family for always being there for me.
# TABLE OF CONTENTS

LIST OF ABBREVIATIONS........................................................................................................iv

LIST OF ILLUSTRATIONS.........................................................................................................vi

ABSTRACT.....................................................................................................................................viii

Chapter

I. INTRODUCTION.................................................................................................................. 1

II. CYTOTOXICITY, GLUTATHIONE DEPLETION AND HNE-PROTEIN ADDUCT FORMATION BY 4-HYDROXY-2-NONENAL (HNE) IN MCF7 AND HepG2 CELL LINES EXPRESSING HUMAN GLUTATHIONE S-TRANSFERASE M1, ALONE OR TOGETHER WITH HUMAN MRP1 OR MRP2........................................................................12

III. REDOX-ACTIVE AND CONJUGATIVE ENZYMES AND THEIR COMPARATIVE EFFECTIVENESS AGAINST TOXICITY OF 4-HYDROXY-2-NONENAL (HNE), AN ALDEHYDE LIPID PEROXIDATION PRODUCT........................................................................41

IV. DISCUSSION......................................................................................................................93

REFERENCES.......................................................................................................................105

SCHOLASTIC VITA..................................................................................................................116
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>Advanced DMEM</td>
<td>Advanced Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>AR</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AOR</td>
<td>alkenal/one oxidoreductase</td>
</tr>
<tr>
<td>AKR</td>
<td>aldo-keto reductase</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>BSO</td>
<td>L-buthionine-[S,R]-sulfoximine</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>CysGly-HNE</td>
<td>cysteinylglycine-HNE</td>
</tr>
<tr>
<td>DHN</td>
<td>1,4-dihydroxynonene</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FA-OOH</td>
<td>fatty acid hydroperoxides</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HNA</td>
<td>4-Hydroxy-2-nonenonic acid</td>
</tr>
<tr>
<td>HNE</td>
<td>4-Hydroxy-2-nonenal</td>
</tr>
</tbody>
</table>
HNE-SG  HNE-glutathione adduct
JNK       c-Jun N-terminal kinase
LDH       lactate dehydrogenase
LPO       Lipid peroxidation
LTB\textsubscript{4}-DH leukotriene B4 dehydrogenase
MAPK      mitogen-activated protein kinase
MDCK      Madin-Darby Canine Kidney II cells
MRP1      multi-drug resistance protein 1
MRP2      multi-drug resistance protein 2
MTT       [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NQO       4-nitroquinoline-1-oxide
OR        oxidoreductase
PBS       phosphate-buffered saline
PL-OOH     phospholipid hydroperoxides
PUFA      polyunsaturated fatty acid
ROS       reactive oxygen species
SAPK      stress-activated protein kinase
SDS       sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA       trichloroacetic acid
### LIST OF ILLUSTRATIONS

#### TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Summary of cell lines/GST activities/GST levels</td>
<td>21</td>
</tr>
<tr>
<td>2.</td>
<td>Cytotoxicity of HNE in MCF7 derived cell lines expressing GSTM1 or MRP1, or both together</td>
<td>23</td>
</tr>
<tr>
<td>3.</td>
<td>Cytotoxicity of HNE in HepG2 parent and GSTM1-expressing cell lines</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>Summary of GSH depletion by HNE in MCF7 derived cell lines</td>
<td>27</td>
</tr>
<tr>
<td>5.</td>
<td>Formation of HNE-protein adducts by HNE in MCF7 derived cell lines</td>
<td>33</td>
</tr>
<tr>
<td>6.</td>
<td>GSH Depletion in V79 Cell Lines Dosed at 20 μM HNE</td>
<td>76</td>
</tr>
<tr>
<td>7.</td>
<td>GSH Depletion in V79 Cell Lines Dosed at 40 μM HNE</td>
<td>77</td>
</tr>
<tr>
<td>8.</td>
<td>GSH Depletion in V79 Cell Lines Dosed at 60 μM HNE</td>
<td>80</td>
</tr>
</tbody>
</table>
## LIST OF ILLUSTRATIONS

### FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HNE chemical reactivity and metabolism ..................... 11</td>
</tr>
<tr>
<td>2</td>
<td>GSH depletion by HNE in MCF7 derived cell lines ............. 29</td>
</tr>
<tr>
<td>3</td>
<td>HNE-protein adduct formation in MCF7 derived cell lines .... 32</td>
</tr>
<tr>
<td>4</td>
<td>pcDNA 3.1 (-) Hyg was selected as a vector for subcloning cDNA inserts .... 46</td>
</tr>
<tr>
<td>5</td>
<td>Western blot of AKR1C1 clones from stable transfection into V79 cells .......... 60</td>
</tr>
<tr>
<td>6</td>
<td>Western blot of AKR1B1 clones from stable transfection into V79 cells .......... 62</td>
</tr>
<tr>
<td>7</td>
<td>Western blot of hGSTA4 clones from stable transfection into V79 cells .......... 64</td>
</tr>
<tr>
<td>8</td>
<td>AKR1C1 enzyme activity for enzymes used in study ............. 66</td>
</tr>
<tr>
<td>9</td>
<td>GST enzyme activity for enzymes used in study ................ 68</td>
</tr>
<tr>
<td>10</td>
<td>ALDH3 enzyme activity for enzymes used in study .............. 70</td>
</tr>
<tr>
<td>11</td>
<td>HNE cytotoxicity in V79 cell lines, IC&lt;sub&gt;50&lt;/sub&gt; Values in μM HNE (n=7) ........ 73</td>
</tr>
<tr>
<td>12</td>
<td>HNE GSH depletion at 20 μM and 40 μM HNE dose in V79 cell lines .......... 79</td>
</tr>
<tr>
<td>13</td>
<td>HNE GSH depletion at 60 μM HNE dose in V79 cell lines .......... 82</td>
</tr>
<tr>
<td>14</td>
<td>HNE-protein adducts in V79 cell lines ......................... 86</td>
</tr>
<tr>
<td>15</td>
<td>HNE-protein adducts in V79 cell lines: repeat blot with higher HNE concentrations .......... 88</td>
</tr>
</tbody>
</table>
ABSTRACT

Comparative protection by Redox-active and Conjugative Enzymes Against Cellular Toxicity of 4-hydroxy-2-nonenal (HNE)

Thesis under the direction of Alan Townsend, Ph.D., Professor of Biochemistry

4-hydroxy-2-nonenal (HNE) is one of the most reactive aldehydes produced during lipid peroxidation (LPO) and has been demonstrated to have cytotoxic and genotoxic effects. Several oxidoreductases (ORs) have been reported to detoxify HNE including aldehyde dehydrogenase (ALDH) and aldo-keto reductases (AKRs). Glutathione S-transferases (GSTs) are known to play a role in the detoxification of HNE by the formation of an HNE-glutathione (GSH) conjugate. We used stable transfection of V79 or MCF7 cell lines to generate transgenic model systems to compare protection across the different enzyme classes represented by ALDH3, GST 5.7, and AKR1C1. ALDH3 showed strong protection against HNE cytotoxicity, protein adduct formation, and GSH depletion. AKR1C1 and GST 5.7 failed to protect against these endpoints.

The hemi-acetal ring of the HNE-SG conjugate can break to form an open chain aldehyde-SG, which can be cytotoxic by reacting with cellular macromolecules. Thus, efflux transporters including multi-drug resistance proteins (MRP1/MRP2) may be required for removal of HNE-SG. The combined role of GSTs with MRP1/2 was studied in stably transfected MCF7 and a HepG2 cell line model. Co-expression of GSTM1 + MRP1 leads to increased HNE-protein adducts formation and sensitization to HNE cytotoxicity (0.44 fold). Expression of GSTM1 alone and MRP1 alone leads to moderate but significant sensitization (0.7- 0.8 fold). GSH depletion was significant in MRP1
expressing cell lines (MCF7/MRP1 and MCF7/MRP1/GSTM1), with only 15-17% GSH remaining after exposure of 60 μM HNE for 60 min. The mechanism by which GST and MRP1/2 modulate HNE cellular toxicity needs to be elucidated.
**CHAPTER I**

**INTRODUCTION**

**Lipid Peroxidation and reactive aldehydes**

Oxidative stress occurs due to an imbalance between pro-oxidants and antioxidants resulting in damage to cellular macromolecules. Proteins, nucleic acids, and lipids are targets of oxidative stress. Lipid peroxidation (LPO) is a particularly damaging consequence since it generates free radicals, which can produce highly reactive compounds. LPO is initiated by hydrogen abstraction of polyunsaturated fatty acids (PUFAs) of biological membranes to form an alkyl radical, which rearranges to a conjugated diene. An autocatalytic cascade can result in the formation of fatty acid (FA-OOH) and phospholipid (PL-OOH) hydroperoxides, which propagate the chain reaction. The process is terminated by $\beta$-scission, a spontaneous cleavage of the fatty acid chain. LPO results in the production of a variety of reactive compounds including $\alpha,\beta$ unsaturated aldehydes. Classes of aldehydes produced are: 2-alkenals, 4-hydroxy-2-alkenals, and ketoaldehydes [1].

4-Hydroxy-2-nonenal (HNE) is a highly reactive 4-hydroxy-2-alkenal and is one of the most cytotoxic aldehydes produced by LPO. HNE has been extensively studied and it’s cytotoxic and genotoxic effects reported [2, 3].

Levels of HNE generated are higher when the cell is undergoing oxidative stress. Normal cellular levels of HNE have been reported to be 0.1 $\mu$M – 3 $\mu$M and levels as high as 10 $\mu$M – 5mM have been reported within the bilayer of peroxidizing lipid membranes [4].
HNE has been proposed as a possible biomarker of disease because there is evidence of elevated levels of HNE in brain tissue in Alzheimer’s disease, atherosclerotic plaques, and in the myocardium during ischemia/reperfusion injury [1].

**Chemical reactivity of HNE**

The high reactivity of HNE is due to the presence of three functional moieties in the molecule, an aldehyde bonded to a C$_2$=C$_3$ double bond linked to a hydroxyl group, forming a conjugated bond system. This conjugated system renders C-3 susceptible to nucleophilic attack in a reaction known as a Michael addition. The hydroxyl group is electron withdrawing and by inductive effects strengthens the Michael addition site. Cysteine, lysine, and histidine residues in protein may react with HNE by Michael addition reactions, as shown in Figure 1. The aldehyde group of HNE can react with primary amines, e.g. lysine residues in proteins, to form a Schiff base product (Figure 1). Michael addition adducts with protein sulfhydryl groups are the most common type of adduct formed with HNE [5, 6].

HNE can form adducts with glutathione (HNE-SG). Since GSH is a tripeptide containing a cysteine residue, HNE-SG adducts may be formed by Michael addition reactions. The HNE-SG thiol adduct forms a cyclic hemiacetal, which can undergo a reversible ring opening reaction yielding a GSH-conjugated open-chain aldehyde. Although the majority of HNE-SG (95%) is in the cyclic hemiacetal form, the 5% that is in the open chain aldehyde form can react via Schiff base formation with cellular proteins resulting in adverse effects on the cell [7]. Glutathione, which contains an SH group, functions as a cellular reducing agent to convert reactive electrophiles to less reactive species. Thus, GSH provides protection against oxidative stress. GSH depletion is a
consequence of oxidative stress and increases vulnerability to xenobiotics. Excessive formation of HNE-SG may also cause depletion of the GSH pool in cells.

HNE can react with nucleic acids to form adducts by two routes. Reaction of HNE with the NH₂ group on C-2 of guanosine will yield the Michael addition product. If peroxides are present, HNE can be oxidized to form an epoxide, which can react with guanosine to form etheno-DNA adducts [6, 7].

HNE-protein adducts and HNE-DNA adducts are thought to compromise function and thereby lead to cytotoxic and genotoxic effects [3, 8]. Adverse effects on cellular function include cytotoxicity, mutation, inhibition of DNA, RNA, and protein synthesis, protein alkylation, GSH depletion, induction of apoptosis as evidenced by caspase activation and DNA fragmentation [9], and aberrant cell signaling [8, 10].

**HNE Metabolic Pathways**

Mammalian cells have adapted different metabolic routes for the inactivation of LPO products to non-reactive or less reactive species, as shown in Figure 1. The two main biotransformation enzyme groups are oxidoreductases (ORs) and the glutathione \textit{S}-transferases (GSTs). There are several cellular oxidoreductase enzymes capable of metabolizing HNE to less reactive compounds as a detoxification mechanism. Enzymes reported to be involved in HNE metabolism include: 1) aldehyde dehydrogenase which oxidizes HNE to form 4-hydroxy-2-nonenoid acid (HNA), 2) alcohol dehydrogenase (AD), and 3) aldo-keto reductase (AKR), both of which reduce HNE to form 1,4-dihydroxynonene (DHN), and 4) leukotriene B4 dehydrogenase (LTB₄-DH), an alkenal/one oxidoreductase (AOR), which reduces alkenals to alkanals [11, 12].
The objective of the studies presented is to compare protection from HNE-mediated toxicity by representative enzymes that oxidize the aldehyde group of HNE (ALDH) relative to those that reduce the aldehyde group of HNE (AKR), and also to compare the oxidoreductases to glutathione S-transferases, which catalyze conjugation of the thiol moiety of GSH at the C₂=C₃ Michael addition site. AD is not believed to be a major pathway for the metabolism of HNE since it is an NADH-dependent enzyme, and although it reacts with HNE with high catalytic efficiency, the NAD⁺/NADH ratio in the cell is high [13, 14]. Siems et al. studied the relative contribution of the metabolizing pathways of HNE in hepatocytes. Approximately 33% was due to GSH conjugation to form HNE-SG, ~32% was due to HNE oxidation to form HNA, and ~8% HNE reduction to form DHN. An additional ~12% of HNE formed secondary metabolites from either HNA or DHN, ~6% of HNE did not react, and the remaining 9% of HNE was metabolized by unidentified pathways. Since oxidation is the predominant redox pathway, ALDH appeared to play a larger role in HNE metabolism than reductive enzymes, AKR or hAOR in transfected cells [15]. The GSH conjugation component also appeared to be quantitatively similar to that of HNA, from HNE oxidation.

ALDH3 is a cytosolic isozyme that oxidizes medium chain length aldehydes, and is expressed in the cornea and upper digestive tract [16, 17]. ALDH3 has been reported to confer resistance to oxidative damage due to UV light and in corneal epithelial cells [16]. Townsend et al. observed in overexpressing cells ALDH3A1 (class 3 ALDH) protection against toxicity of medium-chain aliphatic aldehydes, ranging from 9–25 fold protection, as measured by growth inhibition, GSH depletion, apoptosis, and HNE-protein adducts in V79 cells [18].
In the present studies, the enzymes AKR1B1, AKR1C1 and hAOR were considered likely candidates for protection from HNE toxicity in V79 cells and compared to ALDH3. The two members from the AKR family were selected based on literature reports of the role of AKRs as candidate protective enzymes with broad substrate specificity including hydrophobic, as well as, hydrophilic substrates. Kinetic parameters for HNE as a substrate for AKR1B1 ($k_M = 22 \mu M$, $k_{cat}/K_M = 4.6 \times 10^6 \text{ min}^{-1} \text{M}^{-1}$) and AKR1C1 ($k_M = 34 \mu M$, $k_{cat}/K_M = 2.6 \times 10^5 \text{ min}^{-1} \text{M}^{-1}$) suggested sufficient substrate binding and catalytic efficiency [19, 20]. Kinetic analysis of AKR1C1 compared to four other members of the AKR1C subfamily and AKR1A1 showed high specific activity with HNE and was the only isoform inducible by GSH-depleting agents (ethacrynic acid) and oxidative stress ($\text{H}_2\text{O}_2$) [20].

The enzyme AOR catalyzes the NAD(P)H dependent reduction of the carbon-carbon double bond of alkenals and alkenones. AOR has been proposed as protective against HNE. Kinetic data from rat AOR (rAOR) indicated high catalytic efficiency for HNE reduction ($k_{cat}/K_M = 3.3 \times 10^7 \text{ min}^{-1} \text{M}^{-1}$) [12]. The human AOR enzyme was not chosen for investigation in our current studies due to the much lower catalytic activity with HNE (Dr. Xiang Yu, personal communication), and difficulties with vector construction.

**Glutathione S-transferases**

Glutathione S-transferases (GSTs) are a class of enzymes known to play a role in the detoxification of reactive electrophiles, reactive oxygen species (ROS), and oxidative stress products by catalyzing the conjugation of these species to glutathione, or in some
cases the GSH-dependent reduction of ROS. Glutathione (GSH) is present ubiquitously in most human cell types and can also reduce ROS nonenzymatically [21].

Yang et al. reported the role of human alpha-class GST in reducing products of lipid peroxidation. Fatty acid hydroperoxides and phospholipid hydroperoxides were found to be the preferred substrates for hGSTA1-1 and hGSTA2-2 [4]. Certain members of the alpha-class GST have a substrate preference for HNE and exhibit high catalytic efficiency of HNE-SG formation. These include: hGSTA4, hGST5.8, mGSTA4 (GST5.7), and rGSTA4. Cheng et al. reported kinetic data for Alpha class GST’s: hGSTA1-1 conjugates HNE with a $K_M = 50 \mu M$ and a $k_{cat} / K_M = 3.48 \times 10^6 \text{min}^{-1}\text{M}^{-1}$, while hGSTA4-4 conjugates HNE with a $K_M = 49 \mu M$ and a $k_{cat} / K_M = 1.62 \times 10^8 \text{min}^{-1}\text{M}^{-1}$ [22]. Thus, hGSTA4-4 conjugates HNE with a similar $K_M$ compared to hGSTA1-1, however the catalytic efficiency is 50-fold higher. GSTA4-expressing cells have been observed to have lower steady state levels of HNE [23]. Human GST 5.8 has been reported to have high specificity toward HNE as a substrate. However, this enzyme has not yet been cloned and its primary structure is unknown [4].

Protection by GSTA4 from HNE and $H_2O_2$ cytotoxicity was reported in K562 human erythroleukemia cells. Short-term exposure of cells to 20 $\mu M$ HNE resulted in differentiation and apoptosis. K562 cells transfected with mGSTA4-4 showed resistance to HNE-induced differentiation, apoptosis, and cytotoxicity. Protection against HNE toxicity in mGSTA4-4 (GST5.7) transfected cells was 1.6-fold compared to K562 wild type. K562 cells transfected with mGSTA4-4 ($IC_{50} = 35 \mu M$) had a 1.6-fold protection against $H_2O_2$ toxicity compared to K562 wild type ($IC_{50} = 22 \mu M$) [24].
Previous work has been done in our lab investigating the role of GST 5.7 in cells that were exposed to HNE. GST 5.7 failed to block HNE-protein adducts relative to empty vector-transfected control at HNE concentration of 0-40 μM. In addition, expression of GST 5.7 failed to block HNE toxicity and resulted in activation of caspase-3, accelerating commitment to apoptotic cell death at a dose of 60 μM HNE (R. Haynes and A. Townsend, unpublished results).

