EVALUATION OF DRUG-INDUCED LIVER INJURY USING AN *IN VITRO* OXIDATIVE STRESS INFLAMMATION SYSTEM

by

Abdullah Al Maruf

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Graduate Department of Pharmacology and Toxicology
University of Toronto

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ABSTRACT

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Doctor of Philosophy, 2014

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Drug-induced liver injury (DILI) is a major concern in clinical studies as well as in post-marketing surveillance of drugs. Previous evidence suggests that drug exposure during periods of inflammation can increase an individual’s susceptibility to toxicity. Inflammation caused by infections or endotoxins markedly activates NADPH (nicotinamide adenine dinucleotide phosphate) oxidase that generates superoxide radicals by transferring electrons from NADPH. In the phagosome, superoxide radicals spontaneously form hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS). Neutrophils or Kupffer cells also release myeloperoxidase on activation. The aim of this study was to develop and validate an in vitro oxidative stress inflammation model to identify compounds that may increase hepatotoxicity during inflammation. Toxic pathways were also investigated using the “Accelerated Cytotoxicity Mechanism Screening” techniques. When a non-toxic H₂O₂ generating system (glucose/glucose oxidase) with peroxidase or Iron(II) [Fe(II)] (to simulate in vivo inflammation) was added to freshly isolated rat hepatocytes prior to the addition of the investigated drug, drug-induced cytotoxicity and ROS formation were increased. This was reversed by 6-N-propyl-2-thiouracil (a peroxidase inhibitor) or desferoxamine (an Fe(II) chelator), respectively. Flutamide, nilutamide,
nimesulide, methotrexate, and 6-mercaptopurine were found to form pro-oxidant radicals leading to oxidative stress and mitochondrial injury whereas azathioprine did not form any radicals with this inflammation system. Electron spin resonance (ESR) spectrometry spin trapping studies of 6-mercaptopurine metabolism by HRP (horseradish peroxidase)/H₂O₂ was also investigated. Two radicals were trapped using DMPO (5,5-dimethyl-1-pyrroline-N-oxide) which were previously reported as purine-6-thiyl and superoxide radicals. This oxidative stress inflammation system mimics the respiratory burst (release of H₂O₂) and release of myeloperoxidase by neutrophils or other immune cells (using horseradish peroxidase) or mimics the Fenton reaction, which could occur in vivo as immune cells also release Fe(II) during inflammation. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), a known ROS scavenger and a superoxide dismutase mimetic, and several antioxidants, including DPPD (N,N'-diphenyl-p-phenylenediamine), mesna (2-mercaptoethanesulfonate), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and resveratrol (3,5,4'-trihydroxy-trans-stilbene) were also tested to determine their effect on drug-induced oxidative stress with or without the inflammation system. This system may provide a more robust in vitro pre-clinical tool for screening of drugs for potential hepatotoxicity associated with inflammation.
ACKNOWLEDGEMENTS

All praise and glory are due to Allah for all bounties granted to me and with Allah’s guidance and help this achievement became possible.

I would like to dedicate this work to my parents for their love and encouragement. Although living thousands of miles away in Bangladesh, they kept me going in times of stress and frustration. I hope that I can continue to make them proud.

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<td>ACMS</td>
<td>Accelerated cytotoxic mechanism screening</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>ADR</td>
<td>Adverse drug reaction</td>
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<td>AGE</td>
<td>Advanced glycation end products</td>
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<td>Advanced lipoxidation end products</td>
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<td>ATP</td>
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<td>AZA</td>
<td>Azathioprine</td>
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<td>Bax</td>
<td>Bcl2-associated X protein</td>
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<td>Bcl2</td>
<td>B-cell lymphoma 2 protein</td>
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<td>t-BHP</td>
<td>tert-Butyl hydroperoxide</td>
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<td>BMPO</td>
<td>5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide</td>
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<td>BNPP</td>
<td>bis-p-Nitrophenyl phosphate</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CCAC</td>
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CNS  Central nervous system
COMT  Catechol-O-methyl transferase
COX-2  Cyclooxygenase-2
Cu-ZnSOD  Copper/zinc superoxide dismutase
CYP  Cytochrome P450 enzyme
Cys  Cysteine
Cyss  Cysteine disulfide
DAMPA  2,4-diamino-N-methylpteroic acid
DCF  2′,7′-Dichlorofluorescein
DCFD  2′,7′-Dichlorofluorescein diacetate
DFHR  Dihydrofolate reductase
DILI  Drug-induced liver injury
DMARD  Disease-modifying anti-rheumatic drugs
DME  Drug metabolizing enzyme
DMPO  5,5-dimethyl-1-pyrroline-N-oxide
DMSO  Dimethylsulfoxide
DNA  Deoxyribonucleic acid
DNFB  2′,4′-Dinitrofluorobenzene
DPPD  N,N′-Diphenyl-p-phenylenediamine
DTNB  5,5′-Dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent)
DTPA  Diethylenetriaminopentaacetic acid
EC-SOD  Extracellular superoxide dismutase
ELISA  Enzyme-linked immunosorbent assay
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<td>Electron spin resonance</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<td>Reduced flavin adenine dinucleotide</td>
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<td>FDA</td>
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<td>Fe</td>
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<td>FI</td>
<td>Fluorescence intensity</td>
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<td>4-Nitro-3(trifluoromethyl)phenylamine</td>
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<td>FMN</td>
<td>Flavin mononucleotide</td>
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<td>fmlp</td>
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<td>Glucose</td>
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<td>GGT</td>
<td>Gamma-glutamyl transpeptidase</td>
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<td>GI</td>
<td>Gastrointestinal tract</td>
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<td>GO</td>
<td>Glucose oxidase</td>
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<td>Gp91PHOX</td>
<td>Glycosylated NADPH oxidase subunit</td>
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<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>GR</td>
<td>Glutathione reductase</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HGPRT</td>
<td>Hypoxanthine-guanine-phosphoribosyltransferase</td>
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<td>HIV</td>
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<td>HLM</td>
<td>Human liver microsome</td>
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<td>HO$_2^*$</td>
<td>Hydroperoxyl radical</td>
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<td>Hypochlorous acid</td>
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<td>8-Hydroxyquinoline</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>Lactate dehydrogenase</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<tr>
<td>Mesna</td>
<td>2-Mercaptoethanesulfonate</td>
</tr>
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<td>MMP</td>
<td>Mitochondrial membrane potential</td>
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<td>6-MMP</td>
<td>6-Methyl mercaptopurine</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-Mercaptopurine</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MTX-PGns</td>
<td>Methotrexate polyglutamates</td>
</tr>
<tr>
<td>n</td>
<td>Number of observations</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>NAD</td>
<td>Oxidized nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAD(P)H or NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NIL</td>
<td>Nilutamide</td>
</tr>
<tr>
<td>NIM</td>
<td>Nimesulide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO’</td>
<td>Nitric oxide radical</td>
</tr>
<tr>
<td>NO2</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>N2O3</td>
<td>Dinitrogen trioxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Nox2</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>O$_2^{-}$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>'OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>ONOOCO$_2$$^-$</td>
<td>Nitrosoperoxycarbonate</td>
</tr>
<tr>
<td>$p$</td>
<td>Probability, represents statistical significance</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PS$^*$</td>
<td>purine-6-thiyl radical</td>
</tr>
<tr>
<td>PTU</td>
<td>6-N-propyl-thiouracil</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PUFA$^*$</td>
<td>Lipid radical</td>
</tr>
<tr>
<td>PUFAOO$^*$</td>
<td>Lipid peroxy radical</td>
</tr>
<tr>
<td>QH$_2$</td>
<td>Ubiquinol</td>
</tr>
<tr>
<td>R$^*$</td>
<td>Reactive free radical</td>
</tr>
<tr>
<td>RCS</td>
<td>Reactive carbonyl species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Se</td>
<td>Selenium</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMPOL</td>
<td>4-Hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl</td>
</tr>
<tr>
<td>TNB</td>
<td>5-Thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>6-TG</td>
<td>6-Thioguanine</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine S-methyltransferase</td>
</tr>
<tr>
<td>TNB</td>
<td>2-Nitro-5-thiobenzoic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor – alpha</td>
</tr>
<tr>
<td>Trolox</td>
<td>(±)-6-hydroxy-2,5,7,8-tetramethylchromamane-2-carboxylic acid</td>
</tr>
<tr>
<td>Trx-(SH)2</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>Trx-SS</td>
<td>Thioredoxin disulfide</td>
</tr>
<tr>
<td>UQ•</td>
<td>Ubisemiquinone anion radical</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
<tr>
<td>XOR</td>
<td>Xanthine oxidoreductase</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>Membrane potential</td>
</tr>
</tbody>
</table>
1.1 Drug induced liver injury

Drug-induced liver injury (DILI) is a major concern in pharmaceutical drug development, in clinical studies, as well as in post-marketing surveillance of drugs (Ballet, 1997; Fung et al., 2001). DILI has become a leading cause of severe liver disease in Western countries and therefore poses a major clinical and regulatory challenge (Lee, 2003a,b; Ostapowicz et al., 2002). Thirty to 50% of acute liver failures and 15% of liver transplantations were reported to be due to chemical-induced hepatotoxicity (Andrade et al., 2004; Kaplowitz, 2001; Russo et al., 2004; Tuschl et al., 2008). More than 1000 drugs have been implicated in causing liver disease. It is the most common reason for a drug to be withdrawn from the market and frequently results in a requirement for additional labeling (Temple and Himmel, 2002; Zimmerman, 1999).

Hepatotoxicity may lead to a wide variety of liver pathophysiological e.g. steatosis, cholestasis, fibrosis, hepatitis, necrosis or the formation of liver tumors (Ballet, 1997). The liver is considered as the most important organ in drug toxicity as it is interposed between the sites of absorption and the systemic circulation and it is also a major site of drug metabolism and elimination; thereby rendering it as a preferred target for drug- or xenobiotic-induced toxicity. Proposed pathophysiological mechanisms of most drug-induced hepatotoxicity are: inhibition of mitochondrial function, disruption of intracellular calcium homeostasis, activation of apoptosis and oxidative stress, inhibition of specific enzymes or transporters, and formation of reactive metabolites that cause direct toxicity or immunogenic response, potentially leading to idiosyncratic effects (Boelsterli, 2003a; 2003b; Tuschl et al., 2008).
DILI is broadly classified into intrinsic (Type-1) and idiosyncratic (Type-2) types; intrinsic DILI generally is dose-dependent, has a predictable latent period after exposure, affects all individuals at the same dose, and is predictable using routine animal testing (e.g. acetaminophen toxicity), whereas idiosyncratic DILI (IDILI) does not depend directly on dose, affects only susceptible individuals, has a variable onset (mostly delayed), and is not predictable using routine animal tests relative to exposure (e.g. isoniazid) (Chalasani and Björnsson, 2010; Roth and Ganey, 2010). IDILI occurs at therapeutic doses in 1 in 1000 to 1 in 100,000 patients, with a pattern that is consistent for each drug and for each drug class (Lee, 2003a,b). IDILI is responsible for about 13% of all cases of acute liver failure in North America (Temple and Himmel, 2002). A list of drugs that were withdrawn from the market or were restricted in use (carrying black box warnings) due to IDILI are presented in Table 1.1.
Table 1.1. List of drugs that were withdrawn from the market or carry a black box warning due to IDILI [Reprinted from Idiosyncratic drug-induced liver injury: Mechanisms and susceptibility factors, Volume 9.17, Boelsterli and Kashimshetty, In: Comprehensive Toxicology (Second Edition), edited by C.A. McQueen, pp. 383–402 © 2010, with permission from Elsevier].

<table>
<thead>
<tr>
<th>Drugs withdrawn from the market</th>
<th>Drugs labeled with black box warnings (hepatotoxicity) and therefore restricted in use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumiracoxib (2007), NSAID</td>
<td>Tolcapone, anti-Parkinson</td>
</tr>
<tr>
<td>Ximelagatran (2006), anticoagulant</td>
<td>Trovafloxacin, antibiotic</td>
</tr>
<tr>
<td>Nefazodone (2004), antidepressant</td>
<td>Leflunomide, DMARD</td>
</tr>
<tr>
<td>Troglitazone (2000), insulin sensitizer</td>
<td>Nevirapine, anti-HIV</td>
</tr>
<tr>
<td>Bromfenac (1998), NSAID</td>
<td>Nimesulide, NSAID</td>
</tr>
<tr>
<td>Iproniazid (1997), antidepressant</td>
<td>Felbamate, anticonvulsant</td>
</tr>
<tr>
<td>Benoxaprofen (1982), NSAID</td>
<td>Pemoline, CNS disease</td>
</tr>
<tr>
<td>Tienilic acid (1979), antihypertensive</td>
<td>Valproic acid, antiepileptic</td>
</tr>
<tr>
<td></td>
<td>Penicillamine, antirheumatic</td>
</tr>
<tr>
<td></td>
<td>Stavudine, anti-HIV</td>
</tr>
<tr>
<td></td>
<td>Zileuton, antiasthmatic</td>
</tr>
<tr>
<td></td>
<td>Acitretin, skin diseases</td>
</tr>
<tr>
<td></td>
<td>Bosentan, pulmonary hypertension</td>
</tr>
<tr>
<td></td>
<td>Dacarbazine, anticancer</td>
</tr>
<tr>
<td></td>
<td>Dantrolene, muscle relaxant</td>
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<tr>
<td></td>
<td>Flutamide, antiandrogen</td>
</tr>
<tr>
<td></td>
<td>Gemtuzumab, myeloid leukemia</td>
</tr>
<tr>
<td></td>
<td>Isoniazid, antituberculosis</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole, antifungal</td>
</tr>
<tr>
<td></td>
<td>Naltrexone, opioid antagonist</td>
</tr>
</tbody>
</table>

NSAID, non-steroidal anti-inflammatory drugs; DMARD, disease-modifying antirheumatic drugs; CNS, Central nervous system; HIV, Human immunodeficiency virus
1.2 DILI and oxidative stress

Oxidative stress has been proposed as one of the main and common mechanisms of drug-induced hepatotoxicity, cardiovascular toxicity, nephrotoxicity, retinopathy, neurotoxicity, ototoxicity, and reproductive toxicity (reviewed in Deavall et al., 2012; Pereira et al., 2012). Drugs from several classes of pharmaceutical agents have been reported to have adverse effects related to oxidative stress e.g. anticancer drugs, antibiotics, antiretroviral drugs, anti-tubercular drugs, analgesics including NSAIDs, and antipsychotics (Deavall et al., 2012). Oxidative stress is defined as an increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that exceed cellular adaptive and repair capacities and cause damage to biomolecules such as nucleic acids, proteins, and membrane phospholipids leading to cell death (Chen et al., 2007). Thiol/disulfide couples such as glutathione (GSH/GSSG), cysteine (Cys/CySS) and thioredoxin ((Trx-(SH)2/Trx-SS)) are functionally organized in redox circuits controlled by glutathione pools, thioredoxins and other control nodes that vary little among healthy individuals and are maintained in disequilibrium relative to each other. Although classically oxidative stress is defined as an imbalance of pro-oxidants and antioxidants, under this new concept of “Redox Hypothesis”, oxidative stress is defined as the disruption of these redox circuits (Blokhina and Fagerstedt, 2010; Jones et al., 2010; Mannery et al., 2010; Pereira et al., 2012).
1.2.1 Biochemistry of ROS

Oxygen is the most powerful oxidizing agent in aerobic organisms and readily reacts to form partially reduced species, which are generally short lived and highly reactive. Oxygen free radicals are products of many biological redox reactions. A free radical is a chemical species that has one or more unpaired electrons. The reactivity of free radicals is a consequence of the presence of unpaired electrons which renders them unstable. The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion ($O_2^{•−}$) is the product of one-electron reduction of oxygen (Equation I). It is the precursor of most ROS and a mediator in oxidative chain reactions (Paravicini and Touyz, 2008). It cannot cross cell membranes due to its charge and is very short lived (half-life is $10^{-6}$ sec) (Giorgio et al., 2007; Yu, 1994).

$$O_2 + e^{−} \rightarrow O_2^{•−} \quad (I)$$

Dismutation of $O_2^{•−}$ catalyzed by superoxide dismutases (SOD) produces hydrogen peroxide ($H_2O_2$). $H_2O_2$ is the non-radical ROS formed by several metabolic reactions. This dismutation reaction can also produce an intermediate hydroperoxyl radical ($HO_2^{•}$) (Equation II, III). $H_2O_2$ is less reactive, more stable and has a longer half-life ($10^{-5}$ sec) than other free radicals. $H_2O_2$ can easily diffuse within and between cells in biological systems (Giorgio et al., 2007; Paravicini and Touyz, 2008).

$$O_2^{•−} + H^+ \rightarrow HO_2^{•} \quad (II)$$

$$2HO_2^{•} \rightarrow H_2O_2 + O_2 \quad (III)$$
The hydroxyl radical (’OH) can be formed either from O$_2^-$ (Equation IV) (Haber-Weiss reaction) or from H$_2$O$_2$:

$$\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \cdot \text{OH} \quad \text{(IV) (Haber-Weiss reaction)}$$

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \cdot \text{OH} \quad \text{(V) (Fenton reaction)}$$

The ’OH is considered as one of the most potent oxidants in biological systems (Yu, 1994). Because of its high reactivity and short half-life ($10^{-9}$ sec), it reacts very close to its site of formation and may cause oxidative damage by reacting with adjacent lipids, proteins or nucleic acids. The majority of ’OH produced in vivo comes from the transition metal e.g., iron (Fe)- or copper (Cu)-catalyzed breakdown of H$_2$O$_2$. During oxidative stress or inflammation conditions, as excess presence of O$_2^-$ may trigger the release of unbound Fe(II) from Fe-containing molecules. The “free” Fe(II) may then catalyze the formation of ’OH from H$_2$O$_2$ (Equation V) (Fenton reaction). In biological systems, the Fe(II)-catalyzed Haber-Weiss reaction which makes use of Fenton chemistry is considered to be the main mechanism by which ’OH is generated (Kehrer, 2000). Although other transition metal ions are capable of catalyzing this reaction, the Fe-catalyzed Fenton reaction is now considered to be the major mechanism by which the ’OH is generated in biological systems (Liochev, 1999).

Dietary iron is essential to build the body’s oxygen carriers (blood haemoglobin and muscle myoglobin) and important enzymes such as those within the electron transport chain e.g. catalase, xanthine oxidase, and carriers such as cytochrome c. However, excess iron accumulates in tissues and organs and disrupts their normal function through the process of oxidative stress mediated-toxicity. The pro-oxidant effects of iron may be attributed to its ability to produce
ROS and thereby damaging proteins, lipids, sugars, and nucleic acids (Corpet et al., 2010; Mehta, 2011).

Typical additional radicals formed in the biological systems from oxygen include the peroxyl radicals (ROO\textsuperscript{•}) and alkoxyl radicals (RO\textsuperscript{•}). In addition to H\textsubscript{2}O\textsubscript{2}, some non-radical species are also formed e.g. hypochlorous acid (HOCl), fatty acid hydroperoxides and reactive aldehydes (Halliwell and Gutteridge, 1985). Moreover, O\textsubscript{2}\textsuperscript{−} is highly reactive with nitric oxide (NO) that generates RNS such as peroxynitrite (ONOO\textsuperscript{−}) and further downstream nitrogen species, including NO\textsuperscript{•}, nitrosoperoxycarbonate (ONOOCO\textsubscript{2}\textsuperscript{−}), nitrogen dioxide (NO\textsubscript{2}), and dinitrogen trioxide (N\textsubscript{2}O\textsubscript{3}) via the activity of enzymes such as inducible nitric oxide synthase 2 and NADPH oxidase (Deavall et al., 2012; Turrens, 2003).

1.2.2 Sources of ROS

ROS are produced from both exogenous and endogenous sources. Potential sources are presented in Table 1.2.

<table>
<thead>
<tr>
<th>Table 1.2. Potential exogenous and endogenous sources of ROS.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exogenous sources</strong></td>
</tr>
<tr>
<td>Environmental toxins</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>Dietary toxins</td>
</tr>
<tr>
<td>Microbes</td>
</tr>
<tr>
<td>Metals</td>
</tr>
<tr>
<td>Ultraviolet (UV) light</td>
</tr>
<tr>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>Inflammation</td>
</tr>
</tbody>
</table>
1.2.2.1 Exogenous sources

Exogenous sources include microbes, environmental carcinogens, dietary factors, various xenobiotics, metal ions, UV light, and ionizing radiation (reviewed in Klaunig et al., 2011; Valko et al., 2006). Drugs from several classes of pharmaceutical agents were reported to generate ROS leading to oxidative stress. Quinone containing molecules can also undergo redox cycling, generating large amounts of ROS without themselves being degraded (O'Brien, 1991).

A controlled inflammatory response is generally regarded as being safe, as it provides protection against infection. However, an imbalance in response can be damaging. The inflammatory response in general consists of four components: 1) The inflammatory inducers (e.g. microbes, toxic compounds or their degradation products/metabolites); 2) The sensors that detect and kill inducers by releasing ROS (e.g. macrophages); 3) The inflammatory mediators induced by the sensors (e.g. cytokines-tumor necrosis factor-α (TNF-α), interleukin-1 (IL1) or IL-6), and 4) The target tissues (e.g. liver) that are affected by the inflammatory mediators (Medzhitov, 2008; 2010; Mehta, 2011). How inflammation is related to oxidative stress is discussed later in section 1.3.

1.2.2.2 Endogenous sources

Endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, peroxisomes, NADPH oxidases, xanthine oxidases, and aldehyde oxidase (reviewed in Pérez-Matute et al., 2009).
**Mitochondria** are membrane enclosed organelles regarded as “cell power plants”. Mitochondria consume 90% of the body’s oxygen by oxidative phosphorylation for energy production and cellular respiration (Valko *et al.*, 2004). It is the most important source of ROS *in vivo*. Electrons derived from the oxidation of NADH (nicotinamide adenine dinucleotide) or FADH2 (flavin adenine dinucleotide) can “leak” and directly react with oxygen to produce O$_2^-$ (Ma, 2010; Valko *et al.*, 2006). The major sites of one-electron oxygen reduction in the mitochondrial electron transport chain are NADH dehydrogenase (complex I) and ubiquinone–cytochrome b complex (complex III) (Murphy, 2009). Complex III contributes to O$_2^-$ generation by auto-oxidation of the ubisemiquinone anion radical (UQ$^-$), in which one electron reduction of oxygen by UQ$^-$ causes O$_2^-$ formation (Cadenas and Davies, 2000; Muller *et al.*, 2004). A schematic presentation of ROS production during mitochondrial respiratory electron transfer is presented in Figure 1.1. How mitochondrial functions can be modified by toxicants and how they can contribute to oxidative stress has been reviewed in Maruf and colleagues (2014).
Figure 1.1. ROS production during mitochondrial electron transport chain.

$O_2^-$, superoxide anion; $QH_2$, ubiquinol [Reprinted from Transcriptional responses to oxidative stress: Pathological and toxicological implications, Ma, Pharmacol. Ther., 125(3):376–93 © 2010, with permission from Elsevier].

