



**PROTECTION AGAINST α,β -UNSATURATED ALDEHYDE TOXICITY
BY HYDRAZINOPHTHALAZINE COMPOUNDS.**

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Abstract

α,β -Unsaturated aldehydes are byproducts of lipid peroxidation within the body. These bifunctional aldehydes are capable of inducing cell death by alkylating nucleophilic biomolecules, particularly lysine, histidine, cysteine and guanine residues. It was presumed that this toxicity could be attenuated using nucleophilic compounds that scavenge free aldehydes, thereby sparing nucleophilic biomolecules. Hydralazine, a hydrazine containing anti-hypertensive drug that readily traps biogenic aldehydes and ketones, was previously identified as a good cytoprotective agent against toxicity induced by the short chain 2-alkenal acrolein. The cytoprotection afforded by this arylhydrazine was therefore the main focus of this project, which was aimed at identifying/developing small nucleophilic compounds to prevent aldehyde-mediated toxicity and to determine the mechanism of hydralazine-mediated protection against such toxicity. Acrolein-mediated toxicity was achieved using the precursor allyl alcohol, which induces severe periportal liver damage via its alcohol dehydrogenase catalysed biotransformation to the aldehyde. While hydralazine and related arylhydrazines efficiently scavenged free acrolein from buffered solution and protected against allyl alcohol mediated hepatotoxicity in primary cultured hepatocytes and in whole mice, this scavenging reaction was unrelated to cytoprotection afforded by the arylhydrazines. Alternatively, using electrospray ionisation mass spectrometry and a model lysine-containing peptide (preproenkephalin, sequence GGEVLG**K**RYGGFM, m/z 1370) hydralazine was observed to be capable of trapping reactive acrolein-protein adducts. Thirteen hydralazine-trapped species were identified from cyclic and acyclic adducts of acrolein on both lysine and N-terminal glycine residues. This trapping reaction was shown to correlate well with hydrazine-mediated cytoprotection *in vitro* and *in vivo* by slot blot assays, Western blotting and immunohistochemistry using a rabbit antiserum raised against hydralazine-stabilised acrolein/KLH. Furthermore, by employing a short chain ketone (methylvinyl ketone) with limited cyclising capacity, acrolein-mediated toxicity was judged to be due to acyclic Michael adducts on proteins. Cytoprotection mediated by arylhydrazines was also found to correlate with the radical scavenging properties of the hydrazines, which act as both 1 and 2 electron donors, although to date, the relevance of this radical scavenging reaction to cytoprotection remains to be established in cells. Hydralazine and related arylhydrazines were

therefore proposed to provide protection against aldehyde-mediated toxicity by trapping reactive aldehyde-protein adducts, thereby preventing downstream events in toxicity.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Data 1/7/04.

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I’d also like to acknowledge my supervisors Dr Phil Burcham, who provided assistance with Western blotting experiments, and Dr Simon Pyke who calculated hydrazone coupling constants and contributed to other chemical aspects of this project.

Publications

First Author Journal Publications Arising from this Thesis (Appendix 5):

LM Kaminskas, SM Pyke, PC Burcham (2004) Strong protein adduct-trapping accompanies abolition of acrolein-mediated hepatotoxicity by hydralazine in mice. *Journal of Pharmacology and Experimental Therapeutics*. **In Press**

First Author Publications Submitted for Review:

LM Kaminskas, SM Pyke, PC Burcham (2004) Reactivity of hydrazinophthalazine drugs with the lipid peroxidation products acrolein and crotonaldehyde. *Submitted to Organic and Biomolecular Chemistry*.

Other Publications Arising During this Candidature:

PC Burcham, FR Fontaine, LM Kaminskas, DR Petersen, SM Pyke (2003) Protein adduct-trapping arylhydrazino drugs block the toxicity of acrolein. *Mol Pharmacol*. **65**: 655-664.

PC Burcham, LM Kaminskas, FR Fontaine, DR Petersen, SM Pyke (2002) Aldehyde-sequestering drugs: tools for studying protein damage by lipid peroxidation products. *Toxicology*. **181-182**: 229-236.

Abbreviations

AA	allyl alcohol
Ac	acrolein
AGE	advanced glycation end products
AIQ	aminoisoquinoline
ANOVA	analysis of variance
APH	acrylaldehyde-1-(phthalazin-1-yl)hydrazone
AQL	aminoquinoline
BCA	bicichoninic acid
BH	benzylhydrazine
BHT	butylated hydroxytolulene
BPH	but-2-enal(phthalazin-1-yl)hydrazone
BSA	bovine serum albumin
CA	crotyl alcohol
Crot	crotonaldehyde
Cu DIPS	copper diisopropylsalicylate
CV	coefficient of variation
DH	dihydralazine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPA	diphenylamine
DPH	diphenylhydrazine
DPPH	1,1-diphenyl-2-picryl-hydrazyl
ϵ	extinction coefficient
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
ESI	electrospray ionisation
g	grams
GPT	glutamate pyruvate transaminase
GSH	reduced glutathione
4-HNE	4-hydroxy-2-nonenal
HIQ	hydrazinoisoquinoline
HP	hydrazinopyridine
HPLC	high performance liquid chromatography
HQL	hydrazinoquinoline
HQZ	hydrazinoquinazoline
HYD	hydralazine
Inacc	Inaccuracy
ip	intraperitoneal
IR	infrared
KLH	keyhole limpet hemocyanin
l	litres
LCQ	liquid chromatography quadripole
LDH	lactate dehydrogenase
LDL	low density lipoprotein

MDA	malondialdehyde
ml	millilitres
MS	mass spectrometry
MS/MS	mass fragmentation of an ion of interest
MVK	methylvinyl ketone
<i>m/z</i>	mass/charge ratio
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
Nitro	nitrofurantoin
NMR	nuclear magnetic resonance spectrometry
PBS	phosphate buffered saline
Pent	pentenal
PP	propargyl alcohol
PPE	preproenkephalin fragment 128-140
ROS	reactive oxygen species
SDH	sorbitol dehydrogenase
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
Tempo	2,2,6,6-tetramethyl-1-piperidinyloxy radical
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
U	units
U/l	units per litre
UV	ultraviolet

Chapter 1:

General introduction

1.1 INTRODUCTION TO OXIDATIVE STRESS

1.1.1 Formation of Radicals

Pronounced oxidative stress is associated with a number of diseases prevalent during old age, including the aging process itself (Stadtman and Berlett, 1998). Specifically, the widely accepted definition of oxidative stress is the overproduction of free radicals by cells, coupled with an inability of cellular free radical defence mechanisms to counteract this overproduction. One of the most common sources of free radical generation is oxygen which can undergo a number of splitting, abstraction and rearrangement reactions to produce reactive oxygen intermediates. Among these are the superoxide radical ($O_2^{\bullet-}$), singlet molecular oxygen (O_2^1), the hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$). Several of these species are released by the mitochondria in the incomplete reduction of oxygen during respiration (Wallace, 1997). Radicals are also formed enzymatically via enzymes such as hydroperoxidase which yields $NAD^{\bullet}$ and $NADP^{\bullet}$ from $NADH$ and $NADPH$ respectively, or via the generation of hydrogen peroxide from superoxide dismutase and the superoxide radical (Shapiro, 1998). Among these oxygen derived radicals, the most significant to the destruction of cellular macromolecules is the hydroxyl radical which readily accepts single electrons from weak H-C bonds. Hydroxyl radicals are also formed via the Harber-Weiss reaction between superoxide and hydrogen peroxide and via transition metal (eg. Fe^{+2}) catalysed reduction of hydrogen peroxide.

Normally, radicals are removed from the body by enzymatic and non-enzymatic processes. Enzymes responsible for the termination of radicals include superoxide dismutases, which remove superoxide radicals by the addition of hydrogen ions to produce hydrogen peroxide, and

catalase and glutathione peroxidase which reduce hydrogen peroxide to water. Antioxidants such as α -tocopherol, β -carotene and ascorbate also terminate radical reactions via a secondary antioxidant mechanism, which involves the abstraction of the radical charge from macromolecules. This is due to their capability to act as radical “sinks” via the stabilisation of the radical charge over long conjugated carbon bonds (Hapiot *et al*, 2001).

1.1.2 Formation of Carbonyls from Lipid Oxidation

Oxidative stress consequently mediates the radical attack of cellular macromolecules such as lipids, amino acids and nucleic acids, altering the function of these molecules and leading to the generation of reactive carbonyl species via fragmentation and rearrangement reactions. *In vivo*, the main source of carbonyls, other than through their ingestion and inhalation, is the oxidation of polyunsaturated fatty acids (PUFA) which are long chained lipids with 2 or more double bonds within the structure. Esterbauer *et al* (1987) studied the formation of aldehydic products of lipid peroxidation from low density lipoproteins *in vitro*. They noted that after the initial destruction of α and γ tocopherol (6 hours), a loss of arachidonic and linoleic acid occurred (Esterbauer *et al*, 1987). Within 24 hours of oxidation of LDL in an oxygen saturated buffer, 23% of linoleic acid and 50% of arachidonic acid was oxidised and various aldehydes were generated in the order: malondialdehyde (36.6%) > hexenal (25%) > propanal (8.9%) > 4-hydroxynonenal (8.2%) > butanal (7.6%) > 2,4-heptadienal (4.1%) > pentanal (3.4%) = 4-hydroxyhexenal (3.4%) > 4-hydroxyoctenal (2.5%) as shown in Figure 1.1.

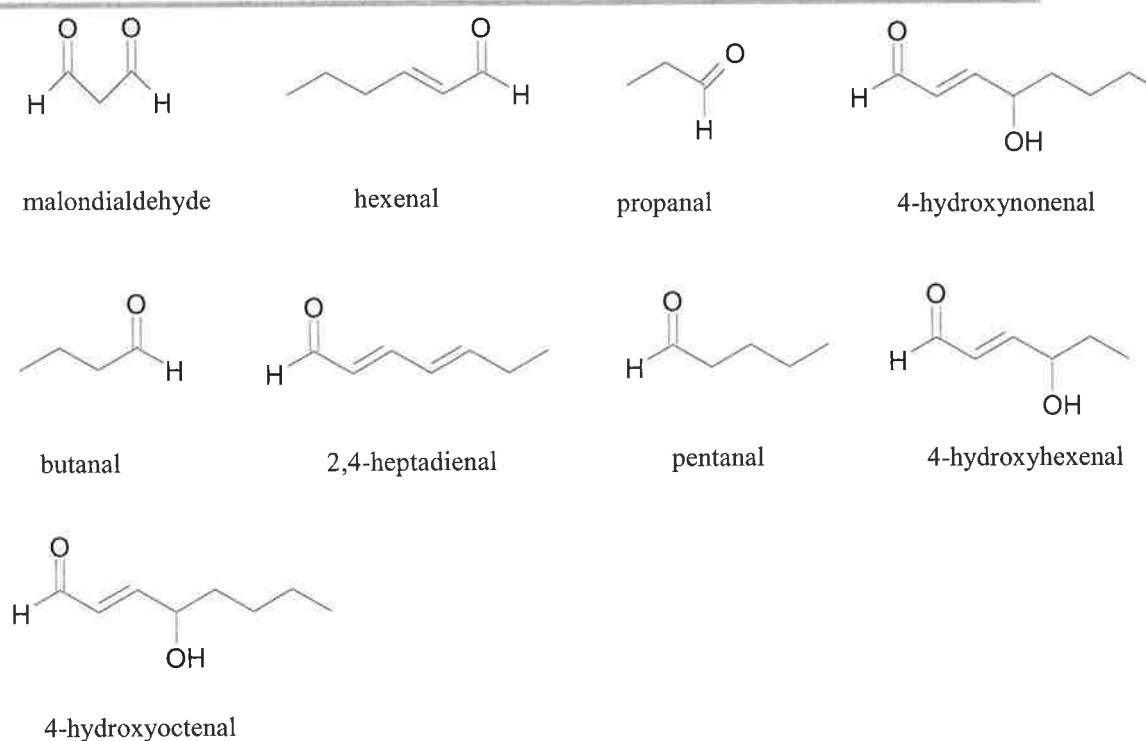
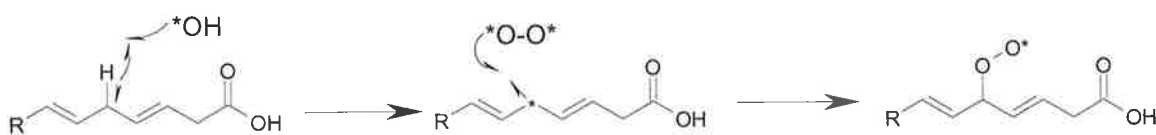


Figure 1.1 Structures of aldehydes detected in the oxidation of human plasma low density lipoprotein by Esterbauer *et al* (1987).

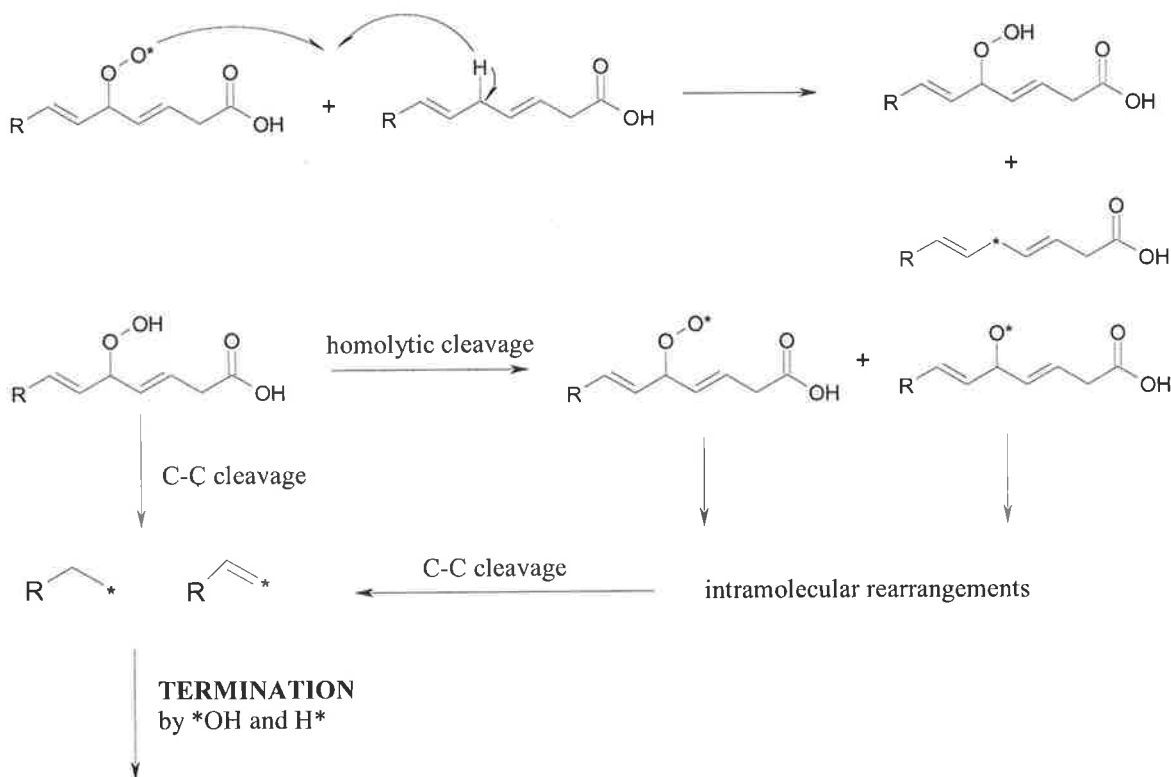
PUFA's are easy targets for radical attack due to the low abstraction energy of H atoms on the methylene bridge between adjacent double bonds. Hence, the more double bonds contained within a lipid structure the more prone it is to H abstraction. This process occurs over a number of steps which can generate multiple new lipid products including carbonyl intermediates (Figure 1.2). The process, which has been reviewed by Pre (1991), begins via initiation reactions which involve the initial abstraction of a H from the methylene bridge between C=C bonds generating lipid radicals, followed by the generation of a peroxy radical via reaction with molecular oxygen (Pre, 1991). In a series of propagation reactions the peroxy radical abstracts another H atom from a further lipid forming a lipid hydroperoxide and another lipid radical. Transition metals further catalyse the conversion of the lipid hydroperoxide to peroxy and alkoxy radicals. Further intramolecular rearrangements can then occur yielding other reaction products. The hydroperoxides produced can then undergo carbon-carbon cleavage generating olefin and alkyl radicals. These radicals can either undergo further propagation reactions or be terminated by the addition of hydroxyl or hydrogen radicals to form aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones. The carbonyls produced are then capable of adducting nucleophilic biomolecules due to their inherent electrophilicity (Shapiro, 1998). Due to this reactivity, carbonyl products of lipid peroxidation are the focus of much recent research

into the pathways of oxidative cell damage in human disease. In the following section, major carbonyl products of lipid peroxidation will be briefly reviewed in terms of their formation, occurrence, toxic properties and reactivity. Since the main focus of work in this thesis is directed towards acrolein, this aldehyde will receive the greatest attention in the following discussion.

INITIATION



PROPAGATION



aldehydes, ketones, alcohols, hydrocarbons,
esters, furans and lactones

Figure 1.2 Process of lipid peroxidation. Radical (*) initiated attack on methylene bridge H's between adjacent carbon double bonds. Based on Pre (1991).

1.2 ALDEHYDES

1.2.1 Malondialdehyde (MDA)

1.2.1.1 Formation and Occurrence of MDA

Among the aldehydes produced by lipid peroxidation, malondialdehyde (MDA) is the most abundant with a normal plasma concentration of approximately 1.3 μM within cells and tissues (Esterbauer *et al*, 1987; Cavalca *et al*, 2001). MDA is mainly formed from the peroxidation of PUFA's containing 3 or more double bonds such as arachidonic acid and docosahexaenoic acid. One mechanism proposed to account for MDA formation from arachidonic acid has been reviewed by Esterbauer *et al* (1991) and is shown below (Figure 1.3). This mechanism involved the peroxidation of arachidonic acid at either carbon-5 or carbon-15 followed by cleavage to a hydroperoxyaldehyde. β -cleavage of the product of peroxidation at carbon-15 may result in MDA formation. Alternatively, an acrolein radical may be produced from the product of peroxidation at carbon-5, which may react with a hydroxyl radical to form the MDA enolate (Esterbauer *et al*, 1991). Other mechanisms of MDA formation from the peroxidation of PUFA's have also been proposed by a number of investigators, although these will not be discussed at length here. At physiological pH the enolate of MDA predominates over the more electrophilic β -hydroxyacrolein, making MDA a rather weak electrophile in biological systems. MDA can also be formed enzymatically from human platelet thromboxane synthase, prostaglandins H_{1-3} , G_2 , 8-iso-prostaglandin H_2 and 13 (S) hydroxy-prostaglandin H_3 as well as polyamine oxidase and spermine (Esterbauer *et al*, 1991).

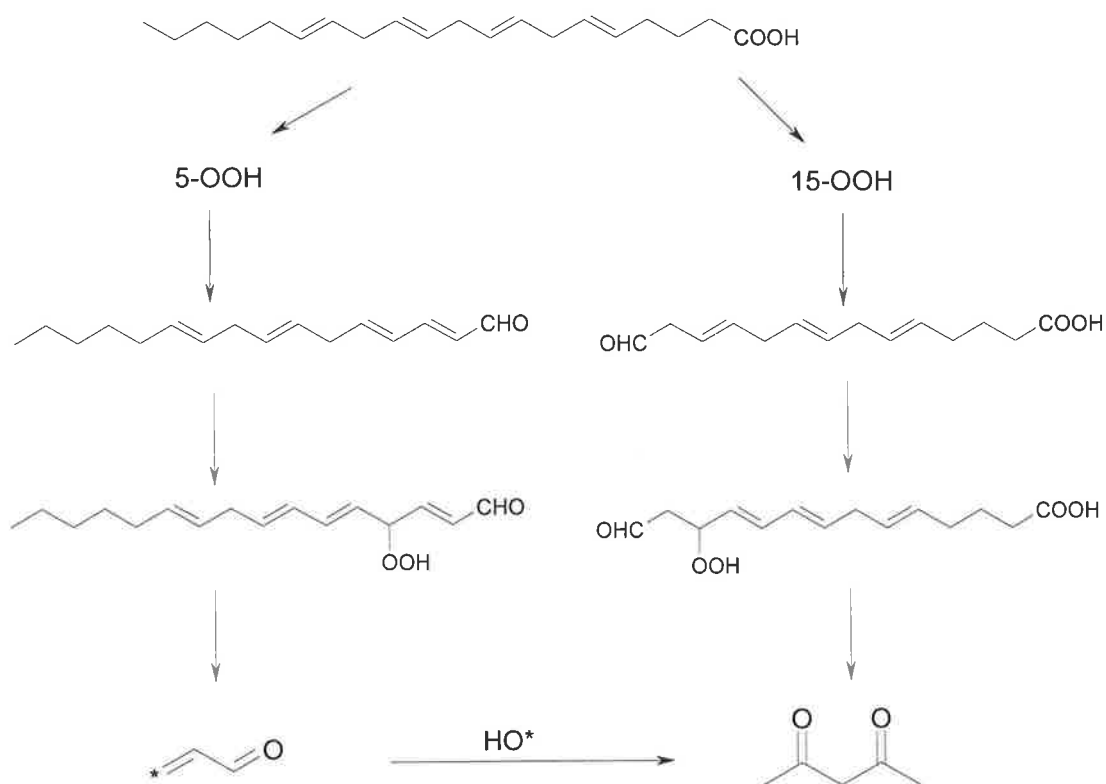


Figure 1.3 Formation of MDA through oxidation of arachidonic acid. Derived from Esterbauer *et al*, 1991.

1.2.1.2 MDA Metabolism and Protein Adduction by MDA

The metabolism of MDA has been summarised by Esterbauer *et al* (1991). Briefly, MDA is converted to malonic acid semialdehyde in the mitochondria by aldehyde dehydrogenases. The semialdehyde then spontaneously decarboxylates to acetaldehyde which is then converted by aldehyde dehydrogenases to acetate. Acetate decomposes to CO₂ and H₂O (Siu and Draper, 1982). Any MDA that escapes metabolism is, however, able to adduct nucleophilic biomolecules, which is another major fate of the aldehyde. For instance, Draper *et al* (1986) identified a variety of enaminals in human urine, including the lysine adducts N^ε-acetyl (2-propenal) lysine and N^α (2-propenal) lysine which is released in the process of digestion (Draper *et al*, 1986). However, it also appears to be formed in cells *in vitro*, suggesting that MDA adducts are not exclusive to whole body systems. Other MDA adducts which have been identified are DHP-lysine, 1-amino-3-iminopropene and pyridium DHP. MDA is also capable of adducting DNA at the nucleosides guanosine, adenosine and cytosine bases as has been reported for other aldehydes (Esterbauer *et al*, 1991). However, the contribution of such adducts to the toxicity of unsaturated aldehydes is beyond the scope of this thesis and will therefore not be discussed at length.

1.2.2 4-Hydroxynonenal (HNE)

1.2.2.1 Formation of HNE and Protein Adduction

Of the 4-hydroxalkenals formed via lipid peroxidation, 4-hydroxy-2-nonenal (HNE) is the most abundant and is also frequently used as a marker of oxidative stress. It is formed in lower amounts than MDA but is far more cytotoxic and is believed to be responsible for the cytopathological effects induced by lipid peroxidation. HNE is formed mainly from the oxidation of omega-6-PUFA's like linoleic acid and arachidonic acid (Winkler *et al*, 1984). Given its strong electrophilicity, which is gained through the presence of its α,β -unsaturated carbonyl group, HNE is capable of readily adducting protein thiol and amine groups (Esterbauer *et al*, 1991). The main reaction products between HNE and protein thiols and amine groups at physiological pH are 5 membered ring hemiacetals, formed via the Michael addition of cysteine, histidine and lysine residues to the carbon double bond at C3 followed by cyclisation (Figure 1.4).

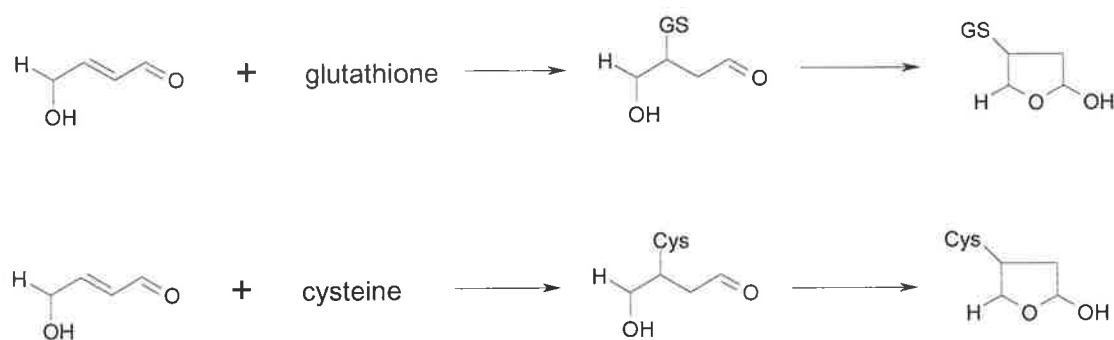


Figure 1.4 HNE adducts formed with glutathione and cysteine. Derived from Esterbauer *et al*, 1991.

1.2.2.2 Metabolism of HNE

HNE is metabolised in the liver to 3 main products, HNE-glutathione and its ring opened carboxylic acid, 4-hydroxy nonenoic acid and 1,4-dihydroxy nonene which are formed by the enzymes glutathione transferase (particularly isozyme μ in humans), aldehyde dehydrogenase and alcohol dehydrogenase respectively. HNE-glutathione can also be metabolised to a mercapturic acid. In rats, HNE is converted to CO_2 by the mitochondria, most likely from the β -oxidation of 4-hydroxynonenic acid (Esterbauer *et al*, 1991).

1.2.2.3 Biological Effects of HNE

Some of the effects mediated by HNE include cytotoxicity, decreased protein and DNA synthesis, enhanced chemotaxis of neutrophils, enzyme inhibition and inhibition of cell growth (Curzio *et al*, 1986; Barrera *et al*, 1987; Esterbauer *et al*, 1991; Moldovan *et al*, 1994; Kreuzer *et al*, 1998; Liu *et al*, 1999). The effects which are relevant to our current work are the cytotoxic and chemotactic effects, as cell death and inflammatory responses are often prominent in disease processes that are accomplished by lipid peroxidation.

Esterbauer *et al* (1991) reviewed the literature on the cytotoxic effects of HNE and the concentrations required to elicit this effect in biological systems and *in vitro*. In general, HNE levels as high as 100 μM may be present within or near peroxidising membranes, where this concentration has been shown to induce generalised, unspecific cell death (Benedetti *et al*, 1984). However, at low micromolar concentrations of HNE cell death is more specific and depends on the cell type and the duration of exposure (Kaneko *et al*, 1988; Herbst *et al*, 1999; Liu *et al*, 2000). The cellular effects reported at low micromolar concentrations of HNE may also be physiologically relevant, since concentrations of HNE in human plasma, low density lipoprotein and monocytes have been reported to be 0.28 – 0.68 μM , 0.14 μM and 3.9 nmol per 100 million cells respectively (Esterbauer *et al*, 1987; Selley *et al*, 1989; Esterbauer *et al*, 1990).

HNE also stimulates chemotaxis of neutrophils to the site of HNE production. Concentrations of HNE exerting this effect are in the nM to μM range and are below the physiological concentration observed in normal healthy individuals (Esterbauer *et al*, 1991). The chemoattractant effect of HNE is important in inflammatory diseases in which HNE concentrations are known to be elevated, as the increased production of this aldehyde may be responsible for exacerbating the inflammatory response, hence enhancing the disease state.

1.2.3 Acrolein

1.2.3.1 Formation and Occurrence of Acrolein

Acrolein occurs in the environment as a pollutant and in the body as a product of lipid peroxidation or via its dietary ingestion or inhalation. Acrolein is the smallest member of the α,β -unsaturated aldehydes and also the most reactive due to its high electrophilicity (Izard and Libermann, 1978; IARC, 1979). It is a highly volatile liquid with a pungent odour which is

irritating to mucous membranes. Its high reactivity allows it to self-polymerise in solution, particularly in the presence of light and strong alkali or acidic conditions. For this reason the compound is stabilised with hydroquinone in commercial sources (IARC, 1979). Given its strong reactivity with nucleophilic biomolecules and its wide occurrence as an environmental pollutant, toxicity induced by acrolein has been the main focus of this thesis.

In the environment acrolein is formed via the incomplete combustion of organic material, such as burning wood, cigarettes, cooking oil and diesel fuel, and as a photochemical reaction product in polluted air (Kensler and Battista, 1963; Izard and Libermann, 1978; IARC, 1979). A number of studies have assessed the levels of acrolein in atmospheric air, most of these reports, however, are from the 1980's. One report by Costa and Amdur (1996) estimated acrolein concentrations at 0.04 – 0.08 ppm (Costa and Amdur, 1996). Other investigators have reported atmospheric concentrations in American air at 0.01 – 0.015 ppm in town air and 0.01 to 0.0018 – 0.0034 ppm in the period 1960 to 1968 (Skog, 1950; Beauchamp *et al*, 1985). A more recent report by Cerqueira *et al* (2003) reported acrolein concentrations in rural Portugal air of up to 1.2 ppb and methylvinyl ketone concentrations up to approximately 0.6 ppb. These aldehydes were reported to be formed via the photochemical oxidation of biogenic organic compounds such as isoprene (Cerqueira *et al*, 2003). The current environmental limit for acrolein in both Australia and America is 0.1 ppm, set as a time weighted average.

Acrolein has also been detected in some foods, including white bread, fried food, alcohol including beer and wine, fish and shellfish, salted pork and chicken breast (Avent, 1961; US EPA, 1978; IARC, 1979; Greenhoff and Wheeler, 1981). However, the main route of environmental exposure to acrolein is via inhalation. The concentration of acrolein in cigarette smoke was determined to be 20 µg/puff by Kensler and Battista (1963), 65.9 µg/cigarette in cigarettes made from black tobacco by Testa and Joigny (1972) and 51-102 µg/cigarette in non-filter cigarettes made from 4 different types of bright tobacco by Rathkamp *et al* (1973) (Kensler and Battista, 1963; Testa and Joigny, 1972; Rathkamp *et al*, 1973). There is a considerable variation in the concentrations of acrolein in cigarettes, however, and levels of acrolein have not always been consistent between studies, reflecting differences in the sensitivity and reliability of the assays used. There does, however, appear to be differences in acrolein content between different tobacco types and it has also been noted that the presence of filters reduces the content of acrolein in the gas phase of cigarettes.

Acrolein also forms from allyl containing drugs such as allyl alcohol, allylamine and allylformate as well as from the biotransformation of the anti-cancer drug cyclophosphamide (Sladek, 1971; Esterbauer *et al*, 1991). Recently, acrolein was found to be formed endogenously by the oxidation of PUFA's, particularly arachidonic acid, linoleic acid, eicosapentaenoic acid and docosahexaenoic acid (Uchida *et al*, 1998a; Uchida *et al*, 1998b). Accordingly, the significance of acrolein formation in humans is the focus of discussion in a later section. An accurate assessment of endogenous acrolein concentrations is difficult, however, due to the pronounced reactivity of the molecule with nucleophiles, particularly thiols such as glutathione and cysteine. Nevertheless, two studies have addressed this issue by measuring the concentrations of free acrolein in human post-mortem brain tissue (Lovell *et al*, 2001) and in a breast cancer cell line (MCF-7) (Kato *et al*, 2002). The former study reported acrolein concentrations in un-demented post-mortem brain tissue between 0.3 and 3.0 nmol/mg of protein, depending on the region of brain examined (Lovell *et al*, 2001). These concentrations were higher in Alzheimer's affected brain tissue. The work by Kato *et al* (2002) employed chemical ionisation mass spectrometry to calculate (from cell diameters) concentrations of acrolein released from Schiff adducts in doxorubicin sensitive (MCF-7) and insensitive (MCF-7/Adr) breast cancer cells (Kato *et al*, 2002). The authors reported intracellular free acrolein concentrations of between 0.54 and 0.68 μM in these cells. These concentrations increased, however, following exposure to the anticancer drug doxorubicin to between 1.0 and 1.37 μM . The reliability of these concentrations is questionable, however, due to the likelihood that chemical reactions occurring post-mortem would have contributed to the acrolein concentrations detected in the former study, whereas the latter study measured free acrolein and acrolein released from Schiff adducts. This latter study therefore did not include Michael adducted acrolein in their calculations, which probably accounts for the majority of intracellular adducts (see below). Hence, since they measured the concentration of acrolein bound to nucleophiles via Schiff base chemistry, these values do not represent "free" acrolein within these cells at the time of analysis. The reported concentrations are therefore only estimates of acrolein concentrations between treated and untreated cells and may not reflect true free acrolein concentrations within these cells.

1.2.3.2 Metabolism of Acrolein

The metabolism of acrolein has been investigated by Patel *et al* (1980) in rat lung and liver preparations and reviewed by Beauchamp *et al* (1985) (Patel *et al*, 1980; Beauchamp *et al*, 1985). A metabolic scheme is depicted in Figure 1.5. Briefly, acrolein is converted to acrylic

acid in liver microsomes and in cytosol by aldehyde dehydrogenase. In the presence of NADPH, acrolein was also converted to the epoxide glycidaldehyde and further to glyceraldehyde by epoxide hydase. Acrylic acid and glyceraldehydes can also be further converted to carbon dioxide. In addition, acrolein can be conjugated to glutathione (GSH) to form a thiol ether. This is believed to be the major detoxification pathway of acrolein, as depletion of GSH ensues before acrolein damage is initiated. However, Kolb *et al* (1994) also observed that acrolein can be reduced by aldose reductase to allyl alcohol (Kolb *et al*, 1994). A more complex metabolic scheme in the rat was recently suggested by Parent *et al* (1998), identifying 3-hydroxypropionic acid, malonic acid, oxalic acid, N-acetyl-S-2-carboxyethylcysteine, N-acetyl-S-3-hydropropylcysteine and N-acetyl-S-2-carboxy-2-hydroxyethylcysteine as major urinary metabolites. They also identified a high molecular weight complex of 2,000-20,000 Da in faeces following intravenous (2.5 mg/kg) or oral (15 mg/kg) acrolein administration. This was believed to be a polymer of acrolein formed following high doses of the aldehyde (Parent *et al*, 1998).

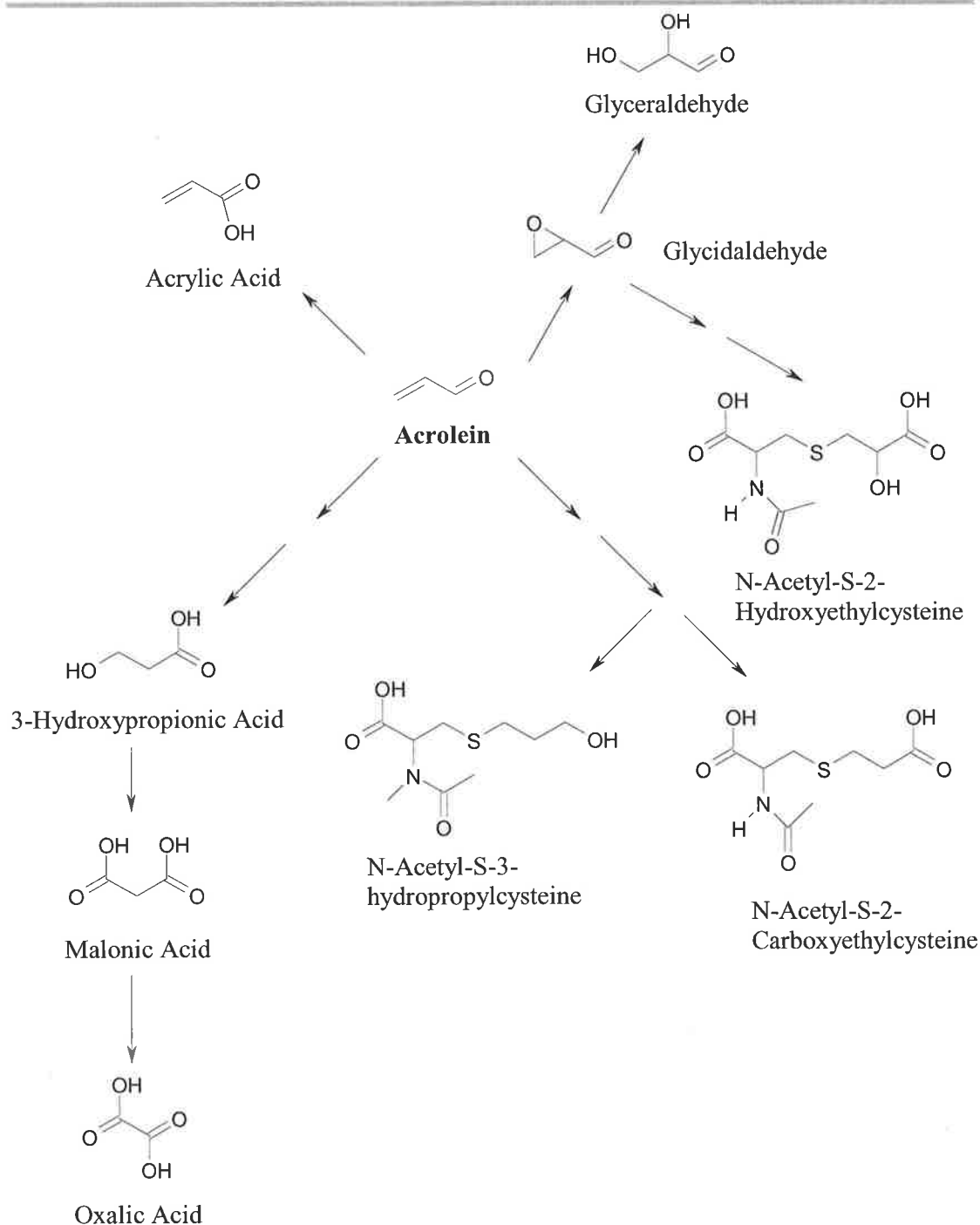


Figure 1.5 Metabolism of acrolein showing major urinary metabolites (Beauchamp *et al*, 1985; Parent *et al*, 1998).

1.2.3.3 Biological Effects of Acrolein

1.2.3.3.1 Introduction

The general biological effects of acrolein are cytotoxicity, inhibition of proliferation, inhibition of chemotaxis of neutrophils (Bridges *et al*, 1977), inhibition of DNA synthesis and a range of additional *in vivo* effects including irritation to mucous membranes, oedema, bronchitis and skin necrosis which have been reviewed by Beauchamp *et al* (1985). There has also been some debate on whether acrolein is carcinogenic, teratogenic and mutagenic. As yet, the data has been conflicting but it appears that in general, acrolein is not carcinogenic or teratogenic *in vivo* or *in vitro*, although it does appear to be weakly mutagenic, more so after activation to glycidaldehyde, which is capable of inducing point mutations, sister chromatid exchange and chromosomal breakage (Beauchamp *et al*, 1985; Barros *et al*, 1994). It has also been convincingly shown that glycidaldehyde is highly carcinogenic (Beauchamp *et al*, 1985).

The conflicting data on these effects may also have been related to the investigators use of serum in the *in vitro* experiments and the type of cells used, as it has been previously shown that the toxicity of acrolein is lower in cultures supplemented with serum, as serum protein thiol groups react preferentially with acrolein (Esterbauer *et al*, 1991). There is also considerable variation in the effects of acrolein in mediating toxicity in various cell lines (Chhibber and Gilani, 1986; Slott and Hales, 1986; Esterbauer *et al*, 1991).

1.2.3.3.2 Cytotoxicity and Cell Proliferation Effects

The form of cell death believed to be initiated by acrolein is oncosis, although apoptosis has been observed in response to acrolein in alveolar macrophages (Li *et al*, 1997; Rudra and Krokan, 1999). As was stated previously, acrolein induces cell death only when the level of GSH has been depleted to a certain threshold limit. For instance, Grafstrom *et al* (1988) showed that in human bronchial epithelial cells, 3-10 μM acrolein induced a significant loss of GSH but had no effect on protein thiol groups until the concentration of acrolein was above 10 μM (Grafstrom *et al*, 1988). However, this was not the case for acrolein induced inhibition of cellular proliferation (Esterbauer *et al*, 1991). The inhibition of proliferation occurred at much lower concentrations than the induction of cell death as highlighted by Grafstrom *et al* (1988). The LC_{50} for acrolein on human bronchial epithelial cells was reported to be 20 μM in the absence of serum although the EC_{50} for inhibition of proliferation was 1 μM .

Acrolein is thought to cause cell death through the induction of oxidative stress in the absence of GSH and/or via the modification of cellular nucleophilic groups (Esterbauer *et al*, 1991). The way in which it induces apoptosis in certain cells and inhibits cell proliferation is less understood.

1.2.3.3.3 Molecular Effects of Acrolein

A review by Kehrer and Biswal (2000) discussed some of the molecular effects of acrolein that may lead to cell death and inhibition of proliferation at low doses. One suggestion is that acrolein induces necrotic cell death by inducing lipid peroxidation following the depletion of cellular GSH. While the loss of GSH itself is unlikely to be responsible for acrolein mediated cell death, disruption of the thiol redox status of cells has been shown to affect a number of cell signalling molecules and cellular functions, including proliferation (Arrigo, 1999).

A number of transcription factors were noted by Kehrer and Biswal (2000) as targets for acrolein, including nuclear factor κ B (NF- κ B), activator protein-1 (AP-1), p53 and c-myc as well as a number of other redox sensitive antioxidant enzymes, kinases, cytokines, ion transporters and phosphatases (Kehrer and Biswal, 2000). NF- κ B, for instance, is a cytoplasmic homo- or hetero-dimer which is translocated to the nucleus following activation (May and Ghosh, 1997). Here, it regulates nuclear transcription and has a role in controlling the expression of growth-regulated genes (Hutter and Greene, 2000). Activation of NF- κ B has also been reported to prevent cells from undergoing apoptosis, whereas its inhibition induces apoptosis in hepatocytes (Wang *et al*, 1996; Bellas *et al*, 1997). Oxidants also appear to regulate NF- κ B activation via the generation of ROS and phosphorylation of the inhibitory κ B subunit (Wang *et al*, 1996). The role of ROS in the activation of NF- κ B is thought to be due to disruption of the cellular thiol redox status, such as through GSH depletion which is also known to activate this protein (Garcia-Ruiz *et al*, 1995). Given that acrolein depletes cellular GSH, it may similarly be expected to activate NF- κ B. Alternatively, adduction of nucleophilic cysteine residues on the P50 and/or P65 subunits may also alter the function of NF- κ B (Kumar *et al*, 1992; Toleando *et al*, 1993).

Another transcription factor which is thought to be affected by acrolein is AP-1, which is involved in the regulation of cell proliferation (Ruppec and Baeurle, 1995). Like a number of cell cycle regulating proteins, its activity is strongly influenced by toxic stimuli and the redox status

of cells (Rupeć and Baurle, 1995). Acrolein is one such toxic stimulus that has been shown to inhibit AP-1 activation (Biswal *et al*, 2000). This effect is most likely through the adduction of Fos or Jun genes whose products comprise the AP-1 protein (Biswal *et al*, 2000). In contrast, antioxidants that maintain the thiol redox status of cells typically activate AP-1 (Meyer *et al*, 1993; Schenk *et al*, 1994).

Given that cysteine residues are prominent targets for acrolein adduction, another likely molecular target for acrolein is p53, an important regulator of the cell cycle that slows DNA replication, allowing for the repair of DNA (Esterbauer *et al*, 1991). The activity of p53 is dependent on a number of cysteine residues present in its DNA-binding domain (Hainaut and Milner, 1993). Also, given that p53 has a κ B site in its promoter region, acrolein may alter p53 function via this region, possibly by blocking its pro-apoptotic effect (Wu and Lozano, 1994).

1.2.3.3.4 Pathophysiological Effects in Humans

Carson and colleagues (1981) were the first group of investigators to comprehensively report the effects of acrolein exposure on humans (Carson *et al*, 1981). They estimated the odour threshold for airborne acrolein at between 0.03 and 0.034 ppm in most acrolein sensitive people. Below this threshold, at concentrations as low as 0.02 ppm, electrocortical activity was affected. At a concentration slightly above the recommended environmental time weighted average of 0.1 ppm, 30% of people experience eye irritation within 2 minutes of exposure. At 0.3 – 0.5 ppm slight eye and nose irritation is experienced. Respiratory effects begin to occur at 0.8 ppm with changes in the amplitude of respiratory movements and an increase in respiratory frequency. At and above 1.2 ppm lacrimation is experienced and medium to severe eye irritation is experienced within 5 minutes. This latter effect is accompanied by subsequent pulmonary oedema and bronchitis after prolonged exposure, although pulmonary oedema usually occurs as a delayed response (Key *et al*, 1977). Direct skin contact can cause either necrosis, dermatitis or sensitisation in a few individuals.

1.2.4 Precursors of Lipid-Derived Aldehydes and Their Use in Toxicity Studies

1.2.4.1 Introduction

Numerous studies have sought to examine aldehyde toxicity *in vitro* by adding these reactive species directly to cell culture media. However this poses several problems that may distort the results obtained. Firstly, most aldehydes are extremely volatile substances, capable of evaporating from the culture media (Esterbauer *et al*, 1991). Secondly, addition of exogenous aldehydes does not accurately represent toxicity by intracellular formation of the aldehydes via lipid peroxidation. Thirdly, α,β -unsaturated aldehydes are highly reactive and will readily react with components of the cell media itself (such as glutathione) or on the extracellular membrane of cells. Consequently, intracellular aldehyde concentrations may not reflect those in the extracellular media. Hence, the cellular effects mediated by exogenously added aldehydes may not mirror the effects produced by endogenously formed aldehydes. To overcome this, the use of aldehyde precursors that enter the cells and then are consequently metabolised to form their reactive aldehydes is an approach to examining the toxicity of aldehydes formed intracellularly.

1.2.4.2 Precursors of 4-Hydroxynonenal

The toxicity of 4-HNE has typically been investigated using exogenously applied aldehyde. Investigation of aldehyde toxicity by this method may not be physiologically relevant due to the likelihood of extracellular adduction by the aldehydes, in contrast to when HNE is formed intracellularly via lipid peroxidation (Siems *et al*, 1995). One group of investigators has sought to overcome this problem by synthesising a triester analogue of HNE that is itself non-toxic. The triester, 1,1,4-tris (acetyloxy)-2(E)-nonene (HNE[Ac]3), was shown to be unreactive with proteins and could be activated by lipase *in vitro* and by a Neuro 2A cell lysate to 4-acetoxy-2-nonenal initially and then to HNE (Neely *et al*, 2002). They further showed that bioactivation of HNE[Ac]3 results in the generation of HNE-protein adducts.

1.2.4.3 Precursors of Malondialdehyde

Precursors for MDA include 1,1,3,3-tetramethoxypropane, which is converted to MDA by acid hydrolysis, and 1,3-propanediol. The former is contained in a reagent kit supplied by OxisResearch which is used to synthesise MDA as the aldehyde itself is rather unstable. The latter, however, is a byproduct of glycerol metabolism in bacteria, including *Escherichia coli*,

Klebsiella pneumoniae and *Clostridium butyricum* (Colin *et al*, 2001; Hartlep *et al*, 2002; Huang *et al*, 2002). Interest in the bacterial production of 1,3-propanediol extends from its use in a number of applications including polymers, cosmetics, food, lubricants and medicines (Huang *et al*, 2002). However, its use as a precursor for MDA has been limited to a study by Summerfield and Tappel (1984). These researchers examined the formation of DNA crosslinks after feeding rats 1,3-propanediol for 15 weeks. They observed an increase in the extent of DNA-protein and interstrand DNA crosslinks in the testes of rats fed the diol, which was attributed to MDA formation (Summerfield and Tappel, 1984). Unfortunately, due to the slow kinetics of 1,3-propanediol oxidation to malondialdehyde, it is not a useful tool for use in isolated cell models such as cultured hepatocytes.

1.2.4.4 Precursors of Crotonaldehyde

Crotonaldehyde is an analogue of acrolein that also forms via the incomplete combustion of organic matter and *in vivo* via lipid peroxidation (IARC, 1995; Ichihashi *et al*, 2001; Fontaine *et al*, 2002). Research conducted by Fontaine *et al* (2002) revealed that crotyl alcohol is rapidly oxidised by alcohol dehydrogenase to crotonaldehyde with a similar $V_{\max}$ to its 3-carbon analogue allyl alcohol which is oxidatively biotransformed to acrolein. This metabolism was demonstrated in isolated primary mouse hepatocytes which showed concentration dependent formation of crotonaldehyde from crotyl alcohol which correlated with cell death. The alcohol dehydrogenase inhibitor 4-methylpyrazole inhibited cell death and protein carbonylation induced by crotyl alcohol. Protein carbonylation results from Michael type reactions of α,β -unsaturated aldehydes with protein nucleophiles, resulting in the insertion of a carbonyl group into the protein molecule. These results indicated that the toxicity of crotyl alcohol is mediated by its oxidative biotransformation by hepatic cytosolic alcohol dehydrogenase to crotonaldehyde and the subsequent adduction of proteins by the 2-alkenal.

1.2.4.5 Precursors of Acrolein

Acrolein has been identified as the metabolite responsible for cyclophosphamide toxicity (Brock *et al*, 1979; Cox, 1979). Cyclophosphamide is an antineoplastic agent which is used as an immunosuppressant in cancer treatment, in diseases with an autoimmune component and also in preconditioning bone for marrow transplantation. However, its use is limited by its conversion to acrolein which causes hemorrhagic cystitis or bleeding in the bladder wall (Cox, 1979). Acrolein, which is formed in the liver, has been postulated to be too reactive to diffuse from the

liver to the bladder and lung where it exerts its toxic effects (Sladek, 1988). One possible explanation for this is that acrolein modified GSH can travel to the bladder where acrolein is then released from the adduct (Ramu *et al*, 1996).

Allylamine induces specific cardiotoxicity in rats, evidently due to its amine oxidase-catalysed biotransformation to acrolein in rat aorta, lung, skeletal muscle and whole heart homogenates (Boor *et al*, 1979; Boor *et al*, 1980; Nelson and Boor, 1982). No acrolein formation was evident in liver, brain, plasma or kidney homogenates, consistent with the theory that allylamine toxicity is due to the formation of the aldehyde as these tissues are deficient in amine oxidase. They also reported that the formation of acrolein and H₂O₂ in the tissue homogenates examined correlated with reported levels of the deaminating enzyme benzylamine oxidase (Lewinsohn *et al*, 1978). In support of the metabolism of allylamine to acrolein via benzylamine oxidase, Nelson and Boor (1982) reported that acrolein formation from allylamine was inhibited by the co-incubation of rat aorta with the amine oxidase substrate benzylamine. Monoamine oxidase did not alter the ability of rat heart homogenates to metabolise allylamine to acrolein.

A more commonly used precursor of acrolein is the specific periportal hepatotoxicant allyl alcohol (Piazza, 1915). The toxicity of allyl alcohol is due to its oxidative biotransformation by hepatic cytosolic alcohol dehydrogenase to acrolein (Reid, 1972; Patel *et al*, 1983; Belinsky *et al*, 1985). Thus, allyl alcohol hepatotoxicity is inhibited by the alcohol dehydrogenase inhibitor 4-methylpyrazole (Reid, 1972). Allyl alcohol toxicity is also augmented by the administration of the aldehyde dehydrogenase inhibitors cyanamide or disulfiram (Rikans, 1987). The by product of acrolein oxidation (acrylic acid) is not overtly toxic (Belinsky *et al*, 1986). These results indicate that hepatotoxicity due to allyl alcohol is mediated by alcohol dehydrogenase but that aldehyde dehydrogenase represents a significant detoxification pathway for acrolein. Allyl alcohol is therefore a useful tool for examining acrolein mediated toxicity in isolated mouse hepatocytes (Burcham *et al*, 2000).

1.2.5 Reactions Between Endogenous Aldehydes and Biomolecules

1.2.5.1 Single Addition of Aldehydes to Biomolecules

Acrolein and related α,β -unsaturated aldehydes readily modify amine groups in proteins, particularly the ϵ -amino group of lysine residues, the imidazole group of histidine residues and the thiol group of cysteine residues (Esterbauer *et al*, 1991). In addition, guanine residues in

DNA and to a lesser extent, adenine residues, are susceptible to modification by these bifunctional electrophiles (Esterbauer *et al*, 1991). This ability to modify proteins and DNA is related to the strong electrophilic character of these aldehydes. An acrolein-lysine adduct has been isolated by Uchida *et al* (1998a) and identified as a dehydrated cyclic bis-acrolein adduct of lysine, N^{α} -acetyl- N^{ϵ} -(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine [Figure 1.6a]). The mechanism of formation of this adduct is similar to the mechanism of adduction of protein amino and thiol groups by other aldehydes. This is predominantly via Michael addition of the nucleophilic thiol and amino groups of proteins on the β -carbon of the carbonyl as shown in Figure 1.7. This mechanism allows the carbonyl group to be retained in the adduct, thereby allowing it to undergo further reactions with other nucleophilic centres, creating a range of reaction products including intra- and intermolecular crosslinks (see below).

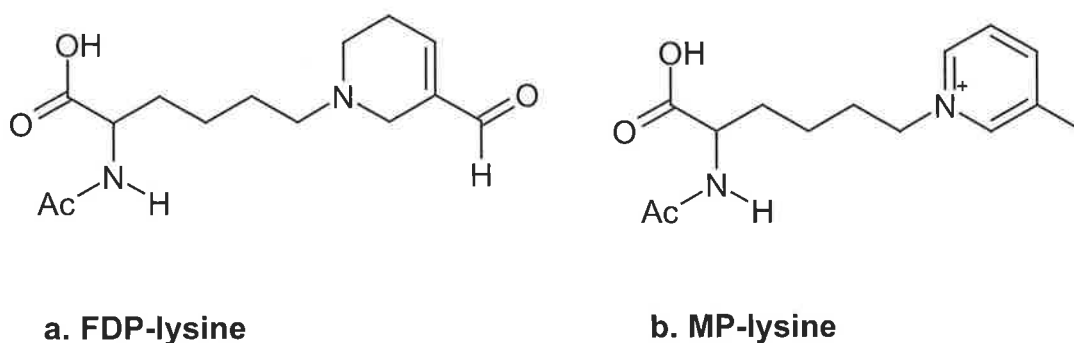


Figure 1.6 Structures of FDP-lysine (a) and MP-lysine (b).

A minor pathway is the Schiff base addition of amines onto the carbonyl carbon which results in an imine and the loss of the oxygen group as water. Interestingly, Schiff base formation had previously been postulated to be the major reaction pathway by which aldehydes reacted with nucleophilic centres (Hoff *et al*, 1989; Hoff and O'Neil, 1991). This has since been disproven by the results of Uchida *et al* (1998a) who exclusively identified the Michael addition product of acrolein and lysine (FDP-lysine) and Bruenner *et al* (1995) who showed that HNE reacts with cysteine, histidine and lysine residues on β -lactoglobulin B and haemoglobin via Michael addition, with less than 1% reacting through Schiff base formation (Brunner *et al*, 1995). In a recent study, the Uchida group identified a further minor product of acrolein addition to N^{α} -acetyl lysine, N^{ϵ} -(3-methylpyridinium)lysine (MP-lysine, Figure 1.6b) (Furuhata *et al*, 2003). This product is formed via initial Schiff addition of acrolein onto the primary amino group of lysine, followed by the Michael addition of another acrolein molecule onto the imine group

generating a positively charged N^{ϵ} group on lysine. This product then condensed with the loss of water and 2 hydrogen ions to form the pyridinium lysine adduct.

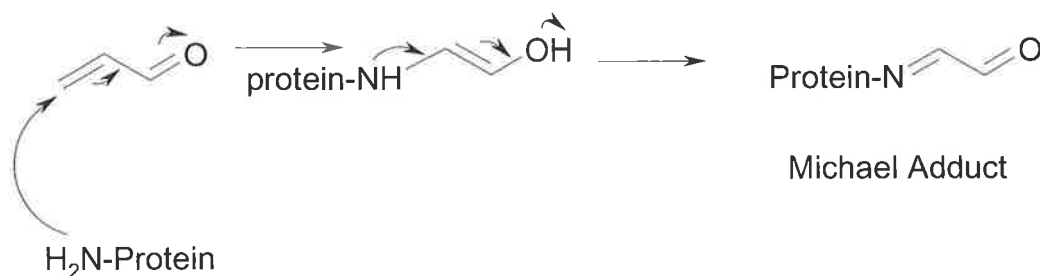


Figure 1.7 Michael addition mechanism for the formation of acrolein adducts on proteins.

1.2.4.2 Crosslinking Reactions Between Aldehydes and Biomolecules

Following the initial adduction of unsaturated aldehydes to nucleophilic biomolecules by Michael type addition, the carbonyl group is then capable of further reactions with neighbouring nucleophiles. This has been reported by a number of investigators who have shown crosslinking between DNA strands and between DNA and neighbouring histones and other peptides (Kuykendall and Bogdanffy, 1992; Kozekov *et al*, 2001; Kurtz and Lloyd, 2003). In addition, multiple studies have revealed intramolecular DNA and protein adducts of α,β -unsaturated aldehydes.

The 4-hydroxyalkenals, such as HNE, have been reported to form diastereomeric cyclised adducts by an initial Michael addition at the N2 position of deoxyguanosine, followed by cyclisation onto N1. This forms 1,N²-substituted propanodeoxyguanosine adducts of HNE (Wang and Rizzo, 2001; Esterbauer *et al*, 1991). MDA, however, is capable of adducting guanine and adenine bases in DNA and to a lesser extent cytosine bases (Seto *et al*, 1981; Stone *et al*, 1990a; Stone *et al*, 1990b; Chaudhary *et al*, 1996). The reaction of MDA involves initial 1,2-addition at the N2 position of guanine, followed by cyclisation and dehydration at N1 to form pyrimidol[1,2- α]purin-10(3H)-one (M₁G; Chaudhary *et al*, 1996). Plataras and colleagues (2002) suggested that due to the N2 atom being contained within the minor groove of DNA, its 3-dimensional structure may affect the adduction of guanine bases by MDA. This was demonstrated, as the minor groove binding agents netropsin and distamycin inhibited the oxopropenylation of guanine whereas the major groove binding agent methyl green had no effect (Plataras *et al*, 2002). They also showed that supercoiled and single stranded DNA were more susceptible to oxopropenylation than linearised and double stranded DNA respectively.

Acrolein has been shown to adduct both guanine and adenine, although the reaction with guanine is quantitatively more significant (Galliani and Pantarotto, 1983; Chung *et al*, 1984; Shapiro *et al*, 1986). The reaction of acrolein with guanine proceeds via initial Michael addition at either the N2 or N1 positions yielding linear hydroxypropano-deoxyguanosine adducts. These then ring close at the N1 and N2 positions respectively, yielding the cyclic adducts 11-hydroxy-tetrahydropyrimidinoguanosine (11-THPG) and 13-hydroxy-tetrahydropyrimido-guanosine (13-THPG) respectively, where 11-THPG predominates due to greater stability and lower heat of formation (Boerth *et al*, 1998). These adducts are stable and do not dehydrate. Guanine adducts of crotonaldehyde form via the same mechanism to that of 11-THPG (Chung and Hecht, 1983). Similar adducts of acrolein on adenine also form following initial addition onto either N1 or N6, with cyclisation occurring at the corresponding nitrogen (Shapiro *et al*, 1986).

Although the formation of intra-molecular cyclised adducts of α,β -unsaturated aldehydes onto nucleobases has been identified in DNA, intra-strand DNA-DNA adducts and DNA-protein adducts have also been characterised. To date, however, protein-protein crosslinks have not been characterised in chemical terms but are expected to occur. For instance, acrolein was shown to cross link axonal cytoskeletal proteins, suggesting that at least in allyl chloride neuropathy, acrolein may be partly responsible for the neuronal damage observed (He *et al*, 1995). Interchain crosslinks between DNA strands have been identified for acrolein by Kozekov *et al* (2001). These crosslinks were reported to be formed by the interruption of the cyclisation step following Michael addition of acrolein onto the N2 of deoxyguanosine. The reaction then proceeded by addition of the carbonyl group of the acrolein-deoxyguanosine moiety onto the N2 of another deoxyguanosine followed by reversible dehydration to an imine crosslink (Kozekov *et al*, 2001).

Crosslinks between DNA and proteins have also been reported to occur following the initial Michael addition of acrolein onto the N2 of deoxyguanosine (Kurtz and Lloyd, 2003). This is via Schiff base addition of lysine or arginine containing peptides onto the free carbonyl group of the acrolein moiety. Such crosslinking has been reported by Kuykendall and Boganffy (1992) to be most efficient for the aldehydes formaldehyde, glutaraldehyde and acrolein, where acrolein formed DNA-histone crosslinks on 1 per 2,700 base pairs at a 150 μ M concentration of aldehyde. Crotonaldehyde induced considerably less crosslinking, requiring concentrations in the millimolar range to induce comparable crosslinking to acrolein (Kuykendall and Bogdanffy, 1992).

Crosslinking has been highlighted as a potential pathway by which unsaturated aldehydes may mediate cellular toxicity. The relative importance of protein versus DNA crosslinking to acrolein toxicity remains to be established, although this thesis is mainly focussed on processes occurring at the protein level. If crosslinking reactions are important in acrolein toxicity, it is predicted that chemical interventions that block the further reaction of residual carbonyl groups from Michael-aldehyde adducted proteins may prevent the subsequent loss of cell viability or protein function. Such interventions would ideally target free carbonyl groups of Michael adducts, blocking their reactivity with susceptible nucleophilic residues. This possibility forms a major focus of the work in this thesis and will be discussed more fully at a subsequent point.

1.2.6 Diseases Manifesting Increased Aldehyde Levels

1.2.6.1 Neurodegenerative Disorders

Evidence for elevated oxidative stress has been obtained in various neurodegenerative disorders. Since one source of reactive aldehydes in the body is lipid peroxidation mediated by the onset of oxidative stress, it is likely that in neuronal diseases in which oxidative stress occurs, elevated levels of these aldehydes can be expected. This has been shown in a number of neuronal disease states where high levels of endogenous aldehydes or their protein adducts have been observed. It is therefore likely that these aldehydes play an important role in neuronal cell death.

Increased levels of MDA have been observed in the cerebrospinal fluid of patients with multiple sclerosis (Calabrese *et al*, 1994). Increases in MDA and HNE adducts have also been found in multiple sclerosis plaques (Newcombe *et al*, 1994), while HNE adducts were identified in the nigral neurons of a patient who died of Parkinson's disease (Yoritaka *et al*, 1996). In contrast, HNE staining was not evident on the surrounding glial cells, which suggested that increased oxidative stress in the substantia nigra, the region of the brain generally affected in Parkinson's disease, occurred in the neurons themselves (Yoritaka *et al*, 1996). This may be due to the fact that the substantia nigra contains a high density of dopaminergic neurons which can contain high cytosolic levels of hydrogen peroxide due to the metabolism of dopamine.

A disease which has attracted a lot of attention with respect to its localised oxidative stress component is Alzheimer's disease. It is therefore reasonable to assume that elevated levels of carbonyl modified proteins would be present in the senile plaques and neurofibrillary tangles of Alzheimer's brains. This was demonstrated by Smith *et al* (1998) who observed, by staining

sections of Alzheimer's affected brains and age matched controls with 2,4-dinitrophenylhydrazine, that the levels of carbonyl containing proteins was increased in the cell bodies of pyramidal neurons in the hippocampus of Alzheimer's brain tissue compared to controls. However, an increase in carbonyl content was not observed in the senile plaques, which was unexpected as the senile plaques are known to be a source of oxygen free radicals in Alzheimer's disease (Butterfield *et al*, 1994). Smith *et al* (1998) attempted to explain this observation by suggesting that the primary source of these radicals may be mitochondria in the neuronal cell body, as neurons consume more oxygen than most other cells in the body (Smith *et al*, 1998).

A drawback of Smith's study is that there was no attempt to identify the types of carbonyls causing neuronal carbonylation. A number of other investigators have, however, examined this issue. MDA, HNE and acrolein or their adducts have been identified in Alzheimer's tissue by various investigators (Yan *et al*, 1994; Sayre *et al*, 1997; Ando *et al*, 1998; Calingasan *et al*, 1999; Lovell *et al*, 2001). These investigators identified these carbonyls or their adducts in neurofibrillary tangles. Lovell *et al* (2001) investigated the concentrations of acrolein in various brain regions affected by Alzheimer's disease and found that acrolein levels were significantly increased in the amygdala and the hippocampus/parahippocampal gyrus but not in the inferior parietal lobule. They also observed that acrolein was 20-30% more toxic to primary hippocampal neurons than HNE and that this toxicity appeared to be linked to an increase in the intracellular concentrations of calcium due to a disruption of membrane polarisation. This is the only study which has reported free brain acrolein concentrations *in vivo* in quantitative terms, as other studies have only qualitatively detected acrolein or measured free plus Schiff bound acrolein. This is likely due to the difficulty associated with assaying acrolein in biological samples, as once the sample is taken acrolein may react rapidly with primary amines and thiol groups. Nevertheless, the concentrations of free acrolein that were reported here in the amygdala, hippocampus/parahippocampal gyrus and inferior parietal lobule in the postmortem brains of normal (age 84 ± 2 years) subjects were 0.3 ± 0.05 , 0.7 ± 0.1 and 3-4 nmol/mg protein respectively.

Increases in carbonyl content have not only been observed in neurodegenerative diseases associated with old age, but also in response to spinal chord injuries. Following spinal chord compression injury, oligodendroglial cells undergo apoptotic cell death. HNE concentrations have been observed to increase within a few hours of injury in spinal chord grey matter and later in white matter as well. Gard *et al* (2001) have therefore investigated the impact of

physiological concentrations of HNE on the ability of oligodendrocyte progenitors to survive, proliferate and migrate in culture. They found that when exposed to HNE, cell death occurred at concentrations that have been measured in neurons undergoing lipid peroxidation, and inhibition of cell chemotaxis at HNE concentrations lower than those required to induce cell death (Gard *et al*, 2001). These findings suggest that HNE can suppress the regeneration capacity of spinal chord neurons. This raises the possibility of the use of anti-HNE treatments such as HNE antibodies or scavengers in the immediate treatment of spinal chord injuries in an attempt to improve the chances of recovery.

1.2.6.2 Diabetes

Advanced glycation end product (AGE) formation, due to an increase in the glycosylation of proteins, is associated with diabetes pathology. A range of reactive carbonyl intermediates are formed during AGE synthesis, the chemistry of which will be discussed in section 1.3. Among the complications associated with diabetes are nephropathy, neuropathy and stiffening of blood vessels (Vlassara *et al*, 1992; Nickander *et al*, 1994; Novotny *et al*, 1994; Suzuki *et al*, 1999). The most prominent complication, however, is nephropathy.

As well as AGE products, elevations in MDA-lysine, HNE-protein and acrolein-protein adducts are prevalent in diabetes, in particular in expanded mesangial matrix and nodular lesions in diabetic nephropathy (Suzuki *et al*, 1999). On the basis of a postulated increase in oxidative stress and glycosylation in diabetes, it is predicted that elevated levels of serum and urine aldehydes occur in diabetic people. This hypothesis has been examined by a number of investigators who assessed the levels of volatile compounds in blood and urine of diabetic and normal subjects. Using gas chromatography/mass spectrometry, one group of investigators observed a difference between the profiles of urinary compounds between the two groups of subjects, although such differences were not observed in plasma samples. Acrolein was one of several compounds identified in plasma, although no attempt was made to quantify it or compare its levels in diabetic and non-diabetic patients (Zlatkis *et al*, 1980).

Noiri *et al*, however, recently reported that patients with end stage renal disease due to type II diabetes mellitus had higher plasma levels of acrolein-adducted proteins compared with control subjects and end stage renal disease patients devoid of diabetes (Noiri *et al*, 2002). These levels were significantly decreased following dialysis with vitamin E bonded hemodialysers, whereas traditional dialysis increased the extent of oxidative stress in diabetic patients. This suggests

that endogenous acrolein formation is significantly elevated in diabetic patients and in patients with end stage renal failure. This association is as yet unclear and further experiments are required to investigate whether elevated plasma concentrations of acrolein exacerbate the extent of diabetic complications.

1.2.6.3 Cardiovascular Diseases

Increased oxidative stress and associated increases in free and protein bound carbonyls have been identified in various cardiovascular diseases, especially atherosclerosis which is initiated by events leading to the oxidative modification of low density lipoproteins (LDL's). LDL's also play a pivotal role in the pathogenesis of these diseases due to susceptibility to oxidative modification and adduction by reactive aldehydes. When incubated with endothelial cells or smooth muscle cells, LDL particles are modified, hence increasing their molecular density (Henriksen *et al*, 1981; Morel *et al*, 1984). This modification promotes LDL uptake into macrophages relative to unmodified LDL, via binding to a macrophage receptor for acetylated LDL (Henriksen *et al*, 1981). MDA-modified LDL, via the binding of the di-carbonyl to the LDL apoprotein, can also be taken up into macrophages more readily than native LDL (Fogelman *et al*, 1980; Esterbauer *et al*, 1993). It was subsequently shown that endothelial and smooth muscle cells modified LDL by inducing lipid peroxidation within the molecule (Steinbrecher *et al*, 1984). Such uptake of modified LDL by macrophages promotes their conversion into foam cells, the predominant cell type in fatty streaks. One theory to explain this is that macrophages may not be able to degrade oxidised LDL as readily as native LDL, as indicated by the increased accumulation of cholesteryl esters in macrophages, giving them a "foamy" appearance (Witztum and Steinberg, 1991).

It is not surprising, therefore, that LDL obtained from atherosclerotic patients are generally more oxidatively modified than those from people without prominent atherosclerosis (Andrews *et al*, 1995). A correlation between the proportion of oxidatively modified LDL and the progression of atherosclerosis in carotid and femoral vessels was also reported by Andrews and colleagues. Oxidised LDL's were also identified in stenotic aortic valves and may contribute to the disease process (Olsson *et al*, 1999). Accumulation of lipids in the aortic valves occurs naturally with ageing, although the patients in this study had higher than normal amounts of aortic lipids. Whether the lipids that are accumulated in patients with stenotic aortic valves is of the oxidised form or whether it becomes oxidatively modified once in the valves is unknown. Uchida *et al* (1998b) have identified the presence of acrolein-lysine adducts (FDP-lysine) in foam cells and

the neointima of human aortic atherosclerotic lesions. Incubation of native LDL with Cu^{+2} ions generated FDP-lysine adducts, suggesting that oxidative modification of LDL generates acrolein which is localised within atherosclerotic lesions.

High levels of MDA and HNE also occur in the serum of patients with prevalent cardiovascular disease and who are undergoing maintenance hemodialysis compared to those not undergoing this treatment (Boaz *et al*, 1999). This suggested an association between the level of oxidative stress in these patients and their accelerated morbidity and mortality rates. Increased levels of MDA and/or HNE occur in the plasma of patients with coronary and peripheral artery diseases and deep vein thrombosis (Belch *et al*, 1995; Re *et al*, 1998; Holvoet *et al*, 1999; Cavalca *et al*, 2001). Plasma MDA and/or HNE may also increase immediately after myocardial or cerebral infarctions, although it appeared that only cardiac sources of cerebral emboli were associated with elevated serum carbonyls (Ozdemirler *et al*, 1995; Re *et al*, 1997).

1.2.6.4 Inflammatory Disorders

Elevated levels of HNE have been detected in the plasma and synovial fluid of patients with rheumatoid arthritis and osteoarthritis (Selley *et al*, 1992). Its presence is likely to be due to increased oxidative stress within the affected area. The production of HNE is likely to exacerbate the disease process as it is chemotactic to neutrophils, which then release inflammatory agents and cytokines resulting in swelling. MDA, however, does not share the chemoattractant properties of HNE. Nevertheless, it has been identified in elevated concentrations in the plasma of patients with systemic lupis erythematosus, scleroderma, periarteritis nodosa, rheumatoid arthritis and fibrosclerosis (Muus *et al*, 1979; Miesel and Zuber, 1993; Amara *et al*, 1995; Leonarduzzi *et al*, 1997). While MDA does not stimulate chemotaxis in inflammatory cells, MDA-protein adducts are immunogenic and stimulate auto-antibody formation (Amara *et al*, 1995). Again, it is unlikely that this aldehyde initiates the inflammatory process, but rather is a consequence of increased oxidative stress and lipid peroxidation occurring at the site of damage. The presence of these reactive aldehydes is likely to exacerbate the disease process by causing crosslinks with proteins, DNA and lipids, hence altering their normal physiological functions.

1.2.6.5 Chronic Lung Diseases

Ogihara *et al* (1999) investigated the relationship between increased aldehyde (hexanal, heptanal, pentanal and HNE) concentrations in the first week of life in premature infants and the incidence of chronic lung diseases (CLD). They observed that there was a significant difference in plasma aldehyde concentrations between infants that developed CLD and those that did not in the first day of birth, where aldehyde concentrations were highest in CLD infants. This effect was not seen 4-6 days after birth. Antioxidant and bilirubin concentrations did not differ significantly between the groups. They found that the optimal predictor of the risk of developing CLD was plasma HNE concentrations above or equal to 200 nM (odds ratio 32, $p < 0.01$). Elevated plasma HNE levels were not associated with the development of perinatal hypoxia. While plasma HNE concentrations may be useful biomarkers of the development of CLD in premature infants, whether HNE is involved in the development of CLD is unknown at this stage (Ogihara *et al*, 1999).