The hemiacetal ring of the HNE-SG conjugate can break to form an open chain aldehyde-SG with a saturated C2-C3 bond, which can be cytotoxic by reaction of the free aldehyde with cellular DNA, RNA, and proteins [7]. Conjugate accumulation has been reported to cause product inhibition of GSTs, which could also lead to increased intracellular levels of HNE and other electrophiles [25]. This observation could also explain the lack of GST protection against HNE toxicity previously observed in our lab. Hence, removal of HNE-SG by an efflux transporter, such as MRP, could be necessary to prevent these consequences.

**Xenobiotics Efflux transporters**

There are three transmembrane efflux pumps reported to be involved in the removal of HNE-SG conjugates: MRP1, MRP2, and RLIP76. Which of these transporters is the most relevant for HNE-SG efflux is a matter of debate [26].

This research project focuses on the role of the well-characterized transporters MRP1 and MRP2 in the efflux of HNE-SG conjugates. MRPs are a family of transporters that function in the ATP-dependent efflux of organic anions and a variety of endobiotics and xenobiotics alone or as glutathione, glucuronate, or sulfate conjugates [27]. Removal of toxic compounds from the cell by MRPs plays a critical role in detoxification. MRP1
is ubiquitously expressed while MRP2 has expression primarily in the liver, kidney, and gut. MRP2 is expressed on the apical membrane of polarized cells, while MRP1 and other members of the MRP family are expressed on the basolateral side of polarized cells [28].

MRP1 can transport the anticancer drugs daunorubicin and vincristine unmodified, a process that involves co-transport of unconjugated GSH, and thus requires adequate levels of GSH [29, 30]. Thus, GSH is an important regulator of MRP1 function. The MRP2 transporter is also known to efflux glutathione-conjugates in an ATP-dependent manner similar to MRP1. The substrates for MRP2 largely overlap with those for MRP1. However, the two transporters differ in kinetic properties [31]. Certain organic anions, such as indomethacin and probenecid have different effects on the ATPase activity and transport by MRP1 and MRP2 [32].

Renes et al. have demonstrated transport of HNE-SG by MRP1 and have suggested that MRP1 is physiologically relevant for HNE detoxification [21]. Three cell lines were studied: the human small cell lung cancer line GLC4 and two multidrug resistant derivative cell lines, selected with doxorubicin [33]. Inside-out membrane vesicles were prepared from these cell lines for transport studies by isolating the membrane fraction from cell lysate and passage through a 25 gauge needle [30]. HNE-[\textsuperscript{3}H]SG was prepared by incubation of a 10:1 molar ratio of HNE with [\textsuperscript{3}H]SG for 1 hr at 37 °C. Kinetic parameters for HNE-SG transport by MRP1 were determined (K\textsubscript{M} = 1.6 ± 0.21 μM, V\textsubscript{max} = 804.5 ± 28.8 pmol/min/mg protein) [21]. This is consistent with HNE-SG being a comparatively high affinity substrate [25]. Inhibition of MRP1 by MK571 resulted in increased sensitivity to HNE as measured by IC\textsubscript{50} values [21]. This suggests that MRP1 may play a role in protection from HNE cytotoxicity.
There is also evidence for the role of MRP2 as a major efflux transporter for HNE-SG conjugates. Ji et al. have demonstrated MRP2 dependent efflux of HNE-SG conjugates. Using MDCK (Madin-Darby Canine Kidney II) cells expressing MRP2, the HNE-SG conjugate was efficiently removed. However, paradoxically these cells become more vulnerable to HNE toxicity, with a 70 % decrease in cell viability observed. GSH levels were consumed to the point of cell necrosis [34].

In order to evaluate the relative efficacy of protection against HNE toxicity, we have compared stably transfected V79 cell lines expressing human AKR1C1 or murine GSTA4 to parental (non-expressing) cells. As a positive control, cells expressing human ALDH3A1 were also compared. These studies have revealed that neither AKR1C1 nor murine GSTA4 are protective against HNE toxicity.

In addition, we have examined the role of MRP1 in protection against HNE toxicity. MCF7 cells expressing either hGSTM1 alone, MRP1 alone, or hGSTM1 and MRP1, were compared with parental MCF7 cells. In these studies, MRP1 paradoxically appears to sensitize MCF7 cells to HNE, particularly in the presence of hGSTM1 expression.
Figure 1. HNE chemical reactivity and metabolism

HNE can react with lysine, cysteine, or histidine residues to form two types of cellular protein adducts, namely, a Michael addition adduct and a Schiff base product. HNE can also bind to and form adducts with DNA and phospholipids. Reaction at the C-3 carbon of HNE results in the formation of the Michael addition adduct, while reaction at the C-1 (aldehyde) position results in a Schiff base product. Oxidoreductases (ORs) and the glutathione S-transferases (GSTs) are the key known enzymes involved in HNE detoxification. ORs detoxify HNE by either reduction (AKR family) or oxidation (ALDH family). hAOR is the only reported enzyme which detoxifies HNE by reduction of the C2-C3 double bond. GSTs catalyze the conjugation of HNE with the thiol group of glutathione. This product is in equilibrium with a hemiacetal product [11, 35, 36].
Figure 1.
CHAPTER II

CYTOTOXICITY, GLUTATHIONE DEPLETION AND HNE-PROTEIN ADDUCT FORMATION BY 4-HYDROXY-2-NONENAL (HNE) IN MCF7 AND HepG2 CELL LINES EXPRESSING HUMAN GLUTATHIONE S-TRANSFERASE M1, ALONE OR TOGETHER WITH HUMAN MRP1 OR MRP2

Introduction

Lipid peroxidation (LPO) is a free radical-mediated process whereby polyunsaturated fatty acids (PUFAs) of biological membranes and membrane-bound organelles are degraded to form reactive and toxic α,β-unsaturated aldehydes. HNE is one of the most highly reactive and cytotoxic aldehydes produced from this process. HNE can react with either nucleic acids or proteins to form adducts [1-3]. The most common type of HNE-protein adduct formed is through the reaction between cysteine, lysine, or histidine residues and the C2=C3 double bond of HNE, resulting in a Michael-addition product at the C3 position [5, 6, 37].

Mammalian cells have adapted different metabolic routes for the inactivation of LPO products to non-reactive or less reactive species [11]. One important pathway of detoxification is through conjugation with glutathione (GSH), catalyzed by glutathione-S-transferases (GSTs). Members of the GST superfamily of conjugases can detoxify a wide range of electrophilic compounds, including cytotoxic drugs and carcinogens, by conjugation with GSH. However, overexpression of GST alone may not be sufficient to provide protection against cytotoxicity of these compounds [38-42]. The primary reasons for this occurrence appear to be product inhibition of the GST by the resulting conjugate
[43, 44], possible reversibility of conjugation [6], and in a few instances, increased toxicity of the conjugate relative to the parent compound [45].

The HNE-SG product rapidly cyclizes to yield a thiohemiacetal derivative that can undergo a reversible ring opening reaction to yield a GSH-conjugated open-chain aldehyde (5% of the total conjugated product), which can react with cellular proteins to form Schiff base adducts, resulting in adverse effects on the cell [7]. Accumulation of HNE-SG has been reported to cause product inhibition of GSTs which could lead to increased intracellular levels of HNE and other substrate electrophiles [27]. Hence, the formation of an HNE-SG conjugate alone may not fully detoxify HNE, thus requiring removal of HNE-SG by an efflux transporter, to prevent toxic consequences [30].

Glutathione, a carrier of SH groups, functions as a cellular reducing agent to inactivate reactive oxygen species. Thus, excessive formation of HNE-SG may cause toxicity via critical depletion of the essential cellular GSH pool.

Multidrug resistance proteins (MRPs) are a family of transporters that function in the ATP-dependent efflux of organic anions and a variety of endo- and xenobiotics either alone or as glutathione, glucuronate, or sulfate conjugates [27]. By removal of toxic compounds from the cell across the plasma membrane, MRPs play a critical role in detoxification. MRP1 is ubiquitously expressed while MRP2 is expressed primarily in the liver, kidney, and gut. MRP2 is expressed on the apical membrane of polarized cells, while MRP1 and other members of the MRP family are expressed on the basolateral side of polarized cells [28]. The MRP2 transporter is also known to efflux glutathione conjugates, in an ATP-dependent manner similar to MRP1. The substrates for MRP2
largely overlap with those for MRP1. However, the two transporters differ in kinetic properties [31].

The possible interaction between GST enzymes and MRPs has been suggested by a number of studies. Morrow et al. reported that expression of GSTA1 in MCF7 cells promoted the detoxification of chlorambucil (CHB), a genotoxic electrophile, but only when MRP1 is co-expressed [46, 47]. In another study, combined expression of both GSTA1 and MRP2 was necessary to confer resistance to CHB toxicity in HepG2 cells [48]. Morrow et al. also demonstrated that GSTP1 protection against 4-nitroquinoline 1-oxide (NQO) toxicity was dependent on MRP2 activity in HepG2 cells [49]. Further, a role of GSTP1 and MRP1 in NQO toxicity in MCF7 cells was reported [41, 50]. Morrow et al. found no protection against NQO cytotoxicity in the GSTP1 expressing cell line, while the MRP1 and GSTP1 ± MRP1 expressing cell lines exhibited a 4-fold and a 10-fold protection against NQO cytotoxicity, respectively. These data support a synergistic interaction of GSTP1 and MRP1 [50].

The role of transporters in removal of HNE-SG has been reported, including members of the ATP-binding cassette (ABC) family of proteins: multi-drug resistance proteins MRP1 and MRP2 [51]. Renes et al. have demonstrated transport of HNE-SG by MRP1 and have suggested that MRP1 plays a physiologically relevant role in HNE detoxification [21]. There is also evidence for the role of MRP2 as an efflux transporter for HNE-SG conjugates [34]. The combined role of GSTs with these transporters has not been directly addressed with lipid aldehydes, such as HNE, as substrates.

To investigate the role of GSTs and MRPs in the detoxification of HNE, we used cell line models derived from MCF7 and HepG2 cells. HNE is effectively conjugated
with GSH by GST isozymes of the Alpha and Mu classes [52]. We determined the effect of GSTM1 and MRP1, either alone or combined in a stably transfected MCF7 cell line model. This cell line was chosen, since MCF7 cells express low endogenous activity of either GSTM1 or MRP1 [46, 53, 54]. As a secondary model, we investigated the effect of GSTM1 and MRP2, either separately or combined in a HepG2 cell line model. The HepG2 cell line was selected, since these cells endogenously express high levels of MRP2 efflux activity and low levels of GST activity [49, 55, 56]. Differences in HNE-induced cellular toxicity were measured using cytotoxicity, GSH depletion, and HNE-protein adduct formation as endpoints.

Materials and Methods

Reagents

All chemicals were of analytical grade and were obtained from Fisher (Atlanta, GA) or Sigma (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (Adv. DMEM) and Advanced Dulbecco’s Modified Eagle’s Medium (Adv. DMEM) were purchased from Gibco/BRL, Grand Island, NY. 4-hydroxynonenal (HNE) was purchased from Cayman Chemical, Inc. (Ann Arbor, MI). Anti-HNE-Michael adducts antibody (reduced) polyclonal was purchased from Calbiochem (San Diego, CA). Goat-anti-rabbit HRP conjugate secondary antibody was purchased from Bio-Rad (Hercules, CA). β-actin mouse monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and Cell Culture

Parental MCF7 human breast carcinoma cells and transduced derivatives were obtained from Dr. Charles Morrow. These cells have been characterized and shown to
have low endogenous expression of MRP1 and GST activity. Cell lines were established as described previously by stable transduction with a control vector (pLNCX) or expression vectors for GSTM1 and/or MRP1 [46, 50, 57, 58]. The MCF7/pLNCX and MCF7/MRP1 cell lines were selected with 1.5 mg/ml G418. MCF7/GSTM1 was selected with 0.2 mg/ml hygromycin and MCF7/MRP1/GSTM1 was selected with 1.5 mg/ml G418 and 0.2 mg/ml hygromycin. Prior to plating experiments, cells were removed from hygromycin selection for one passage (~2 days) but were maintained continuously under G418 selection.

Human hepatoma HepG2 and HepG2/μ-2 cells were obtained from Dr. Charles Morrow. The parental cell line HepG2 was originally obtained from ATCC (HB8065). The HepG2/μ-2 line was constructed by transduction of HepG2 cells with a GSTM1a retroviral vector. These cell lines were characterized for GST and MRP2 expression as described previously [48, 49, 58]. HepG2 (parental) and HepG2/μ-2 (expressing GSTM1a) were grown in DMEM/F12 (1:1) media containing 10 % FBS, penicillin-streptomycin at 37 °C, in 95% air + 5% CO₂. The GST transfectant cell line was maintained in 0.2 mg/ml hygromycin. Hygromycin was removed for 3-4 days prior to experimental set-up.

GST enzyme assay

GST activity in cell lysate was measured as described previously with minor modifications [59, 60]. Cell pellets were collected by scraping into cold phosphate buffered saline (PBS)/5 mM EDTA and centrifuged at 1,000 x g for 5 min at 4 °C. The cell pellet was lysed in 50 mM Tris/5 mM EDTA by sonication (Fisher sonic dismembranator, setting 35 % power, 10 pulses). The lysate was centrifuged at 12,000 x
g for 5 min. GST activity was measured at room temperature using 10-20 μL cell lysate in a reaction buffer composed of 1 mM glutathione (GSH) in 0.1 mM K$_2$PO$_4$, pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in absorbance at 340 nm was monitored for a total of 90 sec at 15 sec intervals. Activity was calculated based on the ΔA/min and extinction coefficient [9.6 (mmol/L)$^{-1}$cm$^{-1}$], corrected for background activity by subtraction of a reagent blank. Total protein concentration in the cell lysate was determined using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Enzyme activities were expressed as nmol/min/mg protein.

**Cytotoxicity determination**

Cytotoxicity was measured using the sulforhodamine B microtiter plate assay as described [61]. MCF7 derived cell lines were plated at a density of 400 cells/well in DMEM with 10 % FBS; HepG2 derived cell lines were plated at a density of 1,200 cells/well in DMEM with 10 % FBS 24 hours prior to dosing in 96-well plates. Cells were then exposed to various concentrations of HNE or vehicle control ethanol (0.1 % final concentration) for 2 hours in serum-free medium. After dosing, the serum-free medium was replaced with serum-containing medium and cells were allowed to incubate for 5 days (MCF7) or 7 days (HepG2), and subsequently fixed in 10% TCA and stained with 0.4 % sulforhodamine B in 1 % glacial acetic acid. Plates were then washed with 1 % glacial acetic acid 4-5 times, oven dried at 55 °C, and then solubilized in 10 mM Tris base. IC$_{50}$ values were obtained from the measured absorbance at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The cytotoxicity values are
expressed as IC$_{50}$ values or fold-difference relative to the control cell line [IC$_{50}$ value transfectant cell line/ IC$_{50}$ value control cell line].

**HNE-protein adduct determination (Western Blot)**

HNE modification of cellular proteins was determined by Western blot. Briefly, cells were plated at a density of 1.5 x 10$^6$/100 mm dish 24 hours prior to treatment with HNE. Following exposure, cells were washed and scraped into cold phosphate buffered saline (PBS)/ 5 mM EDTA and centrifuged at 1,000 x g for 5 min. The cell pellet was lysed in 50 mM HEPES/150 mM NaCl, 1 % Igepal pH 7.5 with 1x Halt Protease Cocktail (ThermoScientific, Rockford, IL) by sonication (Fisher sonic dismembranator, setting 35 % power, 10 pulses). The lysate was centrifuged at 12,000 x g for 5 min and the supernatant assayed for total protein concentration using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Immediately after lysis, cell lysate was chemically reduced with sodium borohydride (Sigma), for 30 min at room temperature, final concentration 100 mM in order to stabilize Michael protein adducts formed as a result of HNE exposure. After reduction HNE-adducted proteins were separated on a 10 % SDS/PAGE gel and transferred to PVDF membrane (Millipore, Bedford, CA) and probed with anti-HNE reduced Michael adduct antibody (Calbiochem/EMD Biochemicals, Inc., San Diego, CA). Primary antibody was used at a dilution of 1:4,000 in 1 % non-fat milk and incubated overnight at 4 $^\circ$C. A goat anti-mouse secondary antibody from Bio-Rad was used (Hercules, CA) at a dilution of (1:10,000) in 1 % non-fat milk and incubated for 2 hrs at RT. Immunoreactive proteins were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) followed either by film exposure or FujiFilm LAS-3000.
Imaging/Gel documentation system. Protein loading equivalency was determined by stripping the blot with 0.2 M glycine pH 2.0 and reprobing with β-actin antibody. For protein-adduct quantification, MultiGauge Version 3.0 (FujiFilm, Corp.) was used.

**GSH depletion**

Cells were plated at a density of 1 x 10^6/100 mm dish 48 hours prior to treatment with HNE. A time-course study of GSH depletion was performed at 60 µM HNE for exposure times up to 1 hour. HPLC analysis of reduced GSH levels in treated cells was described previously [62, 63]. Following HNE treatment, cells were harvested into 5% perchloric acid (wt/vol), containing an internal standard, 10 µM γ-glutamyl-glutamate, and 0.2 M boric acid. The samples were then centrifuged at 14,000 x g for 2 min to separate precipitated proteins from the acid-soluble sample supernatant. The supernatant was treated with iodoacetic acid and subsequently derivatized with dansyl chloride. The derivatized samples were separated by HPLC with a fluorescence detector on a Supelcosil LC-NH2 column, 4.6 x 25 cm, (Supelco, Bellefunk, PA) with the use of a Shimadzu modular HPLC system and a Shimadzu RF-10A fluorescence detector, using an excitation wavelength of 335 nm, and an emission wavelength of 550 nm. GSH levels were quantified based on peak area integration relative to internal standard area and expressed as nmol GSH/mg protein.

Cells were plated for each cell line in a separate plate for analysis of cellular protein using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Alternatively, the protein pellet from each sample was resuspended in 500 µL of 1 M NaOH and analyzed for protein content by the Bradford method with bovine serum albumin (BSA) as a standard.
**Statistical Analysis**

Data analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc. (LaJolla, CA). Cytotoxicity and GSH depletion data are expressed as the mean ± standard deviation (SD) of three or more independent experiments. Statistical differences were evaluated by one-way ANOVA and Tukey’s post-hoc test to evaluate statistical significance. P values < 0.05 were considered significant. For comparison of the HepG2 and HepG2/μ2 cell line data, a two-tailed Student’s t-test was performed for comparison of the two cell lines. Statistical significance was defined as p < 0.05.

