

SHAH, HALLEY, M.S. Protection of HepG2 Cells from Acrolein Toxicity by CDDO-Im Via Glutathione-Mediated Mechanism. (2013)  
Directed by Dr. Zhenquan Jia. 56pp.

Acrolein is an environmental toxicant, mainly found in smoke released from incomplete combustion of organic matter. The compound is ubiquitously found in endogenous as well as exogenous environment. Several studies showed that exposure to acrolein can lead to liver damage. The mechanisms involved in acrolein-induced hepatocellular toxicity, however, are not completely understood. This study examines the toxic effects and cytotoxic mechanisms of acrolein on HepG2 cells.

Acrolein at pathophysiological concentrations was shown to cause a concentration-dependent decrease in cell viability as measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and LDH (lactate dehydrogenase) assays. Acrolein exposure was also found to cause apoptotic cell death and an increase in levels of protein carbonyl and TBARS (thiobarbituric reactive acid substances), markers of protein damage and lipid peroxidation, respectively, in HepG2 cells. Acrolein also rapidly depleted intracellular glutathione (GSH), phase II enzyme GSH-linked glutathione-S-transferases (GST) and aldose reductase (AR) — three critical cellular defenses that detoxify reactive aldehydes.

Results further showed that depletion of cellular GSH by acrolein preceded the loss of cell viability, which suggests that cellular GSH depletion may be an important event in acrolein-induced cytotoxicity. To further determine the role of cellular GSH in protecting against acrolein-mediated cytotoxicity, buthionine sulfoximine (BSO) was

used to inhibit cellular GSH biosynthesis. It was observed that depletion of cellular GSH by BSO led to a marked potentiation of acrolein-mediated cytotoxicity in HepG2 cells.

Furthermore, induction of GSH levels by CDDO-Im, a triterpenoid compound, afforded protection against acrolein toxicity in a concentration-dependent manner. Notably, incubation of HepG2 cells with CDDO-Im at a concentration as low as 10 nM leads to a significant increase in GSH content. To further determine the role of GSH in CDDO-Im-mediated cytoprotection against acrolein-mediated cytotoxicity, BSO was used to inhibit cellular GSH induction by CDDO-Im. Pretreatment of HepG2 cells with BSO and CDDO-Im significantly inhibited CDDO-Im-mediated induction in cellular GSH levels and also reversed cytoprotective effects of CDDO-Im on acrolein-mediated toxicity.

In summary, this study demonstrates that exposure to acrolein results in significant depletion of GSH, an important phase II defense, and causes an increase in apoptosis, lipid peroxidation and protein carbonylation. Furthermore, the endogenous antioxidant GSH can be induced by CDDO-Im. The CDDO-Im-mediated elevated GSH appears to afford a marked protection against acrolein toxicity suggesting that GSH plays a predominant role in CDDO-Im-mediated protection against acrolein-induced toxicity in HepG2 cells. This study may provide understanding on the molecular action of acrolein which may be important to develop novel strategies for the prevention of acrolein-mediated toxicity.

PROTECTION OF HEPG2 CELLS FROM ACROLEIN TOXICITY BY CDDO-IM  
VIA GLUTATHIONE-MEDIATED MECHANISM

by

Halley Shah

A Thesis Submitted to  
the Faculty of The Graduate School at  
The University of North Carolina at Greensboro  
in Partial Fulfillment  
of the Requirements for the Degree  
Master of Science

Greensboro  
2013

Approved by

---

Committee Chair

This thesis is dedicated to my parents and my sister who have supported me all the way since the beginning of my studies.

Also, this thesis is dedicated to my fiancé who has been a great source of motivation and inspiration.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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Date of Final Oral Examination

## ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Zhenquan Jia and committee members John Tomkiel and Paul Steimle for their guidance and support. I would like to thank Dr, Planisamy, Adam Speen, Elizabeth Brooke and Christina Saunders for their assistance throughout this project. I would also like to thank the UNCG biology department for their supportive nature.

## TABLE OF CONTENTS

	Page
LIST OF FIGURES .....	vii
CHAPTER	
I. INTRODUCTION .....	1
Hepatotoxicity.....	1
Acrolein.....	3
Oxidative stress and cell death pathways.....	5
Up-regulation of phase II detoxifying enzymes against acrolein toxicity.....	7
Proposed Study .....	9
II. MATERIALS AND METHODS.....	11
Chemicals and materials .....	11
Tissue culture .....	11
Cell extraction preparation.....	12
Assay for glutathione (GSH) content.....	12
Assay for glutathione-S-transferase (GST) activity.....	12
Aldose reductase assay (AR) .....	13
Cell injury .....	13
Assay for Thiobarbituric acid reactive substances (TBARS).....	14
Protein carbonyl (PC) assay.....	14
Lactate Dehydrogenase (LDH) release assay .....	15
Flow cytometry for cell death.....	15
Real time PCR analysis.....	15
Statistical analysis.....	16
III. RESULTS .....	17
Acrolein-induced cytotoxicity .....	17
Effects of acrolein on cell morphology.....	18
Flow cytometric analysis .....	19
Protein damage and lipid peroxidation caused by acrolein in HepG2 cells .....	20
GSH depletion by acrolein in HepG2 cells.....	21

Effects of BSO pretreatment on acrolein-induced cytotoxicity .....	22
Depletion of intracellular GST and AR by acrolein .....	23
Up-regulation of the endogenous GSH and phase II detoxifying enzyme by CDDO-Im protecting against acrolein-induced cytotoxicity .....	24
Cytoprotective effects of CDDO-Im by flow cytometric analysis.....	26
Effects of CDDO-Im treatment on GSH content and GST, AR activity.....	27
Induction of mitochondrial GSH by CDDO-Im .....	29
Effects of BSO co-treatment on CDDO-Im-mediated cytoprotection against acrolein-induced cytotoxicity.....	30
Effects of CDDO-Im and BSO co-treatment on lipid peroxidation and protein damage.....	32
 IV. DISCUSSION.....	 34
REFERENCES .....	41

## LIST OF FIGURES

	Page
Figure 1. Phase I and II liver detoxification processes .....	2
Figure 2. Chemical structure of acrolein.....	4
Figure 3. GSH synthesis .....	9
Figure 4. Chemical structure of CDDO and CDDO-Im .....	10
Figure 5. Acrolein-induced cytotoxicity in HepG2 cells .....	18
Figure 6. Phase contrast images of HepG2 cells with and without treatment of acrolein .....	19
Figure 7. Acrolein induces apoptosis in HepG2 cells.....	20
Figure 8. Effects of acrolein on protein damage and lipid peroxidation .....	21
Figure 9. Acrolein-induced GSH depletion and time-dependent increase in LDH .....	22
Figure 10. Effects of BSO on cellular GSH and acrolein-induced cytotoxicity in HepG2 cells .....	23
Figure 11. Depletion of intracellular GST and AR by acrolein.....	24
Figure 12. Protective effects of CDDO-Im against acrolein-induced cytotoxicity in HepG2 cells.....	26
Figure 13. CDDO-Im protects against acrolein-mediated cytotoxicity as assessed by the flow cytometric analysis.....	27
Figure 14. Effect of CDDO-Im treatment on GSH, GST and AR.....	28
Figure 15. Real-time PCR analysis of mRNA levels of GCLC.....	29
Figure 16. Induction of mitochondrial GSH by CDDO-Im in HepG2 cells .....	30

Figure 17. Effect of BSO co-treatment on CDDO-Im-mediated cytoprotection .....	32
Figure 18. BSO prevents the CDDO-Im-mediated cytoprotection against acrolein-induced lipid peroxidation and protein damage in HepG2 cells .....	33
Figure 19. Proposed actions of acrolein toxicity and interaction by CDDO-Im .....	40

# CHAPTER I

## INTRODUCTION

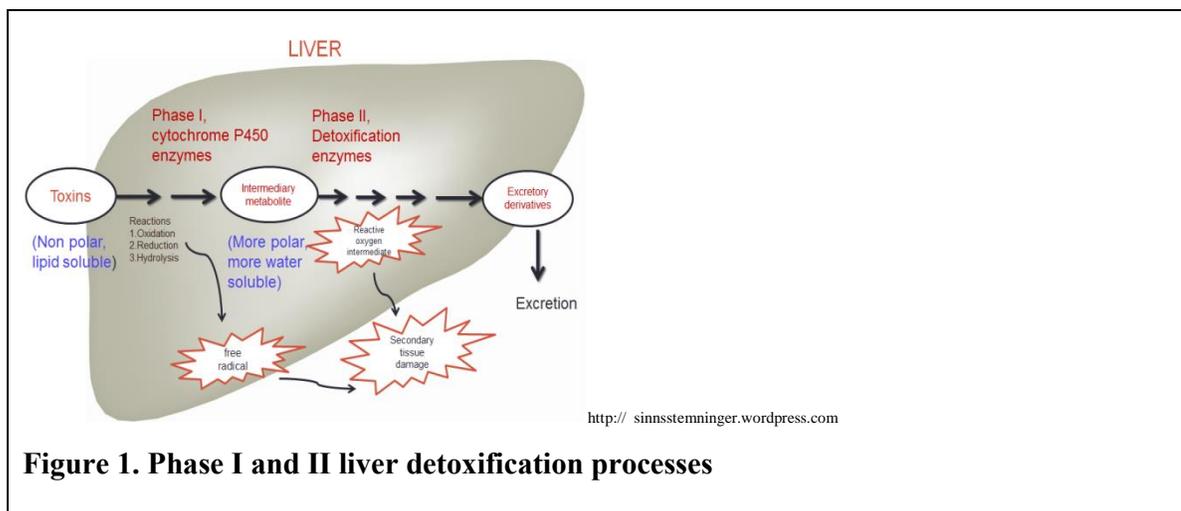
### *Hepatotoxicity*

The liver is the principal organ for xenobiotic transformation. Most exogenous chemicals are metabolized and eventually secreted into the liver. Hence, liver cells, or hepatocytes, are highly exposed to significant concentrations of such chemicals, which can result in liver dysfunction, cellular injury and, in extreme cases, organ failure[1].

There are several explanations as to why hepatocytes are more susceptible to toxic agents. Firstly, the liver is a major site of biotransformation processes for both endogenous and exogenous compounds. Secondly, hepatocytes are exposed to large volume of blood flow per unit of time containing xenobiotics, which are foreign substances, chemicals or drugs. Thus, liver cells are extensively exposed to these compounds when compared to other organs in the body. Thirdly, hepatocytes contain many different metabolizing enzymes that are classified as either phase I or phase II enzymes[2].

Phase I enzymes, such as cytochrome P450s (CYPs), are involved in the oxidation, reduction and hydroxylation of xenobiotics often leading to bio-activation of these xenobiotics[3]. Most chemicals or compounds that are bio-activated by CYPs in the liver have an increased chance of interacting and damaging the liver tissue. CYPs are

known to generate excess reactive oxygen species (ROS) resulting in oxidative stress which leads to liver cell damage. To counteract the detrimental oxidative and electrophilic species generated by CYPs, mammalian cells have evolved an efficient phase II detoxifying enzyme system to deactivate toxic metabolites, which results in the protection of these cells against oxidative and electrophilic damage. The phase II detoxification system includes glutathione (GSH) and GSH-related enzymes such as GSH-transferase (GST), GSH-peroxidase (GPX) and GSH-reductase (GR) [4-6]. Thus, studying cytoprotection via targeting the cytoprotective phase-II detoxifying system in the liver is of particular interest and has been proposed as a therapeutic strategy to counteract liver damage [7]. Numerous chemicals, arising from the external environment as well as from within the cell, can cause liver cell damage. One primary example of such environmental toxin is acrolein.