1.2.6.6 Summary

As the preceding discussion highlights, an overproduction of reactive aldehyde products of lipid peroxidation accompanies a number of disease states including neurodegenerative, cardiovascular, renal and inflammatory disorders. In most cases, the occurrence of these aldehydes is likely a result of oxidative stress mediated lipid damage and that the disease process is related to the increase in oxidative stress and not due directly to an increase in free reactive aldehydes and their adducts. The increase in circulating and local aldehydes is likely, however, to exacerbate the disease due to their cytotoxicity, chemotactic effects and ability to inhibit cell proliferation, DNA and protein synthesis and enzyme activity. Their involvement in crosslinking biomolecules may also contribute to their detrimental effects. It is therefore important to develop agents which are able to remove or scavenge these reactive aldehydes from their sites of formation and inhibit their toxicity in order to prevent, or at least control the effects manifested by these various diseases. Since most of the effort to identify drugs with these properties has focussed on scavengers of sugar derived carbonyls, it is necessary to look at the production of carbonyls during sugar autoxidation in some detail.

1.3 ADVANCED GLYCATION END PRODUCTS

1.3.1 What Are Advanced Glycation End Products and How Are They Formed?

In addition to carbonyls produced by lipid oxidation, the carbonyl group of open chained sugars are also able to adduct protein amino groups. This leads to the accumulation of Maillard reaction products (yellow-brown protein adducts with characteristic fluorescence and immunoreactivity) in long lived tissues such as lens crystallins and tissue collagens (Araki *et al*, 1992; Thorpe and Baynes, 1996). Maillard reaction products have since been termed advanced glycation end products (AGE's), products of the non-enzymatic reaction between the linear, open chained form of carbohydrates and free tissue amines (Figure 1.8). Increases in tissue AGE content have also been observed in diabetic patients whose plasma glucose levels are elevated above normal (Brownlee, 1995). The elevation in AGE's in diabetic patients can be attributed to the reported correlation between blood sugar concentrations and time, particularly within tissues with a low turnover rate (Araki *et al*, 1992; Beisswenger *et al*, 1993; Brownlee, 1995).

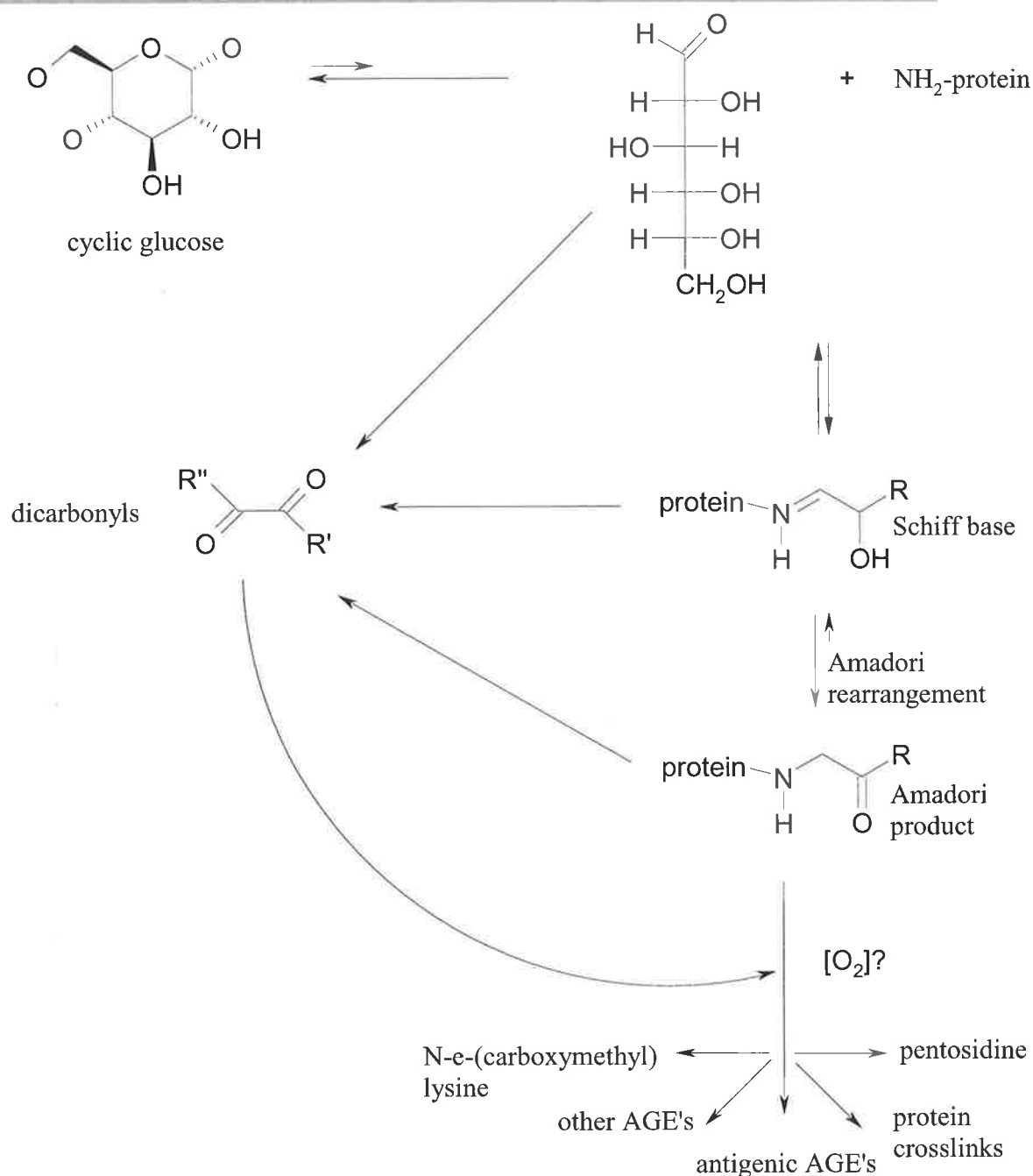


Figure 1.8 Formation of advanced glycation end products. Adapted and modified from Khalifah *et al* (1996).

The mechanism of AGE formation involves the initial nucleophilic attack of carbonyl groups of aldoses and ketoses (open chained sugars) by protein amine groups (Figure 1.8). This reaction forms Schiff base adducts which contain $\text{C}=\text{N}$ groups in place of the sugar carbonyl. These Schiff bases then undergo Amadori rearrangements to Amadori products which contain single carbonyl groups. Up to this stage, the formation of the glycated proteins is reversible by treatment with carbonyl “scavengers” such as carnosine and aminoguanidine, or with insulin intervention (Shapiro, 1998; Chugh *et al*, 1999 1999; Zhou and Decker, 1999). The Amadori

products can then further react with nucleophilic amines to undergo slow (over weeks or months) irreversible intra- or intermolecular rearrangements and cross-linking reactions, oxidative fragmentation and dehydration reactions. Once formed, these products are very stable (Khalifah *et al*, 1996). The Schiff base, Amadori products and open ringed sugars can also undergo oxidative reactions to produce peroxides and dicarbonyl compounds such as glyoxal and glycoaldehyde (Khalifah *et al*, 1996) that undergo further reactions with amine and sulfhydryl groups of proteins, forming AGE-protein complexes. Such compounds can therefore more accurately be described as glycoxidation end products, as oxidation is an integral part of AGE formation by this pathway (Thorpe and Baynes, 1996). To date, the AGE compounds which have been characterised are summarised in Figure 1.9

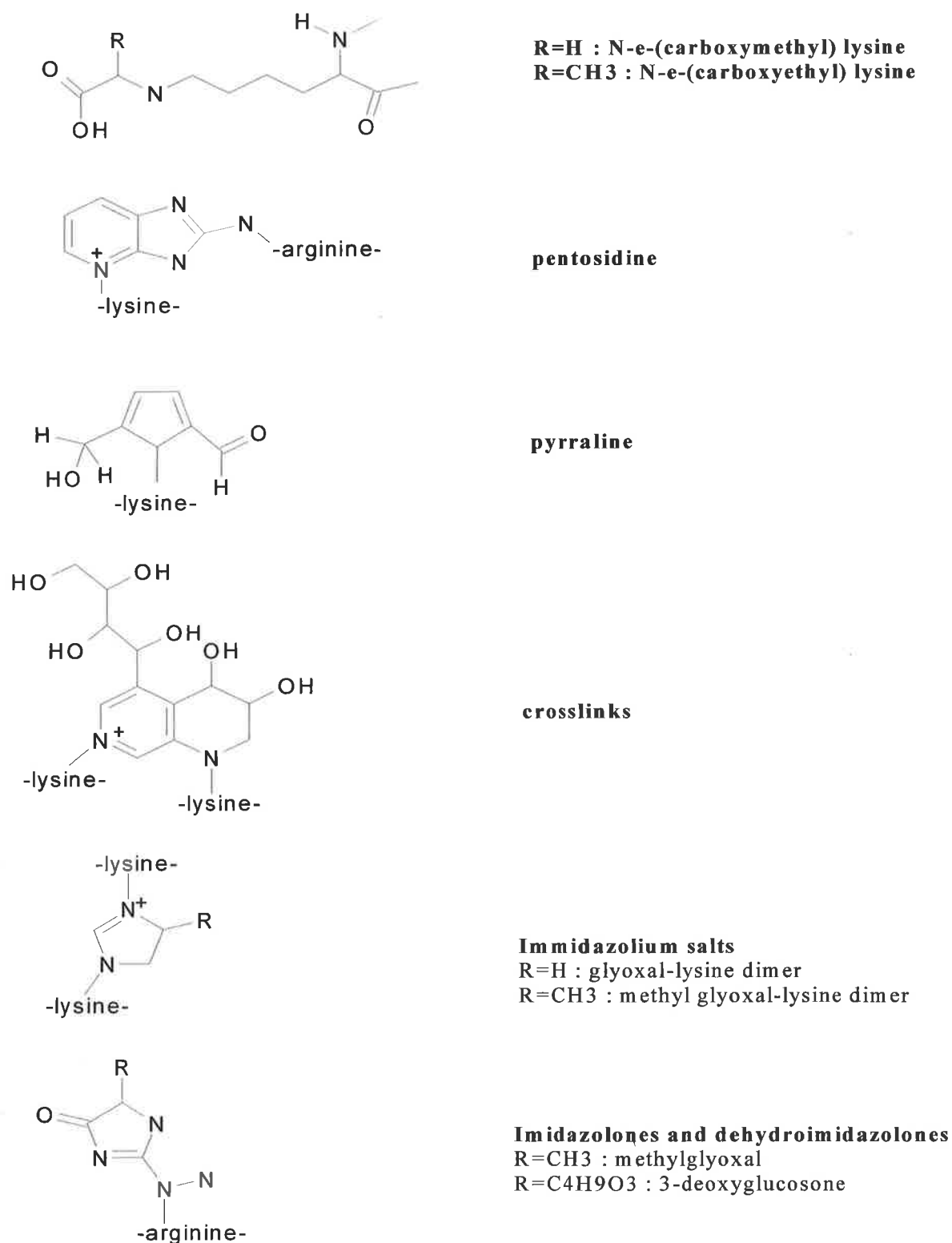


Figure 1.9 Known AGE compounds. Adapted from Baynes and Thorpe (1996).

1.3.2 Pathological Implications of AGE Formation

1.3.2.1 AGE Formation in Diabetes

AGE products in diabetes form as a result of high blood glucose levels and their presence in plasma and tissue is correlated with the severity of diabetic complications (Sell *et al*, 1992; McCance *et al*, 1993). It may therefore be reasonable to assume that the extent of diabetic complications would be correlated with the circulating levels of glucose. This has been shown in humans in such clinical trials as the Diabetes Control and Complications Trial (The Diabetes Control and Complications Trial Research Group, 1993) and in animal models (Brownlee, 1995; Gabir *et al*, 2000). However, the former mentioned clinical trial also noted that some patients with poorly controlled blood glucose had fewer complications than some others with very good control. Brownlee (1995) has suggested that this noted deviation from the normal correlation between blood glucose levels and the severity of diabetic complications may reflect differences in which individuals detoxify AGE intermediates such as 3-deoxyglucosone, which is excreted in the urine as 3-deoxyfructose, indicating the presence of reductive enzymes for AGE intermediates (Knecht *et al*, 1992).

One of the main complications associated with diabetes is renal nephropathy, which can lead to end stage renal disease. The exact biochemical mechanisms associated with the formation of diabetic nephropathy are not well understood, although AGE modification of proteins within the kidney is believed to be a main contributor (Beisswenger *et al*, 1993; Suzuki *et al*, 1995; Suzuki *et al*, 1999). In support of this, Suzuki's group has observed that products of AGE formation are localised in expanded mesangial matrix and nodular lesions in human diabetic nephropathy. This distribution of AGE products co-localised with products of lipid peroxidation such as malondialdehyde-lysine adducts, 4-hydroxynonenal-protein adducts and acrolein-protein adducts (Suzuki *et al*, 1999). However, whereas the AGE products carboxymethyllysine and pentosidine, which are formed by glycoxidation and involve an oxidative stress component, the AGE product pyralline which does not require an oxidative stress component for its formation was not detected in significant amounts in nodular lesions (Suzuki *et al*, 1999). Markers of oxidative stress including lipid peroxidation products and AGE products were not detected in significant amounts in Ig-A nephropathy, which does not involve a hyperglycemic component (Horie *et al*, 1997; Suzuki *et al*, 1999). The reason for this difference in markers of oxidative stress between the two types of nephropathy have not been determined, although possible explanations were suggested by Suzuki *et al* (1999).

1.3.2.2 AGE in Alzheimer's Disease

The neuropathological criteria for the diagnosis of Alzheimer's disease is the presence of aggregates of intra- and extracellular proteins termed neurofibrillary tangles and senile plaques. These protein aggregates are characterised by their general insolubility and resistance to degradation by proteases (Selkoe *et al*, 1982). It has been suggested that the stability of these lesions is due to increased crosslinking between matrix proteins (Perry *et al*, 1991). Such crosslinking is catalysed, in part, by tissue transglutaminase (present within neuronal synapses) and cyclooxygenase-2 (COX-2) (Ho *et al*, 1994; Nagano *et al*, 2004). The former catalyses the formation of dimers and multimers of β -amyloid proteins and of β -amyloid precursor proteins (Ho *et al*, 1994). Interestingly, this reaction can be suppressed by pharmacological intervention with inhibitors of transglutaminase, non-steroidal anti-inflammatory drugs, monoamine oxidase inhibitors, acetylcholinesterase inhibitors and iron chelating agents (Zhang *et al*, 1997). The peroxidase activity of the COX-2 also catalyses the formation of β -amyloid dimers in the presence of H_2O_2 , resulting in β -amyloid-COX-2 crosslinks (Nagano *et al*, 2004). These amyloid-COX complexes were subsequently identified in the post-mortem brains of Alzheimer's patients compared to age matched controls (Nagano *et al*, 2004).

In support of the involvement of AGE's in Alzheimer's pathology, Smith *et al* (1994) immunochemically detected the AGE complexes pyralline and pentosidine in neurofibrillary tangles and senile plaques in human Alzheimer's brains when compared to age matched controls. Whether this occurs as a consequence of Alzheimer's disease pathology, or plays a direct initiatory role has not been determined. Nevertheless, it is reasonable to suggest that the AGE modification present in the brain of Alzheimer's patients contributes to the formation of crosslinks and subsequent stabilisation of neurofibrillary tangles and senile plaques. These localised AGE structures may then increase the oxidative tension present in the lesions (Yan *et al*, 1994).

1.4 SCAVENGERS OF REACTIVE CARBONYLS

1.4.1 Introduction

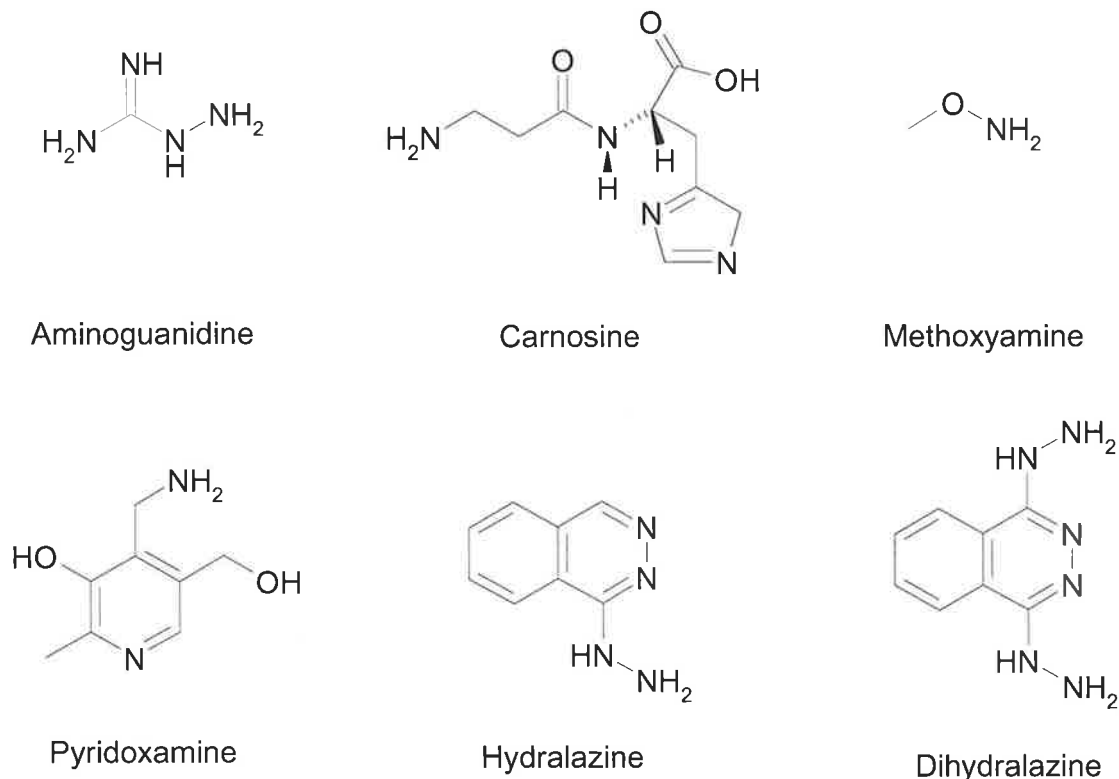


Figure 1.10 Structure of known and currently investigated carbonyl scavengers.

A number of compounds have been investigated for their ability to scavenge reactive aldehydes and suppress the effects elicited by them. Most research has focussed on the compounds carnosine (an endogenous antioxidant in muscles), pyridoxamine (an analogue of vitamin B), the hydrazine compound aminoguanidine and the AGE “breaker” ALT-711 which is being developed by Alteon and the Picower Institute (Figure 1.10). Alteon and the Picower Institute are also currently investigating new compounds for their ability to scavenge aldehydes and break AGE bonds, including compounds such as N-phenacylthiazolium bromide and ALT-946 (Shapiro, 1998). In addition, our laboratory investigated the acrolein scavenging properties of methoxyamine, a known aldehyde trapping reagent (Burcham *et al*, 2000; Burcham *et al*, 2003). Our laboratory also began investigating two other hydrazine compounds, hydralazine and dihydralazine (Figure 1.10), for their ability to scavenge free acrolein. These 6 compounds therefore provide a good starting point in the search for efficient carbonyl scavengers that can be used therapeutically.

1.4.2 Aminoguanidine

Aminoguanidine is a hydrazine compound that has been evaluated in clinical trials at Alteon and the Picower Institute for Medical Research as a treatment for diabetes induced nephropathy, stroke, Alzheimer's disease and inflammatory bowel disease (Bucala *et al*, 1994; Smith *et al*, 1994; Brownlee, 1995; Pfeiffer, 1995; Cockcroft *et al*, 1996). A number of the complications associated with diabetes, including nephropathy, are accompanied by an increase in the AGE content of tissues, including the retina, kidney, nerves, arteries and also individual molecules such as haemoglobin and circulating LDL (Makita *et al*, 1992; Bucala *et al*, 1994; Brownlee, 1995). Aminoguanidine has been shown to slow AGE formation on these tissues and to prevent AGE induced pathologies, including retinopathy (Hammes *et al*, 1994), increased fractional mesangial volume, as well as albuminuria (Soules-Liparota *et al*, 1991), reduced motor and sensory nerve conduction velocities (Cameron *et al*, 1992) and decreased arterial stiffness due to AGE crosslinking (Vlassara *et al*, 1992). Investigators at Alteon also found in a phase II clinical trial that aminoguanidine decreased circulating levels of LDL, total cholesterol, triglycerides and AGE modified LDL (Shapiro, 1998). Cockcroft *et al* (1996) have also observed that when administered within 1 to 2 hours after the induction of focal cerebral infarction in rats, aminoguanidine (320 mg/kg, i.p) reduced the volume of infarction by 88 and 85% respectively. The mechanism by which aminoguanidine has this effect was thought to be unrelated to its ability to inhibit either AGE formation or inducible nitric oxide synthase (iNOS), as normal levels of AGE in stroke do not affect the pathogenesis of brain damage and NOS is not induced until 24 hours after the induction of cerebral ischemia (Bucala *et al*, 1991; Iadecola *et al*, 1994; Iadecola *et al*, 1995). Rather, the ability of aminoguanidine to inhibit polyamine oxidase might have prevented cell death, possibly by inhibiting the formation of reactive aldehydes from polyamines (Brunton *et al*, 1994).

As mentioned above, AGE's form as a consequence of Schiff base adduction of open chained sugars at the primary amine groups of proteins followed by rearrangement to Amadori products and subsequent slow rearrangement to a variety of AGE compounds. A number of investigators have established that aminoguanidine's ability to inhibit AGE formation and subsequent pathogenesis involves scavenging of Amadori derived fragmentation products like 3-deoxyglucosone, yielding 3-amino-5- and 3-amino-6-substituted triazines without removing the Amadori product itself. This effect, however, has been shown by Booth *et al* (1997) to simply slow the formation of AGE's and does not affect their final yields (Booth *et al*, 1997). Aminoguanidine also removes the open chained form of sugars from solution, thereby reducing

their binding to protein amino groups (Brownlee *et al*, 1986; Khatami *et al*, 1988; Edelstein and Brownlee, 1992; Hirsch *et al*, 1992; Chen and Cerami, 1993; Hirsch *et al*, 1995). In support of these findings, Khalifah *et al* (1996) demonstrated that aminoguanidine has little effect on the conversion of Amadori products to AGE compounds, generally requiring concentrations in excess of 50 mM to produce this effect.

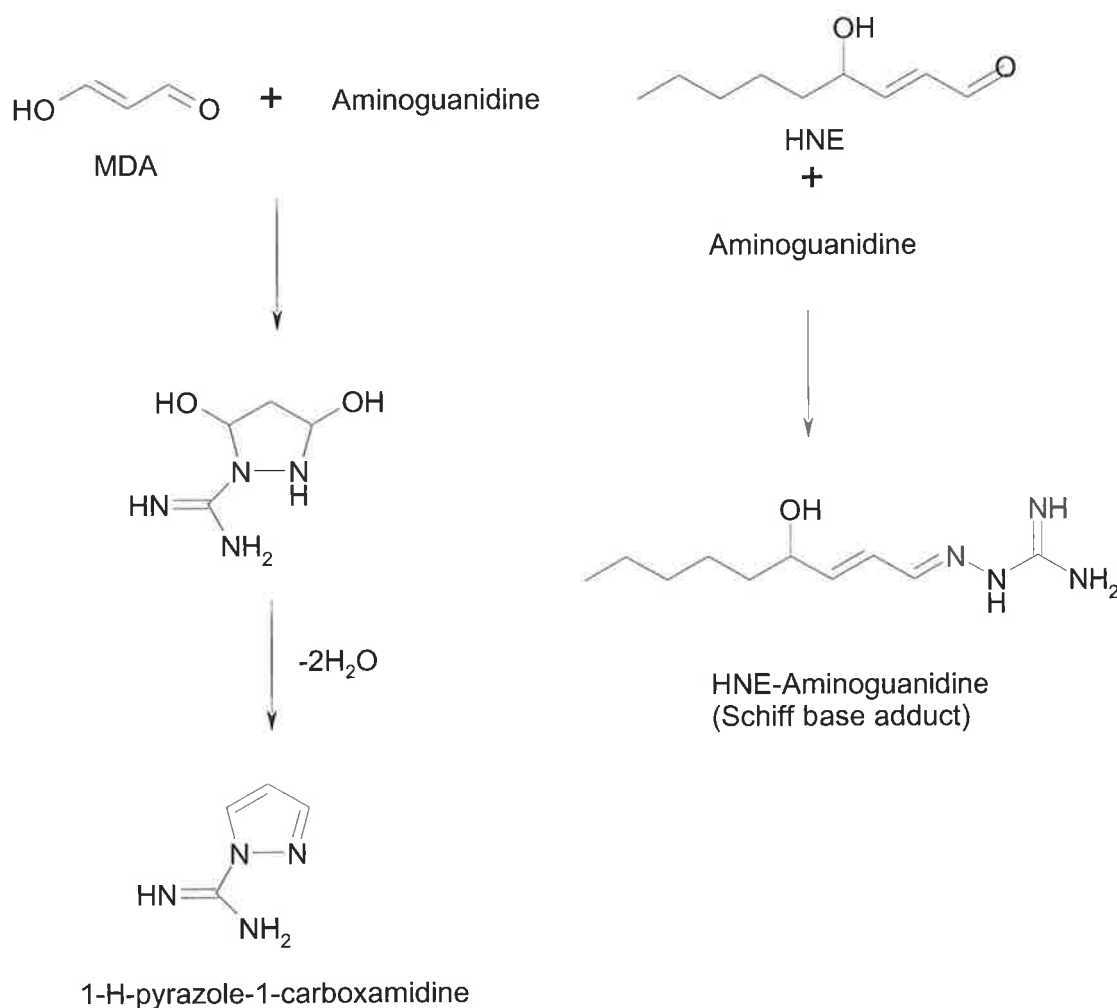


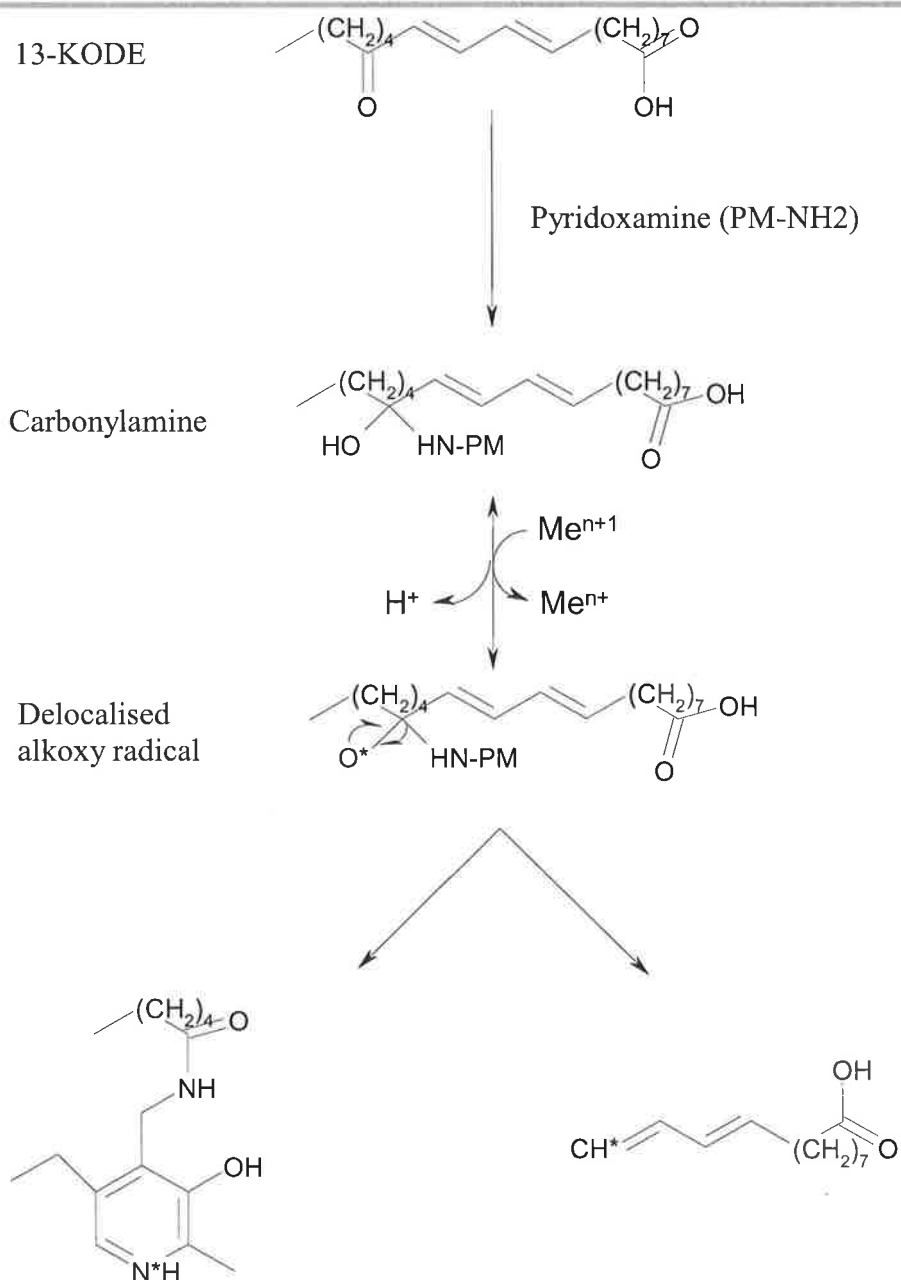
Figure 1.11 Reaction products formed between aminoguanidine and MDA and HNE. Derived from Al Abed and Bucala (1997).

Al-Abed and Bucala (1997) have also shown that aminoguanidine can scavenge aldehydes formed via lipid peroxidation, such as MDA and HNE. The reaction products between MDA or HNE and aminoguanidine were characterised and a reaction scheme proposed as shown in Figure 1.11. The reaction of aminoguanidine with HNE involved addition of the free amine group of aminoguanidine to the β carbon of HNE, forming a Schiff base adduct. MDA, however, added to N1 and 2 of aminoguanidine forming a cyclic structure followed by dehydration to 5-OH-2-pyrazoline-1-carboxamide and 1-H-pyrazol-1-carboxamide. They

also noted that the binding of aminoguanidine to aldehydes occurred in preference to the protein residues lysine, histidine and cysteine, indicating that aminoguanidine can act as a preferential site of carbonyl attack over proteins (Al-Abed and Bucala, 1997).

1.4.3 Pyridoxamine

Pyridoxamine, a derivative of vitamin B₆, is a carbonyl scavenger that is being evaluated in phase II clinical trials for use in the treatment of diabetic complications by Biostratum Inc (USA). Unlike aminoguanidine, pyridoxamine potently suppresses the post-Amadori conversion to AGE compounds in model proteins such as BSA and RNase, and slows the rate of AGE formation and decreases the yield of AGE's formed (Booth *et al*, 1997). Onorato *et al* (2000) have also shown that pyridoxamine inhibits the modification of lysine residues of LDL and RNase by MDA and HNE without forming detectable aminoguanidine-MDA and aminoguanidine-HNE adducts. Instead, they observed the formation of hexanoic acid amide and nonanedioic acid monoamide derivatives of pyridoxamine. These compounds were derived from the binding of pyridoxamine to the 9- and 13- carbons of linoleic acid via reactions with 9- and 13-keto-octadecadienoic acids, formed from the fragmentation of the corresponding peroxides of linoleic acid (Figure 1.12) (Onorato *et al*, 2000).



Hydroperoxyoctadecadienoic acid (HPODE)

Figure 1.12 Trapping of linoleic acid derived KODE by Pyridoxamine (Onorato *et al*, 2000).

1.4.4 Carnosine

Carnosine is an antioxidant dipeptide (β -alanyl-L-histidine) found in the brain and innervated tissue such as skeletal muscle in concentrations up to 20 mM (Chasovnikova *et al*, 1990; Hipkiss *et al*, 1997). Zhou and Decker (1999) reported that it scavenges α,β -unsaturated aldehydes and HNE, although Zhou *et al* (1998) reported that a hydrazine contaminant in commercial sources of carnosine are likely to have contributed to some of these scavenging effects (Zhou *et al*, 1998;

Zhou and Decker, 1999). More recently, however, a number of investigators examined the ability of carnosine to trap free HNE by liquid chromatography/mass spectrometry and electrospray ionisation tandem mass spectrometry. Liu *et al* (2003) observed that the addition of carnosine to a reaction of RNase and HNE prevented the HNE induced formation of dimerised and trimerised RNase. This was postulated to be due to trapping reactions occurring between carnosine and free HNE, which removed HNE available to adduct RNase by forming 2 trapped products: a 13 membered adduct formed via Schiff base addition to the β -amino group on alanine and a Michael adduct at the imidazole nitrogen on the histidine residue (shown in Figure 1.13) (Liu *et al*, 2003). These products have similarly been observed by Aldini and colleagues (Aldini *et al*, 2002a; Aldini *et al*, 2002b). A similar reaction was also seen to occur between acrolein and carnosine, where 13 membered macrocyclic products derived from initial Michael addition to the imidazole group of the histidine residue, followed by cyclisation to form an imine at the amino group of β -alanine were observed (Figure 1.13) (Carini *et al*, 2003). In addition, cyclic FDP-adducts were observed on β -alanine which were derived via the mechanism previously reported by Uchida and colleagues (1998a) (Figure 1.13) (Carini *et al*, 2003).

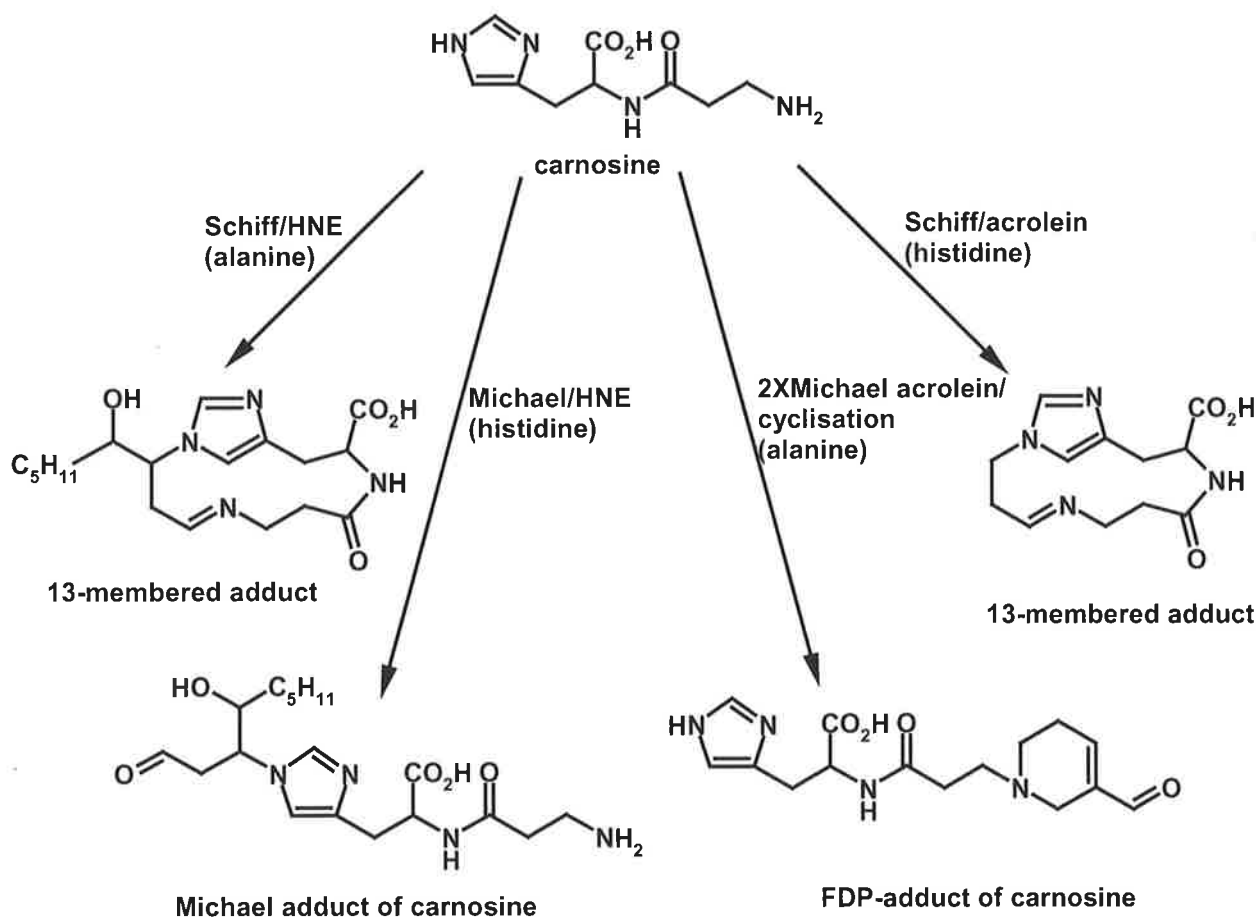


Figure 1.13 Main products of trapping reactions between carnosine and 4-hydroxy-2-nonenal (HNE) or acrolein. Derived from Liu *et al* (2003) and Carini *et al* (2003).

Carnosine is also capable of inhibiting the glycosylation of proteins and the modification of proteins by MDA (Hipkiss *et al*, 1997; Wang *et al*, 2000). This effect is believed to be primarily due to scavenging of the open chained forms of sugars, but can also be complemented by carnosine's antioxidant and metal chelating properties (Brown and Antholine, 1979; Chasovnikova *et al*, 1990; Yoshikawa *et al*, 1991; Decker *et al*, 1992). These effects of carnosine have been suggested to prolong the *in vitro* lifespan of cultured human fibroblasts, protect brain endothelial cells from MDA and β amyloid peptide induced toxicity and delay the impairment of eyesight with ageing (Hipkiss *et al*, 1997; Holliday and McFarland, 2000; Wang *et al*, 2000).

In a review, Hipkiss (2000) further suggested that carnosine binds to the free carbonyl group on carbonylated proteins without removing the carbonyl bearing structure from the protein. Intriguingly, Otteneder and colleagues (2002) also mentioned that the hydrazines benzylhydrazine and phenylhydrazine were similarly capable of trapping the residual carbonyl groups of MDA-adducted deoxyguanosine and stripping the malondialdehyde equivalent from the adduct. These reactions regenerated the deoxyguanosine molecule and formed pyrazoles from the malondialdehyde equivalent (Otteneder *et al*, 2002). This stripping reaction did not occur with pentafluorobenzylhydrazine and alternatively a stable hydrazone species was generated. To date, however, no studies have explored these "adduct-trapping" or "adduct-stripping" mechanisms of drug action. Hipkiss (2000) subsequently suggested that such carnosylated proteins might either undergo proteolysis, form inert lipofuscin or block AGE binding to RAGE receptors and possibly prevent generation of reactive oxygen species.

1.4.5 Hydralazine

Hydralazine (1-hydrazinophthalazine, Apresoline) is an anti-hypertensive drug that has been in clinical use for nearly 50 years. It acts to relax arteriolar smooth muscle without affecting the heart or the venous system, producing a decrease in arterial pressure, decreased peripheral resistance and an increase in splanchnic blood flow (Freis *et al*, 1953; Koch-Weser, 1978). The precise mechanism by which hydralazine has this vasodilatory function is not known. Investigators have suggested molecular effects such as Cu^{+2} chelation and subsequent inhibition of Cu^{+2} containing semicarbazide sensitive amine oxidases, stimulation of adenylate and/or guanylate cyclase, inhibition of inositol triphosphate induced Ca^{+2} release from the sarcoplasmic reticulum, increased ion flux at high conductance Ca^{+2} activated K^{+} channels and inhibiting the conversion of dopamine to noradrenaline (Reece, 1981; Lyles *et al*, 1983; Bang *et al*, 1998;

Ellershaw and Gurney, 2001; Vidrio *et al*, 2003). It is known, however, that the antihypertensive action is due to its 4 nitrogens ($C=N-N=C-NH-NH_2$; Reece, 1981).

The clinical use of hydralazine is accompanied by some unwanted side effects including a systemic lupis erythematosus like syndrome, tachycardia, headache, dizziness, flushing, nasal congestion, anginal attacks and increased stroke volume and cardiac output (Koch-Weser, 1978). Though most of these side effects are due to the pharmacological actions of hydralazine, some have been suggested to be due to the ability of hydralazine to generate free radicals (Weglarz and Bartosz, 1991; Goodwin *et al*, 1996). In particular, the generation of radical species by hydralazine results in DNA scission (Weglarz and Bartosz, 1991; Runge-Morris, 1994). Mutagenic effects of hydralazine and its di-hydrazine analogue dihydralazine have also been reported, presumably due to their ability to intercalate DNA (Williams *et al*, 1980). Strangely, however, hydralazine has also been reported as a scavenger of free radicals (Johnson *et al*, 1998). This latter effect may indicate hydralazine can act as a 1 electron donor to ROS. This topic will be discussed further in Chapter 6 of this thesis.

The metabolism of hydralazine has been extensively studied in man and in rats, although it has not been investigated in mice (Talseth, 1977; Wagner *et al*, 1977; Reece, 1981). The metabolic fate of hydralazine in humans is illustrated in Figure 1.14. N-acetylation and ring closure at the hydrazine group represents the main metabolic fate of hydralazine in humans and rats, leading to formation of triazolo derivatives including triazolophthalazine and 3-methyltriazolophthalazine (Wagner *et al*, 1977). Other metabolic pathways include N-formylation and oxidation, followed by the formation of phase II conjugation products including glucuronides and sulphate metabolites (Wagner *et al*, 1977). However, the formation of hydrazone products with biogenic aldehydes and ketones also represents a major systemic fate of the drug, generating adducts with pyruvate, acetone, acetoactic acid, formaldehyde and acetaldehyde (Talseth, 1977; Wagner *et al*, 1977; O'Donnell *et al*, 1979). *In vitro*, in a cell free system, these products occur in a synthetic yield of greater than 80% following a 30 minute reaction at 37°C in buffered solution. *In vivo*, these hydrazones are eliminated via the urine (Talseth, 1977; O'Donnell *et al*, 1979). Hydralazine also extensively binds to plasma proteins (Talseth, 1977). Given the extensive metabolism and first pass elimination of hydralazine following oral and intravenous dosing, the elimination half life of hydralazine is relatively short, ranging from 1 to 3 hours in humans and rats (Wagner, 1973; Talseth, 1977; Wagner *et al*, 1977; Reece, 1981). However, its antihypertensive effects last considerably longer, possible due to vasodilatory effects mediated by the hydrazones (Talseth, 1977).

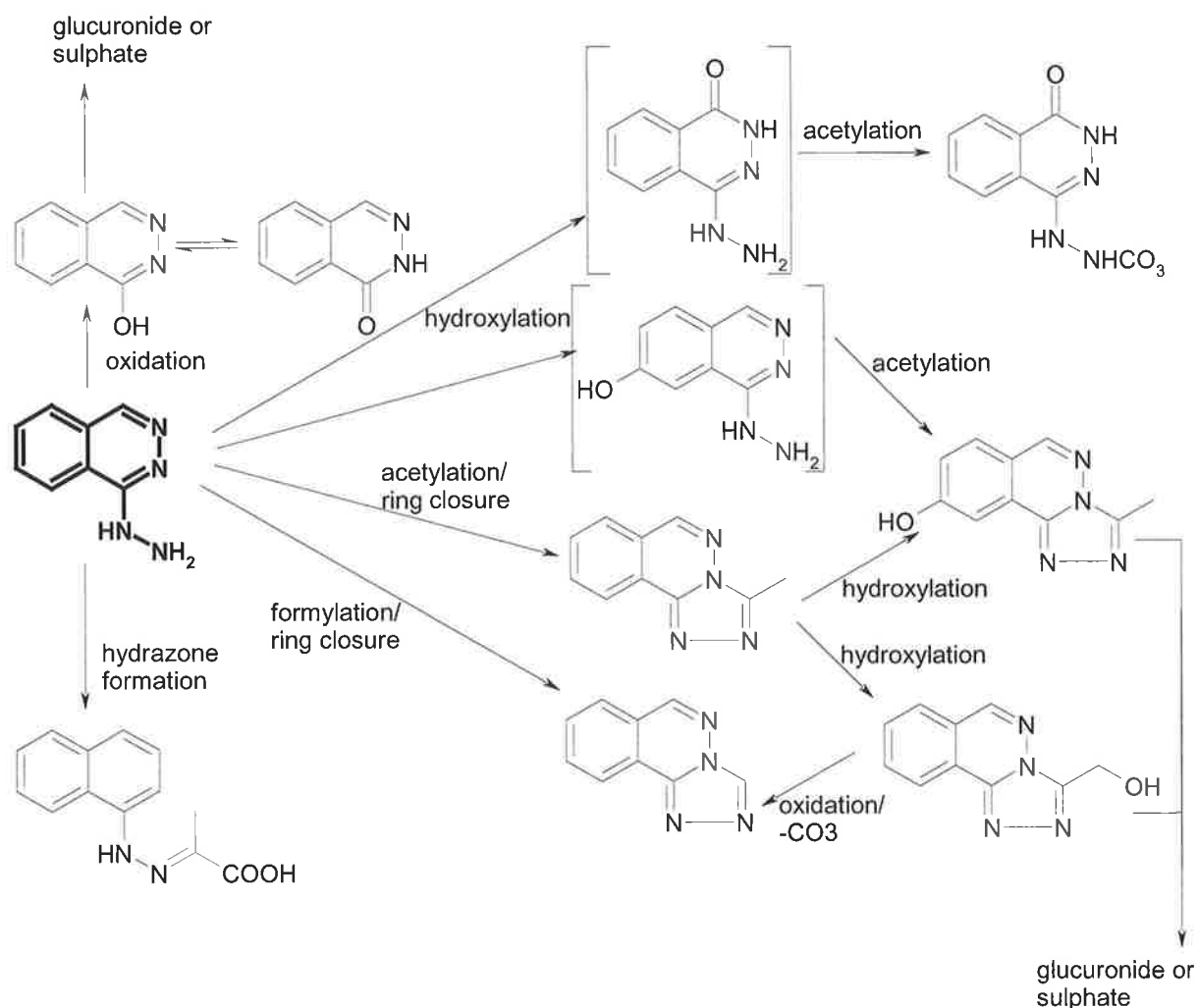


Figure 1.13 Hydralazine metabolism in man. Adapted from Wagner *et al* (1977).

The reported ability of hydralazine to rapidly form hydrazones with biogenic aldehydes and ketones such as acetone, pyruvic acid, acetoacetic acid, formaldehyde and acetaldehyde (O'Donnell *et al*, 1979), indicates that it may be used to alleviate the toxicity of chemically reactive endogenous carbonyl compounds. It has also been found that the reaction of hydralazine and carbonyl compounds critically depends on the structure of the aldehyde or ketone and the pH of the reaction medium (Reece, 1981). Interestingly, Lalich and Paik (1974) found that hydralazine inhibited allylamine induced myocardial fibrosis and hypertrophy in rats. The authors, however, were unsure of the mechanism by which hydralazine had this effect but suggested that it may be related to the ability of hydralazine to alleviate narrowing of the arteries (Lalich and Paik, 1974). It has previously been noted that the primary metabolite of allylamine is acrolein. An alternative mechanism for cardioprotection could be that hydralazine scavenged the acrolein formed, thus preventing its actions on the heart. This mechanism was suggested

tentatively by Nelson and Boor (1982), although definitive data showing trapping of acrolein by hydralazine was not provided.

In support of this, investigators in our laboratory have recently investigated the ability of a variety of known aldehyde scavengers including hydralazine and its analogue dihydralazine, to sequester free acrolein from solution at 37°C in a buffered solution (Burcham *et al*, 2000). Their results showed that not only does hydralazine efficiently remove free acrolein from solution, but that it was by far the most efficient acrolein scavenger tested when compared with carnosine, aminoguanidine and methoxyamine. They also showed that hydralazine prevented acrolein induced carbonylation of bovine serum albumin and that it protected cells from allyl alcohol induced cytotoxicity. These findings suggest that hydralazine may be another agent capable of preventing biogenic aldehyde induced modification of biomolecules and so deserves more attention in this respect. The aim of the work described in this thesis was to extend these preliminary observations, with a view to obtaining a rigorous understanding of the chemistry underlying the ability of hydralazine to abolish the toxicity of acrolein.

The initial findings of Burcham *et al* (2000) suggested that hydralazine blocks protein adduction by scavenging free acrolein, but our laboratory recently discovered that hydralazine also “traps” acrolein adducted BSA and hepatocellular proteins *in vitro* (Burcham *et al*, 2004). This trapping reaction occurred in proteins co-exposed to both acrolein (or its alcohol precursor, allyl alcohol in the case of cultured hepatocytes) and hydralazine. This trapping reaction closely corresponded with hydralazine-mediated cytoprotection against hepatocellular allyl alcohol toxicity. In addition, formation of these ternary trapped adducts blocked the immunorecognition of acrolein adducted proteins by an acrolein/KLH antiserum. Intriguingly, hydralazine only blocked the immunorecognition of acrolein-protein adducts by acrolein/KLH antiserum when the hydrazine was added within 30-60 minutes of commencing incubation of acrolein and BSA. This time dependent blockade also corresponded with cytoprotection, where the administration of hydralazine to allyl alcohol exposed hepatocytes could prevent cell death when the drug was administered 25 minutes after the alcohol. These results suggested that formation of ternary hydrazone products on acrolein-modified proteins may be a potential mechanism by which hydralazine blocks acrolein-mediated cytotoxicity. This mechanism was therefore investigated further in this thesis along side the free acrolein-scavenging mechanism highlighted earlier.

1.5 CONCLUSION

1.5.1 Summary and Rationale for the Study

The preceding review has highlighted the fact that oxidative stress and lipid peroxidation are important in the pathogenesis of a number of disease states including diabetes, neurodegenerative and cardiovascular diseases and inflammatory states. These reactions involve the production of toxic endogenous aldehydes and ketones including malondialdehyde, 4-hydroxynonenal and acrolein. Elevated plasma levels of sugars have also been linked to complications associated with diabetes including neuropathy, vasculopathy and nephropathy due to the formation of advanced glycation end products and carbonyl containing intermediates. Some of the products of lipid peroxidation reactions (MDA and acrolein) have been identified in foods and alcohol and as metabolites of allyl containing compounds and cyclophosphamide. These aldehydic products can react with biomolecules including proteins, DNA and lipids and cause crosslinking, reduced functional capacity, inflammatory reactions, cell death and decreased proliferation and protein and DNA synthesis. Their main targets are generally the nucleophilic primary amino group of lysine, the imidazole ring nitrogen of histidine and the thiol group of cysteine. These reactions occur mainly by Michael addition of the nucleophilic groups to the β carbon of the α,β -unsaturated aldehydes and to a lesser extent by Schiff base addition to the carbonyl carbon.

A range of scavengers of these endogenous aldehyde compound have been investigated for their ability to prevent or alleviate the symptoms of AGE mediated diabetic complications or to decrease the carbonyl-mediated damage associated with Alzheimer's disease, stroke or inflammatory bowel disease. Among the list of scavengers currently being investigated, aminoguanidine has received the greatest attention, having been investigated in phase III clinical trials of diabetic complications. Pyridoxamine has also been investigated in clinical trials. The endogenous skeletal muscle dipeptide carnosine is also showing promise as a scavenger of toxic aldehydes. These compounds generally act by either scavenging AGE intermediates and thereby preventing AGE formation, or by scavenging aldehydes derived from lipid peroxidation reactions, thereby preventing their binding to biomolecules.

Another compound which deserves greater attention as a carbonyl scavenger is hydralazine and its analogue dihydralazine. These hydrazine compounds have proven to be effective at scavenging a range of biogenic aldehydes and ketones including acrolein and appear to be

involved in preventing allylamine induced cardiotoxicity. Preliminary studies conducted so far in this laboratory have shown that among the aldehyde scavengers reviewed above, hydralazine and dihydralazine are much more effective at removing acrolein from solution and preventing BSA carbonylation by this aldehyde. These results suggest that hydralazine may be another potential therapeutic carbonyl scavenger, having a pronounced ability to sequester short chained 2-alkenals compared to other candidate molecules.

While the toxicity of hydralazine may limit its usefulness as a therapeutic agent for diabetic complications and other illnesses, it is an excellent starting point for developing an effective aldehyde scavenger with cytoprotective potency. This study is therefore aimed at determining why hydralazine is such an efficient scavenger of biogenic aldehydes and to use this information to identify an efficient scavenger of biogenic aldehydes without the expected anti-hypertensive and toxic effects of hydralazine. In this respect we will try to dissociate the anti-hypertensive and carbonyl scavenging properties of the compound by examining the acrolein-sequestering and cytoprotective properties of a range of hydralazine analogues.

1.5.2 Experimental Design and Hypotheses

The initial set of experiments will be conducted with the intention of characterising the reaction products of hydralazine with acrolein and crotonaldehyde. Preliminary experiments conducted by Dr's Phil Burcham and Simon Pyke indicated that there is one major reaction product between acrolein and hydralazine. This product was crudely characterised as the hydrazone acrylaldehyde 1-(phthalazin-1-yl)hydrazone, although no effort was made to sufficiently purify the product or do a full structural determination. Reactions will be conducted in phosphate buffer at pH 7.0 under a range of conditions (temperature and time modifications). The product will be characterised by ^1H -NMR, ^{13}C -NMR, mass spectrometry and ultraviolet, fluorescence and infrared spectrometry. The kinetics of product formation will be assessed and related to the rate of disappearance of the free aldehydes. It is hypothesised that kinetic investigations will reveal that the hydrazone is the major product of 1:1 reactions between free acrolein and hydralazine. In addition, the aldehyde trapping abilities of hydralazine and dihydralazine will be compared to trapping reactions by other known aldehyde scavengers (pyridoxamine, carnosine, methoxyamine and aminoguanidine). Scavenging experiments with acrolein will be repeated using a different method to that employed by Burcham *et al* (2000) to minimise loss of acrolein through evaporation.

The likely contribution of 2-alkenal trapping reactions of the abovementioned nucleophiles to their ability to provide protection against allyl alcohol and crotyl alcohol toxicity in isolated mouse hepatocytes will be examined. It is expected that cytoprotection against 2-alkenal toxicity is a direct result of trapping reactions between toxic 2-alkenals and the cytoprotective nucleophiles. This is because trapping, and therefore removal, of reactive 2-alkenals is expected to spare nucleophilic biomolecules, thereby blocking downstream events in cellular toxicity. In addition, to address the hypothesis that hydralazine provides protection against cellular toxicity mediated by a wide range of α,β -unsaturated aldehydes in addition to short chained 2-alkenals, the protection provided by hydralazine against pentenal, MDA and propionaldehyde toxicity will be assessed. The former two compounds will be added as a bolus to the culture media, whereas the latter compound will be generated *in situ* from the precursor propargyl alcohol. Also, given that hydralazine has strong vasoactive properties, a number of hydralazine analogues and related hydrazines devoid of the $-\text{N}=\text{N}-\text{C}-\text{NH}-\text{NH}_2$ group responsible for vasoactivity will be screened for their ability to scavenge acrolein and provide protection against allyl alcohol and crotyl alcohol induced hepatocyte cell death. Given the overall greater reactivity of aldehydes with hydrazines than less nucleophilic amines, it is hypothesised that the hydrazine-containing compounds will show greater reactivity with acrolein than the abovementioned amines examined by Burcham *et al* (2000). The hydralazine analogues that will be examined are 4-hydrazinoquinoline, 2-hydrazinoquinoline, 2-hydrazinopyridine and 1-hydrazinoisoquinoline. These compounds are chosen for study since the $-\text{N}=\text{N}-\text{C}-\text{NH}-\text{NH}_2$ functional group of hydralazine responsible for its antihypertensive effect, is disrupted while still retaining the nucleophilic hydrazine group. Although it will not be directly examined, it is expected that modifying the hydralazine molecule in this manner will result in the loss of its antihypertensive effects while still retaining its cytoprotective properties. This data will be used to further evaluate the structural and physicochemical properties required for cytoprotection with the view to developing more efficient 2-alkenal trapping compounds without pronounced vasoactivity.

Other potential mechanisms of hydralazine mediated protection against 2-alkenal toxicity will similarly be examined. For instance, Otteneder *et al* (2002) showed that various hydrazines can strip MDA from MDA-deoxyguanosine adducts. Alternatively, pentafluorobenzylhydrazine trapped MDA-adducts of deoxyguanosine were stable ternary adducted species. These reactions will similarly be investigated for hydralazine using electrospray ionisation mass spectrometry to monitor the formation of acrolein-peptide adducts as well as the reactions of these species with hydralazine and several structurally related hydrazines. A qualitative assessment will determine whether hydralazine “strips” acrolein from acrolein-protein adducts, or whether it mainly forms

stable ternary complexes. To assist in determining the likelihood of one of these reactions contributing to the cytoprotection afforded by hydralazine against 2-alkenal toxicity, the ability of hydralazine and the abovementioned analogues and related hydrazines to block recognition of acrolein adducted BSA by an anti acrolein/KLH antiserum raised in rabbits will be assessed.

Hydralazine's ability to protect against allyl alcohol hepatotoxicity in whole mice will also be examined, as allyl alcohol induces severe periportal necrosis in the liver (Piazza, 1915). Hepatotoxicity will be assessed by measuring the level of the liver specific enzymes sorbitol dehydrogenase and glutamate pyruvate transaminase in plasma 2 to 6 hours after allyl alcohol administration. It is hypothesised that, in accord with our laboratories previous observations in mouse hepatocytes *in vitro* (Burcham *et al*, 2000; Burcham *et al*, 2004), co-administration of hydralazine will provide protection against the serological changes indicative of liver damage induced by allyl alcohol. The time dependence of hydralazine administration to hepatoprotection will also be examined by administering the drug up to 30 minutes after the alcohol via intraperitoneal injection. This investigation will seek to support the time dependent nature of hydralazine mediated protection against allyl alcohol toxicity observed *in vitro* (Burcham *et al*, 2004) in a whole animal model. It is hypothesised that such protection will follow the pattern seen with *in vitro* protection against allyl alcohol toxicity (ie. that hydralazine could be administered within 25 minutes of the alcohol to afford protection). The extent and localisation of hydralazine-stabilised acrolein-protein adducts in the liver will be assessed by Western blotting and immunohistochemistry using the hydralazine stabilised acrolein/KLH antiserum developed in this laboratory (Burcham *et al*, 2004).

Given that hydralazine acts as both a 1 and 2 electron donor, another cytoprotective mechanism that will be examined is radical scavenging (Johnson *et al*, 1998). This effect of hydralazine has not previously been investigated in depth, thus this study will aim to directly investigate its antioxidant capabilities. This pathway may prove to be important as allyl alcohol is known to induce lipid peroxidation *in vitro* and *in vivo* and its toxicity is sometimes blocked by antioxidants (Miccadei *et al*, 1988). The ability of hydralazine, dihydralazine and a number of known antioxidants to prevent hepatocellular toxicity mediated by the toxic compounds allyl alcohol, tert-butylhydroperoxide and nitrofurantoin (all inducers of oxidative stress) will be examined. This will be for the purpose of examining the likelihood that hydralazine protects against 2-alkenal toxicity by blocking the ensuing oxidative stress. The additional oxidative toxins will be investigated to try to clarify the mechanism of allyl alcohol toxicity and the prevention of this toxicity by hydralazine. To build upon this data, the abovementioned

hydrazines and analogues of hydralazine will be screened for their ability to scavenge the free radicals galvinoxyl and 1,1-diphenylpicrylhydrazyl (DPPH). These studies will indicate whether the cytoprotection afforded by hydralazine against 2-alkenal toxicity involves the 1-electron donating ability of the hydrazine.

Given the apparent predominance of cyclised FDP-lysine adducts following incubation of acrolein and lysine for 24 hours (Uchida *et al*, 1998b), the relative importance of cyclic and acyclic acrolein adducts to the acute toxicity of this aldehyde will be assessed. This will be examined by comparing the hepatocellular toxicity and protein carbonylating ability of acrolein and a non-cyclisable carbonyl, methylvinyl ketone. Methylvinyl ketone has similar toxicological properties to acrolein, although it is expected that due to steric repulsion provided by the additional methyl group on the carbonyl carbon, methylvinyl ketone will not form FDP-like adducts, unlike acrolein (Esterbauer *et al*, 1975; Portoghesi *et al*, 1989; Lash and Woods, 1991; Lash *et al*, 1995; Morgan *et al*, 2000). It is therefore hypothesised that both acrolein and methylvinyl ketone will induce hepatocellular toxicity and protein carbonylation, although the effects of methylvinyl ketone will occur in the absence of cyclised adducts. Such results would tend to suggest that acyclic mono- and bis-acrolein adducts are more important mediators of toxicity than cyclised adducts.

1.5.3 Specific Aims of the Study

- 1- Examine the 2-alkenal trapping properties of hydralazine, related hydrazines and the amines aminoguanidine, pyridoxamine, carnosine and methoxyamine.
- 2- Examine hydralazine's ability to prevent against unsaturated carbonyl toxicity *in vitro*.
- 3- Examine the ability of structurally related hydrazines to protect against allyl alcohol induced hepatocellular toxicity *in vitro*.
- 4- Examine hydralazine's ability to prevent against allyl alcohol induced liver damage *in vivo* and examine the extent and localisation of hydralazine-stabilised acrolein-protein adducts in liver.
- 5- Examine the relative importance of adduct-stripping versus adduct-trapping properties of hydralazine and related hydrazines.
- 6- Examine the antioxidant/radical scavenging properties of hydralazine and related hydrazines with a view to determining whether these properties contribute to cytoprotection against oxidative cell injury.

- 7- Determine which properties of hydralazine make it an efficient cytoprotective compound.**
- 8- Identify a more effective aldehyde sequestering and cytoprotective compound without the anti-hypertensive effects of hydralazine.**
- 9- Determine the relative contribution of cyclised and uncyclised acrolein-protein adducts to its short term toxicity**

Chapter 2:

Aldehyde sequestering reactions

2.1 INTRODUCTION

Given the increasing evidence for the contribution of short-chain 2-alkenals to oxidative cell injury, a preliminary search for nucleophilic drugs that attenuate the toxicity of these substances was conducted by this laboratory (Burcham *et al*, 2000). Hydralazine and dihydralazine were identified as highly efficient scavengers of free acrolein in buffered solution, whereas the amines examined had minimal acrolein scavenging capabilities. They then showed that hydralazine and dihydralazine efficiently protected isolated primary mouse hepatocytes against allyl alcohol toxicity, which is mediated by its intracellular biotransformation to acrolein by hepatic alcohol dehydrogenase. This was shown as concentrations of hydrazine required to prevent cell death by 50% (EC₅₀) were in the low micromolar range. Various amine compounds that are putative carbonyl-trapping agents (aminoguanidine, carnosine, pyridoxamine, methoxyamine) had negligible protective effects against allyl alcohol toxicity, where methoxyamine, which showed the most pronounced acrolein scavenging capacity of all the amines tested, had a protective EC₅₀ of 10 mM. This data supported the mechanism of allyl alcohol protection being due to sequestering of free acrolein within the cells by the nucleophilic compounds.

However, the initial work by Burcham *et al* (2000) was subject to a number of limitations. For instance, the data on acrolein scavenging capacity was devoid of appropriate control acrolein loss, making the significance of the acrolein scavenging data of both the amines and the hydrazines questionable. An initial aim of the work in this Chapter was thus to reassess the acrolein sequestering capacity of the compounds examined by Burcham and colleagues (2000) using a reaction system that minimises acrolein loss via excessive headspace. This system was also used to examine the crotonaldehyde sequestering capability of the abovementioned

compounds. For comparison, the clinically used thiol compound MESNA was examined for its acrolein sequestering and protective capacity in cells. MESNA is used to treat the side effects, namely urotoxicity, associated with acrolein formation from the chemotherapeutic drugs cyclophosphamide, ifosfamide and trofosfamide (Brock *et al*, 1981; Ormstadt *et al*, 1983; Shaw *et al*, 1986). MESNA works by forming the inactive disulphide diMESNA in systemic circulation, which readily enters the bladder and kidneys and regenerates as active MESNA (Brock *et al*, 1981; Ormstadt *et al*, 1983). Since the active form of MESNA is present only in the bladder and kidneys, its acrolein scavenging capacity is restricted to the renal filtrate and urine (Shaw *et al*, 1986). This is due to its low lipophilicity ($\log P = -0.01$, SciFinder Scholar), rendering it unable to efficiently penetrate cellular membranes (Ormstadt *et al*, 1983; Ormstadt and Ohno, 1984).

The chemistry of carbonyl sequestering by hydralazine and dihydralazine was also investigated in the work described in this Chapter. The main reaction products of hydralazine with acrolein and crotonaldehyde were isolated and characterised. These products were then used in kinetic studies to follow their formation, along with the concurrent loss of acrolein and crotonaldehyde, in buffered solutions and in cells.

However, lipid peroxidation *in vivo* results in the production of a variety of structurally diverse α,β -unsaturated aldehydes, not just acrolein and crotonaldehyde (Esterbauer *et al*, 1987). Also, α,β -unsaturated aldehydes are formed as byproducts of the metabolism of a number of clinically used drugs. For instance, the antihypertensive drug Pargyline is associated with hepatotoxicity in humans and rats, via a mechanism involving its biotransformation to the alkynal propiolaldehyde (DeMaster *et al*, 1982; Moridani *et al*, 2001). Therefore, in addition to examining the cytoprotection afforded by hydralazine against allyl alcohol and crotyl alcohol toxicity, the ability of hydralazine to protect against cellular toxicity mediated by a range of unsaturated aldehydes, including malondialdehyde (MDA), pentenal and propiolaldehyde (shown in Figure 2.1), was assessed. While MDA and pentenal were added to the culture media of cells, propargyl alcohol was used as a precursor to propiolaldehyde. This biotransformation of propargyl alcohol to propiolaldehyde by P450 2E1 and alcohol dehydrogenase is responsible for the hepatocellular toxicity of the alcohol (Moridani *et al*, 2001).

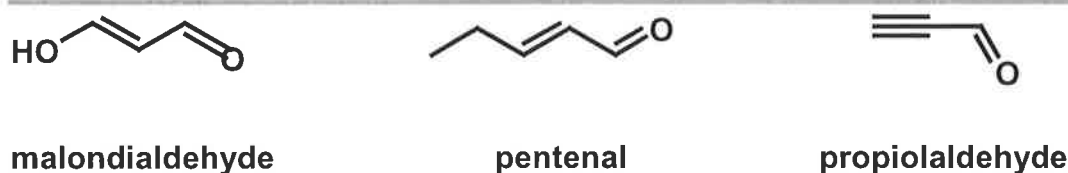


Figure 2.1 Structures of toxic aldehydes mentioned in the text.

The hypothesis proposed in this Chapter to explain hydralazines mechanism of cytoprotection against 2-alkenal toxicity was that direct aldehyde-trapping is the primary protective mechanism. In addition to the abovementioned experiments, it was deemed appropriate to compare the cytoprotective and acrolein sequestering properties of structurally related hydrazines (shown in Figure 2.2), including the phthalazine analogues hydrazinoquinazoline (HQZ), hydrazinoquinoline (HQL), hydrazinoisoquinoline (HIQ) and hydrazinopyridine (HP). Comparison of such structurally similar compounds to hydralazine allowed for a more accurate assessment of whether the 2-alkenal trapping properties of the nucleophilic compounds mediate cytoprotection. It was also expected that the abovementioned phthalazine analogues were devoid of anti-hypertensive effects, as the $-C=N-N=C-NH-NH_2$ pharmacophore present in hydralazine was disrupted. Additionally, naphthylhydrazine (NH) was included as it allowed an evaluation of the significance of ring nitrogens in the cytoprotective and scavenging effects of hydralazine. 1,1- and 1,2-Diphenylhydrazine (1,1-DPH and 1,2-DPH respectively) were examined to evaluate the effect of double substitution at N1 of the hydrazine group as well as the ability of secondary hydrazines to protect against 2-alkenal toxicity. Benzylhydrazine (BH) and phenylhydrazine (PH) were included to compare the cytoprotective properties of alkyl versus phenyl substituted hydrazines. Similarly, dimethylhydrazine allowed a determination of the relative importance of the phenyl groups in cytoprotection. Isoniazid (Iso) and 2,4-dinitrophenylhydrazine (2,4-DNPH) were included as two additional hydrazines with attached phenyl groups. The calculated log P's (SciFinder Scholar) of the abovementioned hydrazines are also shown in Figure 2.2. In addition to comparing the acrolein scavenging and cytoprotective effects of the hydrazines, these effects were compared to their log P's to investigate the significance of compound lipophilicity in the cytoprotective effects of the compounds.

These compounds were used in subsequent Chapters to evaluate the significance of other properties of hydralazine in its cytoprotective effects. It is anticipated that one or more of these compounds represent a good starting point for the development of a compound that provides strong protection against 2-alkenal toxicity without pronounced vasoactivity.

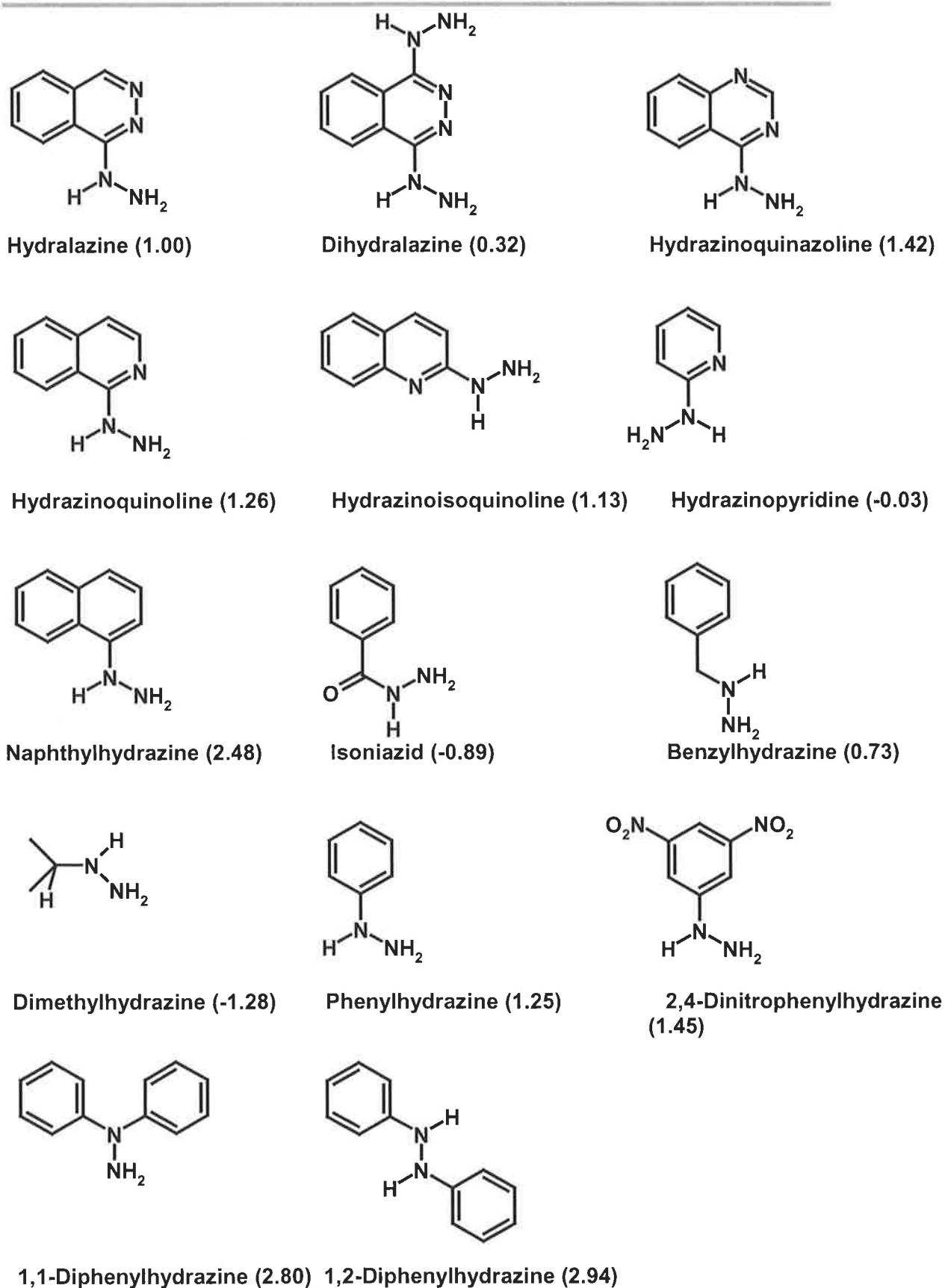


Figure 2.1 Structures of hydralazine analogues and structurally diverse hydrazines tested for their ability to block the toxicity of allyl and crotyl alcohols. Calculated log P values are indicated in parentheses (SciFinder Scholar).

2.2 MATERIALS AND METHODS

2.2.1 Animals

Male Swiss mice aged 4-8 weeks, were obtained from Laboratory Animal Services at the University of Adelaide (Waite campus) and housed in a temperature-controlled room (21°C) on a 12 h light/dark cycle. They received standard rodent pellets and water ad libitum. All animal experimentation was approved by the Animal Ethics Committee at the University of Adelaide.