**Results**

**GST enzyme activity in transfected cell lines**

The GST enzyme activities in transfected cell lines were measured to ensure that the GSTM1 transfected cell line had adequate GST expression and that background GST levels were low in non-expressing cell lines. The HepG2 parental cell line had a GST activity of <5 nmol/min/mg protein, while the HepG2/μ–2 cell line had a GST activity of 313 nmol/min/mg protein (Table 1). Thus, adequate GST expression was obtained with this cell line with a low background activity in the parental cell line.

The MCF7 parental cell line and the MCF7/MRP1 cell line had GST activities of <5 nmol/min/mg protein. Thus, low background GST activity was found in the parental and MRP1-transfected cell lines. The two GST-transfected cell lines both had high levels of GST activity. There was no significant difference (p > 0.05) in activities between the MCF7/GSTM1 (216 nmol/min/mg protein) and MCF7/MRP1/GSTM1 (230 nmol/min/mg protein) cell lines (Table 1). Thus, GST activity was high and closely matched for the MCF7 derived cell lines.
Table 1. Summary of cell lines/GST activities/GST levels

Results for GST activities and basal (resting) GSH levels are the mean ± 1 SD of three or more measurements.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>MRP type</th>
<th>GST</th>
<th>GST activity (nmol/min/mg)</th>
<th>GSH levels (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hep G2</td>
<td>Parental control</td>
<td>(endogeneous) MRP2</td>
<td>none</td>
<td>&lt;5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hep G2/μ−2</td>
<td>Transduced GSTM1</td>
<td>(endogeneous) MRP2</td>
<td>GSTM1</td>
<td>313 ± 20</td>
<td>N.D.</td>
</tr>
<tr>
<td>MCF7</td>
<td>pCLNX transduced empty vector control</td>
<td>none</td>
<td>none</td>
<td>&lt;5</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>MCF7/MRP1/GSTM1</td>
<td>MRP1 and GSTM1 transduced cell line</td>
<td>MRP1</td>
<td>GSTM1</td>
<td>230 ± 18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>44 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCF7/GSTM1</td>
<td>GSTM1 transduced cell line</td>
<td>none</td>
<td>GSTM1</td>
<td>216 ± 21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>65 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCF7/MRP1</td>
<td>MRP1 transduced cell line</td>
<td>MRP1</td>
<td>none</td>
<td>&lt;5</td>
<td>46 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different (p < 0.05) from the mean for the MCF7 control.

<sup>b</sup> Not significantly different (p > 0.05) from the mean for the MCF7 control.

N.D. = Not Determined

<sup>c</sup> GST activities for the two GSTM-1 expressing MCF cell lines were not significantly different (p > 0.05)
Cytotoxicity of HNE

The dose-response of HNE cytotoxicity was compared in the MCF7 control and MRP1 and/or GSTM1 transfected cell lines. Moderate sensitization (p < 0.01), as shown in Table 2, was observed for HNE cytotoxicity in the GST-only expressing cell line (MCF7/GSTM1), which had a mean IC$_{50}$ value of 15.9 ± 1.4 μM, a 0.79-fold decrease in IC$_{50}$ value relative to the control cell line, which had an IC$_{50}$ value of 20.0 ± 1.5 μM (Table 2). A similar degree of moderate sensitization (p < 0.01), as shown in Table 2, was observed for the MRP1-only expressing cell line (MCF7/MRP1) with a mean IC$_{50}$ value of 14.4 ± 1.4 μM, a 0.71-fold decrease in IC$_{50}$ value relative to control (Table 2). These data showed significant sensitization (p < 0.01) in the MCF7/MRP1/GSTM1 cell line relative to MCF7 empty vector control, (Table 2). The mean IC$_{50}$ values for these cell lines were 20.0 ± 1.5 μM and 8.9 ± 1.2 μM, respectively (Table 2), a 0.44-fold decrease relative to control. The comparative sensitization in the GST-only expressing cell line (MCF7/GSTM1) and MRP1-only expressing cell line (MCF7/MRP1) was not significantly different (p > 0.05), Table 2. These data suggest that there is an additive effect of GST and MRP1 in the sensitization of MCF7 cells to HNE cytotoxicity since the greatest degree of sensitization was observed in the MCF7/MRP1/GSTM1 cell line.

The dose-response of HNE cytotoxicity was compared in the HepG2 control and GSTM1 transfected cell lines. The IC$_{50}$ values of the HepG2 and HepG2/μ−2 cell lines were 39.5 ± 3.0 μM and 33.1 ± 3.8 μM, respectively. The HepG2/μ−2 (GSTM1 expressing cell line) had 0.72-fold decrease in IC$_{50}$ value relative to control (Table 3). The IC$_{50}$ values of the HepG2 and HepG2/μ−2 cell lines were significantly different (p < 0.05), as shown in Table 3.
Table 2. Cytotoxicity of HNE in MCF7 derived cell lines expressing GSTM1 or MRP1, or both together

Cytotoxicity was determined using the sulforhodamine B dye method as described in Materials and Methods. Values represent the mean ± 1 S.D. for 4 independent experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ values (μM HNE)</th>
<th>Fold-Difference Relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>20.0 ± 1.5</td>
<td>(N/A)</td>
</tr>
<tr>
<td>MCF7/GSTM1</td>
<td>15.9 ± 1.4ᵃ</td>
<td>0.79</td>
</tr>
<tr>
<td>MCF7/MRP1</td>
<td>14.4 ± 1.4ᵃ</td>
<td>0.71</td>
</tr>
<tr>
<td>MCF7/MRP1/GSTM1</td>
<td>8.9 ± 1.2ᵃ</td>
<td>0.44</td>
</tr>
</tbody>
</table>

ᵃ Significantly different (p < 0.001) from the mean for the MCF7 control.
Table 3. Cytotoxicity of HNE in HepG2 parent and GSTM1-expressing cell lines

Cytotoxicity was determined using the sulforhodamine B dye method as described in Materials and Methods. Values represent the mean ± 1 S.D. for 3 independent experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ values (μM HNE)</th>
<th>Fold-Difference Relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>39.5 ± 3.0</td>
<td>(N/A)</td>
</tr>
<tr>
<td>HepG2/μ−2</td>
<td>33.1 ± 3.8ᵃ</td>
<td>0.84</td>
</tr>
</tbody>
</table>

ᵃ Significantly different (p < 0.05) from the mean for the HepG2 control.
GSH depletion

A GSH depletion time-course study at a 60 μM HNE dose was performed with the MCF7 derived cell lines. In initially characterizing these cell lines it was noted that resting (basal) GSH levels in the MRP-only (MCF7/MRP1, 46 nmol/mg) and the MRP + GSTM1 (MCF7/MRP1/GSTM1, 44 nmol/mg) expressing cell lines were significantly lower (p < 0.05) than the resting GSH levels in either the MCF7 (76 nmol/mg) or MCF7/GSTM1 cell line (65 nmol/mg) (Tables 1 and 4). Resting GSH levels were not significantly different (p > 0.05) in the MCF7/MRP1 versus MCF7/MRP1/GSTM1 cell lines, nor in the MCF7 versus MCF7/GSTM1 cell lines (p > 0.05) (Tables 1 and 4).

MCF7/GSTM1 exhibited similar GSH depletion as that of the control MCF7 cell line throughout the time-course study. The level of GSH reached a plateau at 20 min for the MCF7/GSTM1 cell line with 86 % of the original GSH remaining in the cell. The level of GSH reached a plateau at 10-60 min for the MCF7 cell line with 80 % of the original GSH remaining in the cell (Table 4). The GSH levels were not significantly different (p > 0.05) for MCF7 and MCF7/GSTM1 at all time points studied: 0, 10, 20, 40, and 60 min (Table 4 and Figure 2). The MRP1 expressing cell lines, ± GST, exhibited similar GSH content and degree of GSH depletion at all time points studied relative to the control cell line (Figure 2). Both had nearly identical initial (44-46 nmol/mg protein) and final (7-8 nmol/mg protein, about 17%) levels of GSH remaining after 60 minutes of exposure to 60 μM HNE (Table 4 and Figure 2).

The MCF7/MRP1/GSTM1 cell line did have a greater degree of depletion at the 10, 20, and 40 min time intervals relative to the MCF7/MRP1 (Figure 2 and Table 4). At 10 minutes HNE exposure, the MCF7/MRP1/GSTM1 cell line had 17 nmol/mg protein
GSH (38 % of control), compared to 27 nmol/mg protein GSH (60 % of control) in the MCF7/MRP1 cell line. At 20 minutes HNE exposure, the MCF7/MRP1/GSTM1 cell line had 11 nmol/mg protein GSH (24 % of control), while the MCF7/MRP1 cell line had 20 nmol/mg protein GSH (44 % of control). Similarly, at 40 minutes HNE exposure, the MCF7/MRP1/GSTM1 cell line had 8 nmol/mg protein GSH (19 % of control), while the MCF7/MRP1 cell line had 17 nmol/mg protein GSH (36 % of control). Thus, in the presence of MRP1 expression, the co-expression of GSTM1 resulted in a trend toward greater GSH depletion by HNE than with MRP1 expression alone; however, these differences between the MRP1 versus MRP1 + GSTM1 expressing cell lines were not statistically significant (p > 0.05). The difference between MCF7 and MCF/MRP1 was significantly different (p < 0.001) at all time points studied (Figure 2). Similarly, the difference between MCF7 and MCF/ MRP1/GSTM1 was significantly different (p < 0.001) at all time points studied (Figure 2). Thus, glutathione depletion by HNE in the MCF7 cell line model occurs predominantly in the presence of MRP1 expression with a minor contribution by GSTM1 expression.
Table 4. Summary of GSH depletion by HNE in MCF7 derived cell lines

Cells were exposed to 60 μM HNE for 10, 20, 40, or 60 min. The results represent a mean ± 1 S.D. for 3 independent experiments.

A) Results are expressed GSH concentration (nmol/mg protein).

<table>
<thead>
<tr>
<th></th>
<th>MCF7</th>
<th>MCF7/GSTM1</th>
<th>MCF7/MRP1</th>
<th>MCF7/MRP1/GSTM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>76 ± 6</td>
<td>65 ± 6</td>
<td>46 ± 4 *</td>
<td>44 ± 3 *</td>
</tr>
<tr>
<td>10 min</td>
<td>63 ± 16</td>
<td>60 ± 5</td>
<td>27 ± 3 *</td>
<td>17 ± 5 *</td>
</tr>
<tr>
<td>20 min</td>
<td>64 ± 12</td>
<td>56 ± 6</td>
<td>20 ± 6 *</td>
<td>11 ± 6 *</td>
</tr>
<tr>
<td>40 min</td>
<td>63 ± 17</td>
<td>57 ± 8</td>
<td>17 ± 7 *</td>
<td>8.4 ± 3 *</td>
</tr>
<tr>
<td>60 min</td>
<td>61 ± 14</td>
<td>58 ± 6</td>
<td>7.8 ± 5 *</td>
<td>6.6 ± 4 *</td>
</tr>
</tbody>
</table>

B) Results are expressed as relative to percent control.

<table>
<thead>
<tr>
<th></th>
<th>MCF7</th>
<th>MCF7/GSTM1</th>
<th>MCF7/MRP1</th>
<th>MCF7/MRP1/GSTM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10 min</td>
<td>84</td>
<td>92</td>
<td>60</td>
<td>38</td>
</tr>
<tr>
<td>20 min</td>
<td>85</td>
<td>86</td>
<td>44</td>
<td>24</td>
</tr>
<tr>
<td>40 min</td>
<td>84</td>
<td>87</td>
<td>36</td>
<td>19</td>
</tr>
<tr>
<td>60 min</td>
<td>80</td>
<td>90</td>
<td>17</td>
<td>15</td>
</tr>
</tbody>
</table>

* p < 0.001 for values of GSH levels in transfectant cell lines relative to MCF7 control cell line, indicating a statistically significant difference.
Figure 2. GSH depletion by HNE in MCF7 derived cell lines

Cells were exposed to 60 μM HNE for 10, 20, 40, or 60 min. The results represent a mean ± 1 S.D. for 3 independent experiments. *Significantly different (p < 0.001) from the control cell line. The differences in GSH levels between MCF7 and MCF7/MRP1, as well as MCF7 and MCF7/MRP1/GSTM1 were statistically different (p < 0.001) at each time point measured at 60 μM HNE dose. The difference in GSH levels between MCF7 and MCF7/GSTM1 at each time point measured was found to be statistically different (p > 0.05). The difference in GSH levels between MCF7/MRP1 and MCF7/MRP1/GSTM1 at each time point measured was not found to be statistically different (p > 0.05).
Figure 2.
HNE-protein adducts

The dose-response of HNE-protein adduct formation was compared in the MCF7 control and MRP1 and/or GSTM1 transfected cell lines (Figure 3). HNE adducts were quantitated by peak integration for the two highest doses, 60 μM and 90 μM (Table 5). The cell lines expressing either MRP1 alone or GSTM1 alone yielded similar protein adduct profiles relative to the control MCF7 cell line at all concentrations studied (Figure 3). In contrast, the MCF7/MRP1/GSTM1 line (expressing both MRP1 and GSTM1) had 2-fold more HNE-protein adducts at the 60 μM HNE dose relative to the MCF7 control cell line and 3-fold more HNE-protein adducts at the 90 μM HNE dose relative to the MCF7 control cell line (Figure 3, and Table 5).

At the 60 μM dose, the integrated HNE-protein adduct intensity for MCF7/MRP1/GSTM1 was 230 % relative to the MCF7 control, while at the 90 μM dose, the integrated HNE-protein adduct intensity for MCF7/MRP1/GSTM1 was 330 % relative to the MCF7 control (Figure 3). At the 60 μM dose, the integrated HNE-protein adduct areas for MCF7/GSTM1 and MCF7/MRP1 were both 82 % relative to the MCF7 control, while at the 90 μM dose, the integrated HNE-protein adduct intensities for MCF7/GSTM1 and MCF7/MRP1 were 116 % and 100 % relative to the MCF7 control (Figure 3).

Based on these data, neither MRP1 alone nor GSTM1 alone appear to modify HNE-protein adduct formation. However, in the presence of both MRP1 and GSTM1, synergy is observed; i.e. a greater degree of HNE-protein adducts is formed than would be expected if MRP1 and GSTM1 expression were additive.
Figure 3. HNE-protein adduct formation in MCF7 derived cell lines

Cells were exposed to HNE concentrations of 0, 30 μM, 60 μM, and 90 μM HNE for a 2 hour time interval in serum-free media. Cells were harvested, lysed, and borohydride-reduced cytosolic protein (40 μg/lane) was resolved on a 12 % SDS-PAGE gel and transferred to a PVDF membrane. Following blocking in 5% nonfat milk, protein adducts were detected using a polyclonal antibody (1:4,000 dilution) specific for chemically reduced HNE-Michael adducts as described in Methods. Order of sample loading: 1) MCF7; 2) MCF7/MRP1/GSTM1; 3) MCF7/MRP1; 4) MCF7/GSTM1
Figure 3.
Table 5. Formation of HNE-protein adducts by HNE in MCF7 derived cell lines

Cells were exposed to HNE concentrations of 0, 30 μM, 60 μM, and 90 μM HNE for a 2 hour time interval. The table below lists integrated areas of protein-adducts for the 60 μM and 90 μM doses compared to control MCF7 cell line for each dose. Quantitation performed with MultiGauge Version 3.0 (FujiFilm, Corp.) software.

<table>
<thead>
<tr>
<th>#</th>
<th>Cell line</th>
<th>60 μM HNE</th>
<th>90 μM HNE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCF7</td>
<td>(100 %)</td>
<td>(100 %)</td>
</tr>
<tr>
<td>2</td>
<td>MCF7 /MRP1/GSTM1</td>
<td>230 %</td>
<td>330 %</td>
</tr>
<tr>
<td>3</td>
<td>MCF7/MRP1</td>
<td>82 %</td>
<td>116 %</td>
</tr>
<tr>
<td>4</td>
<td>MCF7/GSTM1</td>
<td>82 %</td>
<td>100 %</td>
</tr>
</tbody>
</table>
Discussion

MCF7 and HepG2 derived cell model systems were used in this study to examine the role of GSTM1 and MRP1/MRP2 in HNE detoxification. These cell lines are a good model to investigate the interaction of conjugation and efflux mechanisms for several reasons. The MCF7 cell line is a good model for examining the role of GSTM1 and MRP1 since this cell line lacks expression of endogenous GSTs including GSTM and also lacks endogeneous expression of MRP1/MRP2. The MCF7 transgenic cell lines expressing GSTM1 ± MRP1 also had closely matched GST activity. The HepG2 cell line was a good model for examining the role of GSTM1 and MRP2 on HNE detoxification, as HepG2 has high levels of endogeneous expression of MRP2, extremely low expression of MRP1 and other members of the MRP family, as well as low expression of GSTs [49].

Removal of the HNE-SG conjugate formed by GSTs may be necessary for complete HNE detoxification. The cyclic hemiacetal HNE-SG conjugate is in equilibrium with an open-chained structure with an exposed aldehyde which may still be toxic to the cell [64]. The HNE-SG conjugate could be toxic to the cell via product inhibition of GSTM1, in addition to Schiff base reactions of the aldehyde. Glutathione conjugates have been reported to cause product inhibition of cytosolic GSTs [43, 44]. Additional reports have also suggested that HNE-SG may be further metabolized by γ-glutamyltranspeptidase (GGT) to form a cytotoxic metabolite, cysteinylglycine-HNE (HNE-CysGly). Thus accumulation of HNE-SG in cells with GGT activity provides another mechanism of HNE-SG toxicity [65].
The observed pattern of sensitization to HNE cytotoxicity in the MCF7/GSTM1
(IC$_{50}$ = 14.4 ± 1.4 μM) and MCF7/MRP1/GSTM1 (IC$_{50}$ = 8.9 ± 1.2 μM) cell lines,
relative to the MCF7 control (IC$_{50}$ = 20.0 ± 1.5 μM) (Table 2), suggests several potential
explanations.

Sensitization by GSTM1 alone suggests that HNE-SG formation and
accumulation may be causing toxicity. The HNE-SG ring-opened tautomer may react
with cellular proteins that are involved in essential or detoxification functions. GSH
depletion observed in the MRP1-expressing cell lines suggests that HNE causes oxidative
stress that may result in GSSG formation and export from the cells. Thus, GSH depletion
is likely the cause of sensitization observed by MRP1. The MCF7/MRP1/GSTM1
expressing cell line had the greatest degree of sensitization and increased HNE protein
adduct formation. This is consistent with the possibility that MRP1 may pump free GSH
or oxidized GSSG out of the cell, compromising GSH levels, and the HNE-SG conjugate
formed by GSTM1 may not be a very good substrate for MRP1, thereby resulting in a
accumulation of this toxic product in the cell. The combined effect of greater GSH
depletion and HNE-SG accumulation could explain the enhanced protein adduct
formation in this cell line.