**Figure 1. Phase I and II liver detoxification processes**

## ***Acrolein***

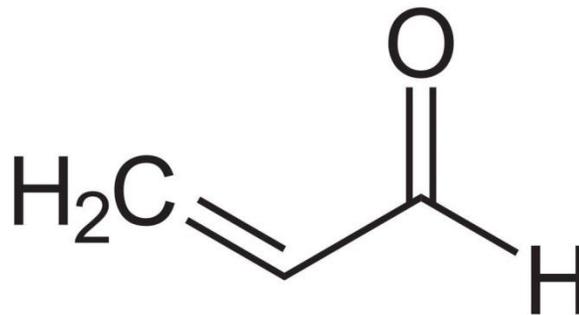
Acrolein is present in varying concentrations in our natural and artificial environments. Chemically, acrolein is a highly reactive  $\alpha,\beta$ -unsaturated aldehyde, with very strong electrophilic characteristics [8]. It is a byproduct of a chemical process forming acrylate polymers and DL-methionine and is industrially used as herbicides and slimicides [9]. Acrolein is found in foods such as fruits, wine, cheese and beer at very low concentrations [10]. Environmentally, acrolein naturally exists in food and is released by incomplete combustion of organic matter. Thus, acrolein is found in smoke released from cigarette smoking, the exhaust pipes of internal combustion engines and in vapors of overheated cooking oil.

Humans are mainly exposed to acrolein via cigarette smoke [11]. A single cigarette can contain 25-50  $\mu\text{g}$  of acrolein and constitutes as one of the many hazardous materials that is a health risk in cigarettes [8, 12]. Smokers are exposed to about 40 times more acrolein than concentrations found environmentally. Normal levels of acrolein in the environment are usually at 0.04 to 0.08 ppm but can be present up-to 90 ppm in cigarette smoke [2].

*In vivo*, acrolein is a metabolic byproduct of anticancer drug cyclophosphamide. It is found at sites of inflammation and formed from threonine via neutrophil myeloperoxidases enzymes [13]. Previous studies have demonstrated that allyl alcohol, a synthetic reactant in production of butanediol or glycerol, is metabolized via alcohol dehydrogenase and NADH to acrolein in the rat liver and lung tissue [14]. Elevated

concentrations of acrolein, of up to 180  $\mu\text{M}$ , have been found in various human tissues [15-17]. The normal concentration of acrolein-protein adducts in the normal human plasma is about 30-50  $\mu\text{M}$  [15], whereas the plasma concentration of acrolein in patients with chronic renal failure has been reported as high as 180  $\mu\text{M}$  [17, 18].

Various studies have shown that acrolein can also cause injury of the respiratory tract and suppression of the pulmonary host defense. It alters gene regulation, mucociliary transport and alveolar barrier integrity, which ultimately causes acute lung injury, chronic obstructive pulmonary disease (COPD) and possibly asthma and lung cancer[19, 20]. Acrolein is reported to induce oxidative stress thereby causing mitochondrial dysfunction in brain cells[21]. The specific toxic effect of acrolein on hepatocytes, however, still remains unclear.



**Acrolein (prop-2-enal)**

**Figure 2. Chemical structure of acrolein**

## ***Oxidative stress and cell death pathways***

Extensive bodies of literary evidence have demonstrated that a number of xenobiotics exert their toxicity via generation of ROS. The subsequent resultant oxidative stress can cause oxidative modification of cellular macromolecules such as lipids, proteins and DNA. Oxidative stress typically occurs in living organisms because of excessive production of ROS. ROS include all oxygen free radicals that can be generated exogenously by radiation, Fenton-type reactions, exposure to environmental pollutants and chemicals and from endogenous sources such as the mitochondria, NADPH oxidase, CYPs and other metabolic reactions[22].

The major types of ROS are the superoxide radicals ( $O_2^-$ ), hydroxyl radicals ( $HO^\cdot$ ) and non-radical hydrogen peroxide ( $H_2O_2$ ) [23, 24]. Mitochondria are considered to be the main source of superoxide ( $O_2^-$ ) which is reduced to hydrogen peroxide ( $H_2O_2$ ) as a consequence of electron transport and ATP synthesis[25].  $H_2O_2$  is not a very reactive free radical alone but can form the extremely reactive  $HO^\cdot$  radical through a Fenton-type reaction. Superoxide also reacts with nitric oxide at a rapid rate forming reactive free radical peroxynitrite.

Oxidative damage to lipids by ROS is known as lipid peroxidation, which can cause increased membrane fluidity, efflux of cytosolic solutes and loss of membrane protein function. Extensive lipid peroxidation can also lead to disintegration of membrane integrity and ultimately cellular death.

There are two major pathways of cell death: apoptosis and necrosis. Apoptosis is a programmed cell death, while necrosis is a sudden cell death. Apoptosis is characterized by retraction of pseudopods, reduction of cellular volume, chromatin condensation, nuclear fragmentation, blebbing of plasma membrane and engulfment by phagocytes [26]. Apoptosis occurs normally during aging and development stages to maintain cellular homeostasis and thereby maintain the cellular population in the tissue. Many environmental toxicants can provide external stimuli and conditions which trigger for apoptotic cell death. Extensive apoptotic cell death has been implicated to cause neurodegenerative disorders, such as Alzheimer's [27]. Necrosis is a term used to define a form of cellular injury that results in sudden and pre-mature death of cells in the living tissue. It is characterized by disintegration of cell wall, reduction in ATP levels and leakage of plasma membrane contents, which ultimately causes dysfunction of cellular organelles [28]. Necrosis is also known to cause inflammation of the surrounding tissue [28]. Necrosis has been suggested to be the result of acute cellular dysfunction in response to exposure to toxic agents. Oxidative stress and its association with cell death (apoptosis and necrosis) have been implicated as a possible mechanism contributing to the toxicity of many external environmental toxins or infections [29]. Whether these events are also involved in acrolein-induced cytotoxicity in hepatocytes remains unclear.

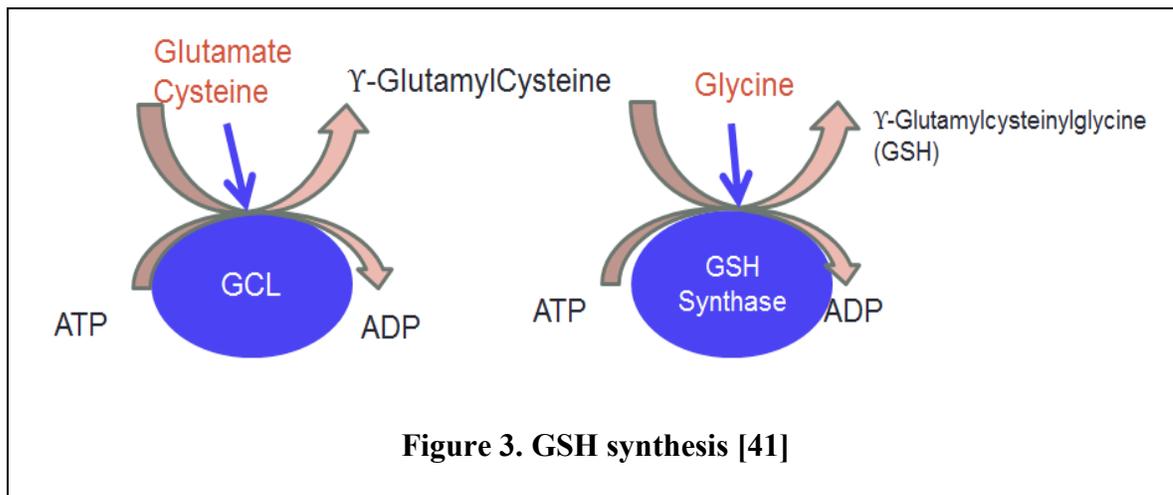
### ***Up-regulation of phase II detoxifying enzymes against acrolein toxicity***

Studies have shown that GSH, glutathione-s-transferase (GST) and aldose reductase (AR) play important roles in the detoxification of aldehydes derived from oxidative processes [30, 31]. GSH is the major endogenous antioxidant and has been found in high amounts in stressed cells. GSH is comprised of three amino acids — glutamine, cysteine and glycine. It forms  $\gamma$ -L-glutamyl-cysteinyl-glycine via 2 step *de novo* synthesis (figure 2). It is naturally present at 0.5-10 mM concentration in cells. In response to oxidative stress GSH is converted to its oxidative state, glutathione disulfide (GSSG), which regenerates reduced GSH via glutathione reductase and NADPH. About 90% of glutathione is present in its reduced form in the cell. While it is found in its disulfide state in its extracellular environment; minute changes in GSH intracellular state can cause very significant effects on redox dependent cell signaling. GSH has also been shown to play a very important role in protecting the cell against cellular damage induced by environmental toxicants, electrophiles and variety of other stress inducers[32, 33].

Apart from maintaining reductive cytosolic environment, glutathione also plays a vital role in detoxifying electrophiles via glutathione-s-transferase (GST) conjugation [34]. GSH has been shown to react with organic hydroperoxides and electrophilic reactive aldehydes, including acrolein, to form a less reactive conjugate that can be facilitated by GST [35]. This suggests that GST, utilizing GSH as a cofactor, plays a critical role in detoxification of reactive aldehydes [33]. GST was further proposed to be an important defense mechanism against pathogenesis of multiple disease processes[36].

Another line of cellular defense against reactive aldehydes has been thought to be AR. It has been reported that AR has a GSH-binding site, with the GSH-aldehyde conjugate serving as a substrate for AR [37]. Overall, these studies suggest that GSH, GST and AR may play a critical role in efficient detoxification of acrolein. Thus, up-regulation of GSH, GST and AR could be an important defense mechanism for the prevention of acrolein-mediated toxicity as acrolein is an important environmentally ubiquitous hepatotoxicant.

In our study we proposed a novel strategy to protect against acrolein-induced liver toxicity through the up-regulation of endogenous detoxification enzymes in HepG2 cells mediated by the triterpenoid compound 2-Cyano-3,12-dioxoleana-1,9-dien-28-imidazolid (CDDO-Im). Triterpenoids are steroid-like compounds derived from plant extracts which have been shown to increase levels of GSH and GST in mouse tissue protecting against aflatoxin-induced toxicity[38]. Several synthetic analogs of CDDO have been used in clinical trials as an anti-cancer proliferation treatment and an anti-inflammatory agent for rheumatoid arthritis [39, 40].

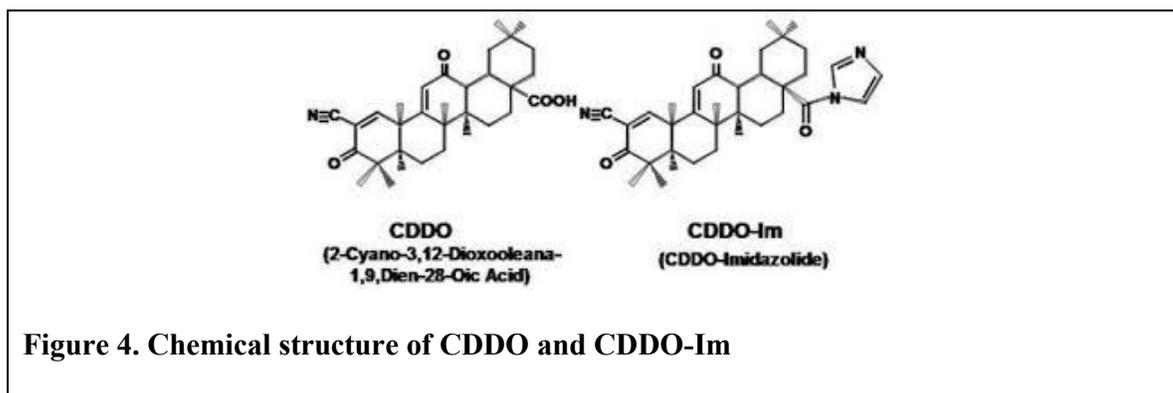


### ***Proposed Study***

This research was initiated to improve understanding of the toxic effects of acrolein on HepG2 cells in culture. HepG2 cells made a good cell line for this study because they possess different phase I and phase II xenobiotic-metabolizing enzymes [42]. The overall hypothesis of the thesis was that acrolein mediates cytotoxicity potentially due to its effects on GSH and other phase II detoxifying enzymes leading to lipid peroxidation and protein damage. The induction of phase II detoxification enzymes by CDDO-Im might then result in cytoprotective effects in HepG2 cells against acrolein toxicity.