2.2.2 Reagents

Sorresen buffer (50 mM, pH 7.0) was made and used for all buffered reactions. Acrolein (90%), allyl alcohol, crotonaldehyde and crotyl alcohol were purchased from Aldrich Chemical Co (Milwaukee, WIS, USA). Hydralazine HCl, 2-hydrazinopyridine, N^α-acetyl lysine, isonicotinic hydrazide (isoniazid), 2,4-dinitrophenylhydrazine (20% H₂O), 1,1-dimethylhydrazine, benzylhydrazine 2HCl, hydrazine hydrate, 1,2-diphenylhydrazine, phthalazine and 1-chloroisoquinoline were also obtained from Aldrich Chemical Co (Milwaukee, WIS, USA). Acetonitrile ("far UV" grade), methanol (HPLC grade), triethylamine (HPLC grade), sodium chloride and 1,1-diphenylhydrazine were purchased from BDH Laboratory Supplies (Poole, England). Phenylhydrazine was obtained from May and Baker Laboratory Chemicals (Dagenham, England). Analytical grade chloroform, dichloromethane, sodium hydroxide, hydrochloric acid, disodium hydrogen phosphate and sodium dihydrogen phosphate were all purchased from Ajax Chemicals (Auburn, Australia). MESNA (2-mercaptoethanesulfonic acid), dihydralazine, carnosine, pyridoxamine, methoxyamine, aminoguanidine, o-phthaldialdehyde, pentenal, propargyl alcohol and glutathione were purchased from Sigma Chemical Co (St. Louis, MO, USA). 2-Hydrazinoquinoline, 4-hydrazinoquinazoline and 2-hydrazinonaphthalene were obtained from Tokyo Kasei Kogyo (Japan), SPECS and BioSPECS BV (Netherlands) and Apin Chemicals (USA) respectively. RPMI culture medium 1640 was purchased from Invitrogen Corp. (NY, USA). Triton-X[®] 100 was purchased from Rohm and Cass Co (Philadelphia, USA). NAD⁺ was from Roche Diagnostics (IN, USA). MDA was synthesised by Dr Phil Burcham by Dowex hydrolysis of tetraethylacetate (Marnett and Tuttle, 1980)(69% purity). All other reagents were of the highest quality commercially available.

The purity of acrolein was assessed by determining its extinction coefficient (ε) following construction of a standard curve. The standard curve was analysed in a UV spectrophotometer

(210 nm, Hitachi U-2000) over a serially diluted concentration range of 10 to 100 μM . The determined ϵ was $10090 \text{ M}^{-1}.\text{cm}^{-1}$ as compared to the reported value of $11220 \text{ M}^{-1}.\text{cm}^{-1}$ (Merck *et al*, 1989). The actual concentration of acrolein in the stock solution was therefore determined to be 80%.

The purity of the hydralazine analogues 2-hydrazinopyridine, 2-hydrazinonaphthalene, 4-hydrazinoquinazoline and 2-hydrazinoquinoline were confirmed by Dr Simon Pyke via ^1H NMR spectroscopy (600 MHz, Varian INOVA, USA, CDCl_3). The purity of 2-hydrazinopyridine was >95%. All other analogues were >99%.

2.2.3 Synthesis of 1-Hydrazinoisoquinoline

1-Hydrazinoisoquinoline was synthesised according a previously described method (Albert and Catterall, 1967). 1-Chloroisoquinoline (1 g), hydrazine hydrate (3.8 ml) and ethanol (4.4 ml) were added to a 50 ml round bottomed flask which was fitted with a condenser attached to a nitrogen adaptor and bubbler. The mixture was refluxed under nitrogen for 1.5 hours. Any excess ethanol was removed under vacuum. The residue was filtered under vacuum, washed with warm water and collected by filtration. After the crude 1-hydrazinoisoquinoline was recrystallised (ethanol) it was further dried on an oil pump (0.1 mmHg) at 80°C (5 hours) to remove excess water and hydrazine. The identity of the product was confirmed (375 mg / 38% yield, mp= $174\text{--}175^\circ\text{C}$ compared to the mp of $172\text{--}173^\circ\text{C}$ reported by Albert and Catterall, 1976, Appendix 1; A1.1).

2.2.4 Sequestering of Acrolein and Crotonaldehyde by Nucleophilic Drugs

2.2.4.1 Protocol for Sequestering Reaction

The method used to compare the 2-alkenal scavenging potencies of various nucleophilic reagents was a modification of a previously reported method (Burcham *et al*, 2000). Briefly, amine and hydrazine nucleophiles, including N-acetyl lysine and MESNA, were dissolved in prewarmed sodium phosphate buffer (pH 7.0) to give a final concentration of 1 M before 0.5 ml volumes of these solutions were added to triplicate 1 ml gas chromatography vials. Reactions were started by the addition of prewarmed 0.5 ml volumes of equimolar concentrations of either acrolein or crotonaldehyde (1 M). Vessels were filled to capacity to minimize headspace loss of aldehydes

(see below). The vials were then placed in a 37 °C mixing incubator for either 10, 20 or 30 minutes. At each time point, an aliquot was taken from one of the triplicate vials, diluted 1/10 to 1/50 in mobile phase before a 100 µl sample was used for the determination of aldehyde concentrations via HPLC.

2.2.4.2 HPLC Assay

The HPLC system comprised an ODS Hypersil column (150 x 4.6 mm, 5 µm, Keystone Scientific Inc, PA, USA) connected to a GBC LC1150 pump (Dandenong, Australia), fitted with an online ERC 3415 degasser and a Hewlet Packard series 1100 UV detector that monitored the absorbance of column eluate at 210 nm. The mobile phase used to analyse free acrolein comprised 20% methanol:water (v/v) while in the case of crotonaldehyde, 30% methanol:water (v/v) was used. The flow rate was maintained at 1 ml/minute. Under these conditions, the retention times for acrolein and crotonaldehyde were 2.7 and 3.1 minutes, respectively. Aldehyde concentrations were determined by comparing sample peak areas to those obtained by analysing standard solutions of acrolein and crotonaldehyde (prepared in mobile phase to give final aldehyde concentrations ranging from 0.1 to 12 µM). Output from the UV detector was collected and analysed using Delta Junior HPLC analysis software (Qld, Australia). The assay validation data for acrolein and crotonaldehyde are reported in Appendix 1; A1.2. As the protocol only measured the % loss of free aldehyde, standard curves were not required for analysis of this data. The reproducibility of multiple injections of a solution of acrolein and of multiple dilutions of acrolein were assessed and are also reported in Appendix 1; A1.2.

2.2.5 Reaction of Acrolein and Hydralazine

The reaction product between acrolein and hydralazine was initially identified via electrospray ionisation mass spectrometry by Dr Phil Burcham. It was subsequently synthesised and characterised by Dr Simon Pyke. Following optimisation of the isolation method, further reaction conditions were trialled to determine if the yield of the apparent main reaction product could be improved. Initial experimentation by Dr Pyke using ¹H NMR showed that a hydrazone product (acrylaldehyde 1-[phthalazin-1-yl]hydrazone, APH) was the predominant (¹H NMR) reaction product between 1:1 (135 mM) acrolein and hydralazine. It was also shown that the reaction between acrolein and hydralazine was almost complete after 10 minutes at 4°C, as hydralazine specific ¹H NMR peaks had disappeared by this time. The reaction method and isolation procedure was therefore developed to specifically isolate and identify APH.

APH was formed by reacting equimolar (135 mM, 50 mM sodium phosphate buffer, Ph 7) acrolein and hydralazine (8 ml total) in a 25 ml round bottomed flask at 4°C (10 minutes). The solution was then extracted with chloroform (3 x 10 ml) and the organic layer washed with brine (15 ml). The chloroform layer was then dried over magnesium sulphate and the solvent removed after filtration of the drying agent. The remaining product was a dark orange oil. An appropriate solvent mixture for column purification of the APH was then determined to be a 1:19 ratio of diethyl ether and dichloromethane (retention coefficient of 0.3). The crude APH was redissolved in diethyl ether:dichloromethane (1:19) and chromatographed on a flash column (2 x 20 cm) with 230 – 400 ASTM mesh silica packing and the same solvent under nitrogen. The APH fractions were combined and the solvent removed, yielding a yellow oil which was further dried on an oil pump (0.1 mmHg, 30 minutes). When left under nitrogen in the freezer (-15°C) for 7 days the yellow oil formed a yellow crystalline product. TLC's of this product (35% yield, mp 61-63°C), however, revealed that it comprised 2 compounds, a low R_f component (approximately 10%) and a high R_f component (approximately 90%). The high R_f component could be isolated by preparative TLC (20 x 20 cm plate, 3 elutions with dichloromethane). The low R_f component could not be isolated in a pure form and showed poor stability. The high and crude low R_f isomers of APH were characterised by ¹H and ¹³C NMR spectroscopy, electrospray mass spectrometry, infrared spectroscopy and UV spectroscopy.

2.2.6 Reaction of Crotonaldehyde and Hydralazine

To characterise products formed during reactions between hydralazine and crotonaldehyde, a 1:1 mixture of hydralazine and crotonaldehyde (135 mM, 20 ml) in 50 mM sodium phosphate buffer (pH 7.0) buffer was stirred for 1 hour at 4 °C. The mixture was then extracted with chloroform (3 x 15 ml) and the combined organic extracts were washed with brine (1 x 20 ml) before they were dried over MgSO₄. The most appropriate solvent mixture for isolating the main hydrazone product by TLC was determined to be 1:29 ethyl acetate:dichloromethane. Following filtration and evaporation to dryness, the reaction product was redissolved in 1:29 ethyl acetate:dichloromethane (v/v) and chromatographed on silica as previously described. Once again two products were isolated; a yellow crystalline high R_f product and a yellow-brown solid low R_f product. The products were found to be isomeric but-2-enal phthalazin-1-ylhydrazone (BPH), (high R_f isomer 34% yield, mp 111-114°C; low R_f isomer 6%, mp 80-85°C). The purity of the products was found to be >95% and 90% for the high and low isomers respectively (¹H NMR). The structures of the isomers were determined by ¹H and ¹³C NMR spectroscopy

(coupling validation by COSY, HMBC and HSQC; spectra not shown), mass spectrometry and IR and UV spectroscopy.

2.2.7 Stability of APH and its Further Reaction with Acrolein and Hydralazine.

2.2.7.1 HPLC Assay for the High R_f APH Isomer

An assay was developed to quantify high R_f APH in the shortest possible run time in the presence of acrolein or hydralazine. The low R_f isomer of APH could not be assessed as it produced a broad, asymmetrical peak in HPLC that could not be accurately quantified. Stock solutions of APH were made up in acetonitrile (5 mM) and stored in a freezer (-15°C) for up to 1 week and aliquots were taken to prepare standards for HPLC analysis. APH solutions were stable during this time interval. The same equipment and column as previously mentioned were again employed in this assay, but a different mobile phase was used. The UV detector was again set at 210 nm. The mobile phase (40% acetonitrile/60% phosphate buffer [50 mM, pH 7.0]/0.2% triethylamine) was pumped at a rate of 1 ml/minute, yielding a retention time for high R_f APH of 4.9 minutes. Details of the validation of this method are given in Appendix 1; A1.3. The reproducibility of multiple injections of the same solution of APH, multiple dilutions from the same stock and multiple stock dilutions of APH are also reported in Appendix 1; A1.3. Acrolein was measured via the method mentioned previously (section 2.2.4.2).

2.2.7.2 Protocol

The protocol for this set of experiments was similar to that employed for measuring the loss of acrolein in solution in the presence of aldehyde scavengers. Equimolar amounts of APH and either acrolein or hydralazine (500 µM) were mixed in prewarmed (37°C) buffer and the loss of APH and/or acrolein monitored every 10 minutes for 4 hours in capped GC vials. Alternatively, APH (500 µM) was incubated in buffered solution alone and its loss was monitored for 4 hours as a determinant of stability in solutions at physiological pH. Once again, at 10 minute intervals an aliquot of reaction solution was taken, diluted in mobile phase and a 100 µl aliquot was injected onto the HPLC. The loss of acrolein when incubated with APH was compared to a control (acrolein alone) loss of acrolein over time. The loss of APH and acrolein was plotted as % of compound remaining in solution (measured as area under the peak) compared with the control (zero time) peak area. Three replicates of each experiment were conducted and values represented as % of control ± SEM.

2.2.8 Kinetics of High Rf APH and BPH Formation

2.2.8.1 HPLC Assays for High Rf APH and BPH

To determine whether kinetic or stability factors contribute to the different hydrazone yields during hydralazine's reactions with acrolein and crotonaldehyde, the time-course of hydrazone formation was explored under the same reaction conditions used in determining the 2-alkenal scavenging potential of hydralazine, except that reactions were followed for up to 24 hours. Also, to explore the role of reaction temperature in product yields and stability, the reactions were performed in parallel at 37 and 4°C. A new assay for measurement of high Rf APH and BPH had to be developed, however, to eliminate contamination by additional products of the reaction of acrolein and crotonaldehyde with hydralazine. Again, the low Rf isomers of APH and BPH could not be assayed as they form broad asymmetrical peaks that could not be accurately quantified. The formation of the high Rf products of hydralazine with acrolein and crotonaldehyde, APH and BPH respectively, were measured at 210 nm on a Zorbax Eclipse XDB C18 column (250 x 4.6 mm) with a 5 µm particle size (Agilent Technologies, USA) using the HPLC system previously mentioned. The column was protected by an Adsorbosil C18 (5 µm particle size) guard column (Adelab Scientific, Adelaide, Australia). The mobile phase (30% acetonitrile in sodium phosphate buffer [50 mM, pH 7] and 40% acetonitrile in sodium phosphate buffer for APH and BPH respectively) was pumped at a flow rate of 1 ml/minute. The retention times for APH and BPH with their respective mobile phases were 17.0 and 13.1 minutes. Details of the validation of these methods are given in Appendix 1; A1.4. Stability of APH and BPH in the reaction buffer was also measured at 37°C and 4°C. Free aldehydes were measured using the assays previously mentioned.

2.2.8.2 Protocol

The concurrent loss of acrolein and crotonaldehyde when incubated with hydralazine and formation of APH and BPH respectively were analysed over 24 hours in buffered solution at 37 and 4°C (3 replicates each). Due to the inability to measure the 2-alkenals and the hydrazones simultaneously under the same HPLC conditions, separate assays were run to assess 2-alkenal loss and hydrazone formation over time. Sequestering reactions were set up as previously described at the required temperature. For acrolein loss and APH formation, samples were taken at 0, 5, 10, 15, 20, 25 and 30 minutes, 1, 3, 6, 12 and 24 hours after the addition of an equimolar amount of acrolein to a solution of hydralazine (500 µM) at 37°C, and at 0, 10, 20 and 30

minutes, 1, 2, 3, 6, 12 and 24 hours later at 4°C. For crotonaldehyde loss and BPH formation, samples were taken at 10 and 30 minutes, 2, 3, 4, 6, 12 and 24 hours after the addition of an equimolar amount of crotonaldehyde to a solution of hydralazine (500 µM) at 37 and 4°C. Standard curves of acrolein and crotonaldehyde (0.5 – 12 µM) or APH (11.25 – 0.9 µM) and BPH (1.0 – 12 µM) were run on each experimental day. The loss of acrolein and formation of APH were plotted as their free concentrations in solution (µM) ± SEM.

2.2.9 Protection by Hydralazine and Dihydralazine Against Toxicity Mediated by Unsaturated Aldehydes or their Precursors.

2.2.9.1 Hepatocyte Isolation

Hepatocytes were isolated via collagenase digestion of the livers of anaesthetised mice using a previously described method (Harman *et al*, 1987). After filtering suspensions through 200 and 100 µm nylon gauze, cells were washed via three rounds of centrifugation and resuspension in Krebs-Henseleit buffer (supplemented with 1 mM CaCl₂). Finally, cells were suspended in RPMI medium (supplemented with 0.03% L-glutamine, 0.2% bovine serum albumin and penicillin/streptomycin (50 units/l and 50 µg/ml respectively) at a density of 1 x 10⁶ cells/ml and were plated on collagen-coated dishes (60 mm diameter, IWAKI, Japan, 3 ml cell suspension per plate). Cells were allowed to attach to the dishes for 2 to 3 h in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C before use.

2.2.9.2 Protocol

Prior to initial experimentation, brief concentration responses for the cytotoxicity of propargyl alcohol, pentenal and MDA were performed on concentrations of toxin ranging from 100 µM to 10 mM for up to 24 hours (data not shown). For propargyl alcohol and pentenal, concentrations were chosen that resulted in maximal or near maximal cell death within 3 hours. MDA did not induce toxicity until 12 hours after the addition of the aldehyde at 10 mM and was therefore assessed at a later time point than for the other aldehydes.

Plated cells were washed with PBS (50 mM, pH 7.4; 3 ml per plate for 60 mm dishes) to remove nonadherent cells before they were incubated with either culture media alone (supplemented with L-glutamine and penicillin/streptomycin as above) or supplemented with one of the

scavengers (1-100 μ M; hydralazine, dihydralazine) for 5 minutes prior to the addition of allyl alcohol (100 μ M), crotyl alcohol (500 μ M), pentenal (1 mM), propargyl alcohol (1 mM) or MDA (10 mM) (cytoprotection by dihydralazine was only examined for allyl and crotyl alcohols). Nucleophilic compounds and unsaturated alcohols were dissolved directly in culture media. Dishes were then returned to the incubator for 3 hours (18 hours for MDA) with aliquots of culture media taken at 1 hourly intervals (2 hourly intervals starting from 12 hours after addition of MDA to the culture media) to assess lactate dehydrogenase (LDH) leakage from the cytoplasm. LDH activity in culture media was assayed using an established method (Richards *et al*, 1975), although it was modified slightly to allow concurrent determination of multiple samples using a fluorescence microplate reader (POLARStar, BMG Laboratories). The reaction followed NADH formation from NAD^+ in tris buffer (50 mM, pH 8) in the presence of 7 mM NAD^+ and 25 mM lactic acid at respective excitation and emission wavelengths of 320 and 460 nm. Cell death was reported as % LDH leakage compared to total cellular LDH, which was determined by sonicating the cells with 0.5% Triton[®] X-100 (1/10 dilution of 5% Triton[®] X-100 in culture medium containing cells).

2.2.10 Effect of Hydralazine and Dihydralazine on Cellular Glutathione Depletion Mediated by Unsaturated Aldehydes or their Precursors

2.2.10.1 Hepatocytes

Hepatocytes were isolated as described above (section 2.2.9.1) except that cells (1×10^6 cells per ml) were plated on 35 mm dishes at a density of 2×10^6 cells per plate (2 ml media). Cells were similarly allowed to attach for 2 to 3 hours in an incubator prior to treatment.

2.2.10.2 Protocol

Hepatocytes were prepared and treated with the abovementioned aldehydes (or their precursors) and hydralazine or dihydralazine (100 μ M) as previously described. Again, dihydralazine was only examined with allyl and crotyl alcohols. Cells were incubated with the aldehydes (or their alcohol precursors) alone or with one of the hydrazines for 30 minutes at 37°C. The cells were then washed once with PBS before ice cold 3% perchloric acid (PCA, 1.5 ml) was added. The cells were then scraped on ice and the homogenate transferred to Eppendorf tubes which were centrifuged at 7000 x g for 5 minutes. The supernatant was removed and a 1 ml aliquot

neutralised with 160 μ l of 2.5 mM sodium hydroxide. A 50 μ l aliquot was then assayed for GSH.

2.2.10.3 Glutathione Determination

The method used for GSH determination was based on the procedure of Hissin and Hilf (1976) with modifications to enable use of a microplate reader (Froschio, 2002). The method measures a fluorescent isoindole formed upon reaction of GSH with o-phthaldialdehyde (Senft *et al*, 2000). Standard curves of GSH (100-1000 ng) were prepared in 3% PCA. These were then neutralised with NaOH (160 μ l per ml of PCA). Samples (50 μ l) were then diluted in 200 μ l volumes of Tris-saline buffer (0.2 M, pH 8.0, containing 1 mM EDTA) into white 96 well microplates (Labsystems, Finland). After the addition of 10 μ l o-phthaldialdehyde (1 mg/ml in methanol) reactions were allowed to proceed at room temperature in the dark for 20 minutes. Sample fluorescence was then measured with a microplate reader (Polarstar Galaxy, BMG Labtechnologies Pty. Ltd, Australia) using excitation/emission wavelengths of 340/420 nm respectively. Cellular GSH content was expressed as μ g/million cells.

2.2.11 Detection of High Rf APH in Culture Media of Allyl Alcohol- and Hydralazine-Treated Hepatocytes.

Mouse hepatocyte monolayers, prepared as outlined in the preceding section, were co-treated for 30 minutes with allyl alcohol (100 μ M) and hydralazine (0-100 μ M) at 37 °C. Next, cells were ruptured by the addition of Triton[®] X-100 (0.3 ml, to give a final concentration of 0.5% v/v) followed by sonication to provide a whole dish lysate. The mixture was transferred to a centrifuge tube and centrifuged at 1600 x g for 5 minutes to pellet cellular debris. The resulting supernatant was then applied to a solid phase extraction column (1 ml, C-18 packing; J.T. Baker Chemical Co., NJ, USA) that had been pre-equilibrated with tetrahydrofuran and then water. The column was then washed with water (1 ml) to remove buffer salts and residual hydralazine. Finally, APH was eluted with 300 μ l tetrahydrofuran and the resulting eluant was diluted 1:1 in water before a 100 μ l aliquot was analysed via HPLC for the presence of high Rf APH. The method employed the Zorbax Eclipse XDB C-18 column and apparatus described above in section 2.2.8.1, except that the mobile phase comprised 40 % acetonitrile/0.01% triethylamine in 50 mM sodium phosphate buffer (pH 7) at a flow rate of 1.2 ml/minute. The retention time for APH was 9.0 minutes. The concentration of APH was determined by comparison of the APH peak areas to those of similarly extracted standard curves (0.1 – 4 μ M). The validation for this assay is given in Appendix 1; A1.5.

2.2.12 Metabolism and Toxicity of High Rf APH in Cultured Hepatocytes

To explore the metabolic fate of products formed during trapping reactions between acrolein and hydralazine, hepatocyte monolayers were exposed to 1 μ M high Rf APH for up to 3 hours. At 30, 60, 120 and 180 minutes following the addition of APH, dishes were removed from the incubator and Triton[®] X-100 was added to the supernatant and cell lysates were prepared as outlined above. Following the isolation of extracts on SPE columns as described above, concentrations of APH were determined in the samples via UV-HPLC as described above. In a related experiment, to determine whether trapping of acrolein by hydralazine truly constitutes detoxication, the toxic potential of APH was assessed by exposing mouse hepatocytes to 1 to 100 μ M APH, and aliquots of culture media were taken for the assessment of LDH leakage at 60, 120 and 180 minutes.

2.2.13 Scavenging Potential and Cytoprotection of Other Hydrazines and Hydralazine Analogues

2.2.13.1 Acrolein Scavenging Ability

Solutions of acrolein were made in phosphate buffer (50 mM) as previously described. Stock solutions (10 mM) of 2-hydrazinoquinoline, 1-hydrazinoquinazoline, 2-hydrazinopyridine, naphthylhydrazine, 1,1-diphenylhydrazine, 1,2-diphenylhydrazine, dimethylhydrazine, isoniazid and 2,4-dinitrophenylhydrazine were made in acetonitrile/buffer mixtures depending on solubility. Phenylhydrazine and benzylhydrazine were diluted in various methanol/buffer mixtures depending on solubility. 1-Hydrazinoisoquinoline was diluted in DMSO. The proportion of solvent in the final reaction mixture was therefore $\leq 5\%$. The presence of 5% of these solvents in the final reaction mixture did not alter the ability of hydralazine to sequester acrolein in solution. The ability of these compounds to scavenge acrolein from buffered solution was then determined as previously described.

2.2.13.2 Allyl Alcohol and Crotyl Alcohol Cytoprotection by Other Hydrazines and Hydralazine Analogues

Hepatocytes were prepared as previously described and resuspended at a density of 4×10^5 cells/ml and plated on collagen coated 96 well plates (IWAKI, Japan, 100 μ l cell suspension per

well) for rapid screening of allyl alcohol toxicity and protection. Cells were then allowed to seed for 2 to 3 hours as previously described. Cells were treated with 100 μ M allyl alcohol and 1-100 μ M of the hydrazines or amines as previously described (with assistance from Dr Frank Fontaine) in a final volume of 100 μ l RPMI. All nucleophilic compounds were prepared in stock solutions of 50 mM in DMSO prior to dilution in culture media. Alternatively, the hydrazines hydrazinoisoquinoline, hydrazinoquinoline, hydrazinoquinazoline, 1,1-diphenylhydrazine and benzylhydrazine were compared for their ability to similarly protect against crotyl alcohol (500 μ M) toxicity in 60 mm dishes as previously reported for hydralazine and dihydralazine. Aliquots of media (10 μ l) were taken 1 and 2 hours after the addition of allyl alcohol and 1, 2 and 3 hours after crotyl alcohol and assayed for LDH activity.

2.2.13.3 Statistical Analyses

Correlation studies were performed between the acrolein scavenging capacity and cytoprotective properties of the various hydrazines and analogues and between cytoprotection and log P of the compounds. Scavenging capacity was calculated as the rate of acrolein loss in the first 10 minutes of the reaction (% acrolein loss per minute). Statistical analyses were via 1-way Pearson's correlation tests (significance set at $p < 0.05$).

2.3 RESULTS

2.3.1 Aldehyde Sequestering Reactions

Several known aldehyde scavengers were compared for their ability to sequester acrolein and crotonaldehyde from buffered solution at physiological temperature (37°C). As lysine groups are particularly susceptible to modification by acrolein, the ability of this amino acid to remove acrolein from solution was also examined.

The ability of various aldehyde scavengers to sequester acrolein from solution is shown in Figure 2.3a. The loss of acrolein due to evaporation into headspace from solution was approximately 4% over 30 minutes. This was similar to the loss of acrolein when co-incubated with an equimolar amount of aminoguanidine, indicating the latter is a very poor acrolein scavenger. The loss of acrolein in the presence of lysine was approximately 13% over 30 minutes followed by carnosine and pyridoxamine which showed similar acrolein scavenging capacity, removing

approximately 20% of free acrolein from solution in 30 minutes. Methoxyamine was the most effective amine at sequestering acrolein from solution, having removed around 40% of available acrolein from solution in the same time period. Second to MESNA, hydralazine and dihydralazine were the most effective scavengers of acrolein, with dihydralazine being approximately twice as efficient as hydralazine. These compounds removed approximately 82 and 92% of acrolein respectively from solution within 30 minutes of incubation. MESNA removed almost all of the acrolein in solution within 10 minutes of incubation (Figure 2.3a) as expected given the high reactivity of the thiol group.

With the exception of N^α-acetyl lysine and MESNA, which were not tested against crotonaldehyde, the ability of these compounds to sequester crotonaldehyde from solution resembled their acrolein sequestering ability with the exception that carnosine appeared to be slightly more effective at removing crotonaldehyde from solution than pyridoxamine (Figure 2.3b). However, in all cases, the ability of these scavengers to remove crotonaldehyde from solution was less than their ability to sequester acrolein (note the different Y-axis in panel B of Figure 2.3). Thus, dihydralazine sequestered only around 28% of crotonaldehyde from solution within 30 minutes of incubation. This is likely due to trapping reactions at the β-carbon being hindered by the additional methyl group.

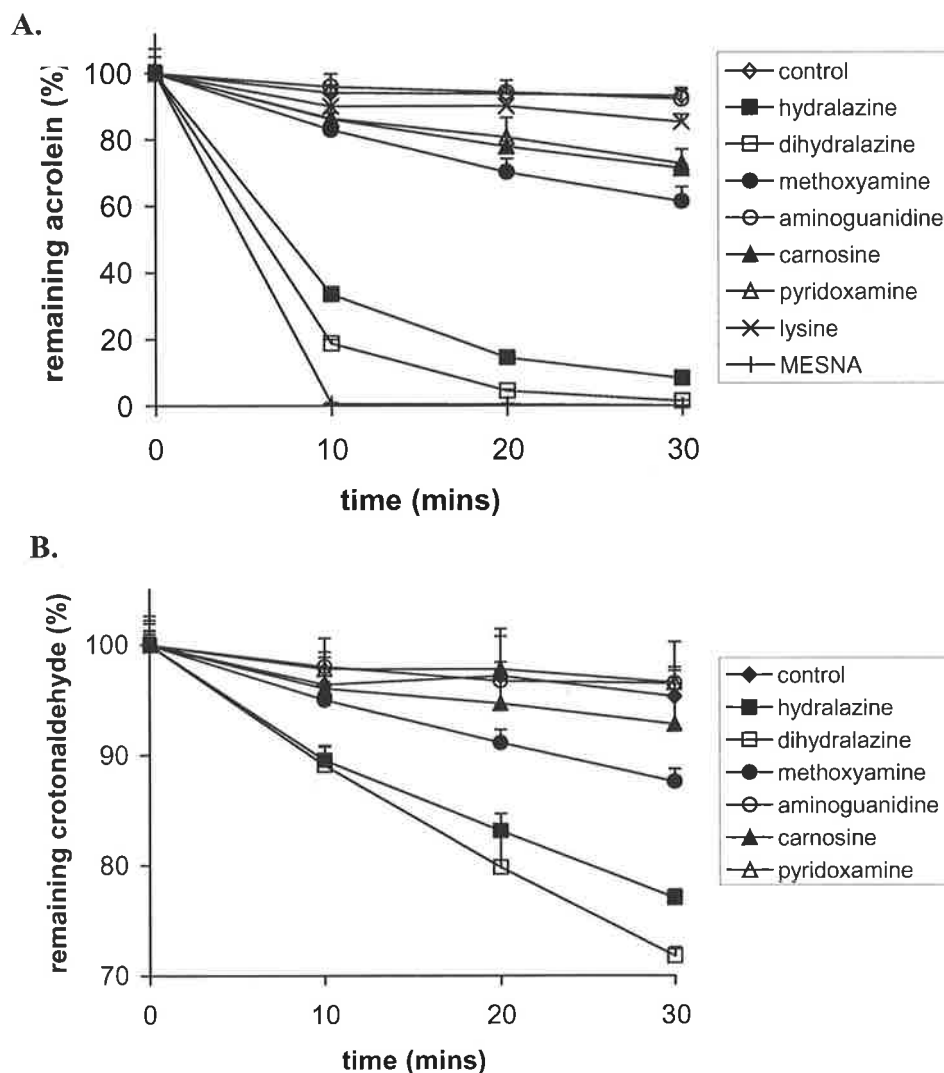


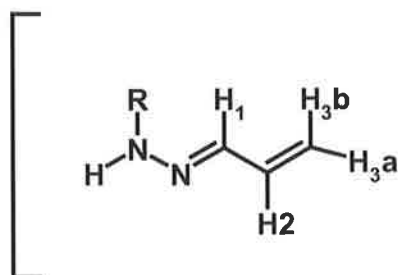
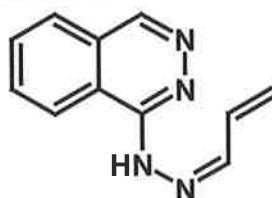
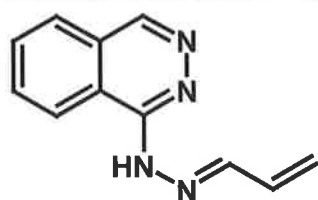
Figure 2.3 Percent of remaining acrolein (A) or crotonaldehyde (B) in solution after reaction with equimolar scavengers at 37°C in buffered solution. Data represented as mean $\pm$ SEM, $n=3$.

2.3.2 Characterisation of the Reaction Products Between Acrolein and Hydralazine

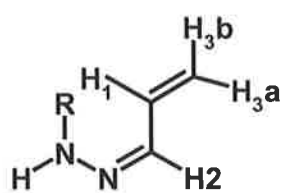
Preliminary studies of products formed during the reaction of acrolein and hydralazine for 10 minutes at 4°C revealed only one predominant hydrazone product by ^1H NMR (SM Pyke, personal communication). This was initially characterised as acrylaldehyde 1-phthalazin-1-ylhydrazone (APH). However, a closer inspection of this product by TLC revealed a relatively minor secondary product which was not initially apparent on ^1H NMR. Electrospray ionisation mass spectrometry of this product also revealed a single MH^+ of m/z 199 (the same as the major product). The major high R_f product could be isolated from the minor low R_f peak although the low R_f peak could not be sufficiently purified. The high R_f compound had a similar IR and UV

spectrum to the Rf mixture (IR; nujol): 3390, 3257, 1624, 1589, 1530 cm^{-1} . UV (acetonitrile) (log ϵ): 202 (5.0), 287 (4.32), 356 nm (4.22). The NMR spectrum of the high Rf compound was different to that of the mixture however. The **high Rf** component was shown to have ^1H NMR (Figure 2.4a and b, 600 MHz / CDCl_3); δ_{H} : 5.58 [dt, $J_{2,3b} = 10.3$ Hz $J_{1,3b} = J_{3a,3b} = 1.1$ Hz, 1 H, H(3b)], 5.61 [ddd, $J_{2,3a} = 17.4$ Hz $J_{1,3a} = J_{3a,3b} = 1.1$ Hz, 1 H, H(3a)], 6.59 [ddd, $J_{1,2} = 9.7$ Hz $J_{2,3a} = 17.4$ Hz $J_{2,3b} = 10.3$ Hz, 1 H, H(2)], 7.41 – 7.43 [m, 1 H, H(5')], 7.54 – 7.59 [m, 2 H, H(6',7')], 7.76 [s, 1 H, H(4')], 8.10 [dt, $J_{1,2} = 9.7$ Hz $J_{1,3a} = J_{1,3b} = 1.1$ Hz, 1 H, H(1)], 8.27 – 8.29 [m, 1 H, H(8')], 10.50 [br s, 1 H, NH]. The ^{13}C NMR spectrum showed (Figure 2.4c, 150 MHz / CDCl_3); δ_{C} : 124.21 [C(8')], 124.75 [C(3)], 126.04 [C(5')], 126.53 [C(8a')], 127.31 [C(4a')], 131.61 [C(7')], 132.12 [C(6')], 135.07 [C(2)], 138.17 [C(4')], 149.15 [C(1')], 156.62 [C(1)].

The **low Rf** component (through analysis of a 1:1 isomeric mixture) was shown to have ^1H NMR (Figure 2.5a and b, 600 MHz / CDCl_3); δ_{H} : 5.56 [dt, $J_{2,3b} = 10.3$ Hz $J_{1,3b} = J_{3a,3b} = 1.1$ Hz, 1 H, H(3b)], 5.60 [ddd, $J_{2,3a} = 17.4$ Hz $J_{1,3a} = J_{3a,3b} = 1.1$ Hz, 1 H, H(3a)], 7.36 [dt, $J_{1,2} = 9.4$ Hz $J_{1,3a} = J_{1,3b} = 1.1$ Hz, 1 H, H(1)], 7.40 – 7.42 [m, 1 H, H(5')], 7.50 [ddd, $J_{1,2} = 9.4$ Hz $J_{2,3a} = 17.4$ Hz $J_{2,3b} = 10.3$ Hz, 1 H, H(2)], 7.54 – 7.59 [m, 2 H, H(6',7')], 7.75 [s, 1 H, H(4')], 8.31 – 8.33 [m, 1 H, H(8')], 10.44 [br s, 1 H, NH]. The ^{13}C NMR spectrum showed (Figure 2.5c, 150 MHz / CDCl_3); δ_{C} : 124.34 [C(8')], 124.97 [C(3)], 125.97 [C(5')], 126.93 [C(8a')], 127.51 [C(4a')], 128.52 [C(2)], 131.48 [C(7')], 131.99 [C(6')], 138.07 [C(4')], 148.35 [C(1')], 153.87 [C(1)]. The high and low Rf components of APH were determined to be *E*- (**1**) and *Z*- (**2**) stereoisomers at the hydrazone groups. The stereochemistry of the hydrazones was determined by the downfield shift of the vinylic proton at δ 8.10 in the high Rf isomer, compared to the proton at δ 7.36 in the low Rf isomer. The method of stereochemical determination of these hydrazones by the downfield shift of H1 was as previously reported (Karabatsos *et al*, 1964).



1. E-APH



2. Z-APH

proton
assignment

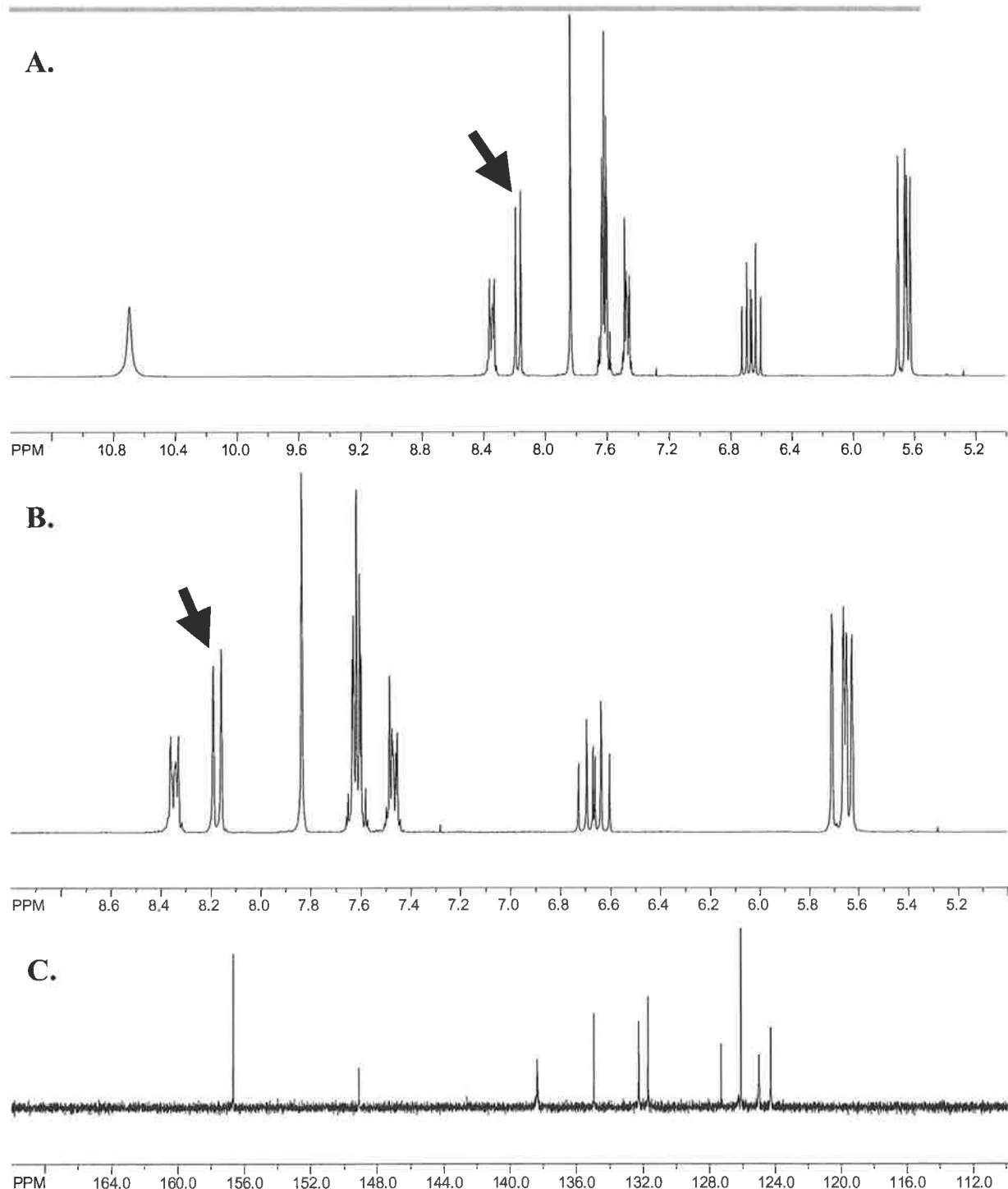


Figure 2.4 The ^1H NMR (A) of high Rf APH (*E*-APH) and an expanded spectrum of the aromatic/vinyl hydrogens (B) are shown in the two upper panels. The H1 proton that was used to assign the stereochemistry around the hydrazone is shown by an arrow. The ^{13}C NMR (C) of high Rf APH is shown in the lower panel.

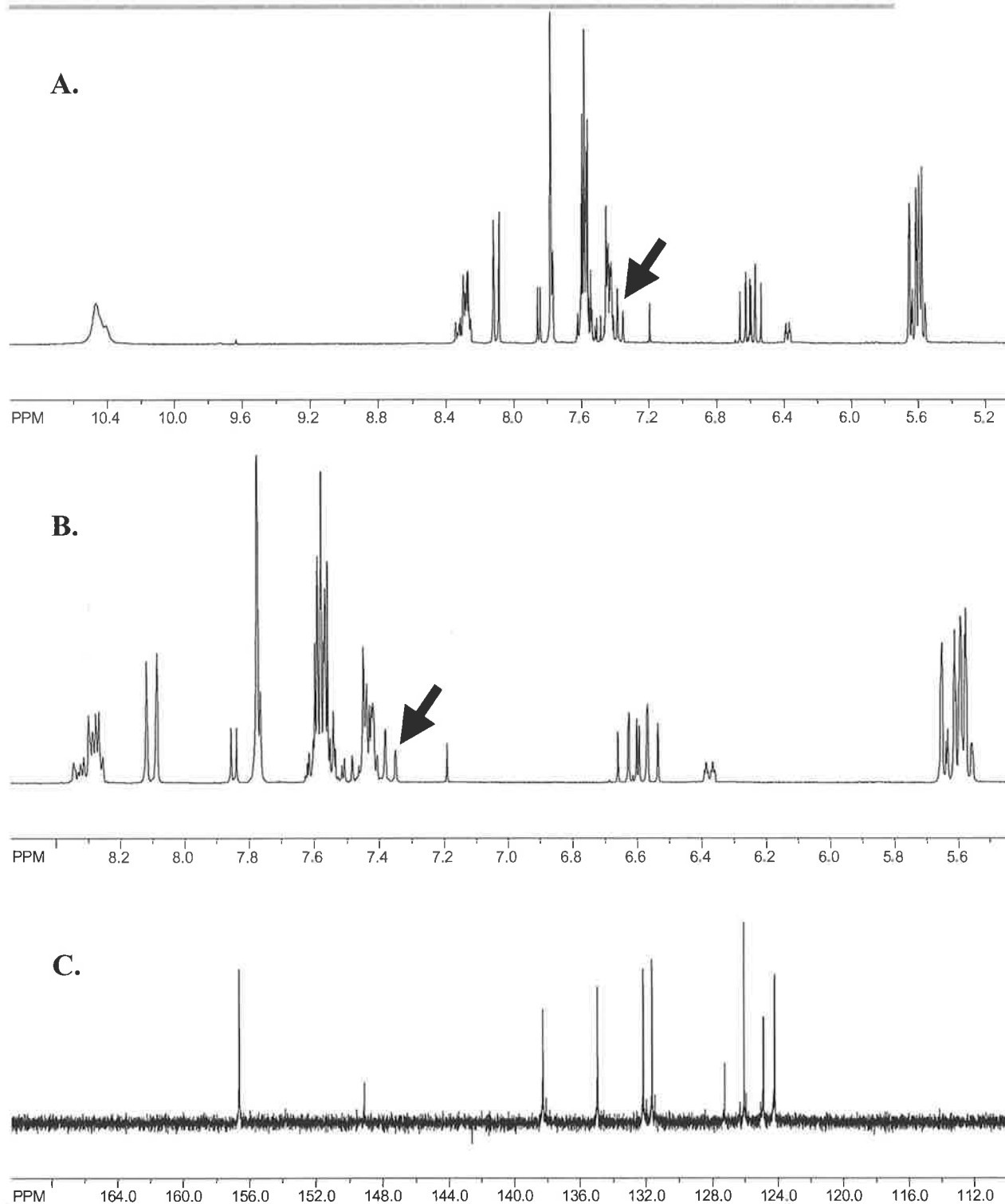


Figure 2.5 The ^1H NMR (A) of an Rf mix of APH (*E* and *Z*-APH) and an expanded spectrum of the aromatic/vinylic hydrogens (B) are shown in the two upper panels. The H1 proton (of *Z*-APH) that was used to assign the stereochemistry around this isomer is shown by an arrow. The ^{13}C NMR (C) of the Rf mix of APH is shown in the lower panel.

In addition, it was noted that a red/orange product remained stuck to silica flash columns during the purification of APH. This product was also present at the origin of analytical TLC plates of crude samples and also of APH samples following prolonged storage (1 week at room temperature or for > 1 month at -20°C). As this product remained bound to the silica of flash columns it could not be purified by this method, even by increasing the polarity of the solvent (100% methanol). Small quantities of this product were crudely isolated by scraping this band off of preparative TLC plates following purification of *E*-APH and back extracting the product into methanol. Following evaporation of the solvent the solid material was analysed by ¹H NMR (deuterated chloroform solvent, Appendix 1; A1.6) and ESI mass spectrometry (acetonitrile solvent, Appendix 1; A1.6). This product contained a ¹H NMR spectra similar to that of APH in the vinylic region, although it also contained several signals in the methyl region. The mass spectrum of the product showed, along with a signal at *m/z* 199 (corresponding to APH), signals at *m/z* 398 and 597 (corresponding to dimer and trimer APH, respectively). This product was therefore determined to be oligomeric APH, although due to the difficulty in detaching this product from silica it could not be quantified.

2.3.3 Characterisation of Reaction Products Between Crotonaldehyde and Hydralazine

Unlike the isomers of APH, the apparent isomers of the reaction product of crotonaldehyde with hydralazine were able to be separated and individually characterised. The **high R_f** product contained the following chemical characteristics: IR (nujol): 3263, 1641, 1607, 1590, 1572, 1530 cm⁻¹; UV (acetonitrile) (log ε): 206 (4.60), 288 (4.44), 360 nm (4.34); ¹H NMR (600 MHz / CDCl₃, Figure 2.6a and b); δ_H: 1.81 [dd, *J*_{2,4} = 1.5 Hz *J*_{3,4} = 6.9 Hz, 3 H, H(4)], 6.11 [dq, *J*_{2,3} = 15.3 Hz *J*_{3,4} = 6.9 Hz, 1 H, H(3)], 6.31 [ddq, *J*_{1,2} = 9.6 Hz *J*_{2,3} = 15.3 Hz *J*_{2,4} = 1.5 Hz, 1 H, H(2)], 7.49 – 7.51 [m, 1 H, H(5')], 7.62 – 7.67 [m, 2 H, H(6',7')], 7.83 [s, 1 H, H(4')], 8.05 [d, *J*_{1,2} = 9.6 Hz, 1 H, H(1)], 8.35 – 8.36 [m, 1 H, H(8')], 10.48 [br s, 1 H, NH]. ¹³C NMR (150 MHz / CDCl₃, Figure 2.6c); δ_C: 18.54 [C(4)], 123.95 [C(8')], 125.83 [C(5')], 126.65 [C(8a')], 127.13 [C(4a')], 129.90 [C(2)], 131.31 [C(7')], 131.72 [C(6')], 137.69 [C(4')], 138.42 [C(3)], 148.36 [C(1')], 156.60 [C(1)]. ESI-MS: *m/z* 214 [MH⁺].

The **low R_f** product was found to differ slightly from the high R_f product with the following chemical characteristics: IR (nujol): 3170, 1633, 1604, 1591, 1569, 1536 cm⁻¹. ¹H NMR (600 MHz / CDCl₃, Figure 2.7a and b); δ_H: 1.87 [dd, *J*_{2,4} = 1.5 Hz *J*_{3,4} = 6.9 Hz, 3 H, H(4)], 6.16 [dq, *J*_{2,3} = 15.3 Hz *J*_{3,4} = 6.9 Hz, 1 H, H(3)], 7.21 [ddq, *J*_{1,2} = 9.6 Hz *J*_{2,3} = 15.3 Hz *J*_{2,4} = 1.5 Hz, 1 H, H(2)], 7.32 [d, *J*_{1,2} = 9.6 Hz, 1 H, H(1)], 7.39 – 7.42 [m, 1 H, H(5')], 7.52 – 7.59 [m, 2 H,

H(6',7')], 7.74 [s, 1 H, H(4')], 8.32 – 8.35 [m, 1 H, H(8')], 10.42 [br s, 1 H, NH]. ^{13}C NMR (150 MHz / CDCl_3 , Figure 2.7c); δ_{C} : 18.65 [C(4)], 123.16 [C(2)], 124.27 [C(8')], 125.91 [C(5')], 127.08 [C(8a')], 127.32 [C(4a')], 131.39 [C(7')], 131.81 [C(6')], 137.81 [C(4')], 139.29 [C(3)], 147.54 [C(1')], 154.01 [C(1)]. ESI-MS: m/z 214 [MH^+].

In addition to the 1 dimensional NMR data, COSY, HMBC and HSQC NMR's were determined on the products to further characterise the coupling of carbon and hydrogen atoms (spectra not shown). As in the spectra from the APH isomers, the proton at δ 8.05 in the high Rf product was shifted downfield in the low Rf product to δ 7.32. The J coupling constants between H2 and H3 (15 Hz) also suggest they are in trans conformation in both products. The short and long range couplings between H2, 3 and 4 are also the same in both products, indicating that only the stereochemistry at the hydrazone bond differs in the two products. It was therefore determined that the high and low Rf products were *E*- and *Z*- isomers at the hydrazone group, but were in the *E*- conformation at the termination of the side chain, where the high Rf product was (1*E*, 2*E*) 1-but-2-enal-1-phthalazin-1-ylhydrazone (*E* BPH, structure 3) and the low Rf product was (1*Z*, 2*E*) 1-but-2-enal-1-phthalazin-1-ylhydrazone (*Z* BPH, structure 4).

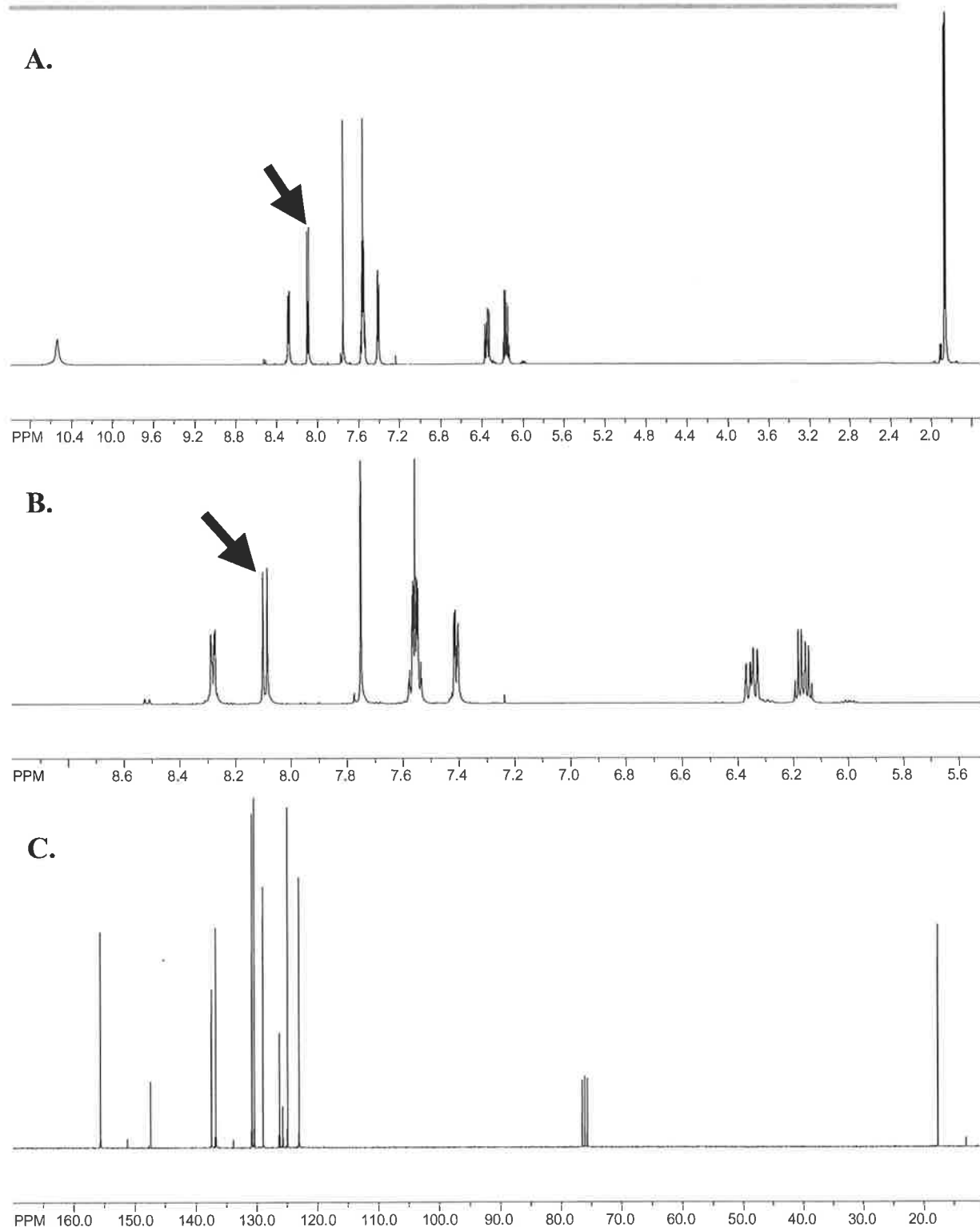


Figure 2.6 The ^1H NMR (A) of high Rf BPH [(1*E*,2*E*)-BPH] and an expansion spectrum of the aromatic/vinyl hydrogens (B) are shown in the 2 upper panels. The H1 proton used to assign the stereochemistry around the hydrazone is shown by an arrow. The ^{13}C NMR (C) of high Rf BPH is shown in the lower panel.

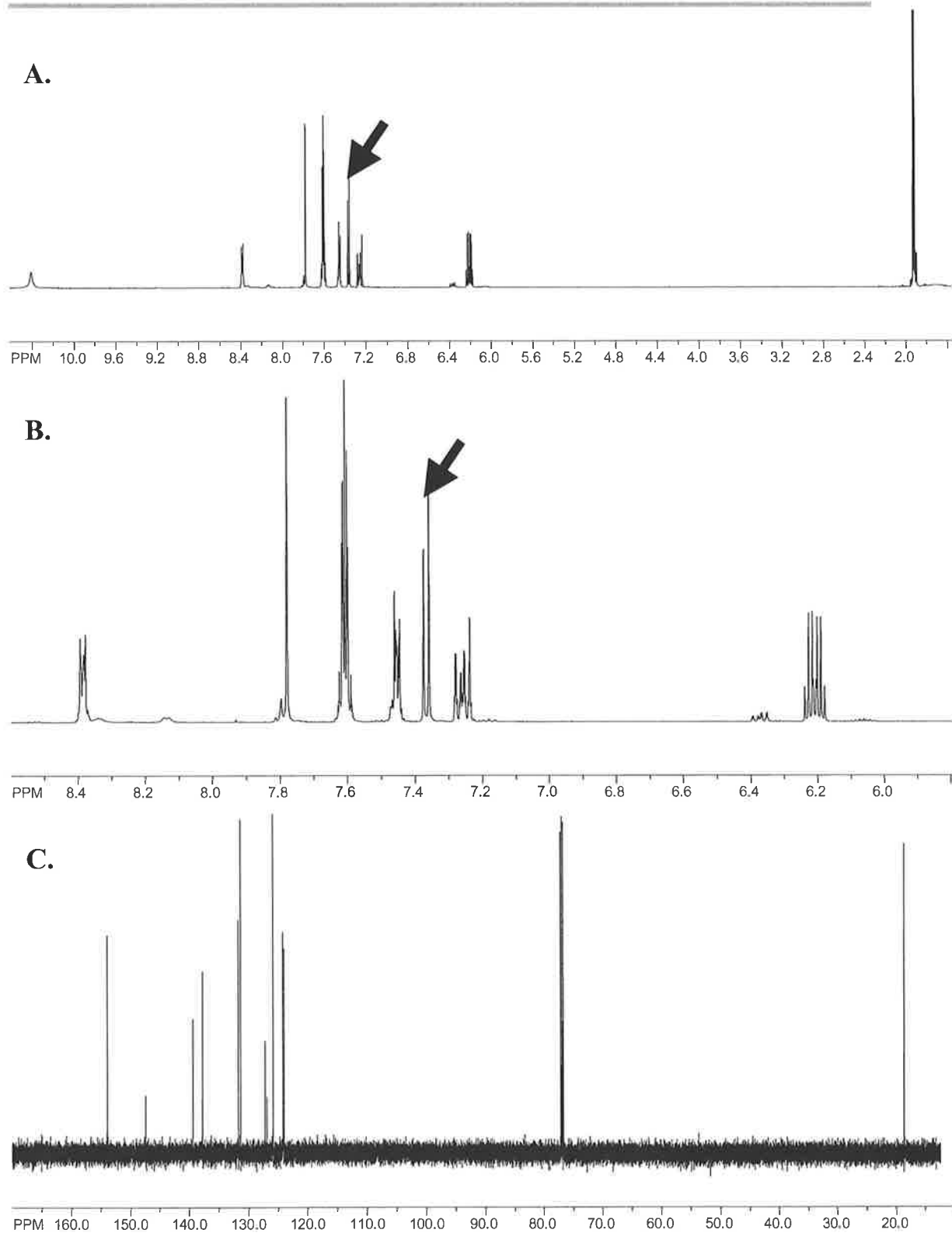
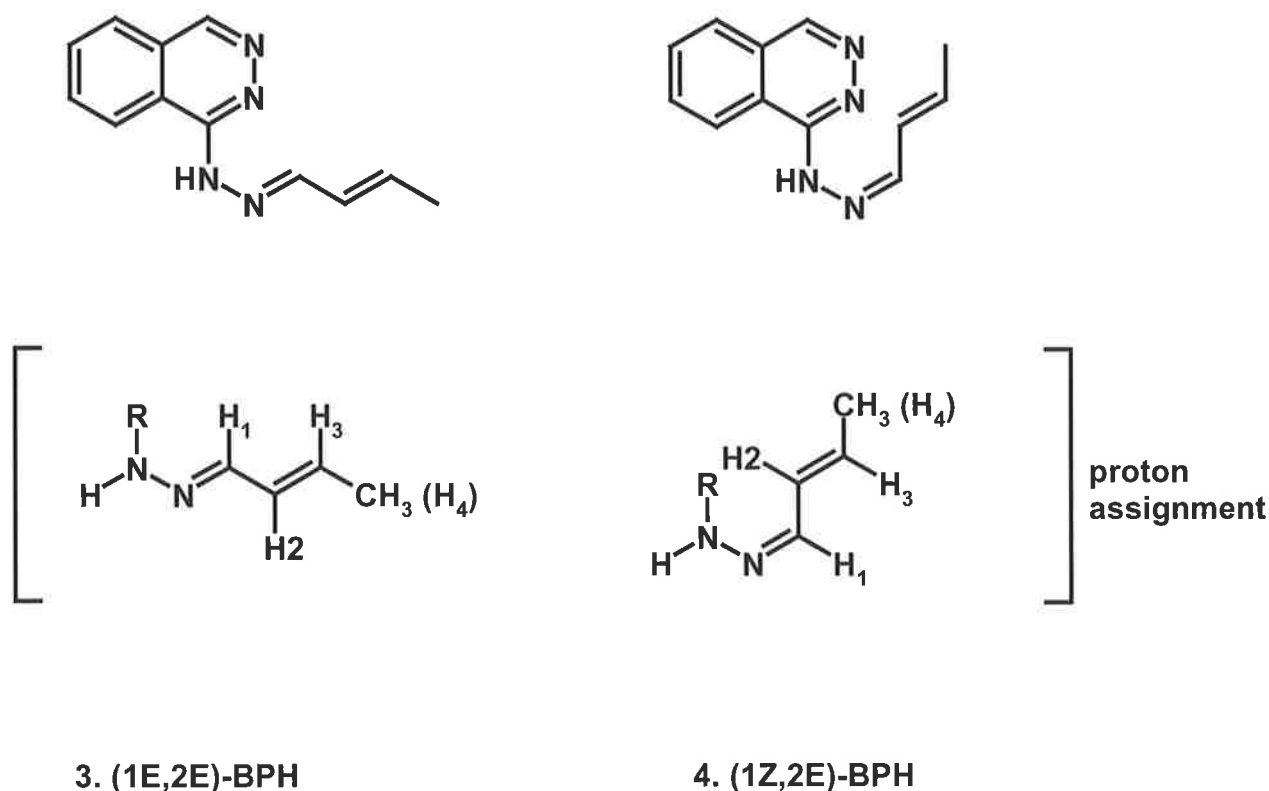


Figure 2.7 The ^1H NMR (A) of low Rf BPH [(1Z,2E)-BPH] and an expansion spectrum of the aromatic/vinyl hydrogens (B) are shown in the two upper panels. The H1 proton used to assign the stereochemistry around the hydrazone is shown by an arrow. The ^{13}C NMR (C) of low Rf BPH is shown in the lower panel.



2.3.4 Stability of APH and its Further Reaction with Acrolein and Hydralazine

The question of whether *E*-APH can reverse back to its starting materials was examined along with the determination of its ability to further react with acrolein or hydralazine. This was examined by incubating APH either alone at 37° or with equimolar acrolein or hydralazine.

Figure 2.8 shows that when incubated alone (▼) or in the presence of acrolein (●) or hydralazine (◆) in buffer at physiological temperature there was no significant loss of APH over 4 hours at 37°C. To confirm that APH does not further react with acrolein, acrolein loss when incubated with APH was compared to the stability of acrolein alone over 4 hours. There were no differences in the profiles between acrolein control (■) and acrolein incubated with APH (▲) (Figure 2.8). This shows that within a 4 hour period, APH does not reverse back to its starting materials, as APH concentrations remain stable while acrolein loss is unchanged compared to control loss due to the absence of nucleophilic reaction centers.

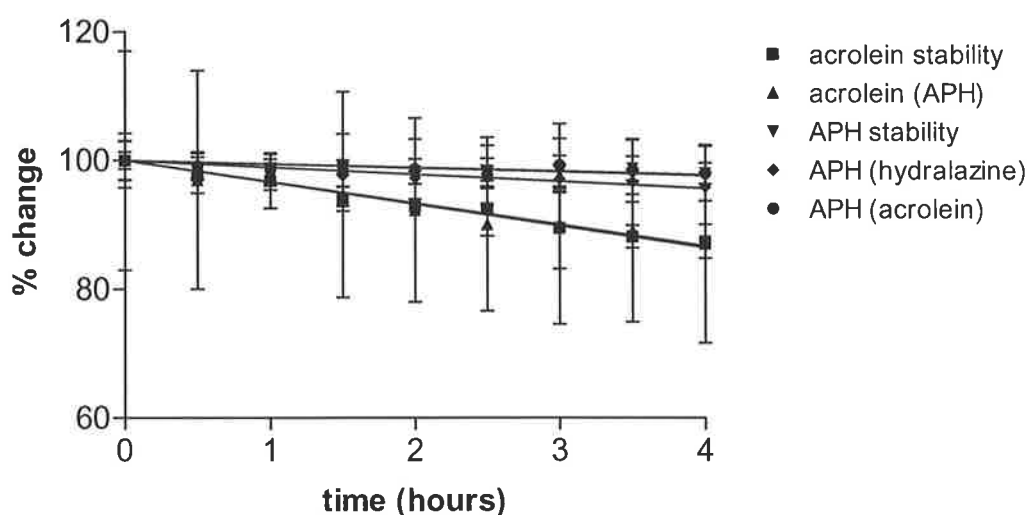


Figure 2.8 Stability of the APh alone and in the presence of acrolein or hydralazine in buffered solution at 37°C. The compounds examined by HPLC are indicated in the figure legend, where the compound that was co-incubated with the one examined is indicated in parentheses. Data are represented as the mean $\pm$ SEM of the % change in acrolein or APh as determined by a HPLC assay (the compound examined is indicated in parentheses). Data are the mean of 3 replicates.

2.3.5 Kinetics of *E*-APh and (1*E*,2*E*)-BPh Formation

Given the relatively low synthetic yield of APh compared to the total consumption of hydralazine as determined by ^1H NMR it was necessary to assess the progressive formation of APh while monitoring the concurrent loss of acrolein to determine the kinetics of APh formation in relation to acrolein disappearance. This was conducted at 37° and at 4°C. The low temperature reaction was followed in an attempt to control the reaction and maximise yield as it was previously determined that, particularly at high reaction temperatures, oligomeric APh is formed between acrolein and hydralazine. The reaction kinetics were examined over a 24 hour period.

Figure 2.9a shows the kinetics of APh formation at 37°C. The stability of acrolein alone was relatively poor over the 24 hour period, but was maintained during the first hour of the reaction, the most important period in terms of APh formation. Acrolein sequestering by hydralazine was very rapid within the first hour of the reaction and essentially reached a plateau within 6 hours. APh formation was also very rapid within the first 30 minutes of the reaction, after which there

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was a marked slowing of APH formation. The total yield of APH was approximately 8% in 24 hours, which was essentially attained within the first hour. APH stability was maintained for the first 12 hours, although it diminished slightly by 24 hours. Based on the observation that APH formation was complete within the first 30 minutes of the reaction, APH instability is unlikely to contribute to the low yield of APH. The disagreement between APH formed in relation to the acrolein consumed indicates that 1:1 trapping is not the major fate of acrolein in this system, suggesting that formation of oligomeric products predominates.

Figure 2.9b shows APH formation kinetics at 4°C. The stability of acrolein at this temperature was much improved, so that loss of acrolein in the reaction was due to sequestering by hydralazine. As expected, the loss of acrolein in the reaction was considerably slower than in the 37°C reaction, with acrolein concentrations dropping by 50% 1 to 2 hours after the start of the reaction and still declining after 12 hours. APH yield was slightly improved at this temperature, although it essentially plateaued after 2 hours. At this temperature acrolein conversion into APH was approximately 12% compared to the amount of acrolein consumed. APH was also stable after 24 hours at this temperature and hence did not contribute to the final yield. Once again, the consumption of acrolein was greater than the 1:1 stoichiometry for APH formation.

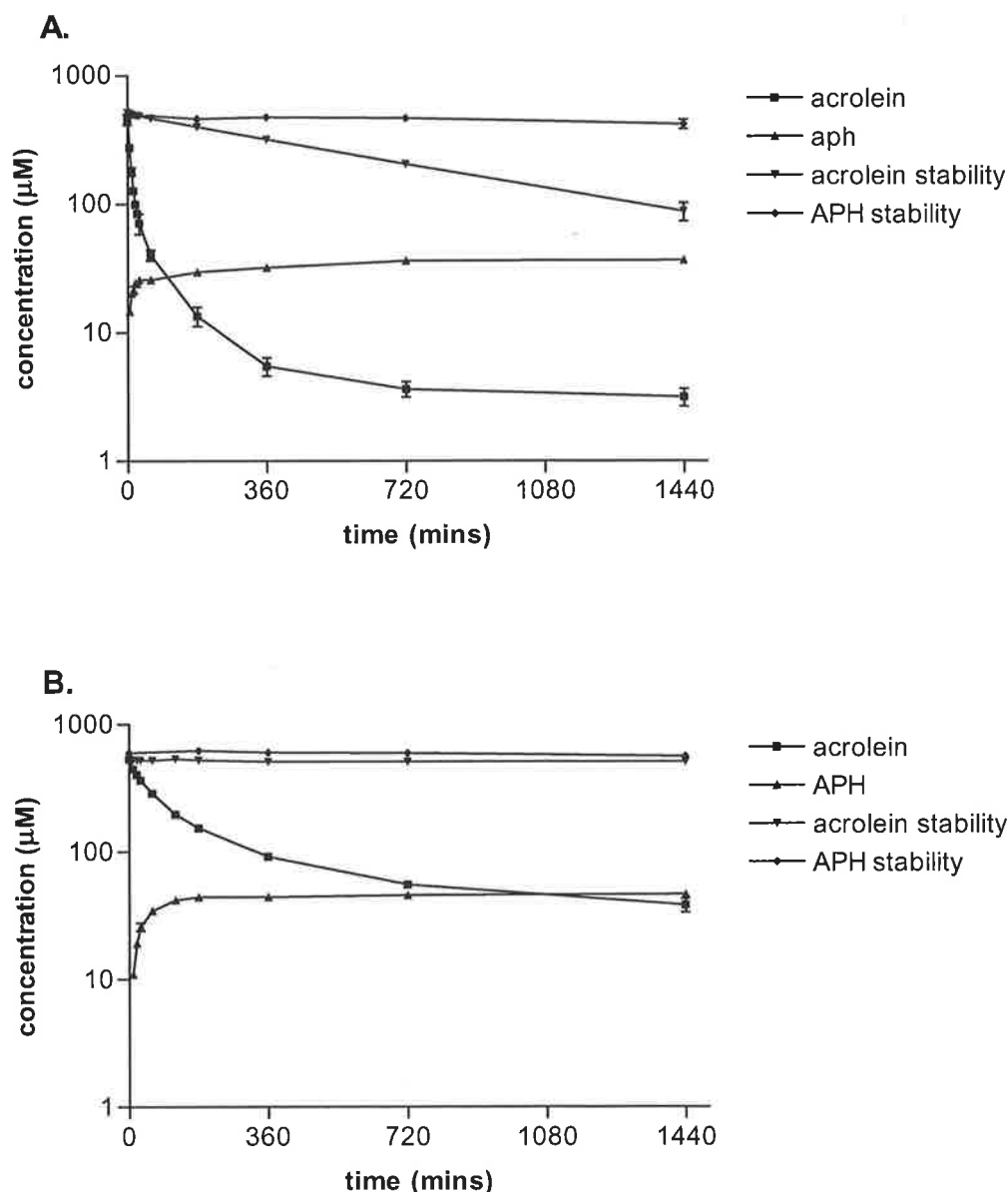


Figure 2.9 Kinetics of *E*-APH formation at 37°C (A) and 4°C (B) and concurrent loss of acrolein. The stability of *E*-APH and acrolein are indicated. The graph represents the log concentration of acrolein or *E*-APH at each time point for up to 24 hours as determined by HPLC. Data are represented as the mean $\pm$ SEM of log acrolein or *E*-APH concentration (μ M), $n=3$ observations.

The kinetics of (1*E*,2*E*)-BPH formation at 37 and 4°C are shown in Figures 2.10a and b respectively. As expected, due to the additional methyl group on the β -carbon relative to acrolein, the rate of formation of (1*E*,2*E*)-BPH was considerably slower than for the reaction of

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acrolein and hydralazine. The yield of BPH formed, however, was considerably higher at both temperatures, namely 47% at 37°C and 63% at 4°C relative to the amount of crotonaldehyde consumed. Reaction of crotonaldehyde with hydralazine was essentially complete after 6 hours at 37°C, after which BPH concentrations began to decline, although when performed at 4°C, the trapping reaction between hydralazine and crotonaldehyde had not reached equilibrium even after 24 hours. Both BPH and crotonaldehyde were essentially stable at 4°C. Therefore, in contrast to the reaction with acrolein, a stronger agreement was seen between the loss of crotonaldehyde relative to the yield of BPH, indicating a lesser tendency towards side reactions such as formation of oligomeric products.

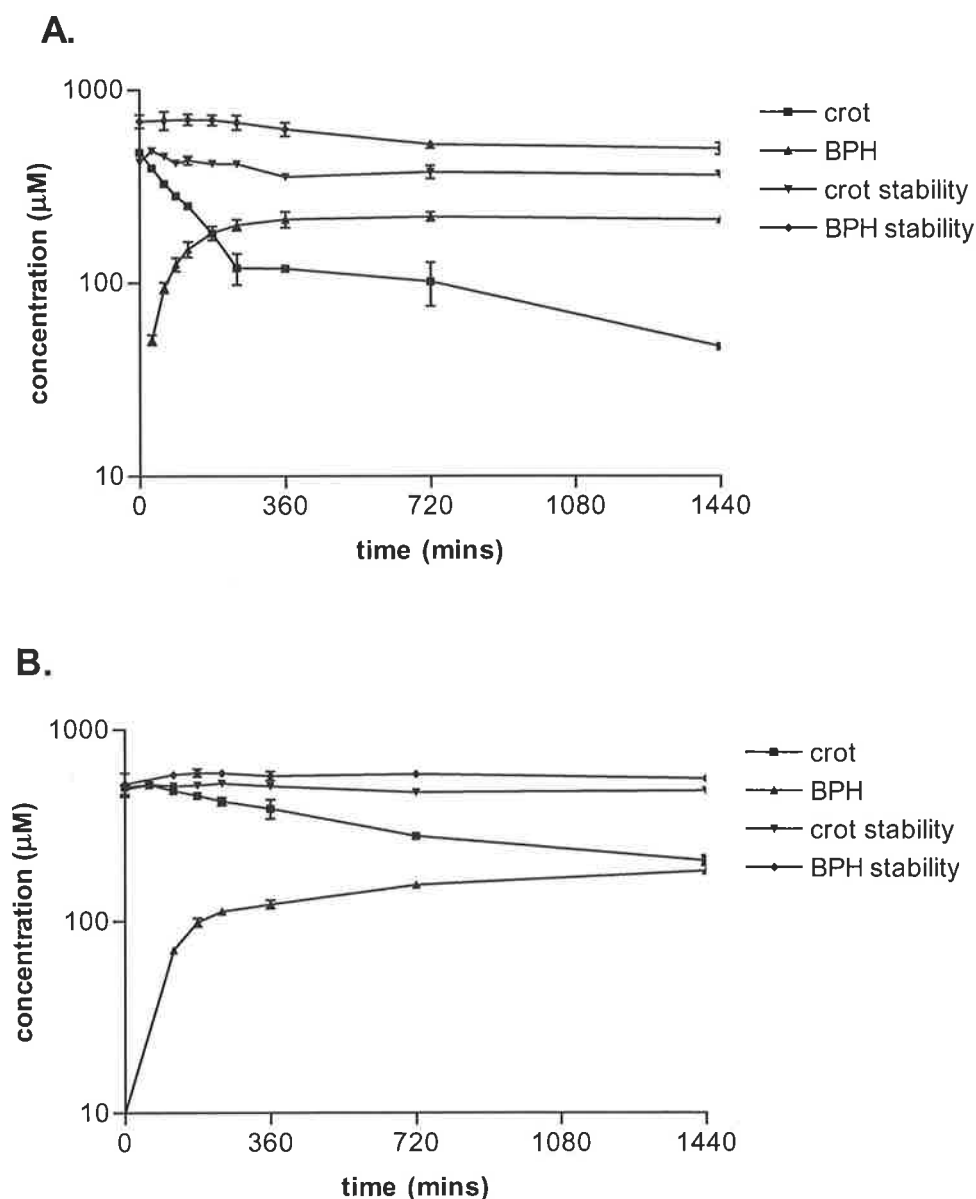


Figure 2.10 Kinetics of (1E,2E)-BPH formation at 37°C (A) and 4°C (B) and concurrent loss of crotonaldehyde (crotonaldehyde). The stability of BPH and crotonaldehyde are indicated. The graph represents the log concentration of crotonaldehyde or BPH at each time point for up to 24 hours as determined by HPLC. Data are represented as the mean $\pm$ SEM of log crotonaldehyde or (1E,2E)-BPH concentration (μM), $n=3$ observations.