**Peroxisomes** are membrane-bound respiratory organelles that are present in virtually all eukaryotic cells and carry out a wide range of essential functions, including $\beta$-oxidation of fatty acids (long-chain, branched-chain and polyunsaturated fatty acids, dicarboxylic acids), biosynthesis of cholesterol, bile acids, and metabolism of ROS (Antonenkov et al., 2010; van den Bosch et al., 1992). They contain more than 100 enzymes and play a key role in the production and utilization of ROS (Antonenkov et al., 2010). Most of the $H_2O_2$ generated by peroxisomal oxidases is generally detoxified within peroxisomes. However, $H_2O_2$ can diffuse out of this cell organelle under conditions of peroxisome proliferation and peroxisomes can then become a significant endogenous source of ROS (Fritz et al., 2007). Peroxisomes are also home
to many antioxidant enzymes, including catalase (described later), which provide protection against ROS through detoxification at the site of ROS formation (Singh, 1996).

**Cytochrome P450 (CYP)** enzymes are another major source of ROS, especially in the liver. These are present mainly in the endoplasmic reticulum (ER) of most mammalian cells as components of a multi-enzyme monooxygenase system. Their main function is to detoxify foreign compounds as well as endogenous substrates into polar, less toxic products by utilizing oxygen to oxidize exogenous compounds. CYPs may produce ROS (O$_2^-$ and H$_2$O$_2$) by two possible ways. The first possibility is the formation of ROS as intermediates in the CYP-mediated catalytic cycle, where O$_2$ is reduced instead of being added to the substrate. The second possibility is that an electron can leak into oxygen molecules from flavins in the NADPH: P450 reductase enzyme (Jezek and Hlavata, 2005).

**NADPH oxidases** are membrane-bound enzyme complexes found in the membranes of phagosomes and are used by neutrophils and white blood cells to engulf microorganisms. Normally the complex, Gp91PHOX (contains heme) (encoded by gene *Nox2*), is latent in neutrophils and is activated during the respiratory burst. They have been implicated as a major source of ROS generation (Pérez-Matute *et al.*, 2009). When a phagocytic cell is exposed to invading foreign compounds, their degradation products or metabolites, the defense enzyme undergoes a series of reactions called the “respiratory burst” that enables the cell to provide oxidizing agents (ROS) to destroy such compounds (Ma, 2010; Valko *et al.*, 2004; 2006). When NADPH oxidase becomes activated, it retrieves cytoplasmic NADPH to reduce cytochrome b558 which catalyzes the NADPH-dependent reduction of oxygen to O$_2^-$ within the plasma membrane or on its outer surface (Figure 1.2). Another strong oxidant and antimicrobial agent,
HOCl can be formed from H₂O₂ catalyzed by myeloperoxidase (MPO) (Equation VI) (Hampton et al., 1998).

\[
2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+ \quad (VI)
\]

During the phagocytosis process intracellular ROS rapidly increase. The protein p47PHOX is activated and integrates with the additional components p67PHOX and p40PHOX into the phagosome membrane, where it combines with flavocytochorme b in the active NADPH oxidase enzyme complex. This enzyme complex catalyzes the generation of ROS and protons, which shift through proton-channels into the interior of the phagosome, where they destroy the internalized particle (Figure 1.2) (Riechelmann et al., 2004).

**Figure 1.2.** Respiratory burst initiated by NADPH oxidase [Adapted from Riechelmann and colleagues (2004), an open access article distributed under the Creative Commons Attribution License].
Xanthine oxidase (XO) is a highly versatile enzyme that is widely distributed among mammalian tissues and is well known to produce ROS (Kelley et al., 2010; Tapner et al., 2004; Valko et al., 2006). The molybdoflavin enzyme xanthine oxidoreductase (XOR) catalyzes the terminal two steps of purine degradation (from hypoxanthine to xanthine and from xanthine to uric acid) in humans. XOR is transcribed as a single gene product, xanthine dehydrogenase (XDH). Under inflammatory conditions, posttranslational modification by oxidation of critical cysteine residues or limited proteolysis converts XDH to XO (Amaya et al., 1990; Kelley et al., 2010). Substrate-derived electrons at the molybdenum (Mb) cofactor of xanthine oxidase reduce O$_2$ at the FAD (flavin adenine dinucleotide) cofactor both univalently, generating superoxide (O$_2^{-}$), and divalectly, forming H$_2$O$_2$. However, conversion to xanthine oxidase is not required for ROS production, as XDH displays partial oxidase activity under some conditions such as the ischemic/hypoxic microenvironment encountered in vascular inflammation (Harris et al., 1997). This same inflammatory milieu leads to enhanced xanthine oxidase levels and thus increased xanthine oxidase-derived ROS formation resulting in activation of redox dependent cell signaling reactions and alterations in vascular function (Kelley et al., 2010). Xanthine oxidase is exemplified by numerous studies in which inhibition of xanthine oxidase attenuated symptoms of several vascular diseases including congestive heart failure, sickle cell anemia, and diabetes (Aslan et al., 2001; Butler et al., 2000; Desco et al., 2002; Farquharson et al., 2002).

Aldehyde oxidase has been reported to cause oxidation of NADH in the presence of O$_2$ that produced large amounts of O$_2^{-}$. Aldehyde oxidase has a broader specificity toward drugs and xenobiotics, and although it catalyzes the oxidation of physiological substrates, the significance of this is not yet clear (reviewed in Beedham, 2010). Aldehyde oxidase also mobilizes iron from ferritin (Shaw and Jayatilleke, 1990) which can catalyze O$_2^{-}$ reduction to
form the highly reactive and more toxic hydroxyl radical (•OH) or the O₂•⁻ radical can directly react with NO to produce the powerful oxidant, ONOO⁻. Thus aldehyde oxidase functions as an important cellular source of ROS under normal physiological conditions. Under various pathological conditions such as ischemia, alcohol-induced liver diseases and diabetes, this ROS production would increase due to the increase in tissue NADH level thus contributing to oxidative stress and free-radical-mediated tissue injury (Kundu et al., 2012).

### 1.2.3 ROS detoxification

Antioxidants, being exogenous or endogenous, are chemical agents that donate an electron to free radical molecules which converts them to a harmless configuration that decreases damaging radical chain reactions (Iannitti and Palmieri, 2009). Many natural and synthetic compounds are currently being used with different claims and are prescribed by physicians or sold over the counter. However, not all of them have clinical effectiveness (reviewed in Mannery et al., 2010). The defense mechanisms are different in the intracellular and extracellular compartments and comprise both enzymatic and nonenzymatic types. The major enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx).

SOD is the first line of antioxidant defense against ROS generated by respiration and free radical damage. SOD catalyzes the dismutation of O₂•⁻ to form H₂O₂. The H₂O₂ is then further detoxified by catalase (Equation VII). There are three forms of SOD, (i) the manganese containing SOD (Mn-SOD) which is located in the mitochondrial matrix, (ii) the copper and zinc (Cu-ZnSOD) containing SOD located in the cytosol, the extracellular space and the mitochondrial inner membrane, (iii) the extracellular SOD (EC-SOD) containing Cu-Zn.
prosthetic group located on the surface of the cells (Fridovich, 1995). In rat hepatocytes, approximately 70% of SOD is found in the cytoplasm (Yu, 1994).

\[
2O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \quad \text{(VII)}
\]

Catalase is a heme containing enzyme located primarily in the peroxisomes and catalyzes the conversion of \(H_2O_2\) to water (\(H_2O\)) (Equation VIII). Catalase has one of the highest turnover rates amongst the enzymes: one molecule of catalase can complete the reaction below up to 6 million times in one minute (Valko et al., 2006).

\[
2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + H_2O \quad \text{(VIII)}
\]

**Glutathione Peroxidase** (GPx) catalyzes the reduction of \(H_2O_2\) and organic hydroperoxides while simultaneously oxidizing GSH with the generation of glutathione disulfide (GSSG) (Equation IX-X). It has two forms, selenium (Se)-dependent or selenium independent glutathione S-transferase (GST). The Se-independent transferases can be functionally distinguished from GPx as they are inactive with \(H_2O_2\) and only exhibit activity with organic hydroperoxides (Arthur, 2001). GSH is an endogenous tripeptide (glutamyl-cysteinyl-glycine) that serves as a cofactor for GST (glutathione S-transferase), which catalyzes the enzymatic detoxification of xenobiotics.

\[
2\text{GSH} + H_2O_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2H_2O \quad \text{(IX)}
\]

\[
2\text{GSH} + \text{ROOH} \xrightarrow{\text{GPx}} \text{GSSG} + H_2O + \text{ROH} \quad \text{(X)}
\]
Figure 1.3. Detoxification reactions of ROS. ROS is generated by one-electron reductions of molecular oxygen ($^3$O$_2$). The spontaneous reaction of superoxide (O$_2^-$) with nitric oxide (NO) yields peroxynitrite (ONOO$^-$) and peroxynitrous acid (ONOOH). NADPH oxidases (NOX) and mitochondria are potential sources of superoxide; NO is generated by nitric oxide synthases (NOS), and H$_2$O$_2$ can be directly formed by various oxidases. The neutrophil-derived enzyme MPO can catalyze the formation of hypochlorite (OCl$^-$). Singlet oxygen ($^1$O$_2$) can be formed during the spontaneous dismutation of superoxide [Reprinted from Antioxidant defense mechanisms, Volume 9.14, Jaeschke, In: Comprehensive Toxicology (Second Edition), edited by C.A. McQueen, pp. 319–337 © 2010, with permission from Elsevier].

Extracellular antioxidants include albumin, transferrin, lactoferrin, ceruloplasmin, haptoglobin, urate, GSH, vitamin E (α-tocopherol), β-carotene, bilirubin, ascorbate (vitamin C), extracellular SOD (EC-SOD), and GPx (reviewed in Jaeschke, 2010).
1.2.4 Detrimental effects of ROS

An imbalance of ROS production and antioxidant defense systems can lead to oxidative stress. Whilst small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signaling (Dröge, 2002), uncontrolled increases in the concentration of these oxidants lead to free radical-mediated reactions which may damage cellular macromolecules e.g. lipid (Rubbo et al., 1994), proteins (Stadtman and Levine, 2000), and DNA (Richter et al., 1988). Oxidative stress has been found to be associated with the pathogenesis of different diseases e.g. cancer, Parkinson’s, Huntington’s, Alzheimer’s, prions, Down’s syndrome, Ataxia, Multiple sclerosis, Creutzfeldt-Jacob disease, Amyotrophic lateral sclerosis, schizophrenia, Tardive dyskinesia, asthma, chronic obstructive pulmonary disease, cataracts, cardiovascular diseases (reviewed in Iannitti and Palmieri, 2009; Kovacic and Somanathan, 2013).

Lipids are critical to the structural and functional integrity of cell membranes. The free radical oxidation of polyunsaturated fatty acids (PUFAs) is known as lipid peroxidation and it is one of the most common mechanisms by which xenobiotics cause cytotoxicity. The biochemical consequences of lipid peroxidation include cell membrane damage (and decreased membrane potential), enzyme inhibition, release of lysosomal enzymes, and protein-protein cross-linking (Smith et al., 1982). Highly reactive free radicals (R’*) derived from some xenobiotics are capable of abstracting hydrogen atoms from PUFA on phospholipid membranes, resulting in the formation of a lipid radical (PUFA’*). Reaction with oxygen yields the corresponding peroxy radical (PUFAOO’*) which initiates a chain propagation step leading ultimately to the degradation of the lipid to a range of products including aldehydes or gases such as ethane and pentane (Tafazoli, 2008). Several hepatotoxicants were reported to cause toxicity that involved lipid
peroxidation, such as carbon tetrachloride (CCl₄) (Comporti et al., 1965), trichlorobromomethane (Slater, 1988), chloroform (Ekström and Högbåck, 1980), and halothane (Tomasi et al., 1983).

Under oxidative stress conditions, proteins may be modified either indirectly by RCS formed by the autoxidation of lipids or carbohydrates or directly by ROS leading to oxidized amino acids. Reaction of proteins with lipid peroxidation-derived RCS results in the formation of adducts known as advanced lipoxidation end products (ALEs) whereas reaction with carbohydrates forms advanced glycation end products (AGEs) (Mehta, 2011; Negre-Salvayre et al., 2008). Protein carbonylation can lead to alterations of protein (enzyme) functions, protein fate and proteolysis, and protein misfolding. Protein carbonylation has been associated with a large number of age-related disorders as protein aggregates can accumulate with age in such diseases as, Parkinson’s disease, Alzheimer’s disease and cancer (Marin-Kuan et al., 2011; Nyström, 2005).

Lipid peroxidation products and RCS can damage DNA by directly oxidizing DNA bases or by forming exocyclic-adducts with DNA bases that induce base-pair substitution mutations (Nair et al., 2007; Valko et al., 2006). 'OH can result in single- or double-strand DNA breaks, base modifications, and DNA-DNA or DNA-protein cross-links (Toyokuni, 1998). DNA repair mechanisms may not work properly when DNA damage occurs at critical sites or when repair processes are interrupted by 'OH (Kehrer, 2000). Mitochondrial DNA is more prone to DNA damage due to its close proximity to the major source of cellular ROS formation and limited DNA repair capacity (Ma, 2010).
1.3 Drug-induced hepatotoxicity and inflammation: research rationale

Individual susceptibility plays an important role in determining whether or not a person develops an untoward drug reaction. There are numerous factors that can contribute to the inter-individual variations in xenobiotic response (pharmacological or toxic effects). These include variations in age, gender, xenobiotic metabolism, immunologic responses, reserve and repair capacity of tissues, xenobiotic absorption, coexisting disease, coexposure to additional xenobiotic agents and nutritional status, as well as underlying inflammation. The majority of these determinants acting at the same time can complicate the mechanism of variation due to xenobiotic agents. Moreover, both genetic and environmental factors have the potential to exert important influences on most of these determinants (Ganey and Roth, 2001).

It has been suggested that drug properties, genetic variation, and environmental factors may contribute to IDILI (Boelsterli, 2003a; Kaplowitz, 2001). Two hypotheses to explain IDILI have been proposed (Deng et al., 2009). The first is based on drug metabolizing enzyme (DME) polymorphisms among patients that result in different levels of toxic drug metabolites. The second one proposes the involvement of an adaptive immune response to proteins bound to the drug or its metabolites (Ju and Uetrecht, 2002; Park et al., 2001). Drug metabolism polymorphisms might also contribute to reactive metabolite formation and consequently to the production of haptens needed for a harmful adaptive immune response. An extension of the latter is the “danger hypothesis” (Pirmohamed et al., 2002; Séguin and Uetrecht, 2003), which suggests that, in addition to immunization and challenge, a second “danger signal” is needed to precipitate an adaptive immune response that becomes hepatotoxic. This signal might be any of a number of factors including some form of cellular stress, underlying disease conditions, or
environmental factors (reviewed in Deng et al., 2009). Direct activation of antigen-presenting cells (APC) is also proposed to be involved (Uetrecht, 2013).

Several experimental models suggested that an episode of inflammation during drug treatment predisposes the animals to tissue injury and may be an important determinant of individual susceptibility (Buchweitz et al., 2002; Luyendyk et al., 2000). This raises the possibility that the presence or absence of inflammation is another susceptibility factor for drug toxicity in humans (reviewed in Ganey and Roth, 2001).

Inflammatory episodes are common in humans and animals and are precipitated by numerous stimuli such as bacteria, viruses and exposure to toxins produced by microorganisms. Moreover, episodes of inflammation can be precipitated by the mammalian gastrointestinal (GI) tract. In particular, endotoxin i.e. lipopolysaccharide (LPS) (a potent inducer of inflammation) components released from the cell walls of gram-negative bacteria can translocate across the intestinal mucosa into portal venous circulation. When these gram-negative bacteria divide or are injured, large amounts of LPS are released in the intestinal lumen. LPS can translocate from the GI lumen into the blood, and thereby the liver and other organs become exposed. The rate of LPS translocation and magnitude of consequent liver exposure can be increased by diseases of GI tract/liver, alterations in diet, alcohol consumption, surgical trauma, xenobiotic agents, and other conditions (reviewed in Roth et al., 1997). However, this type of exposure is only accompanied by a mild inflammatory response.

Individuals experiencing concurrent exposure to a xenobiotic and/or its metabolites and endotoxin may be at greater risk of intoxication than those exposed to either of these alone. LPS in the circulation leads to activation of inflammatory cells and the consequent release of numerous endogenous mediators. However, homeostatic alterations in parenchymal cells
initiated by nontoxic doses of a xenobiotic agent may progress to overt injury through the simultaneous action of these mediators (Figure 1.4) (Roth et al., 1997).

**Figure 1.4.** Augmentation of toxic responses by a bacterial endotoxin LPS. KCs (Kupffer cells); PMNs (polymorphonuclear neutrophils); AA (arachidonic acid) [Reprinted from Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? Roth and colleagues, Toxicol. Appl. Pharmacol., 147(2):300–11 © 1997 with permission from Elsevier].
Inflammation is a necessary response to pathogen invasion. However, inappropriate or unregulated inflammatory reactions may cause tissue injury. Before drug-induced liver injury occurs \textit{in vivo}, an inflammatory response usually occurs and cells other than hepatocytes (e.g. Kupffer cells, macrophages) become activated. Inflammmagens such as LPS can also activate Kupffer cells (macrophages) and other inflammatory cells. Immune cells (e.g. neutrophils and macrophages) also infiltrate the liver. At large doses of inflammmagens, this response can lead to overt injury. At smaller doses, cells become more sensitive to the toxic effects of xenobiotics which otherwise may not occur. Numerous studies with animals have shown that a modest inflammatory response enhanced tissue susceptibility to xenobiotics. Therefore, it was hypothesized that inflammatory episodes during drug therapy decreased the threshold for drug toxicity and, thereby, markedly increased the individual’s susceptibility to some drugs (Roth \textit{et al.}, 1997). The inflammmagen hypothesis has been presented in Figure 1.5.

Kupffer cells and resident liver macrophages normally play a role in protecting hepatocytes from xenobiotics by phagocytosing incoming particles and releasing cytoprotective cytokines (Roberts \textit{et al.}, 2007). Kupffer cell inhibitors, e.g. gadolinium chloride, were reported to prevent hepatotoxicity induced by some hepatotoxic drugs, whereas Kupffer cell activators, e.g. retinol or LPS, markedly enhanced hepatotoxicity induced by acetaminophen, allyl alcohol, diethyldithiocarbamate, halobenzenes, and CCl₄ (Buchweitz \textit{et al.}, 2002; Roth \textit{et al.}, 2003).
It is generally thought that most hepatotoxins are activated by oxidation catalyzed by the endoplasmic reticular mixed function oxidase system (MFO) in the liver. The MFO system consists of hepatocyte cytochrome P450, NADPH, cytochrome P450 reductase and oxygen. However, peroxidase and $\text{H}_2\text{O}_2$ can also oxidatively activate some drugs or detoxify some other drugs at higher peroxidase doses (Tafazoli, 2008). Whilst there is little peroxidase activity in hepatocytes, MPO is located in Kupffer cells that are resident macrophages of the human and
rodent liver (Brown et al., 2001). Furthermore, neutrophil infiltration of the liver in response to inflammation can result in a 50 to 100-fold increase in hepatic MPO activity (Kato et al., 2000). Therefore peroxidase activity is a useful marker for measuring neutrophil/macrophage infiltration as well as the hepatic inflammatory response. Eosinophil infiltration (e.g., following a parasite infection) can also cause a marked increase in liver eosinophil peroxidase activity (Gharib et al., 1999). During the inflammatory response, H$_2$O$_2$ can also be formed by activation of the NADPH oxidase in the infiltrated cells. It is therefore reasonable to suggest that the large increase in drug liver susceptibility could also be attributed to peroxidase catalyzed drug oxidation to form reactive pro-oxidant radicals that are toxic to hepatocytes (Tafazoli, 2008).

Drug-induced tissue toxicity is often preceded by infiltration of the tissues by neutrophils, e.g. indomethacin-induced kidney toxicity. This resulted in a marked increase in hepatic ROS generated by neutrophils and a sevenfold increase in hepatic MPO activity (Basivireddy et al., 2004).

**1.4 Intrinsic versus idiosyncratic drug-induced hepatotoxicity-two villains or one?**

In the paper “Intrinsic versus idiosyncratic drug-induced hepatotoxicity-two villains or one?”, Roth and Ganey (2010) suggested that an acute inflammatory episode could shift the dose-response curve for hepatotoxicity to the left, thereby bringing hepatotoxic doses into the therapeutic range. This hypothesis can account for the bizarre characteristics of IDILI and is supported by recent results showing that several drugs associated with human idiosyncratic reactions can be rendered hepatotoxic to rodents upon interaction with an inflammatory stimulus (a complete list of drugs investigated to date is available in Ganey and Roth, 2001). Given this
hypothesis and observations from recent results, it can be suggested that intrinsic and idiosyncratic reactions may not be that different after all (Ganey and Roth, 2001).

**1.5 Models to study DILI**

Pharmaceutical industries face challenges in selecting new drug candidates with high efficacy and low toxicity. In a retrospective analysis, regulatory animal toxicity testing was able to identify more than 70% of human toxicities whereas hepatotoxicity in humans had the poorest correlation with animal toxicity tests. Only half of the new pharmaceuticals that produced hepatotoxicity in humans had any signals in animal toxicity studies (Olson *et al.*, 2000). It is difficult to develop and validate an animal model due to the overlap and unknown mechanisms involved between the intrinsic DILI and IDILI. Of course, there is no “perfect” animal model that will accurately predict all of the drug–human interactions for any new candidate drug. Both *in vitro* and *in vivo* studies are necessary for safety/metabolism/toxicity studies, since neither alone can fully estimate the potential safety or risk of the new candidate drug.

Significant efforts have been made by the pharmaceutical companies to screen drug candidates for their potential to cause DILI, but were mostly unsuccessful. Therefore, often signals, from both animal testing and clinical trials, turned out to be false negative. This increases the cost and time for drug development. Again if a drug gets withdrawn from the market after its approval due to unpredictable IDILI or some other forms of DILI, the loss of revenue, the cost of litigation, and the time and effort spent dealing with the problem may paralyze a company (Uetrecht, 2013). Indeed, developing better animal models to predict human hepatotoxicity is a critical need for the pharmaceutical industry.
Although the mechanistic basis for IDILI remains poorly understood, attempts at animal model development have been made based on several hypothesis for the IDILI mechanism. These hypotheses have centered on drug disposition polymorphisms, adaptive immunity, mitochondrial dysfunction, failure to adapt to modest injury, inflammatory stress, and multiple other determinants (reviewed in Roth and Ganey, 2011).