The cytotoxicity of HNE in the HepG2 cell line was determined to investigate
whether the MRP2 efflux transporter might play a role in detoxification of HNE (Table
3). The parental cell line HepG2, which expresses MRP2 alone, had an average IC$_{50}$
value 39.5 μM while the HepG2/μ-2 cell line, which expresses both GSTM1 and MRP2
had had an average IC$_{50}$ value 33.1 μM, a 0.84 fold sensitization relative to the HepG2
parental cell line (Table 3). These results paralleled the results of the MCF7 cytotoxicity data.

MRP1 expressing cell lines both with and without GSTM1 had lower basal, resting levels of GSH, (44-46 nmol/mg protein compared to 65-76 nmol/mg protein in the non-MRP1 expressing cell lines) (Table 4). This suggests that GSH, or oxidized GSSG, is a substrate for MRP1, and that MRP1 will efflux GSH out of the cell either as free GSH, GSSG, or as GSH-conjugates. Since MCF7/GSTM1 did not exhibit significant GSH depletion relative to the MCF7 control, this suggests that conjugation of HNE by human GSTM1 does not alter cellular GSH pools.

GSH depletion was significant in MRP1 expressing cell lines (MCF7/MRP1 and MCF7/MRP1/GSTM1), with only 7-8 nmol/mg GSH remaining after 60 minutes of exposure to 60 μM HNE, compared to 58-61 nmol/mg GSH remaining after 60 minutes of exposure to 60 μM HNE in the non-MRP1 expressing cell lines (MCF7 and MCF7/GSTM1), (Table 4). Considering the effects of HNE on GSH levels (Table 4), GSH depletion is plausible as a primary contributing mechanism of the sensitization to HNE cytotoxicity observed in the MRP1 expressing cell lines (Table 2). Since the cell lines expressing MRP1 or GSTM1 alone exhibited modest sensitization (0.79-fold or 0.71-fold decrease in IC₅₀ values, respectively, relative to the control) and the MCF7/MRP1/GSTM1 cell line exhibited a greater degree of sensitization (0.44-fold decrease in IC₅₀ values relative to the control) this indicates that the GSH depletion due to MRP1 expression must somehow interact with the GSTM1 expression. However, the GSTM1 did not deplete GSH in cells exposed to HNE, nor did it significantly exacerbate the GSH depletion in cells expressing both MRP1 and GSTM1. Thus, the enhanced HNE
cytotoxicity in the presence of GSTM1 may be due to enhanced accumulation of HNE-SG in concert with HNE-induced GSH depletion mediated by MRP1.

The results of the HNE-protein adduct data (Figure 3 and Table 5) correlated with the results of HNE cytotoxicity (Table 2) data with some notable differences. The HNE adduct data showed increased adduct formation only in the MCF7/MRP1/GSTM1 cell line relative to the other cell lines tested. At 60 μM, MCF7/MRP1/GSTM1 has approximately 2-fold more adducts than the control MCF7 cell line. At 90 μM, MCF7/MRP1/GSTM1 has approximately 3-fold more adducts than the control MCF7 cell line (Figure 3). These data suggest that while co-expression of MRP1 and GSTM1 contribute to enhance HNE-protein adduct formation, neither GSTM1 nor MRP1 alone appears to have an effect on HNE-protein adduct formation. Again, however, their additivity in the cytotoxicity assay suggests that different mechanisms may have been responsible for the separate but cumulative contributions of the GSTM1 versus the MRP1 expression to cytotoxicity, GSH depletion, and protein adducts.

When HNE-protein adducts are formed the damaged proteins can either be degraded by the 20S proteasome or they can accumulate in the cells resulting in cytotoxicity [66]. The time of HNE exposure, cell harvest and dose range could be important variables that influenced the HNE-protein adduct accumulation, degradation, and cytotoxicity. In both the cytotoxicity and protein adduct assay, cells were treated with HNE for a 2 hour exposure. However, cells were harvested immediately after exposure in the HNE-protein adduct assay. In contrast, in the cytotoxicity assay, cells were allowed to incubate for an additional 4-5 days after dosing before harvesting. The dose range for the HNE-protein adduct experiments was about twice a high as dose range
used for the cytotoxicity assay, 0-90 μM and 0-40 μM, respectively. This may indicate time-dependent differences in sensitivities or rates of repair between the two endpoints.

Some researchers have reported that as a result of the formation of HNE protein adducts, cellular signaling may be affected through altering activities of kinases including MAPKs (mitogen-activated protein kinase), protein kinase C and ASK1 (apoptosis signal-regulating kinase 1) [10, 67-70]. These signal kinases are known to have a role in critical cellular functions including apoptosis and differentiation [10, 67].

MAPK signaling is important in the regulation of cell survival and apoptosis. Several types of cellular stresses can activate JNK (c-Jun N-terminal kinase) and thereby result in a signaling cascade leading toward apoptosis [71]. There are three major MAPK subfamilies: ERK, JNK/SAPK, and p38 MAPK [69]. ASK1, an upstream kinase and activator of p38MAPK, JNK, and c-Jun have been reported to be involved in stress-induced apoptosis through the activation of JNK. Binding of the C-terminal end of mGSTM1-1 to the N-terminus of ASK1 has been demonstrated by in vitro binding assays. Additionally, this interaction resulted in a decrease in ASK1 activity and ASK-1 mediated apoptosis. This physical interaction is thought to inhibit the activation of ASK1 and downstream targets, which include JNK/SAPK, and the transcription factor c-Jun [69, 72].

Treatment of cells Chinese hamster CCL39 cells with heat shock causes the dissociation of GSTM1-1 from ASK1, resulting in activation of ASK1 and p38, a downstream kinase [68]. ASK1 and other downstream kinases in the JNK pathway can be activated by oxidative stress. In turn these kinases can activate by phosphorylation transcription factors such as c-Jun [69, 71].
Also, GSTP1 can form a complex with c-Jun-JNK, which serves a regulatory function in keeping JNK in its inactive form and inhibiting apoptosis. Under non-stressed conditions cells expressing GSTP1 have lower levels of JNK activity. Under conditions of UV-irradiation, JNK inhibition by GSTP1 was abolished. Mouse fibroblasts exposed to H$_2$O$_2$ or UV resulted in both a decrease in JNK-GSTP1 complex formation in addition to elevated JNK activity [70].

One possibility is that treatment of MCF7 cells with HNE could disrupt the GSTM1-1-ASK1 protein-protein interactions, thus activating ASK1. Such activation could enhance HNE induction of apoptosis in the GSTM1 expressing cell lines, MCF7/GSTM1 and MCF7/MRP1/GSTM1. However, while this would theoretically obviate any GSTM1 effect, it should only result in the same sensitivity as the parent MCF7 cells, not an enhanced sensitivity, unless ASK1 expression is increased via an autoregulation mechanism in cells expressing the inhibitory GSTM1. A more likely scenario would involve activation of a cellular death pathway by the HNE-SG that is likely accumulated in the GSTM1 expressing cells, e.g. via cell cycle arrest induced by alkylation of key proteins by the open-chain HNE-SG tautomer.

In summary we have shown that co-expression of GSTM1 together with MRP1 leads to an increased amount of HNE-protein adducts formed and sensitization to HNE induced cytotoxicity compared to either parent MCF7 or cells expressing either MRP1 or GSTM1 alone. Co-expression of both GSTM1 and MRP2 also leads to modest but significant sensitization to HNE cytotoxicity. This suggests a similar role of MRP1 and MRP2 in the cytotoxicity of HNE and parallels the results seen in the MCF7 cell line. GSH depletion in the MCF7 cell line was predominantly due to MRP1 expression, since
the cell line expressing GSTM1 alone did not significantly deplete GSH relative to the control cell line. The effect of GSTM1 + MRP1, although statistically significant between 10-40 min at 60 μM, was not dramatic. Further studies are needed to elucidate the mechanism by which GST and MRP1/2 modulate endpoints of HNE cellular toxicity.
CHAPTER III

REDOX-ACTIVE AND CONJUGATIVE ENZYMES AND THEIR COMPARATIVE EFFECTIVENESS AGAINST TOXICITY OF 4-HYDROXY-2-NONENAL (HNE), AN ALDEHYDE LIPID PEROXIDATION PRODUCT

Introduction

Aldehydes derived from lipid peroxidation have been reported to be toxic to cells and are increased during oxidative stress. There are several major pathological conditions associated with lipid aldehyde products including Alzheimer’s disease, atherosclerosis, aging, alcoholic liver disease, cancer, inflammation, and ischemia-reperfusion injury. HNE has also been reported to be a possible biomarker of disease, since it is elevated in affected tissues, such as the brain in Alzheimer’s patients, in atherosclerotic plaques, and myocardial tissue effected by ischemia-reperfusion [1]. The normal cellular levels of HNE are typically 0.1 µM – 3 µM, however levels as high as 10 µM – 5 mM have been found under conditions of oxidative stress [4].

Aldehyde dehydrogenases (ALDH) are an enzyme superfamily, currently consisting of 17 members, which catalyzes the pyridine nucleotide-dependent oxidation of aldehydes to carboxylic acids [73]. ALDH class 1 iosenzymes are cytosolic and require an NAD\(^+\) cofactor. Typical substrates for ALDH1 are short aliphatic aldehydes. For example, Bunting et. al. reported that ALDH1A1 plays a prominent role in cellular protection against cyclophosphamide (CPA) [74]. ALDH class 3 iosenzymes are cytosolic and are NAD\(^+\)/NADP\(^+\) dependent. Substrates for ALDH3 are benzylic or long-chain lipid aldehydes. ALDH3A1 also protects against toxicity of CPA and lipid
aldehydes (HNE) formed as a result of oxidative stress or UV damage. Previous work in this lab has shown that ALDH3 confers protection against several endpoints of toxicity, including growth inhibition, GSH depletion, protein alkylation, and apoptosis, while ALDH1 provided modest or no protection against similar endpoints induced by HNE [18].

Aldo-keto reductases constitute a cytosolic NADPH-dependent oxidoreductase enzyme superfamily, containing 114 genes within 14 families (AKR1-AKR14) [75]. The largest family is AKR1, which is comprised of aldose reductases, aldehyde reductases, steroid 5β-reductases, and hydroxysteroid dehydrogenases. Aldose reductase (AR; AKR1B1; ALR2) is a cytosolic enzyme, widely expressed in human tissues, especially heart/skeletal muscle [76]. AKR1B1 catalyzes the reduction of lipid-derived aldehydes such as acrolein and HNE [19]. AKR1C1 is inducible by electrophilic Michael acceptors and reactive oxygen species (ROS) and HNE. It is expressed in hepatic and extrahepatic tissues [20]. Kinetic parameters for HNE as a substrate for AKR1B1 (K_M = 22 µM, K_cat/K_M = 4.6 x 10^6 min^{-1}M^{-1}) and AKR1C1 (K_M = 34 µM, K_cat/K_M = 2.6 x 10^7 min^{-1}M^{-1}) indicate good substrate binding and catalytic efficiency for reduction [19, 20].

The overall objective of this study was to conduct comparisons of the protection by different enzymes against HNE-induced toxicity. These experiments were designed to determine which oxidoreductases (ORs) provide the best protection against HNE-induced toxicity endpoints. Additionally, we address how GSTs compare to the oxidoreductases for the relative degree of protection conferred. Prior work in the lab has demonstrated that ALDH3 provides protection against various endpoints of HNE-induced toxicity. In
this study ALDH3 will be used as a benchmark upon which to compare relative protective efficacy of other enzymes expressed in the model.

For these experiments we used a V79 Chinese hamster lung fibroblast cell line model which lacks expression of the ORs, GSTs, and ALDH enzymes investigated in this study. The initial phases of the project involved establishing lines that express the enzymes of interest by stable transfection into parental V79 cells. Clones were characterized for enzyme expression by Western blot and enzyme assay. Those clones exhibiting highest activity were chosen for subsequent experiments including: cytotoxicity, HNE protein adduct formation, and GSH depletion.

In Chapter II of this thesis, the role of MRP1/MRP2 in protection by GSTs was examined. An ultimate goal of this project would be to regulate enzyme activities for chemopreventive function. This could involve downregulation of enzymes which result in sensitization of cells to HNE toxicity or upregulation of enzymes which result in protection of cells against HNE toxicity.

Materials and Methods

Reagents

All chemicals were of analytical grade and were obtained from Fisher (Atlanta, GA) or Sigma (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (DMEM) and Advanced Dulbecco’s Modified Eagle’s Medium (Adv. DMEM) were purchased from Gibco/BRL, Grand Island, NY. 4-hydroxynonenal (HNE) was purchased from Cayman Chemical, Inc. (Ann Arbor, MI). Hygromycin B was purchased from Calbiochem (San Diego, CA). Trans-2-nonenal was obtained from Sigma Aldrich (St. Louis, MO).
Polyclonal antibody directed against reduced HNE-Michael adducts was purchased from Calbiochem (San Diego, CA). Goat anti-rabbit HRP-conjugated secondary antibody was purchased from Bio-Rad (Hercules, CA). β-actin mouse monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Vector Construction

The cDNA for AKR1B1 in the pET23d (+) vector and AKR1C1 in the pcDNA3 vector were obtained from Dr. Trevor Penning (University of Pennsylvania). The cDNA for hAOR in the pCEP4 was obtained from the Dr. Thomas Kensler (Johns Hopkins University). Oligonucleotide primers were designed for each construct to amplify the complete coding region of the appropriate gene. An XhoI site was engineered into the 5’ end of the primers and a NotI site was engineered into the 3’ end of the primers. The following cycle parameters were used: denaturation 94°C, initial annealing temperature 54 °C (first cycle) and 60 °C subsequent cycles, and elongation temperature 72 °C. A total of 28 cycles were run. PCR amplified AKR1B1, AKR1C1, and hAOR cDNAs were obtained in all reactions, and these were subcloned into the multiple cloning site of the pcDNA3.1 (-) Hyg vector (Invitrogen) (Figure 4), following digestion with XhoI and NotI. Expression vectors were amplified in One Shot® Top 10 chemically competent cells (Invitrogen) with ampicillin selection and purified using Qiagen Maxi Prep ion exchange columns. The cDNAs for AKR1C1, AKR1B1, and hAOR were verified for correct sequence and orientation, and correct coding sequences, prior to transfection into V79 cells by automated DNA sequencing performed by the DNA sequencing core lab of Wake Forest University School of Medicine.
**Figure 4.** pcDNA 3.1 (-) Hyg was selected as a vector for subcloning cDNA inserts

The pcDNA 3.1 (-) Hyg vector has the CMV strong constitutive promoter, a poly-A target sequence, and a Hyg resistance selectable marker gene. cDNAs used in this study were modified by PCR to include restriction endonuclease sites and a Kozak consensus sequence. The pcDNA 3.1 plasmid was purchased from Invitrogen.
Figure 4.
Transfection of V79 cells

Parental V79MZ Chinese hamster lung fibroblast cell line has been previously described by Glatt et. al. [77]. Stable transfection of hALDH3 into V79 cells, and characterization of clones has been previously performed in our laboratory, as described by Bunting et al. [74, 78]. Briefly, ALDH3 cDNA was previously subcloned into the nonepisomal mammalian expression vector, ΔpCEP4Δ into the XhoI site. This is a derivative of the pCEP4 vector (Invitrogen, Carlsbad, CA), which has been modified to enhance expression in V79 cells and allow selection of stably transfected clones based on hygromycin resistance [74]. Stable transfection of mGST 5.7 into V79 cells, and characterization of clones has been previously performed in our laboratory. The mGST5.7 cDNA was obtained from Dr. Piotr Zimniak (University of Arkansas for Medical Sciences, Little Rock, AR) and subcloned into the mammalian expression vector, ΔpCEP4Δ and transfected into V79 cells, with clones characterized for activity and expression (R. Haynes and A. Townsend, unpublished data).

The development of cell lines expressing hGSTA4, AKR1B1, and AKR1C1 by stable transfection into V79 cells was performed as a part of this thesis project. hGSTA4 cDNA in pLHCX and hGSTA4 in pET 21(+) were obtained from Dr. Charles Morrow (Wake Forest University, Department of Biochemistry) [79]. Initial transfection of hGSTA4 into V79 cells was performed using the hGSTA4 cDNA in the pLHCX construct. The resulting clones yielded low expression by Western blot and enzyme assay. Therefore, subsequent transfection into V79 cells used the hGSTA4 in pET 21(+). This construct contains ten silent mutations, designed for improved expression in Escherichia coli, as described previously [23, 79].
Vector constructs for AKR1B1, AKR1C1, and hGSTA4 were transfected into V79 Chinese hamster lung fibroblast cells. In addition, a pcDNA 3.1(-) Hyg empty vector without insert was transfected into V79 cells to serve as a control for all experiments in this study. Calcium phosphate precipitation was used for all transfections as described by Gorman et al. [80]. Cells (5 x 10^5) were plated on a 100 mm-plate one day prior to addition of 10-15 μg precipitated plasmid DNA in a CaCl₂ - HEPES buffered saline and incubated 6-8 hours in Advanced DMEM with 2% FBS. The media was removed from the cells and replaced with media without plasmid DNA. Cells were allowed to recover overnight and then split into two 150 mm- plates, one containing 20% and one with 80% of the total cells. Cells were allowed to attach for 24 hours and then selected with 0.6 mg/ml hygromycin 7-10 days until colonies of transformed cells were established. Clones were expanded in 24 well plates and then 100 mm plates and then screened for appropriate enzyme activity levels at 80% confluency. AKR1B1, AKR1C1, and hAOR clones were characterized for enzyme expression. Clones with highest expression were used for subsequent experiments.

Assay for ALDH3 activity

Measurement of ALDH3 has been previously described by Manthey and Sladek [81]. A modification of this method was used to test transfected clones for ALDH3 activity. Cells were plated at a density of 5 x 10^6 - 7.5 x 10^6 on 100 mm plates and harvested at 75 - 90% confluency. Cell pellets were harvested and frozen at -80°C and subsequently lysed by sonication in 50 mM Tris/HCl, 5 mM EDTA (pH 7.4) at 4°C. The activity of ALDH3 was determined by following the enzyme reduction of NAD⁺ using benzaldehyde as a substrate by measuring the change in absorbance at 340 nm.
Assay buffer consists of sodium pyrophosphate, 5 mM EDTA, 0.5 mM pyrazole, and 20 mM NAD\(^+\). Change in absorbance was measured spectrophotometrically every 15 sec over the course of 5 minutes. A kinetics program was used calculate the activity values. Cytosolic protein levels were measured by the bicinchoninic acid (BCA) method [80, 82]. These values were used to normalize activity levels.