2.3.5 Cytoprotection Against Unsaturated Aldehyde-Mediated Toxicity in Isolated Hepatocytes

To assess whether the 2-alkenal sequestering abilities of hydralazine and dihydralazine confer cytoprotection against the toxicity of acrolein and crotonaldehyde, their ability to protect against the toxicity of the corresponding alcohol precursors was explored in isolated mouse hepatocytes. As previously described, alcohol dehydrogenase in mouse hepatocytes readily oxidises allyl and crotyl alcohols to acrolein and crotonaldehyde respectively. In addition, the ability of

hydralazine to protect against the toxicity of three other unsaturated aldehydes was examined. These included the prominent enolate malondialdehyde (MDA), pentenal (a longer chained 2-alkenal) and the 2-alkynal propiolaldehyde (which was examined using the precursor, propargyl alcohol). These aldehydes were examined for the purpose of establishing whether hydralazine protects against the toxicity produced by diverse unsaturated aldehydes (the structures of these are shown in Figure 2.1).

Figures 2.11a and b show the concentration dependent protection against allyl alcohol toxicity by hydralazine and dihydralazine respectively over 3 hours. Allyl alcohol induced 100% cell death within 3 hours of its addition to hepatocytes. Hydralazine and dihydralazine both inhibited this toxicity in a concentration dependent manner, with dihydralazine approximately twice as protective as hydralazine. When screened in 96 well culture plates, MESNA did not afford any protection against allyl alcohol toxicity between concentrations ranging from 1 to 100 μM (Frank Fontaine, personal communication; data not shown). This suggested that MESNA shows poor membrane permeability to intracellular sites of acrolein formation and adduction.

Crotyl alcohol similarly induced 100% cell death by 3 hours of incubation (Figure 2.12a and b for hydralazine and dihydralazine respectively). A similar concentration dependence in hydralazine and dihydralazine protection was seen where dihydralazine was approximately twice as effective as hydralazine at inhibiting this toxicity. This showed that hydralazine and dihydralazine protect against toxicity mediated by two different short chained 2-alkenals.

Pentenal and propargyl alcohol both induced maximal cell death by 3 hours of incubation (Figure 2.13a and b). MDA had a much slower onset of toxicity, taking 12 hours before noticeable toxicity began (Figure 2.13c). Hydralazine protected against cell death induced by all the aldehydes or their precursors in a concentration dependent manner, although the protection was most efficient for pentenal toxicity. Complete protection against propargyl alcohol and MDA toxicity was only achieved at the highest concentration of hydralazine used (100 μM). Nevertheless, the data indicated that hydralazine protects against the cytotoxicity of a range of α,β -unsaturated aldehydes.

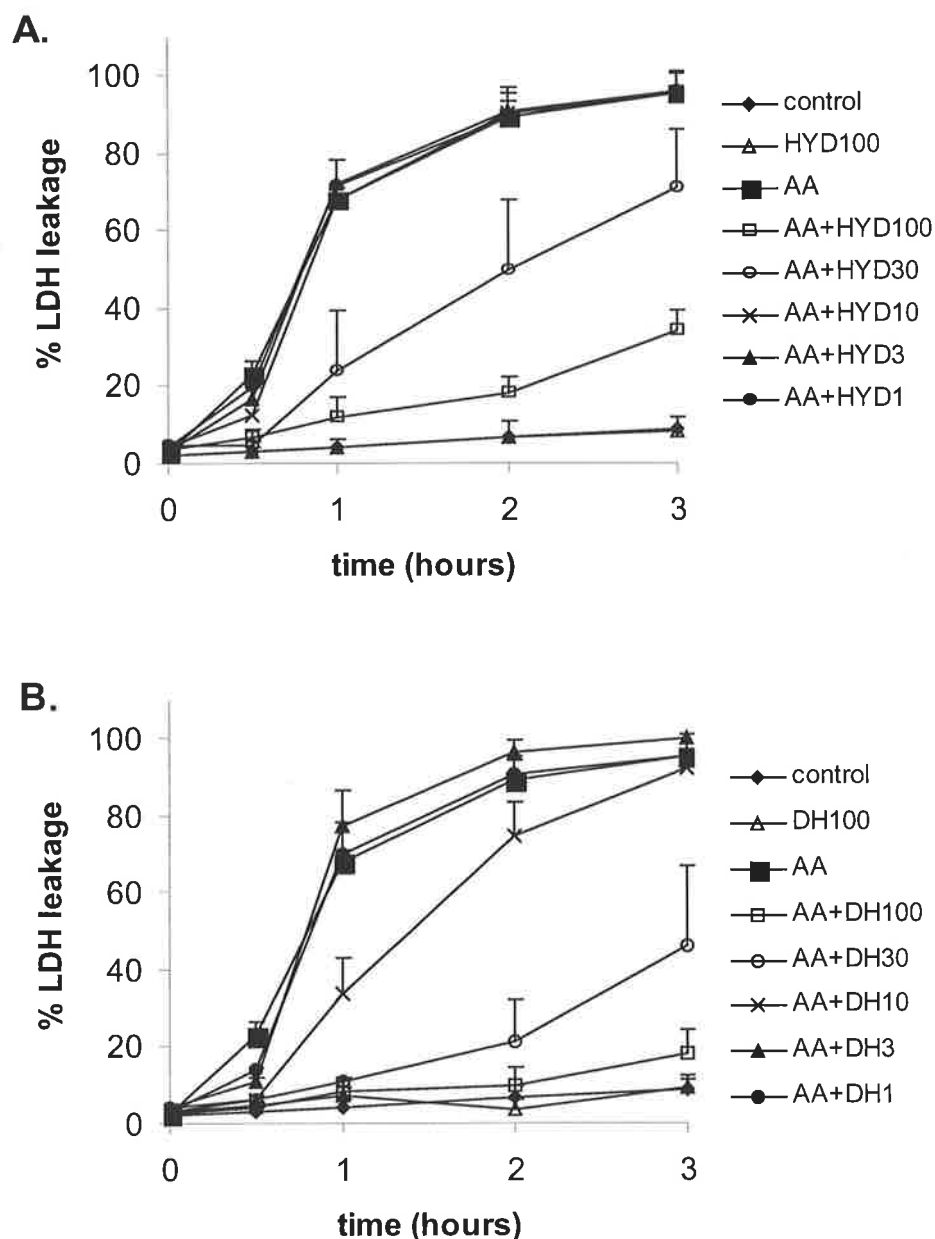


Figure 2.11 Protection against allyl alcohol (AA, 100 μ M) toxicity in isolated primary mouse hepatocytes by hydralazine (HYD, A) and dihydralazine (DH, B). AA and the protective hydrazines were co-incubated in RMPI medium for up to 3 hours. Aliquots of supernatant were taken after 30 minutes and then at hourly intervals for assessment of LDH leakage from the cytoplasm into the culture medium. The concentration of hydrazine used is indicated in the legend as the number next to HYD or DH (μ M). Data are represented as mean $\pm$ SEM of the % of LDH leakage from the cytoplasm at each time point, $n=3$ observations.

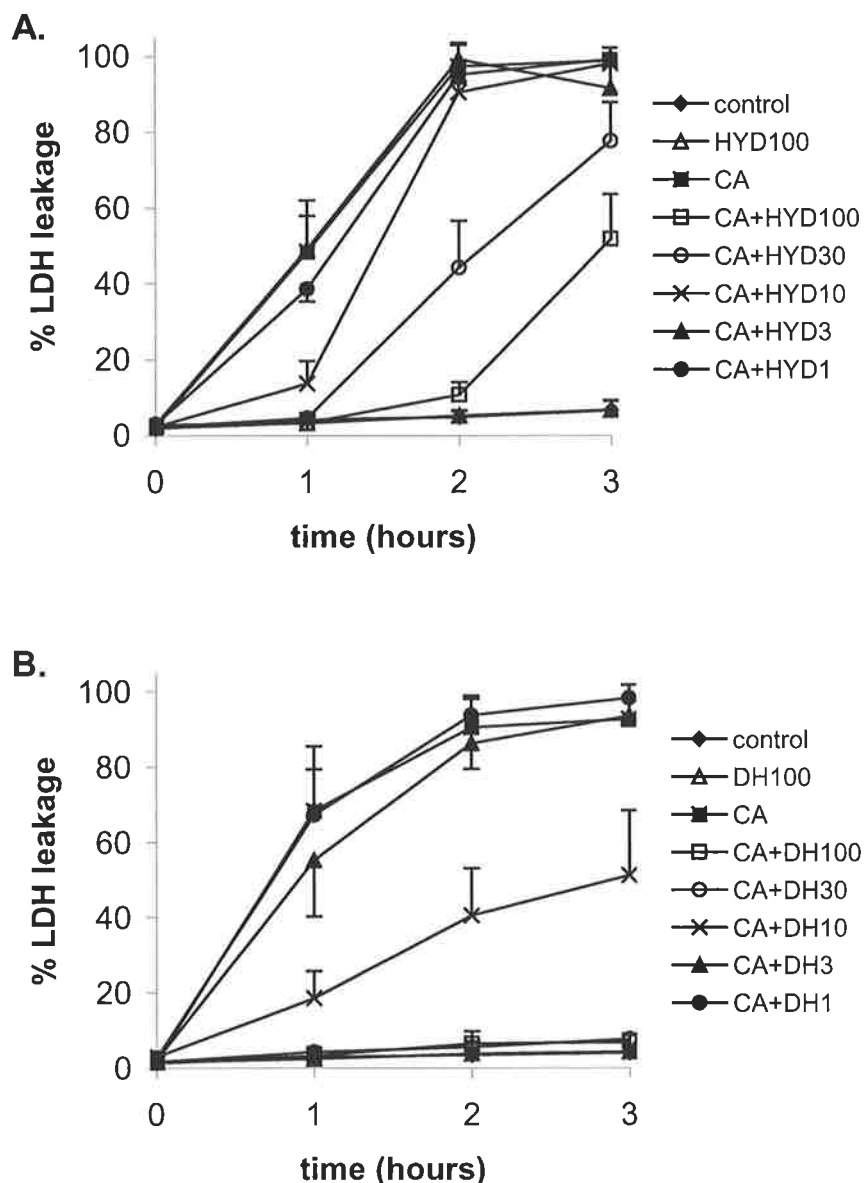
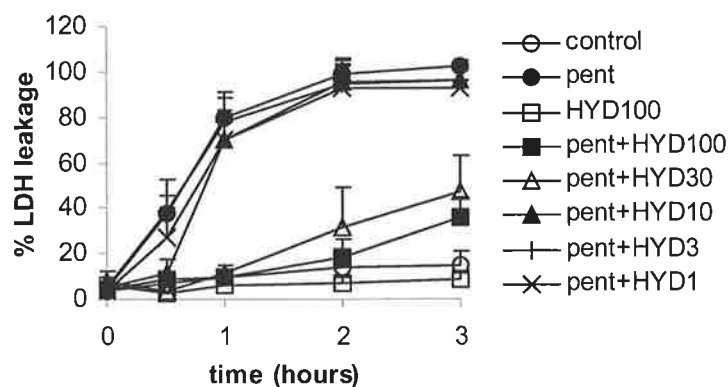
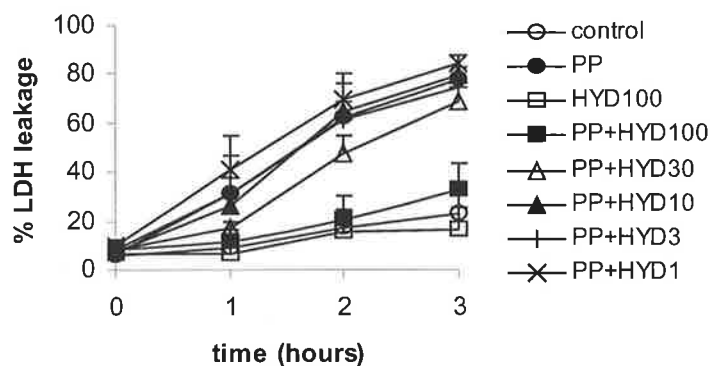


Figure 2.12 Protection against crotyl alcohol (CA, 500 μM) toxicity in isolated primary mouse hepatocytes by hydralazine (HYD, A) and dihydralazine (DH, B). CA and the protective hydrazines were co-incubated in RMPI medium for up to 3 hours. Aliquots of supernatant were taken at hourly intervals for assessment of LDH leakage from the cytoplasm into the culture medium. The concentration of hydrazine used is indicated in the legend as the number next to HYD or DH (μM). Data are represented as mean $\pm$ SEM of the % of LDH leakage from the cytoplasm at each time point, $n=3$ observations.

A.



B.



C.

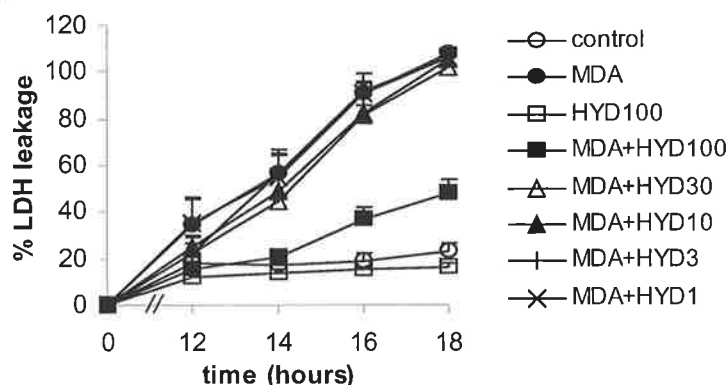


Figure 2.13 Hydralazine (HYD) mediated protection against hepatocellular toxicity induced by pentenal (Pent, 1 mM, A), propargyl alcohol (PP, 1 mM, B) and MDA (10 mM, C). Hydralazine was co-incubated with the aldehydes and culture media from propargyl alcohol and pentenal treated cells taken at hourly intervals for 3 hours to assess lactate dehydrogenase leakage (%LDH) as an indicator of cell death. LDH was assessed at 2 hourly intervals from 12 to 18 hours for MDA. The concentrations of hydralazine added to the cells are indicated in the figure legends (in μ M). Data are expressed as mean $\pm$ SEM (3 replicates) of the % LDH leakage from the cytoplasm at each time point.

2.3.6 Effect of Hydralazine and Dihydralazine on Aldehyde Mediated Decreases in Cellular Glutathione

Glutathione is an important antioxidant molecule in cells that acts as a defence against electrophilic aldehydes. GSH levels were therefore examined following administration of the highest concentration of hydralazine used in cytoprotection studies (100 μ M) to examine whether it prevents against the loss of cellular GSH induced by the aldehydes or their alcohol precursors. Figure 2.14 (Panel a) shows a decrease in cellular GSH levels 30 minutes after addition of allyl alcohol (100 μ M), crotyl alcohol (500 μ M), pentenal (1 mM) or propargyl alcohol (1 mM). Except for propargyl alcohol (which decreased glutathione levels by less than 50%) the compounds decreased GSH levels to approximately one-third of control values. Following a 12 hour incubation of cells with MDA (1 mM; Figure 2.14, Panel B) cellular GSH levels declined to approximately one-third. Hydralazine or dihydralazine alone (100 μ M) had no effect on cellular GSH (Figure 2.14, Panel a and b). Strikingly, fully cytoprotective concentrations of hydralazine and dihydralazine were unable to prevent the decrease in GSH induced by any of the aldehydes or alcohol precursors. This indicates that the cytoprotection afforded by these compounds is not due to prevention of GSH depletion by the various 2-alkenals and that 2-alkenals react with GSH in preference to reacting with these hydrazines.

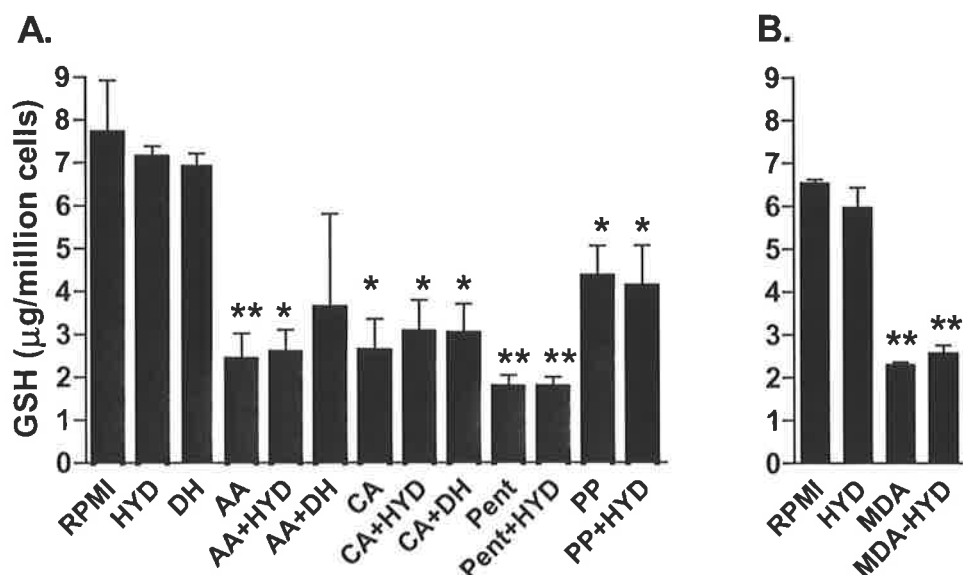


Figure 2.14 GSH in hepatocytes after a 30 minute treatment with hydralazine (HYD, 100 μ M), dihydralazine (DH, 100 μ M), allyl alcohol (AA, 100 μ M), crotyl alcohol (CA, 500 μ M), pentenal (Pent, 1 mM), propargyl alcohol (PP, 1 mM) or toxic compound co-incubated with one of the respective hydrazines (Panel A; note that pentenal and propargyl alcohol were co-incubated with hydralazine only). Panel B shows the concentration of GSH in hepatocytes after a 12 hour treatment with hydralazine (HYD, 100 μ M), MDA (10 mM) or MDA co-incubated with hydralazine. Data are represented as mean $\pm$ SEM of GSH (μ g) in hepatocytes per million cells, $n=3$ observations. Treatment groups were compared to control (RPMI) by 1-way ANOVA with a Dunnett's post test. * $p<0.05$, ** $p<0.01$ cf control.

2.3.7 Hydrazone Formation Accompanies Cytoprotection Against Acrolein-Mediated Toxicity.

To confirm that aldehyde scavenging accompanies hydralazine and dihydralazine-induced cytoprotection, total cellular *E*-APH levels were measured via UV-HPLC after a 30 minute exposure of hepatocytes to allyl alcohol (100 μ M) and hydralazine (0-100 μ M). The 30 minute time point was used since, in hepatocytes exposed to 100 μ M allyl alcohol, acrolein concentrations in extracellular media peak at approximately 4 μ M at this time (PC Burcham, unpublished observation). In the presence of hydralazine and allyl alcohol, cellular *E*-APH levels were below quantifiable levels at drug concentrations of 10 μ M and below, although concentration-dependent acrolein-trapping was evident at 25, 50 and 100 μ M concentrations of hydralazine, yielding cellular *E*-APH levels of 39 ± 37 , 121 ± 55 and 381 ± 97 pmol/ml respectively. Given the low cellular yield of APH (no greater than 0.4% compared with allyl

alcohol) the contribution of metabolism to the low APH yield was examined. The loss of free *E*-APH was followed over a 3 h period when added to culture media to give a starting concentration of 1000 pmol/ml (1 μ M). After a 30 minute incubation, total cellular *E*-APH levels had declined by over 90% to just 84 ± 40 pmol/ml, while by 60 minutes they were below detectable limits. This indicates that the low yield of APH from allyl alcohol and hydralazine was largely due to the rapid loss of detectable hydrazone in cells.

2.3.8 Toxic Potential of *E*-APH in Isolated Hepatocytes.

Since it possesses an isolated unsaturated centre, *E*-APH is potentially a reactive entity that may be targeted by nucleophiles in cellular macromolecules. The issue as to whether *E*-APH represents a true detoxication product was therefore examined by assessing its ability to induce LDH leakage after a 3 hour exposure period in isolated hepatocytes. Exposure to 1 μ M *E*-APH produced no increase in LDH leakage compared to controls over this time, although 10 μ M *E*-APH modestly increased LDH leakage after 3 hours ($23 \pm 6\%$ compared to $10 \pm 2\%$ in controls). Progressively more LDH leakage was evident with increasing APH concentrations (Figure 2.15). Given that the concentration of APH formed in cells co-incubated with allyl alcohol and hydralazine did not exceed 0.4 μ M, APH is not likely to induce toxicity in this system. APH formation therefore apparently represents a detoxification pathway for acrolein.

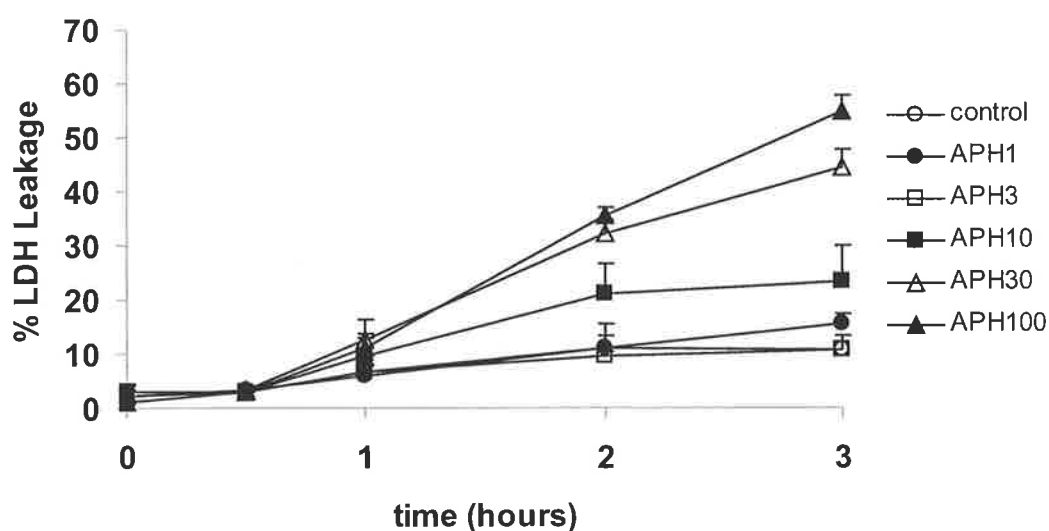


Figure 2.15 Toxicity of *E*-APH in isolated mouse hepatocytes. Samples of culture media were taken at 30 minutes, 1, 2 and 3 hours to assess LDH leakage from the cells. The concentration of *E*-APH added to the cells (μ M) is indicated in the legend. Data are represented as mean $\pm$ SEM ($n=3$) of the % LDH leakage from cells at each sample time point.

2.3.9 Acrolein Scavenging Capacity of Other Hydrazines and Hydralazine Analogues

While the reactivity of most amine nucleophiles with 2-alkenals is due to the nucleophilicity of the reacting amine, the possibility that the greater reactivity of hydralazine and dihydralazine is due to the presence of their hydrazine groups was examined. This was achieved by screening a number of structurally diverse hydrazines and hydrazinophthalazine analogues (shown in Figure 2.2) for their ability to sequester acrolein from solution, as it was expected that the hydrazine functional groups on each of these compounds would confer better 2-alkenal sequestering effects than aminoguanidine, pyridoxamine, methoxyamine and carnosine. The acrolein sequestering effects of the hydrazines (Figure 2.16a) and phthalazine analogues (Figure 2.16b) were directly compared to hydralazine and dihydralazine, by performing the experiments under identical conditions to those reported in Figure 2.2. The following experiments were also expected to yield insights into the significance of 2-alkenal trapping reactions in the cytoprotection afforded by hydralazine and dihydralazine. Although APH was identified as a detoxification product of acrolein in allyl alcohol and hydralazine exposed cells, a direct correlation between 2-alkenal trapping efficacy and cytoprotection is required to confirm this mechanism of protection. In addition, the abovementioned hydrazines and hydrazinophthalazine analogues provided a good starting point for identification of an efficient cytoprotective compound that is devoid of hydralazine's anti-hypertensive effects.

As shown in Figure 2.16a, the most effective hydrazine examined for its acrolein scavenging ability was benzylhydrazine (BH), which removed almost all acrolein from solution within 20 minutes of incubation. In descending order of potency the next most efficient hydrazines were dihydralazine (DH), 1,1-dimethylhydrazine (DMH), hydralazine (HYD), phenylhydrazine (PH), isoniazid, 1,1-diphenylhydrazine (1,1-DPH), 1,2-diphenylhydrazine (1,2-DPH). 2,4-Dinitrophenylhydrazine (DNPH, a commonly used carbonyl trapping agent) was only able to removed approximately 40% of acrolein from solution after 30 minutes of incubation, while 1,2-diphenylhydrazine was the least effective, having removed approximately 10% of acrolein from solution in this time period. However, most of the hydrazine compounds examined were more effective acrolein scavengers than the amine compounds examined in the earlier part of this Chapter (eg. aminoguanidine, carnosine, pyridoxamine and methoxyamine, Figure 2.2).

It is known that the $\text{C}=\text{N}-\text{N}=\text{C}-\text{NH}-\text{NH}_2$ pharmacophore of hydralazine is obligatory for the anti-hypertensive effects of the drug (Reece, 1981). Given that the hydrazine group of hydralazine

and dihydralazine is responsible for their acrolein sequestering capacities, it was therefore hypothesised that the anti-hypertensive functional group could be disrupted while retaining the aldehyde scavenging properties, allowing a non-cardioactive aldehyde scavenger to be identified. Therefore, the hydralazine analogues naphthylhydrazine (NH), 2-hydrazinopyridine (HP), 4-hydrazinoquinazoline (HQZ), 2-hydrazinoquinoline (HQL) and 1-hydrazinoisoquinoline (HIQ) were examined for acrolein scavenging capacity, with the results shown in Figure 2.16b (structures shown in Figure 2.2).

Naphthylhydrazine (NH) was the poorest scavenger of the phthalazine analogues, having removed 28% of free acrolein from solution in 10 minutes. After this time, however, a precipitate became apparent in the reaction mixture, which was accompanied by a decline in the rate of removal of acrolein from solution. 2-Hydrazinoquinoline (HQL) and 1-hydrazinoisoquinoline (HIQ) were the next most effective analogues having identical acrolein scavenging profiles. These compounds removed approximately 70% of free acrolein from solution in 30 minutes. 4-Hydrazinoquinazoline (HQZ), 2-hydrazinopyridine (HP) and hydralazine (HYD) were the next most effective scavengers with 2-hydrazinopyridine (HP) appearing to be slightly less effective than the other 2 compounds. These compounds sequestered approximately 85 to 90% of acrolein in solution in 30 minutes. Dihydralazine (DH) was again the most effective scavenger among the hydrazinophthalazines. This data shows that none of the hydrazinophthalazine analogues tested were better scavengers of acrolein than hydralazine and dihydralazine, although dissociation of hydrazine and phenyl groups by means of a $-\text{CH}_2-$ spacer appears to increase the 2-alkenal trapping efficacy of hydrazines, since benzylhydrazine displayed much stronger acrolein-trapping efficacy than any of the compounds tested in this work.

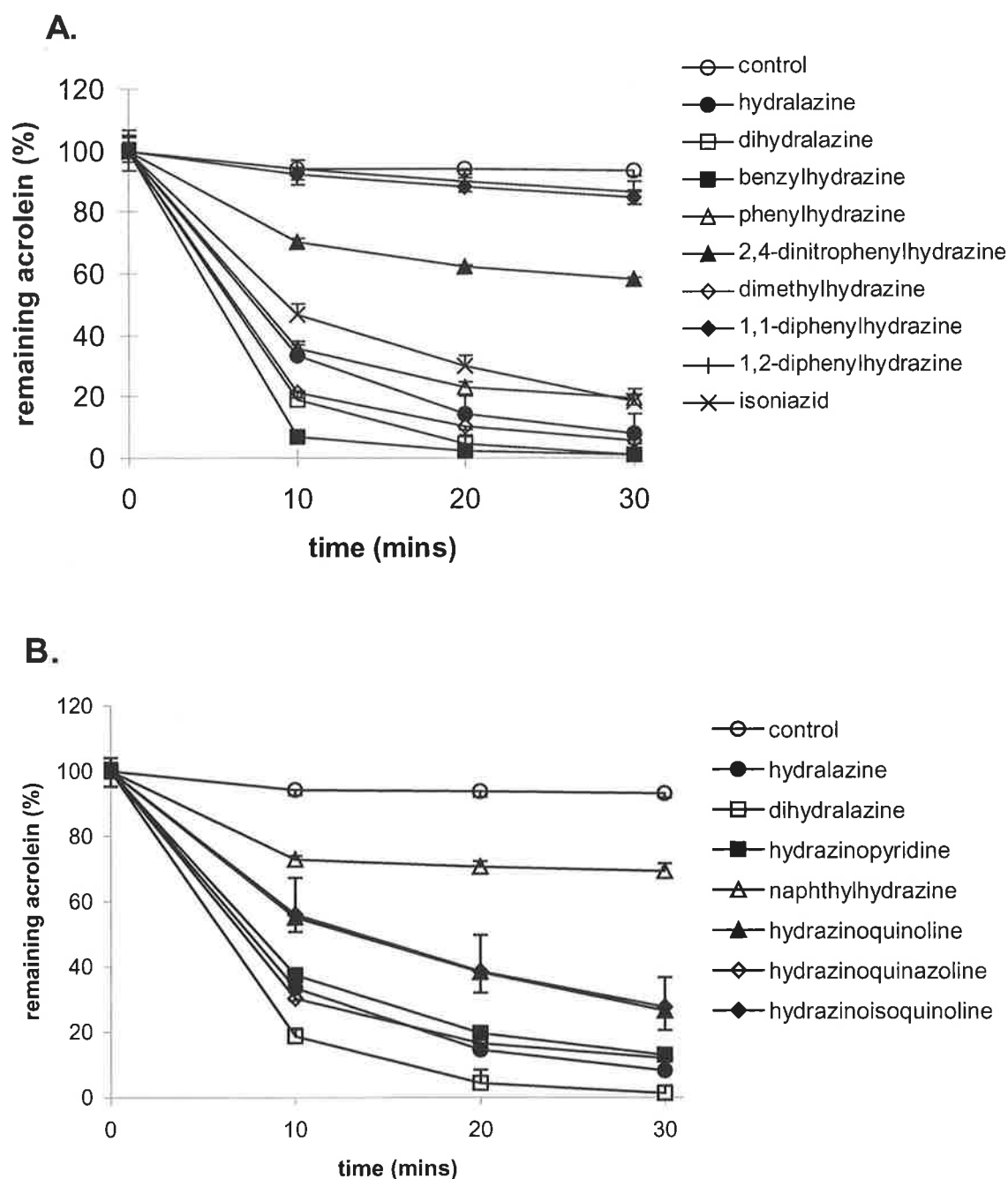


Figure 2.16 Scavenging of free acrolein from buffered solution at 37°C by structurally diverse hydrazines (A) and hydralazine analogues (B). Acrolein (500 μ M) was incubated with equimolar amounts of each of the hydrazine scavengers for up to 30 minutes and aliquots of the reaction mixture taken at 10 minute intervals for assessment of free acrolein by a HPLC assay. Data are represented as mean $\pm$ SEM of remaining acrolein in solution, $n=3$ observations. Note that data for both hydralazine and dihydralazine is included in both panels to facilitate comparisons (see text).

2.3.10 Cytoprotection Against Allyl Alcohol and Crotyl Alcohol Toxicity by Other Hydrazines and Hydralazine Analogues

In order to assess the structural requirements for cytoprotection against 2-alkenal mediated toxicity and to compare the cytoprotective and 2-alkenal scavenging properties of the abovementioned hydrazines and hydralazine analogues, they were screened for cytoprotective efficacy against allyl alcohol toxicity in 96 well culture plates. Use of these multi-well culture plates allowed for high throughput EC₅₀ determinations which were calculated (by Dr Frank Fontaine) by normalising the data at each time point to the % of allyl alcohol toxicity for that time and assessing EC₅₀ using a sigmoidal dose response curve-fitting capability in GraphPad Prism. Samples to determine LDH leakage were taken 1 and 2 hours after allyl alcohol addition to the wells. These time points were used since cell death was essentially maximal within 2 hours in allyl alcohol treated cells. Figure 2.17 shows the EC₅₀ results of the various hydrazines and analogues after 1 and 2 hours of incubation. The data indicates that in contrast to the data on 2-alkenal sequestering reactions, hydralazine and dihydralazine display only moderate cytoprotective potency in comparison to the other hydrazinophthalazine analogues. The order of cytoprotective potency was 1,1-diphenylhydrazine (1,1-DPH) > 1-hydrazinoisoquinoline (HIQ) > naphthylhydrazine (NH) > phenylhydrazine (PH) > 4-hydrazinoquinazoline (HQZ) > 2-hydrazinoquinoline (HQL) > dihydralazine (DH) > hydralazine (HYD) = 1,2-diphenylhydrazine (1,2-DPH) > 2,4-dinitrophenylhydrazine (DNPH) > 2-hydrazinopyridine (HP) > benzylhydrazine (BH). No protection was seen with isoniazid or 1,1-dimethylhydrazine (DMH). Generally, the 2 hour samples showed less protection than the 1 hour samples, with the exception of 1,1-diphenylhydrazine. The protection afforded by benzylhydrazine (BH, 1 hour) was rapidly lost by 2 hours. Also, in contrast to the effect on scavenging efficacy, it appears that dissociation of hydrazine and phenyl groups with a -CH₂- spacer decreases the protective efficacy of the hydrazines against acrolein mediated cytotoxicity (ie. benzylhydrazine is a poor cytoprotective compound in comparison to phenyl hydrazine).

The hydrazines 1-hydrazinoisoquinoline (HIQ), 2-hydrazinoquinoline (HQL), 4-hydrazinoquinazoline (HQZ), 1,1-diphenylhydrazine (1,1-DPH) and benzylhydrazine (BH) were also compared for their ability to protect against crotyl alcohol toxicity in mouse hepatocytes (Figure 2.18). Since crotyl alcohol toxicity was not reproducible in cells cultured in 96 well plates, larger dishes (60 mm diameter) were used for these experiments. As shown in Figure 2.18, the ability of these hydrazines to protect against crotyl alcohol toxicity closely resembled their efficacy against allyl alcohol toxicity, where 1,1-diphenylhydrazine provided the best

protection while benzylhydrazine afforded very poor cytoprotection. This data supports the above observations suggesting that dissociation of hydrazine and phenyl groups decreases the cytoprotective efficacy of the hydrazine and that several analogues of hydralazine show improved cytoprotective efficacy against 2-alkenal mediated toxicity.

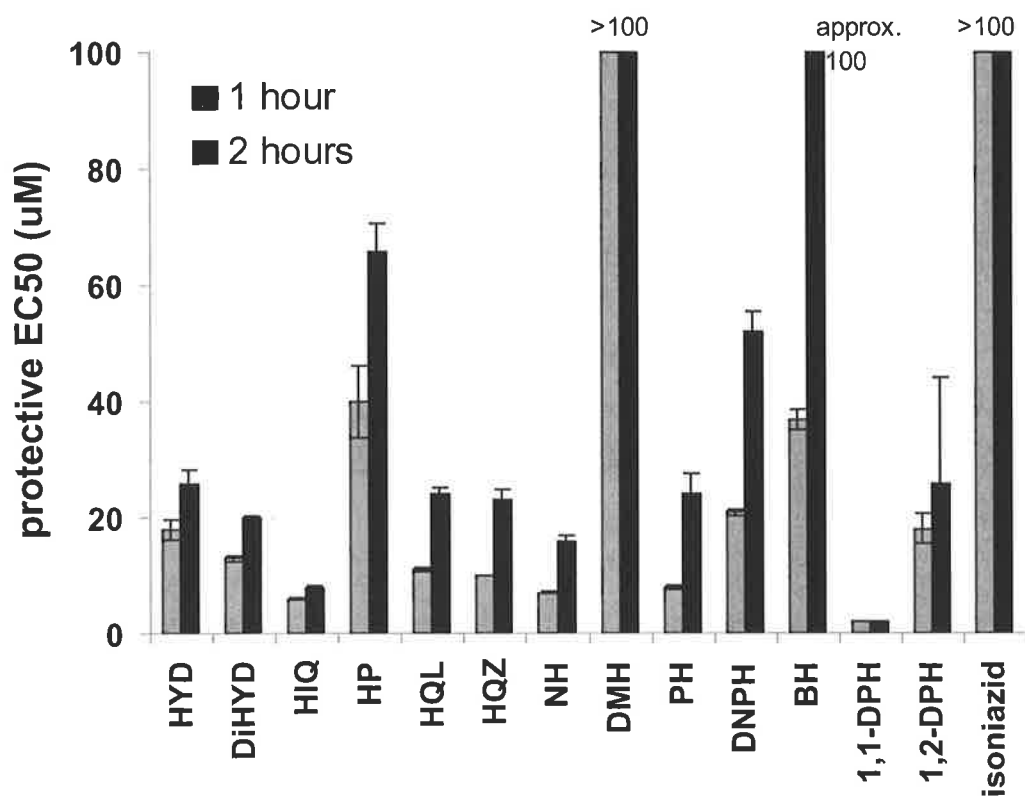


Figure 2.17 Protective efficacies against allyl alcohol (AA, 100 μ M) induced hepatocyte cell death by various hydrazines and hydralazine analogues. Samples of culture media were taken 1 and 2 hours after the co-addition of AA and the hydrazines (1-100 μ M) for analysis of LDH leakage from the cytoplasm. Data are represented as mean $\pm$ SEM of the hydrazine's protective EC_{50} 's, $n=3$ observations.

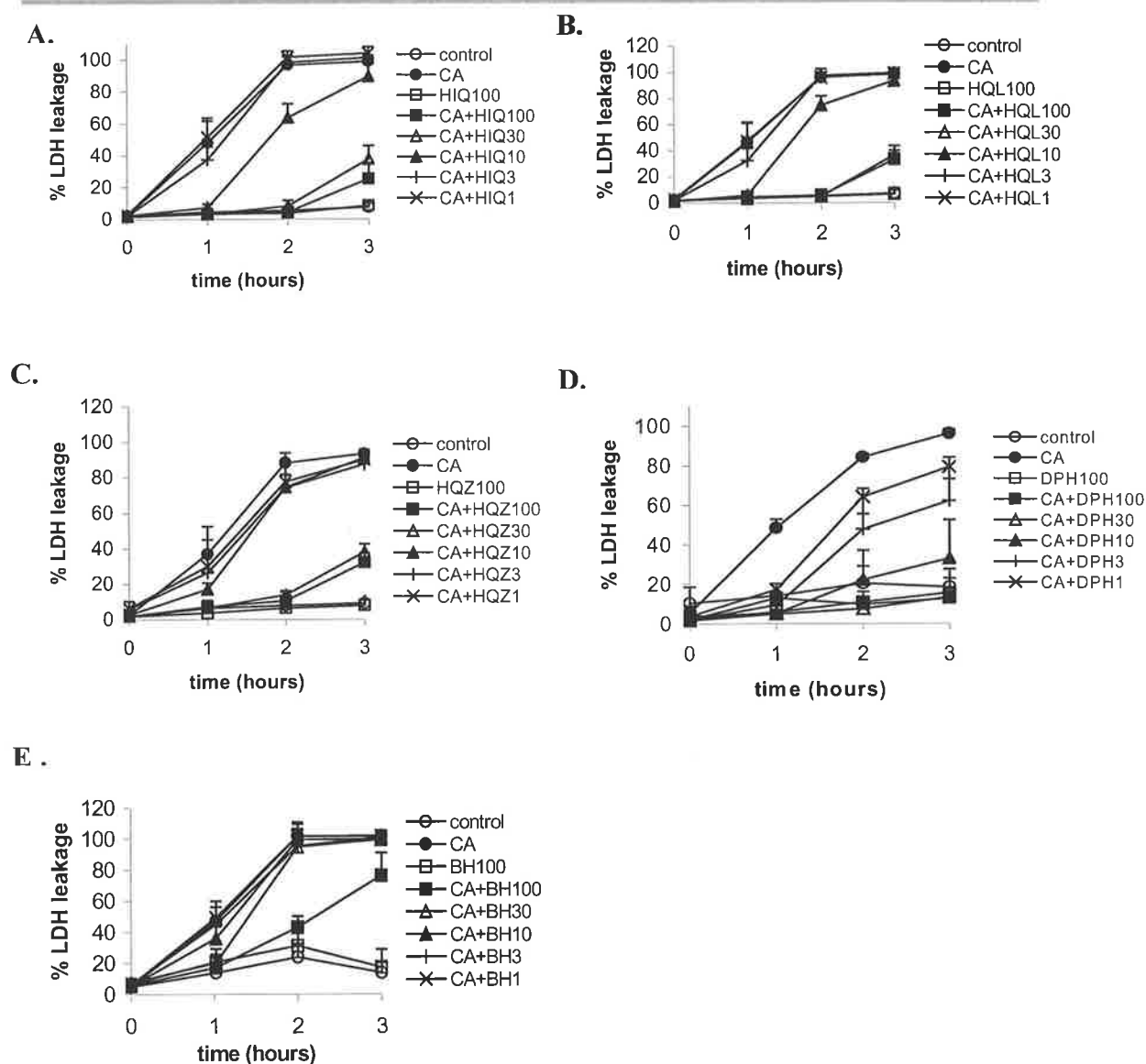


Figure 2.18 Protection against crotyl alcohol (CA, 500 μ M) induced hepatocyte cell death afforded by 1-hydrazinoisoquinoline (HIQ, A), 2-hydrazinoquinoline (HQL, B), 4-hydrazinoquinazoline (HQZ, C), 1,1-diphenylhydrazine (DPH, D) and benzylhydrazine (BH, E). Samples of culture media were taken 1, 2 and 3 hours after the co-addition of CA and the hydrazines (1-100 μ M) for analysis of LDH leakage from the cytoplasm. The concentration of hydrazine used (μ M) is indicated in the Figure legends. Data are represented as mean $\pm$ SEM of the % lactate dehydrogenase leakage, $n=3$ observations.

To assess the relationship between the 2-alkenal trapping and cytoprotective effects of the abovementioned hydrazines, a correlation study was then performed on acrolein scavenging, cytoprotection and log P data, in the expectation that such information would then form the basis for further structure based studies. Figure 2.19 shows the correlation between allyl alcohol cytoprotection and acrolein sequestering efficacy. Cytoprotection is reported as the EC₅₀ at 1 hour for each compound. Acrolein sequestering capacity was determined as the rate of acrolein loss within the first 10 minutes of reaction (% acrolein loss per minute). Due to the fact that no cytoprotection was observed for isoniazid and benzylhydrazine within the concentration range tested (1-100 μ M, ie. cytoprotective EC₅₀'s were not determined) these compounds were excluded from correlation analyses. However, as isoniazid and benzylhydrazine represent compounds which are structurally dissimilar to the remaining arylhydrazines (ie. a hydrazine adjacent to a carbonyl group and an alkyl hydrazine respectively) suggests that they may not fit within the correlation for the remaining hydrazines (Nilsson *et al*, 1989; Ancerewicz *et al*, 1998). As expected, Figure 2.19 shows a significant inverse correlation between cytoprotection and acrolein scavenging capacity ($R^2 = 0.2631$, $p < 0.05$). A stronger correlation exists, however, between cytoprotection and log P (Figure 2.20, $R^2 = 0.6819$, $p < 0.001$). Unexpectedly, these observations suggest that excellent scavengers of 2-alkenals can be quite poor cytoprotective agents, in contrast to our initial hypothesis suggesting that 2-alkenal sequestering reactions underlie the protective effects of hydralazine and dihydralazine. That membrane permeability of the cytoprotective hydrazines contributes to cytoprotective efficacy is suggested by the correlation between log P and cytoprotection, indicating a vital role for cytoplasmic access of nucleophilic compounds in the protection against 2-alkenal mediated toxicity.

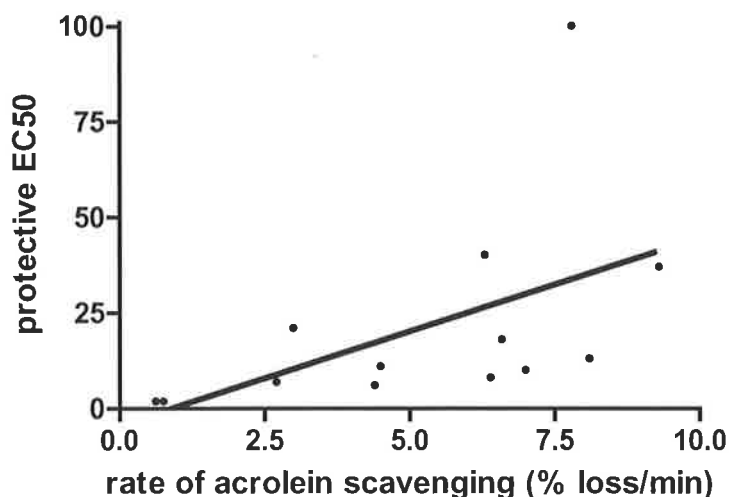


Figure 2.19 Correlation between cytoprotective EC_{50} 's of the hydrazines and hydralazine analogues examined and their ability to sequester free acrolein from buffered solution at 37°C. The rate of acrolein scavenging by each of the hydrazines examined (with the exception of isoniazid and benzylhydrazine whose cytoprotective EC_{50} 's were greater than 100 μ M) is plotted as the % loss of acrolein per minute for the first 10 minutes of reaction. The line represents the line of best fit between the individual points of the graph ($R^2=0.2631$).

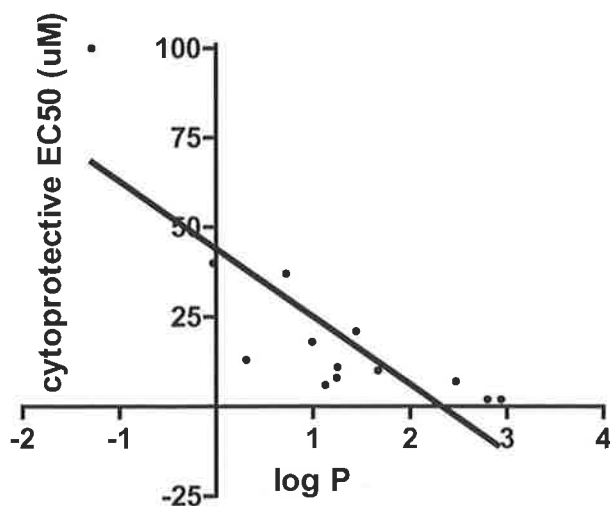


Figure 2.20 Correlation between cytoprotective EC_{50} 's of the hydrazines and hydralazine analogues examined and their log P's. The log P values reported were calculated values. The line represents the line of best fit between the individual points of the graph ($R^2=0.6819$).

2.3 DISCUSSION

In recent preliminary work, our laboratory compared the acrolein sequestering capabilities of hydralazine to those of several reported scavengers of biogenic aldehydes (Burcham *et al*, 2000). However, subsequent investigations revealed that the use of large reaction vessels in this study resulted in significant loss of acrolein into head space (approximately 55% over 30 minutes), confounding accurate assessment of the aldehyde scavenging potency of the nucleophiles. To accurately measure the loss of acrolein, the acrolein scavenging abilities of hydralazine, dihydralazine, carnosine, pyridoxamine, aminoguanidine and methoxyamine were reassessed using an improved method in which trapping reactions were performed using maximal reaction volumes in small reaction vessels. Head space loss was much reduced in this system, with free acrolein levels in control incubations declining by just 6% over 30 minutes. In addition, the crotonaldehyde sequestering abilities of these nucleophiles were also examined.

Somewhat surprisingly, the AGE inhibitor aminoguanidine displayed little or no acrolein or crotonaldehyde-trapping efficacy in these experiments. Aminoguanidine has been investigated in clinical trials with diabetic subjects due to its activity as an inhibitor of advanced glycation end product (AGE) formation (Brownlee *et al*, 1986; Al-Abed and Bucala, 1997). While aminoguanidine readily scavenges Amadori fragmentation products such as 3-deoxyglucosone and also blocks protein modification by sugars, others have shown that it readily traps two important lipid-derived α,β -unsaturated aldehydes, MDA and 4-HNE (Al-Abed and Bucala, 1997). Given that both malondialdehyde and 4-hydroxy-2-nonenal contain a β -hydroxy group, the minimal reactivity of aminoguanidine with acrolein and crotonaldehyde observed may suggest unsaturated aldehydes possessing a β -hydroxy group are most reactive with this drug.

Pyridoxamine, carnosine and methoxyamine displayed modest reactivity with acrolein and crotonaldehyde, whereas, as expected, the clinically-used acrolein-trapping thiol compound MESNA was highly reactive with acrolein, sequestering almost all the free acrolein from solution within 10 minutes. This was expected, given that thiol compounds are typically more nucleophilic than hydrazine containing compounds (Esterbauer *et al*, 1991). Hydralazine and dihydralazine also displayed very strong acrolein and crotonaldehyde-trapping reactivity, confirming our previous observation that hydralazine and dihydralazine are highly efficient scavengers of acrolein, with the improved method for determining scavenging capacity revealing marked distinctions between the two arylhydrazino drugs and the other amine nucleophiles (Burcham *et al*, 2000). Also, the present finding that sequestering of acrolein by N^α-acetyl lysine

was poor in contrast to hydralazine and dihydralazine, suggests that these arylhydrazines could act as sacrificial nucleophiles, preventing the adduction of important cellular nitrogen containing nucleophiles by 2-alkenals.

The reaction products of the 2-alkenals with hydralazine were identified by ^1H NMR analysis. Two major reaction products were isolated and characterised as hydrazones formed by Schiff base addition of the 2-alkenals to the hydrazine group of hydralazine. These were identified as (1*E*) and (1*Z*)-acrylaldehyde phthalazin-1-ylhydrazone (APH), for the reaction with acrolein, and (1*E*,2*E*) and (1*Z*,2*E*)-but-2-enal phthalazin-1-ylhydrazone (BPH), for the reaction with crotonaldehyde. However, during the study of acrolein and hydralazine reactions it was noticed that an orange/brown precipitate would form in some vials. This material was quite difficult to isolate and purify as it was strongly bound to silica stationary phases. This product was identified by ^1H NMR and ESI mass spectrometry as oligomeric forms of APH, with ions detected at m/z 199 (acrolein₁:hydralazine₁), 397 (acrolein₂:hydralazine₂) and 595 (acrolein₃:hydralazine₃, spectra shown in Appendix 1; A1.6).

It was also noticed that upon prolonged standing of the individual isomers of BPH in deuterated chloroform for several weeks at 4°C, *Z*-BPH equilibrated to *E*-BPH, whereas *E*-BPH did not equilibrate appreciably to *Z*-BPH. This suggests that *Z*-BPH is the kinetic product of reactions between crotonaldehyde and hydralazine, whereas *E*-BPH is the thermodynamic product. Also, it was noticed that after 1 week of leaving APH at 4°C (both isomers of *E*-APH), the purified APH formed oligomeric APH. The same occurred after 1 month of leaving solid APH at -20°C. This effect was not seen, however, during incubations of APH alone or with acrolein or hydralazine at 37°C for 4 hours. This indicates that APH polymerises into dimers and trimers over long periods of time but not during short term (up to 3 hours) incubations in isolated mouse hepatocytes. This product did, however, seem to be prominent in reactions between free acrolein and hydralazine, suggesting that it may account for the low synthetic yield of APH (35%) in contrast to the complete loss of hydralazine.

Since efforts to synthesise *E*-APH resulted in quite low yields, and the yield of the oligomeric product could not be determined, the kinetics and stability of *E*-APH was explored over 24 hours. HPLC was used to monitor the kinetics of *E*-APH formation in a 1:1 reaction mixture of hydralazine and acrolein in buffered solution at 37 and 4°C. In contrast to the 35% yield obtained during the synthesis of *E*-APH, the lower concentration of reagents used in the kinetic study (500 μM for both acrolein and hydralazine) gave *E*-APH yields of approximately 10%

after 24 hours at either 37°C or 4°C. The low yield was not due to product instability, since *E*-APH alone was stable in solution over a 24 hour period at 4°C, although some *E*-APH loss was evident with prolonged incubation at 37°C. This was attributed to the formation of dimeric (acrolein₂:hydralazine₂) and trimeric (acrolein₃:hydralazine₃) APH, as the loss of acrolein was not attributed to evaporation or polymerisation. However, due to the difficulties involved in extracting these products, we were unable to determine their yield during these kinetic experiments, but expect that they represent the predominant product between reactions of acrolein and hydralazine due to the abundance of product that remains on silica columns following elution of APH.

A similar kinetic study was also performed to examine the formation of *Z*-BPH from crotonaldehyde and hydralazine. As predicted on the grounds of the earlier aldehyde-trapping experiments, *E*-BPH formation occurred at a slower rate than for *E*-APH, although the yield after 24 hours was considerably higher: 47% at 37°C and 63% at 4°C respectively. The higher yields may indicate a lesser tendency for hydralazine to catalyze the formation of oligomeric adducts from crotonaldehyde compared to acrolein. This is likely due to the additional methyl group on crotonaldehyde hindering polymerisation. However, some material still remained bound to silica columns following the elution of BPH, suggesting that oligomeric products were formed from crotonaldehyde and hydralazine.

To assess whether the hydrazone-forming properties of hydrazino drugs are relevant in a cellular model, we compared the ability of hydralazine and dihydralazine to attenuate the toxicity of various unsaturated aldehydes, or alcohol precursors to 2-alkenals, in isolated mouse hepatocytes. Since they are rich in alcohol dehydrogenase activity, hepatocytes readily convert allyl and crotyl alcohols to their respective oxidation products, acrolein and crotonaldehyde (Rikans, 1987; Fontaine *et al*, 2002). Similarly, P450 2E1 and alcohol dehydrogenase convert propargyl alcohol to the 2-alkynal propiolaldehyde. In mouse hepatocytes, allyl and crotyl alcohol produce time- and concentration-dependent cell death, preceded by extensive GSH depletion and protein carbonylation, effects that are abolished by prior exposure to the alcohol dehydrogenase inhibitor 4-methylpyrazole (Burcham *et al*, 2002). Similarly, GSH depletion precedes propargyl alcohol induced hepatocellular toxicity (Moridani *et al*, 2001). These unsaturated alcohols thus provide a means of exploring unsaturated aldehyde toxicity via their *in situ* generation in cytosol. Pentenal and MDA, however, were added as a bolus to the culture media of cells. For the present experiments, mouse hepatocyte monolayers were exposed to either the aldehydes or their alcohol precursors in the presence of 0 to 100 µM concentrations of

hydralazine. The protection afforded by dihydralazine was examined against allyl and crotyl alcohol mediated toxicity as a means of confirming the cytoprotection achieved by this hydrazinophthalazine. Exposure to the aldehydes or unsaturated alcohols alone was very toxic to the cells, inducing maximal cell death within 3 hours (except for MDA). MDA toxicity was not evident till 12 hours after the addition of the aldehyde to the culture media, consistent with previous reports indicating that exogenously added MDA is not very toxic (Esterbauer *et al*, 1991). Micromolar concentrations of both hydralazine and dihydralazine provided clear, concentration-dependent protection against the toxicity of each of the toxic compounds examined. In keeping with its possession of two nucleophilic hydrazine substituents and hence greater reactivity with aldehydes, dihydralazine was approximately twice as cytoprotective as hydralazine against the toxicity of allyl and crotyl alcohols. Hydralazine was also able to afford protection against the toxicity of propargyl alcohol, the active metabolite of which (propionaldehyde) is capable of complex chemical reactivity with nucleophilic biomolecules due to its electrophilic alkyne bond (Skoldinov *et al*, 1974; Medvedeva *et al*, 1987; Awad and Hassan, 1990). For instance, propionaldehyde forms β -aminoacroleins with amines, which then spontaneously isomerise to enamino ketones (Skoldinov *et al*, 1974; Medvedeva *et al*, 1987).

However, in contrast to the cytoprotection data, hydralazine and dihydralazine were unable to prevent the decrease in cellular GSH that was produced by the aldehydes or their alcohol precursors. Declining GSH levels have been reported to precede cellular necrosis and elevating cellular GSH provides protection against acrolein and allyl alcohol mediated cell death both *in vivo* and *in vitro* (Jaeschke *et al*, 1987; Miccadei *et al*, 1988). However the present study indicates that a decrease in GSH is not critical for cell death to occur. The fact that thiol compounds are typically better scavengers of 2-alkenals than hydrazines may explain why hydralazine did not prevent the decrease in cellular GSH with the aldehydes or alcohol precursors administered.

Burcham and colleagues (2000) previously reported that pyridoxamine, carnosine and methoxyamine were relatively poor acrolein-trapping reagents, in contrast to hydralazine and dihydralazine, and also provided no protection against allyl alcohol induced hepatocyte cell death. Methoxyamine, which showed modest reactivity with acrolein, provided some protection against allyl alcohol toxicity at 10 mM. This data, combined with the observation that hydralazine and dihydralazine are effective 2-alkenal sequestering and cytoprotective reagents, provided initial support for the hypothesis that trapping reactions with 2-alkenals underlies the cytoprotective mechanism of the arylhydrazine compounds. However, both arylhydrazine drugs

afforded stronger cytoprotection against allyl alcohol toxicity than MESNA, which provided only minimal protection at 100 μM (Dr Frank Fontaine, personal communication; data not shown). Since MESNA was a highly efficient acrolein-trapping reagent, the greater cytoprotective potency of hydralazine and dihydralazine in hepatocytes compared to MESNA presumably reflects the low lipophilicity and poor cellular access of the latter (Ormstadt et al, 1983; Ormstadt and Ohno, 1984).

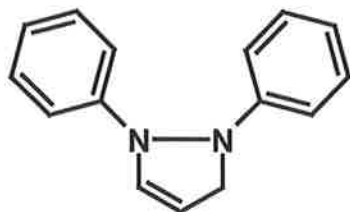
In order to assess the role of trapping reactions with 2-alkenals in the cytoprotection afforded by hydralazine and dihydralazine, the formation of hydrazone products in cells treated with 100 μM allyl alcohol and 0 to 100 μM hydralazine was examined. Concentration dependent formation of *E*-APH was evident in cells, although the maximum yield was just 0.4% (assuming the limiting reagent was 100 μM hydralazine). However, it has previously been observed in our laboratory that after a 30 minute incubation of allyl alcohol (100 μM) with hepatocytes, acrolein concentrations peak at 4 μM . Assuming 4 μM acrolein as the limiting reagent, the yield of *E*-APH becomes 10%, which is more consistent with the reported yield of the hydrazone at 500 μM starting concentrations of acrolein and hydralazine. However, given the rapid rate at which acrolein reacts with thiol compounds such as GSH, the total concentration of acrolein (ie. bound and unbound acrolein) after 30 minutes of incubation of cells with 100 μM allyl alcohol is likely to be higher than 4 μM . This indicates that the amount of APH detected after 30 minutes should have been greater than 0.4 μM , given the expected 10% yield of *E*-APH from equimolar hydralazine and acrolein

To see whether rapid metabolism might contribute to low *E*-APH yields, the rate of disappearance of *E*-APH in cells was monitored when a relevant concentration of *E*-APH was added to cells (1 μM) to account for the lower expected yield of *E*-APH via metabolism or protein binding of the free hydrazone. The disappearance of APH in cells was rapid, having declined to 10% of the starting concentration within 30 minutes. By 1 hour APH was below a quantifiable concentration. It was not determined, however, whether this loss was due to binding of APH to cellular components, or if it was due to metabolism (eg. by cytochrome P450's). Combined with the total yield of APH in cells after 30 minutes, the data indicates that hydralazine efficiently scavenges free acrolein within cells, forming a hydrazone product which was also shown to be non-toxic at the low concentrations likely to be formed in allyl alcohol exposed cells. This indicates that hydrazone formation from toxic aldehydes represents a true detoxification pathway for acrolein, providing further support for the initial hypothesis.

Further evidence of this mechanism of protection was then sought by comparing the acrolein sequestering and cytoprotective effects of a range of hydrazinophthalazine compounds and structurally related hydrazines. Investigation of these compounds achieved 3 aims: 1) to investigate the significance of 2-alkenal sequestering reactions in the cytoprotection afforded by hydrazine compounds, 2) to examine the structural requirements for cytoprotection and 3) to identify a range of similarly cytoprotective analogues of hydralazine without the vasoactivity of the parent compound. The latter aim was achieved by disrupting the $C=N-N=C-NH-NH_2$ pharmacophore that reportedly underlies hydralazine's vasoactivity (Reece, 1981). It was also expected that the cytoprotective and aldehyde sequestering effects of hydralazine and dihydralazine were mediated by their strongly nucleophilic hydrazine functional groups, which are typically better nucleophiles than amino groups. Hence, the hydrazines shown in Figure 2.2 were chosen for comparison of their acrolein sequestering and cytoprotective effects.

In general, it was found that most hydrazines tested were effective scavengers and provided efficient protection against allyl alcohol toxicity compared to the abovementioned amine compounds studied. Unexpectedly, however, the relationship between cytoprotection was not the strict correlative association initially expected. Specifically, hydrazines that were not directly attached to phenyl groups (benzylhydrazine and 1,1-dimethylhydrazine) were highly efficient aldehyde trapping reagents but showed poor cytoprotective effects. This was evident when comparing phenylhydrazine and benzylhydrazine, as phenylhydrazine was a poor acrolein scavenger yet showed better cytoprotection than dihydralazine (Figures 2.17b and 2.18). On the other hand, benzylhydrazine was an excellent scavenger yet it provided only modest protection after 1 hour of incubation. The hydrazines 1,1- and 1,2-diphenylhydrazine provided important information regarding the effects of substitution on the hydrazine nitrogens. 1,1-Diphenylhydrazine was the most efficient cytoprotective compound examined, yet one of the poorest acrolein scavenging reagents. That this compound is a secondary hydrazine indicates that substitution at N2 on hydralazine would not likely hinder the cytoprotective effects of the drug. In contrast, 1,2-diphenylhydrazine showed less cytoprotective efficacy than 1,1-diphenylhydrazine, indicating the importance of a primary nitrogen at N1 of hydralazine. Surprisingly, though, cytoprotection was still afforded by 1,2-diphenylhydrazine, although this hydrazine would not be expected to form hydrazone products. It may, however, form a 3-pyrazoline (1,2-diphenyl-3-pyrazoline, shown below) via initial Schiff base addition to one nitrogen, followed by Michael addition of the second nitrogen to the β -carbon of the bound acrolein (Wagner *et al*, 1981; Dockner and Krug, 1986; Usachev *et al*, 2003). 2,4-Dinitrophenylhydrazine also showed some cytoprotective and 2-alkenal sequestering effects,

though nitro-substitution on the phenyl ring decreased its cytoprotective efficacy compared to phenylhydrazine. The antituberculosis drug, isoniazid, was found to provide no cytoprotection consistent with the electronegative effect of the carbonyl groups. These results suggest that groups that decrease the nucleophilicity of reacting nitrogens decrease the cytoprotective potency of the hydrazines.



1,2-diphenyl-3-pyrazoline

When comparing the analogues of hydralazine it was revealed that simple relocation of N4 from position 3 to position 4 (ie. 4-hydrazinoquinazoline) had a negligible effect on the 2-alkenal trapping effects of hydralazine, but increased the cytoprotective potency approximately 2 fold. A similar effect was seen by removal of N4 along with relocation of the hydrazine group to position 2 (2-hydrazinoquinoline), although the acrolein scavenging effects were slightly diminished. In contrast, complete removal of N4 (ie. 1-hydrazinoisoquinoline) increased cytoprotective potency 3 fold compared to hydralazine, while resulting in similar acrolein sequestering efficacy to that seen with 2-hydrazinoquinoline. Removal of both N4 and the additional phenyl ring (ie. 2-hydrazinopyridine) resulted in decreased cytoprotective potency and an increased acrolein sequestering capacity. Surprisingly, removal of the ring nitrogens altogether (naphthylhydrazine) increased the cytoprotective efficacy of the parent molecule (hydralazine) 3 fold.

The collective data on the cytoprotective capacity of the abovementioned hydrazines suggests that to develop a highly efficient cytoprotective hydrazine, a few key structural features could be considered, including: 1) direct attachment of the hydrazine to a 2-ringed aromatic structure (diphenyl or naphthyl), 2) avoid inclusion of groups that decrease the nucleophilicity of the reacting nitrogen, 3) avoid substitution on the reacting nitrogen and 4) minimise inclusion of ring nitrogens. Whether the addition of more phenyl rings confers better cytoprotective efficacy remains to be determined, although this is expected given the positive correlation between log P and cytoprotection, since polycyclic hydrazines would be expected to exhibit enhanced lipophilicity.

Data collected from the abovementioned hydrazines also suggested that contrary to initial results indicating that the 2-alkenal scavenging capacity of nucleophiles was the key determinant of cytoprotective potency, cytoprotection was found to be negatively correlated with acrolein scavenging in this model. Cytoprotection was, however, positively correlated with log P as mentioned above. This suggests that the carbonyl sequestering capacity of hydralazine was poorly related to its cytoprotective actions, although its ability to permeate cells was a crucial factor in its cytoprotective effects. This indicates that although acrolein scavenging occurs in cells treated with allyl alcohol and hydralazine, the formation of non-toxic hydrazone products does not account for the cytoprotection. The correlation data from the current set of experiments on hydrazine compounds is believed to be more robust than the apparent correlation between scavenging and cytoprotection with the amine compounds. This is because in the current set of experiments, cytoprotection and scavenging data spanning from very good to very poor efficacies was collected, whereas methoxyamine was the only amine compound that showed some intermediate cytoprotection that was vital for the conclusion in the previous study. In addition, while the hydralazine analogues examined were not tested for vasoactivity, they may represent useful starting points for future drug development by acting as experimental tools for examining the mechanism of hydralazine mediated cytoprotection and its limitations.

Collectively, the results reported in this Chapter suggest that although 2-alkenal trapping occurs in cells exposed to allyl alcohol and hydralazine, this trapping reaction does not appear to underlie the cytoprotective effects of hydralazine and structurally related hydrazines. Given that the hydrazines examined provided considerably better cytoprotection than the amines tested, it is reasonable to suggest that the mechanism of hydralazine cytoprotection is still mediated by a chemical interaction between the toxic 2-alkenal and the hydrazine group. One possible interaction may be at the residual carbonyl group of aldehyde-adducted proteins, formed by aldehydes that escape trapping reactions with hydralazine. Such ternary adducts were identified by Otteneder and colleagues via mass spectrometry investigations of MDA adducted deoxyguanosine (Otteneder *et al*, 2002). In the following Chapter, the chemical nature of interactions between hydrazine compounds and acrolein-adducted proteins is explored using mass spectrometry as the main experimental tool. This is followed in subsequent chapters by exploration of the biological significance of such “protein-adduct trapping” reactions of hydralazine.

Chapter 3:

Trapping of acrolein-protein adducts

in vitro

3.1 INTRODUCTION

It has thus far been shown that the hydrazines hydralazine and dihydralazine protect against acrolein mediated toxicity in isolated primary mouse hepatocytes. However, as opposed to the initial expectation that hydralazine protects against acrolein mediated toxicity by sequestering the free aldehyde before it adducts cellular macromolecules, it was found that protection is not strictly related to scavenging efficacy. Protection was, however, positively correlated with log P, indicating that permeation of the hydrazines into the cytosol is important for protection. This latter finding suggests that intracellular targets are important for protection. Moreover, recent work from our group (Burcham *et al*, 2004) has also indicated that hydralazine protection occurs when the drug is added to the culture media of cells that have been subjected to a prior brief exposure to the acrolein precursor allyl alcohol (ie. 25 minutes). This observation provides further support for the mechanism of protection by hydralazine not being simply due to sequestering of free acrolein. This brief exposure resulted in similar cell death to cells exposed continuously to allyl alcohol, indicating that cell death was irreversibly initiated in the early stage, potentially through the modification of cell macromolecules. Yet the fact that hydralazine still afforded protection suggests it interferes with events subsequent to acrolein formation in the cells (eg. adduction).

The current data indicates that hydralazine mediates protection against carbonyl toxicity by blocking a post-adduction event. Given that hydrazines are commonly used reagents for the detection of carbonyl groups, it is possible that hydralazine traps acrolein-protein adducts which, since adduction occurs through the β -carbon, contain residual carbonyl groups. It is therefore possible that hydralazine mediated protection of carbonyl toxicity is due to reactions between

hydralazine and free carbonyl groups on the Michael adducts of proteins. These trapping reactions may prevent further downstream events in toxicity, such as inter- or intra-molecular crosslinking (shown in Figure 3.1) which may initiate cell death pathways. For instance, single Michael-acrolein adducts on proteins may undergo inter- or intra-molecular crosslinking reactions at nearby proteins or DNA, as has been observed for DNA adducts of acrolein (Kuykendall and Bogdanffy, 1992; Kozekov *et al*, 2001). Alternatively, bis-acrolein adducts of proteins may undergo aldol condensation reactions, forming a immunogenic FDP-adduct which may similarly undergo crosslinking reactions via its residual aldehyde group (Uchida *et al*, 1998a; Uchida *et al*, 1998b).

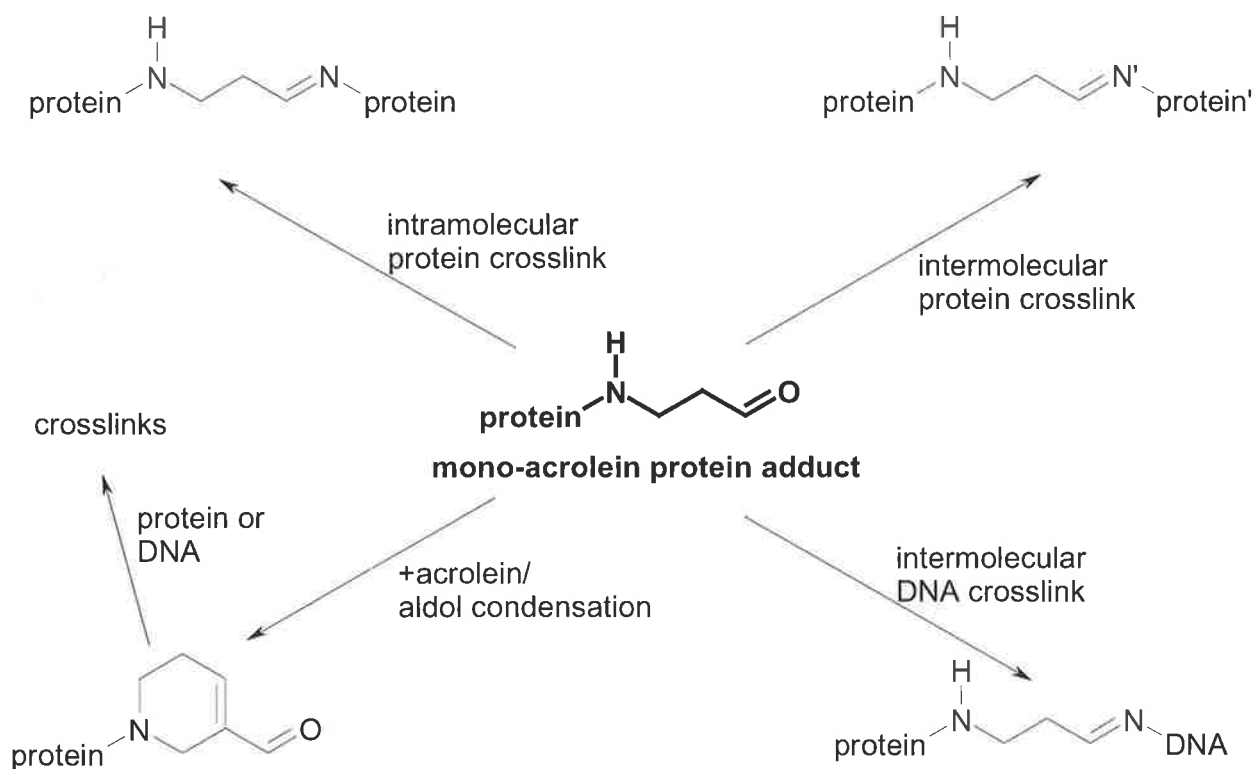


Figure 3.1 Scheme showing possible secondary reactions of acrolein-added proteins.