Accordingly, the first aim was to assess the cytotoxicity of acrolein on the HepG2 cell line evaluated by MTT, LDH, flow cytometric analysis, lipid peroxidation, protein damage and change of phase II detoxifying systems, in particular GSH, GST and AR. The second aim was to determine the cytoprotective effects of CDDO-Im on acrolein-mediated cellular injury in HepG2 cells. The final aim was to determine the mechanistic

role of GSH in CDDO-Im-mediated protection against acrolein-induced cytotoxicity. Uncovering the change in intracellular levels of phase II detoxification enzymes and some of the other molecular events altered by acrolein may have implications for developing protective strategies against reactive aldehyde-induced cytotoxicity.



## CHAPTER II

### MATERIALS AND METHODS

#### *Chemicals and materials*

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/ streptomycin; fetal bovine serum (FBS) and trypsin were purchased from Gibco-Invitrogen. CDDO-Im (2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide) was purchased from Toronto Research Chemicals, Inc. Glutathione (GSH), oxidized glutathione (GSSG), yeast-derived glutathione reductase, 1-chloro-2,4-dinitrobenzene (CDNB), o-phthalaldehyde (OPT), NADPH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bovine serum albumin (BSA) were purchased from Calbiochem. Acrolein and hydrogen peroxide were purchased from Sigma. Tissue culture flasks and 24- and 48-well plates were purchased from Corning.

#### *Tissue culture*

HepG2 cells were purchased from ATCC. Tissue culture flasks were kept at 37<sup>0</sup>C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were fed with DMEM media with 10% FBS, 100 ug/mL of penicillin and 100 ug/mL of streptomycin every other day. Cells were sub-cultured once they reached 80% confluence.

### ***Cell extraction preparation***

After treatment, cells were collected and resuspended in ice-cold 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer containing 2 mM EDTA at pH 7.4. Sonicated cells were centrifuged at 13,000 rpm at 4<sup>0</sup>C for 10 minutes. Supernatants were collected from centrifuged tubes and kept on ice for measurement of glutathione and phase II enzymes.

### ***Assay for glutathione (GSH) content***

Total cellular GSH content was measured using o-phthalaldehyde-based fluorometric method that is specific for determination of GSH at pH 8.0[43]. The cell extract (10 µL) was incubated with 12.5 µL of 25% metaphosphoric acid and 37 µL of 0.1 M sodium phosphate buffer containing 5 mM EDTA at pH 8.0 at 4<sup>0</sup>C for 10 minutes. The samples were centrifuged at 13,000 rpm at 4<sup>0</sup>C for 5 minutes. The resulting supernatant was incubated with 100 µL of o-phthalaldehyde solution and 1.89 mL of 0.1 M sodium phosphate buffer containing 5 mM EDTA for 15 minutes at room temperature. Fluorescence intensity was then measured at excitation 350 nm and emission 420 nm. Cellular GSH content was calculated using GSH standard curve.

### ***Assay for glutathione- S-transferase (GST) activity***

The GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in a freshly prepared final reaction mixture of 0.6 mL[43]. The reaction mix containing GSH, 50 mM CDNB, 3 mg/mL of bovine serum albumin (BSA) in 0.1 M phosphate buffer at pH 6.5 was added to each cuvette. The reaction was started by adding

10  $\mu\text{L}$  of the sample and the rate of formation of CDNB-GSH conjugate was measured on spectrophotometer at 340 nm for 5 minutes at  $25^{\circ}\text{C}$ . The cellular GST activity was calculated using an extinction coefficient of  $9.6\text{ mM}^{-1}\text{ cm}^{-1}$  and was expressed as nmol of CDNB-GSH conjugate formed per min per mg of cellular proteins.

### ***Aldose reductase assay (AR)***

AR was measured at room temperature by following the oxidation of NADPH on spectrophotometer at 340 nm [44]. Reaction mixture for AR activity was made from 50 mM potassium phosphate with 0.4 M lithium sulfate and 10 mM of D-glyceraldehyde. To each cuvette 0.945 mL of assay mixture was added followed by 40  $\mu\text{L}$  of the sample. The reaction was started by adding 15  $\mu\text{L}$  of 10 mM NADPH. AR activity was measured at 340 nm at  $25^{\circ}\text{C}$  for 5 minutes.

### ***Cell injury***

Cell injury was measured using MTT assay, HepG2 cells were plated into 48-well plates. On day one they were treated with cytoprotectant CDDO-Im for 24 hr. On the second day, cells were treated with acrolein for 24 hr. On day three, all the media was removed and cells were fed with media containing 0.5% FBS and MTT (0.2 mg/mL) following two-hr incubation at  $37^{\circ}\text{C}$ . After two hr of incubation all the media was removed and cells were treated with 0.2 mL mixture of dimethyl sulfoxide, isopropanol and deionized water (1:4:5) to solubilize formazan crystals. The amount of dissolved formazan was measured at 570 nm[45].

### ***Assay for Thiobarbituric acid reactive substances (TBARS)***

For the TBARS assay, working standards (25, 50, 100, 150 and 200  $\mu\text{L}$ ) were added to microcentrifuge tubes and volumes were made up to 200  $\mu\text{L}$  with distilled water. A blank standard contained 200  $\mu\text{L}$  of distilled water. Then, 50  $\mu\text{L}$  of 8.1% SDS, 375  $\mu\text{L}$  of 20% acetic acid, 375  $\mu\text{L}$  of 0.8% TBA and 150  $\mu\text{L}$  of distilled water were added to all the tubes. All the tubes were incubated at 95<sup>0</sup> C for 60 minutes followed by a cooling procedure using tap water. The upper organic level was taken for photometric evaluation at 532 nm [46, 47] .

### ***Protein carbonyl (PC) assay***

The standards contained 300  $\mu\text{L}$  of DNPH and 300  $\mu\text{L}$  of 2 M HCl added to microcentrifuge tubes as the control. Then 75  $\mu\text{L}$  of each sample was added to the control and standard tubes followed by one hour incubation in the dark. After that, 375  $\mu\text{L}$  of 20% TCA was added to these tubes followed by 5 minutes of incubation on ice. Tubes were further centrifuged at 10,000 rpm for 10 minutes at 4<sup>0</sup> C. Supernatants were discarded and the above mentioned step was repeated two times. After centrifugation, the supernatant was discarded and pellet was resuspended in 500  $\mu\text{L}$  of ethanol followed by centrifugation at 10,000 rpm for 10 minutes at 4<sup>0</sup> C. This step was repeated one more time before the final wash. After the final wash, the protein pellet was resuspended in 500  $\mu\text{L}$  of 6 M guanidine hydrochloride. Tubes were centrifuged at 10,000 rpm for 10 minutes at 4<sup>0</sup> C. The supernatant was transferred from the sample and control tubes to 96-well plates and absorbance was measured at 370 nm using plate reader [48].

### ***Lactate Dehydrogenase (LDH) release assay***

HepG2 cells were grown up to 60% confluence in DMEM medium with 10% FBS in 24-well culture plate. Cells were exposed to treatment in 200  $\mu$ L non-phenol red DMEM with 0.5% FBS. Media was then collected into microcentrifuge tubes and was centrifuged for 5 minutes at 13,000 rcf at 4<sup>0</sup>C. Supernatant was then collected in another tube and was used for measuring LDH in spectrophotometer at 320 nm[49].

### ***Flow cytometry for cell death***

HepG2 cells, grown upto 85% in 55 cm<sup>2</sup> plates, were treated or untreated with different concentrations of acrolein and 100nM of CDDO-Im. Afterwards, cells were washed with PBS, trypsinized, and diluted to a concentration just under 5 X 10<sup>5</sup> cells/ml. A 25  $\mu$ l sample of cells was added to 475  $\mu$ l of Guava Viacount Reagent and was incubated in dark for 5 mins. Cell viability, apoptosis and cell death were determined with Guava Easycyte Miniflow Cytometer (Millipore). The Guava Viacount Reagent distinguishes between viable and non-viable cells based on differential permeability of DNA binding dyes in Viacount Reagent. The fluorescence of each dye is resolved operationally to allow quantitative assessment of viable and non- viable cells present in suspension.

### ***Real time PCR analysis***

The synthesis of GSH from its constituent amino acids involves the action of two ATP- dependent enzymes,  $\gamma$ -glutamyl cysteine ligase (GCL) and GSH synthase. GCL,

the rate limiting enzyme in the overall pathway, is a heterodimer composed of catalytic (GCLC) and modulatory (GCLM) subunit. GCLC retains all the catalytic activity and GCLM improves the catalytic efficiency. To study the effects of CDDO-Im on the cellular levels of m-RNA for GSH, HepG2 cells were incubated with 100nM of CDDO-Im for 1, 3, 6, and 24 hr. Total RNA from HepG2 cells were isolated using Trizol reagent. 1 µg of RNA from each sample was reverse transcribed to cDNA. The reaction mixture per each well contained 5 µl of distill autoclaved H<sub>2</sub>O, 10 µl of cyber green, 2 µl of forward and reverse primer. The primers used in quantitative real time PCR were GCLC (forward, 5'- ACCATCATCAATGGGAAGGA 3'; reverse, 5'- GCGATAAACTCCCTCATCCA-3') and GCLM (forward, 5'- CTCCTCTCGGGTCTCTCTC-3'; reverse, 5'-ATCATGAAGCTCCTCGCTGT-3'). The mean qualities of GCLC and GCLM were normalized based on the mean of control gene GAPDH [50].

### ***Statistical analysis***

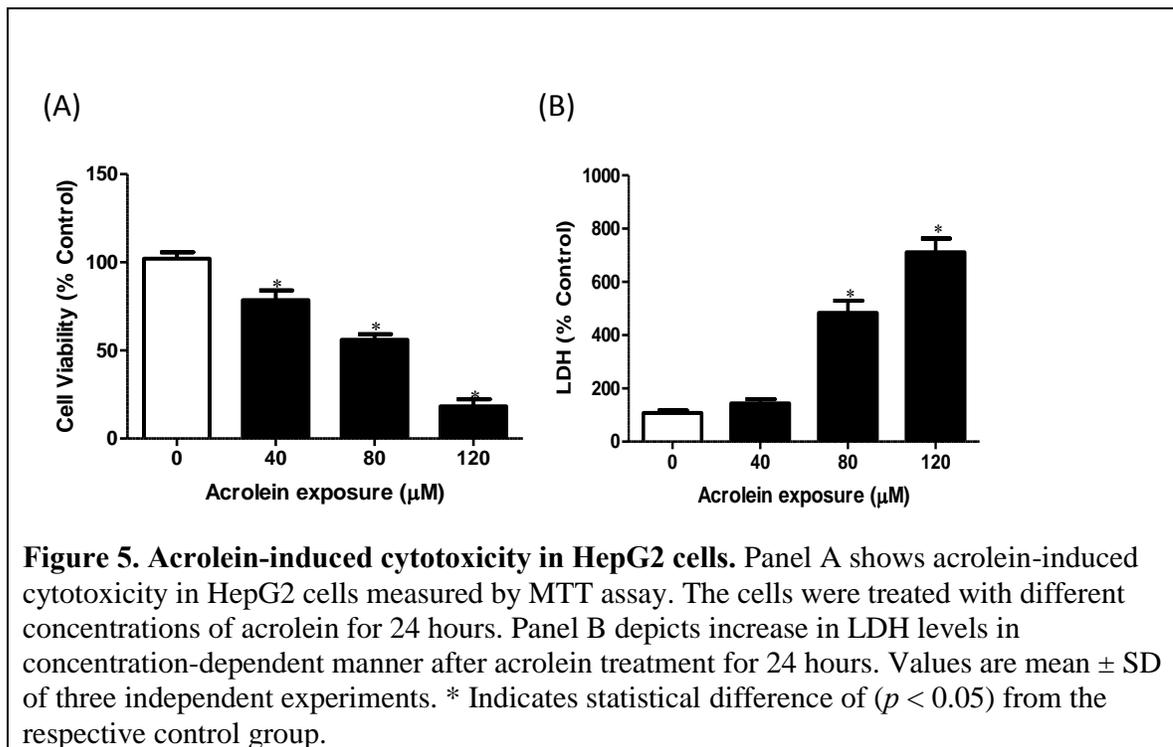
All data are expressed as mean ± SEM from at-least three different experiments. Differences between mean values of multiple groups were analyzed using one way analysis of variance (ANOVA). Differences between two groups were analyzed using student t-test. Statistical significance was considered at p<0.05