**Assay for AKR1C1 activity**

A spectrophotometric assay for AKR1C1 used 1 mM 1-acenaphthenol as a substrate, monitoring NAD\(^+\) dependent oxidation at 340 nm. Reactions were performed in 100 mM potassium phosphate, 2.3 mM NAD\(^+\) (pH 7) at 37 °C [83].

**Assay for AKR1B1 activity**

Cells were plated similarly as described for the ALDH3 assay. Optimal cell density at plating and harvesting was determined for the AKR enzymes. A spectrophotometric assay for AKR1B1 was performed using glyceraldehyde as a substrate and monitoring absorbance at 340 nm. Reactions were in 0.1 M potassium phosphate, 0.15 mM NADPH (pH 7) at 25 °C [84].

**GST enzyme assay**

GST activity in cell lysate was measured with minor modifications as described previously [59, 60]. Cell pellets were collected by scraping into cold phosphate buffered saline (PBS)/ 5 mM EDTA and centrifuged at 1,000 x g for 5 min at 4 °C. The cell pellet was lysed in 50 mM Tris/ 5 mM EDTA by sonication (Fisher sonic dismembranator) setting 35 % power, 10 pulses. The lysate was centrifuged at 12,000 x g for 5 min. GST activity was measured at room temperature using 10-20 μL cell cytosolic supernatant in a
reaction buffer composed of 1 mM glutathione (GSH) in 0.1 M K$_2$PO$_4$, pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in absorbance at 340 nm was monitored for a total of 90 sec at 15 sec intervals. Activity was calculated based on the $\Delta A$/min and extinction coefficient [9.6 (mmol/L)$^{-1}$cm$^{-1}$]; and background activity was corrected for by subtraction of a reagent blank. Total protein concentration in the cell lysate was determined using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Enzyme activities were expressed as nmol/min/mg protein.

**hGSTA4 enzyme assay**

The specific activity of hGSTA4 with CDNB as a substrate is 5.3 ± 1.29 $\mu$mol/min/mg protein, while the specific activity of hGSTA4 with trans-2-nonenal as a substrate is 54.0 ± 3.59 $\mu$mol/min/mg protein [85]. Because CDNB is a poor substrate for hGSTA4, clones were assayed for GST activity using trans-2-nonenal as a substrate.

GST activity of hGSTA4 clones was measured spectrophotometrically by monitoring the absorbance at 225 nm with trans-2-nonenal as a substrate and 0.6 mM GSH. The reaction results in a decrease in absorbance due to the loss of the trans-2-nonenal C2=C3 double bond upon conjugation with GSH [85, 86]. Activity was calculated based on the $\Delta A$/min and extinction coefficient [19.2 (mmol/L)$^{-1}$cm$^{-1}$], and background activity was corrected by subtraction of a reagent blank [86].

**Western Blot of transfectant clones**

For characterizing AKR1C1 transfectant clones, a primary antibody provided by Dr. Trevor Penning (University of Pennsylvania) was used. This antibody is a rabbit anti-
rat AKR1C9 antiserum, which recognizes all human AKR1C isozymes. Primary antibody was diluted 1:1,000 in 1 % non-fat milk and incubated O/N at 4 °C, followed by incubation with goat anti-rabbit, horseradish peroxidase-conjugated secondary antibody, 1:10,000 dilution (Bio-Rad, Hercules, CA). Cell lysate from A549 (human lung epithelial cell line) and HepG2 (human hepatoma cell line) have been reported to express AKR1C1 and were used as positive controls for Western blot analysis [83, 87]. The A549 cell line was obtained from Dr. Griffith Parks (Wake Forest University, Department of Microbiology and Immunology) and the HepG2 cell line was obtained from Dr. Charles Morrow (Wake Forest University, Department of Biochemistry).

For characterizing AKR1B1 transfectant clones, an affinity purified goat polyclonal antibody, from Santa Cruz Biotechnology (DD-119: sc-20425) was used. Primary antibody was diluted 1:400 in 1 % non-fat milk and incubated O/N at 4 °C, followed by incubation with a secondary antibody, donkey anti-goat IgG-HRP, 1:5,000 dilution (Santa Cruz Biotechnology, sc-2020). JAR cell lysate was used as a positive control for Western blots as recommended by Santa Cruz Biotechnology. The JAR cell line is derived from fetal placental trophoblastic tumor, first established by R.A. Pattillo, obtained from ATCC (Manassas, VA) [88]. HepG2 cells have been reported to lack AKR1B1 expression and were used as a negative control for Western blot analysis [89].

Primary antibody against hGSTA4-4 developed in chicken was obtained from Dr. Yogesh Awasthi (University of Texas Medical Branch, Galveston, TX). Clones were transferred to nitrocellulose and probed with primary antibody (1:5,000 dilution) in 1 % non-fat milk/PBS O/N at 4 °C, followed by incubating with a rabbit-anti-chicken HRP
conjugate secondary antibody (1:1,000 dilution) (ICN Biochemicals, Inc., Irvine, CA), for 2 hrs in 1 % non-fat milk/PBS.

**Cytotoxicity determination**

Cytotoxicity was measured using the sulforhodamine B microtiter plate assay as described [61]. V79 derived cell lines were plated at a density of 250 cells/well in Advanced DMEM with 2 % FBS 24 hours prior to dosing in 96-well plates. As a control, V79 cells transfected with an empty vector, pcDNA 3.1 (-) Hyg were used. Cells were then exposed to various concentrations of HNE or ethanol vehicle control (0.1 % final concentration) for 2 hours in serum-free medium. After dosing, the serum-free medium was replaced with serum-containing medium and cells were allowed to incubate for 4 days, rinsed and subsequently fixed in 10 % TCA, then stained with 0.4 % sulforhodamine B in 1 % glacial acetic acid. Plates were then washed with 1 % glacial acetic acid 4-5 times, oven-dried at 55 °C, and then solubilized in 10 mM Tris base. IC$_{50}$ values were obtained from the measured absorbance at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The cytotoxicity values are expressed as IC$_{50}$ values or fold difference relative to the control cell line [IC$_{50}$ value transfectant cell line/IC$_{50}$ value control cell line].

**GSH depletion**

Cells were plated at a density of $5 \times 10^5/100$ mm dish 48 hours prior to treatment with HNE. A time-course study of GSH depletion was performed at 30 µM and 60 µM HNE for exposure times up to 1 hour. HPLC analysis of GSH levels in treated cells was described previously [62, 63]. Following HNE treatment, cells were harvested into
perchloric acid 5 % (wt/vol), containing an internal standard, 10 μM γ-glutamyl-glutamate, and 0.2 M boric acid. The samples were then centrifuged at 14,000 x g for 2 min to separate precipitated proteins from the acid-soluble sample supernatant. The supernatant was treated with iodoacetic acid and subsequently derivatized with dansyl chloride. The derivatized samples were separated by HPLC with a fluorescence detector on a Supelcosil LC-NH2 column, 4.6 x 25 cm, (Supelco, Bellefunk, PA) with the use of a Shimadzu modular HPLC system and a Shimadzu RF-10A fluorescence detector, using an excitation wavelength of 335 nm, and an emission wavelength of 550 nm. GSH levels were quantified based on peak area integration relative to internal standard area and expressed as nmol GSH/mg protein.

Cells were plated for each cell line in a separate plate for analysis of cellular protein using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Alternatively, the protein pellet from each sample was resuspended in 500 uL of 1 M NaOH and analyzed for protein content by the Bradford method with bovine serum albumin (BSA) as a standard.

**HNE-protein adduct determination (Western Blot)**

HNE modification of cellular proteins was determined by Western blot. Briefly, cells were plated at a density of 1 x 10^6/100 mm dish 24 hours prior to treatment with HNE. Following exposure, cells were washed and scraped into cold phosphate buffered saline (PBS)/ 5 mM EDTA and centrifuged at 1,000 x g for 5 min. The cell pellet was lysed in 50 mM HEPES/150 mM NaCl, 1 % Igepal pH 7.5 with 1x Halt Protease Cocktail (ThermoScientific, Rockford, IL) by sonication (Fisher sonic dismembranator) setting 35 % power, 10 pulses. The lysate was centrifuged at 12,000 x g for 5 min and the
supernatant was assayed for total protein concentration using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Immediately after lysis, cell lysate was chemically reduced with sodium borohydride (Sigma), for 30 min at room temperature, final concentration 100 mM in order to stabilize Michael protein adducts formed as a result of HNE exposure. After reduction HNE-adducted proteins were separated on a 10 % SDS/PAGE gel and transferred to PVDF membrane (Millipore, Bedford, CA) and probed with anti-HNE reduced Michael adduct antibody (Calbiochem/EMD Biochemicals, Inc., San Diego, CA). Primary antibody was used at a dilution of 1:4,000 in 1 % non-fat milk and incubated overnight at 4 °C. A goat anti-mouse secondary antibody from Bio-Rad (Hercules, CA) was used at a dilution of 1:10,000 in 1 % non-fat milk and incubated for 2 hrs at RT. Immunoreactive proteins were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) followed either by film exposure or FujiFilm LAS-3000 Imaging/Gel documentation system. Protein loading equivalency was determined by stripping the blot with 0.2 M glycine pH 2.0 and reprobing with antibody to β-actin 1:5,000 dilution (Sigma). For protein-adduct quantification, MultiGauge Version 3.0 (FujiFilm, Corp.) was used.

**Statistical Analysis**

Statistical differences unless otherwise stated were evaluated by one-way ANOVA with Tukey’s post test, performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California USA). As a criterion, p values < 0.05 were considered significant.
Results

Transfections into V79 cells and characterization of clones

Upon sequencing, the hAOR construct was shown to have a point mutation (g682a) and a possible polymorphism present in the cDNA. Transfection of hAOR into the V79 line was not performed, since we were advised by the Kensler lab that the hAOR activity toward HNE was much weaker than rAOR. Additionally, even if the point-mutation were corrected, there is no known lab or commercial source of hAOR antibody available. In this study, we decided not to pursue additional work with hAOR.

AKR1B1, AKR1C1, and hGSTA4 were successfully transfected into V79 cells (Figures 7-9). Western blot of transfectant clones showed protein expression of the correct MW (37 kD) for AKR1C1 (Figure 5). Clones with bands of greatest intensity at ~36 kD, indicative of positive protein expression of AKR1C1, included clones 15, 32, 57, 58, 36, and 39. Some clones with weaker bands at ~36 kD included clones 38, 55, 6, 33, and 52. The positive control, A549 exhibited a band at ~36 kD in the lower gel, and the negative control, mzΔ10, a hygromycin resistant clone of V79MZ, did not have expression of AKR1C1 by Western blot (Figure 5). Also clones were assayed for AKR1C1 enzyme activity (Figure 8) using acenaphthenol as a substrate. The activity of AKR1C1-15 (2.18 ± 0.51 nmol/min/mg protein) was 4-fold higher than the background activity in the non-expressing cell lines: (0.50 ± 0.13 nmol/min/mg protein in pcDNA 3.1 (-) Hyg clone 8, 0.55 ± 0.17 nmol/min/mg protein in GST 5.7-53, and 0.33 ± 0.20 nmol/min/mg protein in ALDH3-19). The activity of AKR1C1-15 was significantly different (p < 0.05) from pcDNA 3.1 (-) Hyg clone 8, ALDH3-19, and GST 5.7-53. The
AKR1C activities in pcDNA 3.1 (-) Hyg clone 8, ALDH3-19, and GST 5.7-53, were not significantly different from each other (p > 0.05).

Western blot of clones showed protein expression of the correct MW (37 kD) for AKR1B1 (Figure 6). A few clones, such as 15 and 25, revealed two bands in the Western blot. The positive control, JAR lysate, was positive for expression of AKR1B1 whereas the negative control HepG2 [89], did not express AKR1B1 by Western. Interestingly, A549 appears to express AKR1B1. The empty vector negative control, mzΔ10, a hygromycin-resistant clone of V79MZ, appeared to have expression of AKR1B1 in the top gel with a faint band at (~ 37 kD) in the bottom gel by Western blot. This indicates possible background activity in the V79 cell line. Clones were screened for AKR1B1 enzyme activity. The activity for the clones screened ranged between 2.4 – 2.9 nmol/min/mg protein, while the V79MZ cell lysate had AKR1B1 activity ranging from 2.6 – 3.0 nmol/min/mg protein. None of the clones, which were positive for expression by Western blot, exhibited significant activity above baseline. Therefore, AKR1B1 was not investigated in this study and AKR1C1 was used instead as a representative member of the AKR family.

Western blot of clones showed protein expression of the correct MW (25.7 kD) for hGSTA4 (Figure 7). Clones resulting from transfection with hGSTA4 cDNA in pLHCX construct yielded weak expression of hGSTA4 (25.7 kD) by Western blot. Clones resulting from the transfection of hGSTA4 in pET 21(+), containing silent mutations, did yield higher expression of hGSTA4 (25.7 kD) by Western blot than observed for the previous transfection, however expression was still not robust.
Clones were screened for GST activity using trans-2-nonenal as substrate. The highest expressing hGSTA4 clone (hGSTA4-57) had an average activity of 122 ± 12 nmol/min/mg protein compared to 72 ± 10 nmol/min/mg protein in the control V79MZ cell line. These values represent the mean ± S.D. for 3 separate measurements. Because expression of hGSTA4 appeared to be modest even with the improved expression construct we decided to perform trial cytotoxicity experiments with hGSTA4-57, to see if this cell line would exhibit HNE resistance. Initial cytotoxicity experiments with hGSTA4-57, performed in our lab showed no protection relative to the V79MZ control cell line (Sandra Kabler, personal communications).

We tested the GST activity of mGST5.7 (mGSTA4) in V79MZ cells which had been previously developed and characterized in our lab. The original cell line was re-transfected to obtain higher GST expression in this study (Figure 9). The highest expressing clone, GST5.7-53, had GST activity with CDNB (344 ± 29.6 nmol/min/mg protein) that was 3-fold higher than the background activity in the empty vector-control cell line, pcDNA 3.1(-) Hyg clone 8, (108.5 ± 25.8 nmol/min/mg protein) and other non-expressing cell lines, AKR1C1-15 (113.2 ± 13.6 nmol/min/mg protein), and ALDH3-19 (103.5 ± 10.3 nmol/min/mg protein). The activity of GST 5.7-53 was significantly different (p < 0.05) from pcDNA 3.1 (-) Hyg clone 8, as well as from the other cell lines in our study: AKR1C1-15, and ALDH3-19. The GST activity in pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and ALDH3-19, were not significantly different from each other (p > 0.05).
Since the mGST5.7-53/ V79 cell line was available and had high expression of GST with CDNB as substrate (Figure 9), we decided to use this mGST5.7 expressing cell line for subsequent cytotoxicity, GSH-depletion, and HNE-protein adduct experiments.

The ALDH3 expressing cell line had been previously developed in our lab. This current study involved repeat transfection of ALDH3 to achieve higher stable enzyme expression. The highest expressing ALDH3 clone was ALDH3-19. ALDH3 enzyme activity of ALDH3-19 was compared to other cell lines in this study (Figure 10). Cell pellets were collected and assayed for ALDH3 enzyme activity as measured with benzaldehyde as a substrate. The activity of ALDH3 was > 400 nmol/min/mg protein, while background activity in the non-expressing cell lines was undetectable (< 1 nmol/min/mg protein). The activity of ALDH3-19 was significantly different (p < 0.05) from pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and GST 5.7-53. The ALDH3 activity in pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and GST 5.7-53 were undetectable.
**Figure 5. Western blot of AKR1C1 clones from stable transfection into V79 cells**

Clones were probed for AKR1C1 expression, using a rabbit anti-rat AKR1C9 antiserum from Dr. Trevor Penning (1:1,000 dilution). Cell lysate from A549 (human lung epithelial cell line) and HepG2 (human hepatoma cell line) have been reported to express AKR1C1 and were used as positive controls for Western blot analysis; V79MZ cells were used as a negative control. Clones with bands of greatest intensity at ~ 36 kD, indicative of positive protein expression of AKR1C1, included clones 15, 32, 57, 58, 36, and 39. Some clones with weaker bands at ~ 36 kD included clones 38, 55, 6, 33, and 52. The positive control, A549 exhibited a band at ~36 kD in the lower blot. However, A549 cell lysate appeared negative on the upper blot (perhaps due to a gel loading issue).

The HepG2 cell lysate, reported to be positive, based on the literature for AKR1C1 expression, [83] appeared negative in the lower gel. The negative control, mzΔ10, a hygromycin resistant clone of V79MZ, did not have expression of AKR1C1 by Western blot.
Western of transfection AKR1C1(V79)

Figure 5.
Figure 6. Western blot of AKR1B1 clones from stable transfection into V79 cells

Clones were probed for AKR1B1 expression, using an affinity purified goat polyclonal antibody from Santa Cruz Biotechnology (DD-119: sc-20425) (1:400 dilution).

JAR cell lysates were used as a positive control for AKR1B1 expression. HepG2 cells were used as a negative control. A549 cells were reported to express AKR1C1, but expression of AKR1B1 was unknown [83]. Since cell lysates were available, these were also probed for AKR1B1 expression. A high percent of clones were positive for protein expression of AKR1B1 (~37 kD). A few clones, such as 15 and 25, had two bands that appeared in the Western blot. The positive control, JAR cell lysate, was positive for expression of AKR1B1 and the negative control HepG2 [89], did not express AKR1B1 by Western. Interestingly, A549 appears to express AKR1B1. The empty vector control, mzΔ10, a hygromycin-resistant clone of V79MZ, appeared to have expression of AKR1B1 in the top gel with a faint band at (~ 37 kD) in the bottom gel by Western blot. This indicates possible background activity in the V79 cell line.
Western of transfection AKR1B1(V79)

Figure 6.
Figure 7. Western blot of hGSTA4 clones from stable transfection into V79 cells

Clones were screened for hGSTA4-4 expression using a primary antibody against hGSTA4-4 developed in chicken, obtained from Dr. Yogesh Awasthi (1: 5,000 dilution). The top two blots represent transfections performed with hGSTA4 cDNA in the pLHCX construct. Expression of hGSTA4 (25.7 kD) in clones was weak using this construct. The bottom blot represents transfection performed with hGSTA4 in pET 21(+), containing silent mutations. This construct yielded higher expression of hGSTA4 (25.7 kD). The negative control, mzΔ10, a hygromycin-resistant clone of V79MZ, did not have expression of hGSTA4 by Western blot. As a positive control, purified hGSTA4-4 enzyme (50 ng top gel, 10-50 ng bottom gel) specific activity 5.1 μmol CDNB/min/mg protein, obtained from Dr. Charles Morrow, was analyzed by Western blot.
Western of transfection hGSTA4 (V79)

Figure 7.
Figure 8. AKR1C1 enzyme activity for enzymes used in study

Cell pellets were collected and assayed for AKR1C enzyme activity as measured with acenaphthenol as a substrate (n = 5). The activity of AKR1C1-15 (2.18 ± 0.51 nmol/min/mg protein) was 4-fold higher than the background activity in the non-expressing cell lines (0.50 ± 0.13 nmol/min/mg protein in pcDNA 3.1(-) 8; 0.55 ± 0.17 nmol/min/mg protein in GST 5.7-53; and 0.33 ± 0.20 nmol/min/mg protein in ALDH3-19. The activity of AKR1C1-15 was significantly different (p < 0.05) from pcDNA 3.1 (-) Hyg clone 8, ALDH3-19, and GST 5.7-53. The AKR1C activity in pcDNA 3.1 (-) Hyg clone 8, ALDH3-19, and GST 5.7-53, were not significantly different from each other (p > 0.05). * indicates p < 0.05
Figure 8.