Burcham *et al* (2004) recently used polyclonal anti-acrolein-KLH antiserum raised in rabbits to examine the effect of hydralazine on acrolein-BSA adducts. They showed that co-incubation of BSA with acrolein and hydralazine resulted in a decrease in the immunoreactivity of acrolein adducts with increasing concentrations of hydralazine. These effects were observed when hydralazine was added either 15 or 30 minutes after starting BSA modification by acrolein, indicating that the immunorecognition of acrolein adducted proteins were being blocked by the addition of hydralazine. However, when hydralazine was added greater than 1 hour after acrolein addition, hydralazine was unable to block acrolein-adducts. These results were

consistent with the idea that hydralazine interfered with early acyclic Michael adducts in acrolein-modified BSA, whereas after an extended period of time cyclised FDP-lysine adducts or intramolecular crosslinks were present which were not targets for hydralazine.

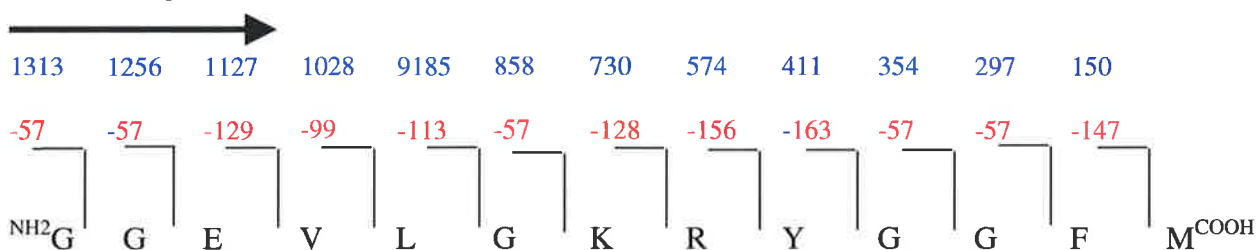
Given that hydralazine mediated protection against carbonyl toxicity still occurs after addition of the drug within 25 minutes of allyl alcohol and that crosslinking reactions are likely to occur predominantly at the sterically less hindered mono- and bis-acrolein adducts, it was hypothesised that hydralazine mediated protection involves targeting of the “early” Michael adducts of acrolein. These typically include the mono- and bis-acrolein adducts of lysine which precede the formation of cyclic adducts (ie. FDP-lysine). Uchida *et al* (1998a), however, reported that FDP-lysine is exclusively formed after a 24 hour reaction of acrolein with N^α-acetyl lysine. However, whether acyclic adducts are dominant at earlier time points has never been explored. Since acrolein toxicity is expressed rapidly in many experimental models “early” reaction products may play a vital role in the onset of toxicity. For example, allyl alcohol toxicity is typically evident within 30 minutes of addition of the toxin to hepatocyte cultures, by which time formation of cyclic bis-adducts might not have occurred.

It is therefore hypothesised that hydralazine mediated cytoprotection against 2-alkenal toxicity is via interactions with “early” acyclic Michael adducts, thereby preventing downstream events in toxicity. The relative kinetics of formation of mono- bis- and FDP-lysine adducts of acrolein were therefore initially examined to assess the likelihood that early acyclic adducts exist independently of the cyclic adducts studied by Uchida and colleagues. Positive ion electrospray ionisation mass spectrometry was chosen as an appropriate tool for examining the relative formation of these adducts on a model lysine containing peptide (preproenkephalin fragment 128-140, sequence NH₂-GGEVLGKRYGGFM-COOH, *m/z* 1370). This peptide was chosen as it contains a single lysine residue which was predicted to be the primary reacting centre on the peptide (this peptide does not contain any histidine or cysteine residues which are known targets for acrolein), indicating that the adduction site for acrolein could be easily identified. However, this peptide does contain an unprotected terminal glycine as well as an arginine. These residues were also assessed for modification, as they represent potential adduction sites. The identity of adducted species was therefore determined via MS/MS analysis.

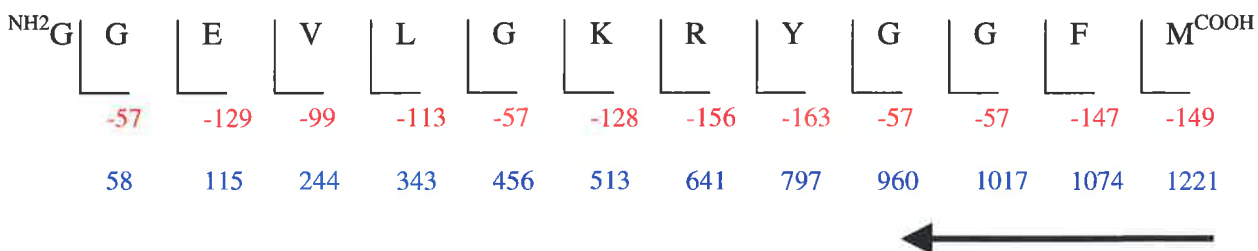
In classic mass spectrometric analyses, the mass of the peptide is determined by bombarding it with an energy source and recording the results as a mass/charge ratio (m/z). The mass spectrometer used in the present studies was an electrospray ionisation mass spectrometer with a double quadrupole. This technique involves the injection of solubilised peptide droplets into the ionisation chamber of the mass spectrometer, where it is ionised by a voltage (typically 3-5 kV) and desolvated (MS mode). This represents a soft ionisation technique that analyses ions in solution (such as peptides which are typically already positively charged at the nitrogen groups of amide bonds). Thus, electrospray ionisation is appropriate for studying peptide sequences and has become a widely used tool for peptide analysis. Once ionised, the ions pass into a quadrupole mass analyser which contains a combination of electric and radio frequency fields that create a window through which ions of a specific mass:charge ratio may pass. These ions are then passed via the continuous flow to the detector which counts the number of ions of the various mass:charge ratios. This produces a histogram of the relative intensity of each ion versus its m/z ratio. Alternatively, the quadrupole may be programmed to trap all ions except those with a particular m/z ratio, allowing only those to pass to a second quadrupole. The selected ions may then be fragmented into daughter ions by collision with an inert gas (MS/MS mode), resulting in a histogram spectrum of the relative intensity of the parent ion and the daughter ions. During MS/MS analysis of peptides, the loss of individual amino acid residues can be monitored, since loss of a residue via cleavage at the weak amide bond ($-\text{NH}-\text{CO}-$) is a predominant fragmentation (see the diagram below)(Biemann and Martin, 1987). This forms Y+2 cleavages, originating via fragmentation at the N-terminus, and B cleavages originating at the carboxy terminus. This allows the structure of a peptide molecule to be determined by examining the molecular cleavages arising from MS/MS analysis. The Y+2 cleavages are so named as the ionised N-terminus contains two additional hydrogens due to the fact that it is unbound to another amino acid residue. With B cleavages, however, the first fragmentation is coupled with the loss of an additional 18 mass units due to the additional loss of $-\text{OH}$ from the free carboxy terminus plus an additional hydrogen on the nitrogen group. As an example, the B and Y+2 fragmentation pattern of preproenkephalin in its unadducted state is shown below, where the mass of the peptide fragment following the indicated cleavage is indicated by the **blue** numbers and the mass lost from the peptide in that cleavage is shown in **red**.

Chapter 3: Trapping of Acrolein-Protein Adducts

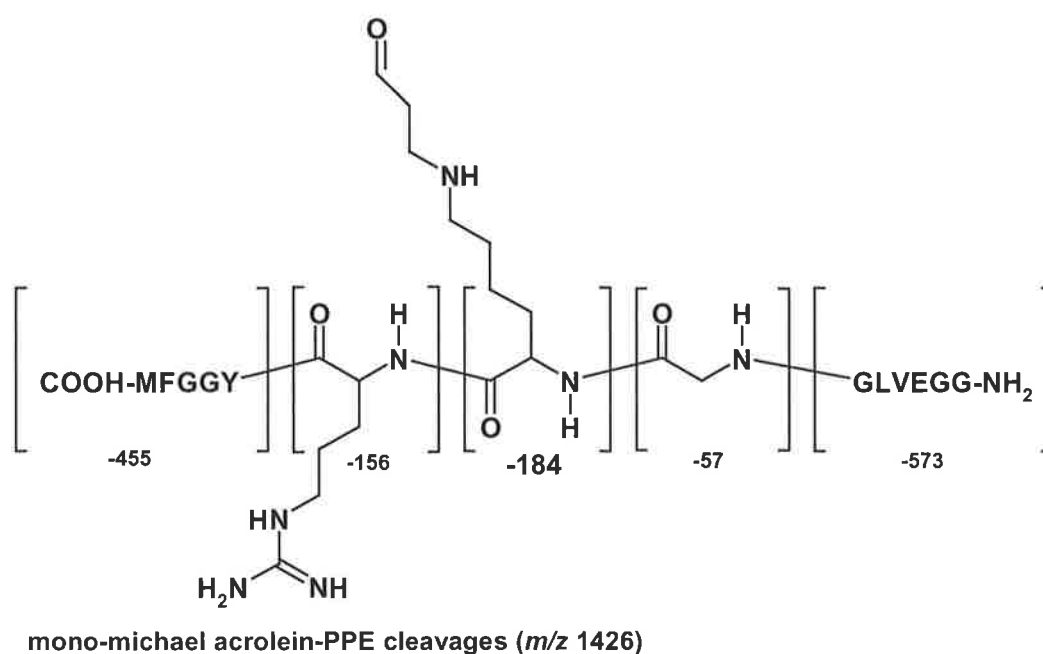
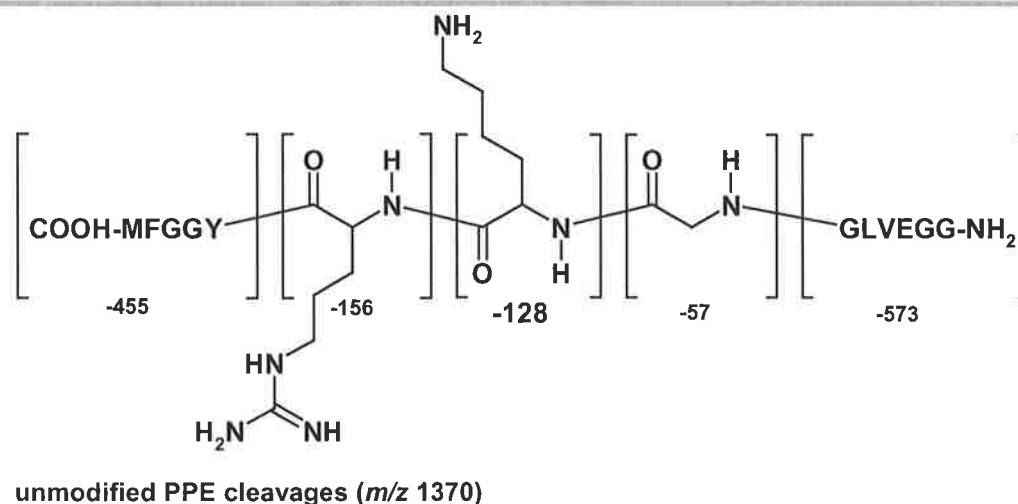
Y+2 cleavage:



B cleavage:



Amino acid modification in the form of acrolein adduction at any of the abovementioned residues would result in an increase in mass of the residue lost as shown in the diagram below. For instance, addition of a single Michael adducted acrolein onto the lysine residue would result in a mass loss of 184 instead of 128 (ie. the mass of lysine plus 56 mass units from the adducted acrolein), while cleavage at non-adducted residues would result in no additional mass loss as shown below.



The chemistry of hydralazine's interactions with acrolein-protein adducts was similarly characterised via this method in order to evaluate the antibody-based observations of Burcham *et al* (2004). At the outset, it was expected that mass spectrometry would reveal either an "adduct-stripping" reactivity of hydralazine (ie. regeneration of the peptide) or an "adduct-trapping" reactivity (ie. formation of hydrazones with carbonyl adducts). In addition, several hydrazines with varying cytoprotective EC_{50} 's (namely 1,1-diphenylhydrazine, benzylhydrazine and 1-hydrazinoisoquinoline; EC_{50} 's are 2, 40, and 6 μ M respectively after 1 hour) were examined for their ability to similarly trap acrolein-protein adducts. It was presumed that if this trapping mechanism represents a true detoxification pathway for acrolein adducts, then efficacious cytoprotective hydrazines (ie. 1,1-diphenylhydrazine and 1-hydrazinoisoquinoline) would readily

trap acrolein-PPE adducts, whereas less cytoprotective hydrazines (eg. benzylhydrazine) would not. To support the observations of the above mass spectrometry experiment, the hydrazines used previously in Chapter 2 were also examined for their ability to interfere with the immunoreactivity of acrolein-BSA adducts using the polyclonal acrolein-KLH antiserum previously generated in this laboratory (Burcham *et al*, 2003). Using this antibody in Western blots, other workers in the laboratory established that “early” reaction products formed during protein adduction by acrolein are unstable to nucleophilic reagents (Burcham *et al*, 2003; Burcham *et al*, 2004). Experiments in the present Chapter incorporated the use of slot blots, which work on a similar principle to Western blots, except that proteins are not separated by mass on a polyacrylamide gel. Instead, solutions of modified proteins (in this case, acrolein modified BSA) are drawn through a nitrocellulose membrane via a vacuum, through narrow slots above the membrane. While BSA remains attached to the nitrocellulose, the solvent is removed by the vacuum. This allows the protein bound to the nitrocellulose membrane to react with the antibody as per typical Western blotting procedures. This experiment involved pre-modification of BSA with acrolein for 25 minutes, followed by the addition of the hydrazines, to determine whether they diminished recognition of acrolein-adducted BSA by the acrolein/KLH antibody, perhaps by stripping or trapping the residual carbonyl group of Michael adducts.

3.2 METHODS

3.2.1 Chemicals

Preproenkephalin fragment 128 - 140 (PPE, m/z 1370) was purchased from ICN Biomedicals (Costa Mesa, CA, USA). The primary antibody for detection of acrolein adducted BSA was a polyclonal acrolein-KLH antiserum raised in rabbits as reported by Burcham *et al* (2003). The secondary antibody was a horseradish peroxidase conjugated goat anti-rabbit antibody (Pierce, Rockford, IL, USA). Detection of acrolein-BSA adducts was by a chemiluminescence luminol reagent (BioRad, CA, USA). Acrylamide/bisacrylamide solution (40%), ammonium persulfate (APS) and Kaleidoscope prestained standards (myosin, 202 kDa; β -galactosidase, 128 kDa; BSA, 80 kDa; carbonic anhydrase, 43.5 kDa; soybean trypsin inhibitor, 33.9 kDa; lysozyme, 19 kDa; aprotinin, 6.8 kDa) were also from BioRad. Bovine serum albumin (BSA), Kodak film, developer solution and fixer, N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS) and Tris base were all purchased from Sigma Chemicals (St Louis, MO, USA).

All other chemicals were obtained as previously described. All solvents used in ESI-mass spectrometry were HPLC grade.

3.2.2 Electrospray-Ionisation Mass Spectrometry

3.2.2.1 Mass Spectrometer Parameters

Samples were analysed by electrospray ionisation (ESI) mass spectrometry on a Finnigan LCQ mass spectrometer in positive ion monitoring mode (Finnigan, San Jose, CA, USA). The spray voltage was set at 4.8 kV with a capillary temperature of 200°C. The cylinder gas (nitrogen) was at 100 psi. Samples were injected at a rate of 8 µl per minute. Spectra were obtained in an m/z range of 400-2000.

3.2.2.2 Modification of PPE by Acrolein

Reactions of acrolein with PPE were performed in 600 µl volumes, in microcentrifuge tubes of the same volume to minimise head space acrolein loss. Acrolein (10 mM) was reacted with PPE (100 µM) in water at 37°C for up to 3 hours, with aliquots (25 µl) taken at 10, 30, 60, 90, 120 and 180 minutes. Aliquots were diluted in 50 µl of 50% acetonitrile/2% glacial acetic acid and 25 µl water (final concentration of PPE = 25 µM) and injected onto the mass spectrometer for analysis of acrolein adducted PPE. Experiments with the hydrazino compounds were run concurrently.

3.2.2.3 Reaction of Acrolein-PPE Adducts with Hydralazine, Dihydralazine, 1,1-Diphenylhydrazine, Benzylhydrazine and 1-hydrazinoisoquinoline

After reaction of acrolein with PPE for 3 hours, one of the hydrazines (hydralazine, dihydralazine, 1,1-diphenylhydrazine, benzylhydrazine or 1-hydrazinoisoquinoline; 100 µl, 100 mM) were added to 100 µl aliquots of the acrolein-PPE reaction mixture, giving final concentrations of 50 µM PPE, 5 mM acrolein and 50 mM hydrazine. Hydralazine and dihydralazine stock solutions were made in water, whereas the remaining hydrazines were made in acetonitrile. Trapping reactions of 1,1-Diphenylhydrazine, benzylhydrazine and 1-hydrazinoisoquinoline were examined as they represent a selection of cytoprotective hydrazines with varying potencies (EC₅₀'s are 2, 40 and 6 respectively, see section 2.3.10). The reaction mixtures with acrolein-PPE and each of the hydrazines were then maintained at 37°C, with

aliquots (25 μ l) taken at specific intervals for up to 240 minutes (depending on the apparent rate of trapping reactions, ie, 10, 30, 60, 240 minutes) and diluted in 25 μ l of the above-mentioned acetonitrile/glacial acetic acid solution for injection onto the LCQ mass spectrometer.

MS/MS spectra of ions of interest were performed at collision energies between 25 – 40 mV.

3.2.3 Slot Blot Analysis of Acrolein-BSA Adducts

3.2.3.1 Protocol

The ability of various hydrazines to block immunorecognition of acrolein-BSA adducts by the primary antibody raised against acrolein-KLH in rabbits was investigated using slot blot analysis. This was performed by a modified method to that reported in Burcham *et al* (2003). A BSA solution (5 ml, 2 mg/ml) was made in phosphate buffer (50 mM, pH 7) and separated into two portions; one for modification by acrolein (4.5 ml) and the other for unmodified BSA controls (0.5 ml). The BSA solutions were warmed to 37 °C prior to the addition of 37 μ l of 120 mM acrolein to the 4.5 ml aliquot of BSA (final acrolein concentration of 1 mM). Acrolein modification of BSA was allowed to proceed for 25 minutes at 37 °C before 100 μ l aliquots of unmodified BSA or acrolein modified BSA was added to prewarmed 96 well microplates containing 100 μ l solutions of the hydrazines previously mentioned in chapter 2 (0, 20, 200, 500 or 2000 μ M initial concentrations in phosphate buffer). Stock solutions of the hydrazines (50 mM) were initially made in DMSO as required. The reactions were then left to proceed for a further 1 hour at 37 °C.

Following this, the plates were cooled on ice prior to transferring the contents of each well (100 μ l) to separate slots of the slot blot manifold containing a 7 x 9 cm piece of 0.2 μ m nitrocellulose membrane (Biorad). The membrane was soaked in 20% methanol/PBS for at least 20 minutes prior to loading onto the slot-blot manifold (Biorad). Prior to the addition of BSA samples, the wells of the slot blot were rinsed with 1 ml water, which was drawn through the membrane via a vacuum pump. Following sample application, the solutions were drawn through the membrane via a vacuum. Each well was then rinsed with 1ml phosphate buffer.

To assess the possibility of the compounds physically interfering with the binding of the acrolein-BSA adducts on the nitrocellulose membrane, solutions of acrolein modified BSA (100

µl) were drawn through the membrane followed by 100 µl of the hydrazines to be tested. Any hydrazines that were shown by this method to block antibody recognition of the acrolein-BSA adducts were eliminated from statistical analysis due to interference in the assay.

Following its removal from the manifold, the nitrocellulose membrane was then blocked in 5% skim milk powder/PBS for at least 1 hour. During this time the primary antibody (anti acrolein-KLH serum, 1/1000) was preincubated with 5% skim milk powder/3% BSA/PBS for 20 minutes. Following blocking in milk, the membrane was incubated with the primary antibody for 1 hour prior to rinsing briefly in 3 water washes and in 3 x PBS washes (5 minutes each) followed by 1 x TBS (Tris buffered saline, 50 mM, pH 7, 10 minute wash). The membrane was then incubated with the secondary antibody (Pierce, goat anti-rabbit conjugated to horseradish peroxidase, 1/5000 in 5% skim milk powder/TBS) for 30 minutes. The membrane was washed again (3 x water and 5 x TBS, 5 minutes each) and developed in 1:1 luminol/enhancer (Pierce, SuperSignal West Pico, 3 ml each) for 5 minutes. The membrane was then placed in a plastic sheet, excess solution removed, and then exposed to photographic film (Kodak Biomax Light) for 5 to 15 seconds. The film was developed by immersing in developer solution (3 minutes) washing in water (1 minute) and immersing in fixer solution (2 minutes).

3.2.3.2 Analysis of Slot Blot Data and EC₅₀ Determination

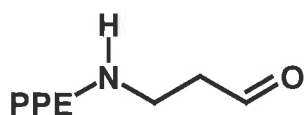
The intensity of the spots on the film was analysed by densitometry via a Kodak imaging program (Kodak Digital Science 1D v 2.0.3, Eastman Kodak Co., New Haven). Replicates from 3 analyses were analysed for EC₅₀'s via a sigmoidal curve fit on GraphPad Prism (version 3). A 1 tailed Pearson's correlation analysis was performed on the slot blot EC₅₀'s and cytoprotective EC₅₀'s of the hydrazines.

3.3 RESULTS

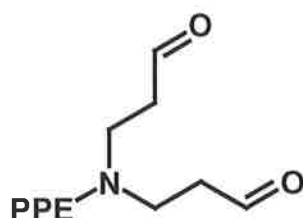
3.3.1 Formation of Acrolein Adducts of PPE

PPE is a 13 amino acid peptide containing a single lysine group which was predicted to be the main residue capable of reacting with acrolein (Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met). The optimum concentration of PPE for ESI mass spectrometry was determined to be 25 µM, giving an ion current of approximately 1×10^7 . The spectra of unmodified PPE is

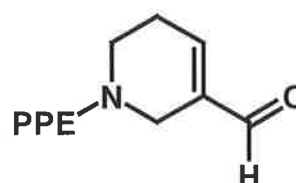
shown in Figure 3.2, with the dominant ion (m/z 1370) corresponding to the MH^+ of the unmodified peptide. However, rather than observing three distinct adducts on lysine, a large number of products were detected due to reactions of acrolein with the N-terminal glycine residue. The addition of a 100 fold excess of acrolein to PPE resulted in the generation of one predominant ion after 30 minutes at m/z 1426 (Figure 3.3). This corresponded to the MH^+ of mono-Michael adducted acrolein-PPE (**1**). Additional ions of lesser relative abundance were also observed at m/z 1464 (**3**) and 1482 (**2**). The ion at m/z 1482 corresponded to the MH^+ of bis-Michael adducted acrolein-PPE. The ion at m/z 1464 corresponded to the MH^+ of FDP-lysine-PPE (**3**). A small signal corresponding to single Schiff adducted acrolein-PPE (**4**, m/z 1408) was also present in reactions of PPE with acrolein, although a signal at m/z 1408 was also present in small amounts in unmodified PPE alone, indicating that this ion may represent a contaminant in commercial PPE (Figure 3.2-3.3).



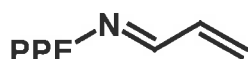
1. mono-acrolein-PPE
(m/z 1426)



2. bis-acrolein-PPE
(m/z 1482)



3. FDP-lysine-PPE
(m/z 1464)



4. Schiff-acrolein-PPE
(m/z 1408)

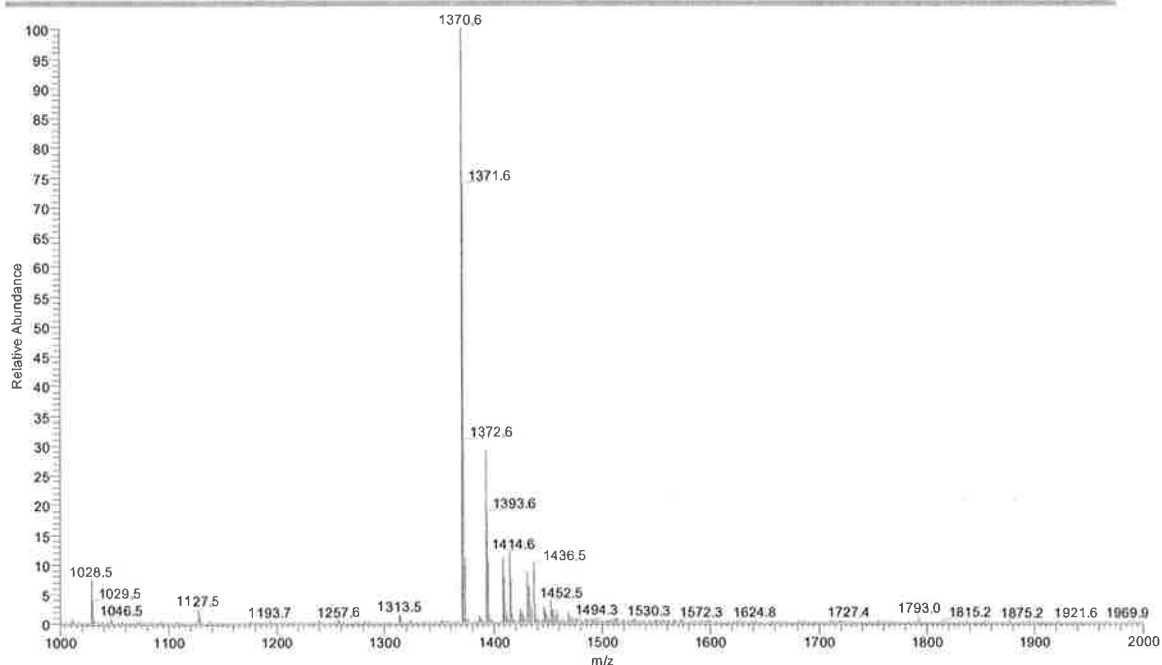


Figure 3.2 Mass spectra of unmodified PPE (25 μ M analysed in 25% acetonitrile/1% glacial acetic acid)

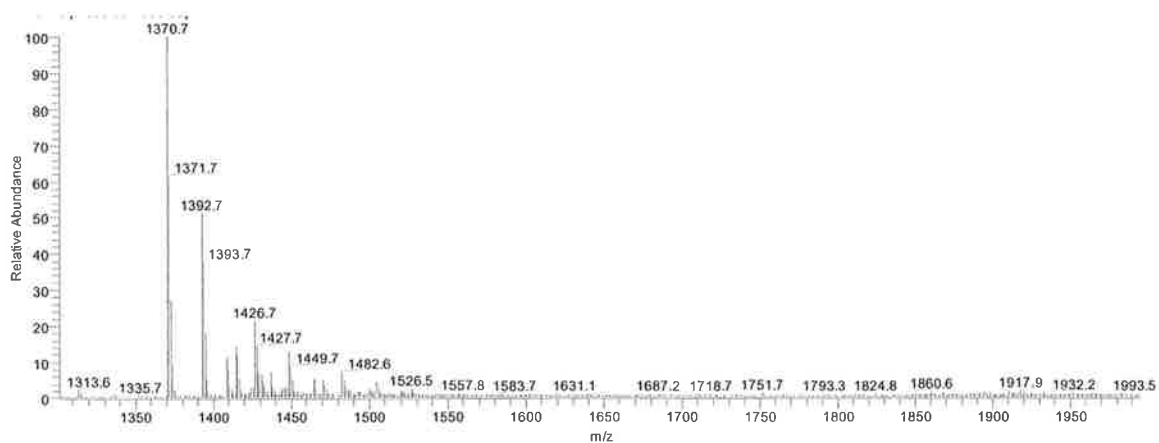


Figure 3.3 Mass spectrum of PPE (25 μ M) modified with a 100 fold excess of acrolein for 30 minutes at 37°C.

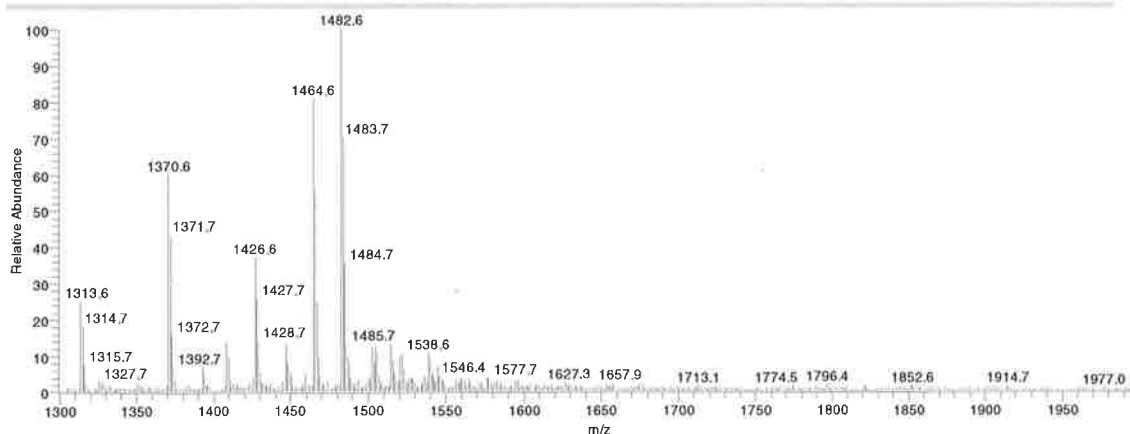
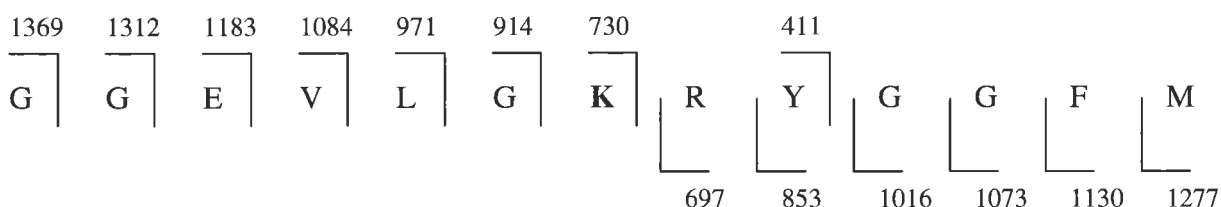
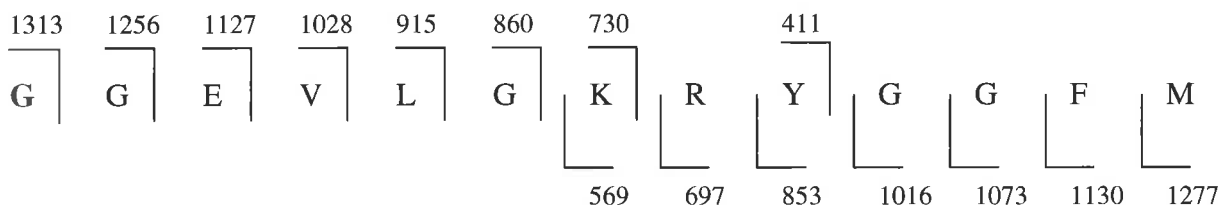


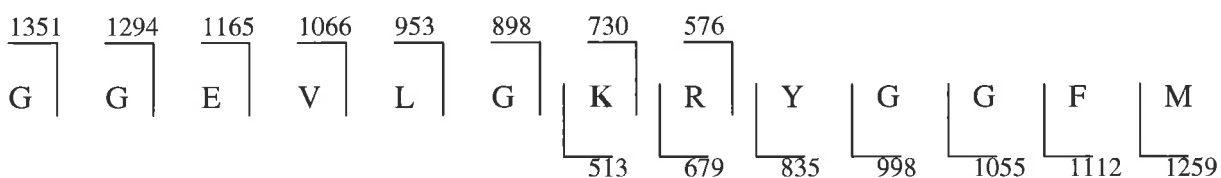
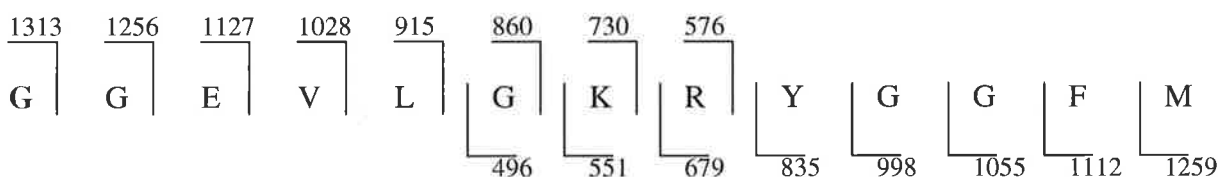
Figure 3.4 Mass spectrum of PPE (25 μ M) modified with a 100 fold excess of acrolein for 3 hours at 37°C

The relative intensities of these ions progressively increased as the modifications proceeded over the following 2.5 hours, where the spectra at 1, 1.5, 2 and 2.5 hours are shown in Appendix 2; A2.1. By 3 hours the major ion is the MH^+ of **(2)** (100 %) with the MH^+ of **(1)** remaining at an abundance of 40% (figure 3.4). The MH^+ of **(3)** is at a relative abundance of 80 % after this time point. At 180 minutes there are also additional ions present at m/z 1538 and 1520. These likely correspond to the MH^+ of tris-acrolein-PPE and tris-acrolein-PPE with the loss of water (m/z 18). The intensities of these ions were not strong enough to characterise by MS/MS, however, but are likely composed of combinations of lysine and terminal glycine acrolein adducts.

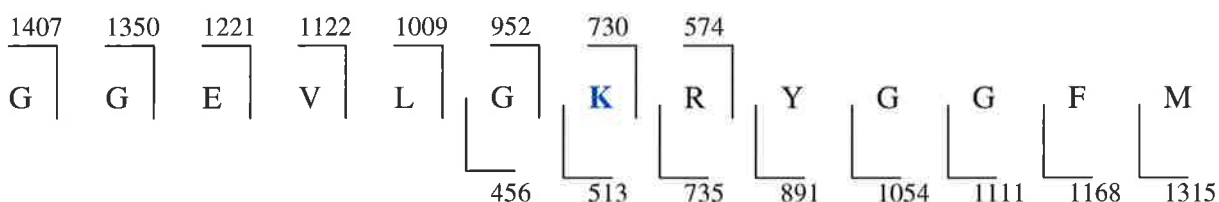
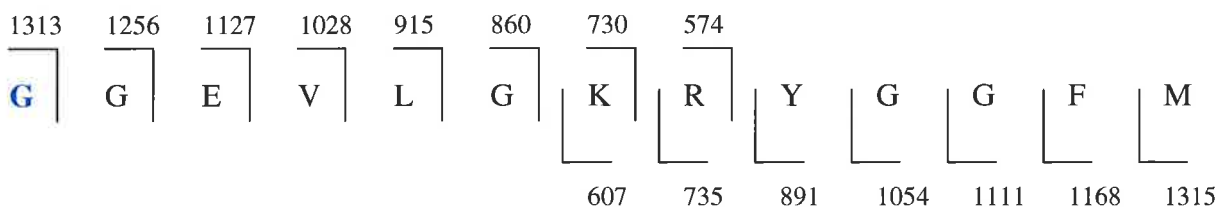
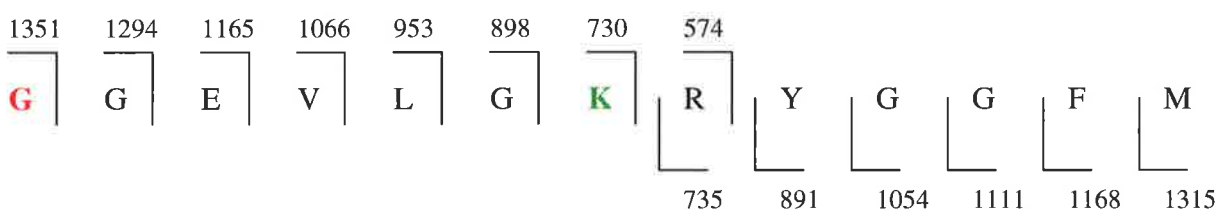
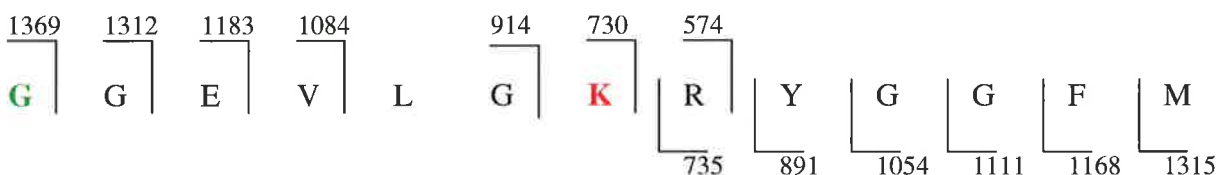
The chemistry underlying the formation of ions corresponding to **(1)**, **(2)** and **(3)** was found to be very diverse. This is in keeping with expectations given the use of a large excess of acrolein relative to PPE in these experiments. In addition to expected adducts on lysine, adducts on the terminal glycine were revealed during MS/MS analyses. The identity and fragmentations of these ions are shown below. The mass of each fragment generated following loss of the residue indicated is shown above the brackets (Y+2 cleavages) or below the brackets (B cleavage). Only the structures of lysine adducts are shown here. As indicated previously in the Introduction to this Chapter, the location of adducted residues were determined by the observed loss of additional mass units from that residue, corresponding to the mass of the adduct. Furthermore, the type of acrolein adducts on lysine and glycine residues in the following MS/MS fragmentations are indicated by colours, where residues containing acyclic Michael-acrolein adducts are highlighted in **red**, Schiff adducts are highlighted in **green** and FDP-adducts are highlighted in **blue**. Residues containing two Michael-acrolein adducts are **underlined**.

m/z* 1426. Mono-acrolein (K)**m/z* 1426. Mono-acrolein (G)**

The fragmentation of the ion at ***m/z* 1426**, which corresponded to Michael type mono-acrolein adducted PPE, shows evidence of two species of adducted peptides contributing to the ion. The top fragmentation shows evidence of a loss of lysine plus an additional 56 mass units, corresponding to the mass of acrolein, in the Y+2 fragmentations. This means that rather than losing 128 mass units from the peptide fragment, corresponding to the mass of lysine, 184 mass units were lost, indicating that lysine had been adducted, resulting in an increase of its mass by 56 units (ie. the ion at *m/z* 860 loses 184 mass units to form the ion at *m/z* 730). The B cleavages show the loss of unmodified residues between methionine and arginine. The lower fragmentation shows evidence of a loss of N-terminal glycine plus an additional 56 mass units in the Y+2 cleavage (loss of 113 mass units from glycine instead of only 57), while the lysine residue loses no additional mass in either the B or Y+2 cleavages. This proved the presence of two populations of mono-adducted PPE, comprising respectively mono-acrolein adducts at either lysine or the N-terminal glycine. No loss of 56 mass units was evident from the arginine residue, indicating the absence of adducts at this residue.

m/z* 1408. Schiff-acrolein (K)**m/z* 1408. Schiff-acrolein (G)**

Given the presence of a small signal at ***m/z* 1408**, the identity of this ion was investigated as it corresponded to the mass of mono-acrolein adducted PPE via Schiff base formation. The upper fragmentation shows evidence of a loss of lysine plus 38 mass units, whereas cleavage of the other residues resulted in the loss of only their individual masses. This cleavage was evident in both the B and Y+2 fragments. The lower fragmentation shows evidence of a loss of an additional 38 mass units only from the terminal glycine. This provided evidence that Schiff base adducts of acrolein were formed on lysine and the N-terminal glycine.

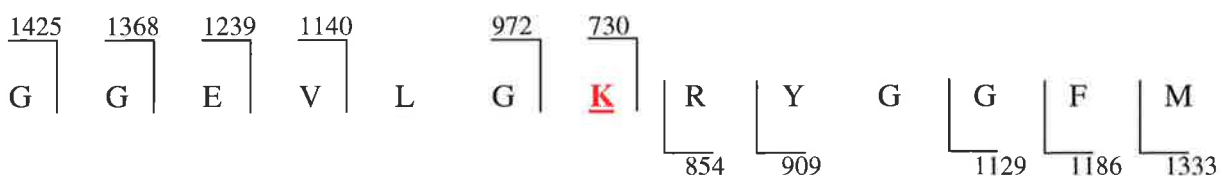
m/z* 1464. FDP-K-PPE**m/z* 1464. FDP-G-PPE*****m/z* 1464. Schiff acrolein-K/Michael acrolein-G*****m/z* 1464. Schiff acrolein-G/Michael acrolein-K**

The ion at ***m/z* 1464** was found to be a complex mixture of 4 species. We initially expected this ion to correspond to FDP-lysine-PPE and FDP-glycine-PPE, formed via the addition of two acrolein molecules to the same residue by Michael type addition, followed by aldol condensation. Evidence of these products is shown by the upper two fragmentation panels, where either the lysine residue or the N-terminal glycine residue lose an additional 94 mass units, corresponding to bis-acrolein adducts minus water (FDP-). This was evident in both the B and Y+2 cleavages. This finding represents the first evidence for FDP-formation on an N-terminal glycine and suggests that other N-terminal residues may also be targets for acrolein, likely to form these cyclised immunogenic species. However, the ion at *m/z* 1464 would also

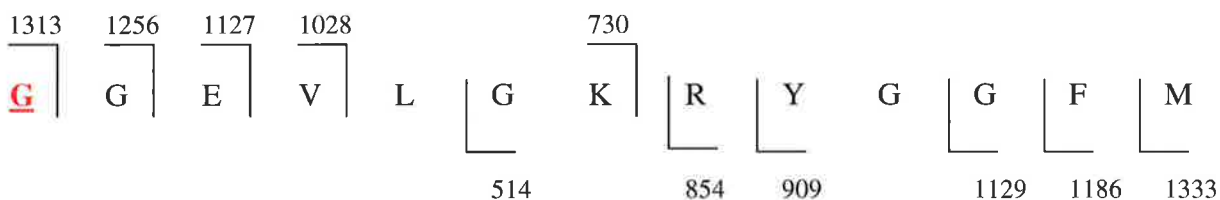
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appear if one Michael addition of acrolein plus one Schiff base addition of acrolein occurred on different residues of the same PPE molecule. Evidence of these adducts was then sought and proof of their identification is shown by the lower two fragmentation panels. Evidence was found for Michael-acrolein adducted lysine (losing an additional 56 mass units) plus Schiff-acrolein adducted glycine (losing an additional 38 mass units). The reverse was also found, where a Schiff-acrolein adduct was present on lysine, while a Michael-acrolein adduct was present on glycine. These fragmentations were apparent in the Y+2 cleavages, while the B cleavages showed the loss of unmodified residues between methionine and arginine. This ion (m/z 1464) was therefore found to be composed of a combination of cyclic FDP-adducts on lysine and the N-terminal glycine as well as single Michael plus single Schiff acrolein adducts on both residues.

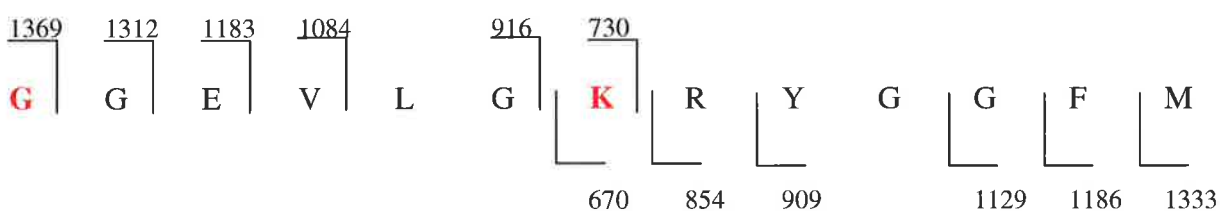
m/z 1482. Bis-acrolein-PPE ($2 \times K$)



m/z 1482. Bis-acrolein-PPE ($2 \times G$)



m/z 1482. Bis-acrolein-PPE ($1 \times K, 1 \times G$)



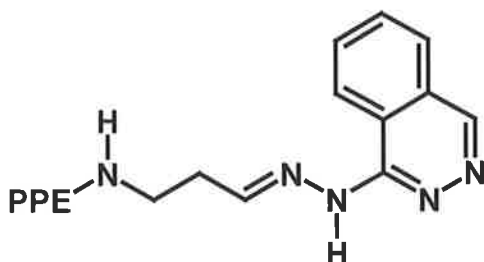
The ion at m/z 1482 was found to be composed of double Michael acrolein adducts on the same lysine and N-terminal glycine residues, as well as a species containing a single Michael-acrolein adduct on both residues. The upper fragmentation panel shows evidence of a loss of lysine plus an additional 112 mass units, with no other residues losing additional mass. This was evident in the Y+2 cleavage, whereas B cleavages showed the loss of unmodified residues between

methionine and arginine. The middle panel shows evidence of a loss of an additional 112 mass units from the N-terminal glycine in both the B and Y+2 cleavages. These losses corresponded to the loss of two Michael adducted acrolein molecules from these residues. In addition, the lower fragmentation panel shows that an additional 56 mass units were lost from both lysine and the N-terminal glycine. Again, this is evident for both B and Y+2 cleavages. Once again, no evidence was found for adduction at the arginine residue.

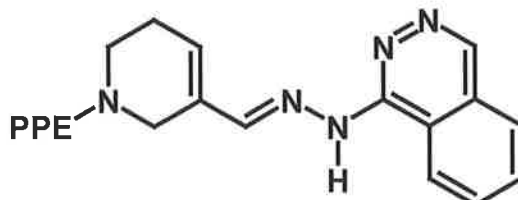
3.3.2 Trapping of Acrolein-PPE Adducts by Hydralazine and Dihydralazine

As hydralazine (HYD) and dihydralazine (DH) are capable of blocking acrolein toxicity during the early stages of adduction, their ability to react with the individual acrolein-PPE adducts was assessed. Following the addition of hydralazine almost no signals were observed at the masses corresponding to acrolein adducts within 10 minutes (ie. only small relative abundances of ions at m/z 1426 and 1464 remained). It was expected that if hydralazine was “stripping” acrolein from PPE, the relative intensity of the ion corresponding to unmodified PPE (m/z 1370) would increase following addition of the hydrazine, while the relative intensity of acrolein adducts would decrease. Instead, a very complex mixture of reaction products was observed with additional ions at m/z 1568, 1606, 1624, 1678 and 1822 (Figure 3.5) within 10 minutes of the addition of a 1000 fold excess of hydralazine to PPE that had been pretreated with acrolein for 3 hours. These ions corresponded to the MH^+ of mono-hydralazine-mono-acrolein-PPE (**5**), mono-hydralazine-FDP-lysine (**6**), mono-hydralazine-bis-acrolein-PPE (**7**), mono-hydralazine-tris-acrolein-PPE and bis-hydralazine-tris-acrolein-PPE. MS/MS spectra of the tris-acrolein-PPE adducts were not obtained as their relative abundances were not high enough. This was expected, as the corresponding tris-acrolein adducts were also shown in the previous section to be relatively minor ions. Therefore, the detection of these hydralazine-trapped adducts may indicate that hydralazine increases the ionisation and detection of these minor adducted species in the mass spectrometer. There is also a small but significant signal at m/z 1767 corresponding to the MH^+ of bis-hydralazine-bis-acrolein-PPE (**8**). The spectra of hydralazine trapped acrolein-PPE adducts is similar at later time points (shown in Appendix 2; A2.2) indicating that they represent stable species that are unlikely to result in “stripping” of acrolein from the PPE molecule. The MS/MS fragmentations of these ions are shown below and are comprised of hydralazine-trapped acrolein adducts at both lysine and the N-terminal glycine, as expected based on the above fragmentation data for acrolein-PPE adducts. B cleavages of the individual residues are again shown in the lower brackets, whereas Y+2 cleavages are shown in upper brackets. The location of hydralazine- or dihydralazine-trapped residues are indicated by a *,

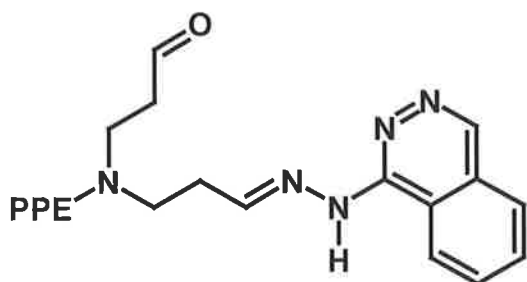
where bis-hydralazine species are indicated by **. In addition, examination of the mass spectra in the m/z 200 region shows a clear ion at m/z 199, corresponding to the reaction product of hydralazine and free acrolein (*E*- and *Z*-APH, data not shown).



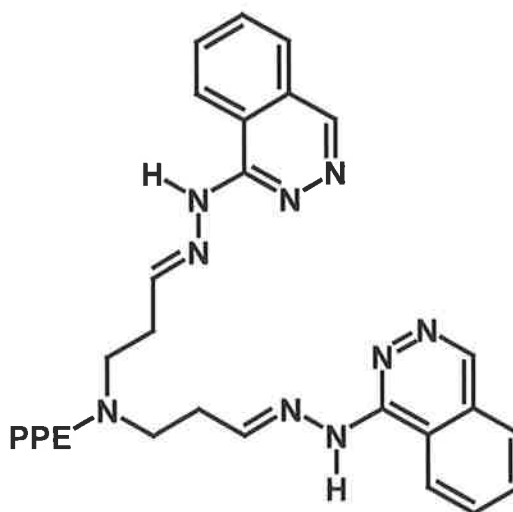
5. mono-HYD-mono-acrolein-PPE
(m/z 1568)



6. mono-HYD-FDP lysine-PPE
(m/z 1606)



7. mono-HYD-bis-acrolein-PPE
(m/z 1624)



8. bis-HYD-bis-acrolein-PPE
(m/z 1766)

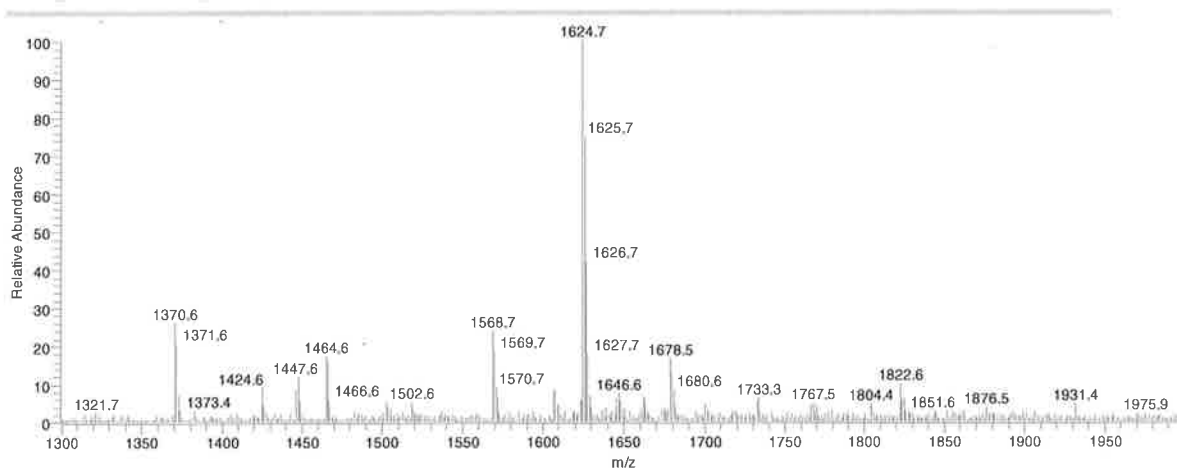
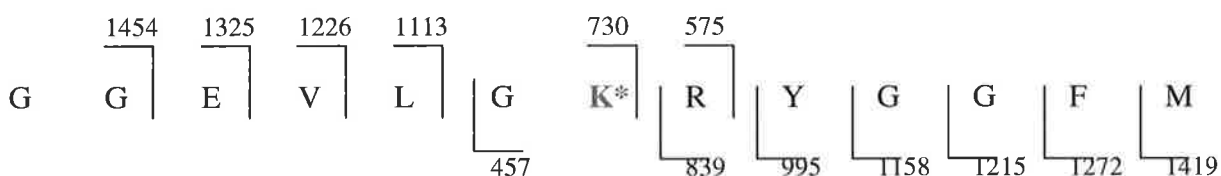
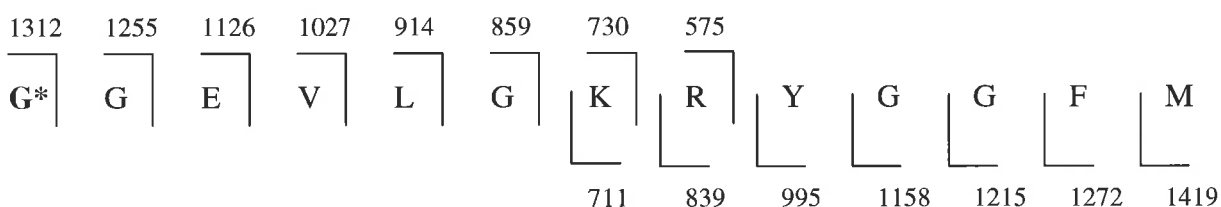


Figure 3.5 Mass spectrum of hydralazine trapped adducts of acrolein-PPE 10 minutes after addition of a 10 fold excess of hydralazine (cf. acrolein) to the acrolein/PPE reaction mixture (37°C). 25 μ M PPE injected onto the LCQ.

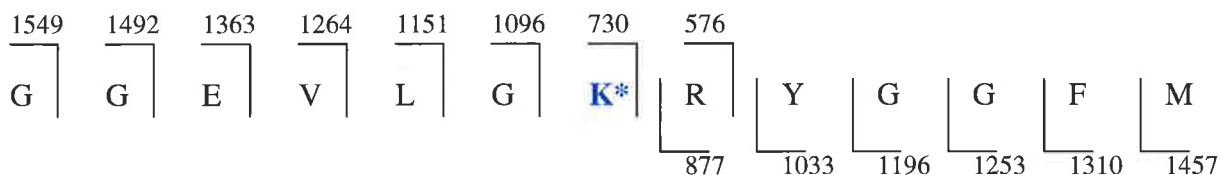
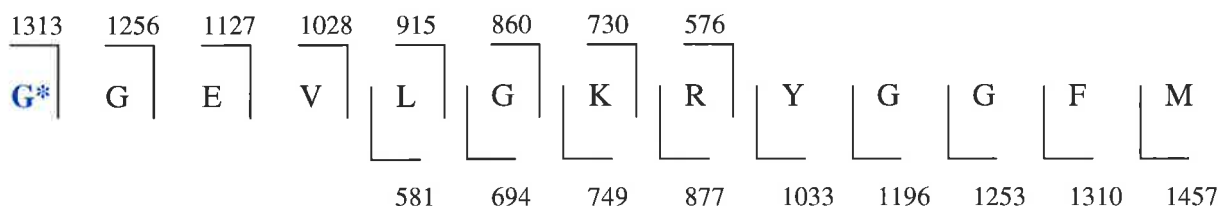
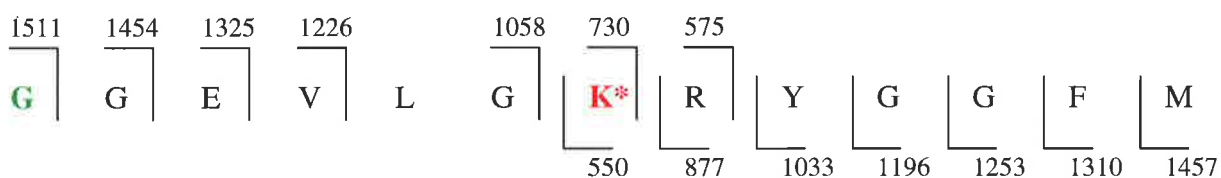
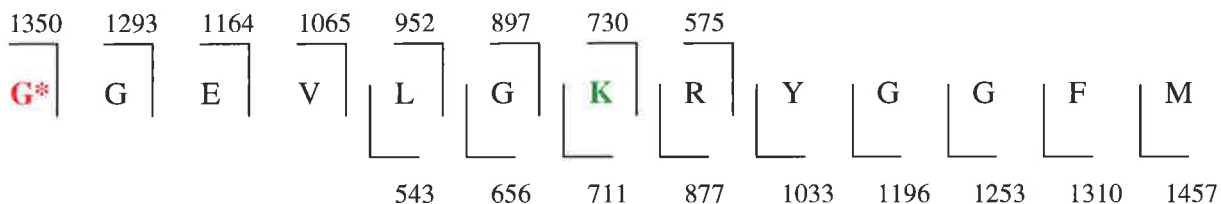
***m/z* 1568. mono HYD-mono acrolein (K)**



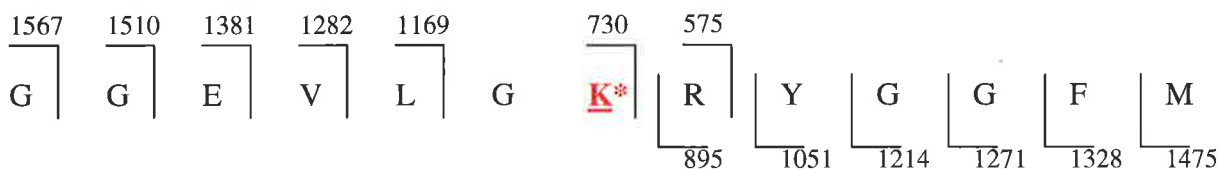
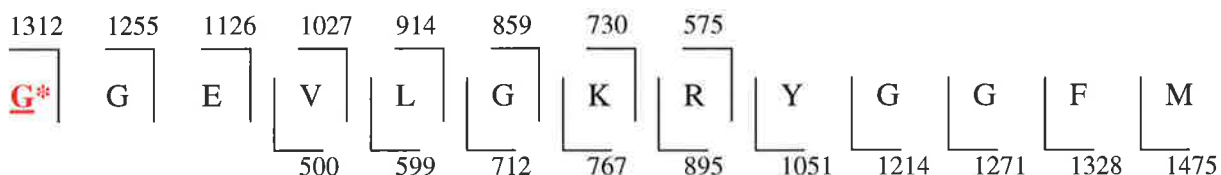
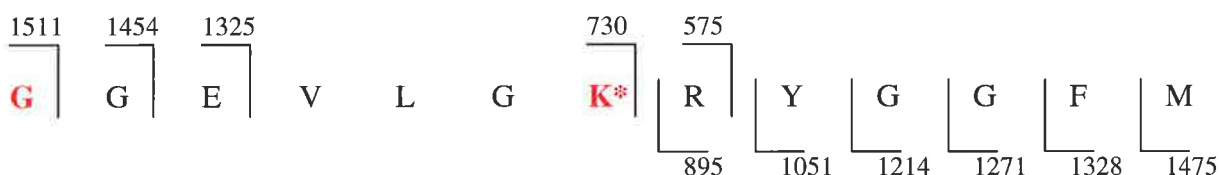
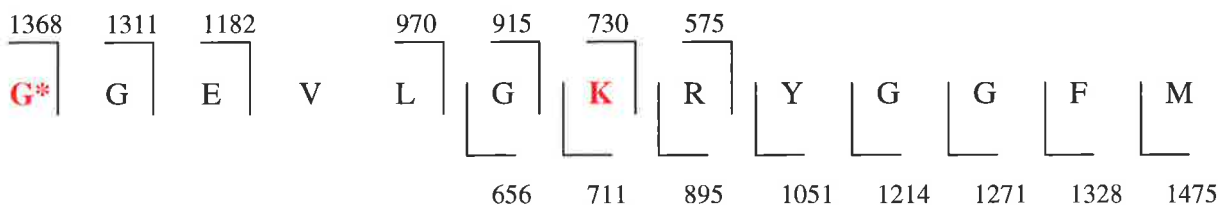
***m/z* 1568. mono HYD-mono acrolein (G)**



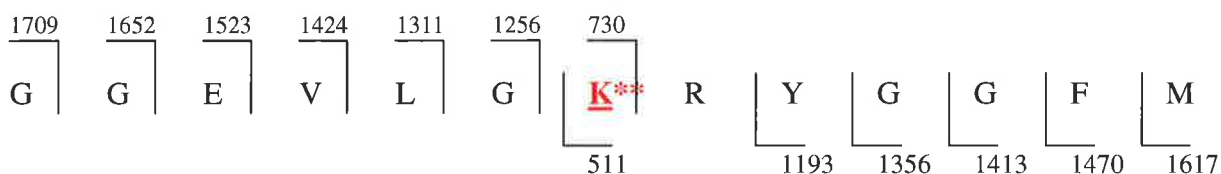
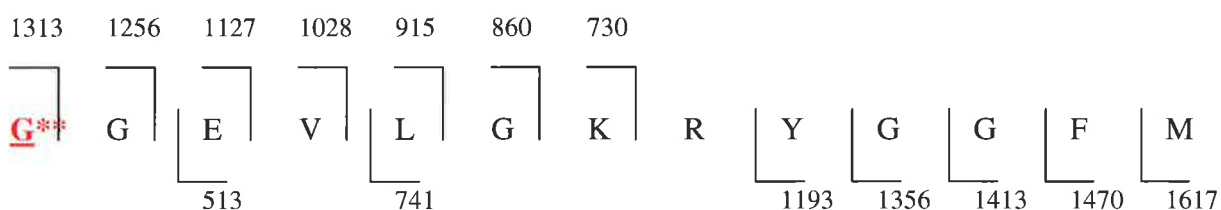
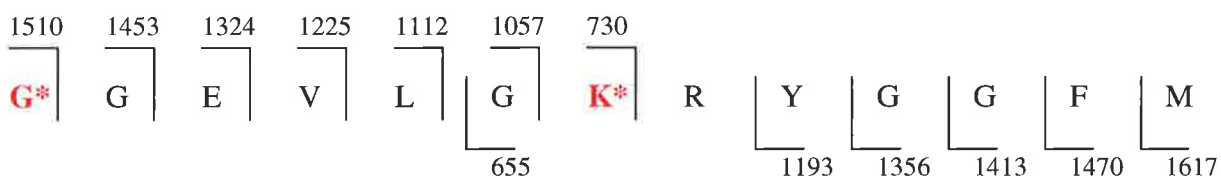
As shown above for acrolein-PPE adducts, the loss of mass corresponding to hydralazine-trapped acrolein-PPE adducts was monitored on lysine and N-terminal glycine residues of the peptide. Peptide modifications were assessed by the same process described above for acrolein modifications. Fragmentations confirming the presence of Hydralazine-trapped mono-acrolein-lysine and glycine adducts were detected for the ion at ***m/z* 1568**. This was shown by the loss of an additional 198 mass units from the lysine (upper fragmentation) or glycine (lower fragmentation) residues, corresponding to the hydrazone product of reactions between hydralazine and Michael adducted acrolein.

m/z 1606. mono HYD-FDP (K)***m/z 1606. mono HYD-FDP (G)******m/z 1606. mono HYD (K)-Schiff G/Michael K******m/z 1606. mono HYD (G)-Schiff K/Michael G***

The ion at *m/z 1606* was found to be composed of 4 distinct species, including hydralazine-trapped FDP-lysine and FDP-glycine (via the additional loss of 236 mass units from either residue) as well as hydralazine-trapped mono-Michael-acrolein adducts on PPE composed of one Michael adducted acrolein plus one Schiff adducted acrolein as observed above. These latter structures were identified by the loss of an additional 198 mass units from either lysine or glycine (corresponding to the hydrazone products of trapping reactions at Michael-acrolein adducted residues) plus loss of an additional 38 mass units from the opposing residue (corresponding to the Schiff adduct).

m/z 1624. mono HYD-bis acrolein (2 × K)***m/z 1624. mono HYD-bis acrolein (2 × G)******m/z 1624. mono HYD (K)-bis acrolein (1 × G/1 × K)******m/z 1624. mono HYD (G)-bis acrolein (1 × G/1 × K)***

The ion at ***m/z 1624*** corresponded to a mixture of peptide species containing two Michael adducts of acrolein plus one hydralazine-trapped adduct at either of the two adducted residues (ie. four species in total). The upper two fragmentations show additional losses of 254 mass units from either lysine or glycine, corresponding to doubly acrolein-adducted residues containing one hydralazine-trapped adduct. The lower two fragmentations show losses of 198 mass units from one residue, plus loss of another 56 mass units from the other residue, indicating single Michael-acrolein adduction on both residues with trapping at one residue.

m/z* 1766. bis HYD-bis acrolein (2 × K)**m/z* 1766. bis HYD-bis acrolein (2 × G)*****m/z* 1766. bis HYD-bis acrolein (1 × G/1 × K)**

Similarly, the ion at *m/z* 1766 corresponded to two Michael adducted acrolein molecules with hydralazine-trapping occurring at each acrolein adduct. Three species were detected based on whether acrolein bis-adduction in a given peptide occurred at lysine only, glycine only or lysine and glycine. Thus, fragmentations corresponding to bis-hydralazine-bis-acrolein adducts on lysine or glycine were observed (shown by the loss of an additional 396 mass units from either residue) or single hydralazine-trapped acrolein adducts on both residues (shown by the loss of an additional 198 mass units from both glycine and lysine).

A similar profile was evident with dihydralazine trapped acrolein-PPE adducts (Figure 3.6). No acrolein-PPE adducts remained after a 10 minute reaction of the above acrolein-PPE mixture with dihydralazine, unlike with hydralazine where some signals remained. Ions are present at *m/z* 1598, 1636, 1654 and 1827. These ions correspond to the MH^+ of mono-dihydralazine-mono-acrolein (**9**), mono-dihydralazine-FDP-PPE (**10**), mono-dihydralazine-bis-acrolein-PPE (**11**) and bis-dihydralazine-bis-acrolein-PPE (**12**). Spectra from dihydralazine incubations with acrolein modified PPE at longer times (up to 1 hour) are not shown as these were similar to the spectra at 10 minutes. This indicated that as with hydralazine-trapped adducts, dihydralazine-trapped adducts are also stable species. These adducts correspond to acrolein adducts on both lysine and the terminal glycine as shown in the MS/MS fragmentations below.

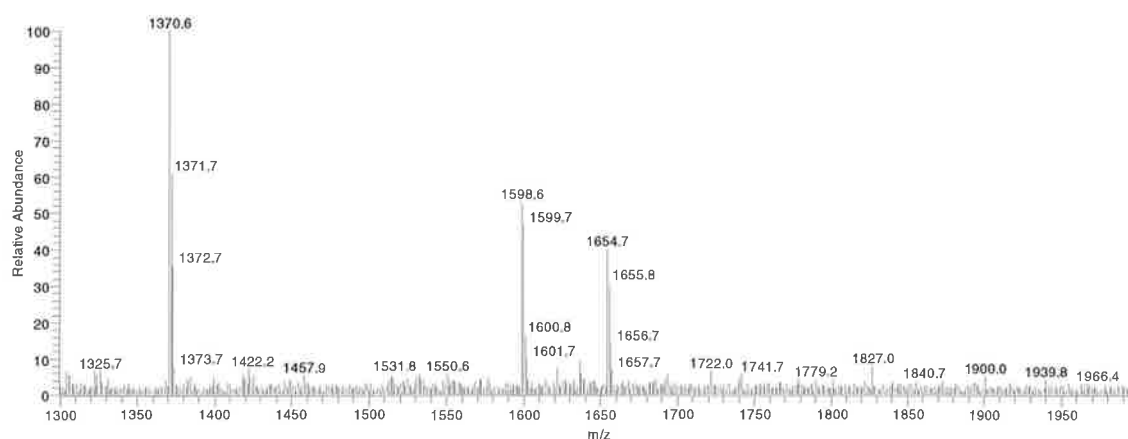
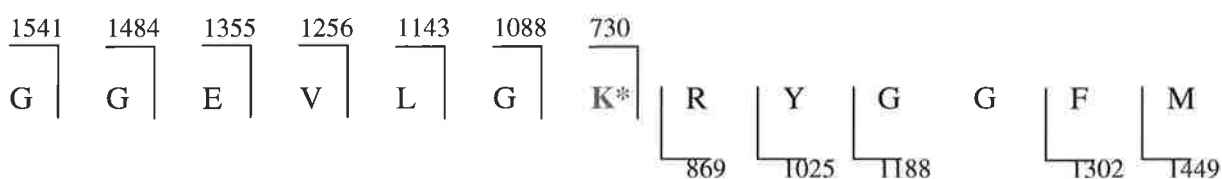
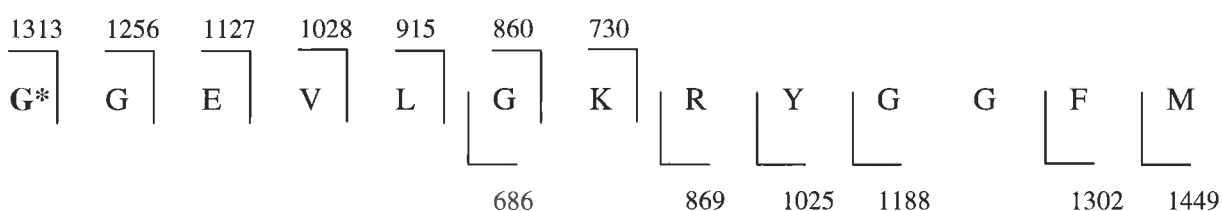


Figure 3.6 Mass spectrum of dihydralazine modified acrolein-PPE adducts following a 10 minute incubation of the acrolein/PPE reaction mixture (37°C) with a 10 fold excess of dihydralazine (cf. acrolein). 25 μ M PPE injected onto the LCQ.

***m/z* 1598. mono DH-mono acrolein (K)**



***m/z* 1598. mono DH-mono acrolein (G)**

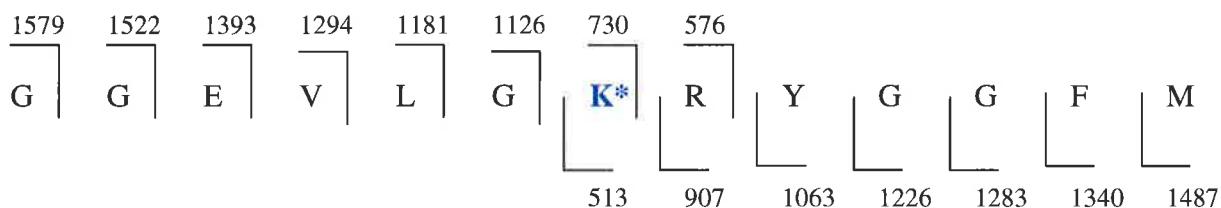


The ion at *m/z* 1598 was found to be composed of 2 products: mono dihydralazine-trapped acrolein-PPE at lysine and mono dihydralazine-trapped acrolein-PPE at the N-terminal glycine residue. These structures were determined as shown previously for acrolein-PPE adducts and hydralazine-trapped acrolein-PPE adducts. Adducts on these two residues were identified by the loss of an additional 228 mass units from the lysine (upper fragmentation) or the glycine (lower fragmentation) residues. This mass corresponded to the anticipated mass of the hydrazone formed from reaction of dihydralazine with a Michael-adducted acrolein molecule. As expected

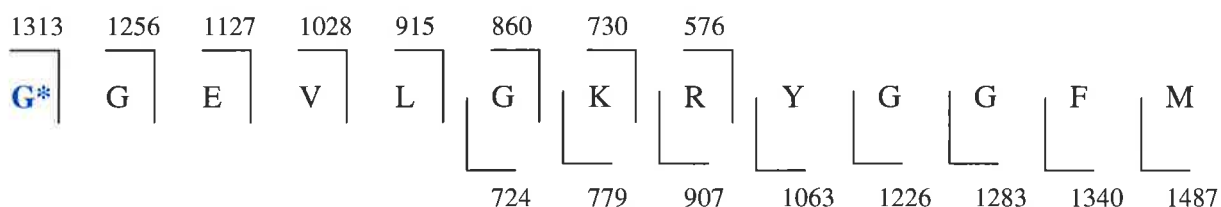
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based on the identity of acrolein adducts of PPE (shown above), no adduction was evident on any of the other residues as indicated by the absence of cleavages larger than the masses of the residues.