*In vitro* cellular models of drug toxicity have unique and important roles to play in order to screen out drugs that have potential for hepatotoxicity and to facilitate understanding of the hepatotoxic mechanisms involved. In the current study, focus has been given to *in vitro* models that could be used to predict drug induced hepatotoxicity. It is difficult to distinguish the primary effects of a compound from those induced secondarily because liver functions are under the influence of various endogenous and exogenous factors that result in complex interactions with other organs in *in vivo* animal studies. Moreover, *in vivo* studies are limited by animal welfare/ethical concerns and the fact that data obtained in animals cannot exactly be extrapolated with certainty to humans due to the frequent idiosyncratic nature of liver toxicity and the inherent differences between the metabolic activity in human and non-human species. Therefore *in vitro* liver systems represent a better experimental approach to screen potential hepatotoxic compounds and investigate mechanism(s) of DILI (Davila *et al.*, 2008; Guillouzo, 1998; Tuschl *et al.*, 2008).
1.6 *In vitro* cytotoxicity assessment

*In vitro* cytotoxicity testing is becoming increasingly recognized as an effective and robust tool for assessing human toxicity potential of pharmaceuticals early in drug discovery in order to maximize the probability of successful progression of compounds into development (Dambach *et al.*, 2005; O’Brien and Haskins, 2006; Perlman *et al.*, 2004; Xu *et al.*, 2004). Conventional *in vitro* cytotoxicity assays have low predictive value for the detection of human hepatotoxicity. However, if these assays identified a compound as a liver-toxicant, there is more than an 80% chance of corresponding findings in humans (Xu *et al.*, 2000). *In vitro* systems are simple and provide the ability to specifically manipulate and analyze a small number of parameters to understand toxicity mechanisms. The most commonly used test systems of the past few decades include, for example, the isolated perfused liver, liver slices, primary hepatocytes in suspension or culture, cell lines, transgenic cells and sub-cellular fractions like S9-mix, microsomes, supersomes or cytosol. However, the isolated hepatocyte is the most widely used model and considered to be the gold standard for *in vitro* DILI study (Guillouzo, 1998; Tuschl *et al.*, 2008).

1.7 Isolated hepatocytes in studying DILI

An ideal *in vitro* test system should adequately represent the *in vivo* situation of drug metabolism and biotransformation in the liver. The isolated perfused liver represents the closest *in vitro* model to simulate *in vivo* situation and has long been used for investigating DILI. It is able to retain phase I and II drug metabolizing enzyme activities, as well as inducibility by xenobiotics (Davila and Morris, 1999). Major advantages of the isolated perfused liver are (a) the three-
dimensional architecture is preserved with cell–cell, cell–matrix interactions and functional bile canaliculi are maintained, leading to in vivo-like metabolism, (b) the bile flow can be collected and analyzed separately, (c) allows consideration of issues related to hemodynamics. Despite all of these advantages, the isolated perfused liver model is difficult to handle and its functional integrity only maintained for a few hours. Therefore it can only be used for toxicants that are expected to have toxic effects at very early ‘time-points’ (acute toxicity). Moreover, its reproducibility is low and relative to other in vitro models, it does not necessarily reduce the number of animals used. Whole organ perfusion of human liver is technically difficult and human liver is rarely available for perfusion (Guillouzo, 1998; Tuschl et al., 2008).

1.8 Accelerated cytotoxicity mechanism screening techniques

The “accelerated cytotoxicity mechanism screening” (ACMS) methods determine the molecular cytotoxic mechanisms of drugs/xenobiotics when incubated at 37°C for 3 hrs using freshly isolated rat hepatocytes. ACMS is a useful tool for identifying the hepatocyte metabolizing enzymes by comparing the effects of specific inhibitors of metabolizing enzymes in modulating the loss of cell viability caused by the drug/xenobiotic being investigated. This functionomic approach is useful for understanding the molecular cytotoxic mechanisms of xenobiotics under investigation. A major assumption with ACMS is that high dose/short time (in vitro) exposure simulates low dose/long time (in vivo) exposure (Chan et al., 2007; O’Brien and Siraki, 2005). With 24 halobenzenes, it was found that the relative lethal concentrations required to cause 50% cytotoxicity in 2 hrs at 37°C (defined as ACMS LC₅₀), as determined in vitro using hepatocytes isolated from phenobarbital-induced Sprague-Dawley rats, correlated with hepatotoxicity in vivo
at 24 – 54 hrs (Chan et al., 2007). Moreover, using these techniques, the molecular hepatocytotoxic mechanisms found in vitro for six classes of xenobiotics/drugs were found to be similar to the rat hepatotoxic mechanisms reported in vivo (O’Brien et al., 2004). The following procedures have been used (O’Brien and Siraki, 2005):

“1) Determination of the concentration of drug/xenobiotic required to induce a 50% loss of membrane integrity (LC50) of freshly isolated rat hepatocytes in 2 hrs using the trypan blue exclusion assay.

2) Drug- or xenobiotic-induced cytotoxicity by inhibiting or inducing different metabolizing enzymes which activate or detoxify the drug/xenobiotic was then determined. In this way, the major metabolic pathways and metabolizing enzymes of xenobiotics can be rapidly identified. ACMS techniques were used to show that the drug metabolic pathways at cytotoxic drug concentrations in vitro in 2 hrs were similar to those that occur in vivo in 24 – 36 hrs.

3) The hepatocyte molecular cytotoxic mechanism of xenobiotics was determined by following the changes in bioenergetics (ATP, mitochondrial membrane potential, respiration, glycogen depletion), oxidative stress (GSH/GSSG levels, lactate/pyruvate ratio, and ROS formation), and electrophile stress e.g. GSH conjugates and protein/DNA adducts. If oxidative stress caused the cytotoxicity, then it should precede cytotoxicity and antioxidants, ROS scavengers or redox therapy should prevent or delay the cytotoxicity. If not, then the oxidative stress likely occurred as a secondary result of the cytotoxicity. If mitochondrial toxicity caused the cytotoxicity, then glycolysis partly compensates and restores the membrane potential (O’Brien et al., 2004; O’Brien and Siraki, 2005)”.

The enzyme inhibitors/activators are chosen on the basis of their selectivity, modulator effectiveness, and their lack of toxicity in the hepatocyte model. Therefore, the ACMS
techniques are a methodological application that may be adaptable for high throughput screening and may be useful for supplementing existing in vitro screening techniques as a means to explore hepatotoxicity mechanisms. Using an in vitro model to help predict in vivo animal hepatotoxicity may clarify mechanisms that can be used to minimize drug-induced toxicity and help determine if alternative drug candidates are likely to be safer. In this way, the ACMS techniques can help accelerate the pre-clinical drug screening process and filter out compounds with DILI potential, thus guiding the development of safer pharmaceutical drugs (MacAllister, 2013).

1.9 In vitro hepatocyte oxidative stress inflammation model

It is important to define the role of inflammation in drug toxicity and to develop models or methods to predict which drugs or drug candidates have the potential to cause toxicity through interaction with inflammation. This knowledge could enable identification of individuals who are susceptible to DILI due to inflammation.

In order to simulate the marked increase of drug-induced hepatotoxicity caused by acute episode inflammation in vivo (as discussed previously), and assess the potential for the in vivo hepatotoxicity risk of various drugs, our laboratory generally uses an in vitro hepatocyte screening system. This “In Vitro Oxidative Stress Inflammation system” includes subjecting freshly isolated rat hepatocytes to a low non-toxic continuous flow of a H₂O₂-generating system using glucose (G) and glucose oxidase (GO) and supplementing it with either horseradish peroxidase (HRP) or Fe(II) (MacAllister et al., 2013a) to simulate in vivo inflammation. HRP/H₂O₂ was used for in situ activation of drugs and to simulate MPO. The H₂O₂ acts by increasing the oxidation state of the ferric ion which then oxidizes the peroxidase substrates
6-N-propyl-2-thiouracil (PTU) was used as a peroxidase inhibitor in this study as evidence for the involvement of HRP/H2O2 catalyzed formation of drug pro-oxidant radicals. PTU inhibits HRP in a noncompetitive fashion (Zatón and Ochoa de Aspuru, 1995). Phagocytes generate O2− and H2O2 and their interaction results in an Fe(II)-catalyzed reaction that forms ‘•OH. Desferoxamine chelates Fe(II) in a catalytically inactive form, and thus inhibition by desferoxamine has been employed as evidence for the involvement of ‘•OH generated by the Fenton reaction (Klebanoff and Waltersdorph, 1988). Using this model, the laboratory was able to mimic the products formed by the inflammatory immune cells and study the mechanism of inflammation-enhanced drug-induced cytotoxicity. A list of drugs/xenobiotics investigated in our lab previously with positive and negative results with the in vitro inflammation system are presented in Table 1.3.
Table 1.3. List of drugs previously investigated in this lab with positive and negative results with the inflammation system.

<table>
<thead>
<tr>
<th>Drugs that formed cytotoxic pro-oxidant radicals</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid, anti-tubercular drug</td>
<td>Tafazoli <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>Amodiaquine, anti-malarial</td>
<td>Tafazoli and O’Brien, 2009</td>
</tr>
<tr>
<td>Hydralazine, smooth muscle relaxant</td>
<td>Tafazoli and O’Brien, 2008</td>
</tr>
<tr>
<td>Chlorpromazine, typical antipsychotic drug</td>
<td>MacAllister <em>et al.</em>, 2013b</td>
</tr>
<tr>
<td>Clozapine, atypical antipsychotic drug</td>
<td>MacAllister <em>et al.</em>, 2013a</td>
</tr>
<tr>
<td>Troglitazone, anti-diabetic drug</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Tolcapone, COMT inhibitor</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Mefenamic acid, NSAID</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Diclofenac, NSAID</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Phenylbutazone, NSAID</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
</tbody>
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<tr>
<th>Drugs that did not formed cytotoxic pro-oxidant radicals</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tribromoethanol, anesthetic drug</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Acetylsalicylic acid, analgesic drug</td>
<td>Tafazoli, 2008</td>
</tr>
<tr>
<td>Naproxen, antipyretic/analgesic drug</td>
<td>Tafazoli, 2008</td>
</tr>
<tr>
<td>Ciglitazone, anti-diabetic drug</td>
<td>Tafazoli, 2008</td>
</tr>
<tr>
<td>Pioglitazone, anti-diabetic drug</td>
<td>Tafazoli, 2008</td>
</tr>
<tr>
<td>Leflunomide, anti-arthritic drug</td>
<td>Tafazoli, 2008</td>
</tr>
<tr>
<td>Entacapone, COMT (catechol-O-methyl transferase) inhibitor</td>
<td>Tafazoli <em>et al.</em>, 2005</td>
</tr>
</tbody>
</table>
1.10 Literature review of the drugs investigated

1.10.1 Nitroaromatics (nimesulide, nilutamide, flutamide)

**Nimesulide** (NIM) \((\text{N-}(4\text{-nitro}-2\text{-phenoxyphenyl})\text{methanesulfonamide})\) is the unique molecule of the sulphonanilide class of NSAIDs having analgesic, anti-pyretic, and potent anti-inflammatory activities and very good gastro-intestinal tolerability (Bessone et al., 2010). Mechanisms include cyclooxygenase-2 (COX-2) inhibition, scavenging of free radicals, and inhibition of various pathways of inflammation. NIM was licensed by Helsinn Healthcare SA (Switzerland) in 1980 and first launched in Italy as a therapeutic agent in 1985. Since 1985, it has been marketed in more than 50 countries with the exception of the United States and a few other countries due to safety concerns. After widespread clinical use of NIM (Boelsterli, 2002), hepatic toxicities, including both acute hepatitis and the more severe fulminant hepatic failure, were reported (Boelsterli, 2002; Dastis et al., 2007; Stadmam et al., 2002; Tan et al., 2007). The relatively high occurrence of these adverse events (9.4 cases per million patients treated) in Finland and Spain caused NIM to be withdrawn from the market in those countries (Marcia et al., 2002; Traversa et al., 2003). However, NIM remains on the market in a number of countries where the rate of NIM-induced injury is reported to be relatively low (about 1 per million patients treated). The hepatotoxicity of NIM is rare, relatively insensitive to accumulated dose, and specific to a patient and is thus considered to induce an idiosyncratic toxicity (Boelsterli, 2002; Boelsterli et al., 2006; Traversa et al., 2003). NIM exhibited liver injury features possibly consistent with an immunoallergic mechanism in a few patients. However, hypersensitivity manifestations were
absent in most patients, suggesting metabolic idiosyncrasy rather than immunoallergy (Van Steenbergen et al., 1998).

The molecular mechanisms involved in hepatotoxicity due to NIM have not been fully elucidated. However, the involvement of ROS formation and mitochondria mediated pathways in NIM-induced apoptotic cell death and resultant hepatotoxicity have been implicated (Boelsterli et al., 2006). NIM is oxidatively metabolized via liver cytochromes P450 (principally by CYP2C9, CYP2C19, and possibly CYP1A2), mainly to the 4-hydroxy nimesulide (4-OH-NIM) which has similar pharmacological properties to the parent drug but with lower potency (Bernareggi and Rainsford, 2005). It has been suggested that NIM is bioactivated by CYP2C to a protein-reactive electrophilic intermediate that activates the nuclear factor-like 2 pathway even at non-toxic exposure levels (Kale et al., 2010).

Recently, it has also been demonstrated that a known NIM metabolite (Figure 1.6) could be bioactivated by MPO through a pathway distinct from human liver microsome-mediated pathways and that the generation of reactive species by the MPO-mediated bioactivation pathway at the site of inflammation may contribute to the toxicity associated with NIM (Yang et al., 2010).

Incubation of hepatocytes with NIM (0.1 – 1 mM) elicited a concentration- and time-dependent decrease in cell viability, a decrease of mitochondrial membrane potential (MMP), and cell adenosine 5’-triphosphate (ATP) depletion. NIM also decreased the levels of NADPH and GSH in hepatocytes, but the extent of the effects was less pronounced in relation to the energetic parameters; in addition, these effects did not imply the peroxidation of membrane lipids. The decrease in the viability of hepatocytes was prevented by fructose and, to a larger extent, by fructose plus oligomycin; it was stimulated by proadifen, a cytochrome P450 inhibitor.
In contrast, the reduced metabolite of NIM did not present any of the effects observed for the parent drug (Mingatto et al., 2000).

**Figure 1.6.** Proposed amine activation of reduced NIM by neutrophils and myeloperoxidase.


NIM (100 µM) treatment resulted in a rapid depletion of GSH (60%) and enhanced generation of ROS in isolated rat liver mitochondria. A significant change in Ca^{2+} dependent mitochondrial permeability transition (MPT) was also observed (Singh et al., 2010). Albumin (Berson et al., 2006) and a combination of natural terpenes (camphene and geraniol) (Singh et al., 2012) were found to prevent NIM-induced hepatotoxicity.
Nilutamide (NIL) (5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl)phenyl]imidazolidine-2,4-dione) is a nonsteroidal antiandrogen derivative that acts as a competitive antagonist of the androgen receptor (Raynaud et al., 1984). This nitroaromatic compound is proposed in the treatment of metastatic prostatic carcinoma in association with castration (Beland et al., 1988; Brisset et al., 1987). Its therapeutic effects are overshadowed by the occurrence of some adverse reactions e.g. loss of visual dark adaptation and the rare development of lung and/or liver lesions. Reversible episodes of interstitial pneumonitis occur in 1 to 2 % of patients whereas hepatotoxicity occurs in approximately 0.5 to 1 % of patients. The hepatitis is hepatocellular in type. Signs of hypersensitivity and autoimmunity are not common, suggesting that hepatitis may be mediated by a toxic rather than an immunoallergic mechanism (Fau et al., 1992). In large clinical trials, ALT (alanine aminotransferase) elevations occurred in 2 to 33 % of patients during NIL therapy. The elevations were usually mild, asymptomatic and transient, rarely requiring drug discontinuation. NIL-induced acute liver injury rarely occurs (McLeod, 1997).

The mechanism of NIL-induced hepatotoxicity is still unknown, but NIL-induced toxic metabolite formation that leads to oxidative stress or interferes with mitochondrial functions has been proposed. NIL is extensively metabolized in the liver, undergoing mainly reduction of the nitro group to the non-toxic primary amine product. Electron spin resonance studies have shown that NIL is first reduced by the NADPH-cytochrome P-450 reductase of rat liver microsomes into a nitro anion-free radical (Berson et al., 1991). NIL itself, or the amine derivative, may also undergo oxidation at several sites (Ojasoo, 1987). The structure and different metabolites of NIL are presented in Figure 1.7.
The initial one-electron reduction of the nitro group forms a reactive nitro anion free radical. Under anaerobic conditions, a further one-electron reduction occurs probably mediated by disproportionation of the nitro anion radical forming a highly reactive nitroso derivative. The nitroso can then be further reduced by two electrons to the hydroxylamine, and again reduced by two electrons, to form a relatively inactive amine. The nitroso and the hydroxylamine are reactive species which can covalently bind to glutathione and cellular macromolecules. Under aerobic conditions, molecular oxygen oxidizes the nitro anion free radical, resulting in a redox cycle with regeneration of the nitro compound and production of superoxide anion, whose dismutation by superoxide dismutase yields $\text{H}_2\text{O}_2$. Therefore the reduction of some nitroaromatic compounds e.g. NIL can lead to the formation of alkylating intermediates and/or potentially...
toxic oxygen metabolites (Ask et al., 2004; Berson et al., 1991). In human recipients, it has been suggested that ROS can be formed during the reductive metabolism of this nitro aromatic drug in aerobic conditions (Berson et al., 1991).

In isolated mitochondria, NIL (100 µM) inhibited respiration, supported by substrates feeding electrons into complex I of the respiratory chain. In sub-mitochondrial particles, NIL (100 µM) decreased both oxygen consumption mediated by NADH and the oxidation of NADH; addition of SOD and catalase had no effect. NIL (100 µM) also decreased the MMP and ATP formation in mitochondria energized by malate plus glutamate (Berson et al., 1994).

Flutamide (FLU) (2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide) is a competitive antagonist of the androgen receptor, which is used, like NIL, in association with castration in the treatment of metastatic prostatic carcinoma (Steinsapir et al., 1991). The efficacy of this antiandrogen is somewhat overshadowed by the occurrence of hepatitis in a few subjects. The incidence of hepatotoxicity (defined as >4-fold increase in serum transaminase activity) was found to be 0.36 % of 1091 consecutively treated patients with prostate cancer (Gomez et al., 1992). Moreover, the incidence of liver disorder (defined as >1.5-fold increase in alanine aminotransferase levels) was reported to be 26 % of 123 men with prostate cancer treated with FLU. The rate was higher in those with previous histories of liver disease and preexisting elevations in GGT (gamma-glutamyl transpeptidase) or ALT (alanine aminotransferase) (Wada et al., 1999). FLU-induced hepatitis was cholestatic and/or cytolytic and fulminant (Alperine et al., 1991; Corkery et al., 1991; Dankoff, 1992; Hart and Stricker, 1989).

Although the mechanisms of FLU-induced hepatotoxicity have not been precisely elucidated, the formation of a reactive toxic metabolite might be responsible for such drug-
induced hepatotoxicity (Matsuzaki et al., 2006; Pessayre and Larrey, 1991). Unlike the related anti-androgen NIL, FLU was not noticeably reduced into a nitroanion free radical by NADPH-cytochrome P450 reductase. Instead, rat and human microsomal cytochrome P450 oxidatively metabolized FLU into electrophilic metabolite(s), which bound covalently to microsomal proteins (Berson et al., 1993). FLU is metabolized into 2-hydroxy FLU (OH-FLU) in liver by the CYP1A2. FLU is also known to be metabolized into FLU-1 (4-nitro-3(trifluoromethyl)phenylamine) (Figure 1.8) (Asakawa, 1995; Radwanski et al., 1989). The plasma concentration of FLU-1 was reported to be usually much lower than that of OH-FLU during clinical pharmacological examination, and it can be metabolized further by hydroxylation and acetylation (Matsuzaki et al., 2006). FLU and OH-FLU were cytotoxic to primary cultured rat hepatocytes at concentrations of approximately 40 µM and 170 µM, respectively (Wang et al., 2002). CYP3A has also been reported to be involved in the metabolism of FLU into toxic electrophilic metabolites (Berson et al., 1993).

Some FLU-induced liver cases were found to be associated with blood eosinophilia and neutropenia, suggesting an involvement of the immune system (Hart and Stricker, 1989; McDonnell and Livingston, 1994). It was suggested that in some individuals FLU simultaneously renders hepatocytes more susceptible to oxidant-mediated injury that can initiate infiltration of PMNs in to the liver and increases PMNs responsiveness to endogenous activators (Srinivasan et al., 1997).
Figure 1.8. Structure of FLU and its metabolites. OH-flutamide (OH-FLU), 2-hydroxy flutamide; FLU-1, 4-nitro-3 (trifluoromethyl)phenylamine) [Reprinted with permission from Matsuzaki and colleagues, Metabolism and hepatic toxicity of flutamide in cytochrome P4501A2 knockout SV129 mice. J. Gastroenterol., 41(3):231–9. Copyright © 2006 Springer].

FLU (1 mM) led to the covalent binding of reactive electrophilic metabolites to male rat hepatocyte proteins. It decreased the GSH/GSSG ratio and total protein thiols. This was associated with an early increase in phosphorylase a activity (a Ca^{2+}-dependent enzyme) and a decrease in cytoskeleton-associated protein thiols, the formation of plasma membrane blebs, the release of lactate dehydrogenase (LDH) and a loss of cell viability. The addition of cystine (a GSH precursor) increased GSH and decreased LDH release in male rat hepatocytes (Fau et al., 1994). A novel GSH conjugate of FLU was reported in human liver microsomes, suggesting that the P450-mediated oxidation of FLU via a nitrogen-centered free radical could be one of several bioactivation pathways of FLU (Kang et al., 2007). FLU also significantly increased lipid
peroxidation in an *in vivo* rabbit model that was prevented by GSH (Ray *et al.*, 2008). FLU (50 µM) markedly inhibited complex I respiration in isolated male rat liver mitochondria and FLU (1 mM) decreased ATP levels in isolated male rat hepatocytes (Fau *et al.*, 1994).

### 1.10.2 Methotrexate

Methotrexate (MTX) ((2S)-2-[(4-\{(2,4-diaminopteridin-6-yl)methyl\}(methyl)amino)benzoyl]amino]pentanedioic acid) is one of the folic acid antagonists (Al-Motabagani *et al.*, 2006) that is widely used in the treatment of psoriasis (Thomas & Aithal, 2005), psoriatic arthritis (Wollina *et al.*, 2001), rheumatoid arthritis in elderly and younger patients (Padeh *et al.*, 1997), acute lymphoblastic leukemia (Aytac *et al.*, 2006), ectopic pregnancy (Chen *et al.*, 2003), inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Siegel and Sands, 2005), and chronic inflammatory demyelinating polyradiculoneuropathy (Fialho *et al.*, 2006). One of the most serious side effects of MTX therapy is hepatic toxicity. Mild hepatitis, cholestasis, fatty changes, fibrosis, and cirrhosis have been reported in patients receiving MTX for malignant disorders (Hersh *et al.*, 1966) whereas Penalva Polo and colleagues (2002) reported an acute liver failure in a patient with MTX therapy. MTX-induced cytotoxicity was reported to be associated with ROS formation and depletion of cellular and mitochondrial GSH and ATP (Babiak *et al.*, 1998; Chang *et al.*, 2013; Chen *et al.*, 1998; Neuman *et al.*, 1999; Phillips *et al.*, 2003). MTX increased H₂O₂ levels in stimulated PMNs in a dose-dependent manner with a maximum increase of 43.7% for 500 µM MTX (Gressier *et al.*, 1994). Addition of MTX (100 nM – 10 µM) to U937 monocytes induced a time and dose dependent increase in cytosolic H₂O₂ from 6 – 16 hr. MTX also caused corresponding monocyte growth arrest, which was inhibited by pre-treatment with N-
acetylcysteine (10 mM) or GSH (10 mM) (Phillips et al., 2003). MTX alone or in combination with Cu(II) also generated ROS in lymphocytes that was inhibited by ROS scavengers (Chibber et al., 2011).