<table>
<thead>
<tr>
<th></th>
<th>pcDNA3.1(-) 8</th>
<th>AKR1C1-15</th>
<th>ALDH3-19</th>
<th>GST5.7-53</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mean</strong></td>
<td>0.50</td>
<td>2.2</td>
<td>0.33</td>
<td>0.55</td>
</tr>
<tr>
<td><strong>std dev</strong></td>
<td>0.13</td>
<td>0.51</td>
<td>0.20</td>
<td>0.17</td>
</tr>
</tbody>
</table>

AKR 1C1 activities (acenaphthenol) n = 5
Cell pellets were collected and assayed for GST enzyme activity as measured with CDNB as a substrate (n = 4). The activity of GST5.7-53 (344 ± 29.6 nmol/min/mg protein) was 3-fold higher than the background activity in the non-expressing cell lines (109 ± 25.8 nmol/min/mg protein in pcDNA 3.1(-) Hyg clone 8; 113 ± 13.6 nmol/min/mg protein in AKR1C1-15; and 104 ± 10.3 nmol/min/mg protein in ALDH3-19. The activity of GST 5.7-53 was significantly different (p < 0.05) from pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and ALDH3-19. The GST activity in pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and ALDH3-19, were not significantly different from each other (p > 0.05).

* indicates p < 0.05
Figure 9.

<table>
<thead>
<tr>
<th></th>
<th>pcDNA3.1(-) 8</th>
<th>AKR1C1-15</th>
<th>ALDH3-19</th>
<th>GST5.7-53</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>109</td>
<td>113</td>
<td>104</td>
<td>344</td>
</tr>
<tr>
<td>std dev</td>
<td>25.8</td>
<td>13.6</td>
<td>10.3</td>
<td>29.6</td>
</tr>
</tbody>
</table>
Figure 10. ALDH3 enzyme activity for enzymes used in study

Cell pellets were collected and assayed for ALDH3 enzyme activity as measured with benzaldehyde as a substrate (n = 4). The activity of ALDH3 was > 400 nmol/min/mg protein, while background activity in the non-expressing cell lines was undetectable (< 1 nmol/min/mg protein). The activity of ALDH3 was significantly different (p < 0.05) from pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and GST 5.7-53. The ALDH3 activity in pcDNA 3.1 (-) Hyg clone 8, AKR1C1-15, and GST 5.7-53, were not significant from each other (p > 0.05). * indicates p < 0.05
Figure 10.
Cytotoxicity

The results of HNE cytotoxicity in V79 cells are shown in Table 6 and Figure 11 based on seven separate experiments. The GST 5.7 expressing cell line had an average IC$_{50}$ value of 13.8 $\mu$M compared to an average IC$_{50}$ value of 14.2 $\mu$M in the empty vector control cell line, pcDNA3.1(-) Hyg clone 8. Thus, GST 5.7 (mGSTA4) does not have any significant effect on cytotoxicity (p > 0.05). The AKR1C1 expressing cell line had an average IC$_{50}$ value of 19.4 $\mu$M compared to an average IC$_{50}$ value of 14.2 $\mu$M in the empty vector control cell line, pcDNA3.1(-) 8. This is a 1.4-fold increase in IC$_{50}$ value relative to control, but was not significantly different, using one-way ANOVA analysis (p > 0.05).

The ALDH3-expressing cell line had an average IC$_{50}$ value of 57.1 $\mu$M compared to an average IC$_{50}$ value of 14.2 $\mu$M in the empty vector control cell line, pcDNA3.1(-) Hyg clone 8. This is a 4.0-fold increase in IC$_{50}$ value relative to control and is significantly different (p < 0.001). This result is consistent with previous results from our lab. These data suggest that ALDH3 is the only enzyme in our study that exhibits significant protection against HNE cytotoxicity. AKRs detoxify HNE by reduction while ALDH3 detoxifies HNE by oxidation. The results of the cytotoxicity data suggest that the more effective pathway for HNE detoxification involves oxidation of HNE to form 4-hydroxynon-2-enoic acid (HNA).
Cells were exposed to HNE for 2 hours in serum-free media. After exposure, 
media was removed and replaced with serum-containing media and cells were grown for 
an additional 4 days prior to harvesting. Cells were assayed for cytotoxicity by the 
sulforhodamine B (SRB) dye method as described in the Materials and Methods section.

Data were analyzed for statistical significance by one-way ANOVA, followed by 
Tukey’s post-hoc test using Graph Pad Prism (San Diego, California USA). The 
difference in average IC$_{50}$ values between the control cell line pcDNA3.1 (-) Hyg clone 8 
and GST 5.7-53, 14.2 ± 1.1 μM and 13.8 ± 3.8 μM, respectively, was not statistically 
significant (p > 0.05). The difference in mean IC$_{50}$ values between the control cell line, 
pcDNA3.1 (-) 8 and AKR1C1-15, 14.2 ± 1.1 μM and 19.4 ± 4.0 μM, respectively, a 1.4-
fold higher IC$_{50}$ value, was also not statistically significant (p > 0.05). The difference in 
average IC$_{50}$ values between the control cell line, pcDNA3.1 (-) 8 and ALDH3-19, 14.2 ± 
1.1 μM and 57.1 ± 9.0 μM, respectively, a 4.0-fold increase, was statistically significant 
(p < 0.0001).
HNE cytotoxicity (n = 7)

*** Denotes mean IC$_{50}$ values are significantly different (p < 0.0001)

N.S. = Denotes mean GSH level are not significantly different (p > 0.05)

Results are from a one-way ANOVA followed by Tukey’s post hoc test

Values shown represent the mean ± 1 S.D. from 7 independent experiments.

Figure 11.
**GSH-depletion**

GSH depletion in V79 cell lines was measured as a time-course study at three different doses 20, 40, and 60 μM HNE and at time points of 0, 10, 20, 40, 60 minutes of HNE exposure. These data are given in Tables 6-8 and Figures 12-13.

Data for 20 μM HNE GSH experiments are shown in Table 6 and Figure 12. At 20 μM HNE, ALDH3-19 shows almost complete protection against GSH depletion, with maximum depletion occurring at 20 minutes of exposure, and GSH levels at 27 ± 6 nmol/mg (or 86 % of resting levels). This compares to GSH levels at 18 ± 2 nmol/mg (or 58 % of resting levels) for the pcDNA 3.1(-) Hyg clone 8 cell line. GSH levels were higher at all time points measured for ALDH3-19 relative to pcDNA 3.1(-) Hyg clone 8 at the 20 μM dose. It is interesting to note that recovery from GSH depletion was observed at 60 minutes (104 % of resting GSH levels) in the ALDH3-19 cell line.

Data for 40 μM HNE GSH experiments are shown in Table 7 and Figure 12. The 40 μM HNE depletion profile was similar to that of 20 μM HNE. ALDH3-19 had more GSH depletion at 40 μM HNE than at 20 μM HNE with maximum depletion occurring at 10 minutes with GSH levels at 21 ± 2 nmol/mg (68 % of control). Recovery from GSH depletion did not appear to occur at 40 μM HNE.

Data for GSH depletion experiments at 60 μM HNE are shown in Table 8 and Figure 13. At 60 μM HNE, differences in GSH between cell lines becomes statistically significant. Data was analyzed by one-way ANOVA, followed by Tukey’s post hoc test, with p < 0.05 considered significant. At 60 μM HNE, the mean GSH levels at all time points measured (0, 10, 20, 40, and 60 min) in the GSH 5.7-53 were significantly lower (p < 0.05) than in the control cell line pcDNA 3.1(-) Hyg clone 8. The mean GSH levels
at all time points measured (0, 10, 20, 40, and 60 min) in the ALDH3-19 were significantly higher (p < 0.001) relative to the control cell line. The mean GSH levels at all time points measured (0, 10, 20, 40, and 60 min) in the AKR1C1-15 were not significantly different (p > 0.05) relative to the control cell line. The GST 5.7-53 cell line had GSH levels that were significantly lower than ALDH3-19 (p < 0.0001) and also significantly lower than AKR1C1-15 (p < 0.05) at all time points measured (0, 10, 20, 40, and 60 min). The GSH levels of ALDH3-19 and AKR1C1-15 were also significantly different (p < 0.001) at the time points measured. These results show that ALDH3 expression can play a significant role in the preservation of GSH levels in V79 cells and that GST5.7 depletes GSH pools at 60 μM HNE. AKR1C1 expression does not preserve cellular GSH levels. These results also suggest that effects on the GSH pool may be a key mechanism for protection against HNE cytotoxicity by ALDH3 as well as a rationale for lack of protection against HNE cytotoxicity by GST 5.7.
Table 6. GSH Depletion in V79 Cell Lines Dosed at 20 μM HNE

Data represent the mean ± S.D. of n = 3 experiments, as described in Figure 12.

GSH depletion in V79MZ derived cell line, time-course dosed at 20 μM HNE

GSH levels (nmol/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>pcDNA 3.1(-) 8</th>
<th>GST 5.7-53</th>
<th>ALDH3-19</th>
<th>AKR1C1-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>31 ± 4</td>
<td>31 ± 1</td>
<td>31 ± 2</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>10 min</td>
<td>23 ± 6</td>
<td>19 ± 5</td>
<td>29 ± 7</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>20 min</td>
<td>18 ± 2</td>
<td>16 ± 3</td>
<td>27 ± 6</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>40 min</td>
<td>16 ± 5</td>
<td>16 ± 2</td>
<td>27 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>60 min</td>
<td>19 ± 6</td>
<td>23 ± 5</td>
<td>32 ± 2</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

GSH levels (expressed as % of control)

<table>
<thead>
<tr>
<th></th>
<th>pcDNA 3.1(-) 8</th>
<th>GST 5.7-53</th>
<th>ALDH3-19</th>
<th>AKR1C1-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10 min</td>
<td>73</td>
<td>61</td>
<td>94</td>
<td>89</td>
</tr>
<tr>
<td>20 min</td>
<td>58</td>
<td>51</td>
<td>86</td>
<td>73</td>
</tr>
<tr>
<td>40 min</td>
<td>50</td>
<td>50</td>
<td>89</td>
<td>70</td>
</tr>
<tr>
<td>60 min</td>
<td>62</td>
<td>73</td>
<td>104</td>
<td>72</td>
</tr>
</tbody>
</table>

p > 0.05 for all transfectant cell lines relative to pcDNA3.1(-) Hyg clone 8 control, for all time points.
Table 7. GSH Depletion in V79 Cell Lines Dosed at 40 μM HNE

Data represent the mean ± S.D. of n = 3 experiments, as described in Figure 12.

GSH depletion in mz derived cell line, time-course dosed at 40 μM HNE

GSH levels (nmol/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>pcDNA 3.1(-) 8</th>
<th>GST 5.7-53</th>
<th>ALDH3-19</th>
<th>AKR1C1-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>30 ± 3</td>
<td>31 ± 4</td>
<td>31 ± 2</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>10 min</td>
<td>25 ± 7</td>
<td>22 ± 5</td>
<td>21 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>20 min</td>
<td>18 ± 3</td>
<td>15 ± 3</td>
<td>24 ± 3</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>40 min</td>
<td>20 ± 4</td>
<td>17 ± 6</td>
<td>24 ± 3</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>60 min</td>
<td>22 ± 1</td>
<td>23 ± 11</td>
<td>22 ± 1</td>
<td>23 ± 5</td>
</tr>
</tbody>
</table>

GSH levels (expressed as % of control)

<table>
<thead>
<tr>
<th></th>
<th>pcDNA 3.1(-) 8</th>
<th>GST 5.7-53</th>
<th>ALDH3-19</th>
<th>AKR1C1-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10 min</td>
<td>83</td>
<td>70</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>20 min</td>
<td>61</td>
<td>49</td>
<td>77</td>
<td>73</td>
</tr>
<tr>
<td>40 min</td>
<td>67</td>
<td>55</td>
<td>78</td>
<td>71</td>
</tr>
<tr>
<td>60 min</td>
<td>74</td>
<td>72</td>
<td>71</td>
<td>74</td>
</tr>
</tbody>
</table>

p > 0.05 for all transfectant cell lines relative to pcDNA3.1(-)8 control, for all time points.
**Figure 12. HNE GSH depletion at 20 μM and 40 μM HNE dose in V79 cell lines**

Cells were exposed to 20 μM HNE (top figure) and 40 μM HNE (bottom figure) for 0, 10, 20, 40, and 60 min in serum-free media. After exposure, cells were harvested in perchloric acid (5 % wt/vol) and processed for analysis by HPLC as described in Materials and Methods. The data represent a mean ± SD of three independent experiments. At 20 μM HNE, differences between cell lines were not statistically significant (p> 0.05) at the dose level and time points measured.
GSH depletion in V79 cell lines, 20 μM HNE (n=3)

GSH depletion in V79 cell lines, 40 μM HNE (n=3)

Figure 12.
Table 8. GSH Depletion in V79 Cell Lines Dosed at 60 μM HNE

Data represent the mean ± S.D. of n = 3 experiments, as described in Figure 13.

**GSH depletion in mz derived cell line, time-course dosed at 60 μM HNE**

**GSH levels (nmol/mg protein)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pcDNA 3.1(-) 8</th>
<th>GST 5.7-53</th>
<th>ALDH3-19</th>
<th>AKR1C1-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29 ± 4</td>
<td>27 ± 4</td>
<td>29 ± 3</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>15 ± 5</td>
<td>6.5 ± 2 *</td>
<td>25 ± 4 **</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>20</td>
<td>11 ± 3</td>
<td>5.8 ± 4 *</td>
<td>23 ± 5 **</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>40</td>
<td>9.3 ± 6</td>
<td>4.2 ± 2 *</td>
<td>20 ± 1 **</td>
<td>9.4 ± 4</td>
</tr>
<tr>
<td>60</td>
<td>9.1 ± 5</td>
<td>3.2 ± 2 *</td>
<td>19 ± 6 **</td>
<td>9.1 ± 5</td>
</tr>
</tbody>
</table>

**GSH levels (expressed as % of control)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pcDNA 3.1(-) 8</th>
<th>GST 5.7-53</th>
<th>ALDH3-19</th>
<th>AKR1C1-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>24</td>
<td>85</td>
<td>45</td>
</tr>
<tr>
<td>20</td>
<td>37</td>
<td>21</td>
<td>79</td>
<td>36</td>
</tr>
<tr>
<td>40</td>
<td>31</td>
<td>16</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>31</td>
<td>12</td>
<td>66</td>
<td>29</td>
</tr>
</tbody>
</table>

* p < 0.05 for values of GSH levels in transfectant cell lines relative to pcDNA3.1(-)8 control cell line.

** p < 0.001 for values of GSH levels in transfectant cell lines relative to pcDNA3.1(-)8 control cell line.
**Figure 13. HNE GSH depletion at 60 μM HNE dose in V79 cell lines**

Cells were exposed to 60 μM HNE for 0, 10, 20, 40, and 60 min in serum-free media. After exposure, cells were harvested in perchloric acid (5 % wt/vol) and processed for analysis by HPLC as described in Materials and Methods. The data represent a mean ± SD of three independent experiments. At 60 μM HNE, the mean GSH levels at all time points measured (0, 10, 20, 40, and 60 min) in the GSH 5.7-53 were significantly lower (p < 0.05) from the control cell line pcDNA 3.1(-) Hyg clone 8. The mean GSH levels at all time points measured (0, 10, 20, 40, and 60 min) in the ALDH3-19 were significantly higher (p < 0.001) relative to the control cell line. The mean GSH levels at all time points measured (0, 10, 20, 40, and 60 min) in the AKR1C1-15 cell line were not significantly different (p > 0.05) relative to the control cell line. The GST 5.7-53 line has GSH levels that were significantly lower than ALDH3-19 (p < 0.0001) and also significantly lower than AKR1C1-15 (p < 0.05) at all time points measured (0, 10, 20, 40, and 60 min). The GSH levels of ALDH3-19 and AKR1C1-15 were also significantly different (p < 0.001) at the time points measured.
GSH depletion in V79 cell lines, 60 μM HNE (n=3)

* Denotes mean GSH level are significantly different (p < 0.05)

** Denotes mean GSH level are significantly different (p < 0.001)

*** Denotes mean GSH level are significantly different (p < 0.0001)

N.S. = Denotes mean GSH level are not significantly different (p > 0.05)

Figure 13.
**HNE-protein adducts**

Results from HNE-protein adduct experiments in V79MZ cell lines are shown in Figures 14-15. HNE-protein adduct band intensity was integrated and the results of data were calculated as % integrated band intensity relative to the V79MZ control. ALDH3-19 results showed complete protection against adduct formation, with less than 5 % of integrated area relative to V79MZ at both the 30 μM HNE and 45 μM HNE dose (Figure 14). GST5.7-53 showed some protection against HNE-protein adduct formation with only 35-36 % of adducts formed relative to control, while AKR1C1-15 showed a lesser degree of protection, with 48-57 % of adducts formed relative to control (Figure 14). Subsequent, repeat experiments showed a similar trend in order of cell line protection against HNE-protein adduct formation, from greatest to least as follows: ALDH3-19 > GST 5.7-53 > AKR1C1-15 > V79MZ control. However, absolute percentage of adduct formation relative to control had some variation between experiments, as well as the required dose needed to obtain a similar adduct intensity.