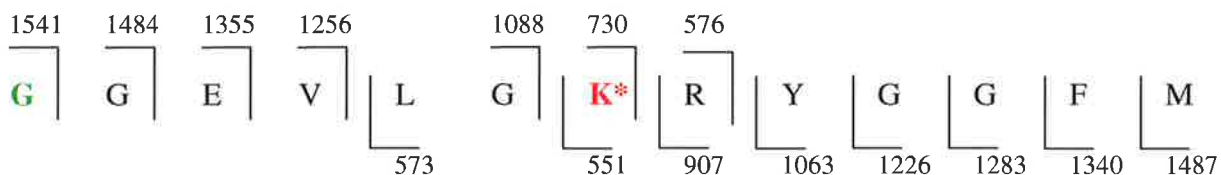
***m/z* 1636. mono DH-FDP (K)**



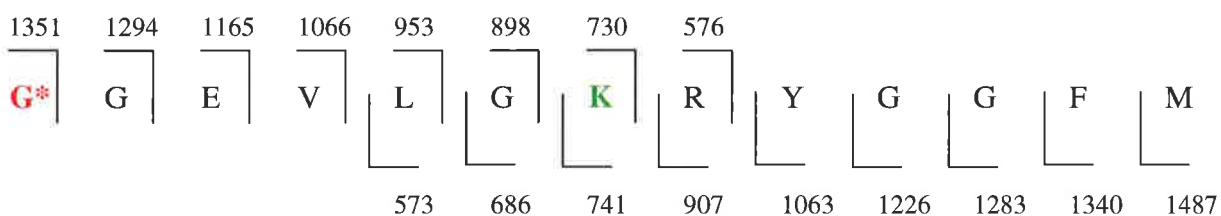
***m/z* 1636. mono DH-FDP (G)**



***m/z* 1636. mono DH (K)-Michael (K)/Schiff (G)**



***m/z* 1636. mono DH (G)-Michael (G)/Schiff (K)**

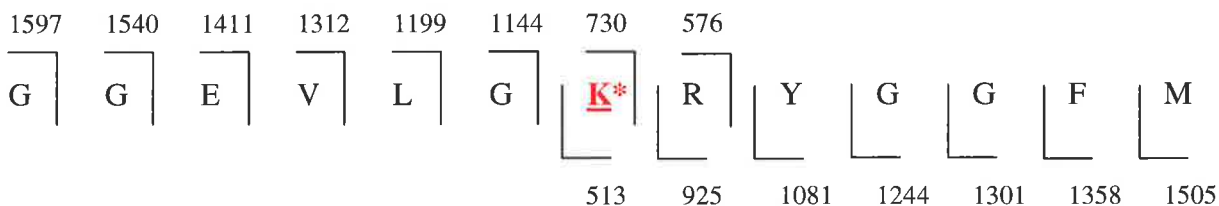


The ion at *m/z* 1636 was comprised of dihydalazine-trapped FDP-lysine and FDP-glycine adducts, shown by the loss of an additional 266 mass units (corresponding to the mass of dihydalazine-trapped FDP-adducts) from either lysine or glycine residues respectively. The ion was also composed of dihydalazine-trapped mono Michael-acrolein adducts at either residue (shown by the loss of an additional 228 mass units, corresponding to the mass of a dihydalazine-

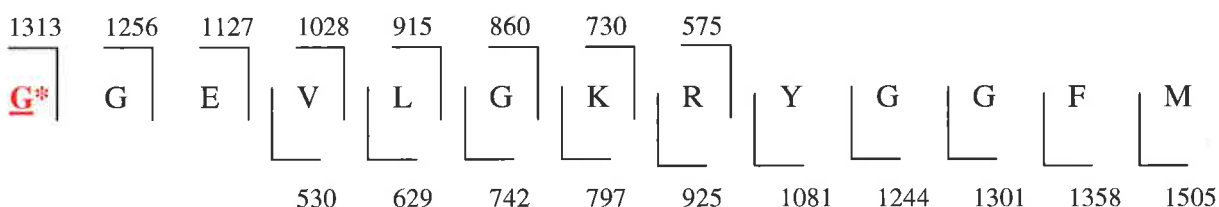
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trapped acrolein adduct) plus a Schiff acrolein adduct at the other residue (shown by the loss of an additional 38 mass units). These structures were consistent with the trapped adducts formed by hydralazine (shown above).

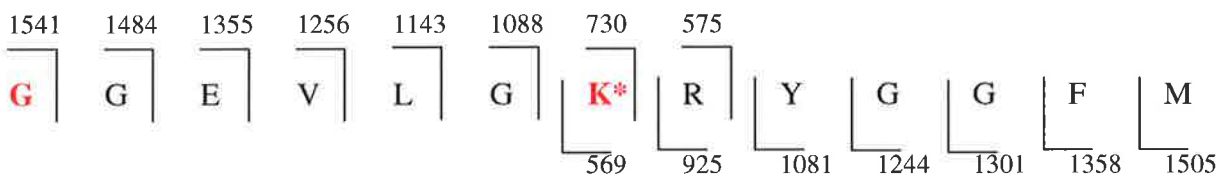
***m/z* 1654. mono DH-bis acrolein (K)**



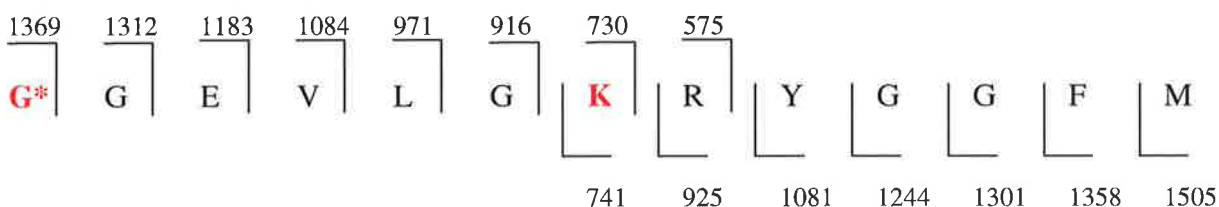
***m/z* 1654. mono DH-bis acrolein (G)**



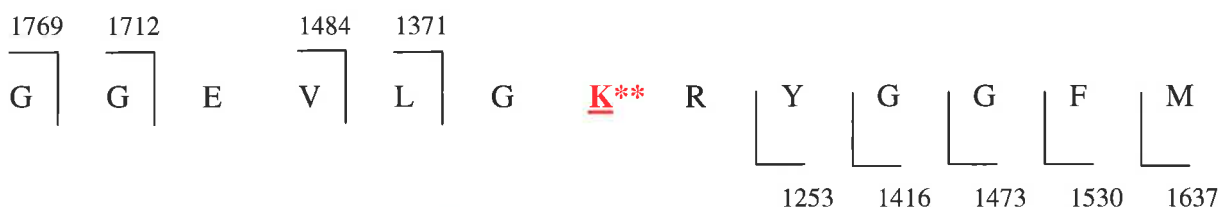
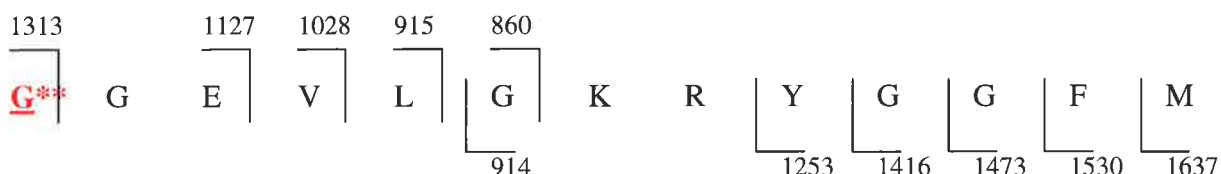
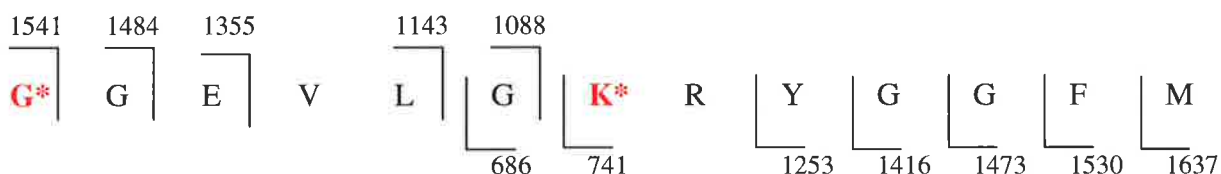
***m/z* 1654. mono DH (K)-bis acrolein (1 × K/1 × G)**



***m/z* 1654. mono DH (G)-bis acrolein (1 × K/1 × G)**



The ion at ***m/z* 1654** corresponded to mono dihydralazine-trapped bis-Michael-acrolein adducts at both lysine and glycine residues. These trapped structures were comprised of double acrolein-adduction at either lysine or glycine (shown by the loss of an additional 284 mass units from either residue) or single acrolein-adducts at both residues with trapping by dihydralazine occurring at one of the residues (shown by the loss of 228 mass units from one residue plus 56 mass units from the other residue).

m/z* 1826. bis DH-bis acrolein (K)**m/z* 1826. bis DH-bis acrolein (G)*****m/z* 1826. bis DH-bis acrolein (1 × K/1 × G)**

B and Y+2 cleavages of the ion at *m/z* 1826 were not strong, given the relatively weak intensity of the signal. Therefore, Y+2 cleavages of lysine were not observed, although two of the apparent structures resulted in cleavages that showed identical fragments from both B and Y+2 cleavages. The identity of these structures were therefore elucidated by showing that each of the B and Y+2 cleavages corresponded to trapped adducts at lysine and/or the N-terminal glycine. Thus, the upper fragmentation panel shows that each of the residue cleavages loses only the mass of the unmodified residue. Given that neither arginine nor the non-terminal glycine residues are adducted by acrolein, the lysine residue was predicted to contain the additional 456 mass units, corresponding to bis hydralazine-trapped bis-acrolein-PPE. The middle fragmentation shows Y+2 cleavage loss of the additional 456 mass units from the N-terminal glycine, while other cleavages contained only the mass of the unmodified residue. This indicated the presence of bis dihydralazine-trapped bis-acrolein-PPE at the N-terminus. The lower fragmentation shows Y+2 cleavage loss of a single dihydralazine-trapped acrolein-glycine residue and B cleavage loss of the same adduct on lysine. The remaining cleavages corresponded with the presence of these adducts.

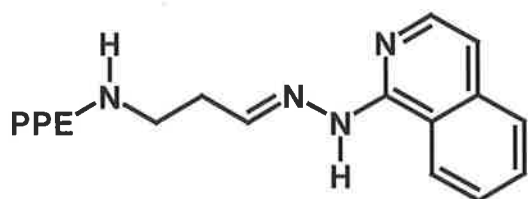
3.3.3 Identification of Trapped Acrolein-PPE Adducts by Hydralazines of Varying Cytoprotective Potencies.

The results shown above for hydralazine and dihydralazine suggest that trapping reactions at reactive acrolein-protein adducts represents a potential detoxification pathway for the adducts. To support these observations it was decided to examine the ability of various cytoprotective hydrazines to trap acrolein-PPE adducts by ESI mass spectrometry. The compounds chosen for examination were 1-hydrazinoisoquinoline (the most efficient cytoprotective analogue of hydralazine, 1 hour $EC_{50} = 6 \pm 0.14 \mu M$), 1,1-diphenylhydrazine (the most efficient cytoprotective hydrazino compound screened, 1 hour $EC_{50} = 2 \pm 0.02 \mu M$) and benzylhydrazine (a relatively poor cytoprotective hydrazine, 1 hour $EC_{50} = 37 \pm 1.75 \mu M$) (each of these EC_{50} values were as reported in Figure 2.17 of Chapter 2). Due to the inability to dissolve these hydrazines in water they were dissolved in acetonitrile, an acceptable practice since the injection solution for mass spectrometry contained this solvent. The trapping reactions were therefore performed in a 50% acetonitrile solution following a 3 hour incubation of PPE with acrolein to generate modified peptide.

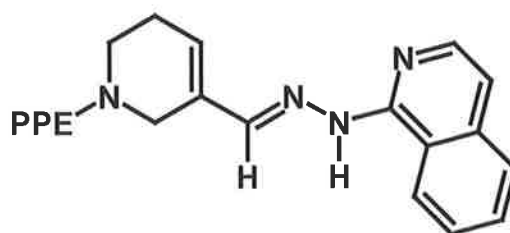
Within 10 minutes of incubation of acrolein modified PPE with 1-hydrazinoisoquinoline (HIQ) only small ion signals remained corresponding to acrolein-PPE (mono-acrolein-PPE, 6% relative abundance; FDP-PPE, 19% relative abundance; bis-acrolein-PPE, 6% relative abundance) (Figure 3.7a). However, a number of additional ions were present at m/z 1567, 1605, 1623 and 1764. These ions corresponded to the MH^+ of mono-hydrazinoisoquinoline-mono-acrolein-PPE (**13**), mono-hydrazinoisoquinoline-FDP-PPE (**14**), mono-hydrazinoisoquinoline-bis-acrolein-PPE (**15**) and bis-hydrazinoisoquinoline-bis-acrolein-PPE (**16**). MS/MS fragmentations of these ions revealed adducts on both lysine and the N-terminal glycine as seen previously with acrolein-hydralazine adducts. MS/MS fragmentations showing evidence for these structures are reported in Appendix 3; A3.1. Only the structures for lysine adducts are shown below. In addition, an ion was present at m/z 1820 which appeared to correspond to the MH^+ of bis-hydrazinoisoquinoline-tris-acrolein-PPE where the three additions of acrolein were via Michael addition. For the following 4 hours the relative intensities of the acrolein adducts remained approximately stable, although it appears that by 4 hours the mass of the acrolein adducts had increased by 16 mass units (Appendix 3; A3.1). This increase of 16 mass units is also evident in the 1-hydrazinoisoquinoline adducts after 1 hour of reaction (Figure 3.7b). MS/MS analysis of the ion at m/z 1639 reveals daughter ions present at m/z 1621 (-16, loss of oxygen), 1480 (-159, loss of HIQ), 1462 (-175, loss of HIQ-O) and 1424 (-213, loss of O-HIQ-acrolein). This

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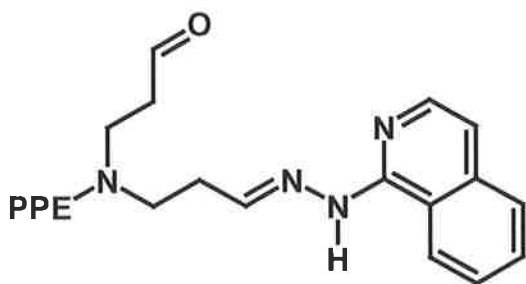
fragmentation appears to indicate that the 1-hydrazinoisoquinoline had become N oxidised. Furthermore, by 4 hours of reaction the N oxidised ions had increased by a further 16 mass units representing a second N-oxidation reaction (Appendix 3; A3.1). Overall, the results indicate that 1-hydrazinoisoquinoline, a highly efficient cytoprotective agent, is also an efficient acrolein adduct-trapping reagent, although the drug is also prone to N-oxidation.



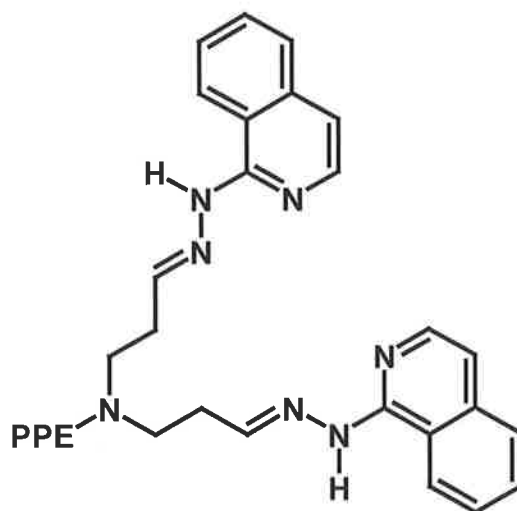
13. mono-HIQ-mono-acrolein-PPE
(*m/z* 1567)



14. mono-HIQ-FDP lysine-PPE
(*m/z* 1605)



15. mono-HIQ-bis-acrolein-PPE
(*m/z* 1623)



16. bis-HIQ-bis-acrolein-PPE
(*m/z* 1764)

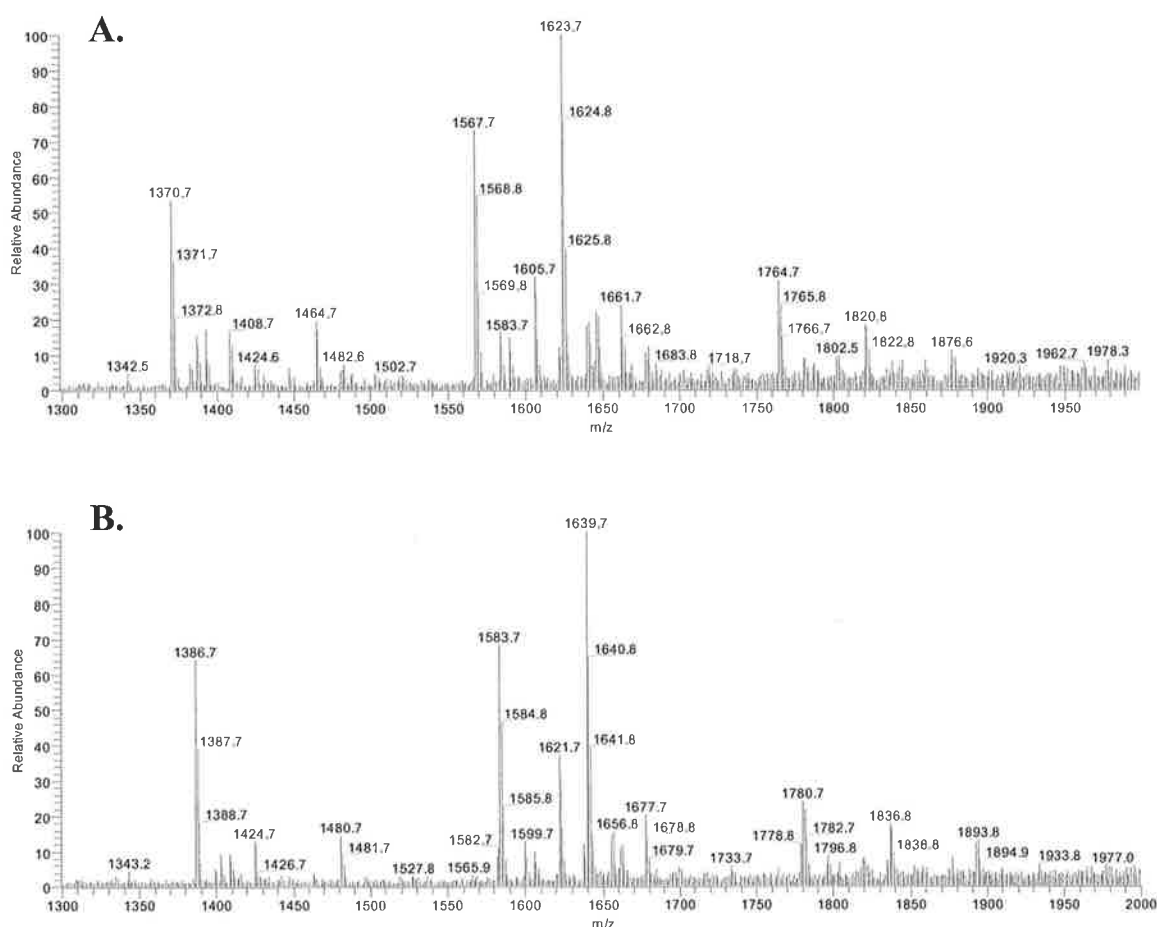
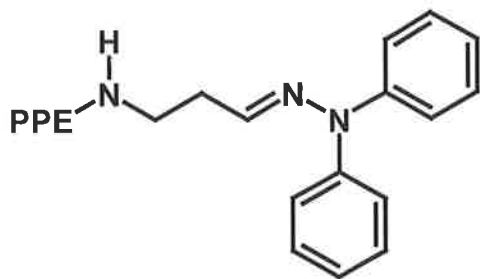


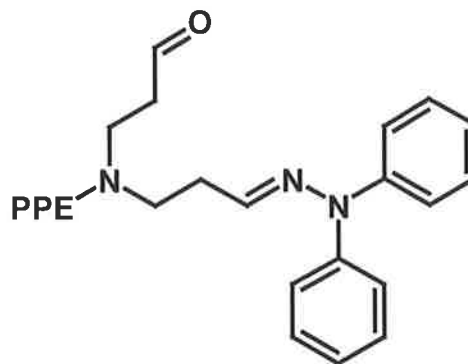
Figure 3.7 Mass spectra of acrolein-PPE adducts trapped by a 10 fold excess of 1-hydrazinoisoquinoline (cf. acrolein) after a 10 minute (A) and 1 hour (B) reaction at 37°C. Reactions were preformed in 50% acetonitrile due to the inability to solubilise 1-hydrazinoisoquinoline in water. Hydrazinoisoquinoline-acrolein-PPE adducts became N-oxidised by 1 hour of reaction, resulting in an increase of 16 mass units of each of the adduct ions. 25 μ M PPE was injected onto the LCQ.

Within 10 minutes of the addition of 1,1-diphenylhydrazine (DPH) the relative abundance of acrolein-PPE adducts had declined by approximately 10 to 15% (Figure 3.8). Several additional ions became apparent at m/z 1592 and 1648, corresponding to the MH^+ of mono-diphenylhydrazine-mono-acrolein-PPE (**17**) and mono-diphenylhydrazine-bis-acrolein-PPE (**18**) respectively. Only the structures of the adducts at lysine are shown below. MS/MS fragmentations of these are shown in Appendix 3; A3.2, where adducts at both lysine and the terminal glycine are present. In addition, two small signals were present at m/z 1631 and 1816, corresponding to the MH^+ of mono-diphenylhydrazine-FDP-PPE (**19**) and bis-diphenylhydrazine-bis-acrolein-PPE (**20**) respectively. Over the following 2 hours the relative intensities of the acrolein adducts did not change appreciably (Appendix 3; A3.2). Within this

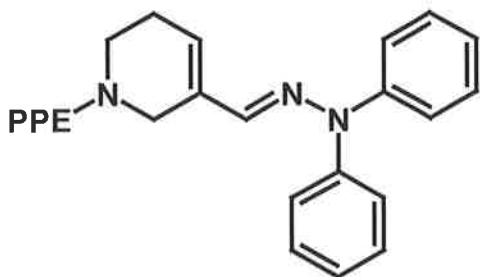
time, the baseline noise also increased, resulting in no significant detection of the ions corresponding to **17** and **18**. This shows that although 1,1-diphenylhydrazine traps acrolein-protein adducts, this reaction is not as efficient as with hydralazine, dihydralazine and 1-hydrazinoisoquinoline. This finding was somewhat unexpected, given that it was the most efficient cytoprotective compound examined. However, it is also possible that the trapped adducts formed by diphenylhydrazine are less prone to ionisation in the mass spectrometer than the corresponding species formed from hydralazine and hydrazinoisoquinoline.



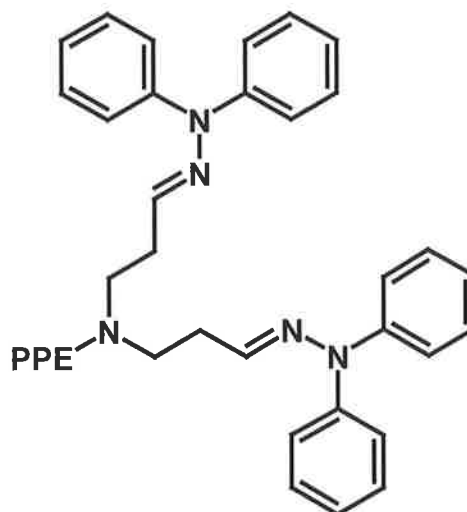
17. mono-DPH-mono-acrolein-PPE
(*m/z* 1592)



18. mono-DPH-bis-acrolein-PPE
(*m/z* 1648)



19. mono-HIQ-FP-lysine-PPE
(*m/z* 1631)



20. bis-HIQ-bis-acrolein-PPE
(*m/z* 1816)

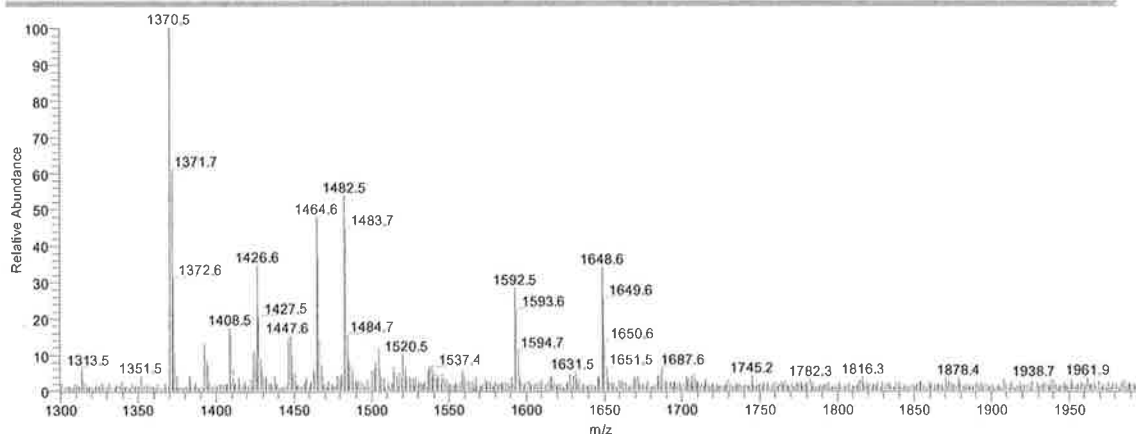
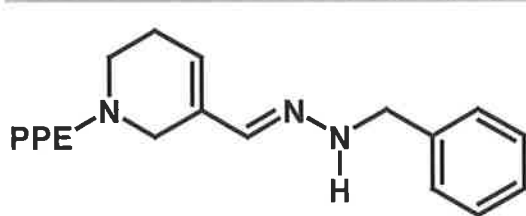
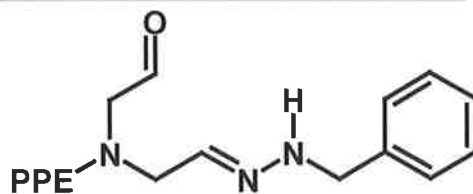


Figure 3.8 Mass spectrum of acrolein-PPE adducts trapped by a 10 fold excess of 1,1-diphenylhydrazine (cf. acrolein) after a 10 minute reaction at 37°C. The reaction was performed in 50% acetonitrile. 25 μ M PPE was injected onto the LCQ.

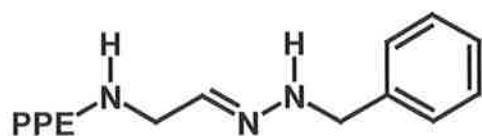
Within 2 hours of the addition of benzylhydrazine (BH) to the acrolein-PPE reaction, the relative abundance of the acrolein-PPE adducts were largely unchanged compared to the level of adducts after 3 hours of reaction of PPE with acrolein (Appendix 3; A3.3). After a 10 minute reaction of acrolein-PPE adducts with a 10 fold excess of benzylhydrazine, two additional ions became apparent at m/z 1568 and 1586 which corresponded to the MH^+ of mono-benzylhydrazine-FDP-PPE (**21**) and mono-benzylhydrazine-bis-acrolein-PPE (**22**) (Figure 3.9). The relative abundances of these ions were 11 and 23% respectively. MS/MS fragmentations of these are reported in Appendix 3; A3.3, and correspond to adducts on both lysine and the N-terminal glycine. A small signal at m/z 1530 was present whose mass corresponded to the MH^+ of mono-benzylhydrazine-mono-acrolein-PPE (**23**), although this ion was not intense enough to obtain MS/MS proof of structure. The spectrum was largely unchanged over the following 2 hours, although two small signals at m/z 1624 and 1643 were present in the spectra at 30 minutes, 1 and 2 hours (Appendix 3; A3.3). The mass of these ions corresponded to the mono-benzylhydrazine adduct of a triple acrolein adducted PPE minus water (m/z 1624) and mono-benzylhydrazine-tris-acrolein-PPE (m/z 1643) where the acrolein molecules were added via Michael type addition. Collectively, these findings suggest that benzylhydrazine does not appreciably trap acrolein-PPE adducts, which possibly explains its poor cytoprotective capacity in cells.



21. mono-BH-FDP-lysine-PPE
(*m/z* 1568)



22. mono-BH-bis-acrolein-PPE
(*m/z* 1586)



23. mono-BH-mono-acrolein-PPE
(*m/z* 1530)

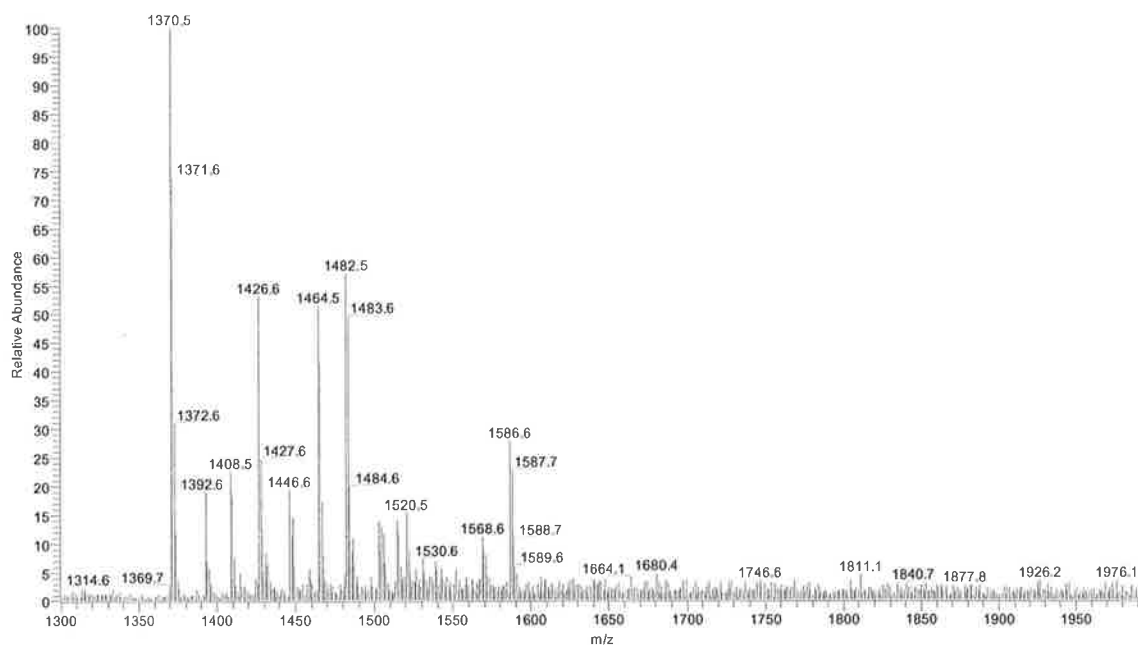


Figure 3.9 Mass spectrum of acrolein-PPE adducts trapped with a 10 fold excess (cf. acrolein) of benzylhydrazine at 37°C after 10 minutes. The reaction was performed in 50% acetonitrile. 25μM PPE was injected onto the LCQ.

3.3.4 Hydrazines and Hydralazine Analogues Block Recognition of Acrolein-BSA Adducts by a Polyclonal Acrolein-KLH Antiserum

The above mass spectrometry results indicated that adduct trapping rather than adduct stripping is a likely mechanism by which hydralazine and other cytoprotective hydrazines interfere with the chemistry of acrolein adduction and toxicity. To confirm that adduct trapping represents a possible mechanism of cytoprotection for hydralazine, slot blot analysis was used to quantify the adduct modifying efficacy of the various hydrazines mentioned previously in Chapter 2. As previously mentioned, this assay works on the basis of monitoring the recognition of acrolein adducted BSA using an anti-acrolein/KLH antiserum, and the ability of the hydrazines to disrupt this recognition by trapping the residual carbonyl groups on adducted proteins, forming non-immunoreactive species.

Slot blot analysis of the recognition of acrolein-BSA adducts following the addition of hydralazine, dihydralazine or one of the hydrazines previously mentioned revealed that all of the compounds tested blocked antibody recognition of acrolein-BSA adducts. A representative slot blot is shown below in Figure 3.10, where increasing concentrations of hydrazine (lanes 3 to 6) blocked immunorecognition of acrolein-BSA adducts (control, lane 2) by the rabbit anti-acrolein/KLH serum.

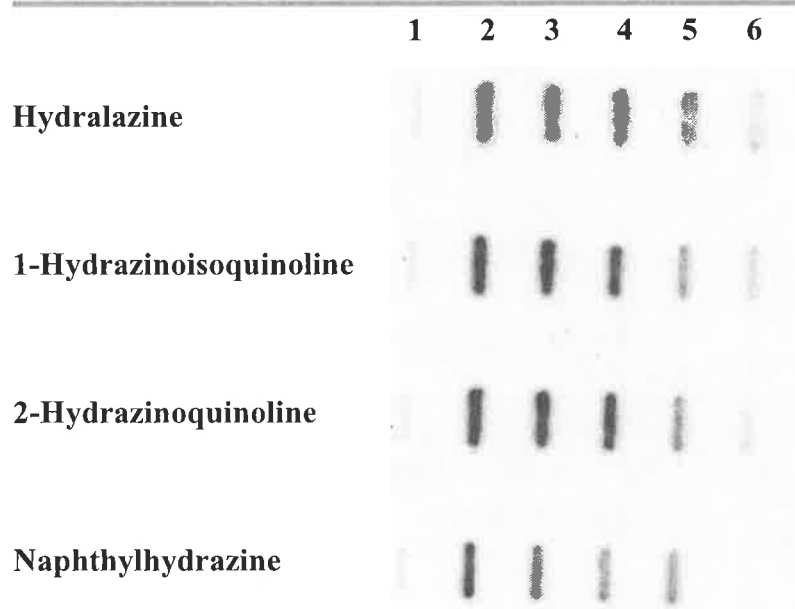


Figure 3.10 Representative slot blot showing disruption of acrolein-BSA immunorecognition by hydralazine, 1-hydrazinoisoquinoline, 2-hydrazinoquinoline and naphthylhydrazine. Immunolabelling of acrolein-BSA adducts was by a rabbit anti-acrolein/KLH serum. BSA (2 mg/ml) was modified with acrolein (1 mM) for 25 minutes at 37°C prior to the addition of hydrazine for an additional 1 hour. Each lane corresponds to: (1) unmodified BSA; (2) acrolein-modified BSA; (3) acrolein-modified BSA + 10 μ M hydrazine; (4) acrolein-modified BSA + 100 μ M hydrazine; (5) acrolein-modified BSA + 250 μ M hydrazine and (6) acrolein-modified BSA + 1000 μ M hydrazine.

The EC_{50} 's for blocking of antibody recognition were (in order of potency) naphthylhydrazine (50 μ M), 1,1-diphenylhydrazine (133 μ M), dihydralazine (150 μ M), benzylhydrazine (158 μ M), 1-hydrazinoisoquinoline (190 μ M), 2-hydrazinopyridine (190 μ M), 2-hydrazinoquinoline (210 μ M), phenylhydrazine (233 μ M), 1,2-diphenylhydrazine (250 μ M), hydralazine (300 μ M) and 1,1-dimethylhydrazine (570 μ M). 4-Hydrazinoquinazoline and 2,4-dinitrophenylhydrazine interfered with acrolein-BSA recognition and could therefore not be accurately quantified. Pearson's correlation analysis between cytoprotective EC_{50} and adduct blocking EC_{50} revealed a significant correlation ($R^2 = 0.62$, $p < 0.01$, graph not shown). Removal of 1,1-dimethylhydrazine from this analysis, however, results in no observable significance between cytoprotective EC_{50} and adduct-blocking EC_{50} (graph not shown). However, this analysis does not take into account the cellular access of the hydrazines, merely whether adduct blocking capacity correlates with cytoprotection. Another analysis was therefore performed between log P of the hydrazines and blocking capacity. A correlation was also observed between these parameters ($R^2 = 0.4851$, $p < 0.05$, graph not shown).

3.4 DISCUSSION

Allyl alcohol toxicity, in the form of irreversible membrane leakage, occurs within 30 minutes of its addition to mouse hepatocytes (Burcham *et al*, 2000). Burcham *et al* (2004) have previously shown that when added within 25 minutes of allyl alcohol addition, hydralazine and dihydralazine are able to prevent allyl alcohol induced cell death. Also, when added within 30 minutes of acrolein, hydralazine is also able to disrupt antibody recognition of acrolein-BSA adducts (Burcham *et al*, 2004). As indicated previously, these immunochemical observations could reflect either “adduct-stripping” or “adduct-trapping” actions of hydralazine. The data in the present Chapter indicates that the latter reaction is a more likely explanation of the effects of hydralazine on the immunoreactivity of acrolein-modified BSA than adduct stripping. The results of these studies raise the possibility that hydralazine may bind with acyclic Michael adducts of acrolein and block their ability to participate in cyclisation and crosslinking, thereby preventing downstream events in toxicity.

The current mass spectrometry results indicate that acyclic acrolein-protein adducts are prominent during the early stages of toxicity (within 60 minutes). Bis-acrolein- and FDP-adducts show strong relative ion intensities 3 hours after commencing acrolein modification. Therefore, during the early stages of acrolein toxicity (within 30 minutes), the reactivity of the early Michael adducts may be critical in determining the fate of cells, whereas cyclised adducts may not contribute significantly to short term acrolein toxicity. Despite its slower rate of formation, FDP-lysine is highly immunogenic and may contribute to later cellular damage by acting as a chemoattractant to macrophages and other immune cells which initiate localised oxidative stress in response to a foreign substance (Uchida *et al*, 1998a). This may prove to be important in chronic illnesses where lipid peroxidation and acrolein formation occur over a longer time frame than is the case during acute toxic syndromes.

Interestingly, rather than simply forming expected lysine adducts, acrolein adduction also occurred on the N-terminal glycine residue. No addition products were observed on the arginine residue, however, indicating that this amino acid does not represent a significant cellular target for acrolein. This is in keeping with the fact that as a basic residue, arginine is ionised at physiological pH and is therefore unlikely to react with electrophiles. Generally, lysine, histidine, cysteine and deoxyguanosine residues have been considered important intracellular targets for acrolein (Esterbauer *et al*, 1991). However, the current data indicates that terminal nitrogen groups in proteins also represent possible sites of adduction. For many proteins, N-

terminal adduction is expected to have subsequent functional consequences. For instance, there are a wide variety of proteins whose active sites lie close to the N-terminus, including p73, the V1a vasopressin receptor and β -amyloid (Muiswinkel *et al*, 1999; Zeng *et al*, 2000; Biswal *et al*, 2002; Hawtin *et al*, 2003; Walker *et al*, 2004). The β -amyloid protein is important in Alzheimer's disease pathology as its aggregation results in neuronal cell death and inflammatory responses that initiate localised oxidative stress. This latter effect is mediated specifically by a macrophage binding site near the N-terminus (Muiswinkel *et al*, 1999). High localised acrolein concentrations have also been observed in the brains of post-mortem Alzheimer's patients compared to age matched controls (Lovell *et al*, 2000). This raises the possibility that acrolein may be responsible for not only enhancing the aggregation of the β -amyloid protein through crosslinking reactions, but may also alter inflammatory responses by macrophages. This latter effect, however, needs to be investigated to determine if acrolein adduction at the N-terminus of β -amyloid enhances or inhibits β -amyloid induced macrophage activation.

A small signal corresponding to the Schiff base adduct of acrolein-PPE was also detected in reaction solutions of acrolein and PPE. This was expected, as Schiff base addition represents a minor pathway for acrolein adduction of proteins (Bruenner *et al*, 1995). The significance of this adduct to allyl alcohol toxicity is unclear. However, given the current data indicating that trapping reactions of hydralazine at reactive acrolein-protein adducts may mediate cytoprotection, Schiff adducts would not be expected to contribute significantly to toxicity. This is due to the fact that these adducts contain no sufficiently electrophilic reactive sites for hydralazine, hence hydralazine would be unlikely to block any toxic consequences arising from Schiff adduction.

The proposed theory that aromatic hydrazines mediate protection by binding to the early acrolein adducts in proteins is supported by results showing that hydralazine, dihydralazine and 1-hydrazinoisoquinoline rapidly trapped all evident acrolein-PPE adducts within 10 minutes, whereas benzylhydrazine barely trapped these adducts. Given that dihydralazine has two hydrazine functional groups capable of trapping acrolein adducts, it was expected that its reactivity would be twice as fast as that of hydralazine. Indeed, stronger adduct trapping occurred for dihydralazine compared to effects seen at the same concentration of hydralazine. This possibly explains the observation that dihydralazine was twice as effective as hydralazine at protecting against acrolein toxicity *in vitro* (Figure 2.12). That a similar effect was not seen for 1-hydrazinoisoquinoline, which was a better cytoprotective hydrazine than dihydralazine, may be due to solvent interference as this reaction occurred in a 50% acetonitrile solution. The less

effective adduct trapping by 1,1-diphenylhydrazine (the most efficient cytoprotective hydrazine identified thus far) was suggested to possibly be due to an inability to ionise these trapped species in the mass spectrometer. However, given the persistence of acrolein-PPE adducts in the presence of 1,1-diphenylhydrazine, it is likely that cytoprotection afforded by this particular hydrazine is not strictly related to its trapping ability. For instance, its amino counterpart (diphenylamine) is a known atypical antioxidant (Sugihara *et al*, 1993), thus the cytoprotective effects of 1,1-diphenylhydrazine might involve similar antioxidative mechanisms. This matter will be examined in Chapter 6.

Although adduct-trapping appears to be unrelated to cytoprotection mediated by 1,1-diphenylhydrazine, there is evidence to suggest that this trapping mechanism does contribute to cytoprotection afforded by other arylhydrazines. It has been noted that the free acrolein sequestering capacity of hydralazine and dihydralazine does not fully explain their cytoprotective effects, as hydralazine is still able to block toxicity after protein carbonylation has occurred. Hydralazine protection may then reflect its ability to trap carbonylated proteins and prevent further downstream events in toxicity. The mechanisms involved in these downstream toxic events need further investigation, although it may involve crosslinking to neighbouring nucleophilic biomolecules in protein or DNA, generating inter- and intra-molecular crosslinks. DNA-DNA crosslinks for instance have been shown to induce rapid cell death, although the significance of protein-protein and protein-DNA crosslinks needs to be addressed (Palom *et al*, 2002).

In order to provide further support for the mechanism of hydralazine induced protection against allyl alcohol and crotyl alcohol toxicity, slot blot analyses were performed to assess the ability of other hydrazines to block recognition of acrolein adducted BSA by a polyclonal acrolein-KLH antiserum. This was to determine whether an ability to abolish adduct immunoreactivity correlates with the cytoprotective potency of the various hydrazines tested in Chapter 2. Correlation analyses revealed a positive correlation between the ability of the hydrazines to block antibody recognition and cytoprotection, as well as between blocking of antibody recognition and log P. That 1,1-diphenylhydrazine (which proved to be a poor trapping agent during mass spectrometry analysis) was effective at abolishing adduct immunoreactivity here suggests that slot blot analysis may not be a strong tool for examining the ability of hydrazines to trap acrolein-protein adducts. Nevertheless, given the above data showing that hydralazine traps acrolein-peptide adducts and that hydralazine blocks antibody recognition of acrolein-protein adducts, the slot blot data likely reflects the ability of the hydrazines to trap acrolein-BSA

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adducts and not scavenge free acrolein. This later point is strengthened by the fact that trapping in slot blots was correlated with cytoprotection and log P, whereas scavenging by the hydrazines is inversely related to these parameters. Given the discrepancy with 1,1-diphenylhydrazine, however, this data must be interpreted cautiously.

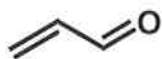
In the present Chapter, mass spectrometry proved to be a very useful tool for exploring the chemical basis of interactions between nucleophilic drugs and aldehyde-adducted peptides. However, given the diverse chemistry of protein adduction by acrolein, interpreting the data was complicated by the huge number of adducted species formed. The current results supported the hypothesis that trapping of the acyclic Michael adducts of acrolein by hydralazine might underlie the cytoprotection against acrolein toxicity. However, given the complex mixture of adducts formed from acrolein, further investigation is needed to evaluate the full significance of cyclised adducts in the induction of short term 2-alkenal toxicity. This issue was therefore addressed in the following Chapter, using the unsaturated ketone, methylvinyl ketone, as a chemical model for acrolein. It was hypothesised that methylvinyl ketone would be less prone to the formation of cyclised adducts than acrolein and so would simplify the study of adduct trapping reactions and their role in cytoprotection.

Chapter 4:

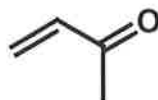
Methylvinyl ketone as a simplified model for acrolein toxicity

4.1 INTRODUCTION

Use of mass spectrometry established in the previous Chapter that in the early stages of modifying a model peptide with acrolein, ions corresponding to acyclic mono- and bis-acrolein adducts of lysine and N-terminal glycine predominated. A significant ion peak corresponding to the cyclised adduct, FDP-lysine was observed after a delay of approximately 1 hour. These observations raised intriguing questions surrounding the role of different adducts in the acute toxicity of acrolein. Hydralazine was previously found to protect against allyl alcohol toxicity only when added *in vitro* within 30 minutes of the toxin, suggesting that hydralazine adduction of early acyclic Michael adducts may mediate protection against allyl alcohol toxicity. However, given that some FDP-lysine adducts were still detected after a 30 minute reaction of acrolein and PPE, their contribution to acrolein toxicity and significance in terms of hydralazine protection could not be fully established. To address this issue, methylvinyl ketone (shown below) was explored in the present Chapter as an acrolein analogue in the expectation that it would exhibit simplified adduction chemistry relative to acrolein.



acrolein



methylvinyl ketone

Methylvinyl ketone is a 4-carbon α,β -unsaturated ketone that has similar toxicological and chemical properties to acrolein, although its alkylating and cytotoxic effects have not been widely studied. It is an environmental toxicant that causes respiratory irritation and lung necrosis at concentrations as low as 8 ppm in rats and mice exposed for 2 weeks (Morgan *et al*, 2000). Its toxicity has only been systematically studied by Lash and colleagues (1995) in tubular cells in rat kidney. It was found to deplete cellular thiols, including GSH (Portoghese *et al*, 1989; Lash and Woods, 1991), although the rate of GSH depletion by methylvinyl ketone was approximately 4 times slower than that of acrolein, with the respective carbonyls yielding rate constants for glutathione depletion of 32 and 120 $\text{mol}^{-1} \text{sec}^{-1}$ respectively (Esterbauer *et al*, 1975). Unlike acrolein, the resulting glutathione-carbonyl adducts are very stable, with half lives of 60.7 and 4.6 days respectively (Esterbauer *et al*, 1975). As with acrolein, methylvinyl ketone is toxic to proximal and distal tubular cells in the rat kidney, producing depletion of cellular GSH, with toxicity diminished by pre-treatment of cells with GSH (Lash and Woods, 1991; McKernan *et al*, 1991; Lash *et al*, 1995).

Although methylvinyl ketone has a similar toxicological profile to acrolein, it was predicted that there would be significant differences in its chemistry of protein adduction. It was thus predicted that its ketone group would make it less susceptible to cyclising reactions involving bis-adducts formed during protein adduction (ie. those that result in the formation of FDP-lysine by acrolein). This is because the terminal methyl group on methylvinyl ketone was expected to hinder the rate of aldol condensation during the formation of FDP adducts formed from bis-adducted entities. At present, nothing is known concerning protein adduction by methylvinyl ketone. Nevertheless, in keeping with its similarity to acrolein, it is capable of adducting deoxyguanosine residues in DNA, forming three main adducts: a cyclic 1, N2 adduct, a linear N7 adduct and a bis-adduct composed of 1, N2 cyclised adduct with a linear N7 adduct. No other nucleosides were susceptible to methylvinyl ketone adduction (Eder *et al*, 1991). These cyclised adducts presumably form over extended incubation periods and were not thought relevant to short term toxicity studies conducted in rat kidney tubular cells (Lash *et al*, 1995).

In the present Chapter, patterns of protein adduction by methylvinyl ketone were compared to those of acrolein, with the intention of establishing whether cyclic, FDP-type adducts are less likely to form from methylvinyl ketone compared to acrolein. Once again, as per the last Chapter, studies were conducted using electrospray ionisation mass spectrometry and PPE as a model peptide. Then, based on the observation of differences in protein adduction by these two closely related carbonyl compounds, their acute toxic potencies were directly compared in mouse

hepatocytes. Also, to directly assess their protein modifying reactivities, the extent of protein carbonylation produced by acrolein and methylvinyl ketone was measured in both BSA and in isolated mouse hepatocytes by a Western blot assay that detects 2,4-dinitrophenylhydrazine-trapped carbonylated proteins. The ability of hydralazine to protect against methylvinyl ketone toxicity was also assessed. Since early acyclic adducts appear to be major targets for hydralazine, the drug was expected to similarly protect against methylvinyl ketone toxicity by trapping acyclic Michael adducts formed by this compound.

4.2 METHODS

4.2.1 Chemicals

Methylvinyl ketone (MVK) was purchased from Aldrich Chemical Co (Milwaukee, WIS, USA). Rabbit anti-DNP primary polyclonal antiserum, Urea, 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (BCIP/NBT) tablets and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co (Milwaukee, WIS, USA). Trichloroacetic acid (TCA) was purchased from BDH Laboratory Supplies Poole, England. All other reagents were obtained as previously described.

4.2.2 Animals

Swiss mice were obtained for hepatocyte isolation as previously described (section 2.2.1).

4.2.3 Adduction of PPE by Methylvinyl Ketone and Trapping of Adducts by Hydralazine

Electrospray ionisation mass spectrometry was used to assess the formation of MVK adducts of PPE as described for acrolein (section 3.2.2). MVK (10 mM) was reacted with PPE (100 μ M) in a reaction volume of 600 μ l at 37 °C for up to 22 hours in capped 600 μ l Eppendorf tubes. Aliquots of the reaction mixture (25 μ l) were taken at 30 minutes, 1, 1.5, 3, 6 and 22 hours after the addition of MVK and diluted in 25 μ l water and 50 μ l 50% acetonitrile/2% glacial acetic acid as described in Chapter 3. This sample was then injected onto the LCQ for analysis as previously described (25 μ M PPE injected onto the LCQ). In some instances, hydralazine (100 μ l, 100 mM) was then added to 100 μ l of the reaction solution and incubated at 37 °C for a

further 3 hours. Samples of this reaction solution (25 μ l) were taken 30 minutes, 1, 1.5 and 3 hours later, diluted in 25 μ l of acetonitrile/glacial acetic acid and injected onto the LCQ.

4.2.4 Toxicity of Methylvinyl Ketone and Acrolein in Isolated Hepatocytes and Protection Against Methylvinyl Ketone by Hydralazine

A concentration response for acrolein and MVK mediated hepatocellular toxicity was established by incubating mouse hepatocytes (60 mm culture dishes as previously described, section 2.2.9.2) with 0, 20, 50, 100, 150 or 300 μ M concentrations of the aldehydes. Samples of culture media were taken immediately after the addition of the aldehydes and then 1, 2 and 4 hours later. Samples of culture media were assessed for LDH leakage as an indicator of cell death as described in Chapter 2 (section 2.2.9.2).

The ability of hydralazine to protect against MVK toxicity was assessed by co-treatment of hepatocytes with MVK (100 μ M) and 1 to 100 μ M hydralazine. Samples of culture media were taken at 0, 1, 2 and 4 hours after the addition of the ketone for assessment of LDH leakage as described in Chapter 2.

4.2.5 Effects of Methylvinyl Ketone and Acrolein on Cellular Glutathione

RPMI alone, hydralazine alone (100 μ M), acrolein or MVK alone (100 μ M) or in combination with hydralazine (100 μ M) were added to isolated mouse hepatocytes (35 mm dishes as described in Chapter 2, section 2.2.10.1) and incubated at 37 °C for 30 minutes as previously mentioned (section 2.2.10.1). The media was removed from the dishes which were then washed with cold PBS before 1.5 ml of cold 3% PCA was added. The cells were then scraped off the dishes and treated as described for GSH determination in section 2.2.10.2.

GSH content in the cells was then measured as described in Chapter 2 and a 1 way ANOVA with a Dunnett's post test performed to compare the individual groups to control GSH (RPMI alone). Significance was at a level of $p < 0.05$.

4.2.6 Carbonylation of BSA by Methylvinyl Ketone and Acrolein

4.2.6.1 Protocol

A 5 mg/ml solution of BSA in phosphate buffer (50 mM, pH 7) was prepared and prewarmed to 37 °C. To 1.5 ml Eppendorf tubes that contained 1 ml of the BSA solution was added acrolein or MVK to give final concentrations of 30, 100, 300 or 1000 µM. The tubes were then incubated for 2 hours at 37 °C prior to chilling on ice.

4.2.6.2 Protein Carbonylation Assay for BSA

The carbonylation assay for MVK and acrolein was via a similar method to that reported by Levine and colleagues (Levine *et al*, 1990). A sample of each of the above BSA solutions (100 µl) was added to 20 µl of TCA (20% in water) to precipitate the BSA. The samples were then centrifuged at 7000 x g for 5 minutes to pellet the protein and the supernatant was discarded. The pellet was resuspended in 8 M urea (1 ml) and vortexed. To a 20 µl aliquot of this was added 20 µl DNPH solution (made by adding 1 ml TFA to 49.5 mg DNPH and diluted to 10 ml in water) and reacted at room temperature for 30 minutes. The samples were then neutralised with 36 µl of Tris/glycerol (40% glycerol in 2 M Tris base). A 7.6 µl sample of this solution (containing 1 µg of BSA) was then added to 2.4 µl water and 10 µl loading buffer (containing 50 mM Tris; pH 6.8, 8% glycerol, 1.6% SDS and 0.4% bromophenol blue). These samples were then heated at 70 to 80 °C for 2 minutes, cooled to room temperature and 10 µl (0.5 µg) were loaded onto a 12% acrylamide mini-gel for electrophoresis (see below for protocol). Immunoblots of carbonylated BSA were then subjected to densitometric analysis using Kodak Digital Science image analysis software (Eastman Kodak).

4.2.7 Carbonylation of Hepatocellular proteins by Methylvinyl Ketone and Acrolein

4.2.7.1 Protocol

Hepatocyte cultures were prepared in 60 mm dishes as previously described. Cells were treated with RPMI (supplemented with 0.03% L-glutamine and 50 U/l and 50 µg/ml of penicillin/streptomycin respectively as described in section 2.2.9.2) alone or 20, 50, 100, 150 or 300 µM MVK or acrolein. Cells were incubated at 37°C for 30 minutes followed by washing in

cold PBS and the addition of 1 ml 3% cold PCA. The dishes were then kept on ice for 10 minutes followed by scraping the cells and transferring them to Eppendorf tubes. The suspension was then centrifuged for 4 minutes at 4000 x g and the supernatant discarded. The pellet was then resuspended in 250 µl urea (8M) and vortexed. A sample of this suspension (50 µl) was mixed with 700 µl urea (8M) and assayed for protein concentration via the 260/280 nm measurement method reported in Bollag *et al* (1996). The volume of cell suspension equating to 40 µg protein was then calculated for each sample.

4.2.7.2 Immunochemical Carbonylation Assay in Cells

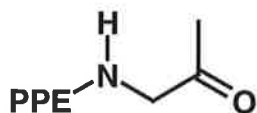
The required volume of cell suspension containing 40 µg protein was made up to a volume of 5 µl in urea (8M). DNPH solution (5 µl) was then added and the reaction mixed and left at room temperature for 30 minutes. Tris/glycerol (9 µl) was then added to neutralise the samples, followed by 6 µl of loading buffer (60 mM Tris-HCl; pH 6.8, 25% glycerol, 2% SDS, 0.5% 2-mercaptoethanol, 0.1% bromophenol blue). The samples were heated at 70 to 80 °C for 2 minutes, cooled and a 12.5 µl sample (20 µg protein) loaded onto 12% acrylamide mini-gels.

The 12% acrylamide resolving gel, 4% acrylamide stacking gel, electrophoresis running buffer and Towbins transfer buffer were prepared as reported in Bollag (1996). Proteins were resolved on the acrylamide gels for 40 to 50 minutes at 200 V, then transferred to nitrocellulose membranes (0.22 µm, Biorad) at 100 V for 30 minutes in Towbins transfer buffer (containing 10% methanol, Bollag; 1996). The membranes were then blocked in 10% nonfat milk powder/PBS for 1 hour followed by incubating in rabbit anti-DNP serum (1/1000 in 10% nonfat milk/PBS) at 4°C overnight. The membranes were then washed in 3 changes of water, 3 changes of PBS (5 minutes each) and 1 change of TBS (10 minutes). They were then transferred to goat anti-rabbit IgG conjugated to alkaline phosphatase (1/4000 dilution in 10% nonfat milk powder/TBS) and incubated for a further 1 hour. Following rinsing in 3 changes of water and 5 changes of TBS (5 minutes each) the membranes were developed with *SigmaFast* BCIP/NBT substrate (1 tablet freshly dissolved in 10 ml water) for at least 5 minutes. Immunoblots of carbonylated hepatocellular proteins were then subjected to densitometric analysis using Kodak Digital Science image analysis software (Eastman Kodak).

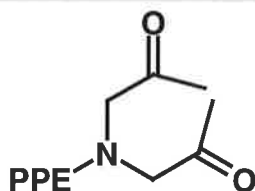
4.3 RESULTS

4.3.1 Adduction of PPE by Methylvinyl Ketone and Trapping by Hydralazine

To identify protein adducts formed by MVK, adduct formation in PPE was followed over a 22 hour incubation period by ESI-mass spectrometry as described for acrolein in Chapter 3. Following a 30 minute incubation of PPE with a 100 fold excess of MVK a minor signal at m/z 1440 was present, corresponding to the MH^+ ion of mono Michael-adducted MVK-PPE (**1**, Appendix 4; A4.1), although no other ions were detected. The intensity of this ion progressively increased over the next 21.5 hours (Appendix 4; A4.1). Also, a small signal at m/z 1510, corresponding to the MH^+ of bis Michael-adducted MVK-PPE (**2**), appeared after 3 hours of reaction (Appendix 4; A4.1). By 6 hours of reaction several ions at m/z 1424 and 1494, corresponding to the MH^+ of mono-MVK-PPE minus CH_4^+ and bis-MVK-PPE minus CH_4^+ respectively, were also present as strong signals at relative intensities of 65 and 15% respectively. The relative intensities of these methyl-deficient adducts declined by 22 hours of reaction (Appendix 4; A4.1; Figure 4.1). Also, at this time, the relative intensities of **1** and **2** had increased to 100 and 40% respectively, although the intensity of the signal corresponding to unmodified PPE was still strong at a relative intensity of 90%. MS/MS analysis of the ions of **1** and **2** revealed a combination of Michael adducts of MVK on both the lysine residue and the N-terminal glycine as shown below. Confirmation of these structures was as reported in Chapter 3 for acrolein adducts. Adducts on the arginine residue were not detected, consistent with PPE adduction sites for acrolein (Chapter 3). The mass of fragments corresponding to the Y+2 cleavages are shown in upper brackets while B cleavages are shown by the lower brackets. As in Chapter 3, the residues adducted by MVK are highlighted in **red**, while doubly adducted residues are **underlined**. Importantly, ions corresponding to either cyclised MVK-PPE adducts or Schiff base adducts of MVK on PPE were not detected during mass spectrometry analysis of reaction mixtures, even after 22 hour reactions. This indicated that the predominant adducts formed by MVK were mono- and bis-Michael adducts of lysine and the N-terminus, in keeping with the prediction that steric factors would render such adducts less prone to cyclisation than their counterparts derived from acrolein.



1. mono-MVK-PPE
(*m/z* 1440)



2. di-MVK-PPE
(*m/z* 1510)

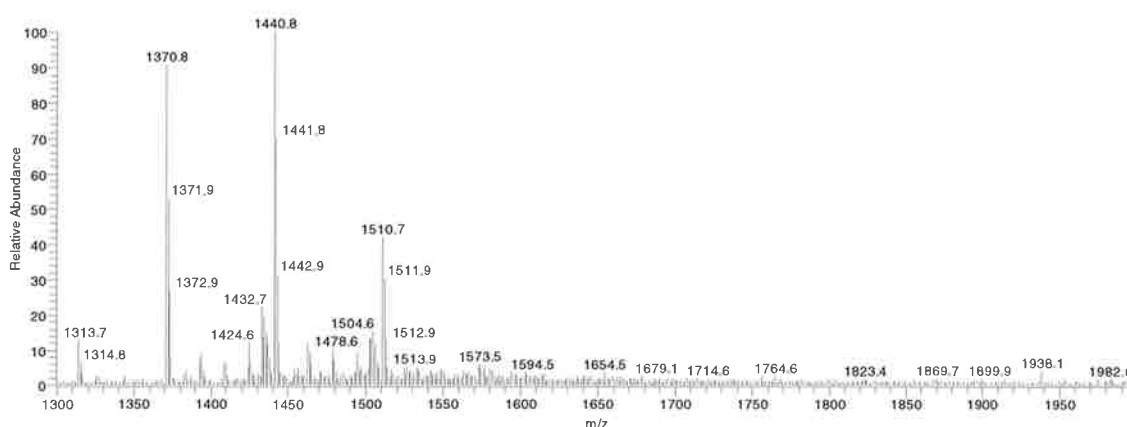
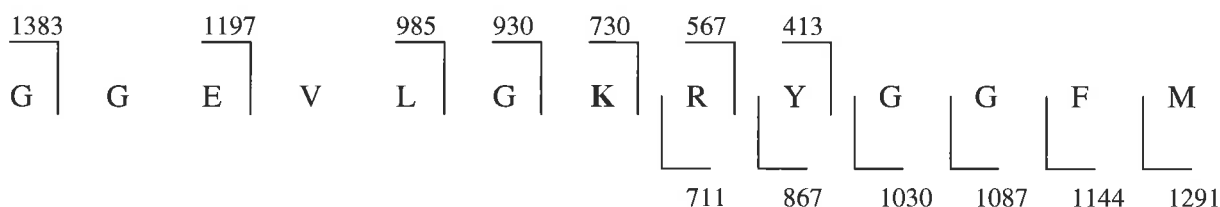
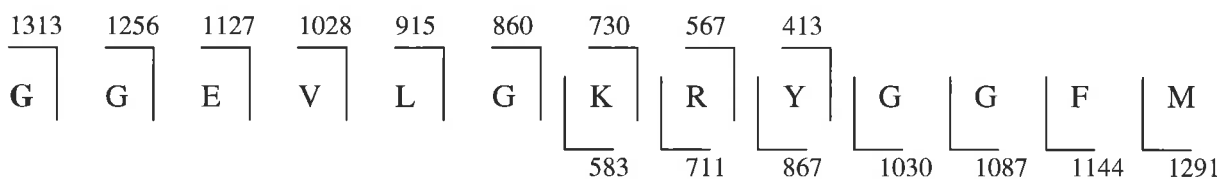


Figure 4.1 Mass spectrum of PPE modified by a 100 fold excess of MVK for 22 hours at 37°C. A final concentration of 25 μ M PPE injected onto the LCQ.

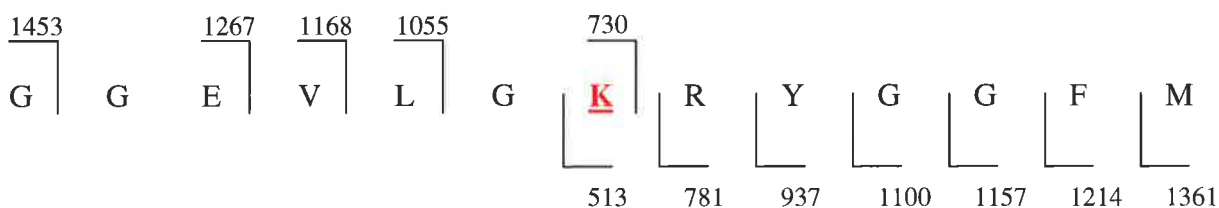
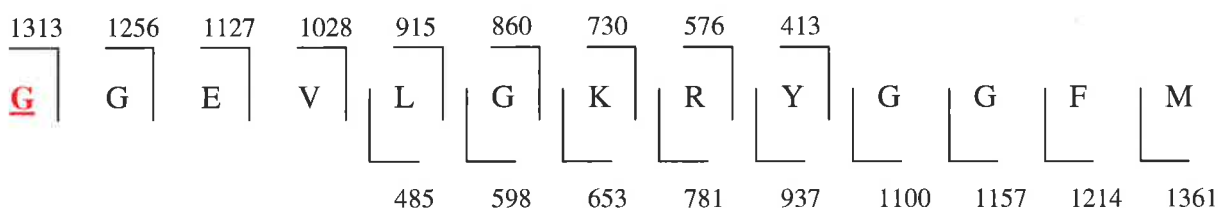
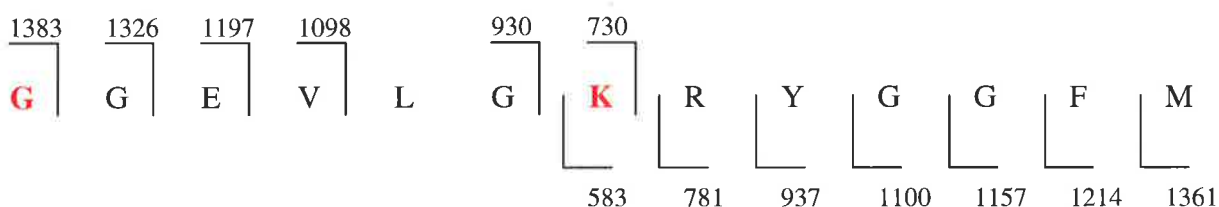
***m/z* 1440. mono-MVK (K)**



***m/z* 1440. mono-MVK (G)**



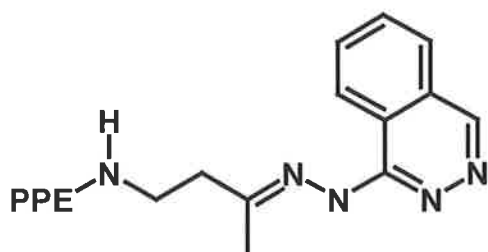
The ion at *m/z* 1440 detected in MVK-modified PPE was found to comprise two different species, namely mono-MVK adducted PPE at the lysine and N-terminal glycine residues. The adduction sites were revealed by the loss of an additional 70 mass units from these cleavages, corresponding to the mass of MVK.

m/z* 1510. bis-MVK (2 × K)**m/z* 1510. bis-MVK (2 × G)*****m/z* 1510. bis-MVK (1 × G/1 × K)**

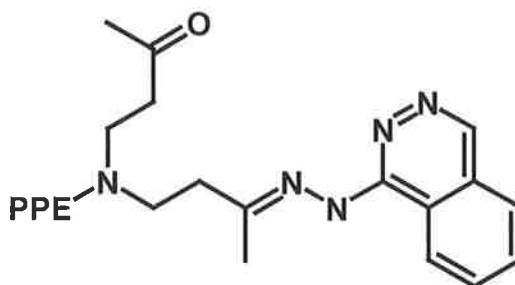
The ion at *m/z* 1510 comprised three species, a bis-MVK adducted PPE at lysine (upper fragmentation) or glycine (middle fragmentation) and one MVK adduct on both residues (lower fragmentation). These were identified by the loss of either 140 additional mass units from either lysine (upper fragmentation) or glycine (middle fragmentation) or the loss of 70 mass units from both lysine and glycine (lower fragmentation).

It was then determined whether these acyclic Michael adducts of MVK were capable of trapping reactions with hydralazine, as reported for acrolein adducts in Chapter 3. Reaction of the above mixture comprising **1** and **2** with a 10 fold excess of hydralazine (relative to MVK) resulted in the detection of several new ions at *m/z* 1582, 1654 and 1794 within 30 minutes, corresponding to hydrazones that represented the MH⁺ of mono-hydralazine-mono-MVK-PPE (**3**), mono-hydralazine-bis-MVK-PPE (**4**) and bis-hydralazine-bis-MVK-PPE respectively (**5**) (Figure 4.2). The relative intensities of these signals were 32, 5 and 5% respectively, although ions corresponding to **1** and **2** were still present with relative intensities of 82 and 30% respectively. By 3 hours of reaction the spectra had not changed significantly, although the relative intensity of MVK-PPE ions had decreased by approximately 10% while the relative intensity of the ion at *m/z* 1582 increased by approximately the same amount (Appendix 4; A4.2). This indicated that

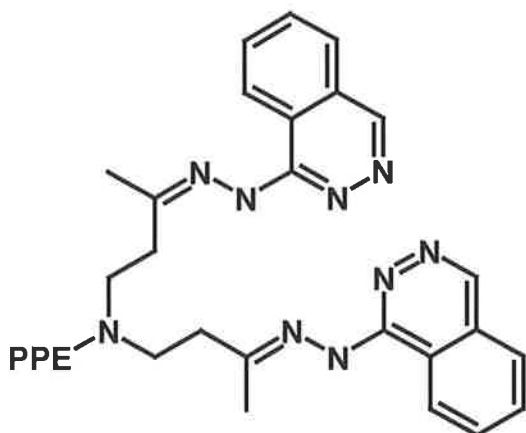
while hydralazine does trap acyclic Michael adducts of MVK, this reaction appears to be slower than the corresponding reaction with acrolein-PPE adducts. MS/MS fragmentations corresponding to hydralazine trapped MVK-PPE adducts are shown below, where adduct-trapping occurred on both lysine and N-terminal glycine residues as expected given the localisation of MVK-PPE adducts. However, while strong evidence for the formation of mono-hydralazine-mono-MVK-PPE adducts on lysine and glycine was obtained, MS/MS fragmentations corresponding to hydralazine adducts on bis-MVK-PPE were less prevalent. Mass fragments corresponding to Y+2 cleavages are shown as upper brackets while B cleavages are shown by the lower brackets. Residues containing a single hydralazine-trapped species are indicated by a *, while bis-hydralazine trapped adducts are shown by a **.



3. mono-hydralazine-mono-MVK-PPE
(*m/z* 1582)



4. mono-hydralazine-bis-MVK-PPE
(*m/z* 1654)



5. bis-hydralazine-bis-MVK-PPE
(*m/z* 1794)

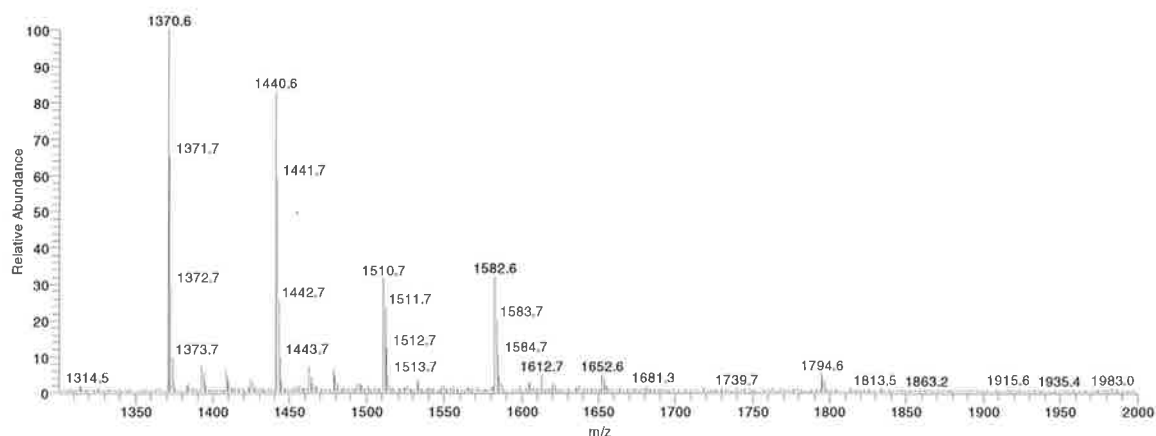
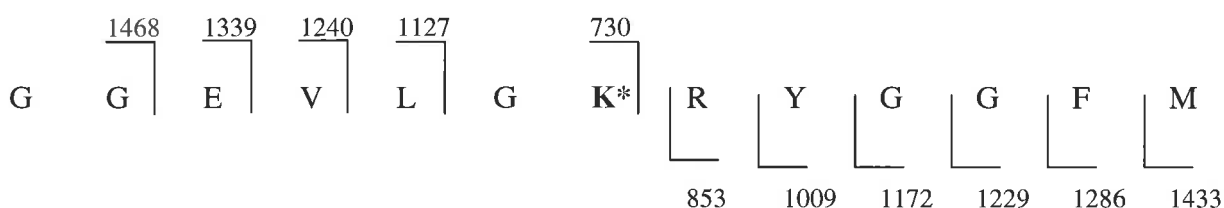
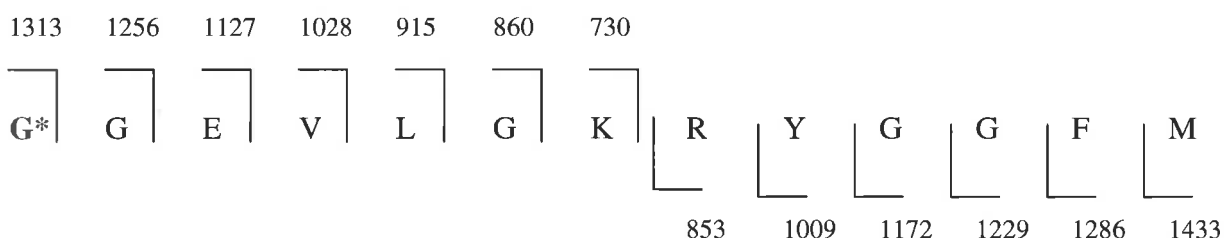


Figure 4.2 Mass spectrum of MVK-PPE adducts modified by a 10 fold excess of hydralazine (cf. MVK) after a 30 minute reaction at 37°C. A final concentration of 25 μ M PPE was injected onto the LCQ.