It was found that MTX caused a significant increase in TBARS (thiobarbituric acid reactive substance) levels (an important marker of lipid peroxidation) in the group in which MTX was administered in vivo compared to the control group. The activities of SOD, catalase, and glutathione reductase were significantly decreased in the MTX groups when compared with the control group. The results therefore indicate that MTX caused oxidative stress by decreasing the activities and effectiveness of the antioxidant enzyme defense system (Coleshowers et al., 2010).

MTX, like naturally occurring folates is converted by folypolyglutamyl transferase into polyglutamates (PGns) (Sczesny et al., 1998). MTX undergoes metabolic degradation to 7-hydroxy methotrexate (7-OH-MTX) by the action of unspecific aldehyde oxidases in the rat liver (Bremnes et al., 1991a; Wolfrom et al., 1990). A proposed metabolic pathway for MTX is shown in Figure1.9. In human liver, both aldehyde oxidase and xanthine oxidase have been proposed to be involved in MTX-oxidation (Chládek et al., 1997). Enterohepatic circulation by the action of bacteria from the intestinal flora produces another MTX metabolite, 2,4-diamino-\textit{N}-methylptericoic acid (DAMPA) at a rate of 4%. However, since this metabolite has only 1/200 of MTX activity, it is of minor importance clinically (Donehower et al., 1979). DAMPA was not cytotoxic and did not significantly alter MTX-induced cytotoxicity in human leukemic cell line (Widemann et al., 2000). Although 7-OH-MTX is an active metabolite of MTX, it exhibited only 1/100 – 1/200 of the original MTX activity in the inhibition of dihydrofolate reductase (DHFR) (Farquhar et al., 1972). Although 7-OH-MTX cytotoxicity was increased several-fold by
polyglutamylation (Sholar et al., 1988), the inhibition of DHFR, the main target enzyme, was
minute as compared with that of equivalent intracellular levels of MTX polyglutamates (MTX-
PGns) (Seither et al., 1989).

Figure 1.9. A proposed metabolic pathway of MTX. (1) Hydroxylation: 7-hydroxy methotrexate
(7-OH-MTX), (2) Cleavage: 2,4-diamino-\(N\)-methylpterolic acid (DAMPA), (3) Polyglutamation:
MTX-PGn and 7-OH-MTX-PGn [Reprinted from Capillary electrophoretic drug monitoring of
methotrexate and leucovorin and their metabolites, Sczesny and colleagues, J. Chromatogr. B

7-OH-MTX has been proposed as a mediator of MTX associated clinical toxicity. Renal
failure due to the precipitation of the metabolite in renal tissues of mammals and precipitation of
7-OH-MTX at high concentrations in rat bile \textit{in vivo} and \textit{in vitro} were also reported (Bremnes et
al., 1989; Bremnes et al., 1991b). It has also been suggested that this metabolite may play a role
in clinically acute renal and hepatic toxicity frequently encountered after a high-dose MTX
therapy (Bremnes et al., 1991c; Smeland et al., 1994; 1996).

MTX also decreased the ionic conductivity of the mitochondrial inner membrane and
membrane potential (\(\Delta \Psi\)), and inhibited state III respiration in rat liver mitochondria. The effect
on the steady-state of cytochrome b, as well as the inhibitory effect on state III of respiration with NAD$^+$-linked substrates, offers a reasonable explanation that the inhibition site of MTX could be in a place anterior to the cytochrome b region, not linked to respiratory chain.

(Yamamoto et al., 1888; 1989).

Several *in vitro* and *in vivo* animal studies were carried out to find molecules which could be hepatoprotective with MTX therapy e.g. misoprostol (Asci et al., 2011); resveratrol (Dalaklioglu et al., 2013; Tunali-Akbay et al., 2010); propolis (Badr et al., 2011); curcumin (Banji et al., 2011; Hemeida et al., 2008); taurine (Issabeagloo et al., 2011); omega-3 and selenium (Mohammad et al., 2011); dexamethasone (Fuksa et al., 2010); melatonin (Jahovic et al., 2003); β-carotene (Vardi et al., 2010); lipoic acid (Tabassum et al., 2010); caffeic acid phenolic ester (CAPE) (Cakir et al., 2011); milk thistle (Ghaffari et al., 2011); and β-glucan (Sener et al., 2006).

### 1.10.3 Thiopurines

Three thiopurine drugs (Figure 1.10) have been commonly used in the last forty years (Petit et al., 2008). 6-mercaptopurine (3,7-dihydro-6$H$-purine-6-thione) (6-MP) and 6-thioguanine (2-amino-6-mercaptopurine) (6-TG) are used in the treatment of acute leukaemia whereas azathioprine (6-[(1-methyl-4-nitro-1$H$-imidazol-5-yl)sulfanyl]-7$H$-purineglucose) (AZA) is widely used as an immunosuppressant for the treatment of diseases such as inflammatory bowel diseases (IBD), autoimmune conditions and following transplantation to avoid organ rejection (El-Azhary, 2003; Dubinsky, 2004; Lennard et al., 1997). In most of the cases, hepatotoxicity is an unpredictable side effect of AZA and 6-MP, whose pathogenic mechanism remains unknown.
AZA has been reported to conjugate with GSH to form 6-MP, catalyzed by GSTs (DeLeve et al., 1996; Kaplowitz, 1977; Lee and Farrell, 1999; 2001) (Figure 1.11). This consumes GSH, which is normally present in abundance in hepatocytes. Previous studies performed with rat hepatocyte primary cultures showed that toxic concentrations of AZA (25 – 250 µM) led to profound intracellular GSH depletion, mitochondrial injury, metabolic activity reduction, decreased ATP levels, and cell death due to necrosis, not apoptosis. Toxic effects were acute and dose-dependent. Hepatocyte death was prevented by GSH or N-acetylcysteine, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a vitamin E analogue), high dose allopurinol (acts as an antioxidant), cyclosporine A and glycine (Lee and Farrell, 1999; 2000). Similar effects were observed by Menor and colleagues (2004) where AZA (150 µM) decreased the viability of rat hepatocytes and induced intracellular GSH depletion, metabolic activity reduction, and LDH release. However, the cell death was not accompanied by DNA laddering, procaspase-3 cleavage, or cytochrome c release. AZA caused mitochondrial dysfunction and

**Figure 1.10.** Chemical structures of azathioprine, 6-mercaptopurine, and 6-thioguanine.
activation of stress-activated protein kinase pathways leading to necrotic cell death in intact isolated rat mitochondria (Menor et al., 2004).

**Figure 1.11.** The pathways of AZA metabolism. HGPRT, hypoxanthine-guanine-phosphoribosyltransferase [Reprinted from Mechanism of azathioprine-induced injury to hepatocytes: Roles of glutathione depletion and mitochondrial injury, Lee and Farrell, J. Hepatol., 35(6), 756–764 © 2001, with permission from Elsevier].

Clinically relevant concentrations of AZA (0.5 – 5 µM) were also found to be toxic to rat hepatocyte cultures and involved oxidative stress, mitochondrial injury and ATP depletion that led to cell death by necrosis (Tapner et al., 2004). Allopurinol (a xanthine oxidase inhibitor) and Trolox together provided near complete hepatocyte protection from AZA (Tapner et al., 2004).
Xanthine oxidase is proposed to be involved in several steps of AZA metabolism such as in the direct metabolism of AZA to form an inactive metabolite, 1-methyl-4-nitrothioimidazole, in the conversion of AZA to 6-MP, and formation of 6-thiouric acid from 6-MP (Lee and Farrell, 1999; 2001; Tapner et al., 2004) (Figure 1.11). The possibility that xanthine oxidase may play a role in AZA-induced tissue injury has been raised by the observation that patients taking allopurinol, a xanthine oxidase inhibitor, experienced less nephrotoxicity during rejection episodes after renal transplantation (Chocair et al., 1994).

Thiopurine S-methyltransferase (TPMT) converts 6-MP to 6-methyl mercaptopurine (6-MMP) and elevated 6-MMP levels were reported to be associated with dose dependent elevations of liver enzymes such as ALT, AST (alanine aminotransferase), GGT (reviewed in Bradford and Shih, 2011; Katsanos and Tsianos, 2007). However, several studies reported AZA-induced hepatotoxicity had no relationship with 6-MMP levels (Andrejic et al., 2010; Gupta et al., 2001; McGovern, 2002). AZA-induced myelosuppression and skin reactions were related to TPMT polymorphisms (Lennard et al., 1997; Snow and Gibson, 1995). However, TPMT polymorphisms did not appear to be involved in AZA-induced hepatotoxicity (King and Perry, 1995).

Several in vitro and in vivo animal studies were carried out to find molecules which could be hepatoprotective for AZA or 6-MP therapy e.g. green tea polyphenols (El-Beshbishy et al., 2011); L-arginine (Moustafa and Badria, 2010); lycopene (Issabeagloo et al., 2011); aminoguanidine (Raza et al., 2003); quercetin (Shanmugarajan et al., 2008); and Vit-E (Tabrizi et al., 2009).
1.11 Aims of the study and hypothesis

The aims of this thesis are:

1. To develop an in vitro system using isolated rat hepatocytes to investigate the effects of inflammation and oxidative stress on the development of DILI. We use either a low non-toxic continuous flow of a H₂O₂-generating system using G and GO, supplemented with either (a) HRP or (b) Fe(II) to simulate in vivo inflammation.

2. To investigate molecular mechanisms of drug-induced hepatotoxicity using the ACMS techniques and the in vitro oxidative stress inflammation system.

3. To investigate what compounds or antioxidants may act as antidotes to prevent or delay drug-induced cytotoxicity.

General hypothesis:

*Exposure of drugs such as flutamide, nilutamide, nimesulide, methotrexate, azathioprine, 6-mercaptopurine to an in vitro oxidative stress inflammation system will increase hepatotoxicity through the formation of pro-oxidant radicals and other reactive oxygen species leading to oxidative stress.*

A simplified schematic representation of the aims of the study is presented in Figure 1.12.
Figure 1.12. Simplified schematic representation of the hypotheses and aims the study.
1.12. Summary of introduction

Drug-induced liver injury is a major concern in clinical studies as well as in post-marketing surveillance of drugs. Previous evidence suggests that drug exposure during periods of inflammation can increase an individual’s susceptibility to toxicity. Inflammation caused by infections or endotoxin markedly activates NADPH oxidase that generates superoxide radicals by transferring electrons from NADPH. In the phagosome, superoxide radicals spontaneously form H$_2$O$_2$ and other ROS. Neutrophils or Kupffer cells also release MPO on activation. The aim of this study was to develop and validate an *in vitro* oxidative stress inflammation model to identify compounds that may increase hepatotoxicity during inflammation. Toxic pathways of tested drugs were also investigated using the “Accelerated Cytotoxicity Mechanism Screening” techniques routinely used in our laboratory. Early in drug discovery, *in vitro* cytotoxicity is becoming increasingly recognized as an effective indicator of human toxicity potential that must be addressed in order to maximize probability of successful progression of compounds into development. The ACMS technique may be useful for supplementing existing *in vitro* screening techniques as a means to accelerate the pre-clinical screening process.
CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals

Nimesulide (N-(4-nitro-2-phenoxyphenyl)methanesulfonamide); nilutamide (5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl)phenyl]imidazolidine-2,4-dione); flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide); methotrexate ((2S)-2-[(4-[[2,4-diaminopteridin-6-yl]methyl](methyl)amino]benzoyl)amino]pentanedioic acid); azathioprine (6-[(1-methyl-4-nitro-1H-imidazol-5-yl)sulfanyl]-7H-purineglucose); 6-mercaptopurine (3,7-dihydro-6H-purine-6-thione); isoniazid (isonicotinic hydrazide); entacapone ((2E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethylprop-2-enamide); glucose oxidase from *Aspergillus niger* (type II, 15,000–50,000 units/g solid); catalase from bovine liver (2,000–5,000 units/mg protein); peroxidase from horseradish (type II, 150–250 units/mg solid); bovine superoxide dismutase (≥2500 units/mg protein); hydrogen peroxide; diethylenetriaminopentaacetic acid (DTPA); 1-bromoheptane; trichloroacetic acid; thiobarbituric acid; 2',7'-dichlorofluorescin diacetate; 3-amino-1,2,4-triazole; 6-N-propyl-2-thiouracil; N-acetyl-L-cysteine; allopurinol (1H-pyrazolo[3,4-d]pyrimidin-4(2H)-one); menadione (2-methylnaphthalene-1,4-dione), amsacrine (N-(4-(acridin-9-ylamino)-3-methoxyphenyl)methanesulfonamide); 1-aminobenzotriazole; fructose; Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid); TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), DPPD (N,N'-diphenyl-p-phenylenediamine); mesna (2-mercaptopethanesulfonate); resveratrol (3,5,4'-trihydroxy-trans-stilbene); DMPO (5,5-dimethyl-1-pyrroline-N-oxide), and all other chemicals were purchased from Sigma-Aldrich Corp. (Oakville, ON, Canada). Type II collagenase (from *Clostridium histoloticum*) was
purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) was purchased from Boehringer-Mannheim Ltd. (Montreal, Quebec, Canada). Stock solutions of chemicals were made up in either H$_2$O or DMSO (dimethyl sulfoxide).

2.2 Animal treatment

Male Sprague–Dawley rats (Charles River Laboratories International Inc., Wilmington, Massachusetts, USA) weighing 275 – 300 g were used for experimental purposes. Experiments were carried out in compliance with the Guide to the Care and Use of Experimental Animals by Canadian Council on Animal Care (Olfert, Cross and McWilliam, 1993) and the University of Toronto Animal Use Protocol, which was reviewed and approved by the Faculty of Medicine and Pharmacy Animal Care Committee. Rats were housed in ventilated plastic cages with 12 air changes per hr, 12 hr light photoperiod (lights on at 0800 hr) and an environmental temperature of 21 – 23°C with a 50 – 60% relative humidity. The animals were fed a normal standard chow diet and water *ad libitum*.

2.3 Hepatocyte preparation and treatment

Rat liver was perfused with collagenase and hepatocytes were isolated by a method previously developed by Moldeus and colleagues (1978) with slight modifications in our lab.
**Isolation of hepatocytes:**

Rat liver was preperfused with a chelator to remove Ca$^{2+}$ and then perfused with collagenase. With this approach good quality and quantity of hepatocytes were obtained characterized by single hepatocytes with a smooth, spherical appearance, and essentially free of nonparenchymal cells.

**Gas Mixtures and solutions:**

All solutions were heated to 37°C and conditioned with 95% O$_2$ and 5% CO$_2$ gas that was used during liver perfusion as well as hepatocyte incubation. Several buffers were used for the purpose. The composition of the buffers are given below:

- **Krebs-Henseleit Buffer** (volume to 1 L ddH$_2$O, pH 7.4)
  
  3.0 g, HEPES; 2.1 g, NaHCO$_3$; 6.95 g, NaCl, 0.355 g, KCl, 0.16 g, KH$_2$PO$_4$, 0.295 g, MgSO$_4$·7H$_2$O, 0.287 g, CaCl$_2$.

- **Buffer A** (Perfusion buffer, volume to 100 mL ddH$_2$O, pH 7.4)
  
  8.0 g, NaCl; 0.4 g KCl, 0.2 g, MgSO$_4$·7H$_2$O; 0.06 g, Na$_2$HPO$_4$·2H$_2$O; 0.06 g, KH$_2$PO$_4$; 2.19 g, NaHCO$_3$ containing 0.5 mM ethanedioxybis(ethylamine)tetraacetate (EGTA) and 2% albumin.

- **Buffer B** (Collagenase buffer, volume to 100 mL ddH$_2$O, pH 7.4)
  
  8.0 g, NaCl; 0.4 g KCl, 0.2 g, MgSO$_4$·7H$_2$O; 0.06 g, Na$_2$HPO$_4$·2H$_2$O; 0.06 g, KH$_2$PO$_4$; 2.19 g, NaHCO$_3$; 0.8 g, collagenase (type II); and 4 mM, Ca$^{2+}$.

- **Buffer C** (Washing buffer, volume to 100 mL ddH$_2$O, pH 7.4)
  
  NaCl, 6.9 g; KCl, 0.36 g; KH$_2$PO$_4$, 0.13 g; MgSO$_4$·7H$_2$O, 0.295 g; CaCl$_2$·7H$_2$O, 0.374 g; NaHCO$_3$; albumin (2%).
Surgical procedure

Male rats of the Sprague-Dawley strain (275 – 300 g) were anesthetized with xylazine: ketamine (1:1) mixture. Heparin (500 units in 0.1 ml) was injected in the inferior venacava. Portal vein was tied with a loose ligature; the mesenteric part of the vein was incised (vena mesenterica superior), cannula was inserted and secured with the ligature. To resume normal shape of the liver the perfusate flow rate was then adjusted. The liver was excised by first removing the ventricle and intestine in one piece followed by removal of the liver from the diaphragm and finally by cutting the dorsal ligaments with the rat in a tilted position. The duration of total procedure was less than 2 min.

Perfusion and washing procedure

The buffer A was allowed to drip out of the cannula before cannulation of the vein to avoid perfusing bubbles into the liver. In case of a good perfusion, liver cleared immediately and completely. After liver removal from the body, it was suspended in the buffer in the reservoir and the cannula was fixed to the horizontal bar. After 4 min of perfusion with buffer A, the plastic rack (with the liver) was removed from the reservoir beaker. By compressing the shunt, the gas chamber was almost emptied of perfusate. The rack was then placed in another beaker containing buffer B. Keeping the constant pressure (10-15 cm H2O) buffer B was recirculated for approximately 8 min. At the end of perfusion, the liver appeared swollen and pale, but no blebs were seen on the surface. The liver was then kept in buffer C (in a wide, low beaker), the capsule was cut open, and the cells were scattered with a pair of scissors and gentle stirring movements, followed by filtration through cotton gauze to remove remaining connective tissue and clumps of cells. The filtrate was collected in a beaker and allowed to settle down the cells to form a loose pellet and the supernatant was removed by aspiration. The volume of the pellet was
estimated with a pipette and the cells (200-300 × 10^6, corresponding to about 3 g of liver) were counted in a hemocytometer.

A continually rotating 50 mL round bottom flask was conditioned with 95% O_2 and 5% CO_2 in a 37°C water bath for 30 min and then isolated hepatocytes (10 mL, 1 × 10^6 cells/mL) were added to flask to mix with in Krebs-Henseleit buffer (pH 7.4).

At various time points, cell viability assays [ROS assay, FOX1 assay (H_2O_2 determination), lipid peroxidation assay, mitochondrial membrane potential assay (% MMP), GSH and GSSG assay, and ATP assay] were carried out using isolated rat hepatocytes (Figure 2.1).

GSH-depleted hepatocytes were obtained by pre-incubating the hepatocytes with 200 µM 1-bromoheptane for 30 min (Khan and O'Brien, 1991). 1-Bromoheptane rapidly conjugates...
hepatocyte GSH without affecting the hepatocyte viability (Figure 2.2). N-acetylcysteine (1 mM), a GSH precursor, was added 30 min prior to the addition of any agents.

Catalase inhibition was achieved by incubating hepatocytes with 3-amino-1,2,4-triazole (3-AT) (20 mM) for 30 minutes prior to adding other chemicals. 3-AT, an irreversible inhibitor of the enzyme catalase markedly reduces the capacity of isolated hepatocytes to metabolize H$_2$O$_2$ (Boutin et al., 1989).

**Figure 2.2.** Depletion of GSH with 1-bromoheptane. 1-Bromoheptane attaches to GSH with the aid of glutathione-s-transferase (GST), forming an irreversible conjugate, heptyl-s-glutathione (Heptyl-S-GSH) [Adapted from Lip (2014), M.Sc. dissertation, University of Toronto with permission].

Cytochrome P450-inhibited hepatocytes were prepared by adding 1-aminobenzotriazole (ABT, 200 µM) to hepatocytes 60 min prior to the addition of other agents (Balani et al., 2002). ABT is a mechanism-based, non-specific inactivator that seems to inactivate all CYP isoenzymes *in vitro* (Balani et al., 2002) or *in vivo* (Mico et al., 1988).

Xanthine oxidase-and aldehyde oxidase-inhibited hepatocytes were obtained by preincubating hepatocytes with 20 µM allopurinol and 20 µM menadione for 30 min,
respectively (Shaw and Jayatilleke, 1990). Aldehyde oxidase was also inhibited using amsacrine (10 μM) (preincubated for 30 min) (Bremnes et al., 1991a).

Fructose (10 mM) (an ATP generator) was added to hepatocytes 30 min prior to the addition of other agents (Wu et al., 1990).

2.4 Oxidative stress inflammation model

A H₂O₂ generating system (Antunes and Cadenas, 2001) was employed by adding 10 mM glucose (G) to the hepatocyte suspension followed by glucose oxidase (GO, 0.5 Unit/mL). This G/GO system continuously supplied H₂O₂ during the 3 hr experimental period, without affecting GSH levels or cell viability (Antunes and Cadenas, 2001; Tafazoli and O’Brien, 2008). MTX was co-incubated with the H₂O₂-generating system and HRP (0.5 μM) in vitro to simulate inflammation in vivo. HRP was preincubated with hepatocytes for 15 min prior to the addition of other agents. Peroxidase activity was inhibited by PTU (6-N-propyl-2-thiouracil, 5 μM) (Lee et al., 1990) by preincubating it with hepatocytes for 15 min prior to the start of the experiment. MTX was also incubated with the hydroxyl-radical generating Fe(II)-mediated Fenton model, which consisted of non-toxic Fe(II) [2 μM Fe(II) and 4 μM 8-hydroxyquinoline (HQ)] with the H₂O₂ generating system (MacAllister et al., 2013a). Desferoxamine (200 μM) was added to chelate Fe (II) and was preincubated with hepatocytes for 30 min prior to the addition of other agents.

The concentrations of enzyme-modulators/antioxidants/H₂O₂ generating system/ROS-scavengers/ATP-generator/Fenton system/Fe (II)-chelator used in the experiments had no significant effect on hepatocyte viability.
2.5 Cell viability

Hepatocyte viability was tested by the trypan blue exclusion test (Moldeus et al., 1978). Hepatocyte viability was assessed at every 60 min during a 3 hr incubation period. Inclusion criteria of cell viability for the experiment were 80 – 90%. The cell suspension was diluted to 100-fold in Krebs-Henseleit buffer containing 0.1 % (w/v) trypan blue for cell counting in a hemocytometer. Within 5 min, 1-2 % of the cells were stained. This test measures the integrity of the plasma membrane since damage to the plasma membrane is the critical step in the sequence of events leading to cell death. Trypan blue is a negatively charged dye and does not interact with the cell unless the membrane is damaged. Living cells do not take up the dye while dead cells do (Moldeus et al., 1978).