## CHAPTER III

### RESULTS

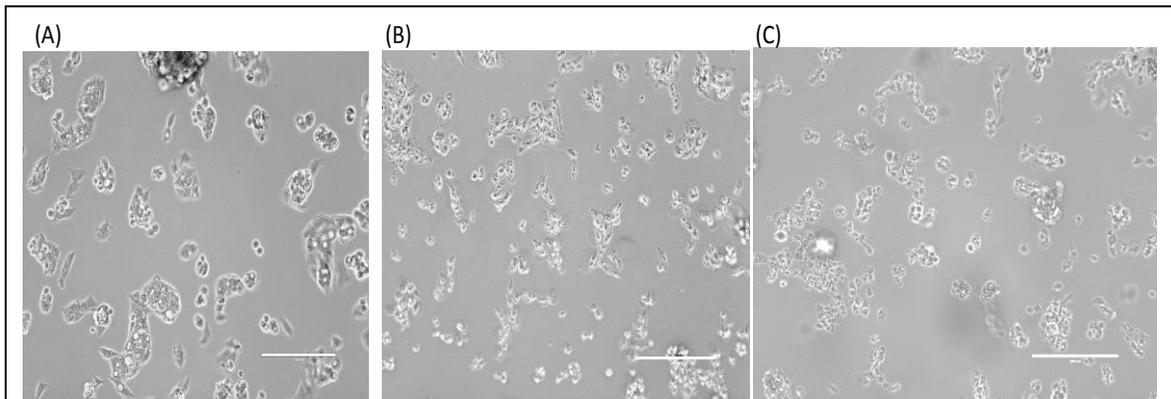
#### *Acrolein-induced cytotoxicity*

The cellular toxicity of different concentrations of acrolein on HepG2 cells was assessed by MTT and LDH release. HepG2 cells were treated with 40, 80 and 120  $\mu\text{M}$  of acrolein for 24 hours in order to obtain a dose-dependent response for acrolein exposure. Figure 5A shows the relationship between different concentrations of acrolein and the cell survival rate. Figure 5B shows LDH release with different concentrations of acrolein. Incubation of cells with various concentrations of acrolein for 24 hours caused a significant decrease in cell viability (Figure 5A) and increase in release of LDH (Figure 5B).



### *Effects of acrolein on cell morphology*

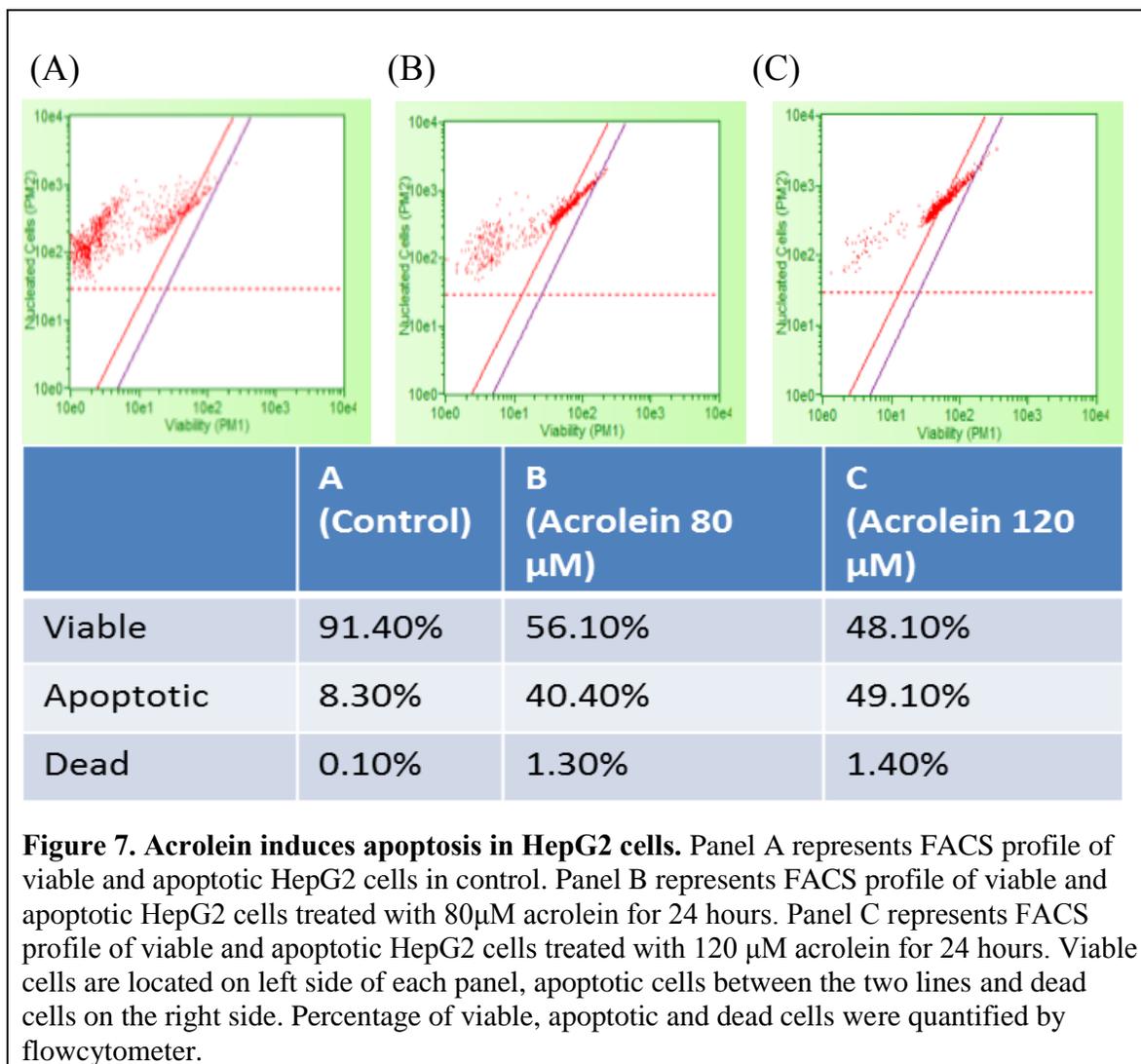
The acrolein-induced cytotoxicity was further investigated by examining the morphological changes of the cells using phase-contrast microscopy. Figure 6A represents healthy control cells. As shown in Figures 6B-C, cells treated with either 80  $\mu$ M (Figure 6B) or 120  $\mu$ M (Figure 6C) for 24 hours displayed significant changes in cell morphology including loss of uniformity and dead cell debris surrounding cell clusters. Cells treated with 120  $\mu$ M of acrolein showed more rounding of cells with larger amount of dead cell debris compared to cells treated with 80  $\mu$ M concentration of acrolein.



**Figure 6. Phase contrast images of HepG2 cells with and without treatment of acrolein.** Panel A shows control HepG2 cells, panel B and C show HepG2 cells treated with 80 and 120  $\mu\text{M}$  of acrolein for 24 hours.

### *Flow cytometric analysis*

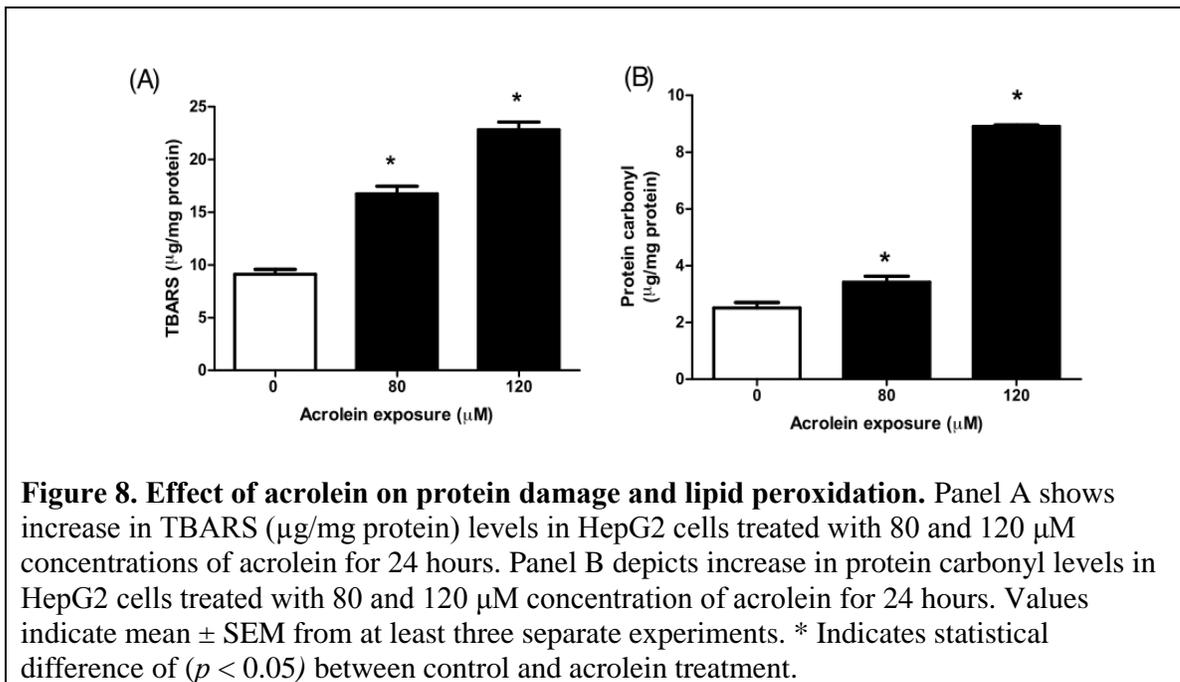
To examine the mechanism of cell death caused by acrolein exposure in HepG2 cells, the apoptotic and necrotic cell death were investigated by flow cytometric analysis (Figure 7). HepG2 cells were treated with 80 and 120  $\mu\text{M}$  concentrations of acrolein for 24 hours followed by analysis using guava count flow cytometer. As shown in Figure 7, cells exposed to acrolein had increased shift of cells towards apoptosis and late apoptosis/necrosis (Figure 7B and 7C) compared to control (Figure 7A).