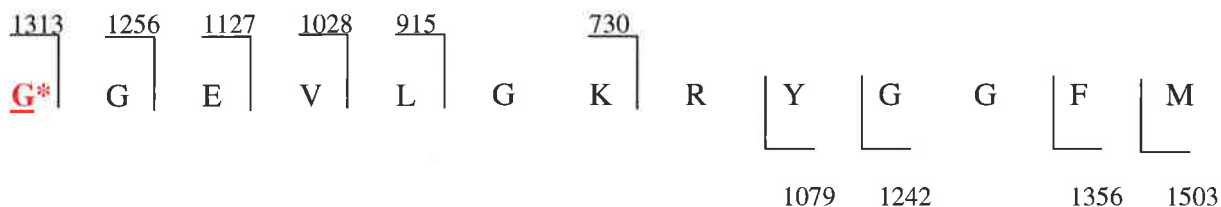
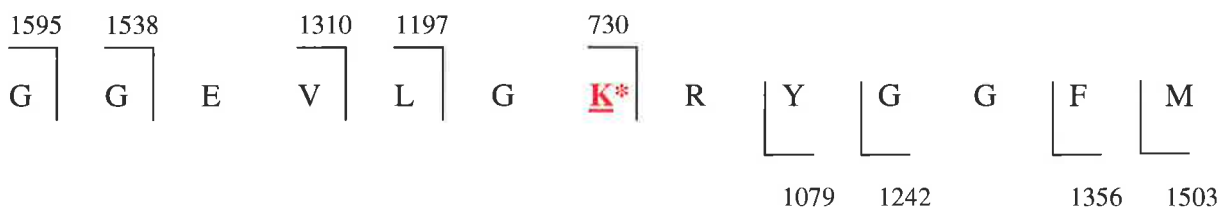
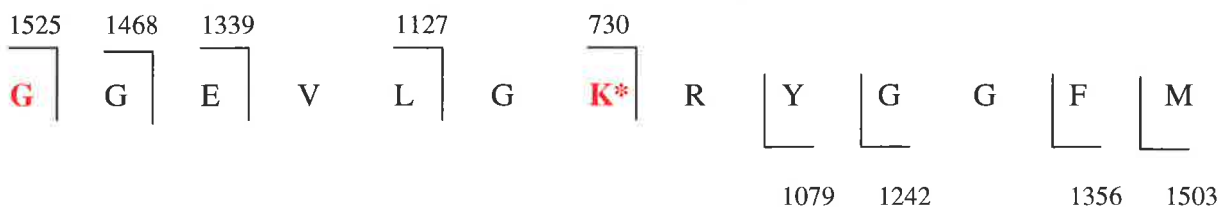
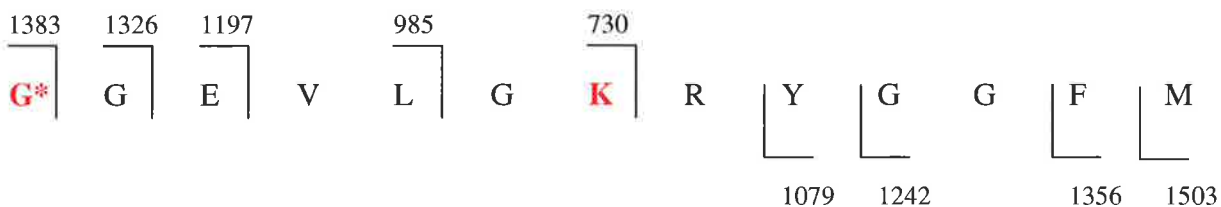
***m/z* 1582. mono HYD-mono MVK (K)**



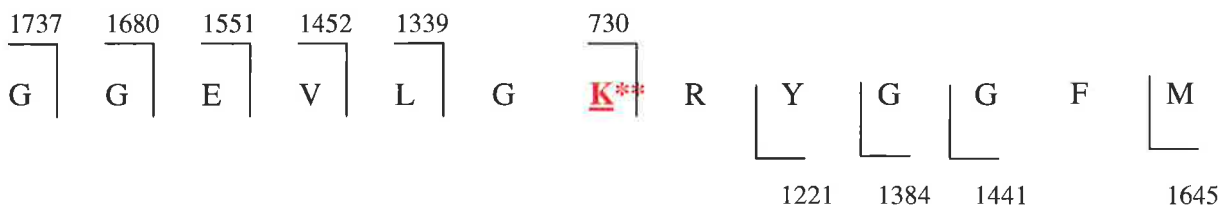
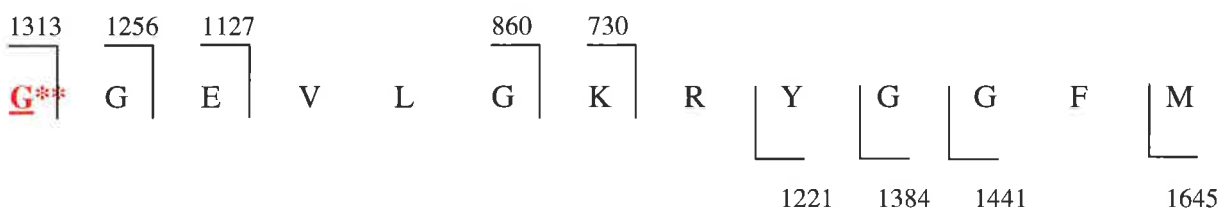
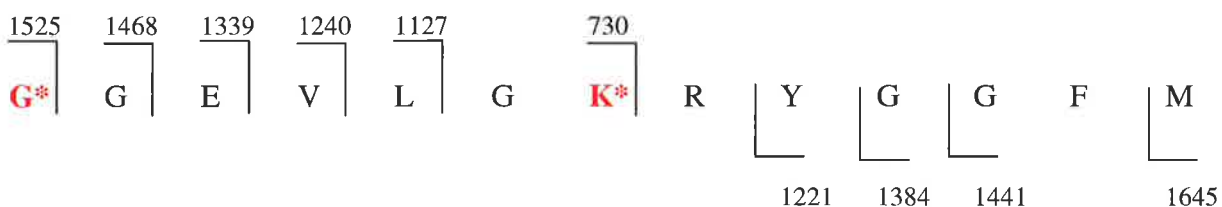
***m/z* 1582. mono HYD-mono MVK (G)**



The ion at ***m/z* 1582** was found to comprise mono hydralazine-trapped mono-MVK adducts at either lysine or glycine residues, as shown by the loss of an additional 212 mass units (corresponding to the hydrazone product of reaction between hydralazine and MVK) upon loss of either lysine or the N-terminal glycine.

m/z* 1654. mono HYD-bis MVK (2 × G)**m/z* 1654. mono HYD-bis MVK (2 × K)*****m/z* 1654. mono HYD (K)-bis MVK (1 × K/1 × G)*****m/z* 1654. mono HYD (G)-bis MVK (1 × K/1 × G)**

The ion at *m/z* 1654 was a mixture of four distinct species, namely mono hydralazine-trapped bis-MVK adducts on lysine or glycine (upper two fragmentations), or mono hydralazine-trapped mono-MVK adducts at one residue plus a mono-MVK adduct at the other residue (lower two fragmentations). The structures for the upper two fragmentations were identified by the loss of an additional 282 mass units from either lysine or glycine (corresponding to one hydralazine-trapped MVK plus one non-trapped MVK molecule). The structures for the lower two fragmentations were confirmed by the loss of an additional 212 mass units from lysine or glycine plus loss of an additional 70 mass units from the other residue.

m/z* 1794. bis HYD-bis MVK (2 × K)**m/z* 1794. bis HYD-bis MVK (2 × G)*****m/z* 1794. bis HYD-bis MVK (1 × K/1 × G)**

The ion at *m/z* 1794 was identified as either bis hydralazine-trapped bis-acrolein-PPE at the lysine (upper fragmentation) or glycine (middle fragmentation) residues, or as single-trapped adducts on both residues (lower fragmentation). These structures were diagnosed by the loss of an additional 424 mass units from either residue (corresponding with two-trapped adducts) or loss of 212 mass units from both residues.

4.3.2 Concentration Response for Methylvinyl Ketone and Acrolein Toxicity and Hydralazine Mediated Protection Against Methylvinyl Ketone Toxicity

The data obtained during study of the chemistry of protein adduction revealed important differences between acrolein and MVK. To see whether these differences are of relevance to the toxicological properties of these substances, their acute toxicity in isolated mouse hepatocytes was compared. For direct comparison of MVK and acrolein toxicity in isolated mouse hepatocytes, concentration response profiles for LDH leakage induced by both carbonyls were determined. This was for the purpose of assessing the likely contribution of acyclic Michael adducts to the toxicity of these two α,β -unsaturated carbonyl compounds. Thus, it was anticipated that if FDP-lysine plays a major role in the acute toxicity of acrolein, since the

corresponding adduct was not formed by MVK, the latter would be expected to possess lower toxic potency than acrolein. The data in Figure 4.3 confirms that MVK induced time and concentration dependent cell death, where submaximal cell death was achieved by 4 hours of incubation with 20 to 100 μ M MVK (Figure 4.3a). The highest concentrations of MVK (150 and 300 μ M) induced maximal cell death by 4 hours. Surprisingly, the cells seemed slightly less susceptible to acrolein than they were to MVK, with acrolein inducing 100% cell death after 4 hours only at the highest concentration examined (300 μ M) (Figure 4.3b). This indicated that although MVK was incapable of forming FDP-lysine-like adducts, it was nevertheless capable of inducing pronounced cell death comparable to that produced by equimolar concentrations of acrolein.

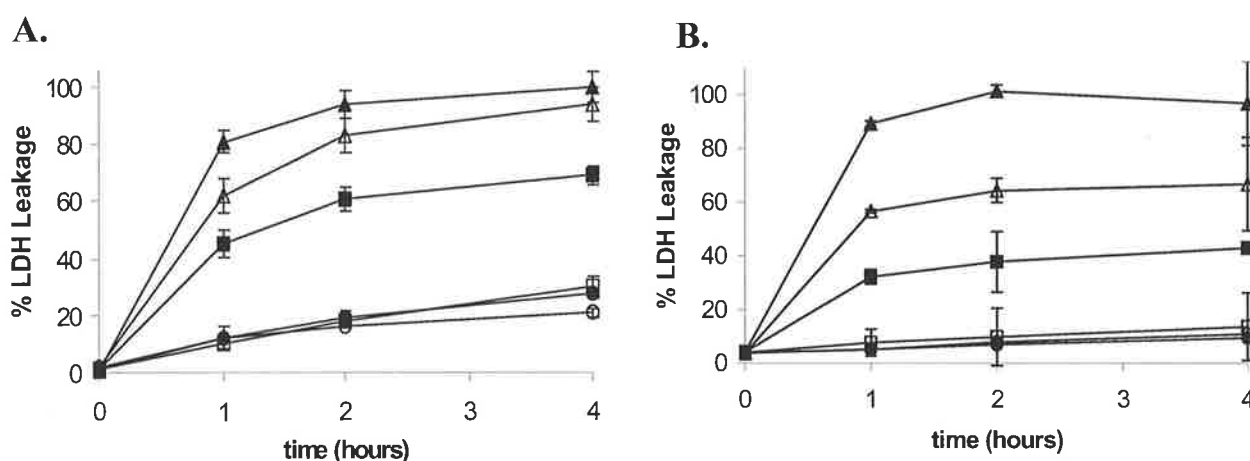


Figure 4.3 Concentration and time dependence of methylvinyl ketone (MVK, A) and acrolein (B) toxicity in primary isolated mouse hepatocytes. Hepatocytes were treated with RPMI alone (○) or 20 μ M (●), 50 μ M (□), 100 μ M (■), 150 μ M (△) or 300 μ M (▲) MVK or acrolein. Cell death was assessed by lactate dehydrogenase leakage (%LDH) into the culture media at 1, 2 and 4 hours after the addition of the ketone. Data are represented as mean $\pm$ SEM of 3 replicates.

To determine whether hydralazine was capable of protecting mouse hepatocytes against MVK toxicity, the cytoprotection studies discussed in Chapter 2 (2.3.5) were repeated using the ketone. Hydralazine protected against cytotoxicity induced by a 100 μM concentration of MVK in a concentration dependent manner (Figure 4.4), with 100 μM hydralazine completely protecting against MVK toxicity while 30 μM hydralazine prevented cell death by over 40% at 3 hours. Lower concentrations of hydralazine provided only partial protection at the first time point studied. Thus, the protection against MVK toxicity afforded by hydralazine was comparable to that seen against allyl alcohol toxicity. This suggests that the two carbonyl compounds induce cell death via comparable mechanisms and also that the mechanism of hydralazine mediated protection is similar for toxicity induced by both MVK and acrolein.

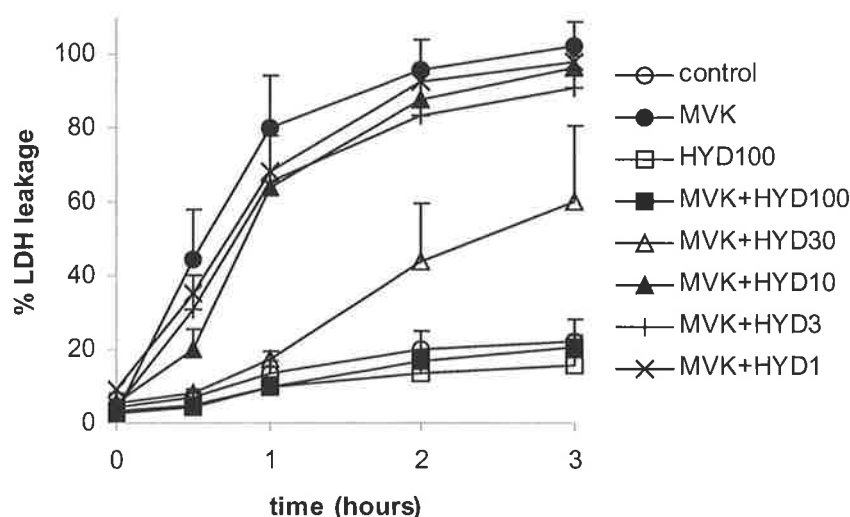


Figure 4.4 Protection against MVK (100 μM) induced hepatocyte cell death by hydralazine (HYD). Cells were co-incubated with MVK and various concentrations of hydralazine (indicated in μM as the numbers represented in the legend) and samples of culture media taken 0, 30 minutes and 1, 2 and 4 hours later for assessment of LDH leakage from the cytoplasm. Data are represented as mean $\pm$ SEM (3 replicates) of the % LDH leakage from cells after various incubation times.

4.3.3 Depletion of Cellular Glutathione by Methylvinyl Ketone and Acrolein

Previous studies have established that a decrease in cellular GSH precedes induction of cell death by both acrolein and MVK (Beauchamp *et al*, 1985; Lash and Woods, 1991). It was also shown in Chapter 2 that hydralazine was unable to prevent the decrease in cellular GSH induced by crotyl and allyl alcohols, suggesting that hydralazine does not protect against toxicity by sparing GSH. It was therefore sought to compare the GSH depleting ability of MVK and

acrolein and to determine if hydralazine was able to block any depletion of this tri-peptide by either unsaturated carbonyl compound. Unexpectedly, MVK (100 μ M) did not significantly deplete cellular GSH within 30 minutes of its addition, whereas an equivalent concentration of acrolein (100 μ M), which was less toxic than MVK to the hepatocytes, induced significant depletion of cellular GSH within this time (by approximately 66%, $p < 0.01$, Figure 4.5). The addition of hydralazine (100 μ M) to cell culture dishes either alone or with acrolein or MVK did not affect GSH levels. This suggests that depletion of cellular GSH is not vital in the induction of unsaturated carbonyl toxicity in this cellular model and confirms that hydralazine, while cytoprotective, does not spare the tri-peptide during acrolein toxicity.

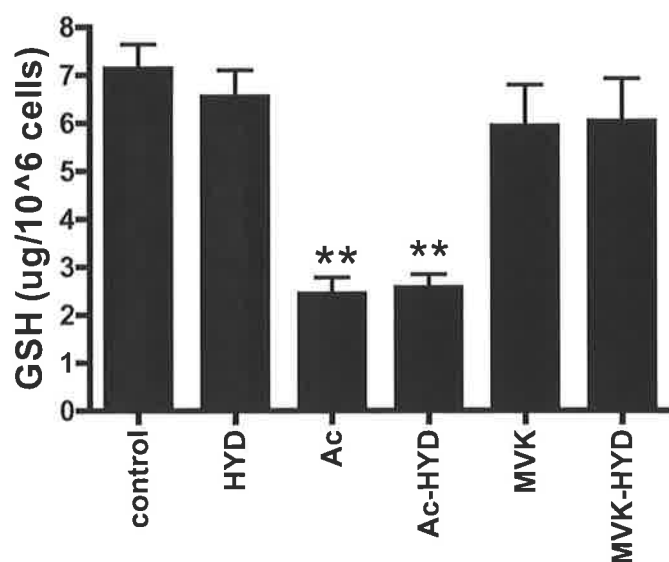


Figure 4.6 Hepatocyte GSH levels 30 minutes after treatment with acrolein (Ac, 100 μ M), MVK (100 μ M), hydralazine (HYD, 100 μ M) or hydralazine co-incubated with one of the carbonyls. GSH was reported as mean $\pm$ SEM in μ g/million cells (3 replicates). A 1 way ANOVA with a Dunnett's post test (compared to control, RPMI alone treated cells) was used to compare the groups. ** $p < 0.01$ cf. control.

4.3.4 Carbonylation of BSA and Hepatocytes by Methylvinyl Ketone and Acrolein

Work described thus far in this thesis has been conducted according to the paradigm that the toxicity of unsaturated carbonyl compounds reflects their ability to alkylate cellular macromolecules such as proteins. To assess the alkylating potencies of MVK and acrolein their ability to carbonylate a model protein, BSA, were therefore compared. This assay offers an advantage over the use of ESI-mass spectrometry since the extent of Michael adducted proteins can be quantified via the measurement of protein carbonyl groups. However, it does not assess the extent of secondary crosslinks or Schiff adducts. For this reason, BSA and hepatocytes were

exposed to the carbonyls for only 30 minutes to minimise the extent of secondary reactions that would confound accurate comparison of the carbonylating capacity of the carbonyls.

BSA is a 67 kDa protein containing multiple lysine, histidine and cysteine groups that provide ample adduction sites for α,β -unsaturated carbonyls. BSA was modified for 30 minutes with 30 to 1000 μ M acrolein or MVK at 37°C. Following labelling of protein carbonyl groups with the trapping reagent 2,4-dinitrophenylhydrazine, the protein was resolved on a 12% acrylamide gel before it was transferred to nitrocellulose and assessed for adducts using anti-DNP sera. A resulting immunoblot is shown in Figure 4.6, where lane 1 corresponds to unmodified BSA. Lanes 2 to 5 correspond to BSA treated with increasing concentrations of acrolein, where a concentration dependent increase in protein carbonylation is evident (30, 100, 300 and 1000 μ M acrolein produced a respective increase in band intensity of 1.4, 3.0, 5.7 and 9.1 compared to BSA alone). Lanes 6 to 9 correspond to BSA modified with increasing concentrations of MVK, where a concentration dependent increase in BSA carbonylation is also evident (30, 100, 300 and 1000 μ M MVK produced a respective increase in band intensity of 2.2, 3.1, 5.0 and 5.6 compared to BSA alone). However, the intensity of BSA carbonylation by the two highest concentrations of MVK was slightly lower than that of acrolein. This was in contrast to the expectation that carbonylation would be greater for MVK than acrolein given the greater toxicity of the ketone in mouse hepatocytes.

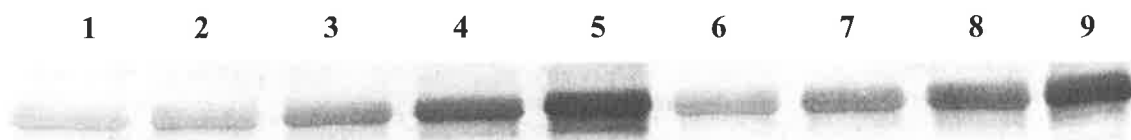


Figure 4.7 Comparisons of protein carbonylating potencies of acrolein and MVK. BSA (5 mg/ml) was modified for 2 hours with either PBS (control, lane 1); 30, 100, 300 or 1000 μ M acrolein (lanes 2, 3, 4 and 5 respectively; or 30, 100, 300 or 1000 μ M MVK (lanes 6, 7, 8 or 9 respectively). DNPH-labelled carbonylated BSA was loaded onto a 12% acrylamide gel (0.5 μ g protein) and separated by electrophoresis. These groups were then labelled using an anti-DNP antiserum conjugated to alkaline phosphatase and developed using *SigmaFast* BCIP/NBT substrate tablets.

The ability of MVK and acrolein to carbonylate cellular proteins was then compared following a 30 minute incubation of hepatocytes with the same concentrations of carbonyl compounds used for the LDH leakage experiment in Figure 4.3 (section 4.3.2). This time point was chosen as both it precedes significant cell death, which would result in the loss of cytosolic proteins

through a ruptured membrane. Cell proteins were derivatised with DNPH then resolved on 12% acrylamide mini-gels. The ability of equivalent concentrations of acrolein and MVK to carbonylate cell proteins is shown in Figures 4.7a and b respectively. Lane 1 in both figures corresponds to control (RPMI alone) treated cells. A concentration dependent increase in protein carbonylation is evident for both acrolein (Figure 4.7a) and MVK (Figure 4.7b). This is clearer when examining the intensity of carbonylation of proteins smaller than approximately 80 kDa (shown in brackets). In particular, densitometric analysis of the protein band at approximately 55 kDa (indicated by the arrow) reveals that modification of this protein band with 20, 50, 100, 150 and 300 μ M acrolein increases in intensity by approximately 1.5, 1.7, 8.1, 9.6 and 22.2 times respectively compared to the control band (unmodified protein). Similar modification with MVK results in an increase in the intensity of this band by 2.6, 3.3, 4.7, 8.0 and 6.8 times respectively. Although the extent of carbonylation was not strictly linear, it appears more modification occurs following incubation with acrolein than MVK at the highest concentrations (particularly following addition of the higher concentrations of carbonyl), consistent with the results of BSA modification. Interestingly though, at the lower carbonyl concentrations, MVK appeared to modify proteins to a greater extent than acrolein, consistent with observations that MVK induces greater hepatocyte cell death than acrolein.

As a means of finding evidence for hydralazine trapped MVK-protein adducts in cells, an attempt was made to detect these ternary adducts using the rabbit antiserum raised against hydralazine/acrolein-modified KLH used in the previous Chapter. Unfortunately, these adducts could not be detected in proteins from hepatocytes treated with MVK (20 minutes) followed by an additional 20 minutes with hydralazine. Subsequent work revealed that this might be due to the fact that hydralazine-trapped MVK adducts are poor epitopes for the rabbit antiserum compared to their acrolein-derived counterparts.

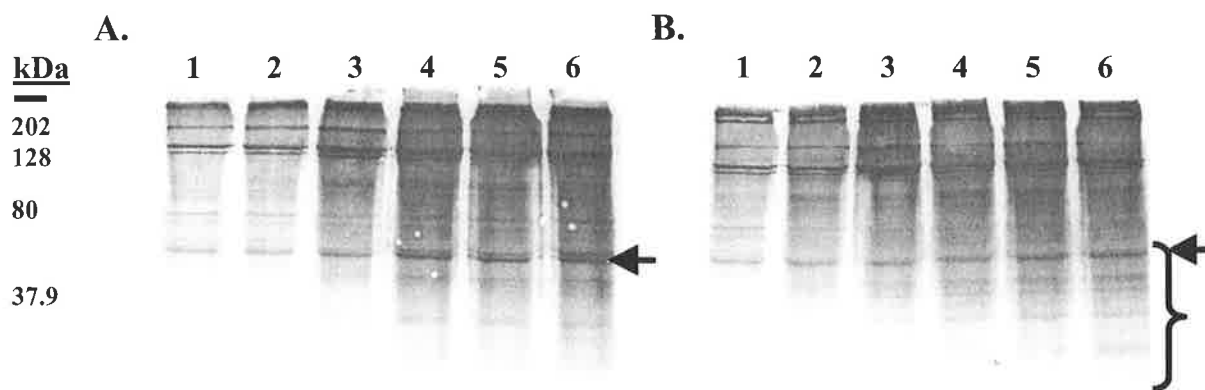


Figure 4.7 Western blot of acrolein (A) and MVK (B) carbonylated proteins in primary cultured mouse hepatocytes. Hepatocytes were incubated with RPMI alone (lane 1) or 20, 50, 100, 150 or 300 μ M acrolein or MVK (lanes 2, 3, 4, 5 and 6 respectively) for 30 minutes. DNP labelled carbonylated hepatocellular proteins (40 μ g) were loaded onto 12% acrylamide gels and separated by electrophoresis. Derivatised proteins were then detected using rabbit anti-DNP antiserum conjugated to alkaline phosphatase and the colour developed using *SigmaFast* BCIP/NBT substrate tablets. The bracket indicates the range of proteins that best highlight the concentration dependent increase in protein carbonylation by acrolein and MVK.

4.4 DISCUSSION

In keeping with theoretical predictions, MVK did not form cyclised products with PPE, even after an extended 22 hour reaction, forming only mono- and bis-Michael adducts at lysine and the N-terminal glycine. The formation of adducts at these 2 residues and the lack of adduction at arginine is comparable to the localisation of adducts formed by acrolein (Chapter 3). The lack of evidence of cyclised or Schiff base adducts over 22 hours provides strong evidence that these adducts are unlikely to be generated during the considerably shorter exposure periods used for toxicity studies (3 to 4 hours). In contrast, Eder and colleagues detected the formation of N2 cyclised products of MVK on deoxyguanosine (Eder *et al*, 1991) although these species do not form via aldol condensations required for FDP-type adducts. DNA adducts presumably form over long periods of time and are perhaps not relevant to the current short term toxicity studies. The findings of Eder *et al* therefore suggest that the β -carbon of MVK is capable of crosslinking and cyclisation reactions on DNA over extended reaction times. The finding that MVK was just as acutely toxic to hepatocytes as acrolein, despite its inability to form FDP-like cyclic adducts, seems to rule out any contribution of these species to the short term toxicity of these unsaturated carbonyl compounds.

The lack of GSH depletion in MVK treated cells was unexpected, but may reflect MVK's slower rate of reaction with GSH compared to the corresponding reaction with acrolein (Esterbauer *et al*, 1975; Eder *et al*, 1991). The differences in GSH depletion by MVK versus acrolein are intriguing given that the two carbonyl compounds exhibited comparable protein carbonylating efficacy. If cysteine residues were major participants in protein carbonylation by these species, we might expect comparable differences to those seen with regards to GSH depletion, since the cysteine group of GSH often displays comparable reactivity to protein cysteine residues. Clearly, future work should address the nature of the residues involved in protein carbonylation by acrolein and MVK.

In light of data showing that less protein adduction occurs with MVK compared to acrolein at the highest concentrations examined (100 to 300 μ M), it is intriguing that MVK was more toxic to hepatocytes than exogenously added acrolein. This is a surprising finding, as typically it would be expected that toxicity correlates with the extent of macromolecule adduction. However, the enhanced toxicity of MVK over acrolein may also reflect slower detoxification of the ketone via the formation of thiol ethers and mercapturic acids and also by oxidative metabolic pathways. Another possibility is the existence of specific sites within the cell that is targeted more effectively by MVK than acrolein. This seems feasible given the discrepancy between the extent of protein carbonylation and toxicity for the two carbonyls. Moreover, perhaps the type of adducts formed (ie. cyclic versus acyclic) is a more important determinant of toxicity than the extent of adduction per se. Since MVK predominantly forms mono-Michael adducts, these may be more toxic to cells than cyclised adducts. This concurs with the results of a study by Palom and colleagues who showed, using mitomycin C, that the proportion of mono-adducted DNA is a more important determinant of toxicity than the proportion of crosslinked species or the extent of adducts formed (Palom *et al*, 2002).

Hydralazine was found to trap MVK adducts formed on PPE and protect against MVK toxicity in isolated hepatocytes. This further supports our hypothesis that hydralazine mediates protection against unsaturated carbonyl toxicity by trapping acyclic Michael adducts, given that these were the only adducts of MVK formed during this time frame. Evidence for this theory would have been stronger had the hydralazine-acrolein/KLH antiserum been able to detect these adducts in hepatocellular proteins. It is of interest that the trapping of MVK-PPE adducts by hydralazine appeared considerably slower than the trapping of acrolein adducts. This may have been due to poor ionisation and detection of hydralazine trapped MVK adducts during mass spectrometric analysis, although this is unlikely given that the relative intensity of MVK-PPE

adducts to unmodified PPE was not significantly changed by the addition of hydralazine. This apparently slower reaction of hydralazine with MVK adducts compared to acrolein adducts, despite strong protection afforded for both carbonyls, may also represent a slower rate of crosslinking by MVK. For instance, if crosslinking was a major determinant in carbonyl toxicity the slower rate of trapping reactions by hydralazine may not hinder its protective effects if MVK crosslinks form slower than acrolein crosslinks. This would result in sufficient hydralazine being present within the cells to trap MVK carbonylated proteins before formation of secondary crosslinks and the initiation of downstream events in toxicity.

Collectively, the data presented in this Chapter supports the hypothesis that hydralazine protects against acute acrolein toxicity by trapping acyclic Michael adducts. It remains possible, however, that FDP-lysine is important in mediating long term toxicity, as it is a highly immunogenic adduct capable of eliciting chronic inflammatory responses that are known to induce oxidative stress through the production of H_2O_2 and cell damage (Klebanoff, 1980; Uchida *et al*, 1998b). These adducts may therefore be very relevant to chronic diseases such as Alzheimer's disease, diabetic nephropathy and atherosclerosis (Uchida *et al*, 1998a; Uchida *et al*, 1998b; Calingasan *et al*, 1999; Suzuki *et al*, 1999). Moreover, although the data thus far has provided evidence that hydralazine protects against 2-alkenal toxicity *in vitro* by trapping reactive carbonylated proteins, no evidence has been obtained to show that this occurs *in vivo*. Such evidence was therefore sought in the following Chapter, using a mouse model of allyl alcohol induced liver damage.

Chapter 5:

Hydralazine mediated protection against allyl alcohol induced hepatic toxicity *in vivo*

5.1 INTRODUCTION

Allyl alcohol is a classic hepatotoxicant that causes severe periportal necrosis in the livers of rats and mice (Piazza, 1915). The mechanism underlying the periportal localisation of allyl alcohol toxicity is unclear and hence requires some discussion. One very early report that assessed alcohol dehydrogenase levels in the liver lobule suggested that alcohol dehydrogenase content is higher in periportal than pericentral areas (Greenberger *et al*, 1965). However, two subsequent reports indicted that alcohol dehydrogenase levels are higher in pericentral areas and that the rate of allyl alcohol metabolism is greater in pericentral than periportal areas of the perfused rat liver (Morrison and Brock, 1967; Belinsky *et al*, 1986). Alternatively, the periportal localisation of allyl alcohol hepatotoxicity may be a reflection of the different isoforms of alcohol dehydrogenase in different regions of the liver lobule. Thus far, six murine isoforms of alcohol dehydrogenase have been identified with varying specificities for different organs (Jornvall and Hoog, 1995). The intra-lobular distribution of the various isoforms in rodent liver, however, has not been explored.

An alternative explanation for the zonal localisation of allyl alcohol toxicity involves the distribution of the acrolein detoxifying enzyme aldehyde dehydrogenase, rather than the allyl alcohol metabolising enzyme. Hence, lower expression of aldehyde dehydrogenase in the periportal region of the liver lobule may contribute to the periportal localisation of allyl alcohol toxicity. However, this issue has been poorly examined in the mouse, and data in rats is

inconsistent. For instance, Yamazaki *et al* (1988) reported higher mitochondrial and microsomal aldehyde dehydrogenase in the centrilobular region of rat liver (Yamazaki *et al*, 1988). Chen *et al* (1992) alternatively reported that mitochondrial aldehyde dehydrogenase was higher in periportal cells, while Maly and Sasse (1987) reported that both isozymes were expressed mainly in periportal cells in female rats and approximately evenly expressed in male rats (Maly and Sasse, 1987; Chen *et al*, 1992). These results generally indicate that aldehyde dehydrogenase distribution in the liver lobule does not contribute to the periportal localisation of allyl alcohol hepatotoxicity. This then raises the possibility that other differences between periportal and perivenous cells may contribute to the zonal heterogeneity in allyl alcohol toxicity.

Given that lipid peroxidation accompanies cell death by allyl alcohol *in vitro* and *in vivo*, the difference in partial pressure of oxygen between periportal and pericentral zones may influence the periportal localisation of allyl alcohol toxicity, as these zones contain higher partial pressures of oxygen than pericentral zones. Investigations of this possibility to date, however, have proved somewhat inconclusive. For instance, Badr and colleagues reported greater periportal (than pericentral) toxicity when the rat liver was perfused with allyl alcohol in the retrograde direction (Badr *et al*, 1986). However, in a follow up study published in the same year, the authors reported that this toxicity was in part an artefact resulting from liver perfusion (Belinsky *et al*, 1986) suggesting that only limited conclusions could be drawn from the earlier study. However, a subsequent study by Brown *et al* (1991) suggested that following allyl alcohol perfusion, the oxygen dependent conversion of xanthine dehydrogenase to the ROS generating xanthine oxidase was higher in periportal than pericentral areas (Brown *et al*, 1991). Collectively, these results tend to suggest some dependence of oxygen in allyl alcohol hepatotoxicity, although such toxicity also appears to involve immune cells such as neutrophils and Kupffer cells (Przybocki *et al*, 1992; Ganey and Schultze, 1995; Kinser *et al*, 2002).

It was reported in Chapters 3 and 4 that protein adducts formed by acrolein can be targeted using carbonyl-trapping drugs such as hydralazine and structurally related hydrazines. Also, together with a number of hydrazine-containing structural analogues, hydralazine strongly attenuated allyl alcohol toxicity in isolated mouse hepatocytes (Chapter 2). Using rabbit antiserum raised against hydralazine-stabilised acrolein adducts in the carrier protein KLH, Burcham and colleagues detected clear concentration-dependent adduct-trapping in proteins from allyl alcohol-pretreated hepatocytes exposed to 2 to 50 μM concentrations of hydralazine (Burcham *et al*, 2004). Also, as shown in Chapter 3, hydralazine traps the early acrolein Michael adducts of a model lysine containing peptide, PPE. These findings suggested that hydralazine might protect cells by

attacking early products of reactions between acrolein and cell proteins, thereby blocking subsequent deleterious chemical reactions.

The aim of the experiments in this Chapter was to explore the *in vivo* relevance of these findings. It was hypothesised that hydralazine blocks liver toxicity induced by allyl alcohol administration by interfering with protein adduction by acrolein, while not preventing the decrease in hepatic GSH which precedes allyl alcohol intoxication. This latter point arises from the finding that hydralazine attenuated allyl alcohol induced hepatocellular toxicity *in vitro*, but did not prevent the decrease in GSH (Chapter 2). It was also hypothesised that this protection would involve strong hydralazine mediated adduct trapping, particularly in periportal regions of the liver lobule where allyl alcohol toxicity is localised. Hydralazine's ability to block liver toxicity induced by allyl alcohol was examined in whole mice by assessing the activity of the liver specific enzymes sorbitol dehydrogenase and glutamate pyruvate transaminase in plasma. Although these enzymes are found within other organs, including the heart and kidney, they are considered organ-specific indicators of liver damage as they are absent from the plasma of healthy subjects with no liver disease (Gerlach, 1957; Gerlach, 1967; Gerlach, 1968; Kreuzer *et al*, 1998). As such, they are used clinically as indicators of liver damage, and by researchers to assess the extent of liver damage induced by liver specific toxins and diseases. Second, it was determined whether any hepatoprotection afforded by hydralazine minimised the hepatic glutathione depletion occurring in these animals. Finally, using antiserum raised against hydralazine-stabilised acrolein-protein adducts, Western blotting and immunohistochemical analysis was used to determine whether adduct-trapping accompanied any effects of hydralazine on acrolein-mediated hepatotoxicity.

5.2 METHODS

5.2.1 Chemicals

L-alanine was supplied by Aldrich Chemical Co. (Milwaukee, WIS, USA). NADH, α -ketoglutarate, fructose, phenylmethanesulphonyl fluoride (PSMF), 3-aminopropyltriethoxy silane (APES), benzamidine and type II lactate dehydrogenase were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glycerol was from May and Baker (Vic, Australia). EDTA was purchased from Amresco (Solon, Ohio, USA). The primary polyclonal antibody used to detect hydralazine trapped acrolein-protein adducts in mouse livers *in vivo* was raised in

rabbits against hydralazine stabilised acrolein/KLH (Burcham *et al*, 2004). The secondary antibody and luminescence reagent used for Western blots was as previously described. The secondary antibody for immunohistochemistry was a FITC-conjugated goat anti-rabbit (Pierce, Rockford, IL, USA). All other chemicals used were as previously mentioned.

5.2.2 Animals

Male Swiss mice (aged 4 to 6 weeks) were used in this study. The animals were obtained and housed as mentioned previously. Food (rodent chow), but not water, was withheld for 18 hours prior to and during experiments to minimise variations in initial liver GSH levels. The Animal Ethics Committee of the University of Adelaide approved the use of mice for these experiments.

5.2.3 Dose Response and Time Course Determination

5.2.3.1 Protocol

The mice were caged into their respective treatment groups at the time fasting began. All mice were given their respective treatments between 0900 and 1100 hours to minimise the influence of diurnal variations in hepatic GSH levels at the time of sacrifice. Allyl alcohol (AA) solutions were freshly prepared in PBS each day. To determine the most appropriate duration over which to treat the animals 5 to 6 mice for each time point were dosed with 80 mg/kg allyl alcohol and anaesthetised with 0.05 – 0.10 ml Nembutal 2, 4 or 6 hours later. The chest and abdominal cavity were then opened and blood was withdrawn into heparinised syringes by cardiac puncture. The blood was stored on ice until all mice had been sacrificed and then centrifuged at 7000 x g for 3 minutes, after which the plasma was extracted and stored at -20°C until analysed for sorbitol dehydrogenase (SDH) or glutamate pyruvate transaminase (GPT) activities. The liver was removed, weighed and homogenised in 9 volumes of 3% PCA with an Ultra-Turax homogeniser (John Morris Pty. Ltd, Adelaide, Australia). The liver homogenate was then stored at 4°C until analysed for GSH as previously described (section 2.2.10.3).

The effect of varying allyl alcohol dose on liver toxicity in mice was determined by injecting a group of mice with either 80, 90 or 100 mg/kg allyl alcohol or PBS vehicle intraperitoneally, in an injection volume of 10 ml/kg. Mice were then sacrificed 4 hours later and blood collected as described above.

5.2.3.2 Statistics

Serum enzyme data were analysed by non-parametric Kruskal-Wallis analysis with Dunn's post hoc tests. GSH data was analysed by parametric 1 way ANOVA's as there was consistent variance in the data. All groups were then compared by Tukey's post tests. Significance was determined at a level of $p < 0.05$.

5.2.4 Protection Against Allyl Alcohol Hepatotoxicity by Hydralazine

5.2.4.1 Protocol

The ability of hydralazine to protect mouse livers against allyl alcohol toxicity was explored using 80 and 90 mg/kg doses of allyl alcohol. The doses of hydralazine used were 100, 200 and 300 $\mu\text{mol/kg}$ (20, 40 and 60 mg/kg). Hydralazine was made up in PBS fresh on the day of use. Allyl alcohol and hydralazine were co-injected in a final injection volume of 10 ml/kg. The 2 compounds were mixed together within 5 minutes of injection. It was previously determined that allyl alcohol and hydralazine do not react together (data not shown). The mice were anaesthetised with Nembutal 4 hours after receiving allyl alcohol and their blood and liver extracted and treated as previously mentioned.

In addition, 12 mice were treated with 90 mg/kg allyl alcohol (10 ml/kg) followed 20 or 30 minutes later by 200 $\mu\text{mol/kg}$ hydralazine in order to determine the time dependence for the loss of hydralazine mediated hepatoprotection. Blood and liver were collected and analysed 4 hours later as described above.

5.2.4.2 Statistics

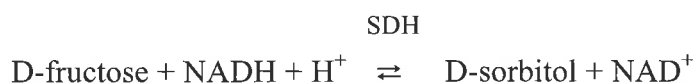
Plasma enzyme data were compared by Kruskal-Wallis analysis with a Dunn's post hoc test. Liver GSH levels were compared to control GSH via a 1 way ANOVA with a Dunnett's post test. Significance was at a level of $p < 0.05$.

5.2.5 Plasma Enzyme Assays

5.2.5.1 Analysis of Plasma Sorbitol Dehydrogenase (SDH)

Plasma SDH activity was analysed based on the method of Asada and Galambos (1963). The principle behind this assay is illustrated in Equation 1. A 100 μ l sample of plasma was mixed with 500 μ l of 0.4 mM NADH in Tris buffer (0.1 M, pH 7.5) and incubated for at least 10 minutes at room temperature to allow the removal of interfering metabolites, particularly ketoses (Asada and Galambos, 1963). Where dilution of plasma was required this was performed in Tris buffer. The reaction was started by the addition of 100 μ l fructose (4 M in Tris buffer) and the loss of NADH was monitored for 3 minutes at room temperature in a spectrophotometer (Metertek SP-830, Analytical Equipment Co, South Australia, Australia) at 340 nm. SDH content was reported as U/l of plasma, where 1 U is defined as the amount of enzyme required to produce 1 μ mol of NAD^+ per minute at 25°C. This was determined using Equation 2 (Asada and Galambos, 1963).

Equation 1:



Equation 2:

$$\text{Enzyme activity (U/l)} = \frac{\text{Final volume (ml)} \times 1000}{6.22 \times \text{volume of plasma (ml)}} \times \frac{\text{change in absorbance}}{\text{Time (mins)}}$$

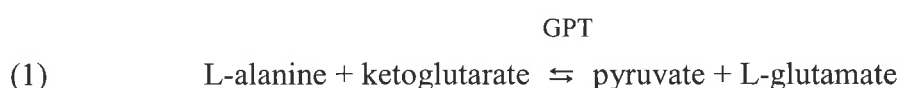
5.2.5.2 Analysis of Plasma Glutamate Pyruvate Transaminase

The LDH used in this method was dialysed against 3 changes of 10 mM potassium phosphate buffer (pH 7.4) over night at 4°C to remove ammonium ions in suspension. The resulting solution was diluted 1:1 in glycerol to make up a final concentration of approximately 4.75 mg protein/ml and stored at 4°C (Bergmeyer and Bernt, 1974).

Plasma GPT activity was analysed according to the method described by Bergmeyer and Bernt (1974). The principle behind this assay is illustrated in Equation 3. A 100 μ l sample of plasma

(whole or diluted in PBS), was added to a mixture composed of 600 µl of 1 M alanine in potassium phosphate buffer (0.1 M, pH 7.4), 10 µl of LDH (0.5 mg protein/ml in 50% glycerol) and 10 µl of 13 mM NADH (made up in 0.12 M NaHCO₃). The alanine/phosphate buffer was prepared fresh each day. The ketoglutarate and NADH solutions were stored for up to 1 month at 4°C. The reaction mixture was incubated at room temperature for at least 3 minutes to remove interfering substances from plasma. The reaction was then started by the addition of 20 µl ketoglutarate solution (0.66 M in water, pH approximately 7.0). The loss of NADH was monitored for 3 minutes. Plasma GPT was reported in U/l of plasma, where the definition of 1 U was the same as for SDH. Equation 2 was also used to determine plasma GPT levels.

Equation 3:



5.2.6 Western Blot Analysis of Hydralazine Stabilised Acrolein-Protein Adducts in Liver

5.2.6.1 Preparation of Liver Homogenate

In addition to the mice that received allyl alcohol and hydralazine treatment for analysis of hepatoprotection, 8 mice were treated with either PBS, allyl alcohol alone (90 mg/kg), hydralazine alone (100 or 200 µmol/kg) or allyl alcohol (90 mg/kg) plus hydralazine (100 or 200 µmol/kg) to assess the extent of hydralazine trapped acrolein-protein adducts in liver. This procedure was performed with assistance from Dr Phil Burcham and David Ascher. Mice received the treatments in a single ip injection (10 ml/kg) and sacrificed 1 hour later. The livers were perfused with 25% sucrose solution for further immunohistochemical processing of the tissue. The right medial lobe was removed for immunohistochemical staining and the remainder of the liver was homogenised for 1 minute using an Ultra-Turrax homogeniser in 9 volumes (w/v) of a chilled lysis buffer that contained 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.2 mg/mL phenylmethylsulfonyl flouride and 1 mM benzamidine. The resulting homogenate was then centrifuged for 10 minutes at 1500 x g at 4 °C. An aliquot of the resulting supernatant was diluted with 0.1 M NaOH and assayed for protein content using the BCA assay

(Bollag *et al*, 1996). After mixing aliquots of supernatant with 4 volumes of dilution buffer (1% SDS, 25% glycerol and 1% Igepal CA-630) samples were further diluted with SDS/PAGE loading buffer (as reported in Chapter 4, section 4.2.7.2). After heating at 80 °C for 5 minutes, aliquots containing 125 µg protein were loaded onto a 12.5% acrylamide gel (4% stacking gel).

5.2.6.2 Electrophoresis Separation of Liver Proteins and Western Blotting Procedure

Electrophoresis of liver proteins and Western Blotting was performed by Dr Phil Burcham. The electrophoresis and transfer buffers were made as described in Bollag (1996). Proteins were resolved overnight on a 12.5% acrylamide gel using a BioRad Protean II electrophoresis tank at 120 V. After soaking gels in transfer buffer for at least 20 minutes, proteins were transferred to a nitrocellulose membrane (MSI *NitroBind*, 0.22 µm, BioRad) during a 30 minute transfer at 100 V in Towbins transfer buffer. The membrane was then blocked for 30 minutes using 5% nonfat milk powder in PBS.

5.2.6.3 Immunodetection of Hydralazine/Acrolein-Modified Protein

The blocked membrane was then treated with antiserum from rabbits immunised with hydralazine/acrolein-modified KLH [1:1000, 60 minutes, Burcham *et al*, 2004]. The membrane was then washed as previously described (section 3.2.3.1) and treated with horseradish peroxidase-coupled goat anti-rabbit IgG (1:10,000; Pierce, *Immunopure*). After washing, membranes were treated with Pierce *SuperSignal West Pico* Chemiluminescence Reagent (described in section 3.2.3.1). The resulting membranes were then exposed to Kodak BioLight film and developed as previously mentioned (section 3.2.3.1).

5.2.7 Immunohistochemical Identification of Hydralazine Trapped Acrolein-Protein Adducts in Mouse Liver

5.2.7.1 Coating of Microscope Slides with 3-Aminopropyltriethoxy Silane (APES)

An APES working solution was prepared by dissolving 10 ml APES in 500 ml absolute ethanol and stored at 4°C when not in use. Glass microscope slides (76.2 x 25.4 mm, 1.0 – 1.2 mm thick, Livingstone, Sydney, Australia) were rinsed twice in absolute ethanol, dipped in the APES working solution for approximately 3 seconds and rinsed in absolute ethanol followed by deionised water. The slides were dried at 37°C overnight and stored in the dark until used.

5.2.7.2 Immunohistochemical Detection of Hydralazine Trapped Acrolein-Protein Adducts in Mouse Liver

Mice were treated with allyl alcohol and hydralazine as described above (section 4.2.4.1). Additional mice were sacrificed an hour after receiving either a single dose of *E*-APH, the main product formed during trapping reactions between free acrolein and hydralazine (300 $\mu\text{mol/kg}$ in 10% dimethyl sulphoxide/PBS) or allyl alcohol (90 mg/kg) plus hydralazine (300 $\mu\text{mol/kg}$) as a single ip injection. Livers were perfused with 25% sucrose in phosphate buffer (50 mM, pH 7) and the right medial lobes were removed as previously described, frozen in liquid nitrogen and then stored at -20°C until sectioned. Liver sections (5 μm) were cut using a cryostat maintained at -20°C and then fixed in methocarn solution (methanol:chloroform:acetic acid, 6:3:1) for 20 minutes. Following rehydration through ethanol, slices were blocked in 10% skim milk powder/PBS for 1 hour. They were then treated overnight at 4°C with the primary antibody (1:750, rabbit anti-hydralazine/acrolein-modified KLH) or non-immune rabbit serum in 10% nonfat milk powder in PBS. In addition, 2 liver sections from a mouse treated with allyl alcohol and 100 $\mu\text{mol/kg}$ hydralazine were treated with primary antibody that had been preincubated with 2 mg/ml of hydralazine/acrolein-modified poly-L-lysine or poly-L-histidine (2 hours, 37°C). These immunohistochemical experiments were used to assess the identity of amino acid residues modified by acrolein/hydralazine *in vivo*. The sections were then washed thoroughly in PBS before they were treated with FITC-labelled goat anti-rabbit IgG (Pierce, 1:400 in 10% nonfat milk powder/PBS) at room temperature for 1 hour. Sections were then washed in 0.05% Tween 20 in PBS (3x) followed by 1x PBS wash, mounted in 50% glycerol/PBS and then viewed using an Olympus BX50WI fluorescence microscope with a 10x objective lens (Olympus Optical Co, Japan). Images were collected using a SPOT advanced V3.0 imaging system (SciTech, Vic, Australia) and with assistance by David Ascher.

5.3 RESULTS

5.3.1 Determination of Appropriate Exposure Duration for Allyl Alcohol Hepatotoxicity

To determine the most appropriate dose of allyl alcohol to be used for toxicity studies in Swiss mice, a dose response for acute liver damage was performed. However, prior to this, it was necessary to choose an appropriate duration for examining allyl alcohol induced liver damage. An allyl alcohol dose of 80 mg/kg was used in this study based on previous reports showing that this dose elicits pronounced hepatic injury in mice (Jaeschke *et al*, 1987; Rikans, 1987; Ferrali *et al*, 1989). Treatment times ranging from 2 to 6 hours were examined based on previous reports showing acute allyl alcohol induced elevations in serum enzymes are maximal over this time frame (Pompella *et al*, 1988). Liver injury was assessed by measuring plasma SDH activity at each time point. Figure 5.1a shows the levels of plasma SDH at each of the time points examined following allyl alcohol dosing (ip). Plasma SDH activity was not significantly elevated from control values in mice 2 hours after allyl alcohol treatment. However, a significant increase in plasma SDH was seen 4 hours after allyl alcohol administration, where the plasma concentration was 144.1 ± 34.3 U/l ($p < 0.001$) compared to 10.7 ± 1.0 U/l in controls. Following this, plasma SDH moderately decreased 6 hours after allyl alcohol administration to 83.8 ± 23.5 U/l which was not statistically significantly compared to control SDH. Corresponding GSH concentrations in liver are shown in Figure 5.1b. Liver GSH levels decreased significantly by approximately 85%, from 1.69 ± 0.18 μ g/mg liver to 0.28 ± 0.11 μ g/mg liver within 2 hours of allyl alcohol administration ($p < 0.001$). GSH levels then progressively recovered over the next 4 hours to approximately one-third and one-half of control GSH, 4 and 6 hours after allyl alcohol dosing respectively. The levels of hepatic GSH 4 and 6 hours after allyl alcohol were still significantly lower than those of control animals ($p < 0.001$ and 0.01 respectively), although they were not significantly different to GSH 2 hours after allyl alcohol.

Based on this data, 4 hours was chosen as the most appropriate time following allyl alcohol dosing to examine liver damage induced by the alcohol, as it appeared that maximal elevation of plasma SDH occurred at this time point. Allyl alcohol induced hepatic toxicity was therefore examined at this time point in future experiments examining the ability of hydralazine to attenuate this damage.

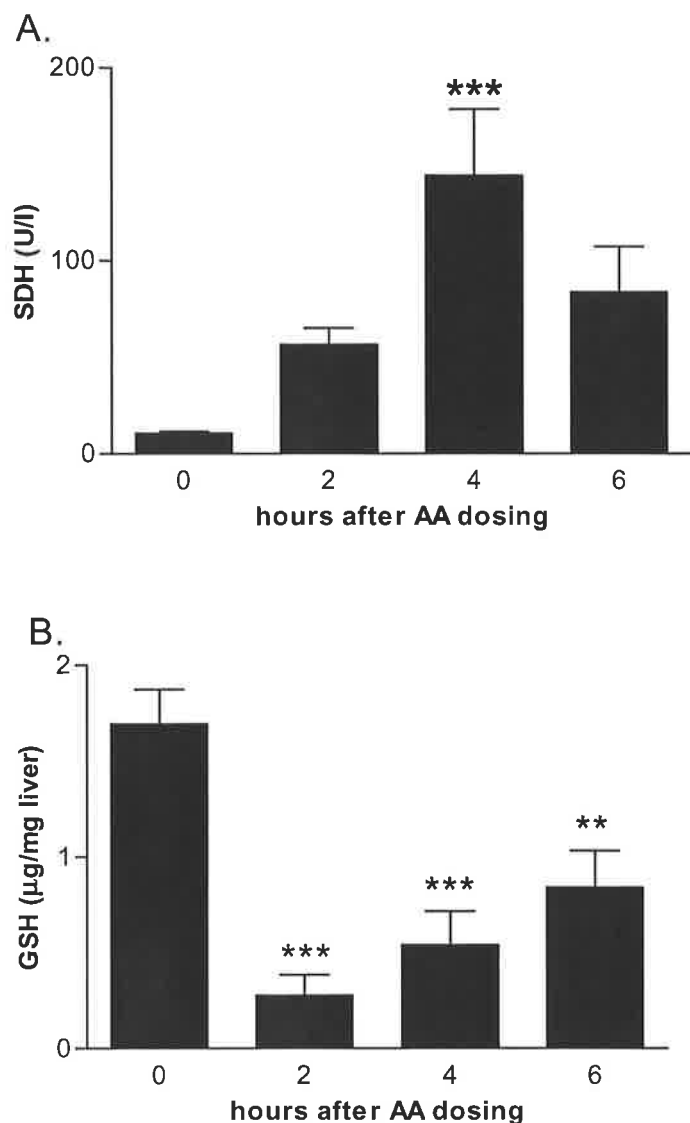


Figure 5.1 Time dependence for changes in plasma SDH (A) and liver GSH (B) following dosing of Swiss mice with 80 mg/kg allyl alcohol (AA). Mice were sacrificed 0, 2, 4 or 6 hours after AA and blood collected by open cardiac puncture. Data are represented as mean $\pm$ SEM of plasma SDH (U/l) and liver GSH (μ g/mg liver). Plasma SDH following AA and control were compared by Kruskal Wallis analysis with a Dunn's post test. Liver GSH results were compared by 1 way ANOVA with a Tukey's post test. ** $p < 0.01$, *** $p < 0.001$ cf control, $n = 5-11$.

5.3.2 Dose Response for Allyl Alcohol Induced Increases in Plasma SDH and GPT in Mice

To identify a dose of allyl alcohol producing reproducible liver toxicity, a second pilot experiment was conducted. Mice received various doses of allyl alcohol and were sacrificed 4 hours later. Significant elevations in plasma SDH activity were evident in mice 4 hours after they received 100 mg/kg allyl alcohol (Figure 5.2a), while GPT was significantly increased by 90 and 100 mg/kg doses of the alcohol (Figure 5.2b). While 80 mg/kg allyl alcohol did not

significantly alter the activity of either plasma marker, this dose decreased hepatic GSH levels to approximately one-third of control values 4 hours after allyl alcohol administration (Figure 5.2c, $p < 0.01$). Comparable hepatic GSH depletion was also produced by the two higher doses of allyl alcohol (90 and 100 mg/kg). Although the 100 mg/kg dose of allyl alcohol produced an appropriate degree of enzyme leakage for use in protection studies using hydralazine, approximately 30% of mice that received this dose died within 4 hours (data not shown). This dose of allyl alcohol was therefore deemed inappropriate for further experimentation based on animal ethics considerations. The 90 mg/kg dose of allyl alcohol was therefore used in the subsequent study, as it induced a significant increase in plasma markers of liver damage while no deaths occurred within 4 hours of its administration (data not shown).

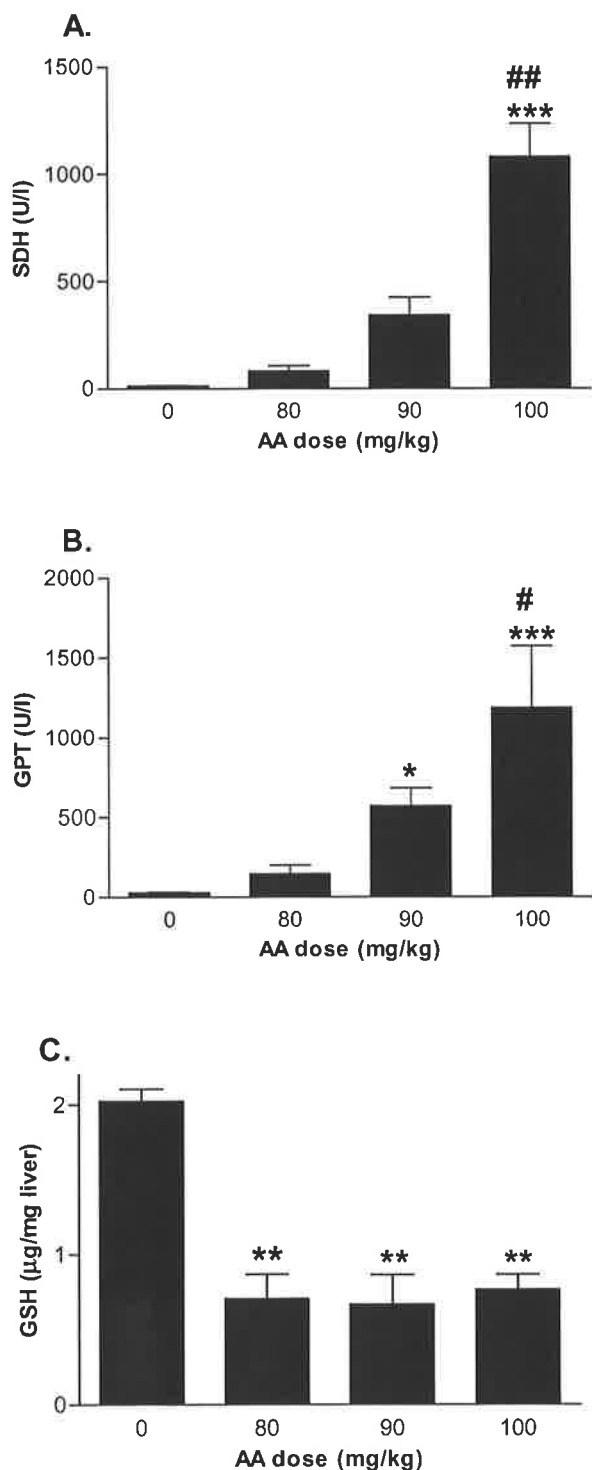


Figure 5.2 Allyl alcohol (AA) dose response for increased plasma SDH (A), GPT (B) and decreased liver GSH (C). Swiss mice were sacrificed 4 hours after AA dosing. Data are represented as mean $\pm$ SEM for plasma SDH and GPT (U/l) and for GSH (μ g GSH/mg liver). SDH and GPT data were compared by Kruskal Wallis analysis with a Dunn's post test. GSH data were compared by 1 way ANOVA with a Dunnett's post test. # $p < 0.05$, ## $p < 0.01$ cf 80 mg/kg dose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ cf control (no AA), $n = 6-13$.

5.3.3 *In Vivo* Protection Against Allyl Alcohol Hepatotoxicity by Hydralazine

For studies of the ability of hydralazine to protect against allyl alcohol induced liver injury, hydralazine was co-administered with allyl alcohol as a single intraperitoneal injection. As shown below, allyl alcohol (90 mg/kg) produced 30 to 24 fold increases in the plasma activity of SDH and GPT 4 hours after ip administration (Figure 5.3a and b respectively). Co-administration of hydralazine with allyl alcohol afforded strong, dose-dependent protection against the changes in the plasma enzymes (Figures 5.3a and b). The top hydralazine dose (300 $\mu\text{mol/kg}$) provided almost total protection against the serological changes induced by allyl alcohol ($p < 0.01$). The 200 $\mu\text{mol/kg}$ dose of hydralazine significantly protected against the rise in SDH, but not GPT, induced by allyl alcohol ($p < 0.05$). This was due to the presence of an outlier GPT value in one mouse at the 200 $\mu\text{mol/kg}$ dose of hydralazine, which could not be eliminated as the plasma sample in question did not contain unusually high SDH activity. This highlights the value of examining two plasma markers of liver damage instead of only one. Hydralazine doses affording half-maximal protection against liver injury (EC_{50}) were calculated as approximately 160 and 80 $\mu\text{mol/kg}$ for SDH and GPT respectively. This shows that hydralazine provides half-maximal protection against allyl alcohol mediated hepatic toxicity at doses equating to approximately $1/9^{\text{th}}$ to $1/18^{\text{th}}$ the dose of the alcohol (ie. approximately 120 $\mu\text{mol/kg}$ hydralazine compared with 1552 $\mu\text{mol/kg}$ allyl alcohol).

Following allyl alcohol administration, hepatic GSH levels were depressed by approximately two-thirds compared to control GSH four hours after ip administration ($p < 0.01$, Figure 5.3c). Although hydralazine significantly protected against the rise in plasma SDH and GPT, it was unable to prevent the decrease in liver GSH induced by allyl alcohol at any of the doses examined. This is consistent with the *in vitro* results reported in Chapter 2.

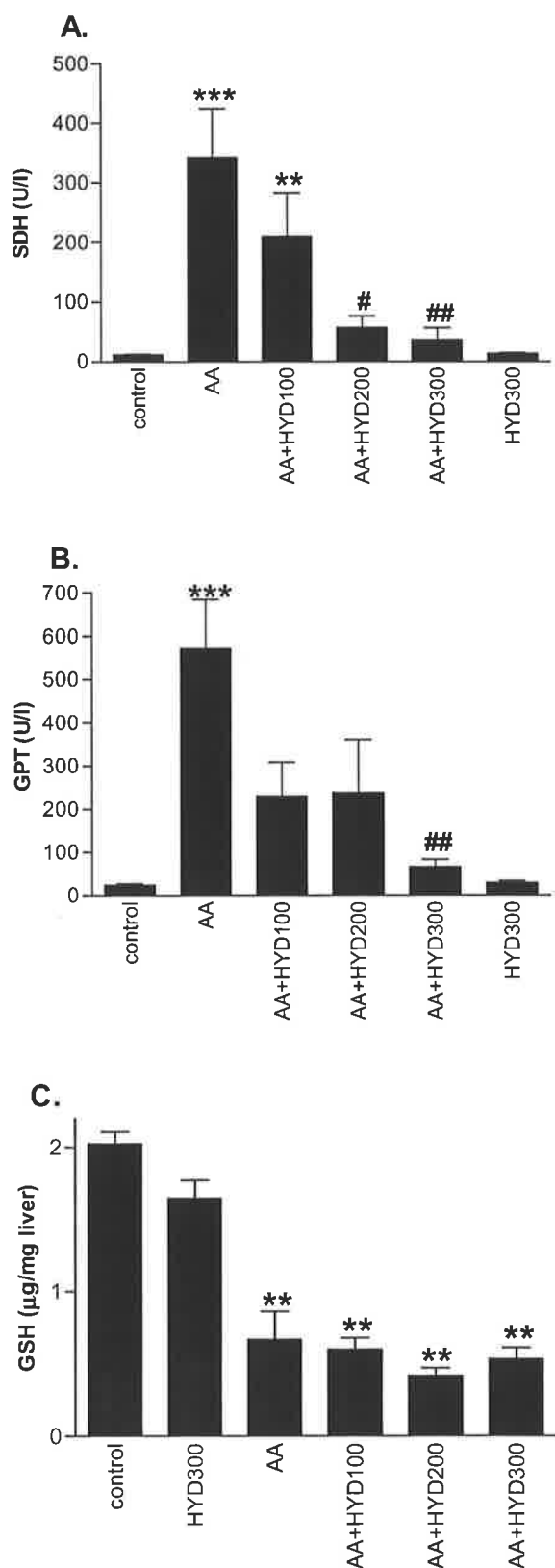


Figure 5.3 Effect of hydralazine (HYD) co-administration on allyl alcohol (AA) induced changes in plasma SDH (A), GPT (B) and liver GSH (C). AA (90 mg/kg) was administered (ip) alone or co-administered with 100-300 $\mu\text{mol}/\text{kg}$ HYD (HYD100, HYD200, HYD300). Mice were sacrificed 4 hours after treatment. Data are represented as mean $\pm$ SEM of plasma SDH or GPT (U/l) or liver GSH (μg GSH/mg liver). Plasma SDH and GPT were compared by Kruskal Wallis analysis with a Dunn's post test. Liver GSH was compared by 1 way ANOVA with a Dunnett's post test. # $p < 0.05$, ## $p < 0.01$ cf AA, ** $p < 0.01$, *** $p < 0.001$ cf control (PBS), $n = 6-8$.

5.3.4 Time Dependence of Hydralazine Mediated Protection Against Allyl Alcohol Hepatotoxicity

Previous studies conducted by Burcham *et al* (2004) revealed that hydralazine affords protection against allyl alcohol toxicity *in vitro* when administered within 25 minutes of the toxin. The time dependence for this protection was therefore studied *in vivo* to determine the significance of the *in vitro* hepatocellular data to a whole animal model.

As shown previously in this Chapter, allyl alcohol (90 mg/kg) induced a significant increase in plasma SDH activity 4 hours after ip administration. Co-treatment with 200 μ mol/kg hydralazine significantly protected against these changes. Similarly, when hydralazine was administered 20 minutes after allyl alcohol, protection was comparable to that seen with the co-administration protocol (Figure 5.4a). However, when this dose of hydralazine was administered 30 minutes after allyl alcohol, no significant protection was afforded. As expected, the administration of hydralazine had no effect on the decrease in GSH induced by allyl alcohol under each of these dosing regimens (Figure 5.4b). This data concurs with the *in vitro* results of Burcham and colleagues, suggesting that hydralazine must be administered within 20 to 25 minutes of a large, hepatotoxic dose of allyl alcohol.

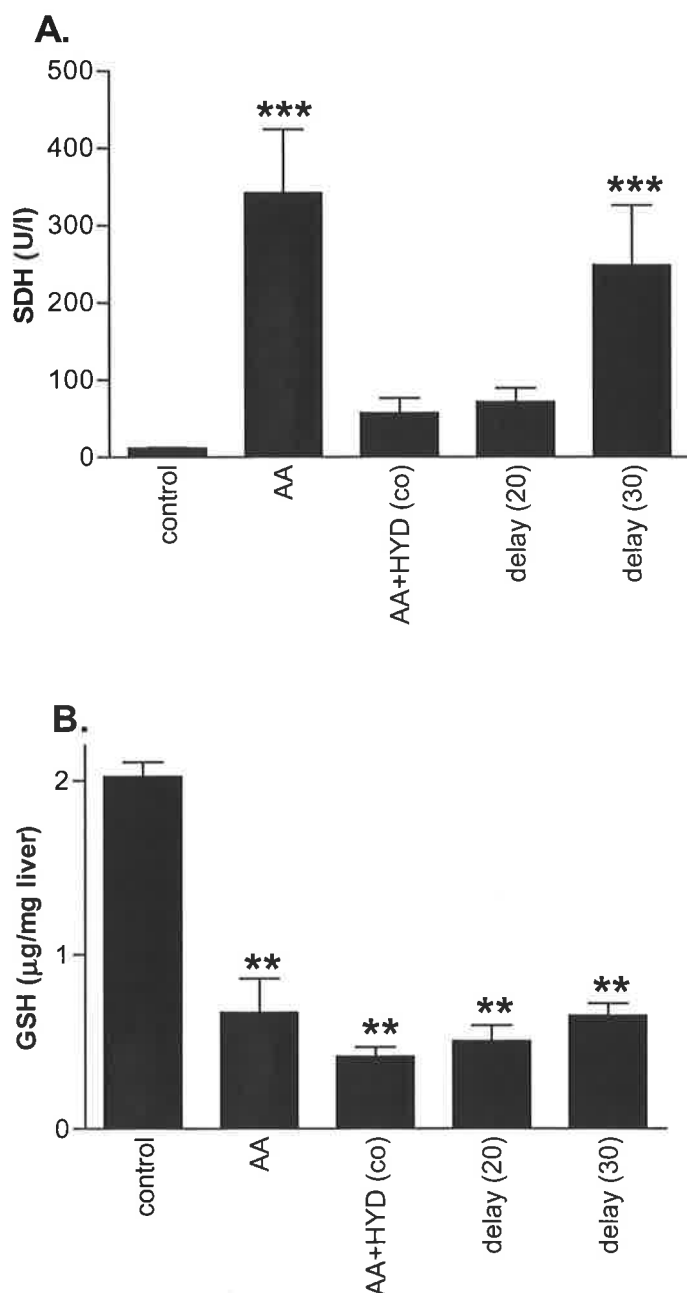


Figure 5.4 Time dependence of hydralazine (HYD, 200 $\mu\text{mol}/\text{kg}$) mediated protection against allyl alcohol (AA, 90 mg/kg) induced increase in plasma SDH (A) and decreased liver GSH (B). HYD was administered as a single injection with AA (co) or after 20 to 30 minutes of the AA dose (delay). The time delay in minutes between AA and HYD administration is indicated in parentheses on the graphs. Data are represented as mean $\pm$ SEM for plasma SDH (U/l) and liver GSH (μg GSH/mg liver). Plasma SDH data were analysed by Kruskal Wallis analysis with a Dunn's post test. Liver GSH data were compared by 1 way ANOVA with a Dunnett's post test. ** $p < 0.01$, *** $p < 0.001$ cf control (PBS), $n = 5-9$.

5.3.5 Western Blot of Hydralazine Trapped Acrolein-Protein Adducts *In Vivo*.

Trapping of acrolein-protein adducts by hydralazine in isolated mouse hepatocytes treated with allyl alcohol was previously reported by Burcham *et al* (2004). Given the current hypothesis suggesting that protection by hydralazine involves trapping reactions at acrolein-protein adducts, evidence of this phenomenon was sought *in vivo*. This was achieved by examining the levels of hydralazine-trapped acrolein/protein adducts in the livers of mice treated with allyl alcohol and hydralazine by Western blotting and immunohistochemistry. Mice were administered PBS, hydralazine alone (100 or 200 $\mu\text{mol/kg}$), allyl alcohol alone (90 mg/kg) or allyl alcohol plus hydralazine and sacrificed 1 hour later.

Figure 5.5 shows intense dose dependent trapping of acrolein-protein adducts in the livers of mice treated with allyl alcohol and hydralazine. Lanes 1 to 4 correspond to proteins from the livers of mice treated with PBS, allyl alcohol alone, 100 $\mu\text{mol/kg}$ hydralazine alone and 200 $\mu\text{mol/kg}$ hydralazine alone respectively. No significant adduct trapping was detected in these livers. Lanes 5 and 6, however, reveal strong adduct trapping in 2 mice treated with allyl alcohol (90 mg/kg) and 100 $\mu\text{mol/kg}$ hydralazine. Protein bands corresponding to masses in the 20 to 60 kDa range were clear targets for the drug. Proteins larger than this were generally not well resolved. Lanes 7 and 8 correspond to 2 mice that received allyl alcohol and 200 $\mu\text{mol/kg}$ hydralazine. These two lanes clearly show a dose dependent increase in the immunorecognition of hydralazine trapped acrolein-protein adducts compared to lanes 5 and 6. At the highest dose of hydralazine (200 $\mu\text{mol/kg}$), while bands larger than 40 kDa are unresolved, bands smaller than 40 kDa show dose dependent increases in adduct trapping, relative to the lower dose of hydralazine (100 $\mu\text{mol/kg}$). This data shows that dose dependent formation of hydralazine trapped acrolein-protein adducts occurs in the livers of allyl alcohol and hydralazine treated mice.

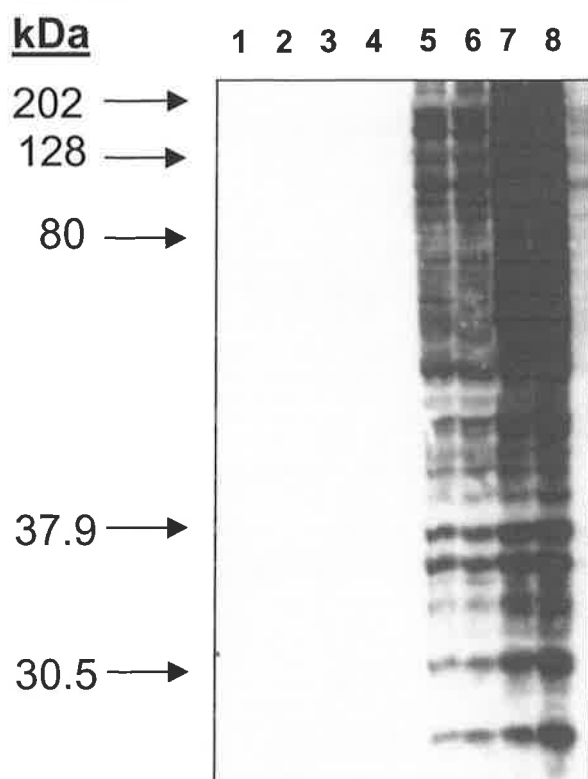


Figure 5.5 Western blot of hydralazine labelled acrolein-protein adducts in the livers of Swiss mice treated with PBS (lane 1), allyl alcohol alone (AA, 90 mg/kg; lane 2), hydralazine alone (HYD, 100 and 200 μ mol/kg; lanes 3 and 4 respectively), AA and HYD (100 μ mol/kg HYD; lanes 5 and 6) and AA and HYD (20 μ mol/kg HYD; lanes 7 and 8). Animals co-treated with AA and HYD were performed in duplicate, such that lanes 5 to 8 represent the livers of individual mice. Mice were heparinised and anaesthetised 1 hour after dosing to allow for perfusion of the livers with 25% sucrose solution.