2.6 ROS formation assay

Hepatocyte ROS generation was determined using of 2', 7'-dichlorofluorescein diacetate (DCFDA) which can permeate hepatocytes and be deacetylated by intracellular esterases to form non-fluorescent dichlorofluorescin (DCF). Dichlorofluorescin is oxidized by intracellular ROS to form the highly fluorescent dichlorofluorescein (Figure 2.3). ROS formation was assayed by withdrawing 1 mL hepatocyte samples at 30 and 90 mins, which were then centrifuged for 1 min at 5000 x g. The cells were resuspended in Krebs–Henseleit buffer and 1.6 μM DCFDA was added. The cells were incubated at 37°C for 10 min, and the fluorescent intensity of dichlorofluorescein was measured using a SPECTRAmax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) set at 490 nm excitation and 520 nm emission.
wavelengths (McAllister et al., 2013; Possel et al., 1997) and were expressed as FI (fluorescence intensity) units.

**Figure 2.3.** Measurement of ROS generation using 2',7'-dichlorofluorescein diacetate (DCF). DCFD enters the cell and is hydrolyzed to form a non-fluorescent dichlorofluorescin (DCF).

DCF is oxidized by ROS to form a fluorescent dichlorofluorescein (DCF) which effluxes the cell [Adapted from Lip (2014), M.Sc. dissertation, University of Toronto with permission, originally from Gomez et al., 2005].
2.7 Lipid peroxidation measurement

Lipid peroxidation was determined by measuring the amount of TBARS formed during the decomposition of lipid hydroperoxides, mostly formed from malondialdehyde (MDA) with the pink adduct being measured at 532 nm (Figure 2.4). Each test tube containing 1 mL aliquots of hepatocyte suspension (withdrawn at 30 and 90 mins) was treated with 250 μL of trichloroacetic acid (TCA) (70% w/v) and 1 mL of thiobarbituric acid (TBA) (0.8 % w/v). The suspension was then boiled for 20 min in a boiling water batch. The samples were cooled for 5 min and then centrifuged at 5000 x g for 5 min. The supernatant was measured at 532 nm to detect the amount of TBARS formed during the decomposition of lipid hydroperoxides, using a SPECTRAmax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as µM concentration of MDA (156 mM⁻¹cm⁻¹) (Smith et al., 1982; Tafazoli and O’Brien, 2008). TBARS commercial assay kits from Cayman Chemical, Ann Arbor, MI, USA were also used to determine lipid peroxidation according to manufacturer’s instructions.

![Figure 2.4. Formations of MDA-TBA adduct. MDA, malondialdehyde; TBA, thiobarbituric acid (TBARS Assay Kit booklet, Cayman Chemical, Ann Arbor, MI, USA).](image-url)
2.8 Endogenous H$_2$O$_2$ measurement

H$_2$O$_2$ was measured in hepatocytes by adding FOX1 reagent. The FOX1 reagent consisted of 25 mM sulfuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange, and 100 mM sorbitol. 50 μL of hepatocyte suspension (withdrawn at 30 and 90 mins) were added to 950 μL of the FOX1 reagent and incubated for 30 min at room temperature. Samples were then spectrophotometrically analyzed at 560 nm using a SPECTRAmax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). An extinction coefficient 2.35×10$^5$ M$^{-1}$ cm$^{-1}$ was used to measure the concentration of H$_2$O$_2$ (Ou and Wolff, 1996; Tafazoli and O’Brien, 2008), which was expressed as nmoles/10$^6$ cells.

2.9 GSH and GSSG determination

GSH and GSSG in hepatocytes were determined colorimetrically at 30 and 90 mins by commercial kits from Cayman Chemical, Ann Arbor, MI, USA according to the manufacturer’s instructions at 412 nm which utilizes an optimized enzymatic recycling method (Eyer and Podhradský, 1986). The sulfhydryl group of GSH reacts with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, also known as Ellman’s reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) is reduced by glutathione reductase (GR) to recycle the GSH and produce more TNB (Figure 2.5). The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample. GSSG was determined by first derivatizing the
samples with 2-vinyl pyridine (Griffith, 1980). GSH and GSSG values were expressed as nmoles/10⁶ cells.

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**Figure 2.5.** GSH recycling mechanism. GSH, glutathione; GSSG, glutathione disulfide; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid thiobarbituric acid; TNB, 5-thio-2-nitrobenzoic acid; GR, glutathione reductase (Glutathione Assay Kit booklet, Cayman Chemical, Ann Arbor, MI, USA).

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### 2.10 Mitochondrial membrane potential (MMP) assay

MMP was estimated by measuring the uptake of the cationic fluorescent dye, rhodamine 123 into hepatocytes. This assay is based on the fact that rhodamine 123 accumulates selectively in the mitochondria by facilitated diffusion. However, when the mitochondrial potential is decreased, the amount of rhodamine 123 that enters the mitochondria is decreased as there is no facilitated diffusion. Thus the amount of rhodamine 123 in the supernatant is increased and the amount in the pellet is decreased. Aliquots (500 μL) of the cell suspension were separated at 30 and 90 mins from the incubation medium by centrifugation at 5000 x g for 1 min. The cell pellet was resuspended in 2 mL of fresh incubation medium containing 1.5 μM rhodamine 123 and incubated at 37°C in a thermostatic water bath for 10 min with gentle shaking. Hepatocytes were
then separated by centrifugation and the amount of rhodamine 123 remaining in the incubation medium was measured at 490 nm excitation and 520 nm emission wavelengths using a SPECTRAmax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference in fluorescence intensity between control and treated cells and was expressed as % MMP compared to control hepatocytes (Andersson et al., 1987).

2.11 Cellular ATP determination

ATP in hepatocytes was determined colorimetrically by commercial ATP Assay kits from Abcam Inc., Boston, MA, USA according to manufacturer’s instructions at 570 nm using a SPECTRAmax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The method utilizes the phosphorylation of glycerol to generate a product that is easily quantified by absorbance at 570 nm. ATP concentration was expressed as nmoles/10^6 cells.

2.12 Electron resonance spectroscopy (ESR) spin trapping study

ESR spin trapping studies of AZA and 6-MP metabolism by HRP/H2O2 were investigated according to Arvadia and colleagues (2011) in the laboratory of Dr. Arno Siraki (Assistant Professor, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta). ESR spectra were obtained using Elexsys E500 series spectrometer (Bruker Corporation, Billerica, Massachusetts, USA). Instrument parameters were set to the following: frequency: 9.81 GHz, center field = 3497 gauss, microwave power = 20 mW, modulation amplitude = 1 gauss,
modulation frequency = 100 kHz, sweep time = 60 s, number of scans = 6 (6 min total recording time). All reactions were carried out at room temperature in 0.1 M phosphate buffer pH 7.4 containing 10 μM DTPA. Where indicated, reactions contained 5 mM AZA or 5 mM 6-MP, 100 mM DMPO, 5 μM HRP, 500 μM H₂O₂, and 40 μg/mL SOD. The samples were vortexed and transferred from microtubes to a quartz flat cell and the resulting spectrum was recorded.

2.13 Statistical analysis

The SPSS software package (version 14.0, SPSS Inc., Chicago, IL, USA) was used to analyze the data. Values were rounded and were expressed as mean ± standard error of the mean (S.E.M.) from 3 independent experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test to assess significance between control and treatment groups in these experiments. p < 0.05 was considered significant.
3.1 Nitroaromatics (flutamide, nilutamide, nimesulide)

3.1.1 ACMS LC$_{50}$ determination

A concentration and time dependent increase in cytotoxicity, ROS formation, and a decrease in % MMP were observed for FLU (50 – 100 µM), NIL (100 – 500 µM), and NIM (100 – 500 µM) (data not shown) compared to control hepatocytes over a 3 hr incubation period. Incubation of freshly isolated rat hepatocytes for 2 hrs at 37°C with 75 µM FLU, 300 µM NIL, and 300 µM NIM induced an approximate 50% loss in hepatocyte viability as measured by the trypan blue exclusion assay (LC$_{50}$, according to the ACMS technique). FLU was found to be more cytotoxic than NIL and NIM.

3.1.2 Effects of non-toxic H$_2$O$_2$ and peroxidase on nitroaromatic drugs

In the presence of a non-toxic G/GO + HRP with NIM (300 µM), NIL (300 µM), and FLU (75 µM), a 2.1-, 1.5-, and 1.5-fold increase in toxicity at 2 hrs were observed, respectively. In the absence of drugs, none of the inflammatory modulators at the doses used, affected hepatocyte viability significantly (Table 3.1). ROS formation was also increased with all of the nitroaromatic drugs when G/GO + HRP was added.
Table 3.1. Nitroaromatics-induced cytotoxicity and oxidative stress in a hepatocyte oxidative stress inflammation model (MacAllister et al., 2013a).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity (% Trypan blue uptake)</th>
<th>Mitochondrial Membrane Potential (% MMP)</th>
<th>Reactive Oxygen Species Formation (FI units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min</td>
<td>120 min</td>
<td>180 min</td>
</tr>
<tr>
<td>Control hepatocytes</td>
<td>18 ± 1</td>
<td>22 ± 1</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>H$_2$O$_2$-generating system</td>
<td>25 ± 1</td>
<td>26 ± 2</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>HRP+H$_2$O$_2$-generating system</td>
<td>22 ± 2</td>
<td>23 ± 2</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>300 µM Nimesulide + H$_2$O$_2$-generating system</td>
<td>39 ± 2$^a$</td>
<td>51 ± 2$^a$</td>
<td>62 ± 4$^a$</td>
</tr>
<tr>
<td>+ 0.5 µM HRP</td>
<td>63 ± 3$^{abcd}$</td>
<td>78 ± 3$^{abcd}$</td>
<td>84 ± 2$^{abcd}$</td>
</tr>
<tr>
<td>300 µM Nilutamide + H$_2$O$_2$-generating system</td>
<td>39 ± 3$^a$</td>
<td>57 ± 3$^a$</td>
<td>62 ± 3$^a$</td>
</tr>
<tr>
<td>+ 0.5µM HRP</td>
<td>46 ± 1$^{ab}$</td>
<td>66 ± 1$^{ab}$</td>
<td>71 ± 5$^{ab}$</td>
</tr>
<tr>
<td>75 µM Flutamide + H$_2$O$_2$-generating system</td>
<td>37 ± 3$^a$</td>
<td>50 ± 1$^a$</td>
<td>67 ± 4$^a$</td>
</tr>
<tr>
<td>+ 0.5 µM HRP</td>
<td>43 ± 3$^{ab}$</td>
<td>60 ± 4$^{abf}$</td>
<td>71 ± 6$^{ab}$</td>
</tr>
</tbody>
</table>
| Means ± S.E.M. for three separate experiments are given (n = 3). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. HRP, horseradish peroxidase. *Due to oxidation of 2'-7'-dichlorofluorescin by peroxidase (Rota et al. 1991) $^a$significant as compared to control hepatocytes (p<0.05). $^b$significant as compared to H$_2$O$_2$-generating system (p<0.05). $^c$significant as compared to HRP + H$_2$O$_2$-generating system (p<0.05). $^d$significant as compared to 300 µM nimesulide (p<0.05). $^e$significant as compared to 300 µM nilutamide (p<0.05). $^f$significant as compared to 75 µM flutamide (p<0.05).
A significant decrease in % MMP was observed ($p<0.05$) with a ranking of NIL > FLU > NIM compared to control hepatocytes. A significant decrease of % MMP was also observed for NIM, NIL and FLU when incubated with G/GO + HRP ($p<0.05$) compared to respective drug controls (Table 3.1).

### 3.1.3 Effects of non-toxic H$_2$O$_2$ and peroxidase or Fe(II) on FLU-induced cytotoxicity

Addition of a non-toxic H$_2$O$_2$ generating system (to simulate inflammation in vitro as described in the introduction section) with HRP (0.5 µM) caused a significant increase in FLU-induced cytotoxicity that was significantly decreased by the addition of PTU (5 µM) (a peroxidase inhibitor) (Figure 3.1). Furthermore, with the addition of a H$_2$O$_2$ generating system and peroxidase caused a significant increase in lipid peroxidation (MDA equivalents, µM) and a decrease in % MMP (Table 3.2) and GSH levels (Figure 3.2) compared to control hepatocytes (Table 3.2). Similar results were obtained when we used the Fenton model (a non-toxic H$_2$O$_2$ generating system and Fe(II) that generates hydroxyl radicals). An iron chelator, desferoxamine (200 µM, pre-incubated for 30 min) delayed FLU-induced cytotoxicity likely by inhibiting the Fenton reaction (Figure 3.1). All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used (data not shown).
Figure 3.1. Effects of non-toxic H$_2$O$_2$ and peroxidase or Fe(II)-mediated Fenton model on FLU-induced cytotoxicity. Data are presented as Mean ± S.E.M. (n = 3). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. FLU, Flutamide; HRP, horseradish peroxidase; G, glucose; GO, glucose oxidase; PTU, 6-N-propyl thiouracil.

All data points were significant compared to control hepatocytes (p<0.05).

*Significant compared to 75 µM FLU (p<0.05);

#Significant compared to 75 µM FLU + HRP (0.5 µM) (p<0.05);

¶Significant compared to 75 µM FLU + Fe(II)-mediated Fenton model (p<0.05).
3.1.4. Protection against FLU-induced cytotoxicity in rat hepatocytes

A significant decrease in GSH levels was observed when FLU was administered with the H$_2$O$_2$ generating system and peroxidase to rat hepatocytes. This was prevented by 1 mM N-acetylcysteine (a GSH precursor). Potent antioxidants, Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (1 mM) and resveratrol (3,5,4'-trihydroxy-trans-stilbene) (50 µM), and DPPD (N,N'-diphenyl-p-phenylenediamine) (2 µM) significantly decreased FLU-induced cytotoxicity, ROS and LPO formation, and increased % MMP (Table 3.2) and GSH levels (Figure 3.2) compared to control hepatocytes. Significant protection against FLU-induced cytotoxicity with the H$_2$O$_2$ generating system and peroxidase was also achieved by a ROS scavenger, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) (200 µM) (Table 3.2). All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used.
Figure 3.2 Effects of FLU on GSH and GSSG levels (nmoles/10⁶ cells, measured at 30 min).

Refer to the materials and methods section for a description of the experiments performed and experimental conditions.

*Significant compared to control (only hepatocytes).

#Significant compared to 75 µM FLU ($p<0.05$).

¶Significant compared to 75 µM FLU + H₂O₂ generating system (G+GO) + PTU (5 µM) ($p<0.05$).
Table 3.2. Protection against FLU-induced cytotoxicity using an oxidative stress inflammation system in isolated rat hepatocytes.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (Trypan blue uptake, %)</th>
<th>Lipid Peroxidation (µM, MDA)</th>
<th>Mitochondrial Membrane Potential (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation time</td>
<td>60 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>19 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>+ H₂O₂ generating system + peroxidase</td>
<td></td>
<td>21 ± 1</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>+ 75 µM FLU</td>
<td></td>
<td>30 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 75 µM FLU + H₂O₂ generating system + peroxidase</td>
<td>53 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>72 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>88 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 5 µM PTU</td>
<td></td>
<td>41 ± 2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>56 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 1 mM NAC</td>
<td></td>
<td>33 ± 2&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>53 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 1 mM Trolox</td>
<td></td>
<td>30 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>47 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 50 µM Resveratrol</td>
<td></td>
<td>31 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>50 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM TEMPOL</td>
<td></td>
<td>31 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>52 ± 2&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 2 µM DPPD</td>
<td></td>
<td>29 ± 2&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>53 ± 1&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± S.E.M. (n = 3). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. PTU, 6-N-propyl-2-thiouracil; NAC, N-acetylcysteine; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; resveratrol (3,5,4′-trihydroxy-trans-stilbene); TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; DPPD (N,N′-diphenyl-p-phenylenediamine).

<sup>a</sup>Significant compared to control (only hepatocytes) (p<0.05).

<sup>b</sup>Significant compared to 75 µM FLU (p<0.05).

<sup>c</sup>Significant compared to 75 µM FLU + H₂O₂ generating system and peroxidase (p<0.05).
3.2 Methotrexate

3.2.1 MTX-induced cytotoxicity

A concentration and time dependent increase in cytotoxicity (Figure 3.3), ROS formation, and a decrease in % MMP were observed for MTX (50 – 500 µM) compared to control hepatocytes over a 3 hr of incubation period. Incubation of freshly isolated rat hepatocytes for 2 hrs at 37°C with 300 µM MTX induced an approximate 50% loss in hepatocyte viability as measured by the trypan blue exclusion assay (LC50, according to the ACMS technique).

![Figure 3.3. Concentration-response curve of MTX towards isolated rat hepatocytes.](image)

*significant compared to control hepatocytes ($p<0.05$).
3.2.2. Involvement of GSH in MTX-induced cytotoxicity

A significant increase in MTX-induced cytotoxicity, ROS formation, and a significant decrease in % MMP were observed when GSH was depleted by using 1-bromoheptane (200 µM) in hepatocytes whereas addition of 1 mM N-acetylcysteine significantly delayed (cytoprotective at 60 min and 120 min, not at 180 min) MTX-induced cytotoxicity (Figure 3.4), ROS formation and increased % MMP (measured at 30 min) (p<0.05) (Table 3.3). N-acetylcysteine was added 30 min prior to the addition of MTX or other agents.

MTX treatment (300 µM) significantly depleted GSH and increased GSSG formation (Figure 3.5) is rat hepatocytes whereas N-acetylcysteine significantly increased GSH. However, N-acetylcysteine (1 mM) did not restore GSH values to control levels when compared to MTX-treated hepatocytes (Figure 3.4). All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used (data not shown).
Figure 3.4. Involvement of GSH, catalase and aldehyde oxidase in MTX-induced cytotoxicity in isolated rat hepatocytes. Refer to the materials and methods section for a description of the experiments performed and experimental conditions. GSH-depleted hepatocytes were obtained by pre-incubating the hepatocytes with 1-bromoheptane (200 µM) for 30 min. N-acetylcysteine was added 30 min prior to the addition of MTX or other agents. Catalase inhibition was achieved by incubating hepatocytes with 3-amino-1,2,4-triazole (20 mM) for 30 minutes and aldehyde oxidase-inhibited hepatocytes were obtained by preincubating hepatocytes with 20 µM menadione for 30 min prior to adding other chemicals. All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used (data not shown).

All data points were significant compared to control hepatocytes ($p<0.05$).

*Significant compared to 300 µM MTX ($p<0.05$).
Table 3.3. MTX-induced ROS formation and % MMP in isolated rat hepatocytes with various enzyme modulators.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Reactive Oxygen Species Formation (FI Unit)</th>
<th>Mitochondrial Membrane Potential (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time</td>
<td>30 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>+ MTX (300 µM)</td>
<td>150 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ GSH-depleted hepatocytes</td>
<td>188 ± 6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>78 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ N-Acetylcysteine (1 mM)</td>
<td>133 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>94 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Catalase-inhibited hepatocytes</td>
<td>258 ± 5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>73 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Catalase (0.25 mg/mL)</td>
<td>131 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>92 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Aldehyde oxidase-inhibited hepatocytes</td>
<td>135 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>91 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± S.E.M. (n = 3). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. GSH-depleted hepatocytes were obtained by pre-incubating the hepatocytes with 1-bromoheptane (200 µM) for 30 min. N-acetylcysteine was added 30 min prior to the addition of MTX or other agents. Catalase inhibition was achieved by incubating hepatocytes with 3-amino-1,2,4-triazole (20 mM) 30 min and aldehyde oxidase-inhibited hepatocytes were obtained by preincubating hepatocytes with 20 µM menadione for 30 min prior to adding other chemicals. All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used (data not shown).

<sup>a</sup>Significant compared to control (only hepatocytes) (p<0.05).

<sup>b</sup>Significant compared to 300 µM MTX (p<0.05).
Figure 3.5. Effects of MTX on GSH and GSSG levels (nmoles/10^6 cells, measured at 30 min).

Refer to the materials and methods section for a description of the experiments performed and experimental conditions.

*Significant compared to control (only hepatocytes) \((p<0.05)\).

#Significant compared to 300 \(\mu\)M MTX \((p<0.05)\).
3.2.3 Involvement of catalase in MTX-induced cytotoxicity

In this study, MTX (300 µM) treatment significantly increased H₂O₂ levels compared to control rat hepatocytes (Figure 3.6). When we inhibited catalase in hepatocytes using 3-AT (20 mM) (pre-incubated for 30 min), a significant increase in MTX-induced cytotoxicity (Figure 3.3), ROS formation, and a significant decrease in % MMP (Table 3.3) was observed. Direct addition of catalase (0.25 mg/mL) significantly decreased MTX-induced cytotoxicity at 60 min but was not protective at 120 min and 180 min (Figure 3.4). Addition of catalase to MTX-treated hepatocytes also significantly decreased ROS formation and increased % MMP (Table 3.3) and H₂O₂ levels (Figure 3.6).
Figure 3.6. MTX-induced H$_2$O$_2$ generation (measured at 30 min.). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. Catalase inhibition was achieved by incubating hepatocytes with 3-AT (3-amino-1,2,4-triazole) (20 mM) for 30 min prior to adding other chemicals. All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used (data not shown).

*Significant compared to control (only hepatocytes) ($p<0.05$).

#Significant compared to 300 µM MTX ($p<0.05$).

3.2.4 Cytochrome P450 and xanthine oxidase inhibition

Xanthine oxidase inhibition (by pre-incubating hepatocytes with 20 µM allopurinol for 30 min) and cytochrome P450 inhibition (by pre-incubating hepatocytes with 200 µM 1-ABT for 60 min)
had no significant effect on MTX-induced cytotoxicity and ROS formation ($p>0.05$) (data not shown).

### 3.2.5 Involvement of aldehyde oxidase in MTX-induced cytotoxicity

Aldehyde oxidase inhibition using 20 µM menadione (pre-incubated for 30 min) resulted in a significant decrease in MTX-induced cytotoxicity at 60 min and 120 min (Fig. 3.4). Menadione also significantly decreased ROS and H$_2$O$_2$ formation and increased % MMP (Table 3.3). A similar result was also found with another aldehyde oxidase inhibitor, amsacrine (data not shown).

### 3.2.6 MTX-induced lipid peroxidation and mitochondrial toxicity

MTX (300 µM) had no significant effect on lipid peroxidation (as measured by TBARS assay). MTX (300 µM) significantly decreased % MMP (Table 3.3) and ATP levels at 30 min (Figure 3.7) in rat hepatocytes whereas addition of 10 mM fructose (pre-incubated for 15 min) significantly increased % MMP (Table 3.3), ATP (Figure 3.7), and delayed MTX-induced cytotoxicity (Table 3.4).
Figure 3.7. MTX-induced ATP depletion (measured at 30 min.). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. Fructose (10 mM) was added 30 min prior to adding other chemicals.

*Significant compared to control (only hepatocytes) ($p<0.05$).

#Significant compared to 300 µM MTX ($p<0.05$).