### ***Protein damage and lipid peroxidation caused by acrolein in HepG2 cells***

To examine the cellular damage caused by acrolein, the amount of protein and lipid damage caused by various concentrations of acrolein in HepG2 cells was studied. For measuring protein damage and lipid peroxidation, HepG2 cells were incubated with 80 and 120 μM concentrations of acrolein for 24 hours. Panel A of Figure 8 shows the amount of thiobarbituric acid reactive substances (TBARS), a marker of lipid

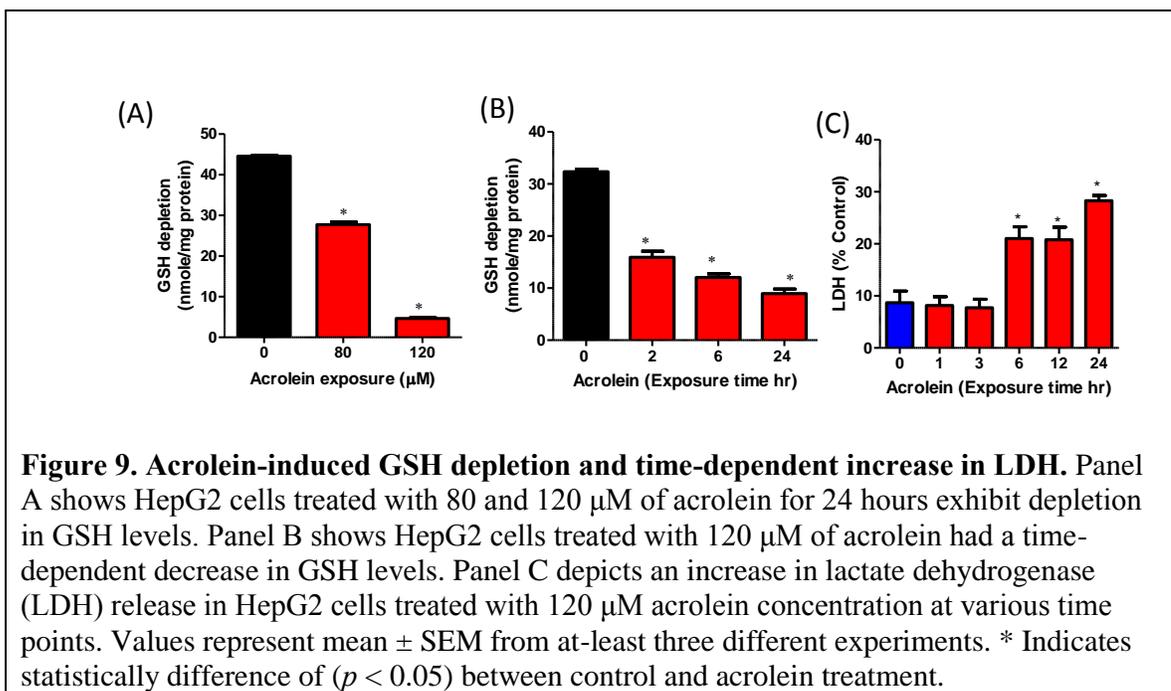
peroxidation, and Panel B shows protein carbonyl levels, a marker of protein damage, caused by acrolein in HepG2 cells. As shown in Figure 8, significant increases in TBARS and protein carbonyl levels were observed in cells treated with acrolein in a concentration-dependent manner.



### ***GSH depletion by acrolein in HepG2 cells***

Acrolein has been shown to react with GSH to form a less reactive conjugate [35]. To examine the role of GSH on acrolein-induced cytotoxicity, it was investigated if acrolein could cause depletion of cellular GSH content in HepG2 cells. HepG2 cells were incubated with 80 and 120 μM of acrolein for 24 hours. As shown in Panel A of Figure 9, incubation of cells with 80 μM and 120 μM acrolein resulted in a significant depletion of cellular GSH. Panel B of Figure 9 depicts the time-dependent decrease in GSH content in

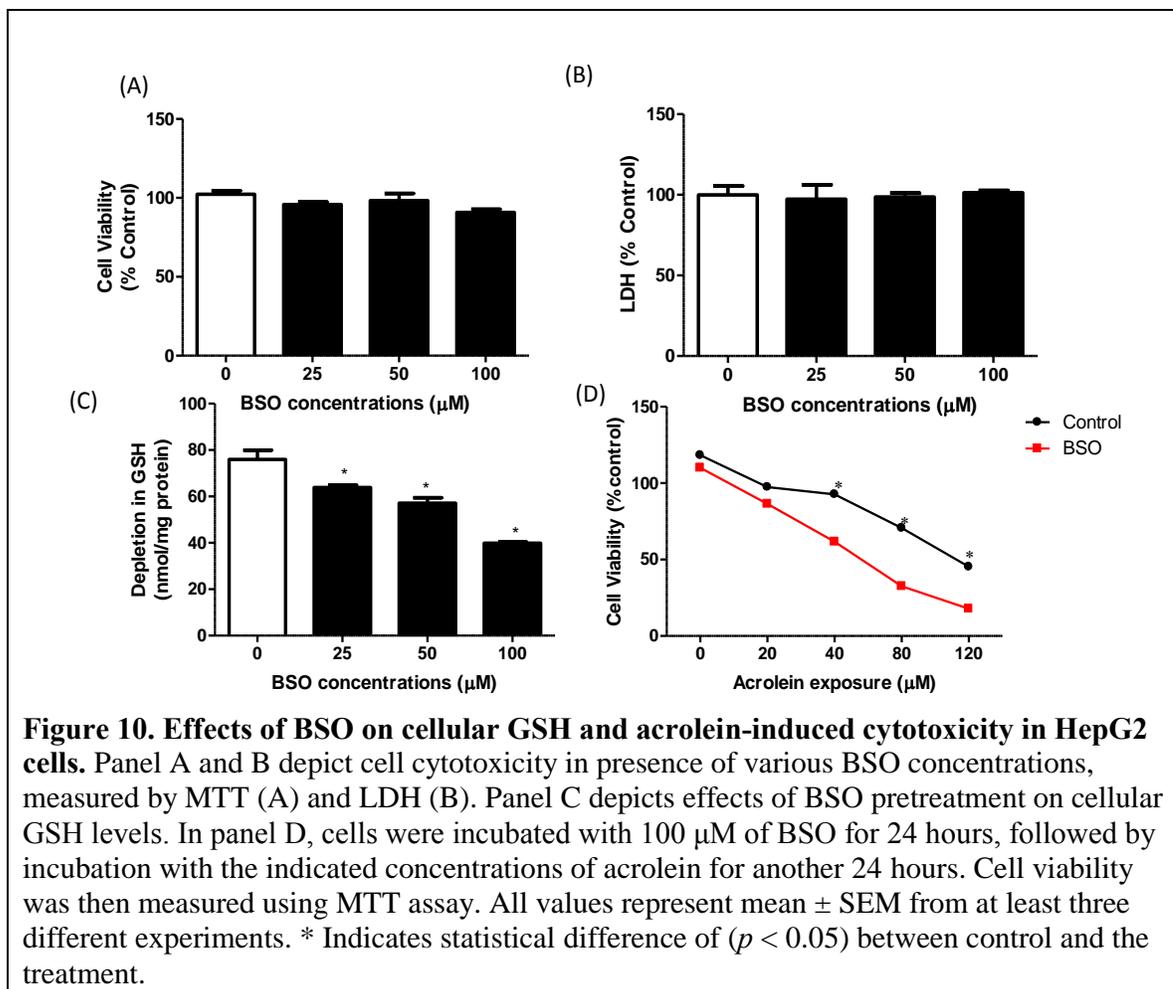
HepG2 cells treated with acrolein (120 $\mu$ M). Significant decrease in cellular GSH content can be observed after 2 hour incubation with 120  $\mu$ M of acrolein which preceded the decrease of cell viability as indicated by release of LDH to the culture media (Figure 9C). Both decrease in GSH content and release of LDH were time dependent (Figure 9).



### *Effects of BSO pretreatment on acrolein-induced cytotoxicity*

BSO (buthionine sulfoximine) is a very well-known inhibitor of GSH subunit gamma-glutamylcysteine synthetase (GCL). It is used widely for GSH depletion in various cells[51]. As shown in Figure 10C, incubation of HepG2 cells with 25, 50 and 100  $\mu$ M of BSO for 24 hours causes significant depletion in cellular GSH levels without altering cell viability (Figures 10A and 10B). To determine if depletion of cellular GSH by BSO could potentiate acrolein-induced toxicity, HepG2 cells were pretreated with 100

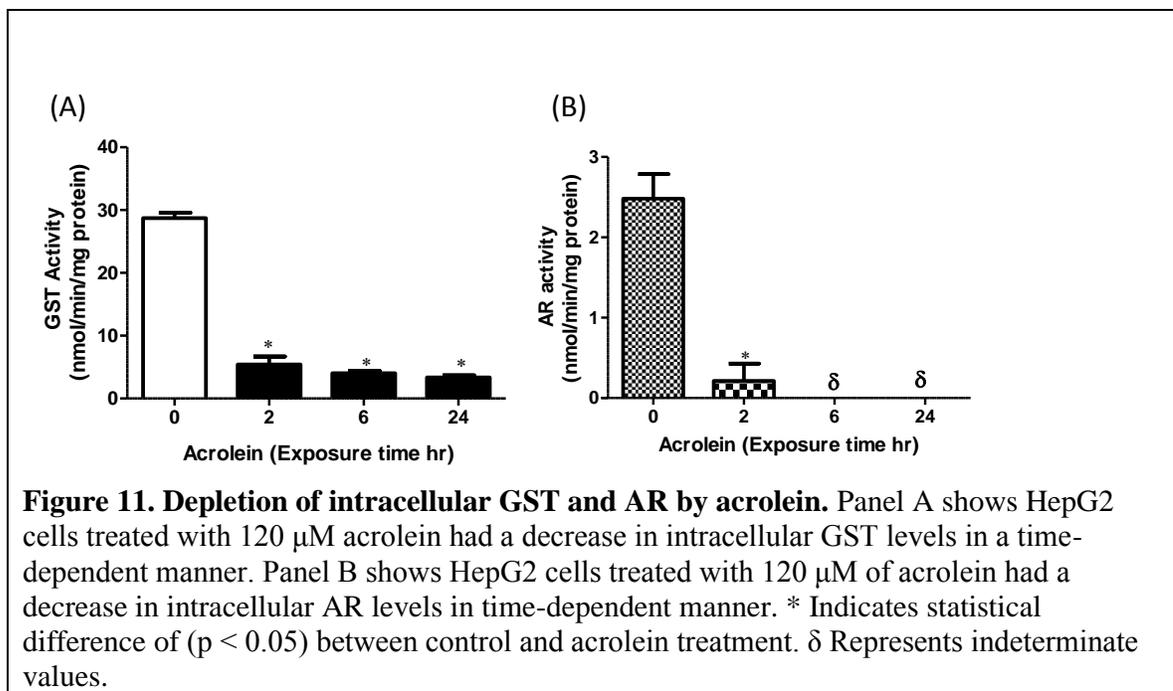
$\mu\text{M}$  BSO for 24 hours followed by treatment with various concentrations of acrolein for another 24 hours. As shown in Figure 10D, the observed MTT assay results indicate that pretreatment with 100  $\mu\text{M}$  of BSO potentiated acrolein-induced toxicity in HepG2 cells.



### *Depletion of intracellular GST and AR by acrolein*

Studies have shown that phase II detoxifying enzyme GST and AR are involved in the detoxification of aldehydes. To investigate effects of acrolein on intracellular levels of AR and GST in HepG2 cells, cells were treated with 120  $\mu\text{M}$  acrolein at various time

points followed by measurement of the activities of cellular GST and AR. As shown in Figure 11, a striking decrease in intracellular levels of GST and AR were observed after 2 hour incubation with 120  $\mu$ M of acrolein in HepG2 cells.