In Figure 15, ALDH3-19 again showed complete protection against adduct formation, with less than 5 % of integrated area relative to V79MZ at both the 60 μM HNE and 80 μM HNE dose. GST5.7-53 showed some protection against HNE-protein adduct formation with 48 % and 64 % of adducts formed relative to control at 60 μM HNE and 80 μM HNE dose respectively (Figure 15). AKR1C1-15 showed the least protection against HNE-protein adduct formation with 72 % and 107 % of adducts formed relative to control at 60 μM HNE and 80 μM HNE dose respectively (Figure 17). It is unclear based on these data whether AKR1C1 provides even modest protection against HNE-protein adduct formation in the V79MZ cell line.
HNE-protein adduct formation at the 40 μM dose followed the same pattern as the 60 μM and 80 μM doses (from greatest to least): ALDH3-19 > GST 5.7-53 > AKR1C1-15 > V79MZ control. Percent adducts formed relative to V79MZ control were: 13 %, 41 %, 100 %, and 100 % respectively. Adducts at the 40 μM dose in the control were similar to the 60 μM HNE dose for GST 5.7-53 and was similar to the 80 μM HNE dose for AKR1C1-15. The HNE-adducts for ALDH3-19 calculated to be 13 % of control at the 40 μM dose (Figure 15), may be overestimated due to difficulty in integration of adducts near the detection limit of the method. Results at 60 μM HNE and 80 μM HNE doses are more likely to have greater accuracy. In Figure 15, approximately double the HNE dose was required to yield a similar degree of adduct formation compared to Figure 14. Thus, comparisons are only valid between cell lines within, but not between, each experiment.

The complete protection by ALDH3 against HNE-protein adduct formation is consistent with previous studies in the lab. In the experiments performed in the current project, GST5.7 and AKR1C1 provided either modest protection or no protection against HNE-protein adduct formation. In the V79 cell line model low molecular weight bands were detected in all cell lines except ALDH3. These bands typically increased in intensity with increasing HNE dose. The identity of these bands is unknown. Also, whether they are the result of the experimental procedures such as harvesting, chemical reduction, or sample storage, or alternatively, degradation of HNE-adducted protein in the intact live cells is undetermined. Potentially the presence of these bands may influence adduct quantification and interpretation of results. The MCF7 HNE-protein adduct experiments (Chapter II) did not exhibit these low molecular weight bands.
**Figure 14. HNE-protein adducts in V79 cell lines**

Cells were exposed to 0, 15, 10 and 45 μM HNE for 2 hours in serum-free media. Cells were harvested, lysed and borohydride-reduced cytosolic protein (40 μg/lane) was resolved on a 12 % SDS-PAGE gel and transferred to PVDF. Protein adducts were detected using a polyclonal antibody (1:4,000 dilution) specific for chemically reduced HNE-Michael adducts as described in Methods. The order of loading is as follows:

1) V79MZ; 2) GST 5.7-53; 3) ALDH3-19; 4) AKR1C1-15
<table>
<thead>
<tr>
<th>#</th>
<th>Cell line</th>
<th>30 μM HNE</th>
<th>45 μM HNE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V79MZ</td>
<td>(100 %)</td>
<td>(100 %)</td>
</tr>
<tr>
<td>2</td>
<td>GST 5.7-53</td>
<td>35 %</td>
<td>36 %</td>
</tr>
<tr>
<td>3</td>
<td>ALDH3-19</td>
<td>1.9 %</td>
<td>3.5 %</td>
</tr>
<tr>
<td>4</td>
<td>AKR1C1-15</td>
<td>57 %</td>
<td>48 %</td>
</tr>
</tbody>
</table>

**Figure 14.**
**Figure 15. HNE-protein adducts in V79 cell lines: repeat blot with higher HNE concentrations**

Cells were exposed to 0, 40, 60 and 80 μM HNE for 2 hours in serum-free media. Cells were harvested, lysed and borohydride-reduced cytosolic protein (40 μg/lane) was resolved on a 12 % SDS-PAGE gel and transferred to PVDF. Protein adducts were detected using a polyclonal antibody (1:4,000 dilution) specific for chemically reduced HNE-Michael adducts as described in Methods. The order of loading is as follows: 1) V79MZ; 2) GST 5.7-53; 3) ALDH3-19; 4) AKR1C1-15
Figure 15.

<table>
<thead>
<tr>
<th>#</th>
<th>Cell line</th>
<th>40 μM HNE</th>
<th>60 μM HNE</th>
<th>80 μM HNE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V79MZ</td>
<td>(100 %)</td>
<td>(100 %)</td>
<td>(100 %)</td>
</tr>
<tr>
<td>2</td>
<td>GST 5.7-53</td>
<td>41 %</td>
<td>48 %</td>
<td>64 %</td>
</tr>
<tr>
<td>3</td>
<td>ALDH3-19</td>
<td>13 %</td>
<td>3.1 %</td>
<td>3.6 %</td>
</tr>
<tr>
<td>4</td>
<td>AKR1C1-15</td>
<td>100 %</td>
<td>72 %</td>
<td>107 %</td>
</tr>
</tbody>
</table>
Discussion

Several oxidoreductases and conjugases have been proposed to afford protection from HNE induced toxicity. However, a comprehensive study comparing protection among several major classes of enzymes has not been explored. We have chosen the selected enzymes in this study based on previous studies supporting protection by these enzymes, high catalytic efficiency for conjugation with HNE, substrate preference, degree of possible product inhibition, tissue expression, nucleotide cofactor dependence, and mechanism of detoxification.

Haynes et al. reported Structure-Activity Relationships (SAR) for HNE in RAW 264.7, a murine alveolar macrophage cell line. HNE and analogous compounds differing by one or more functional groups were assessed for cytotoxicity/growth inhibition. Results of that study showed that the aldehyde functional group had the greatest effect on toxicity followed by the C₂=C₃ double bond, and the hydroxyl group had the least effect [90]. Elimination of the electron withdrawing aldehyde group will dramatically reduce reactivity of the Michael addition site leaving the C₂=C₃ which by itself is not highly electrophilic.

The ALDH enzyme family is involved primarily in hepatic metabolism, and can utilize either NAD⁺ or NADP⁺ [19]. Furthermore, previous data by Bunting et al. demonstrated that ALDH3 confers strong protection from HNE induced cytotoxicity in transfected V79 cells [78]. ALDH3 oxidizes the –CHO group of HNE to form 4-hydroxynon-2-enoic acid (HNA). HNA is much less reactive than HNE since the C₃ Michael addition site is no longer conjugated to the aldehydic carbonyl double bond.
Two members of the AKR family were selected for this study, AKR1C1 and AKR1B1 for comparison to ALDH3. AKR1B1 and AKR1C1 are cytosolic, NADPH-dependent enzymes that have been reported to be expressed in a wide range of tissues, and are primarily involved in extrahepatic metabolism [19]. These enzymes reduce α,β unsaturated aldehydes to alcohols, which also greatly reduces reactivity at the Michael addition site. The major product of AKR-catalyzed reduction of HNE is 1,4-Dihydroxy-2-nonene (DHN). Burczynski et al. report that AKR1C1 can be induced in HepG2 cells by ROS (such as H$_2$O$_2$) and reactive electrophiles, including HNE. This induction suggests that AKR1C1 might play a role in the detoxification of HNE [20].

In this thesis the contribution of these enzymes (ALDH3, AKR1C1, and GST 5.7) toward protection or sensitization against HNE induced cytotoxicity was determined by measuring cytotoxicity, HNE-protein adduct formation, and GSH depletion of transfected cell lines, relative to an empty-vector transfected control V79 cell line. In agreement with previous work done in our lab, ALDH3 expression conferred protection against all endpoints of HNE toxicity investigated. Specifically, a four-fold protection against HNE cytotoxicity was observed relative to the empty vector control cell line (IC$_{50}$ values of 57.1 μM for ALDH3-19 and 14.2 μM for pcDNA 3.1(-) Hyg clone 8). A statistically significant difference in GSH levels was observed among the cell lines at the 60 μM HNE dose. GST 5.7 expression was found to significantly deplete GSH at all time points measured relative to control. In contrast, ALDH3 expression was found to preserve significantly higher GSH at all time points relative to control. AKR1C1 expression was found to have no effect on GSH levels. Complete protection against HNE-protein adducts by ALDH3 was also observed. GST 5.7 and AKR1C1 may inhibit HNE-protein adducts by ALDH3 was also observed.
adduct formation, but the results are not conclusive. These findings indicate that preservation of GSH levels may be a key mechanism of protection against HNE-cytotoxicity and HNE-protein adduct formation by ALDH3.

Previous work in our lab with mGST5.7 also showed lack of protection against HNE induced cytotoxicity and HNE-protein adducts in the murine macrophage cell line, RAW 264.7. In addition, mGST 5.7 expression resulted in activation of caspase-3 and accelerated progression to apoptotic cell death following HNE exposure (R. Haynes, A. Townsend, unpublished data). Results from this thesis are consistent with these findings. Additionally, the data presented in this thesis on GSH depletion in the V79 cell line may provide insight as to why GST5.7 fails to protect against these endpoints. GSH is important for normal cellular processes and a decrease in GSH level can impair many of these essential processes and render the cell more vulnerable to toxicants or ROS, including HNE. The disruption of GSH homeostasis has been linked to cause and progression of a number of disease states including Alzheimer’s disease, Parkinson’s disease, and cancer [91]. Yadav et. al. have reported that exposure to L-buthionine-[S,R]-sulfoximine (BSO), a GSH depleting agent, resulted in HNE-induced DNA damage in K562 (human erythroleukemia cells), whereas repletion of GSH with GSH-ethyl ester resulted in a reversal of this effect [92]. This suggests that exogenous thiol supplementation and/or stimulation of GSH synthesis could be therapeutic in preserving GSH homeostasis, thereby reducing HNE-induced oxidative stress.

It is plausible that a reduction in GSH levels could explain the lack of cellular protection against HNE-cytotoxicity by GST 5.7, which would logically appear to have a potential role in protection against HNE-protein adduct formation. Thus perhaps if GSH
depletion were prevented, or if GSH or other thiol donors were supplemented, potentially GST 5.7 may protect against HNE cytotoxicity.

Since no protection against HNE cytotoxicity was observed among the AKR1C1 and GST 5.7 cell lines relative to control at the IC_{50} level, clonogenic survival might be used as an alternative assay for accurate comparison of protection at a higher fractional kill range, e.g. IC_{90}. At the IC_{90} level, differential effects could be observed between cell lines not seen at the IC_{50} value if there is a threshold effect for HNE toxicity or for enzymatic protection. A reason for lack of protection from cytotoxicity, as measured by IC_{50} values could be that HNE may be causing aberrant cellular function at this concentration range without causing cell death. For example, Bunting et al. described differences in sensitivity to mafosfamide at the IC_{90} level for ALDH3 transfected cells relative to control V79/SD cells, which was not observed at the IC_{50} level [78].

Based on previous data in our laboratory, we expected ALDH3 to provide a high level of protection against HNE toxicity and to be the benchmark for evaluating protective efficiency of other ORs in this study. Results from this study showing protection against cytotoxicity, GSH depletion, and, HNE-protein adduct formation by ALDH3 thus were not surprising. It was unexpected that AKR1C1 would provide little or no protection against the endpoints studied based on previous reports of protection by this enzyme against HNE toxicity. The results with mGST5.7 were consistent with previous studies in our lab but were not in agreement with other reports in the literature which propose that GSTs are important in protection in the detoxification of HNE. More experiments need to be done to reconcile some of the differences between the expected and observed effects in this study and to provide a mechanistic explanation of the results.
CHAPTER IV
DISCUSSION

Lipid peroxidation produces $\alpha,\beta$-unsaturated aldehydes, which have been reported to exert cellular toxicity. HNE is one of the most reactive and cytotoxic of these products and its production has been associated with a variety of pathological conditions including Parkinson’s disease [93], diabetes [94, 95], atherosclerosis [96], alcoholic liver disease [97], and neurodegenerative disorders [1, 98].

The objective of this thesis has been to gain an understanding of the pathways involved in the detoxification of HNE. Representative members of the following enzyme families were studied: oxidoreductases (ORs), aldo-keto reductases (AKRs), aldehyde dehydrogenases (ALDHs), and glutathione S-transferases (GSTs). V79 Chinese hamster fibroblast cells stably transfected with ALDH3, AKR1C1, and mGST 5.7 were used for this model.

MCF7 and HepG2 cell line models were used to investigate the role of GST and MRP in the detoxification of HNE. The role of GSTM1 and MRP1 was evaluated in a stably transfected MCF7 cell line model, while a HepG2 cell line model was used to evaluate the role of GSTM1 and MRP2.

The contribution of these enzymes and efflux transporters toward protection against or sensitization to HNE-induced cellular damage was determined by measuring cytotoxicity, HNE-protein adduct formation, and GSH depletion.
Detoxification of HNE by ALDH3, GST 5.7, and AKR1C1

ALDH3 provided protection against the endpoints of HNE toxicity studied, which is in agreement with previous work done in our lab. In the ALDH3-expressing cell line, a four-fold protection against HNE cytotoxicity relative to the empty vector control cell line was observed. Complete protection against HNE-protein adducts and preservation of GSH levels by ALDH3 was also found in this study. AKR1C1 and GST 5.7 provided limited, if any protection, against these endpoints of toxicity.

Based on the literature, AKR1C1 efficiently catalyzes the reduction of HNE ($k_m = 34 \mu M, k_{cat}/K_m = 2.6 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$) [19, 20]. AKR1C1 is also inducible by GSH-depleting agents (e.g. ethacrynic acid) and oxidative stress (e.g. $H_2O_2$). Burczynski et. al. reported that AKR1C1 can be induced in HepG2 cells by ROS (e.g. $H_2O_2$) and reactive electrophiles, including HNE. This induction suggests that AKR1C1 plays an important role in the detoxification of HNE [20]. However, protection against HNE toxicity by AKR1C1 has not been directly demonstrated in cell line models.

Protection by GSTA4 from HNE and $H_2O_2$ cytotoxicity was reported in K562 human erythroleukemia cells. Exposure of cells to 20 $\mu M$ HNE resulted in differentiation and apoptosis. K562 cells expressing transfected mGSTA4 (also known as mGST5.7) showed resistance to HNE-induced differentiation, apoptosis, and cytotoxicity. K562 cells expressing transfected mGSTA4 ($IC_{50} = 35 \mu M$) had a 1.6-fold protection against $H_2O_2$ toxicity compared to K562 wild type ($IC_{50} = 22 \mu M$) [24].

Previous work in our lab also showed lack of protection against HNE-induced cytotoxicity and HNE-protein adducts by murine GST 5.7 (mGSTA4) in the RAW 264.7 cell line (R. Haynes, unpublished data). Data presented in this thesis on GSH depletion
in V79 cells may provide insight as to why GST5.7 fails to protect against cytotoxicity and HNE-protein adduct formation. At 60 μM HNE, GST 5.7-expressing cells had GSH levels that were significantly lower than in the control cell line pcDNA 3.1 (-) Hyg clone 8. GSH homeostasis is important for normal cellular functions and therefore GSH depletion could compromise the overall cellular defense against electrophiles [92].

An alternative explanation for lack of protection by GST 5.7 against HNE toxicity may be due to metabolism of HNE-SG to a toxic metabolite. Enoiu et. al. studied the role of γ-glutamyltranspeptidase (GGT) on the metabolism of HNE-SG in a V79 cells which overexpress GGT (V79/GGT) relative to mock-transfected V79 cells. They showed that GGT metabolizes HNE-SG to form cysteinylglycine-HNE (HNE-CysGly), using mass spectrometry. Cytotoxicity studies were performed by incubating V79 and V79 GGT cell lines with either HNE-SG or HNE-CysGly at concentrations of 50-200 μM for 3-24 hours. Incubation of V79 cells expressing GGT with HNE-SG showed a dose- and time-dependent decrease in cell viability relative to V79 control using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and trypan blue assays. Lactate dehydrogenase (LDH) release, a measure of cytolysis, was also increased in V79/GGT cells relative to V79 cells, following incubation with HNE-SG [65].

Exposure of cells to HNE-CysGly (200 μM) for 12 hours results in 95-97 % inhibition of cell viability in both V79/GGT and V79 parental cell lines, while exposure of cells to 200 μM HNE-SG for 12 hours lead to 82 and 23 % inhibition of cell viability for V79/GGT and V79 cell lines respectively, as measured by MTT assay. HNE-CysGly was more cytotoxic to both V79/GGT and V79 control cell lines compared to HNE-SG. Also, HNE-CysGly treatment resulted in similar degrees of cell viability inhibition for
V79 GGT and V79 cell lines but HNE-SG resulted in a more profound decrease in cell viability for V79/GGT compared to V79. This suggests that the HNE-CysGly metabolite was primarily responsible for HNE toxicity and not HNE-SG [65].

We hypothesize that HNE-CysGly formation from the HNE-SG product of GST5.7 conjugation together with the resulting GSH depletion may explain the lack of protection by GST5.7. However, in our studies cells were exposed to 40 - 60 μM HNE, and thereby levels of HNE metabolites, such as HNE-SG or HNE-CysGly should be even less than that of the parent compound. Since the levels of cellular exposure to HNE, HNE-SG or HNE-CysGly are considerably less in our studies relative to levels studied by Enoiu, the mechanisms of toxicity could be different [65].

**Detoxification of HNE by GSTM1 and MRP1/2**

The role of GST and MRP in the detoxification of HNE was examined using a cell line model derived from MCF7 and HepG2 cells. MCF7 cells were stably transfected with GSTM1 and MRP1, either separately or combined. The role of GSTM1 and MRP2 was studied in the HepG2 cell line, which has constitutive expression of MRP2. The purpose of these studies was to determine whether the removal of the HNE-SG conjugate by MRP1/2 is essential for the detoxification of HNE.

In this study, both GSTM1 and MRP1 failed to provide protection against HNE cytotoxicity. Indeed, expression of either GSTM1 or MRP1 sensitized cells toward HNE cytotoxicity (MCF7/GSTM1 IC$_{50}$ = 15.9 ± 1.4 μM, and MCF7/MRP1 IC$_{50}$ = 14.4 ± 1.4 μM, relative to the MCF7 control IC$_{50}$ = 20.0 ± 1.5 μM). Moreover, the combined expression of GSTM1 and MRP1 further enhanced sensitization to HNE.
(MCF7/MRP1/GSTM1 IC$_{50}$ = 8.9 ± 1.2 μM). Since an additive sensitization effect of co-expression of GSTM1 with MRP1 was observed, we hypothesize that MRP1 may transport free GSH out of the cell, consistent with the observed depletion of GSH levels. Furthermore, the HNE-SG conjugate likely formed by GSTM1 may not be a good substrate for efflux by MRP1. This would result in an accumulation of HNE-SG in the MCF7/MRP1/GSTM1 cell line. HNE cytotoxicity in the HepG2 cell line was also examined with a 0.84-fold sensitization observed in the HepG2/μ-2 cell line (IC$_{50}$ = 33.1 μM) relative to the HepG2 parental cell line (IC$_{50}$ = 39.5 μM). This agrees with the moderate sensitization by MRP1 in MCF7 and suggests that MRP1 and MRP2 have a similar role in the paradoxical enhancement of the toxic effects of HNE exposure.