3.2.7 Effects of non-toxic H$_2$O$_2$ and peroxidase or Fe(II) on MTX-induced cytotoxicity

Addition of a non-toxic H$_2$O$_2$ generating system with HRP (0.5 µM) caused a significant increase in MTX-induced cytotoxicity that was significantly decreased by the addition of PTU (5 µM) (a peroxidase inhibitor) (Fig. 3.8). Furthermore, the addition of a H$_2$O$_2$ generating system and peroxidase caused a significant increase in lipid peroxidation, ROS formation and a decrease in % MMP (data not shown). Similar results were obtained when we used the Fenton model (a non-toxic H$_2$O$_2$ generating system and Fe(II) that generates hydroxyl radicals). An iron chelator, desferoxamine (200 µM, pre-incubated for 30 min) delayed MTX-induced cytotoxicity likely by inhibiting the Fenton reaction by chelating Fe(II) (Figure 3.8).
Figure 3.8. Effects of non-toxic H$_2$O$_2$ and peroxidase or Fe(II)-mediated Fenton model on MTX-induced cytotoxicity. A H$_2$O$_2$ generating system was employed by adding 10 mM glucose to the hepatocyte suspension followed by glucose oxidase (0.5 unit/mL). MTX was co-incubated with the H$_2$O$_2$-generating system and horseradish peroxidase (HRP) (0.5 μM). HRP was preincubated with hepatocytes for 15 min prior to the addition of the other agents. Peroxidase activity was inhibited by 6-N-propyl-2-thiouracil (PTU, 5 μM) by preincubating them with hepatocytes for 15 min prior to the start of the experiment. MTX was also incubated with a non-toxic Fe(II) [2 μM Fe(II)/4 μM 8-hydroxyquinoline] with a H$_2$O$_2$ generating system (G + GO). Desferoxamine (200 μM) was preincubated with hepatocytes for 30 min prior to the addition of other agents. All data points were significant compared to control hepatocytes ($p<0.05$).

*Significant compared to 300 μM MTX ($p<0.05$); #Significant compared to 300 μM MTX + HRP (0.5 μM) ($p<0.05$); ¶Significant compared to 300 μM MTX + Fe(II)-mediated Fenton model ($p<0.05$).
3.2.8 Protection against MTX-induced cytotoxicity using ROS scavengers and antioxidants

Significant cytoprotection against MTX-induced cytotoxicity in isolated rat hepatocytes was achieved by the ROS scavenger and superoxide dismutase mimetic, TEMPOL (200 µM) (Table 3.4). Antioxidants such as Trolox (1 mM), mesna (2-mercaptoethanesulfonate, 1 mM), and resveratrol (50 µM) also significantly protected hepatocytes which decreased ROS and H₂O₂ formation, and significantly increased % MMP (Table 3.4).
Table 3.4. Protection against MTX-induced cytotoxicity and oxidative stress in isolated rat hepatocytes with various antioxidants, radical scavengers and an ATP generator.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (%) (Trypan blue uptake)</th>
<th>ROS (FI unit)</th>
<th>% MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min 120 min 180 min 30 min 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19 ± 1 22 ± 2 25 ± 2 101 ± 3 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ MTX (300 µM)</td>
<td>35 ± 2&lt;sup&gt;a&lt;/sup&gt; 51 ± 2&lt;sup&gt;a&lt;/sup&gt; 64 ± 3&lt;sup&gt;a&lt;/sup&gt; 148 ± 2&lt;sup&gt;a&lt;/sup&gt; 86 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Trolox (1 mM)</td>
<td>28 ± 2&lt;sup&gt;a,b&lt;/sup&gt; 37 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 50 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 125 ± 1&lt;sup&gt;a,b&lt;/sup&gt; 92 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Mesna (1 mM)</td>
<td>20 ± 1&lt;sup&gt;b&lt;/sup&gt; 25 ± 2&lt;sup&gt;b&lt;/sup&gt; 38 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 115 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 96 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ TEMPOL (200 µM)</td>
<td>24 ± 2 35 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 45 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 128 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 93 ± 1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Resveratrol (50 µM)</td>
<td>21 ± 2&lt;sup&gt;b&lt;/sup&gt; 30 ± 1&lt;sup&gt;a,b&lt;/sup&gt; 43 ± 2&lt;sup&gt;a,b&lt;/sup&gt; 118 ± 4&lt;sup&gt;a,b&lt;/sup&gt; 92 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Fructose (10 mM)</td>
<td>27 ± 3&lt;sup&gt;a,b&lt;/sup&gt; 41 ± 2&lt;sup&gt;a,b&lt;/sup&gt; 53 ± 2&lt;sup&gt;a,b&lt;/sup&gt; 145 ± 4&lt;sup&gt;a&lt;/sup&gt; 98 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as Mean ± S.E.M. (n = 3). Fructose (10 mM) was added 30 min prior to adding other chemicals. All modulating agents were non-cytotoxic compared to control hepatocytes at the concentrations used (data not shown). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; mesna, 2-mercaptoethanesulfonate; resveratrol (3,5,4'-trihydroxy-trans-stilbene).

<sup>a</sup>Significant compared to control (only hepatocytes).

<sup>b</sup>Significant compared to 300 µM MTX (p<0.05).
3.3 Thiopurines (azathiopurine, 6-mercaptopurine)

3.3.1 Azathioprine

3.3.1.1 AZA-induced cytotoxicity

A concentration and time dependent increase in cytotoxicity and reactive oxygen species (ROS) formation and a decrease in % MMP was observed with AZA (100 – 500 µM) compared to control hepatocytes (Figure 3.9) incubated for 3 hrs.

![Concentration-response curve of AZA towards isolated rat hepatocytes.](image)

*significant compared to control hepatocytes ($p<0.05$) (Maruf et al. 2014c).
3.3.1.2 Functions of GSH and xanthine oxidase in AZA-induced hepatotoxicity

GSH and xanthine oxidase dependence of AZA in isolated rat hepatocytes are presented in Figure 3.10. AZA treatment (400 µM) significantly depleted hepatocyte GSH (Figure 3.11). A significant increase in AZA-induced cytotoxicity and ROS formation were observed when hepatocyte GSH was depleted by using 1-bromoheptane (200 µM) whereas addition of N-acetylcysteine (1 mM), a cysteine precursor which generates GSH, prevented AZA-induced cytotoxicity, ROS and H$_2$O$_2$ generation, and increased % MMP (Table 3.5) and hepatocyte GSH.

When we inhibited xanthine oxidase by preincubating hepatocytes with 20 µM allopurinol, AZA-induced cytotoxicity was significantly prevented (Figure 3.10). A significant decrease in ROS and H$_2$O$_2$ formation and an increase in % MMP were observed compared to control hepatocytes with AZA-treated and xanthine oxidase-inhibited hepatocytes (Table 3.5). In this study, the combined addition of N-acetylcysteine (1 mM) and allopurinol (20 µM) showed nearly complete protection against AZA-induced cytotoxicity in rat hepatocytes.

3.3.1.3 Effects of aldehyde oxidase and cytochrome P450 inhibition on AZA-induced cytotoxicity

Aldehyde oxidase inhibition (by 20 µM menadione) and cytochrome P450 inhibition (by 200 µM 1-ABT) had no significant effect on AZA-induced cytotoxicity in rat hepatocytes (data not shown).
Figure 3.10. GSH and xanthine oxidase dependence of AZA towards rat hepatocytes. Refer to the materials and methods section for a description of the experiments performed and experimental conditions. GSH-depleted hepatocytes were obtained by pre-incubating the hepatocytes with 1-bromoheptane (200 µM) for 30 min. NAC was added 30 min prior to the addition of AZA or other agents. NAC, N-acetylcysteine.

\( ^a \)Significant compared to control hepatocytes \((p<0.05)\).

\( ^b \)Significant compared to AZA \((400 \, \mu\text{M}) \,(p<0.05)\) (Maruf et al. 2014c).
Table 3.5. AZA-induced oxidative stress with GSH depletion and protection with a GSH precursor, a xanthine oxidase inhibitor, various antioxidants, and a radical scavenger.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ROS (FI Unit)</th>
<th>MMP (%)</th>
<th>H$_2$O$_2$ (nmoles/10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>30 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Control</td>
<td>102 ± 1</td>
<td>100</td>
<td>6.34 ± 0.07</td>
</tr>
<tr>
<td>+ 400 µM AZA</td>
<td>139 ± 3$^a$</td>
<td>86 ± 1$^a$</td>
<td>8.11 ± 0.08$^a$</td>
</tr>
<tr>
<td>+ GSH depleted hepatocytes</td>
<td>174 ± 5$^{a,b}$</td>
<td>75 ± 1$^{a,b}$</td>
<td>9.21 ± 0.13$^{a,b}$</td>
</tr>
<tr>
<td>+ 1 mM N-acetylcysteine</td>
<td>124 ± 3$^{a,b}$</td>
<td>89 ± 1$^{a}$</td>
<td>6.97 ± 0.04$^{a,b}$</td>
</tr>
<tr>
<td>+ 20 µM Allopurinol</td>
<td>132 ± 3$^a$</td>
<td>92 ± 1$^{a,b}$</td>
<td>7.75 ± 0.19$^a$</td>
</tr>
<tr>
<td>+ 1 mM NAC + 20 µM Allopurinol</td>
<td>107 ± 1$^b$</td>
<td>97 ± 2$^b$</td>
<td>6.54 ± 0.14$^b$</td>
</tr>
<tr>
<td>+ 1 mM Mesna</td>
<td>121 ± 3$^{a,b}$</td>
<td>93 ± 2$^{a,b}$</td>
<td>7.10 ± 0.18$^{a,b}$</td>
</tr>
<tr>
<td>+ 1 mM Trolox</td>
<td>113 ± 3$^b$</td>
<td>94 ± 2$^{a,b}$</td>
<td>7.12 ± 0.02$^{a,b}$</td>
</tr>
<tr>
<td>+ 200 µM TEMPOl</td>
<td>123 ± 4$^{a,b}$</td>
<td>93 ± 1$^{a,b}$</td>
<td>7.46 ± 0.18$^{a,b}$</td>
</tr>
<tr>
<td>+ 2 µM DPPD</td>
<td>124 ± 2$^{a,b}$</td>
<td>92 ± 2$^{a,b}$</td>
<td>7.26 ± 0.06$^{a,b}$</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. (n = 3). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. FI, fluorescence intensity; Mesna, 2-mercaptoethanesulfonate; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TEMPOl, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; DPPD, N,N'-diphenyl-p-phenylenediamine.

$^a$Significant compared to control (only hepatocytes) (p<0.05).

$^b$Significant compared to 400 µM AZA (p<0.05).
**Figure 3.11.** Effects of AZA on GSH and GSSG levels (nmoles/10⁶ cells, measured at 30 min).

Refer to the materials and methods section for a description of the experiments performed and experimental conditions.

*Significant compared to control (only hepatocytes) ($p<0.05$).

#Significant compared to 400 µM AZA ($p<0.05$).
3.3.1.4 Protection against AZA-induced cytotoxicity using ROS scavengers and antioxidants

A significant decrease in AZA-induced cytotoxicity (Table 3.6) and ROS formation (Table 3.5) in isolated rat hepatocytes was achieved by the ROS scavenger and the superoxide dismutase mimetic TEMPOL (200 µM). Potent antioxidants like Trolox (1 mM), DPPD (2 µM), and mesna (1 mM), also significantly decreased AZA-induced cytotoxicity (Table 3.6), ROS and H₂O₂ formation and increased % MMP (Table 3.5).
Table 3.6. Effects of a ROS scavenger and various antioxidants on AZA-induced cytotoxicity in isolated rat hepatocytes (Maruf et al. 2014c).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (trypan blue uptake) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min</td>
</tr>
<tr>
<td>Control hepatocytes</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>+ 400 µM AZA</td>
<td>31 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 1 mM Mesna</td>
<td>23 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 1 mM Trolox</td>
<td>24 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM TEMPOL</td>
<td>24 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 2 µM DPPD</td>
<td>26 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. (n = 3). Refer to the materials and methods section for a description of the experiments performed and experimental conditions. Mesna, 2-mercaptoethanesulfonate; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; DPPD, N,N'-diphenyl-p-phenylenediamine.

<sup>a</sup>Significant compared to control (only hepatocytes) (p<0.05).

<sup>b</sup>Significant compared to 400 µM AZA (p<0.05).
3.3.2 6-Mercaptopurine

3.3.2.1 6-MP-induced cytotoxicity

A concentration and time-dependent increase in cytotoxicity was observed for 6-MP compared to control hepatocytes (data not shown). Incubation of isolated rat hepatocytes for 2 hr with 1 mM 6-MP induced an approximate 50% loss in hepatocyte viability as measured by the trypan blue exclusion assay (LC50, according to ACMS).

3.3.2.2 Effects of non-toxic H2O2 and peroxidase on 6-MP-induced cytotoxicity

Addition of a non-toxic concentration of H2O2 generated with peroxidase caused a significant increase in 6-MP cytotoxicity \( (p<0.05) \) which was reversed by the addition of PTU (5 µM) (Table 3.7). Furthermore, the addition of a H2O2 generating system and peroxidase caused a significant increase in lipid peroxidation, ROS and a decrease in % MMP (Table 3.7) and GSH levels (Figure 3.12) compared to control hepatocytes. A significant decrease in GSH levels was observed when 6-MP was administered with the H2O2 generating system and peroxidase to rat hepatocytes. This was prevented by 1 mM \( N \)-acetylcysteine (a GSH precursor).
3.3.2.3 Protection against 6-MP-induced cytotoxicity in rat hepatocytes

Significant protection against 6-MP-induced cytotoxicity with the \( \text{H}_2\text{O}_2 \) generating system and peroxidase was achieved by a ROS scavenger, TEMPO (200 \( \mu \text{M} \)) and an antioxidant, DPPD (2 \( \mu \text{M} \)). TEMPO and DPPD also significantly increased % MMP and % GSH compared to 6-MP treated hepatocytes with the \( \text{H}_2\text{O}_2 \) generating system and peroxidase (Table 3.7).

Table 3.7. Protection against 6-MP-induced cytotoxicity in rat hepatocytes.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (Trypan blue uptake, %)</th>
<th>MMP (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Control</td>
<td>18 ± 1</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>+ H(_2)O(_2) generating system + peroxidase</td>
<td>21 ± 1</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>+ 1 mM 6-MP</td>
<td>30 ± 3(^a)</td>
<td>49 ± 3(^a)</td>
</tr>
<tr>
<td>+ 1 mM 6-MP + H(_2)O(_2) generating system + peroxidase</td>
<td>53 ± 3(^{a,b})</td>
<td>72 ± 2(^{a,b})</td>
</tr>
<tr>
<td></td>
<td>+ 5 ( \mu \text{M} ) PTU</td>
<td>41 ± 3(^{a,c})</td>
</tr>
<tr>
<td></td>
<td>+ 1 mM NAC</td>
<td>33 ± 2(^{a,c})</td>
</tr>
<tr>
<td></td>
<td>+ 200 ( \mu \text{M} ) TEMPOLO</td>
<td>31 ± 2(^{a,c})</td>
</tr>
<tr>
<td></td>
<td>+ 2 ( \mu \text{M} ) DPPD</td>
<td>29 ± 4(^{a,c})</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± S.E.M. Refer to the materials and methods section for a description of the experiments performed and experimental conditions. PTU, 6-\( N \)-propyl thiouracil; NAC, \( N \)-acetylcysteine; TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; DPPD, \( N,N' \)-diphenyl-\( p \)-phenylenediamine.

\(^a\)Significant compared to control (only hepatocytes) (\( p < 0.05 \)).

\(^b\)Significant compared to 1 mM 6-MP (\( p < 0.05 \)).

\(^c\)Significant compared to 1 mM 6-MP + \( \text{H}_2\text{O}_2 \) generating system and peroxidase (\( p < 0.05 \)).
Figure 3.12. Effects of 6-MP on GSH and GSSG levels (nmoles/10^6 cells, measured at 30 min).

Refer to the materials and methods section for a description of the experiments performed and experimental conditions.

*Significant compared to control (only hepatocytes).

#Significant compared to 1 mM 6-MP ($p<0.05$).

¶Significant compared to 1 mM 6-MP + H₂O₂ generating system (G+GO) + PTU (5 µM) ($p<0.05$).
3.3.2.4 Electron spin resonance spectrometry spin trapping

Electron spin resonance (ESR) spectrometry spin trapping studies of 6-MP metabolism by HRP/H2O2 were also conducted. A mixture of two radicals were trapped (A) using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) which were previously reported (Moore et al., 1994) as purine-6-thiyl (PS•) and superoxide radicals in a photooxidation system containing 6-MP with superoxide dismutase. In the absence of H2O2 (C), HRP (D), and DMPO, HRP, and H2O2 (E), the spectrum in A was not observed. When SOD was added to the mixture, a more intense spectrum of 6-MP thiyl radical was observed. When the reaction was recorded after an additional 6 minutes (F), only one spectrum remained which corresponded to DMPO-OH (B), (Figure 3.13).
Figure 3.13. ESR spectrometry spin trapping studies of 6-MP metabolism by HRP/H₂O₂.
An individual’s sensitivity to xenobiotics may be increased by the presence of an inflammatory response. Numerous studies have shown that a modest inflammatory response enhanced tissue susceptibility to drugs/xenobiotics (reviewed in Roth et al., 1997). This raises the possibility that the presence or absence of inflammation is another susceptibility factor for drug toxicity in humans. This knowledge could allow identification of individuals who are susceptible and provide a better understanding of the confluence of events required for this type of adverse response (Tafazoli and O’Brien, 2008). Inflammatory episodes are common in people and animals and are precipitated by numerous stimuli such as bacteria, viruses, and exposure to toxins produced by microorganisms. Moreover, episodes of inflammation can be precipitated by the mammalian gastrointestinal tract. In particular, endotoxin e.g. LPS released from gram-negative bacteria can translocate across the intestinal mucosa into portal venous circulation (Roth et al., 1997). Before drug-induced liver injury occurs in vivo, an inflammatory response usually occurs and cells other than hepatocytes (e.g., Kupffer cells, macrophages) become activated. Immune cells (e.g., neutrophils and macrophages) also infiltrate the liver. Therefore, it was hypothesized that inflammatory episodes during drug therapy decreased the threshold for drug toxicity and, thereby markedly increased the individual’s susceptibility to some drugs/xenobiotics. Kupffer cells and resident liver macrophages normally play a role in protecting hepatocytes from xenobiotics by phagocytosing incoming particles and releasing cytoprotective cytokines (Roberts et al., 2007). Whilst there is little peroxidase activity in hepatocytes, MPO is located in Kupffer cells that are resident macrophages of the human and rodent liver (Brown et al., 2001). Furthermore, neutrophil infiltration of the liver in response to
inflammation can result in a 50 to 100-fold increase in hepatic MPO activity (Kato et al., 2000). For these reasons, peroxidase activity is a useful marker for measuring neutrophil/macrophage infiltration as well as the hepatic inflammatory response. Eosinophil infiltration (e.g., following a parasite infection) can also cause a marked increase in liver eosinophil peroxidase activity (Gharib et al., 1999). During the inflammatory response, \( \text{H}_2\text{O}_2 \) was also formed by activation of the NADPH oxidase in the infiltrated cells. It is therefore reasonable to suggest that the large increase in drug liver susceptibility could also be attributed to peroxidase catalyzed drug oxidation to form reactive pro-oxidant radicals that could also be toxic to hepatocytes.

The present study aimed to validate an in vitro model to identify compounds that increase hepatocyte susceptibility to drug/xenobiotic-induced toxicity. The in vitro inflammatory model includes subjecting freshly isolated rat hepatocytes to a low non-toxic flow of a \( \text{H}_2\text{O}_2 \)-generating system using G/GO and supplementing it with either peroxidase or Fe(II) (MacAllister et al., 2013a). HRP/\( \text{H}_2\text{O}_2 \) was used to effect in situ activation of drugs and to simulate MPO. Although HRP and MPO are not homologous in structure, the catalytically active amino acid residues are positioned in a similar manner (Welinder, 1985) and the metabolites produced are qualitatively similar (Eastmond et al., 1986). Furthermore, HRP is a glycoprotein that is believed to be taken up by fluid-phase endocytosis by hepatocytes (Scharschmidt et al., 1986; Straus, 1981). PTU was used as a peroxidase inhibitor in this study as evidence for the involvement of HRP/\( \text{H}_2\text{O}_2 \) catalyzed formation of drug pro-oxidant radicals. PTU inhibits HRP in a noncompetitive form (Zatón and Ochoa de Aspuru, 1995). Phagocytes generate \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) and their interaction results in an Fe(II)-catalyzed reaction to form \(^\cdot\text{OH} \). Desferoxamine chelates Fe(II) in a catalytically inactive form, and thus inhibition by desferoxamine has been employed as evidence for the involvement of hydroxyl radicals generated by the Fenton reaction (Klebanoff and
Waltersdorph, 1988). Hydroxyl radical scavengers such as mannitol prevented hepatocyte cytotoxicity (Tafazoli et al., 2008). Previously this laboratory used a nontoxic H2O2 generating system with or without HRP to simulate inflammation in vivo. Recent examples from this laboratory include chlorpromazine (MacAllister et al., 2013b), methanol and formaldehyde (MacAllister et al., 2011), polychlorinated biphenyls (Chan et al., 2010), amodiaquine (Tafazoli and O’Brien, 2009), isoniazid (Tafazoli et al., 2008), hydralazine (Tafazoli and O’Brien, 2008). The system has recently been modified by adding Fe(II) to simulate the Fenton reaction that may occur during an in vivo inflammation (MacAllister et al., 2013a).

4.1 Nitroaromatics (flutamide, nilutamide, nimesulide)

Drugs containing a nitroaromatic moiety (FLU, NIL, NIM) have been associated with organ–selective toxicity, including rare cases of idiosyncratic hepatotoxicity. Nitroaromatic drugs are mostly metabolically activated by various N-reductases to form reactive intermediates that reduce oxygen to superoxide radicals, which cause oxidative stress. At low oxygen concentrations, reduction to reactive nitrosoarylamine and hydroxylamine intermediates occurs which also cause oxidative stress (Boelsterli et al., 2006). Incubation of freshly isolated rat hepatocytes for 2 hrs at 37°C with 300 µM NIM, 300 µM NIL, and 75 µM FLU induced an approximate 50% loss in hepatocyte viability as measured by the trypan blue exclusion assay (LC50, according to ACMS). All of the tested drugs significantly increased cytotoxicity, ROS formation, and decreased % MMP compared to controls (p<0.05) (Table-3.1). This laboratory uses the LC50 to investigate potential cytotoxic mechanisms of drug or xenobiotic under investigation. Although this in vitro study is limited for use at higher concentrations of the drug,
the ACMS techniques assume that the drug metabolic/toxic pathways at cytotoxic drug concentrations *in vitro* at 2 hrs are similar to those that occur *in vivo* in 24 – 36 hrs i.e. high dose/short time (*in vitro*) exposure simulates low dose/long time (*in vivo*) exposure (O’Brien and Siraki, 2005). With 24 halobenzenes, it was found that the relative lethal concentrations required to cause 50% cytotoxicity in 2 hrs at 37°C *in vitro* using hepatocytes isolated from phenobarbital-induced Sprague-Dawley rats) correlated with hepatotoxicity *in vivo* at 24 – 54 hrs (Chan *et al.* 2007). Moreover, using these techniques, the molecular hepatocytotoxic mechanisms found *in vitro* for seven classes of xenobiotics/drugs were found to be similar to the rat hepatotoxic mechanisms reported *in vivo* (O’Brien *et al.*, 2004).

In the presence of a non-toxic G/GO + HRP with NIM (300 µM), NIL (300 µM), and FLU (75 µM), a 2.1-, 1.5-, and 1.5-fold increase in toxicity at 2 hrs were observed, respectively. From the observed results (Table 3.1), it can be suggested that NIM is the drug most susceptible to inflammation. It had been demonstrated previously that a known NIM metabolite (hydroxy-NIM) could be bioactivated by neutrophil and MPO, which generated ROS at the site of inflammation. This may contribute to the toxicity associated with NIM (Yang *et al.*, 2010) and is in agreement with the current study.