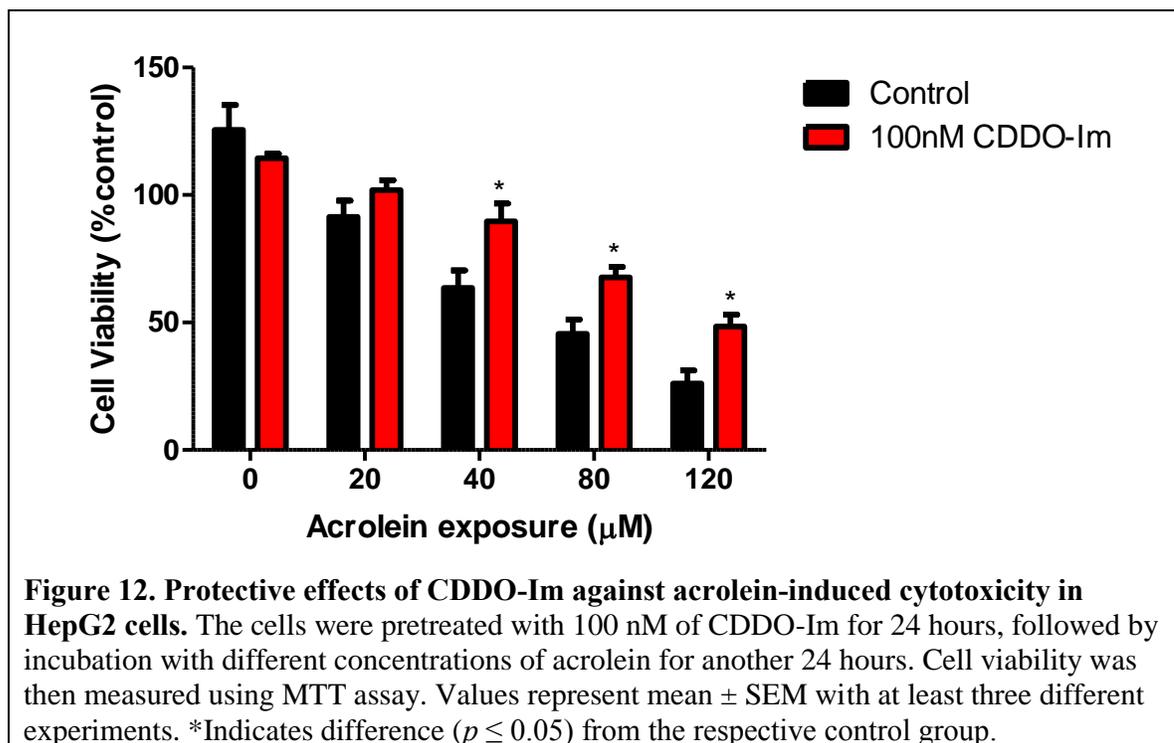


***Up-regulation of the endogenous GSH and phase II detoxifying enzymes by CDDO-Im protecting against acrolein-induced cytotoxicity***

Data shows that exposure to acrolein results in decrease in cellular GSH content and depletion of intracellular GST and AR activities (Figures 9-11). To further assess the contribution of these events to acrolein-induced cytotoxicity, it was investigated whether up-regulation of endogenous GSH and phase II detoxifying enzymes by a triterpenoid compound CDDO-Im could afford protection against acrolein toxicity in HepG2 cells.

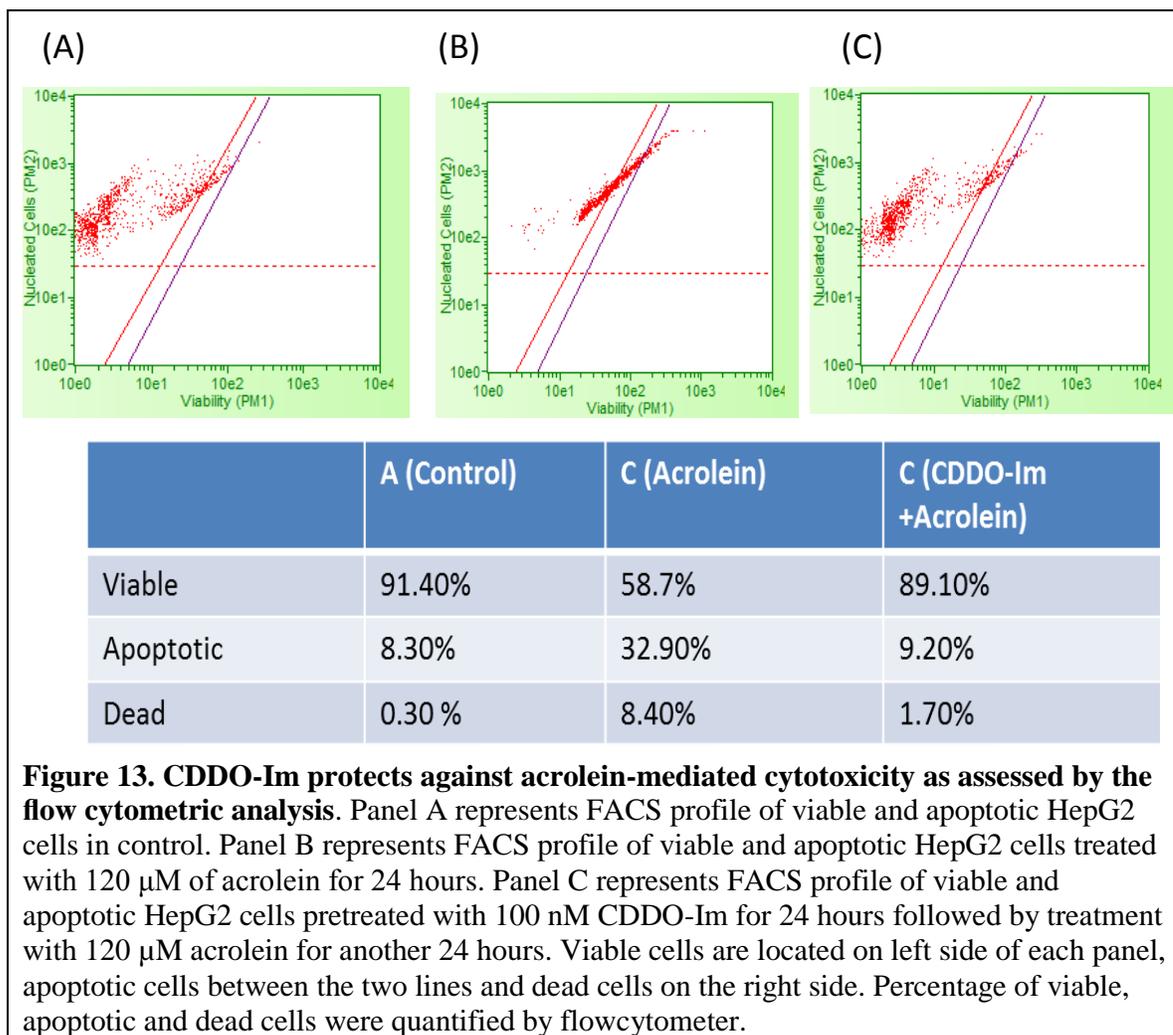
Triterpenoids are steroid-like compounds derived from plant extracts which have been shown to increase intracellular GSH and regulate phase-II enzymes protecting cells against oxidative damage. 2-Cyano-3,12-dioxoleana-1,9-dien-28-imidazolide (CDDO-Im) is a synthetic triterpenoid designed to be more potent to various types of oxidative stress [52]. However, there are no studies involving its protective capabilities against acrolein-mediated injury in HepG2 cells.

To investigate the cytoprotective effects of CDDO-Im on acrolein-mediated cytotoxicity, HepG2 cells were tested in response to acrolein exposure for 24 hours with or without a 24-hour pretreatment of 100 nM CDDO-Im. As shown in Figure 12, control cells showed a decrease in cell viability when treated with acrolein at concentrations of 40, 80 and 120  $\mu$ M for 24 hours. Conversely, the cells pretreated with 100 nM CDDO-Im for 24 hours showed a significant concentration-dependent increase in cell viability when treated with acrolein under the same conditions, as determined by the MTT assay.



### *Cytoprotective effects of CDDO-Im by flow cytometric analysis*

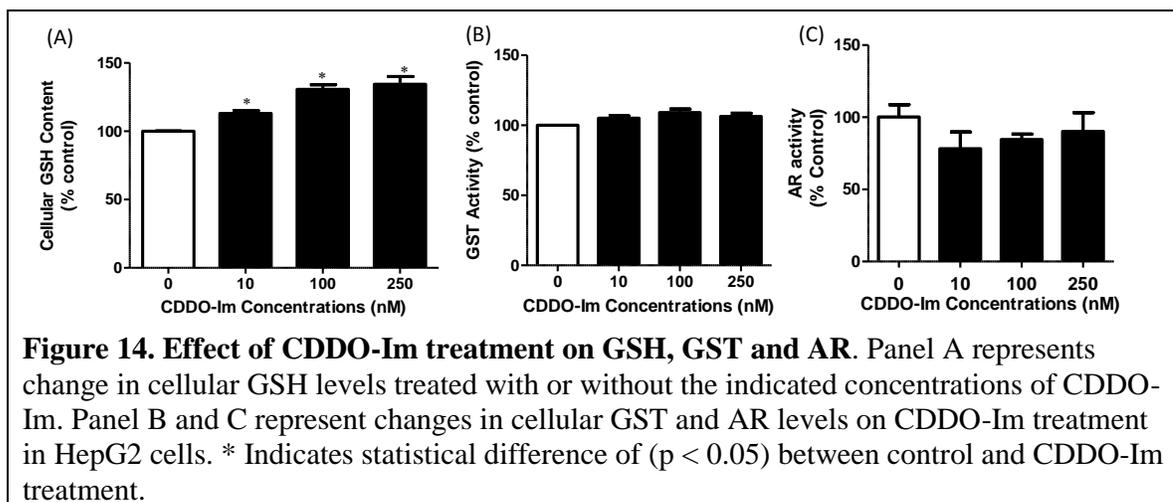
Flow cytometric analysis was further employed to examine the cytoprotective effects of CDDO-Im. To this end, HepG2 cells were treated with and without CDDO-Im for 24 hours followed by acrolein treatment for another 24 hours. As shown in Figure 13, HepG2 cells treated with 120 µM of acrolein showed a significant increase in apoptosis compared to control. Cells pretreated with CDDO-Im (100 nM) reduced the number of cells moving towards apoptosis compared to cells only treated with acrolein. This further supports results that CDDO-Im provides cytoprotection to HepG2 cells against acrolein-mediated toxicity.



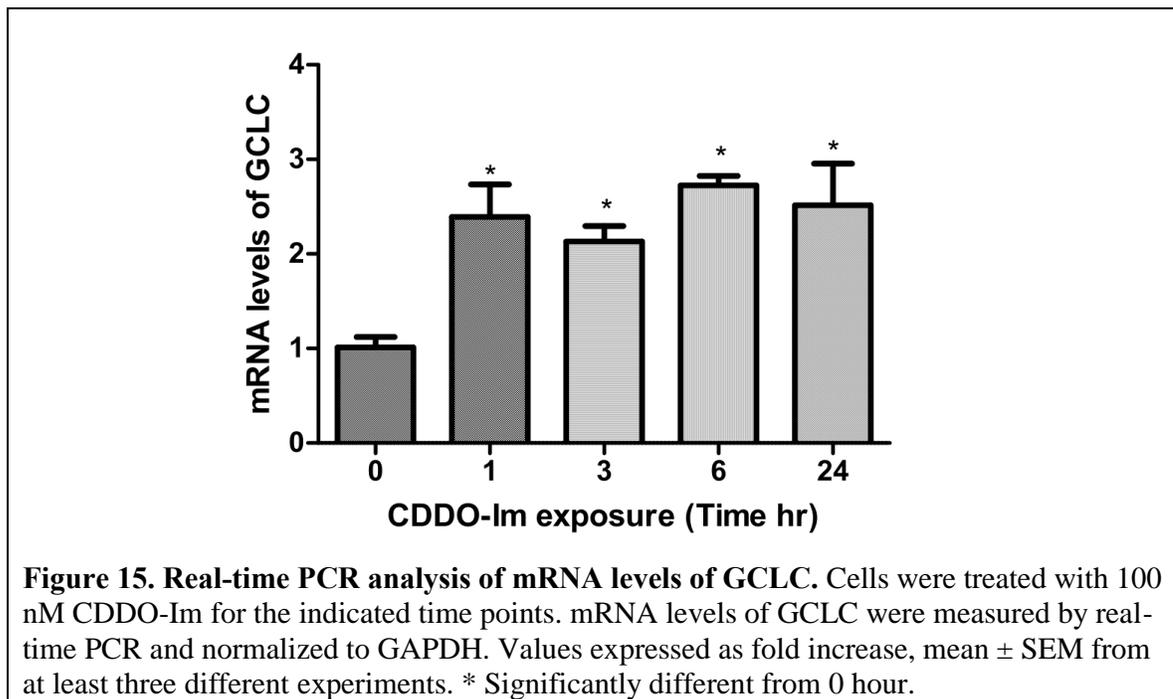
### ***Effects of CDDO-Im treatment on GSH content and GST, AR activity***

Results show that acrolein significantly decreased intracellular levels of GSH, GST and AR (Figures 9-11). Because GSH, GST and AR have been suggested to play a critical role in detoxification of acrolein, it was determined whether intracellular GSH and the activities of GST and AR are altered by CDDO-Im. Results showed that incubation of HepG2 cells with 100 and 250 nM of CDDO-Im for 24 hours resulted in

dramatic increase in the intracellular GSH content in a concentration-dependent manner (Figure 14A). Notably, incubation of HepG2 cells with CDDO-Im at a concentration as low as 10 nM lead to a 20-30% increase in cellular GSH content. However, the activities of GST and AR were not altered by these doses of CDDO-Im (Figure 14-B and C).