MRP1 ± GSTM1 expressing cell lines had lower resting levels of GSH, (44-46 nmol/mg protein than MCF7 cell lines without MRP expression (65-76 nmol/mg protein). Also, GSH depletion in MRP1± GSTM1 expressing cell lines was significantly different from the MCF7 control, whereas depletion in the cell line expressing only GSTM1 was not. Overall, however, MRP1 expression appears to result in GSH depletion under resting or HNE-challenged conditions, and the GSH depletion after HNE exposure is even greater when the cells express both MRP1 and GSTM1, presumably due to HNE conjugation with GSH by GSTM1. Because MCF7/MRP1 depletes GSH, this implies that either reduced GSH or oxidized GSSG is pumped out of cells by MRP1. The GSTM1 expression alone did not appear to alter cellular GSH pools, since similar GSH depletion was observed in the MCF7/GSTM1 and control MCF7 cell lines. However, GSTM1 expression did exacerbate the GSH depletion by HNE in cells expressing MRP1.
Although this is likely due to conjugation of HNE by GSTM1, this remains speculative and will require verification by analysis of HNE-SG levels in cells and medium.

Experiments to determine whether GSH and/or GSSG are transported out of cells by MRP1 or MRP2 would provide complementary data for our GSH depletion results. Typically, charged and polar molecules such as GSH, GSSG, or X-SG conjugates do not readily cross cell membranes due to charge and polarity - hence the need for active transport by MRPs. A direct approach would be to measure GSH, GSSG and HNE-SG in the extracellular medium of these cell lines, without and with exposure to HNE. This could be done by HPLC analysis of the dansylated GSH or conjugate, concentrated from medium using ion-exchange solid phase extraction mini-columns. Efflux of HNE-SG is not likely since that would result in resistance rather than sensitization; nevertheless, if significant HNE-SG, GSSG or GSH levels were found in medium, then further analysis of MRP1 transport kinetics would be warranted. The preparation of inside-out vesicles is required for MRP1/2 transport studies [99]. HNE-SG transport studies using inside-out vesicles by the rapid filter method has been described by Paumi et al. [100]. Uptake of HNE-[\(^3\)H]-SG can be measured by liquid scintillation counting to provide a quantitative measure of HNE-SG transport. GSH has been suggested to serve a role in the function of MRP1, either as a necessary activator or co-transported molecule or both [91]. Peklak-Scott et al. have described a method for assessing co-transport of GSH with QO-SG. Transport of \[^3\)H\)]\(-\)GSH was measured in the presence of unlabeled QO-SG in membrane vesicles prepared from an MRP1 expressing cell line [101]. This method can be adapted for determining the role of GSH in the HNE-SG transport by MRP1. Demonstration of
either HNE-SG or GSH/GSSG efflux from cells would also suggest the possibility that ATP depletion might contribute to the enhancement of HNE toxicity by MRP expression.

The MCF7/MRP1/GSTM1 cell line had increased HNE protein-adduct formation, relative to the other cell lines tested. This indicates that MRP1 and GSTM1 together play a cooperative role to enhance HNE-protein adduct formation, although expression of either one alone made little difference. In contrast, for HNE cytotoxicity some sensitization was observed in the MCF7/GSTM1, as well as the MCF7/MRP1 cell lines, with a fold decrease in IC\(_{50}\) of 0.79 and 0.71, respectively, compared to MCF7. MCF7/MRP1/GSTM1 had a fold decrease in IC\(_{50}\) relative to MCF7 of 0.44. One possible explanation for the greater sensitization in the MCF7/MRP1/GSTM1 expressing cell line could be accumulation of the HNE-SG formed by GSTM1, and increased protein adduct formation by the ring-opened tautomer due to the lower GSH levels caused by MRP1. However, since the Michael addition site of HNE is already conjugated with GSH, this latter explanation would imply a different negative effect of lowering GSH levels, such as reduced competition for Schiff base alkylation of the aldehyde of open-chain HNE-SG.

The MCF7/GSTM1 expressing cell line exhibited mild sensitization to HNE in the cytotoxicity assay, but did not exhibit significant GSH depletion by HNE relative to the MCF7 control cell line. This suggests that GSH may play a role in HNE cytotoxicity, however, GSH depletion is not the sole determinant and there is likely an additional component or contribution involved. Further studies are needed to determine how GSTM1 and MRP1/2 separately and coordinately modulate GSH depletion, HNE-protein adduct formation and HNE-induced cytotoxicity.

Ji et al. have reported that MRP2 exports HNE-SG conjugates into the
extracellular space in Madin-Darby canine kidney II (MDCK II) cells expressing human MRP2 [102]. Treatment of cells for 12 min with 50 μM HNE resulted in a 7-fold higher level of accumulation of HNE-SG conjugates (20.26 nmol/mg protein) in MDCK II cells, relative to MRP2-MDCK II cells (2.75 nmol/mg protein). Paradoxically, the lower HNE-SG level was associated with sensitization of MRP2-MDCK II cells toward HNE cytotoxicity. MDCK-MRP2 cells treated with 62.5 μM HNE for 2 hours resulted in a reduction in cell viability to 70 % of control, while MDCK II cells did not have a significant decrease in viability. The rate of GSH consumption was measured in both cell lines. MDCK-MRP2 cells had a half-life of GSH of 12.2 minutes, for MDCK cells, the half-life of GSH was double, 30.1 minutes. In MRP2-MDCK II cells, GSH consumption following HNE exposure was extreme, to the point of causing necrosis.

In this thesis, it was found that expression of MRP1 in the MCF7 cell line model and expression of MRP2 in the HepG2 cell line model resulted in sensitization to HNE toxicity. Our results are consistent with studies by Ji et al. indicating MRP2 expression exacerbates HNE toxicity in MDCK-II cells [34], most likely via GSH depletion.

Future directions and studies

Several researchers have studied the effects of GSH levels on cellular toxicity. Higher levels of GSH have been reported to protect against HNE-induced genotoxicity and cytotoxicity, while a decrease in intracellular GSH levels was associated with an increase in cellular toxicity [103-105]. Further, addition of GSH ethyl ester (a GSH precursor that can be taken up into cells) resulted in reduced HNE toxicity [92, 105].
GST and GSH have been shown to be induced by 1,2-dithiole-3-thione (D3T) in A10 rat aortic smooth muscle cells. Additionally, D3T pretreatment of A10 cells prior to dosing with HNE resulted in a decrease in cytotoxicity, measured by the MTT assay. To determine the relative contribution of GSH and GST in conferring protection against HNE cytotoxicity A10 cells were treated with either an inhibitor of GST activity, sulfasalazine, or an inhibitor of GSH biosynthesis, buthionine sulfoximine (BSO). Either inhibition of GST activity or reduction in GSH synthesis resulted in an increase in HNE-induced cytotoxicity. Additionally, in A10 cells treated with both BSO and D3T, the BSO was shown to block the protective effects of D3T treatment alone [105].

Yadav et. al. reported that treatment of K562 human erythroleukemia cells with BSO, thereby depleting GSH, resulted in significant sensitization of cells towards HNE-induced genotoxicity as measured by Comet assay [92]. Further, Nakajima et. al. studied the effects of pre-treating PC12 cells with 1 mM GSH ethyl ester, a membrane permeable GSH precursor, for 2 hours before dosing on HNE toxicity. Cells treated with 50 μM HNE exhibited 32.5 ± 12.1 % viability as measured by MTT reduction compared to 89.1 ± 13.4 % viability in cells treated with 1 mM GSH ethyl ester prior to 50 μM HNE exposure [103].

It would be interesting to extend our studies to look at either supplementing GSH levels, by the addition of a GSH precursor such as GSH ethyl ester, or exacerbating the amount of GSH depletion by the addition of BSO (a known GSH depleting agent). After these two treatments are performed, the endpoints of cytotoxicity and HNE-protein adduct formation should be examined alongside measurement of GSH levels to confirm that the two treatments are preserving and exhausting GSH levels respectively. These
types of experiments can further address the role of GSH on other endpoints of toxicity and specifically answer whether these endpoints correlate with each other or possibly cause or result from the other observed effects.

Other potential experiments to assess the pathways/enzymes involved in the detoxification of HNE include investigation of other enzymes in the aldo-keto reductase family compared to ALDH3. The role of AKR 7A5 in V79 cells in protection against HNE induced cytotoxicity, mutagenicity, and caspase-3 cleavage has been reported [98]. AKR7A5 expression resulted in significant protection against HNE cytotoxicity compared to vector-transfected control cells, as measured by the MTT assay. A transgenic V79 cell line expressing AKR7A5 retained 96% cell viability following exposure to 40 μM HNE for 4 hours. In contrast, the empty-vector control V79 line showed only 20% cell viability under the same experimental conditions. Protection against HNE-induced mutagenicity was observed in the AKR7A5-expressing cell line. Treatment of the vector control line with 10 μM HNE resulted in a 1.5-fold increase in mutation frequency, whereas, mutation frequency remained unchanged in cells expressing AKR7A5. Also, a dose and time-dependent decrease in caspase-3 cleavage was observed in V79-AKR7A5 compared to the control V79-pCINeo [98].

Recent literature reports on AKR 7A5 reveal that perhaps other enzyme classes are more relevant to in vitro and possibly in vivo metabolism of HNE [98]. An interesting extension of this thesis would be to compare the relative protection of ALDH3 versus AKR7A5 against endpoints of HNE damage including cytotoxicity, HNE-protein adduct formation, and GSH depletion. Our model comparing ALDH3 to other enzyme classes
provided a positive control as a benchmark for protection, and this approach could be extended to include other aldo-keto reductases.

Other unanswered questions include identifying factors that influence expression and induction of ALDH3A1 in vivo and identifying the protective function of ALDH3A1 in tissues during normal and certain pathological conditions. Although we observed strong protection by ALDH3 against cytotoxicity, HNE-protein adduct formation, and GSH depletion, ALDH3A1 is limited in tissue expression. ALDH3A1 is expressed constitutively in certain specialized tissues including cornea, esophagus, stomach, urinary bladder, and lung. However, ALDH3A1 is not expressed in the liver, a major organ of detoxification [106, 107]. ALDH3A1 expression in the cornea is thought to provide protection against UV damage from sun exposure [108]. A more complete understanding of why ALDH3 expression is specific to a narrow range of tissues and the mechanism by which expression is regulated is still needed.

In summary, the results of this project have yielded new and interesting findings as well as posed some new questions. The role of GGT in detoxification of HNE-SG remains to be explored in our V79 and MCF7 cell line models. Although GSH depletion appears to be an important contributing factor, a more detailed mechanistic understanding of the role of GSTM1 and MRP1/2 in the sensitization of cells to HNE toxicity in the MCF7 and HepG2 cell line model is still needed. Still unknown, for example, is the mechanism of GSH depletion, which could be via efflux of GSH, GSSG, or HNE-SG, or cotransport of GSH with HNE or other cellular metabolites. Transport studies using inside-out vesicles could be used to determine the effect of GSH on MRP1 and MRP2 mediated transport of HNE-SG. Additionally, the relationship of GSH depletion to other
parameters measured including cytotoxicity and HNE-protein adduct formation should be 
examined in more detail. Because HNE has been implicated in a number of disease 
processes, an understanding of pathways and mechanisms of HNE detoxification may 
lead to future prevention and/or treatment of these diseases.
References


37. Witz, G., Biological interactions of alpha,beta-unsaturated aldehydes. (0891-5849 (Print)).


81. Manthey, C.L. and N.E. Sladek, *Kinetic characterization of the catalysis of "activated" cyclophosphamide (4-hydroxycyclophosphamide/aldophosphamide)


SCHOLASTIC VITA

LISA PAULA RUDD

EDUCATION:

8/2009  
*Wake Forest University*, Winston-Salem, NC  
Master of Science in Biochemistry and Molecular Biology  
Thesis Title: “Comparision of Redox-active and Conjugative Enzymes with and without Efflux Transporters and their Comparative Effectiveness in Protection Against Cellular Toxicity of 4-hydroxy-2-nonenal (HNE), an Aldehyde Lipid Peroxidation Product”

*University of North Carolina at Greensboro*, Greensboro, NC  
5/2003  
Master of Science in Chemistry  
Thesis Title: “Analysis of commercial products by capillary zone electrophoresis and micellar electrokinetic chromatography”

5/1996  
Bachelor of Science in Chemistry, magna cum laude

HONORS AND AWARDS:

2004-2008  
Graduate Fellowship, Wake Forest University School of Medicine

1996  
Member of the Phi Beta Kappa honorary society

1996  
Sherri R. Forester Undergraduate Chemistry Award

EMPLOYMENT:

10/00 – 8/02  
*Targacept, Inc.*, Winston-Salem, NC  
**SCIENTIST II**  
Analysis of in process and QA samples by GC-MS analysis, backup analyst for LC-MS samples, Ion Chromatography (IC) analysis of drug substance counter-ions, some experience with chiral HPLC. IC and GC-MS instrument maintenance and troubleshooting. Performed FTIR characterization of compounds used in Certificate of Analysis and stability studies. Synthesized pyridylquinuclidine intermediates/final compounds.

2/98 – 10/00  
*R. J. Reynolds Tobacco Company*, Winston-Salem, NC  
**CHEMIST**  
Responsibilities included extraction, cleanup, and GC-FPD analysis of organophosphorus (OP) pesticide residues in tobacco. Cross trained on herbicide pesticide analysis by GC-ECD. Data acquisition and analysis was performed by EZ Chrom and results reported by LIMS. GC operation and troubleshooting were integral aspects of performing this method.
LABORATORY TECHNICIAN
10/97 - 2/98 Characterization of pyrazine flavor/aroma compounds produced from the reaction of sugar and ammonia by GC-MS and GC-FID, assessed variables of sugar/tobacco type, stalk position, and leaf development.

Glaxo Wellcome, Inc., Research Triangle Park, NC
5/97 - 8/97 MEDICINAL CHEMISTRY/ ANALYTICAL SCIENCES (INTERN)
Responsibilities included the synthesis of anilines used in the preparation of Beta3 receptor agonist analogs in the development of an anti-diabetic drug candidate. Monitored reactions using HPLC and TLC; products were analyzed using NMR, LC-MS, and GC-MS.

5/96 - 8/96 Performed solubility and drug recovery assays from a tablet formulation. Gained experience with analytical techniques including: HPLC, GC, IR, UV/VIS spectroscopy, and KF titrations.

ABSTRACTS:

S. Ahmad, S. Leone-Kabler, L. Rudd, and A.J. Townsend. Wake Forest University School of Medicine, Biochemistry Department, Winston-Salem, NC 27157. Cytotoxicity and mutagenicity of 5-Methylchrysene and its dihydrodiol metabolite in V79MZ cells stably transfected with human CYP1B1 and/or human GSTPi/Mu. Abstract P7, Genetics and Environmental Mutagenesis Society, 23rd Annual Fall Meeting, October 26, 2005, UNC Friday Center, Chapel Hill, NC

Sarfaraz Ahmad, Sandra Leone-Kabler, Lisa Rudd, Johannes Doehmer, Alan J. Townsend. Wake Forest University Medical Center, Winston-Salem, NC, GenPharm Toxicology, Munich, Germany. Cytotoxicity and mutagenicity of 5-Methylchrysene in V79MZ cells stably transfected with human CYP1B1 or CYP1A1 and/or human GSTpi. Abstract 5242, AACR (American Association for Cancer Research), 97th Annual Meeting, April 1-5, Washington, DC

117
PUBLICATIONS:

L. Rudd, S. Leone-Kabler, C.S. Morrow, and A.J. Townsend. Cytotoxicity, glutathione depletion, and HNE-protein adduct formation by 4-hydroxynonenal (HNE) in MCF-7 and HepG2 cell lines expressing human glutathione-S-transferase M1, alone or together with human MRP1 or MRP2. [In Preparation].


PROFESSIONAL AFFILIATIONS:

1/06-12/07 Associate member AACR (American Society for Cancer Research)
10/05-10/08 GEMS Student Member (Genetics and Environmental Mutagenesis Society)

TEACHING:

1/09-5/09 Forsyth Technical Community College, Winston-Salem, NC
ADJUNCT INSTRUCTOR
Instructor for the following courses: General Chemistry, CHM151 and Analytical Chemistry, CHM 263
Responsibilities include preparation, delivery, and grading of all lectures, labs, quizzes, exams, and assignments to undergraduate students.

Wake Forest University, Winston-Salem, NC
8/03 – 5/04 TEACHING ASSISTANT
Provided teaching assistance for undergraduate labs, including, general Chemistry and Quantitative Analysis. Set up experiments, provided lab technique instruction, and graded student lab reports.

TRAINING COURSES:

10/04 Waters U Connections “Q-TOF Operation/Bio Applications”, Winston-Salem, NC (Wake Forest University)
On-Site training course on the acquisition and analysis of data using Q-TOF LC-MS-MS.

8/98 Chroma-Skills “Operator Troubleshooting” (Instructor Linda Green), Baton-Rouge, LA
Two-day in depth course on GC troubleshooting involving hands-on lab exercises. Common GC problem such as septum leaks, incorrect flow, temperature and/or pressure settings were detected and fixed.

PRESENTATIONS:

2/07/08  Wake Forest University Department of Biochemistry and Molecular Biology, Winston-Salem, N.C.
Departmental Research Student Seminar: PI Dr. Alan Townsend
“Redox-active and conjugative enzymes and their comparative effectiveness in protection against cellular toxicity of 4-hydroxy-2-nonenal (HNE), an aldehyde lipid peroxidation product”

1/18/07  Wake Forest University Department of Biochemistry and Molecular Biology, Winston-Salem, N.C.
Departmental Research Student Seminar: PI Dr. Alan Townsend
“Protection from Toxicity of the Aldehyde Lipid Peroxidation Product 4-Hydroxy-2-Nonenal (HNE) in Transfected Cell Lines Expressing Redox Active and Conjugative Enzymes”

11/08/05  Wake Forest University Department of Biochemistry and Molecular Biology, Winston-Salem, N.C.
Departmental Research Student Seminar: PI Dr. Alan Townsend
“Mechanisms of Toxicity of Aldehyde Lipid Peroxidation Products and Their Detoxification in Cells Expressing Transfected Aldehyde Dehydrogenases”

4/14/05  Wake Forest University Department of Biochemistry and Molecular Biology, Winston-Salem, N.C.
Departmental Research Student Rotation Seminar: PI Dr. Alan Townsend
“Cytotoxicity and Mutagenicity of 5-Methylchrysene in V79 Chinese hamster cell line expressing human cytochrome P4501A1, 1B1, 1A2, and 3A4”

7/08/05  Wake Forest University Department of Molecular and Cellular Pathology, Winston-Salem, N.C.
MALT Literature Seminar Presentation: PI Dr. Mary Sorci-Thomas and Dr Mike Thomas

11/18/04  Wake Forest University Department of Biochemistry and Molecular Biology, Winston-Salem, N.C.
Departmental Research Student Rotation Seminar: PI Dr. Mary Sorci-Thomas and Dr Mike Thomas