A significant decrease in % MMP was found for all of the nitroaromatic drugs in this study compared to the control hepatocytes indicating that all of the nitroaromatic compounds were mitochondrial toxins (Table 3.1). A significant decrease in % MMP was also observed for all of the drugs when G/GO+HRP was added and compared to the drug controls. NIM, due to its nitro group, also acted as a potent protonophoretic uncoupler and NADPH oxidant on isolated rat liver mitochondria and induced Ca2+ efflux or mitochondrial permeability transition (MPT) within a concentration range which could be reached *in vivo* (Mingatto *et al.*, 2000). Tay and
colleagues (2005) also showed that mitochondrial uncoupling causing MPT and ROS production was a secondary effect. NIM exposure increased intracellular ROS, translocation of Bax (Bcl2-associated X protein) and Bcl2 (B-cell lymphoma 2 protein) followed by mitochondrial depolarization and cytochrome c release along with caspase-9/-3 activity confirming the involvement of mitochondria in NIM-induced apoptosis (Tripathi et al., 2010). This study found similar results with or without the inflammation system indicating that NIM is a mitochondrial toxin (Table 3.1). In isolated mitochondria, NIL (100 µM) inhibited respiration that was supported by substrates feeding electrons into complex I of the respiratory chain but did not inhibit respiration that was supported by substrates donating electrons to complexes II, III or IV (Berson et al., 1994). In sub mitochondrial particles, NIL (100 µM) decreased both oxygen consumption mediated by NADH and the oxidation of NADH. However, the addition of SOD and catalase did not alleviate this inhibition (Berson et al., 1991; 1993). These references need to be separated out based on findings. A significantly decreased % MMP was found ($p<0.05$) in our study with NIL indicating its mitochondrial toxin potential (Table 3.1).

Lipid peroxidation may also cause oxidative stress (Masaki et al., 1989; Starke and Farber, 1985). However, if oxidative stress is due to redox cycling mediated by NADPH-cytochrome c reductase, lipid peroxidation may be unchanged or even decreased (Comporti, 1989; Dubin et al., 1987, 1990, 1991; Engineer and Sridhar, 1989; Fau et al., 1992). NIL (0.5 mM) had been shown to decrease the formation of TBA-reactants by 70% when induced by NADPH (0.2 mM) in rat liver microsomes (Berson et al., 1991). TBA-reactants in hepatocytes were found to be unchanged after 4 hr of incubation with 0.5 mM NIL, but were significantly decreased after 8 hr of incubation with 0.5 mM NIL. Mingatto and colleagues (2002) showed that NIM both oxidized NADPH and uncoupled oxidative phosphorylation. The decrease of the
cell antioxidant defense due to NIM could be counterbalanced by the low generation of ROS caused by uncoupling which prevents membrane lipid peroxidation. A similar increase/no change in lipid peroxidation due to NIL or NIM was found in our study at doses used ($p>0.05$).

Addition of a non-toxic H$_2$O$_2$ generating system (to simulate inflammation in vitro as described in the introduction/methods section) with HRP (0.5 µM) caused a significant increase in FLU-induced cytotoxicity, ROS formation, lipid peroxidation, and a decrease in % MMP (Table 3.2) and GSH levels (Figure 3.2) that was significantly decreased by the addition of PTU (5 µM) (a peroxidase inhibitor) (Figure 3.1). Similar results were obtained when we used the Fenton model. An iron chelator, desferoxamine (200 µM) decreased FLU-induced cytotoxicity (Figure 3.1). This suggests that using the Fenton system to generate hydroxyl radicals increased hepatocyte susceptibility to FLU-induced cytotoxicity, similar to that of the non-toxic H$_2$O$_2$ and HRP inflammation system. Srinivasan and colleagues (1997) showed that FLU altered the response of PMNs to other stimuli but did not by itself activate PMNs. FLU did not produce superoxide radicals on PMNs but was cytotoxic to the hepatocytes in the presence of PMNs at concentrations that were not toxic to hepatocytes alone. It was suggested that in some individuals FLU simultaneously renders hepatocytes more susceptible to oxidant-mediated injury that can initiate infiltration of PMNs into the liver and increases PMN responsiveness to endogenous activators (Srinivasan et al., 1997). The incidence of FLU-induced hepatotoxicity was found in only 0.36% of 1091 consecutively treated patients with prostate cancer indicating that most patients do not experience liver toxicity. FLU-induced hepatotoxicity is often associated with inflammation (Gomez et al., 1992; Rosman et al., 1993). Moreover, serum transaminases are elevated but return to normal in some patients without alteration of treatment, whereas for other patients reduction of FLU dose is required (Dourakis et al., 1994; Wysowski et al., 1993). Some
cases were found to be associated with blood eosinophilia (Hart and Stricker, 1989). Therefore it can be speculated that an episode of inflammation during FLU therapy could decrease the threshold for FLU toxicity, and thereby renders an individual susceptible to a toxic reaction that would otherwise not occur.

Drug-induced liver injury is often caused by the formation of reactive metabolites, which may lead to either toxic or immune liver toxicity (Pessayre and Larrey, 1991). Unlike the related antiandrogen NIL, FLU was not noticeably reduced to a nitroanion free radical by NADPH-cytochrome P450 reductase. Instead, rat and human microsomal cytochrome P450 oxidatively metabolized FLU to electrophilic metabolite(s), which bound covalently to microsomal proteins (Berson et al., 1993). However, the production of nitro-anion free radical was also shown by Núñez-Vergara and colleagues (2001). When FLU was added to rat hepatocyte and PMN co–cultures, a significant increase in cytotoxicity was observed which was not observed when FLU was added to rat hepatocytes and PMNs separately. This suggests that a cytochrome P450-generated metabolite of FLU produced by hepatocytes is a more potent stimulus for activation of PMNs than FLU itself (Srinivasan et al., 1997). Further studies are required to confirm that FLU and/or its metabolite(s) are responsible for FLU-induced hepatotoxicity with or without inflammation.

FLU may also target hepatic mitochondria and may exert oxidative stress that can lead to overt hepatic injury (Kashimshetty et al., 2009). In the current study, a decreased % MMP with the inflammation model was found for FLU, indicating its potential role as a mitochondrial toxin (Table 3.2). FLU (50 µM) markedly inhibited respiration (mainly at the level of complex I) in isolated male rat liver mitochondria and at higher concentrations (1 mM) decreased ATP levels in isolated male rat hepatocytes (Fau et al., 1994).
A significant decrease in GSH levels was also observed when FLU was administered with the H₂O₂ generating system and peroxidase to rat hepatocytes, potentially increasing hepatocyte susceptibility to oxidant-mediated injury. This was prevented by 1 mM N-acetylcysteine (Table 3.2). N-acetylcysteine is frequently used as an acetylated precursor for GSH in hepatocytes. N-acetylcysteine has been used as a tool for investigating the role of ROS in numerous biological and pathological processes. The usefulness of N-acetylcysteine in modulating different diseases that include cardiovascular diseases, cancer, and chemical/metal toxicity has been reviewed by Zafrullah and colleagues (2003). FLU (1 mM) was reported to covalently bind reactive electrophilic metabolites to male rat hepatocyte proteins. FLU also decreased the GSH/GSSG ratio and decreased total protein thiols. This was associated with an early increase in phosphorylase a activity (a Ca²⁺ dependent enzyme) and a decrease in cytoskeleton-associated protein thiols, the formation of plasma membrane blebs, the release of lactate dehydrogenase and a loss of cell viability (Berson et al., 1993; Fau et al., 1994).

Potent antioxidants, Trolox (1 mM) (the water-soluble vitamin E analogue) and resveratrol (50 µM) (a polyphenolic compound) significantly decreased FLU-induced cytotoxicity, ROS, and LPO formation, and increased % MMP (Table 3.2) and GSH levels (Figure 3.2) compared to control hepatocytes. Resveratrol is found in a wide variety of dietary sources including grapes, plums, and peanuts. It is also present in wines, especially red wines (Tunali-Akbay et al., 2010). Resveratrol is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidant enzymes (Gusman et al., 2001; Leonard et al., 2003). It has three different antioxidant mechanisms: (i) competition with coenzyme Q to decrease the oxidative chain complex, the site of reactive oxygen species generation, (ii) scavenging of O₂⁻ radicals formed in the mitochondria, and (iii) inhibition of
lipid peroxidation induced by Fenton reaction products (Zini et al., 1999). Trolox is a hydrophilic analogue of vitamin E and an established intracellular free radical scavenger (Hamad et al., 2010).

A significant protection against FLU-induced cytotoxicity with the H₂O₂ generating system and peroxidase was also achieved by a ROS scavenger, TEMPOL (200 µM) and an antioxidant, DPPD (2 µM). Protective agents also significantly increased % MMP and GSH levels and decreased lipid peroxidation compared to FLU treated hepatocytes with the H₂O₂ generating system and peroxidase (Table 3.2). This definitely suggests the involvement of ROS in FLU-induced hepatotoxicity. TEMPOL and other stable nitroxide radicals have long been known to protect from a variety of oxidative stress-mediated injury in laboratory animals. It can mimic superoxide dismutase activity. It can also inhibit Fenton chemistry by the ability to oxidize transition metal ions, termination of radical chain reactions by radical recombination, and acceptance of electrons from mitochondrial electron transport chains (Kagan et al., 2007; Soule et al., 2007). TEMPOL was also reported to inhibit MPO-mediated protein nitration (Vaz and Augusto, 2008). The potent antioxidant DPPD has been reported to enlarge the pool size of lipid soluble antioxidants in the whole liver cell and especially in the cytoplasmic membranes. It has also been reported to decrease lipid peroxidation (Di Luzio and Hartman, 1969). A preliminary treatment with DPPD prevented liver fatty infiltration after carbon tetrachloride poisoning (Torrielli et al., 1974).

Data obtained from this study suggests that FLU-induced cytotoxicity involves a H₂O₂ and peroxidase/Fe(II)-catalyzed production of a FLU pro-oxidant radical leading to glutathione depletion, lipid peroxidation, and mitochondrial toxicity.
4.2 Methotrexate

One of the most serious side effects of MTX therapy is hepatic toxicity (Mardini & Record, 2005). Mild hepatitis, cholestasis, fatty changes, fibrosis, and cirrhosis have been reported in patients receiving MTX for malignant disorders (Hersh et al., 1966) whereas Penalva Polo and colleagues (2002) reported an acute liver failure in a patient with MTX therapy. A concentration and time dependent increase in cytotoxicity, ROS formation, and a decrease in % MMP were also observed for MTX (50 – 500 µM) compared to control hepatocytes over a 3 hr incubation period (Figure 3.3). The LC50 (ACMS) was 300 µM for MTX. MTX-induced cytotoxicity was reported to be associated with ROS formation and the depletion of cellular and mitochondrial GSH and ATP (Babiak et al., 1998; Chang et al., 2013; Chen et al., 1998; Neuman et al., 2001; Phillips et al., 2003).

In its reduced form, intracellular GSH is an effective antioxidant and is necessary for the detoxification of xenobiotics (Neuman et al., 2001). A significant increase in MTX-induced cytotoxicity, ROS formation, and a significant decrease in % MMP was observed when GSH was depleted by adding 1-bromoheptane (200 µM) to hepatocytes whereas addition of 1 mM N-acetylcysteine significantly decreased MTX-induced cytotoxicity (Figure 3.4), ROS formation and increased % MMP (p<0.05) (Table 3.3). This indicates that GSH was involved in MTX detoxification. MTX treatment (300 µM) significantly depleted GSH (Figure 3.5) in rat hepatocytes whereas N-acetylcysteine significantly increased GSH. Several studies have reported that MTX-induced cytotoxicity resulted from the depletion of cellular and mitochondrial GSH leading to oxidative stress (Babiak et al., 1998; Chang et al., 2013; Neuman et al., 2001; Phillips et al., 2003). Silymarin, a natural antioxidant that increases mitochondrial GSH, was reported to be protective against MTX-induced cytotoxicity when ethanol or acetaminophen was added.
along with MTX in human hepatocytes *in vitro* (Phillips *et al.*, 2003). GST enzymes detoxify xenobiotics or ROS by catalyzing the nucleophilic attack by GSH on electrophilic carbon, sulfur, or nitrogen atoms on drug/xenobiotic substrates or ROS. In the process of donating an electron, GSH itself becomes reactive and then reacts with another reactive GSH to form GSSG. Genetic polymorphism of the *GST* gene (*GSTM1* positive/null) was reported to be associated with MTX-induced hepatotoxicity in patients with childhood acute lymphoblastic leukemia and malignant lymphoma (Imanishi *et al.*, 2007). These results along with our study results suggest the definite involvement of GSH in the detoxification of MTX.

In this study, MTX (300 µM) treatment significantly increased H$_2$O$_2$ levels compared to control rat hepatocytes (Figure 3.6). Catalase catalyzes the decomposition of H$_2$O$_2$ to water and oxygen without the production of free radicals. Without catalase, H$_2$O$_2$ can form hydroxyl radicals that are highly toxic to cellular macromolecules. When we inhibited catalase in hepatocytes using 3-AT (20 mM), a significant increase in MTX-induced cytotoxicity (Figure 3.4), ROS formation, and a significant decrease in % MMP (Table 3.3) were observed. Direct addition of catalase significantly decreased MTX-induced cytotoxicity at 60 min but was not protective at 120 min and 180 min (Figure 3.4). Addition of catalase to MTX-treated hepatocytes also significantly decreased ROS formation and increased % MMP (Table 3.3) and H$_2$O$_2$ levels (Figure 3.6). MTX-induced generation of H$_2$O$_2$ was previously reported in stimulated peripheral polymorphonuclear neutrophils (Gressier *et al.*, 1994) as well as monocytes (Phillips *et al.*, 2003). Several *in vitro* and *in vivo* animal studies also reported that MTX administration significantly altered antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase in liver, intestinal mucosa, and spinal cord tissues.
(Coleshowers et al., 2010; Uz E et al., 2005; Uzar et al., 2006). Our study results suggest that MTX-induced generation of H₂O₂ caused oxidative stress and mitochondrial injury.

MTX undergoes metabolic degradation to 7-OH-MTX by nonspecific aldehyde oxidases in the rat liver (Bremnes et al., 1991a; Wolfrom et al., 1990). In human liver, both aldehyde oxidase and xanthine oxidase have been proposed to be involved in MTX-oxidation (Chládek et al., 1997). However, no involvement of aldehyde oxidase or xanthine oxidase in hydrolyzing MTX in rat and human has also been reported (Johns and Valerino et al., 1971; Yu et al., 1989) in earlier studies. When we inhibited aldehyde oxidase using menadione (20 µM), a significant decrease in MTX-induced cytotoxicity was observed at 60 min and 120 min (Figure 3.4). Menadione also significantly decreased ROS and H₂O₂ formation and increased % MMP (Table 3.3). Xanthine oxidase inhibition and cytochrome P450 inhibition had no significant effect on MTX-induced cytotoxicity and ROS formation. This suggests that the conversion of 7-OH-MTX from MTX by aldehyde oxidase increased MTX-induced cytotoxicity and ROS formation in rat hepatocytes that can be decreased by the aldehyde oxidase inhibitor, menadione. A similar result was also found with another aldehyde oxidase inhibitor, amsacrine.

Both aldehyde oxidase and xanthine oxidase are known to produce ROS. Aldehyde oxidase has been reported to cause oxidation of NADH in the presence of O₂ producing large amounts of O₂•⁻. Aldehyde oxidase also mobilizes iron from ferritin (Shaw and Jayatilleke, 1990) which can catalyze O₂•⁻ radical reduction to form the highly reactive and more toxic •OH radical that could directly react with NO to produce the powerful oxidant, ONOO⁻. Thus aldehyde oxidase functions as an important cellular source of ROS under normal physiological conditions. Under various pathological conditions such as ischemia, alcohol-induced liver diseases, and diabetes, this ROS production would increase due to the increase in tissue NADH.
level, thus contributing to oxidative stress and free-radical-mediated tissue injury (Kundu et al., 2012).

7-OH-MTX, a major metabolite of MTX in the liver, was once considered to be a detoxification product due to its lower cytotoxic action in proliferating cells (Johns et al. 1966). However, this metabolite has been found to induce acute renal and hepatic toxicity in rats in several studies after high dose MTX therapy (Bremnes et al., 1991a; Smeland et al., 1994; 1996). Rats with 7-OH-MTX levels of 1 mM, which is lower than the levels of this metabolite routinely found in patients on high-dose MTX, demonstrated intolerable toxicity and some rats died within 8 hr (Fuskevåg et al., 2000). 7-OH-MTX has also been reported to influence cellular entry, polyglutamation, and efflux of MTX in vitro (Fabre et al., 1984). Amsacrine (Bremnes et al., 1991a) and vindesine (Bremnes et al., 1991b) were reported to reduce MTX-induced clinical toxicities in the rat model by inhibiting MTX-hydroxylation i.e. by inhibiting aldehyde oxidase. In two clinical studies, MTX and vindesine co-treatment were found to be effective (Bore et al., 1986; Lena et al., 1984). Similar cytoprotection was found in this study using menadione, which inhibited aldehyde oxidase in isolated rat hepatocytes. Thus inhibition of aldehyde oxidase could be beneficial in combination MTX chemotherapy by reducing (a) the generation of ROS and (b) the formation 7-OH-MTX. However, clinical studies are required to establish the effect of 7-OH-MTX levels on aldehyde/xanthine oxidase activity as a marker for MTX associated hepatic and renal toxicity.

Although several in vivo studies reported increased lipid peroxidation in rat livers after administration of MTX (Banji et al., 2011; Coleshowers et al., 2010; Sener et al., 2006; Tunali-Akbay et al., 2010), this study observed no significant effect of MTX on lipid peroxidation probably due to the higher in vitro concentration of MTX used in the study.
MTX (300 µM) significantly decreased % MMP (Table 3.3) and ATP levels (Figure 3.7) in rat hepatocytes whereas addition of 10 mM fructose (pre-incubated for 15 min) significantly increased % MMP (Table 3.3) and ATP (Figure 3.7) and delayed MTX-induced cytotoxicity (Table 3.4). Fructose is a glycolytic substrate that has been reported to increase % MMP and ATP and subsequently decreased cell death in hepatocytes when exposed to the known mitochondrial toxicant, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Wu et al., 1990). This suggests that MTX caused a decrease in % MMP and ATP concentration in our study, leading to hepatocyte cell death in isolated rat hepatocytes. Studies on isolated rat mitochondria reported that MTX inhibited state III respiration (Yamamoto et al., 1988), decreased membrane potential (ΔΨ), and ionic conductivity of the mitochondrial inner membrane (Yamamoto et al., 1989). Tabassum and colleagues (2010) also reported that MTX caused a significant decrease in mitochondrial GSH levels and a significant increase in mitochondrial lipid peroxidation, protein carbonyl content, and superoxide generation in isolated rat liver mitochondria.

Addition of a non-toxic H₂O₂ generating system with HRP (0.5 µM) caused a significant increase in MTX-induced cytotoxicity that was significantly decreased by the addition of PTU (5 µM) (Figure 3.8). Furthermore, the addition of a H₂O₂ generating system and peroxidase caused a significant increase in lipid peroxidation, ROS formation and a decrease in % MMP (data not shown). Similar results were obtained when the Fenton model was used. An iron chelator, desferoxamine (200 µM) decreased MTX-induced cytotoxicity likely by inhibiting the Fenton reaction through chelation of iron (Figure 3.8). This suggests that using the Fenton system to generate hydroxyl radicals increased hepatocyte susceptibility to MTX-induced cytotoxicity, similar to that of the non-toxic H₂O₂ and HRP inflammatory model. Therefore, an episode of inflammation during MTX therapy could decrease the threshold for MTX toxicity, and thereby
render an individual susceptible to a toxic reaction that would otherwise not occur. Increased MPO activity during MTX therapy has been reported in several in vivo animal studies in hepatic tissues (Sener et al., 2006; Tunali-Akbay et al., 2010). Kolli and colleagues (2009) provided evidence that neutrophil infiltration and oxidative damage led to renal damage in rats and suggested that administration of inhibitors of MPO as an adjuvant therapy could be beneficial in patients with MTX-induced renal damage. This study suggests a similar recommendation for adjuvant therapy for treating MTX-induced hepatotoxicity.

Significant cytoprotection against MTX-induced cytotoxicity in isolated rat hepatocytes was achieved by the ROS scavenger and superoxide dismutase mimic, TEMPOL (200 µM) (Table 3.4). Antioxidants such as Trolox (1 mM), mesna (1 mM), and resveratrol (50 µM) also significantly protected hepatocytes, decreased ROS and H₂O₂ formation, and significantly increased % MMP (Table 2). This definitely suggests the involvement of ROS in MTX-induced hepatotoxicity. Mesna (a thiol containing effective antioxidant) was previously found to be protective in stimulated peripheral blood neutrophils against MTX-induced H₂O₂ production and has been proposed for use in combination with cancer therapy during the oxidative burst (Gressier et al., 1994). Trolox (≤ 0.5 mM) was reported to inhibit apoptosis induced by MTX in keratinocytes (Barry et al., 1998). Previous in vivo rat studies showed the protective effects of resveratrol against MTX-induced hepatotoxicity (Dalaklioglu et al., 2013; Tunali-Akbay et al., 2010).

Data obtained from the ACMS techniques suggests that MTX-induced cytotoxicity towards isolated rat hepatocytes may involve several pathways: an aldehyde oxidase-catalyzed production of a toxic metabolite (7-OH-MTX), a H₂O₂ and peroxidase/Fe(II) catalyzed production of a MTX pro-oxidant radical and MTX-induced generation of H₂O₂ and other ROS.
Oxidative stress leads to mitochondrial injury and ultimately hepatocyte death. The GSH precursor, \( N \)-acetylcysteine; the aldehyde oxidase inhibitor, menadione; and several other antioxidants significantly prevented hepatocyte death suggesting that combination therapy with these agents could be beneficial therapeutically to prevent or reduce MTX-induced hepatotoxicity. *In vivo* animal and clinical studies are warranted to test their therapeutic effectiveness against MTX-induced hepatotoxicity.

Possible routes of MTX-induced cytotoxicity and oxidative stress in isolated rat hepatocytes are presented in Figure 4.1.

**Figure 4.1.** Proposed pathways of MTX-induced cytotoxicity in isolated rat hepatocytes. PTU, 6-\( N \)-propyl-2-thiouracil; GSH, reduced glutathione; GSSG, Glutathione disulfide; GST, glutathione S-transferase; SOD, Superoxide dismutase.
4.3 Thiopurines (azathioprine, 6-mercaptopurine)

Three thiopurine drugs (AZA, 6-MP, 6-TG) have been commonly used in the last forty years (Petit et al., 2008). In most of the cases, hepatotoxicity is an unpredictable side effect of these drugs, whose pathogenic mechanism still remains unknown. Previous studies performed with rat hepatocyte primary cultures showed that AZA metabolism leads to intracellular GSH depletion, mitochondrial injury, metabolic activity reduction, decreased ATP levels, and cell death (Lee and Farrell, 2001; Menor et al., 2004; Petit et al., 2008). In the current study, a concentration and time dependent increase in cytotoxicity and ROS formation and a decrease in % MMP were observed with AZA (100 – 500 µM) compared to control hepatocytes (Figure 3.9). The LC50 according to ACMS was found to be 400 µM.