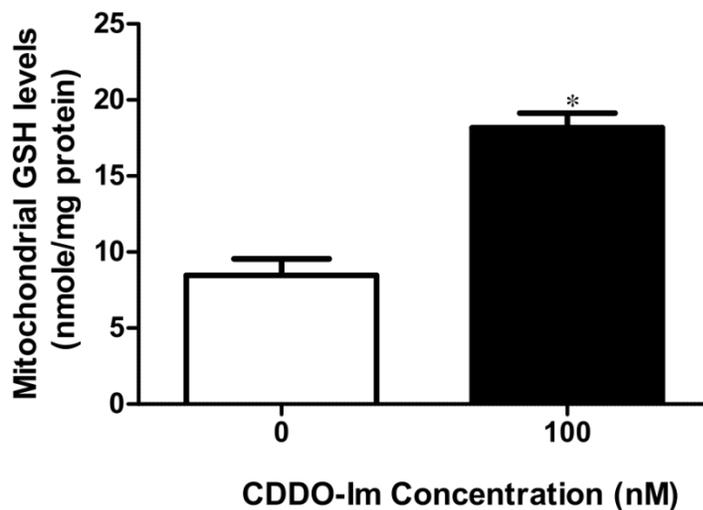


As intracellular GSH content was significantly induced by CDDO-Im, it was next determined if CDDO-Im treatment could also result in increased levels of mRNA for GCLC, which encodes for the peptide GCL, using a highly sensitive and specific real-time PCR assay. As shown in Figure 15, the mRNA levels for GCLC were induced. A maximum 2.72-fold elevation of the GCLC mRNA level was reached after incubation of HepG2 cells with CDDO-Im (100 nM) for 6 hours.



### *Induction of mitochondrial GSH by CDDO-Im*

GSH is known to be present in mitochondria of mammalian cells, and the mitochondria are major targets of ROS generated by reactive aldehydes such as acrolein. For these reasons, GSH levels from mitochondria isolated from HepG2 cells after CDDO-Im treatment for 24 hours were examined. Figure 16 shows significant increase in GSH content of mitochondria on 100 nM CDDO-Im treatment.



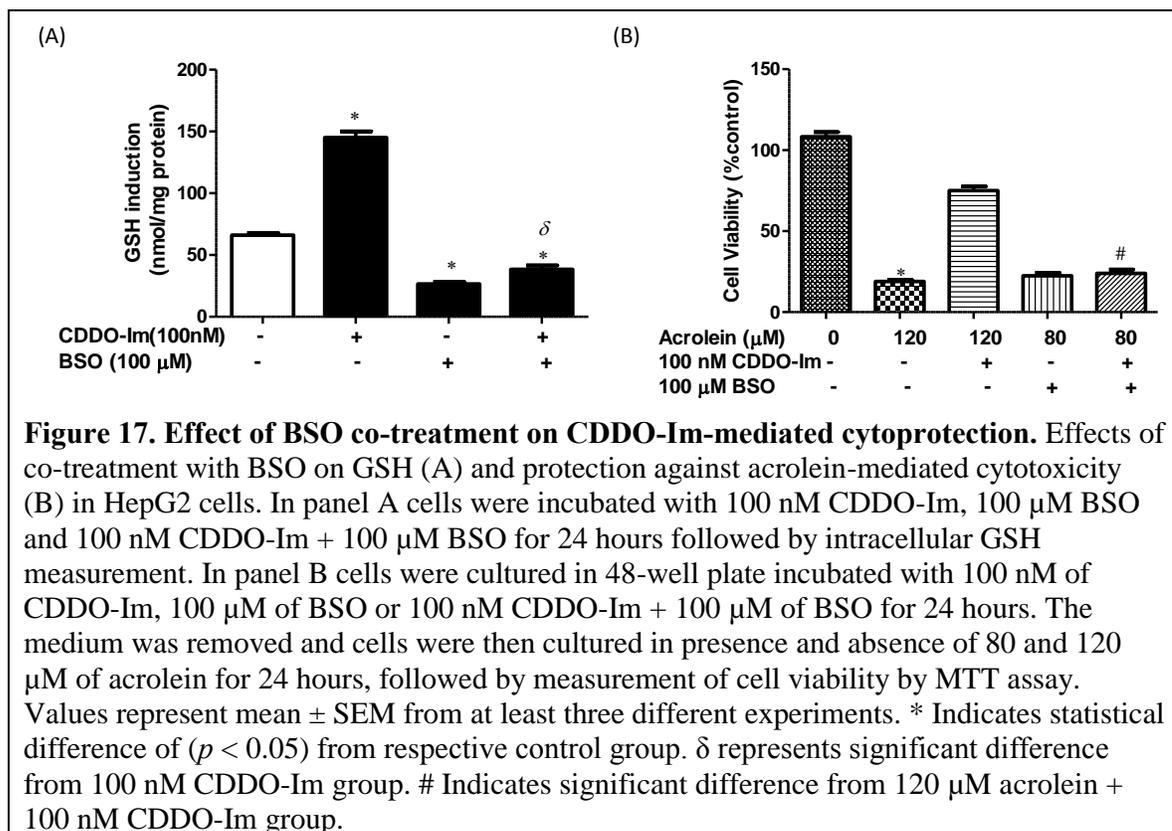
**Figure 16. Induction of mitochondrial GSH by CDDO-Im in HepG2 cells.** The cells were incubated with 100 nM of CDDO-Im for 24 hours. Mitochondria was then isolated and mitochondrial GSH levels were measured as described in “materials and method” session. All values represent mean  $\pm$  SEM from at least three different experiments. \* Indicates statistical difference of ( $p < 0.05$ ) between control and 100 nM CDDO-Im.

### ***Effects of BSO co-treatment on CDDO-Im-mediated cytoprotection against acrolein-induced cytotoxicity***

To further examine the role of GSH on CDDO-Im-mediated cytoprotection, the cell viability by co-treating HepG2 cells with CDDO-Im, BSO and acrolein was examined. HepG2 cells were pretreated with 100 nM of CDDO-Im in presence and absence of 100  $\mu$ M BSO, followed by acrolein exposure (80 or 120  $\mu$ M). Acrolein concentration of 120  $\mu$ M alone or 80  $\mu$ M with 100  $\mu$ M BSO were chosen as they were the lowest concentration to induce at least a 60% decrease in total cell viability (Figure 10D).

Figure 17A shows a decrease in GSH levels on co-treatment of HepG2 cells with CDDO-Im and BSO, which demonstrates that BSO prevented induction in cellular GSH

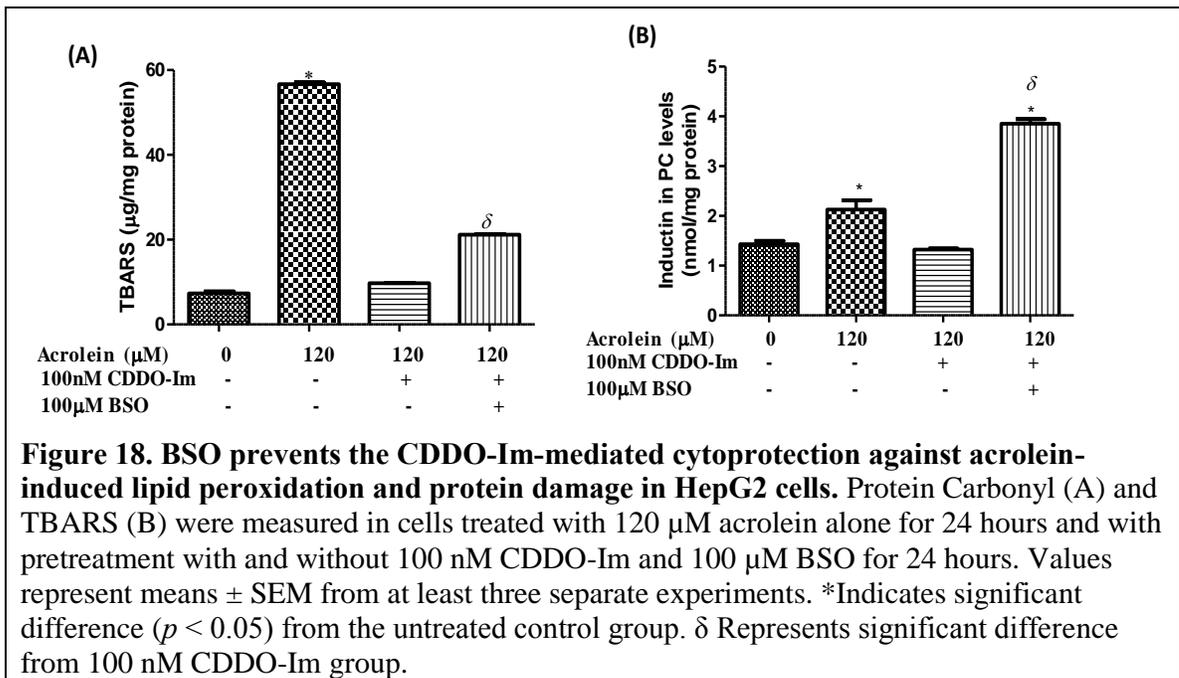
content by CDDO-Im. In Figure 17B, cell viability was measured using MTT assay, a comparative study in GSH induction in presence of acrolein. As shown in Figure 17B, incubation of cells treated with 120  $\mu$ M of acrolein for 24 hours showed a significant decrease in cell viability compared to cells pre-treated with 100 nM of CDDO-Im for 24 hours followed by acrolein treatment (120  $\mu$ M) for 24 hours. Incubation of HepG2 cells with 100  $\mu$ M BSO followed by 80  $\mu$ M acrolein exposure resulted in a significant decrease in cell viability. Similarly, cells pretreated with CDDO-Im (100 nM) and BSO (100  $\mu$ M) followed by 80  $\mu$ M acrolein treatment showed a significant decrease in cell viability, indicating a reduced cytoprotective effect of CDDO-Im. These results demonstrate that GSH played a predominant role in CDDO-Im-mediated cytoprotection against acrolein toxicity.



### ***Effects of CDDO-Im and BSO co-treatment on lipid peroxidation and protein damage***

To further examine the role of GSH on CDDO-Im-mediated cytoprotection against acrolein-induced lipid peroxidation and protein damage, the amount of protein and lipid peroxidation in HepG2 cells were measured. Cells were pre-treated with and without CDDO-Im (100 nM) and BSO (100 μM) for 24 hours followed by treatment with 120 μM of acrolein for another 24 hours. A significant increase in TBARS and protein carbonyl levels in cells treated with 120 μM acrolein compared to control (Figure 18) was observed. Pretreatment of HepG2 cells with 100 nM of CDDO-Im affords significant protection against acrolein-mediated lipid peroxidation (Figure 18A) and protein damage

(Figure 18B). When cells were pretreated with 100  $\mu$ M of BSO in addition to CDDO-Im, the TBARS and protein carbonyl levels were dramatically elevated. These results further confirm the role of GSH in CDDO-Im-mediated cytoprotection against acrolein-induced toxicity.



## CHAPTER IV

### DISCUSSION

Acrolein is a highly reactive  $\alpha$ ,  $\beta$ -unsaturated aldehyde ubiquitously found in the natural and artificial environment. It is also found naturally in food and water and, therefore, human exposure to this toxicant is very common. Reported or calculated levels of acrolein in humans vary greatly. The normal serum levels of acrolein in humans are estimated around 50  $\mu\text{M}$  [15]. However, acrolein concentrations in fluid lining the respiratory tract of smokers have been reported at levels as high as 80  $\mu\text{M}$  [53]. Due to its solubility in water and ability to easily cross cell membranes, elevated concentrations of acrolein of up to 180  $\mu\text{M}$  have been found in various human tissues [15, 16, 18]. The accumulated acrolein in patients with chronic renal failure has been reported to be equivalent to 180  $\mu\text{M}$ , causing damage to cellular macromolecules[18].

Since humans can be exposed to acrolein from the external environment as well as by endogenous generation, tissue-specific and localized levels of acrolein in the human body are more likely to be even higher than 180  $\mu\text{M}$ . Notably, liver is the major biotransformation organ, and phase I enzymes are most highly concentrated in the hepatocytes. Thus, compared to other organs of the body, liver is more susceptible to elevated levels of acrolein and tissue injury from the toxic effects of acrolein-mediated oxidative stress. It is important to note that the acrolein concentrations used in this *in*

*vitro* study (40-120  $\mu\text{M}$ ) are greater than expected in normal conditions but are in a range anticipated in the liver under pathological conditions.

Many clinical studies have linked cigarette smoking to hepatotoxicity, where smoking causes increase in liver fibrosis and hepatocellular carcinoma. A major source of acrolein is the smoke released from incomplete combustion of organic matter. Studies show that exposure to acrolein can lead to the liver damage and heavy cigarette smokers are repeatedly exposed to high concentrations of acrolein, contributing to severe liver problems [54]. However, the mechanisms involved in acrolein-induced hepatocellular toxicity are not completely understood.

This study examines the toxic effects and cytotoxic mechanisms of acrolein on HepG2 cells. The HepG2 cell line is widely used for studying hepatotoxicity/cytoprotection due to the high amount of phase I and phase II drug metabolizing enzymes in its cells. Results show dramatic decrease of intracellular GSH, GSH-linked phase II enzyme GST and phase II enzyme AR (Figures 9-11). This suggests that acrolein exerts its toxic effects in HepG2 cells by depleting phase II detoxifying system. Among them, GSH is a major intracellular non-protein antioxidant that plays an important role in attenuating the oxidative pathophysiology.

Many of the previous studies have suggested that depletion in cellular GSH levels leads to oxidative damage causing various types of liver disorders[33]. GSH has been suggested to directly react with acrolein to form a less reactive conjugate. The marked depletion of cellular GSH by acrolein in a concentration and time dependent fashion

(Figures 9) is also in agreement with the previous report that GSH may be a first line of cellular defense against acrolein-induced toxicity[6]. Results further show that depletion of cellular GSH by acrolein preceded the loss of cell viability (Figure 9) suggesting that that cellular GSH depletion may be an important event in acrolein-induced cytotoxicity.

To further investigate the involvement of GSH depletion in acrolein-induced cytotoxicity, BSO was used to deplete cellular GSH in HepG2 cells. BSO is a potent inhibitor of GSH without affecting cell viability. Results show that depletion of cellular GSH by BSO resulted in a dramatic potentiation of acrolein-induced cytotoxicity (Fig. 10). This further confirms that GSH plays a critical role in the detoxification of acrolein in HepG2 cells.

Apoptosis and necrosis are two mutually exclusive ways of cell death. Most toxicants, if not all, have been shown to induce apoptosis at low doses and are capable of producing necrosis at higher doses. The study further examined the type of cell death caused by acrolein in HepG2 cells by utilizing flow cytometry. Results show that acrolein-induced apoptosis in a concentration dependent manner indicating that the apoptosis may, at least in part, be associated with early GSH depletion and acrolein cytotoxicity.

Due to its high reactivity, acrolein toxicity has been suggested to result from covalent interactions of acrolein with critical target molecules such as lipids, proteins and DNA leading to altered target cell function [55]. Interestingly, acrolein is both an initiator and an end-product of lipid peroxidation. It has been suggested that acrolein causes the alteration of target molecules via oxidative stress, generation of excess reactive oxygen

species and alteration of endogenous antioxidants, such as depletion in GSH level in target cells[55]. In experiments, treatment of HepG2 cells with acrolein resulted in an accumulation of cellular MDA, an indicator of lipid peroxidation measured by TBARS assay (Figure 8). As lipid peroxidation is one of the major outcomes of oxidative stress-mediated injury that directly damages lipid membranes, this result indicated that acrolein toxicity could be due to generation of the lipid peroxidation.

In addition to lipid peroxidation, acrolein toxicity may be attributed to the covalent binding to side-chain amine groups (i.e., lysine, arginine, proline or histidine) of protein into carbonyls. The results of protein carbonyl increase by acrolein as shown in Figures 8 and 18 clearly indicate that cellular protein are modified, which is correlated with the increase in acrolein toxicity. It has been suggested that acute early depletion of GSH, GST and AR may cause an increase in oxidative stress leading to an abrupt onset of initiation of lipid peroxidation and protein damage [55].

To further check whether induction in GSH levels can provide cytoprotection against acrolein-mediated toxicity, HepG2 cells were pretreated with CDDO-Im followed by treatment with acrolein (Fig 12). Results demonstrated that CDDO-Im pretreatment protects cells against acrolein-induced cytotoxicity. Incubation with low nanomolar concentration of CDDO-Im results in a significant increase in cellular GSH levels (Figure 14 A) suggesting an important role played by GSH in acrolein toxicity. However, the same CDDO-Im treatment of HepG2 cells under present experimental condition did not induce intracellular AR and GST levels (Figure 14), indicating that these two aldehyde-

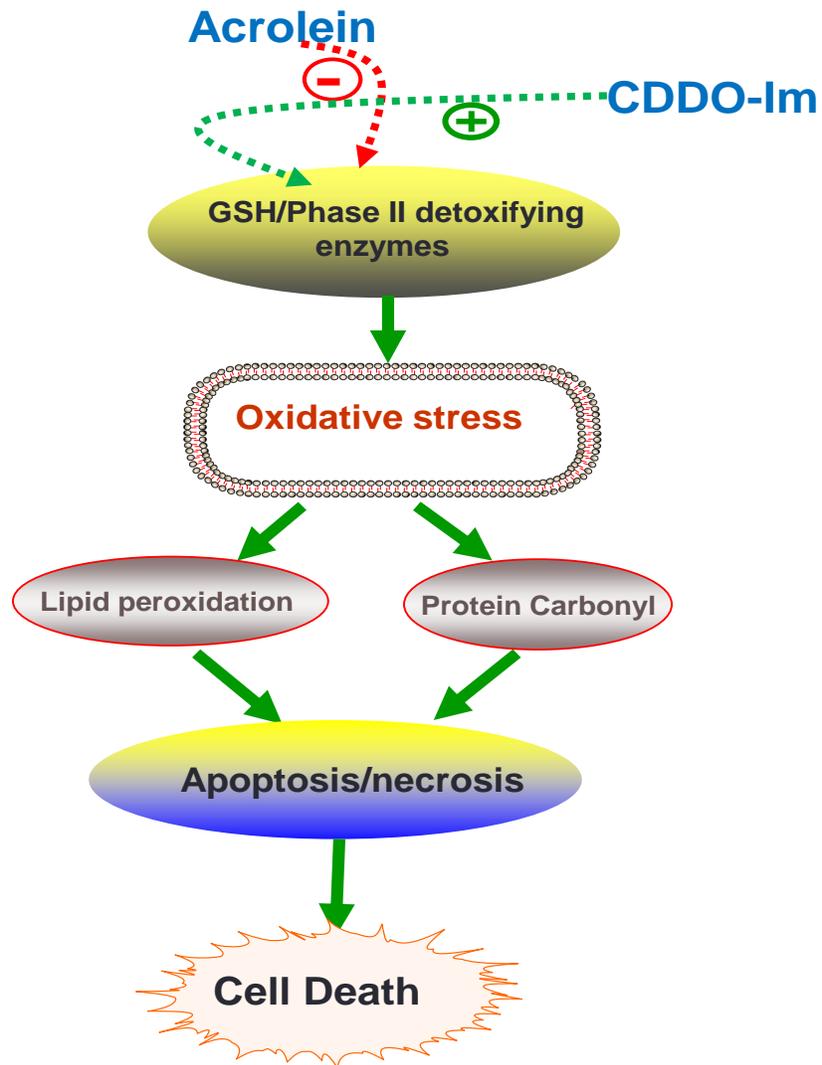
detoxifying enzymes in HepG2 cells might be regulated via distinct signaling pathways. GCLC, one of the subunits of GCL, is responsible for catalytic activity in GCL enzyme during GSH biosynthesis. The marked elevation of GCLC mRNA levels measured by real time PCR (Figure 15) indicated that induction of GSH by CDDO-Im in HepG2 cells appeared to occur via increased transcription of one of its subunit genes.

To further provide additional and more direct evidence for the involvement of GSH in CDDO-Im-mediated protection against acrolein detoxification process, HepG2 cells were treated with CDDO-Im in presence or absence of BSO followed by acrolein exposure. In this particular experiment HepG2 cells were treated with BSO to reverse the effect of GSH elevation caused by CDDO-Im (Figure 17). Co-treatment with BSO prevented the GSH induction caused by CDDO-Im and reversed the cytoprotective effects of CDDO-Im (Figure 17). This observation strongly indicates that elevation of cellular GSH content by CDDO-Im pretreatment provided significant protection against acrolein-mediated cytotoxicity in HepG2 cells. Data further showed that co-treatment with BSO also completely reversed CDDO-Im-mediated cytoprotective effects on acrolein-induced lipid peroxidation and protein damage in HepG2 cells (Figure 18).

Overall, these results demonstrated that induction of cellular GSH, while not GST or AR, is a primary mechanism underlying CDDO-Im-mediated protection against acrolein toxicity in HepG2 cells. This study for the first time suggests that induction of non-protein antioxidant GSH by synthetic triterpenoid CDDO-Im could be a novel strategy against acrolein-mediated cytotoxicity. These results further suggest that CDDO-Im may be a promising agent to provide protecting against electrophilic injuries causing

liver disorders. CDDO-Im is a lipophilic compound and hence should easily be able to cross the cell membrane. Although the cytoprotective effects of CDDO-Im against acrolein-mediated hepatotoxicity are largely unexplored, several synthetic analogs of CDDO as an anti-inflammatory agent for rheumatoid arthritis are currently under clinical investigation [52, 56, 57]

In summary, this study demonstrates that exposure to acrolein results in a rapid depletion of GSH, non-protein phase II enzyme and causes increase in apoptosis, lipid peroxidation and protein carbonylation. Furthermore, the endogenous antioxidant GSH can be induced by CDDO-Im and the CDDO-Im-mediated elevated GSH appears to afford a marked protection against acrolein toxicity suggesting that GSH plays a predominant role in CDDO-Im-mediated protection against acrolein-induced toxicity in HepG2 cells (Figure 19). This study may provide understanding on the molecular action of acrolein, which is important to develop novel strategies for the prevention of acrolein-mediated toxicity.



**Figure 19. Proposed actions of acrolein toxicity and interaction by CDDO-Im**

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