AZA has been reported to conjugate GSH to form 6-MP, catalyzed by GSTs. This consumes GSH, which is normally present in abundance in hepatocytes. (DeLeve, 1996; Kaplowitz, 1997; Lee and Farrell, 1999; 2001). AZA treatment (400 µM) significantly depleted hepatocyte GSH (Figure 3.11) in this study. A significant increase in AZA-induced cytotoxicity and ROS formation were observed when hepatocyte GSH was depleted using 1-bromoheptane (200 µM) whereas addition of N-acetylcysteine (1 mM, a cysteine precursor which generates GSH) prevented AZA-induced cytotoxicity (Figure 3.10), ROS and H2O2 generation, and increased % MMP (Table 3.5) and hepatocyte GSH, which indicates that GSH was required for AZA detoxification. A similar depletion of GSH levels and mitochondrial toxicity during AZA metabolism was observed in previous studies in primary cultures of rat hepatocytes at both toxic (25 – 250 µM) and clinically relevant AZA concentrations (0.5 – 5 µM) (Lee and Farrell, 2001; Menor et al., 2004; Petit et al., 2008). However, human liver parenchymal cells were reported to
be much less sensitive than rat hepatocytes to thiopurine treatments (Petit et al., 2008). Protective effects of N-acetylcysteine against AZA-induced hepatotoxicity have been reported in several *in vitro* studies (Lee and Farrell, 2001; Menor et al., 2004; Wu et al., 2006) and *in vivo* animal studies (Raza et al., 2003) which clearly indicate a potential role of GSH in AZA-induced cytotoxicity.

Xanthine oxidase is proposed to be involved in several steps of AZA metabolism such as in the direct metabolism of AZA to form an inactive metabolite, 1-methyl-4-nitrothioimidazole, in the conversion of AZA to 6-MP, and formation of 6-thiouric acid from 6-MP (Lee and Farrell, 2001; Menor et al., 2004; Tapner et al., 2004). Xanthine oxidase is well known to produce ROS (Kelley et al., 2010; Tapner et al., 2004). The molybdoflavin enzyme xanthine oxidoreductase (XOR) catalyzes the terminal two steps of purine degradation (from hypoxanthine to xanthine and from xanthine to uric acid) in humans. XOR is transcribed as a single gene product, xanthine dehydrogenase (XDH). Under inflammatory conditions, posttranslational modification by oxidation of critical cysteine residues or limited proteolysis converts XDH to xanthine oxidase (Amaya et al., 1990; Kelley et al., 2010). Substrate-derived electrons at the molybdenum (Mb) cofactor of xanthine oxidase reduce O₂ at the FAD cofactor both univalently, generating O₂⁻, and divalently, forming H₂O₂. However, conversion to xanthine oxidase is not a requisite for ROS production, as XDH displays partial oxidase activity under conditions such as the ischemic/hypoxic microenvironment encountered in vascular inflammation (Harris et al., 1997). This same inflammatory milieu leads to enhanced xanthine oxidase levels and thus increased xanthine oxidase-derived ROS formation, resulting in activation of redox dependent cell signaling reactions and alterations in vascular function (Kelley et al., 2010). The adverse effect of xanthine oxidase is exemplified by numerous studies in which inhibition of xanthine oxidase
attenuated symptoms of several vascular disease including congestive heart failure, sickle cell anemia, and diabetes (Aslan et al., 2001; Butler et al., 2000; Desco et al., 2002; Farquharson et al., 2002).

The possibility that xanthine oxidase may play a role in AZA-induced tissue injury has been raised by the observation that patients taking allopurinol, a xanthine oxidase inhibitor, experience less nephrotoxicity during rejection episodes after renal transplantation (Chocair et al., 1994). When we inhibited xanthine oxidase by preincubating rat hepatocytes with allopurinol (20 μM), AZA-induced cytotoxicity was significantly prevented (Figure 3.10). A significant decrease in ROS and H₂O₂ formation and an increase in % MMP were observed compared to control hepatocytes with AZA-treated and xanthine oxidase-inhibited hepatocytes (Table 3.5). Xanthine oxidase inhibition by allopurinol was also found to be protective in primary rat hepatocytes (Tapner et al., 2004). A recent case study reported that the addition of allopurinol with appropriate AZA dose reduction may correct AZA-induced hepatotoxicity and can induce remission of IBD (Al-Shamma et al., 2013). In AZA or 6-MP non-responders, addition of allopurinol also demonstrated safety and efficacy for long-term maintenance in IBD patients (Leung et al., 2009; Sparrow et al., 2007). In our study, the combined addition of N-acetylcysteine (1 mM) and allopurinol (20 μM) showed nearly complete protection against AZA-induced cytotoxicity in rat hepatocytes. The combination of these two agents for improved AZA therapeutic efficacy needs future clinical experimentation.

Aldehyde oxidase inhibition (by 20 μM menadione) and cytochrome P450 inhibition (by 100 μM 1-ABT) had no significant effect suggesting that, in rat hepatocytes, aldehyde oxidase and cytochrome P450 are not involved in AZA metabolism pathways at the AZA concentrations used in this study.
A significant decrease in AZA-induced cytotoxicity (Table 3.6) and ROS formation (Table 3.5) in isolated rat hepatocytes was achieved by the ROS scavenger and the superoxide dismutase mimic TEMPOL (200 µM) suggesting the involvement of ROS. Potent antioxidants like Trolox (1 mM), DPPD (2 µM), and mesna (1 mM), also significantly decreased AZA-induced cytotoxicity (Table 3.6), ROS and H₂O₂ formation and increased % MMP (Table 3.5) suggesting the involvement of oxidative stress in AZA-induced cytotoxicity in hepatocytes.

Data obtained from the ACMS technique suggests that AZA toxicity in isolated rat hepatocytes involves two distinct pathways (i) a xanthine oxidase-catalyzed production of an inactive metabolite (1-methyl-4-nitrothioimidazole), (ii) a GST-catalyzed pathway leading to GSH depletion followed by xanthine oxidase-catalyzed formation of inactive metabolites. Addition of a GSH precursor, N-acetylcysteine and the xanthine oxidase inhibitor, allopurinol, together significantly reversed cytotoxicity which raises the possibility of using these two agents therapeutically. Several antioxidants also prevented hepatocyte death suggesting antioxidant therapy may be useful therapeutically to prevent or decrease AZA-induced hepatotoxicity. In vivo animal and clinical studies are warranted to test their therapeutic effectiveness against AZA-induced hepatotoxicity. Possible routes of cytotoxicity of AZA in isolated rat hepatocytes are presented in Figure 4.2.
Figure 4.2. Proposed routes of AZA-induced cytotoxicity in isolated rat hepatocytes. XO, xanthine oxidase; GST, glutathione S-transferase; NAC, N-acetylcysteine; HGPRT, hypoxanthine guanine phosphoribosyl transferase; TPMT, thiopurine S-methyl transferase (Maruf et al. 2014c).

Addition of a non-toxic H$_2$O$_2$ generating system with peroxidase caused a significant increase in 6-MP (1 mM, LC$_{50}$ according to ACMS) cytotoxicity ($p<0.05$) which was reversed by the addition of PTU (5 µM) (Table 3.7). Furthermore, the addition of a H$_2$O$_2$ generating system and peroxidase caused a significant increase in ROS formation and a decrease in % MMP compared to control hepatocytes (Table 3.7). A similar effect was also found for AZA but it
raised the question whether AZA or 6-MP forms the pro-oxidant radical that leads to toxicity as AZA is readily converted to 6-MP. To answer this question, electron spin resonance spectrometry spin trapping studies of AZA and 6-MP metabolism by HRP/H₂O₂ were also conducted.

Direct detection of some short-lived free radicals (e.g., superoxide and hydroxyl radicals) is very difficult or impossible in solution at room temperature. Therefore, the technique of spin trapping of short-lived free radical intermediates has become a valuable tool for characterizing free radicals in chemistry, biology, and medicine by electron paramagnetic resonance (EPR) spectroscopy. Spin trapping is a technique developed in the late 1960s where a nitrone or nitroso compound reacts with a target free radical to form a stable and distinguishable free radical that can be detected by EPR spectroscopy. The most popular spin trap is 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Britigan et al., 1987; Buettner et al., 1990; Janzen, 1984; Khan et al., 2003).

ESR spectra of 6-MP metabolism by HRP/H₂O₂ are presented in Figure 3.13. A mixture of two radicals were trapped (A) using DMPO, previously reported (Moore et al., 1994) as purine-6-thiyl (PS⁺) and superoxide radicals in a photooxidation system containing 6-MP with SOD. In the absence of H₂O₂ (C), HRP (D), and DMPO, HRP, and H₂O₂ (E), the spectrum in A was not observed. When SOD was added to the mixture, a more intense spectrum of 6-MP thiyl radical was observed. When the reaction was recorded after an additional 6 minutes (F), only one spectrum remained which corresponded to DMPO-OH (B), suggesting that the thiyl radical adducts decayed (Figure 3.13). AZA did not produce any signal in the ESR spin trapping study. It was previously reported that thiol containing drugs may produce thiyl radicals when oxidized by MPO via one-electron oxidations. At physiological pH, MPO was found to be an active catalyst of thiol oxidation for aliphatic and aromatic thiols (Burner et al., 1999). Thiol oxidation
was accompanied by oxygen consumption, superoxide formation and regeneration of H₂O₂
(Burner and Obinger, 1997; Obinger et al., 1996) which is in agreement with our study results.

A significant decrease in GSH levels was observed when 6-MP was administered with
the H₂O₂ generating system and peroxidase to rat hepatocytes. This was prevented by 1 mM N-
acetylcysteine (Table 3.7). A similar result was found by Tapner and colleagues (2004) where 6-
MP depleted hepatocyte GSH and increased GSSG formation at clinically relevant
concentrations (0.5 – 5 µM).

Significant protection against 6-MP-induced cytotoxicity with the H₂O₂ generating
system and peroxidase was achieved by a ROS scavenger, TEMPOL (200 µM) and an
antioxidant, DPPD (2 µM). Protective agents also significantly increased % MMP (Table 3.7)
and GSH levels (Figure 3.12) compared to 6-MP treated hepatocytes with the H₂O₂ generating
system and peroxidase only (Table 3.7).

Data obtained from the ACMS technique suggests that 6-MP cytotoxicity may involve
H₂O₂ and peroxidase catalyzed production of thyl and superoxide radicals leading to glutathione
depletion and mitochondrial toxicity. Possible routes of 6-MP cytotoxicity in isolated rat
hepatocytes are presented in Figure 4.3.
Figure 4.3 Possible routes of 6-MP-induced cytotoxicity in isolated rat hepatocytes. XO, xanthine oxidase; HGPRT, hypoxanthine guanine phosphoribosyl transferase; TPMT, thiopurine S-methyl transferase; PTU, 6-N-propyl-2-thiouracil; GSH, reduced glutathione; GSSG, glutathione disulfide.
4.4 Significance and rationale of the research

The *in vitro* oxidative stress inflammation system used in this study based on ACMS techniques to evaluate drug-induced hepatotoxicity is a very simple, robust, and cheap method. It can guide and accelerate the development of safer pharmaceutical agents and reduce the cost and even deaths associated with adverse drug reactions.

Utilizing the ACMS techniques and using the *in vitro* inflammation system, it is possible:

(a) To identify possible cytotoxic mechanisms of drugs,

(b) To identify drug-metabolizing enzymes involved in either detoxifying or activating pathways of a drug,

(b) To investigate the effects of inflammation as a susceptibility factor leading to drug-induced hepatotoxicity,

(c) To identify potential hepatotoxic drugs and possible effects of inflammation during the preclinical stages of drug discovery and development,

(d) To identify possible protective agents or antidotes to reverse such drug-induced hepatotoxicity.

By broadening our thinking regarding the basis for drug idiosyncrasy, doors may open to increase our understanding of toxicity mechanisms and to find ways for predicting or avoiding such untoward reactions to otherwise useful drugs.
4.5 Limitations

ACMS techniques assume that the drug metabolic/toxic pathways at cytotoxic drug concentrations *in vitro* at 2 hrs will be similar to those that occur *in vivo* in 24 – 36 hrs i.e. high dose/short time (*in vitro*) exposure will simulate low dose/long time (*in vivo*) exposure. However, *in vitro* results do not always correlate to *in vivo* results. The method uses higher concentrations of the drug under investigation. The mechanism of toxicity at higher drug concentrations than clinical drug concentrations is not always the same as at clinically relevant drug concentrations. Caution should be taken in interpretation of the results in humans.

This study used freshly isolated rat hepatocytes. Although they are the gold standard for studying drug-induced hepatotoxicity, they are functional for only a few hours and therefore permit the study of acute toxicity only. Isolation of hepatocytes involves multiple steps which may cause mechanical disruptions of the hepatocytes. Isolating hepatocytes with reproducible viability of 80-90% is difficult.

The *in vitro* oxidative stress inflammation system is based on the hypothesis that an acute episode of inflammation during drug therapy could decrease the threshold for drug toxicity and thereby render an individual susceptible to a toxic reaction that would otherwise not occur (Ganey and Roth, 2001; 2003; Roth *et al.*, 1997). The inflammmagen hypothesis is based on an animal model in which rats treated with drugs e.g. ranitidine, trovafloxacin or LPS developed an acute liver injury with histology dominated by neutrophils (Ganey and Roth, 2001). This model is fundamentally different in every respect from IDILI that occurs in humans: the injury is acute, occurring in a few hours after administration of the drug whereas IDILI is generally delayed in onset. DILI is characterized by a mononuclear infiltrate, sometimes with eosinophil and/or...
plasma cells. In contrast, the inflammmagen model is characterized by an infiltrate of neutrophils, which is typical of liver injury caused by LPS but not IDILI. Although the mechanism of liver injury in this model is clearly different from the mechanism of IDILI, it is possible that an inflammatory stimulus could act as a danger signal and increase the risk of immune-mediated DILI by APCs (reviewed in Uetrecht, 2013).

The spin trapping agent, DMPO, used in the study cannot distinguish superoxide and hydroxyl radical easily because of spontaneous decay of the DMPO-superoxide adduct ($t_{1/2} = 45$ seconds) into the DMPO-hydroxyl adduct (Zhao et al., 2001).
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions
The overall goal of the research was to develop a simple and robust in vitro oxidative stress inflammation system using isolated rat hepatocytes to investigate the effects of inflammation and oxidative stress on drug toxicity. This could then be used to screen out potential hepatotoxic molecules during the early phase of drug development. The thesis also investigated the molecular mechanisms involved in drug-induced liver injury for selected drugs (nimesulide, nilutamide, flutamide, methotrexate, azathioprine, and 6-mercaptopurine) and tested compounds that can be used to prevent or decrease drug-induced hepatotoxicity.

5.1.1 Nitroaromatics (nimesulide, nilutamide, flutamide)

All of the nitroaromatic drugs significantly increased cytotoxicity as compared to the controls ($p<0.05$). The cytotoxicity was found to be concentration and time dependent. In the presence of a non-toxic G/GO+HRP system with NIM (300 µM), NIL (300 µM), and FLU (75 µM), a 2.1-, 1.5-, and 1.5-fold increase in toxicity at 2 hrs were observed, respectively. In the absence of drugs, none of the inflammatory modulators affected hepatocyte viability significantly at the doses used. ROS formation was also increased with all of the nitroaromatic drugs with and without G/GO+HRP. A significant decrease in % MMP was observed ($p<0.05$) with a ranking of NIL > FLU > NIM compared to control hepatocytes indicating that all of these studied drugs are mitochondrial toxins. A significant decrease in % MMP was also observed for NIM, NIL and FLU when incubated with G/GO+HRP compared to respective drug controls. These results
implicate \( \text{H}_2\text{O}_2 \), a cellular mediator of inflammation, as a potential risk factor for drug-induced hepatotoxicity.

This study suggests that FLU-induced cytotoxicity may involve a \( \text{H}_2\text{O}_2 \) and peroxidase/Fe(II)-catalyzed production of a FLU pro-oxidant radical leading to glutathione depletion, lipid peroxidation, and mitochondrial toxicity. Potent antioxidants, resveratrol (a polyphenolic compound), and Trolox (the water-soluble vitamin E analogue) significantly decreased FLU-induced cytotoxicity, ROS and LPO formation, and increased % MMP. TEMPOL, a known ROS scavenger and superoxide dismutase mimetic, and DPPD, a potent antioxidant, also reversed toxicity caused by FLU. These results raise the possibility that the presence or absence of inflammation may be another susceptibility factor for drug-induced hepatotoxicity.

### 5.1.2 Methotrexate

The potential molecular cytotoxic mechanisms of MTX towards isolated rat hepatocytes using the \textit{in vitro} oxidative stress inflammation system were investigated in this study using ACMS techniques. MTX-induced cytotoxicity towards isolated rat hepatocytes may involve several pathways: an aldehyde oxidase-catalyzed production of a toxic metabolite (7-OH-MTX), a \( \text{H}_2\text{O}_2 \) and peroxidase/Fe(II) catalyzed production of MTX pro-oxidant radicals, and MTX-induced generation of \( \text{H}_2\text{O}_2 \) and other ROS. The overall effect is oxidative stress that leads to mitochondrial injury and ultimately hepatocyte death. The GSH precursor, the \( \text{N} \)-acetylcysteine, aldehyde oxidase inhibitor, menadione, and several antioxidants such as mesna (a synthetic organosulpher compound), resveratrol (a polyphenolic compound), and Trolox (the water-
soluble vitamin E analogue) significantly prevented hepatocyte death suggesting combination therapy with these agents could be beneficial therapeutically to prevent or reduce MTX-induced hepatotoxicity. *In vivo* animal and clinical studies are warranted to test their therapeutic effectiveness against MTX-induced hepatotoxicity.

**5.1.3 Thiopurines (azathioprine, 6-mercaptopurine)**

Data obtained from the ACMS technique suggests that AZA toxicity towards isolated rat hepatocytes involves two distinct pathways (i) a xanthine oxidase-catalyzed production of an inactive metabolite (1-methyl-4-nitrothiimidazole), (ii) GST-catalyzed pathway leading to GSH depletion followed by xanthine oxidase-catalyzed formation of inactive metabolites. Addition of a GSH precursor, *N*-acetylcysteine, and a xanthine oxidase inhibitor, allopurinol, together significantly reversed cytotoxicity which raises the possibility of using these two agents therapeutically. A ROS scavenger (TEMPOL) and several antioxidants (Trolox, mesna, and DPPD) also prevented hepatocyte death suggesting antioxidant therapy may be useful therapeutically to prevent or decrease AZA-induced hepatotoxicity.

Addition of a non-toxic H₂O₂ generating system with peroxidase caused a significant increase in 6-MP cytotoxicity (*p*<0.05) which was reversed by the addition of PTU. Furthermore, the addition of a H₂O₂ generating system and peroxidase caused a significant increase in lipid peroxidation, ROS and a decrease in % MMP and GSH levels compared to control hepatocytes. ESR spin trapping study of 6-MP metabolism by HRP/H₂O₂ is also investigated. A mixture of two radicals were trapped using DMPO which were previously reported as purine-6-thiyl (PS⁺) and superoxide radicals in a photooxidation system containing 6-MP with SOD. AZP did not
produce any signal in ESR spin trapping study. Data obtained from this study suggests that 6-MP cytotoxicity may involve H$_2$O$_2$ and peroxidase catalyzed production of thiyl and superoxide radicals leading to glutathione depletion and mitochondrial toxicity.

5.1.4 Hypothesis revisited

“Exposure of drugs such as nimesulide, flutamide, nilutamide, methotrexate, azathioprine, 6-mercaptopurine to an in vitro oxidative stress inflammation system will increase hepatotoxicity through the formation of pro-oxidant radicals and other reactive oxygen species leading to oxidative stress”.

From the findings reported in this thesis, it can be concluded that all the investigated drugs (except for azathioprine) formed pro-oxidant radicals and other reactive oxygen species when exposed to the in vitro oxidative stress inflammation system in isolated rat hepatocytes and caused oxidative stress leading to cell death.
5.2 Future directions

We are planning to incorporate the ESR spin trapping studies into this system for all of the tested drugs so that we can confirm the formation of drug pro-oxidant free radicals (due to oxidation by HRP/H$_2$O$_2$) and other ROS formation. In the current study, DMPO (5,5-dimethyl-1-pyrroline N-oxide) was used as a spin trap agent. However, this agent has some limitations. It cannot distinguish superoxide and hydroxyl radical easily because of spontaneous decay of DMPO-superoxide adduct (t$_{1/2}$ = 45 seconds) into the DMPO-hydroxyl adduct. BMPO (5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide) can be used as its alternative to easily distinguish superoxide and hydroxyl radical as BMPO-superoxide adduct does not decay into a hydroxyl adduct and has a much longer half-life (t$_{1/2}$ = 23 minutes) (Zhao et al., 2001). This method may also be used in in vivo systems to detect short lived radicals.

We are planning to compare and validate our study results with other currently available in vitro systems to study endotoxin-induced hepatotoxicity. Commonly used in vitro systems are:

(a) Co-culture of stimulated PMNs with hepatocytes:
Co-culturing of isolated rat hepatocytes with PMNs stimulated with phorbol myristate acetate (PMA) or f-methionyl-leucyl-phenylalanine (fmlp) in the presence or absence of xenobiotic, and evaluation of cytotoxicity to hepatocytes from the release of alanine aminotransferase into the medium (Srinivasan et al., 1997)

(b) Co-culture of stimulated Kupffer cells with hepatic parenchymal cells (HPC):
“A rat Kupper cell-hepatocyte coculture system”. Treatment of cocultures with various concentrations of either LPS or Interleukin 2 (IL2), the xenobiotic under investigation, vehicle
(culture medium) or a combination compared to Kupffer cells and HPC alone (Tukov et al., 2006).

Although our in vitro model can screen out drug molecules having a potential for toxicity associated with inflammation, this needs to be translated in an in vivo system. Confirmation from both in vitro and in vivo studies can help to detect drugs having potential for idiosyncratic reactions. Several models are available based on different hypotheses of IDILI (reviewed in Roth and Ganey, 2011). However, we are interested in adapting one model developed by Roth and colleagues (Roth et al., 1997) in our laboratory so that we can include it in an in vitro – in vivo correlation in our system. The hypothesis of the model was that altered tissue homeostasis initiated by small, otherwise nontoxic doses of xenobiotic agents can progress to overt toxicity in the presence of inflammatory factors generated by concurrent endotoxin exposure such as LPS. This hypothesis is supported by studies in animals, in which considerable evidence has accumulated indicating that endotoxin exposure can influence the magnitude of responses to toxic chemicals. In this model, rats or mice are treated with nonhepatotoxic doses of LPS and the xenobiotic under investigation alone or in combination. Liver histopathology and liver enzymes are checked after 6 hrs of xenobiotic and/or LPS administration (Roth et al., 1997).

The “In Vitro Oxidative Stress Inflammation System” is a simple and robust system which, after further validation, can be used as a powerful screening tool to screen out potential hepatotoxic drug molecules associated with inflammation during pre-clinical stages of drug development.
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