5.3.6 Immunohistochemical Localisation of Hydralazine Trapped Acrolein-Protein Adducts in Liver

Allyl alcohol hepatotoxicity is typically localised to periportal regions of the liver lobule (Piazza, 1915). This was also confirmed in the current study, where hematoxylin and eosin stained liver sections revealed necrotic hepatocytes in periportal zones 24 hours after administration of 90 mg/kg allyl alcohol (data not shown). These zones were surrounded by hepatocytes undergoing balloon degeneration, indicative of reversible cell damage, and areas of phagocytic neutrophil migration (data not shown). It was therefore expected that hydralazine trapped acrolein-protein adducts would be localised to these regions of the liver lobule, as it was expected that these areas would be richer in acrolein-adducted proteins than pericentral regions. The right medial lobe was therefore taken from mice for immunohistochemical localisation of adducts.

Livers from PBS, allyl alcohol alone and hydralazine alone treated mice contained a low level of background immunofluorescence as shown in Figure 5.6a. This background was not due to inadequate removal of FITC conjugated secondary antibody, as liver sections that had not been treated with serum or FITC labelled antibody also exhibited comparable fluorescence (data not shown). Also, liver sections from the mouse treated with *E*-APH alone contained no significant immunofluorescent labelling compared to PBS control (Figure 5.6b). However, the livers of mice treated with allyl alcohol and 300 $\mu\text{mol/kg}$ hydralazine showed very strong fluorescent labelling, which made identification of cellular structure difficult as the section was saturated with the fluorescence label (Figure 5.6c). This labelling was not present in the same liver treated with non-immune rabbit serum (similar immunofluorescent labelling as seen in Figure 5.6a). Due to the saturation of the fluorescence signal in Figure 5.6c, this dose of hydralazine provided limited insight into the localisation of hydralazine labelled acrolein-protein adducts. Use of a lower dose of hydralazine (100 $\mu\text{mol/kg}$) resulted in less immunofluorescent labelling that was still clearly more intense than in control sections (Figure 5.6d). Unexpectedly, these sections did not reveal appreciable periportal localisation of the adducts, with drug-trapped adducts distributed almost evenly throughout the liver lobule (Figure 5.6d, note the lower magnification compared with the control section). Slight pericentral staining was occasionally seen in the livers of some mice, an outcome that appeared related to incomplete sucrose perfusion of the liver. A higher magnification of the section in Figure 5.6d (400X compared with 100X, Figure 5.6e) is shown to highlight the intracellular localisation of hydralazine labelled acrolein-protein adducts. This section reveals strong immunofluorescent labelling in the cytosol and cellular membranes (CM). No labelling is seen in the nucleus (N). This indicates that strong global adduct trapping occurs in the cytosol and cell membranes of mouse livers exposed to allyl alcohol and hydralazine

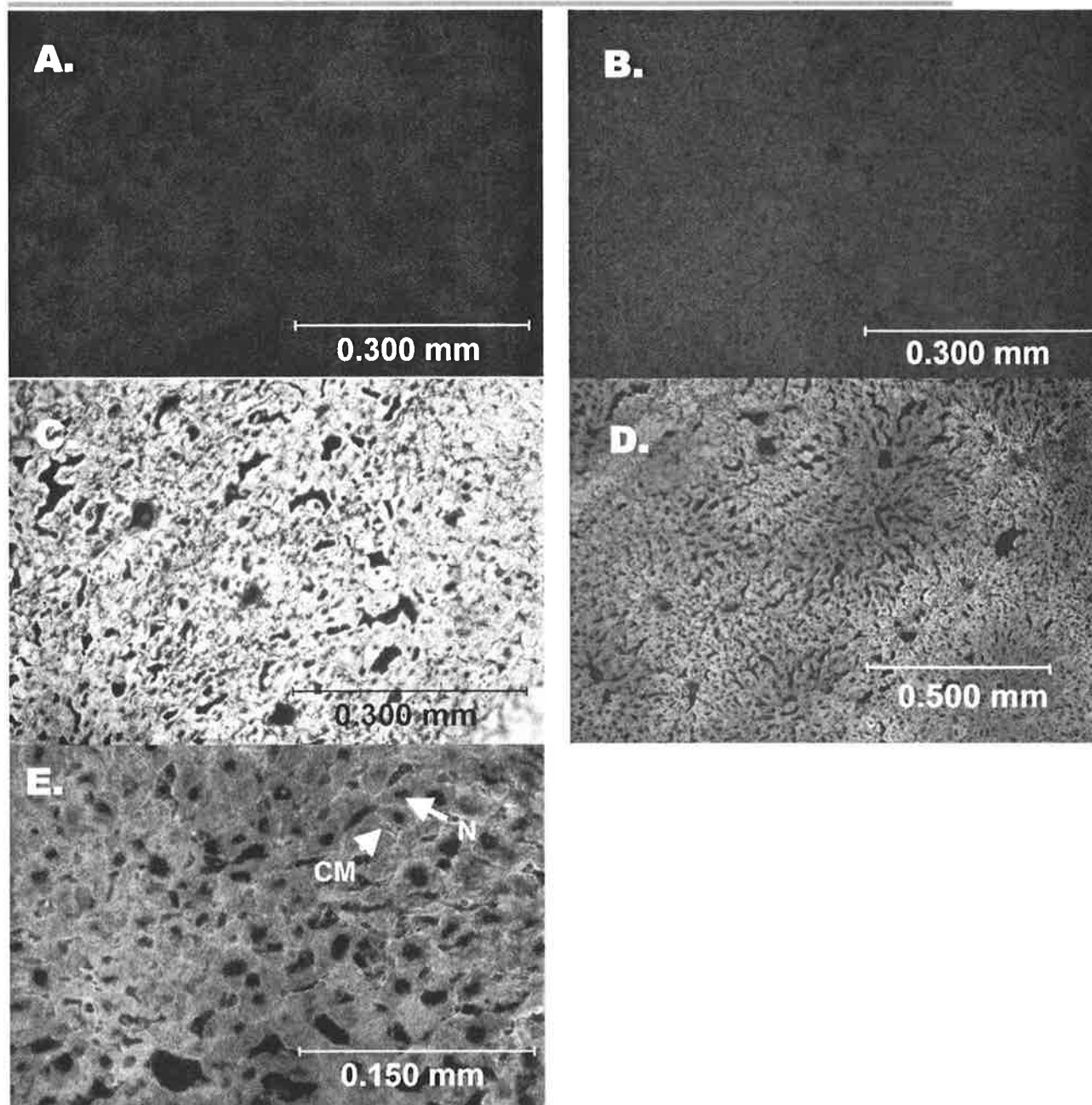


Figure 5.6 Sections of right medial liver lobes of mice administered allyl alcohol (AA, 90 mg/kg) and hydralazine (HYD) and sacrificed 1 hour later for immunohistochemical localisation of hydralazine trapped acrolein-protein adducts. Hydralazine labelled acrolein-protein adducts were detected using a polyclonal hydralazine-acrolein/KLH antiserum raised in rabbits (1/750), or non-immune serum (1/750), followed by visualisation using a fluorescent FITC conjugated goat anti-rabbit antibody (Pierce). Panels correspond to: (A) Liver section representative of control (ie. PBS, HYD alone, AA alone, AA+HYD/non-immune serum) sections (PBS, 200X); (B) 300 μ mol/kg *E*-APH (200X); (C) AA + 300 μ mol/kg HYD (200X); (D) AA + 100 μ mol/kg HYD (100X); (E) AA + 100 μ mol/kg HYD (400X). CM = cell membrane; N = nucleus.

As acrolein is known to adduct lysine and histidine residues in proteins, the primary antiserum was pre-incubated with hydralazine labelled acrolein adducted poly-L-lysine and poly-L-histidine in a competition experiment. These modified polyacyl compounds have been found recently to abolish immunorecognition of trapped adducts (Burcham *et al*, 2004). Pre-incubation of the primary antiserum with these modified poly-aminoacyl compounds inhibited adduct detection by the primary antiserum in the livers of mice treated with allyl alcohol and 300 μ mol/kg hydralazine (Figure 5.7a and b for poly-L-lysine and poly-L-histidine respectively). This suggested that hydralazine trapped acrolein adducts of lysine and histidine in the livers of whole mice administered allyl alcohol.

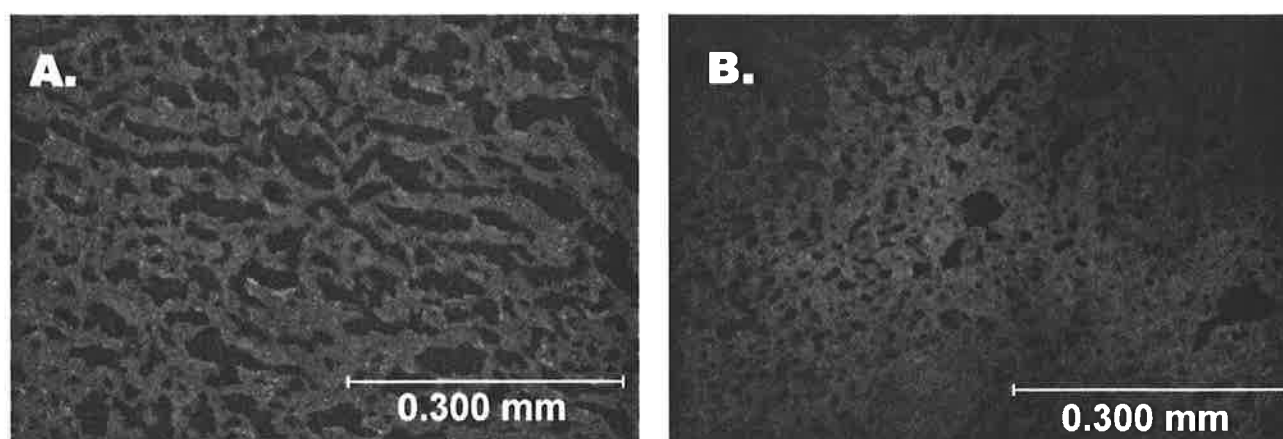


Figure 5.7 Sections of the right medial liver lobe taken from a mouse administered allyl alcohol (90 mg/kg) and hydralazine (100 μ mol/kg) and sacrificed 1 hour later for binding competition with hydralazine-acrolein-poly-L-lysine (A, 200X) and hydralazine-acrolein-poly-L-histidine (B, 200X). The hydralazine-acrolein/KLH antiserum (1/750) was preincubated with the hydralazine-labelled acrolein-modified polyaminoacyl reagents (2 mg/ml, 2 hours, 37°C) prior to primary treatment of liver sections. The labelled adducts were then viewed using an FITC conjugated goat anti-rabbit antibody.

5.4 DISCUSSION

The steep dose response curve for the induction of hepatotoxicity by allyl alcohol observed in Swiss mice in this study is consistent with previous reports of allyl alcohol hepatotoxicity in rodents (Belinsky *et al*, 1985). However, allyl alcohol induced increases in plasma markers reported by others are typically much greater than the levels reported here. For instance, Jaeschke *et al* (1992) reported that serum alanine aminotransferase was elevated 5 fold (from 30 U/l to 152 U/l) 1 hour after 35 mg/kg allyl alcohol in male NRM1 mice. In contrast, no

significant increase in plasma SDH was observed 2 hours after double this dose of allyl alcohol in the Swiss mice used in this study. In two similar studies on NRMI mice, the authors reported increases in GPT to 3428 U/l 3 hours after 87 mg/kg allyl alcohol and to 1283 U/l of SGOT approximately 1 hour after the same dose of allyl alcohol (Jaeschke *et al*, 1987; Pompella *et al*, 1988). However, Jaeschke *et al* (1987) also reported that all of the mice died within an average time of 1 hour (it was initially intended that the experiment would continue for 4 hours). Since only 30% of mice died within 4 hours of a 100 mg/kg dose of allyl alcohol in this study, Swiss mice are clearly more resistant to allyl alcohol induced liver toxicity than NRMI mice. Also, the NRMI mice used in these previous studies exhibited higher control activities of serum enzymes than the Swiss mice used in this study. When the relative differences between control enzymes and allyl alcohol induced increases in GPT were compared in the study by Pompella *et al* (1988), 87 mg/kg allyl alcohol increased serum GPT 31 fold 3 hours after administration of the alcohol. This is consistent with the increase in serum GPT 4 hours after allyl alcohol observed in this study.

The lack of dose-response relationships for hepatic GSH depletion over the range of 80 to 100 $\mu\text{mol/kg}$ is consistent with the findings of others and confirms that GSH depletion is highly saturable. Several explanations might account for this observation, but the fact that acrolein can inhibit several enzymes involved in the biotransformation of either acrolein or allyl alcohol, including aldehyde dehydrogenase (Ren *et al*, 1999) and glutathione S-transferase (Haenen *et al*, 1988; Berhane and Mannervik, 1990) may contribute to this phenomenon. Preferential inactivation of glutathione-S-transferase relative to alcohol dehydrogenase at low doses of allyl alcohol might also explain the lack of dose-dependency, since in the presence of glutathione-S-transferase inhibition, sufficient alcohol dehydrogenase activity might remain to allow bioactivation of the unsaturated alcohol at higher doses of allyl alcohol. However, non-enzymatic reactions between acrolein and GSH are still likely to occur to detoxify the aldehyde.

Consistent with the observation of strong cytoprotection against allyl alcohol toxicity in isolated hepatocytes, hydralazine provided strong protection against liver damage caused by allyl alcohol. The strong hepatoprotection afforded by hydralazine did not, however, involve protection against the hepatic GSH depletion induced by allyl alcohol, even though allyl alcohol toxicity is abrogated by pharmacological interventions that either increase GSH levels or upregulate the expression of glutathione-S-transferases (Jaeschke *et al*, 1987; Haenen *et al*, 1988). This concurs with the findings reported in Chapter 2, showing hydralazine's protection against allyl alcohol toxicity in isolated mouse hepatocytes occurs despite not protecting against the loss of

cellular GSH. Alternatively, the fact that hydralazine did not alter GSH depletion suggests other mechanisms underlie the hepatoprotective phenomenon, with protein adduction a very likely candidate as *E*-APH was not detected in the plasma of animals treated with allyl alcohol and hydralazine (data not shown).

Burcham and colleagues recently observed that hydralazine protected against acrolein-mediated toxicity when it was added to cells that had been briefly exposed to allyl alcohol for 25 minutes, yet were washed free of the toxin prior to the onset of irreversible cell death (Burcham *et al*, 2004). This was consistent with the idea that hydralazine targets “early” chemical events involved in the modification of cellular macromolecules by acrolein, and was supported by immunochemical observations using a model protein and an antibody that recognised hydralazine-modified acrolein adducts at lysine residues (Burcham *et al*, 2004). The results from the *in vivo* experiments reported here confirm these predictions, as hydralazine was just as protective when administered to mice 20 minutes after allyl alcohol as when the drug and allyl alcohol were co-administered to the animals. However, if a 30 minute delay was allowed between the administration of hydralazine and allyl alcohol pretreatment, the drug’s hepatoprotective potency was strongly diminished. Consequently, these findings reinforce the earlier *in vitro* observations reported by Burcham and colleagues (2004) and are consistent with the hypothesis that the drug interferes with chemical events occurring in the early stages of allyl alcohol toxicity. It is therefore suggested that a critical event occurs within 30 minutes of allyl alcohol administration that determines the fate of cells exposed to the toxin. This may involve crosslinking of adducted macromolecules with other proteins or with DNA, thereby initiating the cell death cascade. According to this scenario, hydralazine would be unable to trap the crosslinks present at later time points, making the drug ineffective at preventing cell death as previously discussed.

To explore the role of adduct trapping, an antibody raised against the products of trapping reactions between hydralazine and acrolein adducts was used to determine whether such chemistry accompanied the hepatoprotection afforded by hydralazine *in vivo* during allyl alcohol exposure. The results of Western blot analysis of liver proteins revealed that strong adduct-trapping by hydralazine occurred in the livers of two allyl alcohol-treated mice exposed to 100 $\mu\text{mol/kg}$ hydralazine, while doubling the dose of hydralazine correspondingly increased the intensity of adduct-trapping. Due to saturation of the intense signals at the high dose, bands corresponding to proteins with masses greater than 40 kDa were poorly resolved. At the lower dose of hydralazine, some 20 to 25 proteins can be distinguished as targets for trapping reactions

by hydralazine, consistent with the likelihood that as a strong, indiscriminate electrophile, acrolein will alkylate a wide range of intracellular proteins.

While the above Western blotting data confirmed that intense adduct-trapping reactions accompany hepatoprotection by hydralazine against acrolein-mediated liver injury, they yielded no information on the spatial or zonal heterogeneity of trapping reactions in the intact liver. To explore this issue, the localisation of hydralazine-stabilised acrolein adducts was assessed using immunohistochemistry on sections of right medial liver lobes collected from hydralazine and allyl alcohol treated mice. Strong adduct-trapping was evident at both lysine and histidine residues in liver sections from mice treated with allyl alcohol and hydralazine, particularly in the cytoplasm and cell membranes indicating that these sites represent major targets for adduct-trapping reactions. Regarding the identity of trapped residues, however, it remains to be established if the primary epitope for the antiserum is the hydralazine moiety per se, or whether it recognises other portions of ternary drug-acrolein-protein complexes. Hydralazine-trapped acrolein-protein adducts were not detected in the nucleus, in apparent contrast to the findings of one group of investigators who reported predominant ^3H acrolein localisation in the nucleus of *Dunaliella bioculata* via ultrastructural autoradiography (Marano and Demestere, 1976). However, it is possible that these workers were measuring DNA-adducted acrolein in their experiments, while the present findings suggest that strong adduct trapping does not occur in the nuclear proteome. Whether such trapping reactions occurred on DNA is not known as the primary antiserum was raised and characterised against protein and not DNA adducts. Recent demonstrations, however, suggest that the scope of these investigations should be widened to address the possibility that blockade of reactive acrolein adducts in the genome may be an effect of adduct-trapping drugs. The demonstration by Kurtz and Lloyd (2003) that carbonyl-retaining deoxyguanosine adducts formed during the modification of DNA by acrolein efficiently generate DNA-DNA and DNA-protein crosslinks raise the possibility that low molecular weight hydrazino drugs might also inactivate reactive centres introduced into DNA during acrolein toxicity (Kuykendall and Bogdanffy, 1992; Kozekov *et al*, 2001; Kurtz and Lloyd, 2003).

Unexpectedly, little or no zonal variation was evident in the intensity of adduct-trapping across the liver lobule. Since allyl alcohol toxicity is typically localised in periportal regions, it was expected that immunoreactivity would be most intense in these areas. In contrast, however, the entire liver lobule was nearly uniformly immunoreactive for drug-trapped adducts. The diffuse nature of adduct-trapping reactions across the liver lobule suggests not only that allyl alcohol

bioactivation (and hence protein adduction) occurs throughout the liver lobule, but also that hydralazine is freely distributed throughout the liver lobule. Enhanced sensitivity of periportal cells to the damaging effects of allyl alcohol also does not explain the zonal specificity of allyl alcohol damage, as Penttilä (1988) reported that perivenous cells are more susceptible to allyl alcohol than periportal cells. It was suggested that this may be due to the higher GSH content of the periportal cells and hence greater protection afforded against acrolein toxicity. The author made the somewhat improbable suggestion that the zonal specificity of allyl alcohol toxicity may be due to rapid uptake and oxidation of the alcohol in periportal zones, which seems unlikely as the two cell types metabolise allyl alcohol at similar rates (Penttilä, 1988). The reason for the periportal specificity of allyl alcohol toxicity therefore remains unknown.

The results described in this Chapter confirmed the proposed hypothesis, namely that hydralazine would protect against acrolein-mediated hepatotoxicity when administered within the early stages of the toxic process. The finding that strong, dose-dependent trapping of acrolein-adducted proteins accompanies the inhibition of hepatotoxicity was consistent with the proposal that an ability to block downstream chemical events, such as macromolecular crosslinking reactions, affords protection against allyl alcohol toxicity. Further work, including the development of sensitive analytical tools to detect crosslinked species in biological systems, are clearly needed in order to fully evaluate this hypothesis. An ability to detect inter- and intra-molecular crosslinking in macromolecules might establish whether such chemistry is responsible for some of the documented molecular effects of acrolein in exposed cells and tissues, such as protein kinase C activation (Maddox *et al*, 2003) or alterations in the activity of transcription factors such as AP-1, NF κ B or Nrf2 (Horton *et al*, 1999; Biswal *et al*, 2002; Tirumalai *et al*, 2002). These investigations are beyond the scope of this thesis, however, and will hopefully be examined by our laboratory at a later stage.

However, an aspect of allyl alcohol toxicity that has not been addressed thus far is its ability to induce oxidative stress and lipid peroxidation via its biotransformation to acrolein. Given results showing that antioxidants, such as the iron chelator desferrioxamine, also block allyl alcohol induced hepatic toxicity, many investigators have suggested that allyl alcohol toxicity is primarily due to the induction of oxidative stress rather than macromolecular adduction by acrolein (Pompella *et al*, 1988; Ferrali *et al*, 1990; Jaeschke *et al*, 1992). Interestingly, hydralazine has been proposed to have antioxidant properties, including an ability to donate single electrons to radical species (Johnson *et al*, 1998). Although considerable evidence exists to support the proposed mechanism of adduct trapping by hydralazine in protection against

Chapter 5: Hydralazine Mediated Protection Against Allyl Alcohol *In Vivo*

aldehyde toxicity, the following Chapter will explore the likelihood of antioxidant properties of the drug contributing to protection against acrolein mediated toxicity.

Chapter 6:

Role of antioxidant effects in hydralazine mediated protection against unsaturated carbonyl toxicity

6.1 INTRODUCTION

The toxicity of allyl alcohol is mediated by its oxidative biotransformation to the bifunctional electrophile acrolein, which rapidly adducts cellular macromolecules (Belinsky *et al*, 1985; Esterbauer *et al*, 1991). The findings reported in this thesis thus far have suggested that protection provided by the nucleophilic drug hydralazine against allyl alcohol toxicity involves its ability to trap the early acyclic Michael adducts of acrolein before they are capable of activating downstream cell death pathways. At present the nature of the cellular processes that are triggered by acrolein during induction of cell death are poorly understood.

However, a number of authors have suggested that allyl alcohol induced cell death is mediated by the induction of oxidative stress following its biotransformation to acrolein. This has been shown by the increased formation of malondialdehyde, a classic product of lipid peroxidation, following allyl alcohol administration (Miccadei *et al*, 1988; Pompella *et al*, 1988; Ferrali *et al*, 1990; Jaeschke *et al*, 1992). Furthermore, antioxidants such as the silybin-phospholipid complex IdB 1016, dithiothreitol and N,N'-diphenyl-p-phenylenediamine protect against allyl alcohol toxicity *in vitro* and *in vivo* (Miccadei *et al*, 1988; Carini *et al*, 1992; Rikans *et al*, 1994). Typically, a loss of cellular GSH precedes the generation of lipid peroxidation and cell death (Jaeschke *et al*, 1987). Rikans and Cai (1994) observed a similar time dependent reversal of allyl alcohol cytotoxicity in rat hepatocytes to that observed in mouse liver cells by Burcham *et*

al (2004). The addition of dithiothreitol 30 minutes after addition of allyl alcohol, followed by washing the toxin from the cells resulted in similar cytoprotection to that obtained during co-addition of the compounds. Protection also occurred without restoring GSH levels in the cells (Rikans and Cai, 1994). They also noted that protection against allyl alcohol only occurred in the continuous presence of dithiothreitol.

However, not all studies have confirmed a central role for oxidative stress in allyl alcohol induced hepatotoxicity. For example, Matsumoto *et al* (1987) discovered that the antioxidant (+)(3S)-1,2,3,4-tetrahydro-3-hydroxy-methyl- β -carboline-2-carbodithioate (THC) protected rat livers against hepatotoxicity induced by carbon tetrachloride, bromobenzene and D-galactosamine but did not protect mice against liver damage by allyl alcohol. Although THC exerted no protection against allyl alcohol induced cell death, it did, however, prevent the induction of lipid peroxidation in rat liver microsomes by allyl alcohol. This may indicate that allyl alcohol induced hepatocyte cell death is not dependent on lipid peroxidation and may in fact be due to other mechanisms.

The mechanism of allyl alcohol induced lipid peroxidation has been extensively studied, since decreased GSH levels alone do not induce a significant degree of reactive oxygen species (ROS) formation and lipid peroxidation (Jaeschke *et al*, 1987). Reports have demonstrated that allyl alcohol and the acrolein metabolite acrylic acid are not themselves toxic, but that acrolein and NADH, both products of allyl alcohol metabolism, are responsible for toxicity (Jaeschke *et al*, 1987; Ferrali *et al*, 1989; Jaeschke *et al*, 1992). In addition, acrolein itself is not likely to function as a pro-oxidant and previous studies examining the effect of acrolein on soy bean phospholipid liposomes and mouse liver microsomes have reported that it does not induce lipid peroxidation (Jaeschke *et al*, 1987). Also, our laboratory has confirmed that acrolein does not consistently induce lipid peroxidation in Menhaden fish oil or in a suspension of linoleic acid (LM Kaminskis, unpublished observation). This suggests that an additional cellular factor is required for acrolein to induce lipid peroxidation.

Allyl alcohol induced toxicity and lipid peroxidation are also inhibited by the iron chelator desferrioxamine (Jaeschke *et al*, 1987; Miccadei *et al*, 1988; Ferrali *et al*, 1989; Ferrali *et al*, 1990). To explain this observation, Jaeschke *et al* (1992) administered ferrous sulfate alone or in combination with allyl alcohol to NRMI mice and observed the induction of lipid peroxidation and liver injury. Both ferrous sulfate and allyl alcohol alone induced mild lipid peroxidation and liver damage, although when administered together they produced a marked increase in both

parameters. They then examined the ability of acrolein and NADH, both by products of allyl alcohol metabolism, to mobilise iron from ferritin, a key iron binding protein in the liver. In a cell-free system, acrolein (3 mM) and NADH (1 mM) individually induced the release of approximately 1% of iron from ferritin (approximately $10.2 \mu\text{g Fe}^{+2}$) within 15 minutes (Jaeschke *et al*, 1992). This indicates that acrolein may generate adducts in ferritin in hepatocytes, resulting in the release of iron which, in the absence of appropriate cellular antioxidant defences, results in the generation of ROS and ensuring cell death as suggested by Miccadei *et al* (1988).

The proposal that allyl alcohol mediated cell death is due to adduction of ferritin resulting in the release of free iron is supported by observations that acrolein is also responsible for allyl alcohol induced haemolysis in erythrocytes. *In vivo*, allyl alcohol, along with the induction of hepatotoxicity, results in lipid peroxidation in erythrocytes followed by haemolysis. Antioxidants such as trolox and desferrioxamine prevent lipid peroxidation and haemolysis in erythrocytes (Ferrali *et al*, 1989; Ciccoli *et al*, 1994). Ferrali *et al* (1990) further suggested that acrolein induces the release of iron from hemoglobin in erythrocytes which is then responsible for the ensuing lipid peroxidation and membrane rupture (Ferrali *et al*, 1990). To date, however, this mechanism has not been investigated in hepatocytes and therefore deserves further attention.

Based on observations with allyl alcohol, it appears that acrolein may induce cell death via an iron dependant pathway that involves the production of ROS, initiation of lipid peroxidation and cell death. Given that one study indicated that hydralazine has antioxidant properties, the possibility that these actions play a role in protection against unsaturated carbonyl toxicity by hydralazine needs further examination (Johnson *et al*, 1998). For instance, given that antioxidants can protect against allyl alcohol toxicity, the contribution of hydralazine's radical scavenging properties to its protective effects need to be tested. This was therefore briefly examined in the current Chapter by comparing the cytoprotective capacity of the various hydrazines and hydralazine analogues highlighted in Chapter 2 to their radical scavenging properties. This was achieved by assessing the ability of the hydrazines and hydralazine analogues to scavenge two model radicals, galvinoxyl and 1,1-diphenyl-2-picryl-hydrazyl. These radicals are chromogenic species that lose their colour when they abstract a hydrogen radical from a donor antioxidant (Noguchi *et al*, 1998; Ezure *et al*, 2001). This allows rapid and facile measurement of the 1 electron donating capacity of putative antioxidants. It was expected that if antioxidant actions were a factor in protection against allyl alcohol toxicity, a correlation

would be achieved between the radical scavenging properties of the hydrazines and their cytoprotective potencies.

In addition, various antioxidants and hydrazine compounds were compared for their ability to protect against the toxicity induced by allyl alcohol and two other oxidants, tert-butylhydroperoxide (tBuOOH) and nitrofurantoin. tBuOOH and nitrofurantoin are both known to generate radicals, cause depletion of cellular glutathione and promote lipid peroxidation, similar to allyl alcohol (Rush *et al*, 1985; Rossi *et al*, 1988; Biel *et al*, 1993). However, compared to allyl alcohol, the mechanism of oxidative cell injury induced by these toxins is better understood. Nitrofurantoin is a redox cycling compound that is activated by a NADPH nitroreductase to a nitro anion radical which transfers a single electron to molecular oxygen under aerobic conditions, forming superoxide radicals and hydrogen peroxide (Mason and Holtzmann, 1975; Sasame and Boyd, 1979; Carbonera *et al*, 1988). Under anaerobic conditions nitrofurantoin undergoes covalent binding to microsomal macromolecules (Minchin *et al*, 1989). The importance of hydrogen peroxide formation to the oxidative stress and cytotoxicity of nitrofurantoin is highlighted by the fact that catalase inhibitors, which prevent detoxification of hydrogen peroxide, enhance nitrofurantoin toxicity in diverse cell types including rat hepatocytes (Lyng *et al*, 1988; Rossi *et al*, 1988). The nitro anion radical is also capable of initiating radical chain reactions and reducing hydroperoxides (Carbonera *et al*, 1988). TBuOOH, however, is an organic peroxide that is metabolised by glutathione peroxidase to t-butyl alcohol and also fragments to form peroxy and alkoxy radicals which are responsible for triggering lipid peroxidation reactions (Rush *et al*, 1985; Glascott *et al*, 1995).

If oxidative mechanisms are central in allyl alcohol toxicity, it was predicted that the protection against allyl alcohol toxicity by the various antioxidants and hydrazines would be similar to the protection seen against toxicity mediated by other prooxidant compounds. The antioxidants chosen for investigation were mechanistically diverse in order to provide insight into the antioxidant mechanisms important for protection against allyl alcohol toxicity. The formation of hydralazine-trapped acrolein-protein adducts were also assessed in hepatocytes treated with hydralazine and nitrofurantoin or tBuOOH to address the role of adduct-trapping in any protection afforded by hydralazine.

6.2 METHODS

6.2.1 Chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were from Sigma Chemical Co (St Louis, MO, USA). Galvinoxyl, Tempo, ascorbic acid, butylated hydroxytolulene (BHT), diphenylamine, tert-butylhydroperoxide (tBuOOH) and nitrofurantoin were from Aldrich Chemical Co (Milwaukee, WIS, USA). Cu^{+2} (3,5-diisopropylsalicylate)₂ (Cu-DIPS) was purchased from Calibochem Novabiochem Corp. Keyhole limpet-hemocyanin was purchased from Pierce Chemical Co (Rockford, ILL, USA). 2-Aminoquinoline and 1-aminoisoquinoline were a donation from Steve Inglis (Department of Chemistry/Molecular Biosciences at the University of Adelaide). All other reagents were as previously described.

6.2.2 Animals

Mice (6 to 8 weeks) were used for hepatocyte isolation and were obtained and housed as previously described in Chapter 2 (section 2.2.1).

6.2.3 Cytoprotection Against Allyl Alcohol, tert-Butylhydroperoxide and Nitrofurantoin Toxicity by Antioxidants

Isolation of mouse hepatocytes was performed as previously described (section 2.2.9.1). Cells were then plated on collagen coated 60 mm dishes for studies of nitrofurantoin toxicity. For examination of short term allyl alcohol toxicity and tBuOOH toxicity, cells were plated on collagen coated 96 well plates for screening of protective compounds as described in section 2.2.13.2. This experiment was conducted with assistance provided by David Ascher. Allyl alcohol (147 mM), hydralazine (50 mM), dihydralazine (50 mM) and ascorbic acid (50 mM) stock solutions were prepared fresh in RPMI (without BSA). tBuOOH solutions (250 μM) were also prepared directly in RPMI. Stock solutions of nitrofurantoin, diphenylamine, Trolox, Tempo, Cu-DIPS and BHT (50 mM), were prepared in DMSO.

To each of the 60 mm dishes, 3 ml of the following solutions were added: RPMI alone, protective compound alone (100 μM for all except Cu-DIPS; 10 μM), nitrofurantoin alone (600 μM) or nitrofurantoin with protective compound (1 to 100 μM). Larger dishes were necessary

for nitrofurantoin toxicity as toxicity was not seen in 96 well plates. Dishes were incubated at 37 °C for 3 hours. Samples of culture media (20 µl) were taken at hourly intervals for assessment of LDH leakage by the method previously described (section 2.2.9.2).

The antioxidants were screened for their ability to protect against allyl alcohol (100 µM) and tBuOOH (250 µM) toxicity in 96 well microplates to allow for rapid determination of cytoprotective EC₅₀'s as reported in Chapter 2 (section 2.2.13.2). Each microplate well was treated with the toxic compounds and antioxidants as described in section 2.2.13.2 and samples of culture media (10 µl) taken at hourly intervals for up to 2 hours to assess LDH leakage into the supernatant by the assay mentioned previously (section 2.2.9.2).

Cytoprotective EC₅₀'s were determined by David Ascher following normalisation of the data using a sigmoidal curve fitting capacity on the statistics program GraphPad Prism (version 4).

6.2.4 Effect of Antioxidants on the Loss of Glutathione Mediated by Allyl Alcohol, tert-Butylhydroperoxide and Nitrofurantoin

Hepatocytes were isolated and plated on collagen coated 35 mm dishes for GSH determination as previously described (section 2.2.10.1). Dishes were treated with RPMI alone, the abovementioned toxins alone, the cytoprotective antioxidants alone or the toxins plus each of the antioxidants (100 µM). Dishes were incubated for 30 minutes for allyl alcohol and tBuOOH or 1 hour for nitrofurantoin, washed in cold PBS and the cells treated for analysis of cellular GSH as previously described in section 2.2.10.2. The experiment was performed with 3 replicates with assistance from David Ascher.

A 1-way ANOVA with a Dunnett's post test was used to compare treated cells to control cellular GSH (RPMI alone) and also to compare toxin and antioxidant treated cell GSH to toxin alone. Data was represented as mean ± SEM in µg GSH per million cells. Significance was at p<0.05.

6.2.5 Scavenging of Galvinoxyl by Protective Hydrazines, Hydralazine Analogues and Antioxidants

Galvinoxyl is an oxygen centred radical which is shown in Figure 6.1a. Scavenging of galvinoxyl by the previously mentioned hydralazine analogues (hydralazine, dihydralazine, 1-hydrazinoisquinoline, 2-hydrazinopyridine, 4-hydrazinoquinazoline, 2-hydrazinoquinoline,

phthalazine, naphthylhydrazine as well as 1-aminoisoquinoline and 2-aminoquinoline) including the hydrazines benzylhydrazine and 1,1-diphenylhydrazine and antioxidants were investigated by a modification of a previously reported procedure (Noguchi *et al*, 1998). The modification incorporated the use of a standard spectrophotometer (Hitachi U-2000) to monitor the loss of galvinoxyl. Where possible, cytoprotective compounds (50 mM) were diluted in acetonitrile or water. The compounds that were not soluble in acetonitrile were dissolved in DMSO. Solutions of galvinoxyl were prepared in methanol (5 μ M). From stock solutions, 250 μ M solutions of cytoprotective agents were prepared in acetonitrile. Reactions of galvinoxyl with the cytoprotective compounds were performed at room temperature in a quartz cuvette ($l = 1$ cm). Galvinoxyl (1 ml) was added to the cuvette and the reaction started by the addition of 10 μ l of 250 μ M (final concentration 2.5 μ M) solutions of cytoprotective compound. The loss of galvinoxyl was monitored for 8 minutes at 30 second intervals at 429 nm in the spectrophotometer. In addition, the control loss of galvinoxyl (loss of galvinoxyl in the absence of scavenger) and loss in solutions where 10 μ l DMSO had been added were tested to assess the stability of the radical. The rate of galvinoxyl scavenging by the scavengers is reported as the % loss of galvinoxyl per minute as measured over the linear portion of the scavenging curve (1 minute). A correlation analysis (Pearson's correlation test) was performed on the ability of the hydralazine analogues to scavenge galvinoxyl radicals and their cytoprotective EC_{50} 's for allyl alcohol toxicity. The statistical significance threshold was $p < 0.05$.

6.2.6 Scavenging of DPPH by Protective Hydrazines, Hydralazine Analogues and Antioxidants

The ability of the abovementioned hydrazines, hydralazine analogues and antioxidants to scavenge DPPH radicals (shown in Figure 6.1b) was analysed by a modified method to that previously reported (Ezure *et al*, 2001). DPPH solutions (1 mM) were prepared in ethanol. The reaction was performed in 20 mM acetate buffer (pH 5.5) containing 60% ethanol. Stock solutions of protective compounds were made up in water or DMSO (10 mM), as the reaction solution may contain up to 10% DMSO without affecting the assay (Ezure *et al*, 2001). The stock solutions were then further diluted 1/10 in acetate buffer/ethanol. The ability of the cytoprotective compounds to scavenge DPPH was assayed by monitoring the loss of DPPH at 517 nm on a spectrophotometer in a quartz cuvette ($l = 1$ cm). The reaction was performed by adding 10 μ l of 1 mM solutions of the protective compounds to 900 μ l acetate buffer/ethanol and mixing, followed by the addition of 100 μ l of 1 mM DPPH. The final concentration of protective compound was 10 μ M while the final concentration of DPPH was 100 μ M.

The loss of DPPH over time by the hydrazines and hydralazine analogues was plotted as the absorbance of the reaction at 517 nm. The rate of DPPH scavenging by the antioxidants was calculated as the % loss of radical per second over the linear portion of the curve (12 seconds) as DPPH radicals are scavenged faster than galvinoxyl radicals. A correlation analysis (Pearson's correlation test) was performed on the ability of the hydralazine analogues to scavenge DPPH radicals and their cytoprotective EC_{50} 's for allyl alcohol toxicity. Statistical significance was at $p < 0.05$.

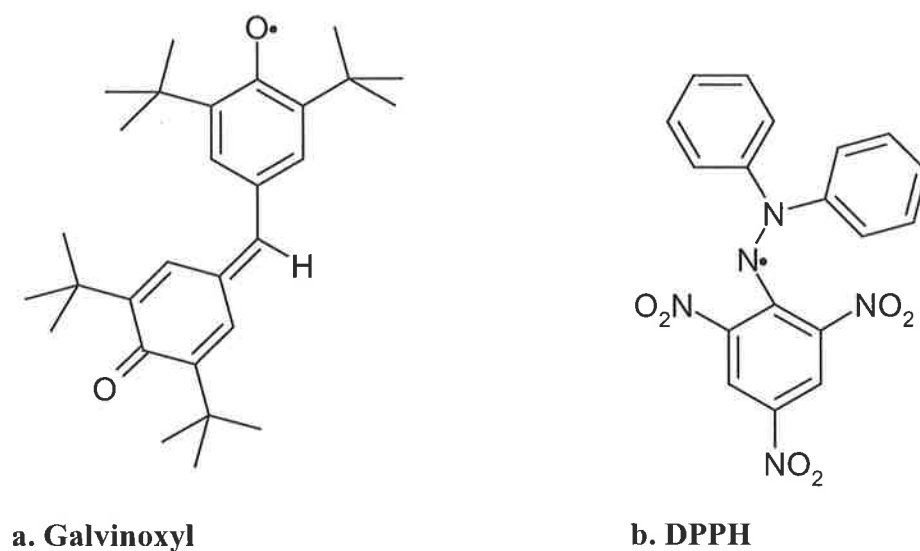


Figure 6.1 Structures of the oxygen centred radical galvinoxyl (a) and the nitrogen centred radical DPPH (b).

6.2.7 Immunodetection of Hydralazine-Trapped Aldehyde-Protein Adducts Generated From tert-Butylhydroperoxide and Nitrofurantoin

The presence of carbonylated proteins generated from tBuOOH and nitrofurantoin was assessed by trapping them with hydralazine. This also allowed an examination of whether adduct-trapping accompanied cytoprotection by hydralazine using these toxins. Cells plated on 60 mm dishes were co-incubated by David Ascher with hydralazine (100 μ M) and the toxins (allyl alcohol, 10 μ M; tBuOOH, 250 μ M; nitrofurantoin, 600 μ M) for 30 minutes (allyl alcohol and tBuOOH) or 1 hour (nitrofurantoin) prior to the addition of lysis buffer (300 μ l). A lower concentration of allyl alcohol than used previously was added to cells to avoid poor resolution of

the proteins due to intense immunorecognition of highly modified proteins. Cells were chilled on ice and prepared for Western blotting which was performed by Dr Phil Burcham. The media was removed from plates and 300 μ l lysis buffer (see section 5.2.6.1) added. Cells were then scraped from the plates and transferred to 1.5 ml Eppendorf tubes which were sonicated (50 V) on ice for 20 seconds each. Following centrifugation (10,000 $\times$ g, 5 minutes) to remove cellular debris and insoluble material, the supernatant was assessed for protein content via the BCA assay. Next, 60 μ g of protein from allyl alcohol, tBuOOH and nitrofurantoin treated cells were loaded onto a 12% acrylamide minigel and resolved at 200 V for 40 minutes. Proteins were then transferred to a nitrocellulose membrane and assessed for hydralazine labelled acrolein-protein adducts as previously described (section 5.2.6.3).

6.3 RESULTS

6.3.1 Cytoprotection Against the Toxicity of Allyl Alcohol, tert-Butylhydroperoxide and Nitrofurantoin

Allyl alcohol toxicity is mediated by the formation of acrolein and its subsequent adduction of nucleophilic macromolecules and the induction of oxidative stress. tBuOOH and nitrofurantoin, on the other hand, generate radicals through self-fragmentation (tBuOOH) or redox cycling (nitrofurantoin) (Rush *et al*, 1985; Lim *et al*, 1986; Glascott *et al*, 1995). The ability of various antioxidants: hydralazine, dihydralazine, the superoxide dismutase mimetic Cu-DIPS, the lipid hydroperoxide stabiliser BHT and the radical scavengers diphenylamine, Tempo, Trolox and ascorbic acid to protect against toxicity of these toxins were compared. This was for the purpose of trying to elucidate the likely significance of antioxidant effects of hydralazine in its protection against allyl alcohol toxicity.

Figure 6.2 shows the ability of the antioxidants to protect against allyl alcohol toxicity. Allyl alcohol induced near maximal cell death within 2 hours of its addition to 96 well culture plates (data not shown). Hydralazine, dihydralazine, diphenylamine and Tempo all protected against allyl alcohol in the low micromolar range, where diphenylamine was the most efficient protective compound, yielding protective EC₅₀'s of 1.2 ± 0.9 and 6.0 ± 1.7 μ M at 1 and 2 hours respectively. Trolox afforded limited protection at 1 hour, yielding an EC₅₀ of 60 ± 8.0 μ M at this time. At the concentrations tested, ascorbic acid, BHT and Cu-DIPS did not protect against allyl alcohol toxicity. This shows that while protection against allyl alcohol toxicity by

hydralazine and dihydralazine is similar to the protection afforded by some radical scavengers, it does not appear to be mediated by trapping superoxide radicals, or by stabilising lipid hydroperoxides since antioxidants with these capabilities didn't block toxicity.

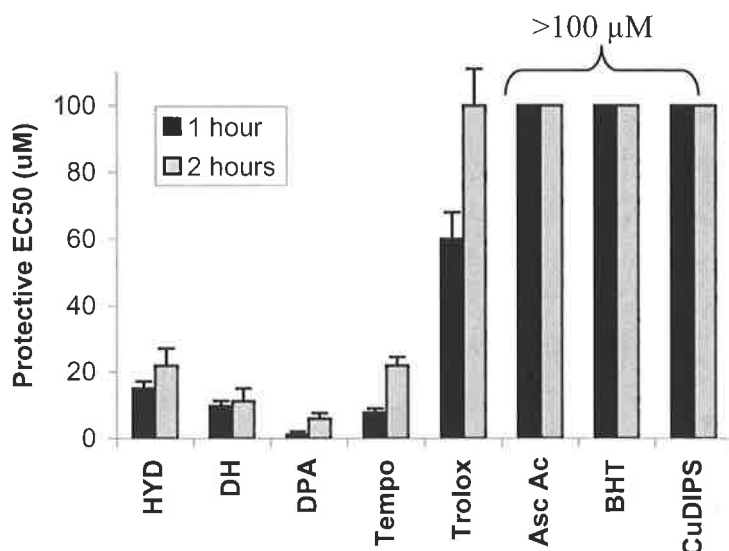


Figure 6.2 Protective EC_{50} of various antioxidants against allyl alcohol (100 μM) toxicity in isolated mouse hepatocytes. Mouse hepatocytes were treated with allyl alcohol alone or co-incubated with 1-100 μM concentrations of hydralazine (HYD), dihydralazine (DH), 1,1-diphenylamine (DPA), tempo, trolox, ascorbic acid (Asc Ac), butylated hydroxytolulene (BHT) or Cu DIPS and samples of culture media taken after 1 and 2 hours to assess lactate dehydrogenase (LDH) leakage from the cytoplasm. Data are represented as the mean $\pm$ SEM of the EC_{50} (μM) for cytoprotection as assessed by the % LDH leakage at each time point ($n=3$ observations).

tBuOOH induced 100% cell death within 1 hour of its addition to 96 well culture plates (data not shown). The ability of the antioxidants to protect against tBuOOH toxicity was examined to compare their abilities to block the toxicity of this radical-forming hydroperoxide to their cytoprotective efficacy against toxicity induced by allyl alcohol. The results are shown in Figure 6.3. Similar to the protection against allyl alcohol toxicity, hydralazine, dihydralazine, diphenylamine and Tempo protected against tBuOOH toxicity, although the protective EC_{50} 's for tBuOOH protection were higher than for allyl alcohol. Trolox again showed a weak protective effect at 1 hour, yielding a protective EC_{50} of $90 \pm 8.7 \mu\text{M}$ at this time. Ascorbic acid, BHT and Cu-DIPS did not provide any protection against tBuOOH toxicity. These results show that toxicity induced by allyl alcohol and tBuOOH may involve comparable mechanisms, although the antioxidants and hydrazines provided slightly less pronounced protection against

tBuOOH toxicity compared to that against allyl alcohol. This may reflect the more intense oxidative damage induced by tBuOOH, necessitating higher concentrations of antioxidants to afford the same protection provided against allyl alcohol toxicity.

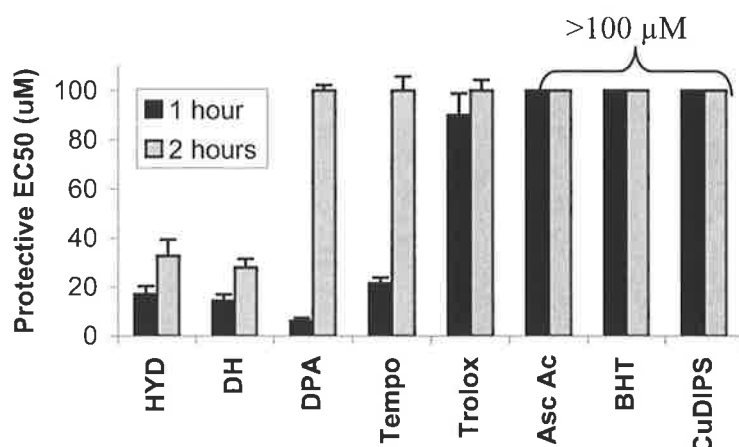


Figure 6.3 Protective EC_{50} of various antioxidants against tBuOOH ($250 \mu\text{M}$) toxicity in isolated mouse hepatocytes. Mouse hepatocytes were treated with tBuOOH alone or co-incubated with 1-100 μM concentrations of hydralazine (HYD), dihydralazine (DH), 1,1-diphenylamine (DPA), tempo, trolox, ascorbic acid (Asc Ac), butylated hydroxytoluene (BHT) or Cu DIPS and samples of culture media taken after 1 and 2 hours to assess lactate dehydrogenase (LDH) leakage from the cytoplasm. Data are represented as the mean $\pm$ SEM of the EC_{50} (μM) for cytoprotection as assessed by the % LDH leakage at each time point ($n=3$ observations).

Nitrofurantoin induced oxidative stress is mediated by a different mechanism to tBuOOH, since whereas tBuOOH fragments to form oxygen based radicals, nitrofurantoin undergoes redox cycling within the cell, generating a range of radicals including $\text{NAD}^\bullet$ and oxygen based radicals such as $\text{O}_2^{\bullet-}$. Figure 6.4 illustrates the ability of the antioxidants to protect against nitrofurantoin toxicity. The onset of nitrofurantoin toxicity occurred more slowly than for tBuOOH, inducing approximately 69 and 80% cell death after 2 and 3 hours respectively. No significant cell death was seen after a 1 hour exposure of hepatocytes to nitrofurantoin (data not shown). The protection afforded against nitrofurantoin toxicity by hydralazine, dihydralazine and diphenylamine was less pronounced than that against tBuOOH toxicity, but still yielded EC_{50} values within the low to mid micromolar range. Tempo was best able to protect against nitrofurantoin toxicity with a protective EC_{50} of $10 \pm 2.3 \mu\text{M}$ at 2 hours. Trolox also protected against nitrofurantoin toxicity to a similar extent as with allyl alcohol and tBuOOH ($EC_{50} = 94 \pm$

4.4 μM after 2 hours). Ascorbic acid was unable to protect cells against nitrofurantoin toxicity. However, BHT and Cu-DIPS provided some protection against nitrofurantoin with protective EC_{50} 's of 100 ± 3.2 and $40 \pm 1.6 \mu\text{M}$ respectively after 2 hours. This may suggest that the over-production of superoxide radicals and subsequent lipid peroxidation are more important contributors to nitrofurantoin toxicity than to tBuOOH and allyl alcohol toxicity. The poorer protection afforded by hydralazine against nitrofurantoin toxicity in contrast to protection against allyl alcohol or tBuOOH toxicity, may indicate that hydralazine is less efficient at scavenging superoxide radicals compared to other oxygen centred radicals such as peroxy and alkoxy radicals. Alternatively, the production of toxic carbonyl by-products may be important in allyl alcohol and tBuOOH toxicity, whereas this may not be a significant factor governing nitrofurantoin toxicity.

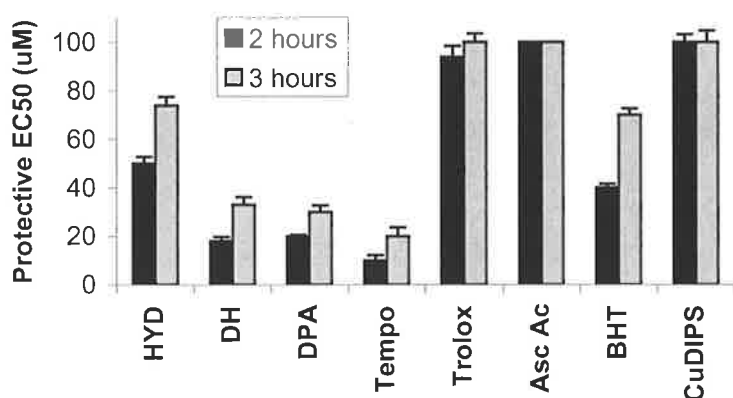


Figure 6.4 Protective EC_{50} of various antioxidants against nitrofurantoin (600 μM) toxicity in isolated mouse hepatocytes. Mouse hepatocytes were treated with nitrofurantoin alone or co-incubated with 1-100 μM concentrations of hydralazine (HYD), dihydralazine (DH), 1,1-diphenylamine (DPA), tempo, trolox, ascorbic acid (Asc Ac), butylated hydroxytolulene (BHT) or Cu DIPS and samples of culture media taken after 1 and 2 hours to assess lactate dehydrogenase (LDH) leakage from the cytoplasm. Data are represented as the mean $\pm$ SEM of the EC_{50} (μM) for cytoprotection as assessed by the % LDH leakage at each time point ($n=3$ observations).

6.3.2 Antioxidant Protection Against Glutathione Loss by Allyl Alcohol, tert-Butylhydroperoxide and Nitrofurantoin

GSH, one of the main antioxidant molecules in cells, contributes to cytoprotection during exposure to prooxidants. The ability of the antioxidants to prevent the decrease in GSH mediated by the abovementioned toxic compounds was therefore examined. This is illustrated in Figure 6.5a to c. Allyl alcohol (100 μ M, Figure 6.5a), tBuOOH (250 μ M, Figure 6.5b) and nitrofurantoin (600 μ M, Figure 6.5c) induced approximately 78, 87 and 72% loss of GSH respectively at times that precede noticeable cell death. One-way ANOVA's revealed that the level of GSH in cells treated only with the antioxidants were not significantly different to the level of GSH in control (RPMI alone) cells. Hence, GSH values from cells treated with antioxidants alone were not included in Figure 6.5 to improve the clarity of this Figure. In doing this, statistical significance was observed for all tBuOOH treated groups ($\pm$ antioxidants) and most allyl alcohol treated groups ($\pm$ antioxidants). No statistical significance was observed within the nitrofurantoin group, even when comparing control to nitrofurantoin alone, although a clear decrease in GSH levels is evident. Co-treatment with the antioxidants had no effect on the toxin induced loss of GSH, although an increase is seen in cells treated with nitrofurantoin and most of the antioxidants. This observation, though, is matched by a substantial increase in the error bars in these treatment groups. The data therefore shows that as with hydralazine and dihydralazine, antioxidants do not prevent against the loss of GSH mediated by allyl alcohol and tBuOOH. It is currently unclear, however, whether antioxidants protect against GSH loss induced by nitrofurantoin.

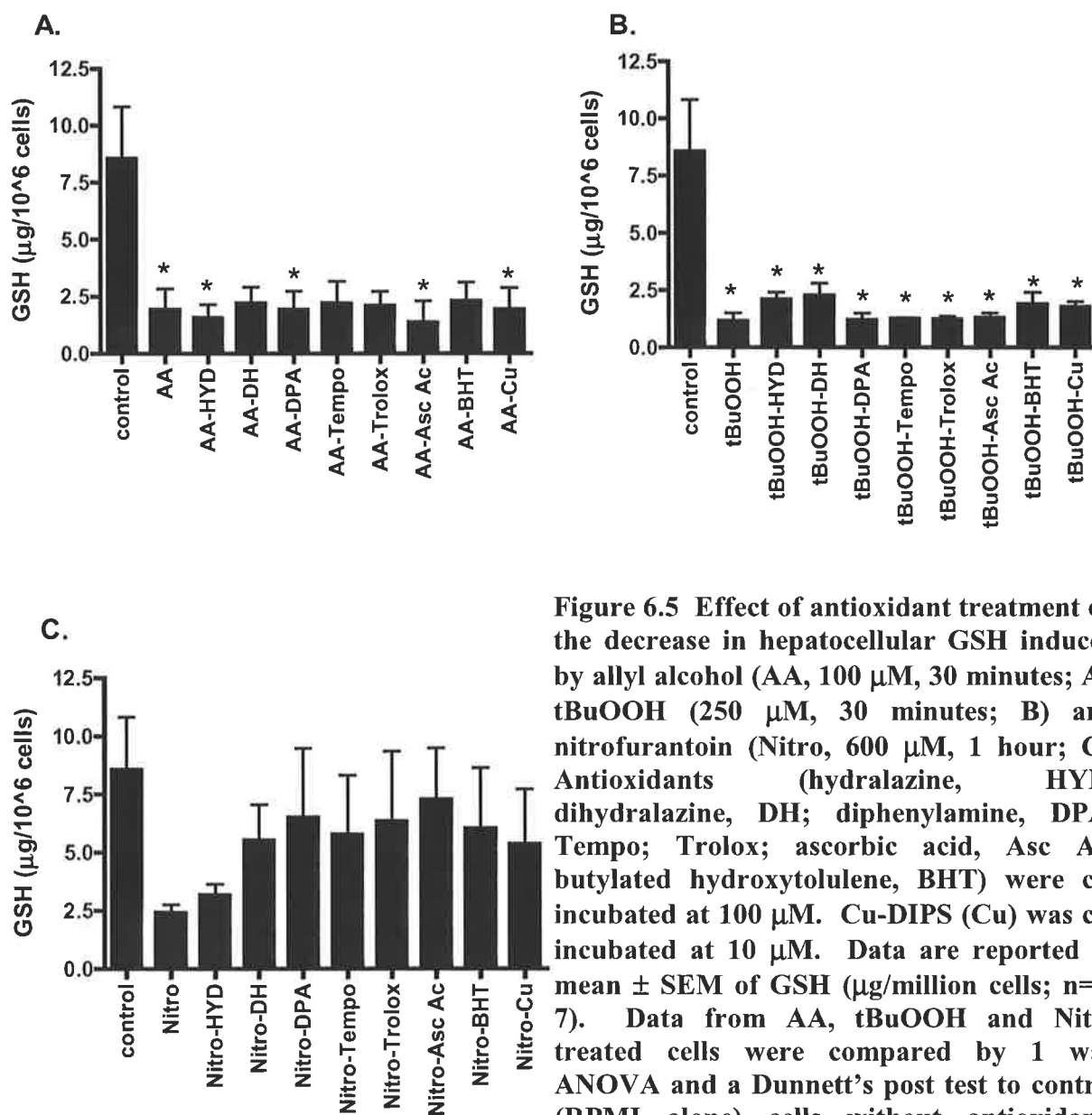


Figure 6.5 Effect of antioxidant treatment on the decrease in hepatocellular GSH induced by allyl alcohol (AA, 100 μ M, 30 minutes; A), tBuOOH (250 μ M, 30 minutes; B) and nitrofurantoin (Nitro, 600 μ M, 1 hour; C). Antioxidants (hydralazine, HYD; dihydralazine, DH; diphenylamine, DPA; Tempo; Trolox; ascorbic acid, Asc Ac; butylated hydroxytolulene, BHT) were co-incubated at 100 μ M. Cu-DIPS (Cu) was co-incubated at 10 μ M. Data are reported as mean $\pm$ SEM of GSH (μ g/million cells; n=3-7). Data from AA, tBuOOH and Nitro treated cells were compared by 1 way ANOVA and a Dunnett's post test to control (RPMI alone) cells without antioxidants alone in the data set. * p<0.05.

6.3.3 Scavenging of Galvinoxyl and DPPH Radicals by Antioxidants

The above results show that hydralazine mediated protection exhibited similarities to protection afforded by a number of radical scavengers. To address this issue more directly, the radical scavenging abilities of hydralazine were compared to the abovementioned antioxidants by examining their ability to donate 1-electron to the radicals galvinoxyl and DPPH. These results were used to examine whether any correlation exists between the cytoprotective efficacy of the compounds and their radical scavenging effects. Scavenging reactions proceeded as shown for

galvinoxyl in Figure 6.6. The rate of radical scavenging for each antioxidant was calculated over the initial portion of each curve (the linear portion). The concentration of antioxidants used to calculate their rate of DPPH scavenging was 10 μM , whereas the concentration deemed appropriate to calculate the rate of galvinoxyl scavenging was 2.5 μM . The galvinoxyl and DPPH assays are based on the ability of antioxidants to donate $\text{H}^\bullet$ to the chromogenic radical species, generating a non-radical compound which is non-chromogenic. The assays therefore measure the loss of galvinoxyl absorbance as a function of time. Hence, an example of galvinoxyl scavenging by the antioxidants is shown in Figure 6.6.

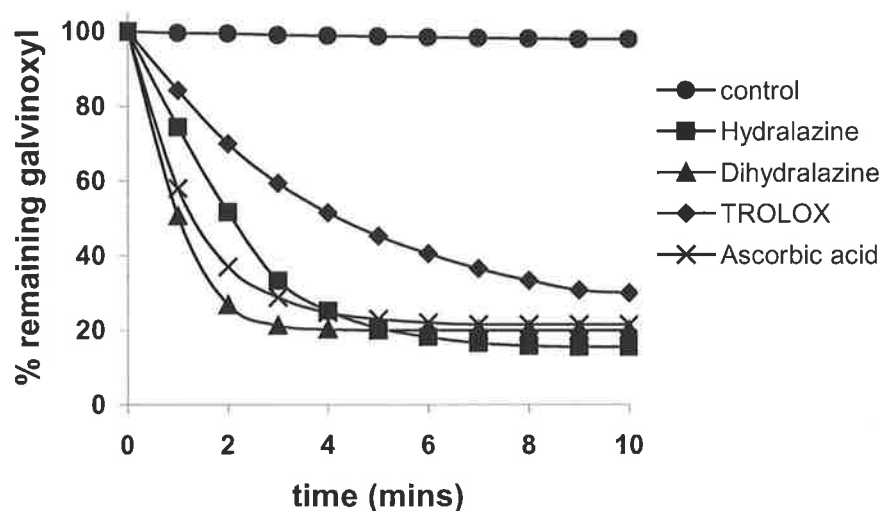


Figure 6.6 Rate of galvinoxyl (5 μM) scavenging by antioxidants (2.5 μM) over a 10 minute period. For clarity, the loss of galvinoxyl in the presence of diphenylamine, BHT and Tempo has not been included here, as these compounds did not appreciably scavenge galvinoxyl at the concentration used.

Table 6.1 shows the rate of galvinoxyl scavenging by 2.5 μM concentrations of hydralazine, dihydralazine and the antioxidants within the first minute of reaction. The control loss of galvinoxyl over this period was insignificant. No galvinoxyl scavenging was evident for diphenylamine, Tempo or BHT. Trolox was also a weak scavenger of galvinoxyl radicals. In contrast, hydralazine, dihydralazine and ascorbic acid rapidly sequestered these radicals. These results are intriguing given that ascorbic acid provides no protection against allyl alcohol, tBuOOH or nitrofurantoin toxicity but is a very efficient scavenger of galvinoxyl radicals. In contrast, while diphenylamine, Tempo and Trolox provide protection against toxicity mediated by these compounds, they proved to be poor scavengers of galvinoxyl.

The rate of basal DPPH loss was similar to the rate of galvinoxyl loss, except that DPPH scavenging by the antioxidants was considerably faster than for galvinoxyl and as a consequence the rate of DPPH scavenging is reported per second (Table 6.1). In this assay, diphenylamine and Tempo interfered with the detection of DPPH by the spectrophotometer, producing a slight but constant increase in the absorbance curve within 12 seconds. Therefore, the rate of DPPH scavenging by these compounds could not be measured. The remaining antioxidants, however, showed similar relative rates of DPPH scavenging as with galvinoxyl, where BHT was a poor scavenger of DPPH radicals and ascorbic acid and Trolox showed rapid scavenging. Once again, the results of radical scavenging experiments with the abovementioned antioxidants revealed no apparent correlations between the radical scavenging properties of the compounds and their cytoprotective abilities against allyl alcohol toxicity.

	Galvinoxyl	DPPH
Control	2.1	1.0
Hydralazine	64.0	5.7
Dihydralazine	61.2	6.3
Diphenylamine	0.4	ND
Trolox	15.8	3.6
Tempo	1.15	ND
Ascorbic Acid	42.0	5.1
BHT	1.14	0.1

Table 6.1 Rate of galvinoxyl and DPPH scavenging by antioxidants. Cu-DIPS was not included here as it is merely a superoxide dismutase mimetic. Data are reported as the % loss of galvinoxyl per minute and the % loss of DPPH per second. Rate was determined over the linear portion of the curve (12 seconds for DPPH, 1 minute for galvinoxyl). Compounds which interfered with the DPPH assay and whose rates of scavenging could therefore not be determined are represented by an ND in the table.

6.3.4 Scavenging of Galvinoxyl and DPPH Radicals by Hydralazine Analogues

The ability of the hydralazine analogues and 2 other hydrazines (1,1-diphenylhydrazine and benzylhydrazine) as well as 2 amine analogues of hydralazine and the basic ring structure of hydralazine (2-aminoquinoline, 1-aminoisoquinoline and phthalazine) to scavenge galvinoxyl radicals was then investigated. The additional hydrazines were included in the analysis as they represent one with a poor acrolein scavenging capacity yet good cytoprotective potency (1,1-diphenylhydrazine) and one with a good acrolein scavenging capacity but poor cytoprotective potency (benzylhydrazine). The amine analogues were examined as they do not provide protection against allyl alcohol toxicity (96 well culture plates, data not shown) and were expected to be poorer 1 electron donors than the hydrazines. Phthalazine was included as an additional negative control since it did not provide any protection against allyl alcohol toxicity (96 well culture plates, data not shown) and was similarly expected to be incapable of donating electrons as it lacks a reactive centre.

The ability of the analogues to scavenge galvinoxyl radicals is shown in Table 6.2. The Table shows 2 distinct groups of compounds: poor galvinoxyl scavengers and efficient galvinoxyl scavengers. The poor galvinoxyl scavengers included the amines (1-aminoisoquinoline and 2-aminoquinoline), phthalazine and benzylhydrazine. The remaining analogues rapidly removed most of the galvinoxyl from solution within 1 minute, after which time the curves plateaued (graph not shown). These results suggest that hydrazines, in general, are efficient 1-electron donors to galvinoxyl radicals, whereas the amine compounds and phthalazine were poor 1-electron donors, as expected.

The ability of the hydralazine analogues to scavenge DPPH radicals is also shown in Table 6.2. Hydralazine analogues were used at $1/10^{\text{th}}$ the molar equivalent of DPPH, although they still exhibited rapid and almost complete removal of the radicals within 12 seconds. However, the ability of the analogues to scavenge DPPH radicals was more varied than for galvinoxyl scavenging. Rather than there being 2 distinct groups of radical scavengers, no DPPH scavenging was evident with the amine compounds and phthalazine whereas partial radical scavenging potency was seen with benzylhydrazine and 2-hydrazinopyridine. 1,1-Diphenylhydrazine interfered with the assay, producing an increase in absorbance, rather than a decrease. This may have been due to formation of a product that strongly absorbed at 517 nm. As a result, the rate of radical scavenging could not be calculated for 1,1-diphenylhydrazine. The remaining analogues showed very rapid and complete scavenging of DPPH within 12

seconds. These results therefore concurred with the results of galvinoxyl scavenging by the compounds.

	Galvinoxyl	DPPH
Control	2.6	1.0
Hydralazine	64.0	5.7
Dihydralazine	61.2	6.3
2-Hydrazinopyridine	68.1	2.7
4-Hydrazinoquinazoline	64.1	6.4
1-Hydrazinoisoquinoline	63.3	5.8
2-Hydrazinoquinoline	68.8	4.6
Naphthylhydrazine	62.1	6.3
Phthalazine	2.4	0.8
1-Aminoisoquinoline	2.0	0.8
2-Aminoquinoline	7.4	0.6
Benzylhydrazine	5.2	1.3
1,1-Diphenylhydrazine	46.1	ND

Table 6.2 Rate of galvinoxyl and DPPH scavenging by analogues of hydralazine and cytoprotective hydrazines. Data are reported as % loss of galvinoxyl per minute and % loss of DPPH per second per second. Rate was determined over the semi-linear portion of the curve (1 minute for galvinoxyl, 12 seconds for DPPH). Compounds which interfered with the DPPH assay and whose rates of scavenging could therefore not be determined are represented by an ND in the table.

Galvinoxyl and DPPH were then used in a correlation analysis between the cytoprotective EC_{50} of the hydralazine analogues and their radical scavenging efficacy. This correlation is shown in Figure 6.7a and b. As the amines and phthalazine did not protect against allyl alcohol toxicity within the concentrations tested they were not included in the correlation analyses (unknown EC_{50} 's). A positive correlation was observed between the cytoprotective EC_{50} 's (reported in section 2.3.10) and DPPH radical scavenging, with an R^2 for linear regression of 0.8978, which was statistically significant ($p < 0.001$). No statistically significant correlation was observed for galvinoxyl, however, which had an R^2 for linear regression of 0.4307. This indicates that the DPPH assay is a better tool for measuring the rate of radical scavenging by aromatic hydrazines

than the galvinoxyl assay. The results obtained with DPPH therefore indicate that the mechanism of cytoprotection against allyl alcohol toxicity may be due to the ability of hydralazine and dihydralazine to sequester oxygen and nitrogen centred radicals formed as a consequence of the biotransformation of allyl alcohol to acrolein. Thus, although the galvinoxyl and DPPH data did not correlate with cytoprotective efficacy for a group of structurally diverse antioxidants (section 6.3.3), it appears the DPPH assay is informative for comparing compounds within a chemical class (ie. arylhydrazino compounds).

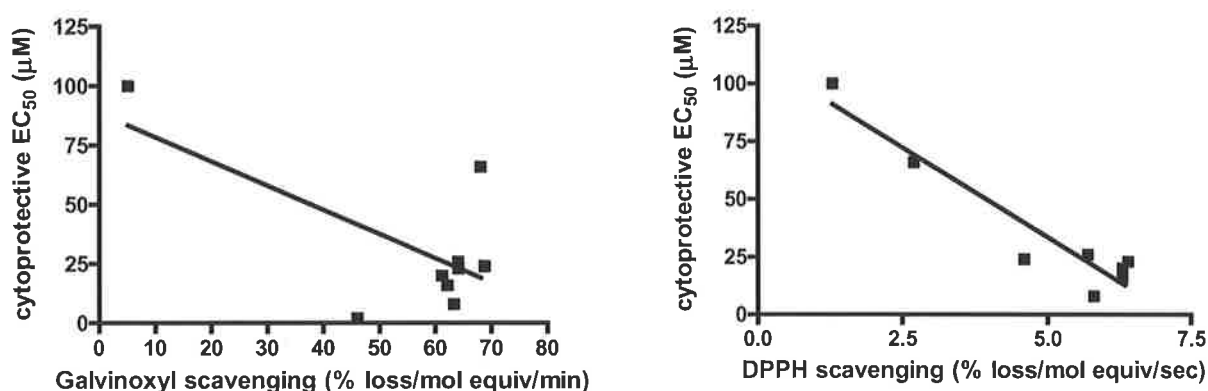


Figure 6.7 Correlation between cytoprotective EC_{50} for allyl alcohol toxicity and the rate of galvinoxyl (A) or DPPH (B) scavenging (% loss of galvinoxyl per minute or % loss of DPPH per second). Rate of radical scavenging was determined over the semi-linear portion of the curve (1 minute for galvinoxyl and 12 seconds for DPPH). Cytoprotective EC_{50} was determined after 2 hours of the addition of allyl alcohol to 96 well microplates (100 μM). A 1-way Pearson's correlation analysis revealed no significant correlation between galvinoxyl scavenging and cytoprotection (R^2 of 0.4307). A significant correlation was achieved, however, for DPPH (R^2 of 0.8978, $p < 0.001$).

6.3.5 Identification of Hydralazine Trapped Aldehyde-Protein Adducts in Hepatocytes Following Incubation with *tert*-Butylhydroperoxide and Nitrofurantoin.

A substantial effort has been made in the course of this project to characterise the reactivity of hydralazine with carbonyl-retaining adducts in proteins formed by α,β -unsaturated aldehydes including acrolein. As numerous unsaturated aldehydes are formed during lipid peroxidation reactions, it was expected that protein carbonylation would accompany tBuOOH and nitrofurantoin toxicity. If so, adduct-trapping might accompany cytoprotection by hydralazine. Hence, hydralazine-trapped acrolein-protein adducts were measured to determine if this trapping reaction occurred in nitrofurantoin and tBuOOH treated cells and whether it contributed to the protection achieved by the hydrazine against nitrofurantoin and tBuOOH toxicity. Figure 6.8

(lane 2) shows the results of Western blot analysis of hepatocellular proteins isolated from mouse hepatocytes treated with 10 μ M allyl alcohol and 100 μ M hydralazine. Clear immunorecognition of hydralazine-trapped acrolein-protein adducts is evident in this lane, even at a lower concentration of allyl alcohol than that used previously to induce hepatocellular toxicity (10 μ M compared to 100 μ M). As expected, no signal was present in lanes 1, 3 or 4 corresponding to allyl alcohol alone, nitrofurantoin alone and tBuOOH alone respectively. Lanes 4 and 6 shows the results of treating hepatocytes with hydralazine (100 μ M) and either nitrofurantoin or tBuOOH. Intriguingly, low levels of hydralazine trapped aldehyde-protein adducts were detected in a single protein corresponding to a mass of approximately 55 kDa. This indicates that although lipid peroxidation was likely initiated by these toxic compounds as reported by others, the generation of toxic aldehydes and their subsequent adduction products on proteins was relatively minor.

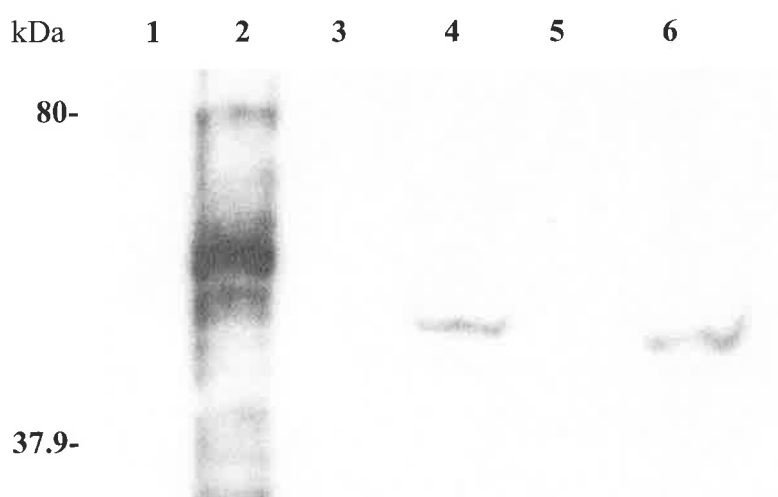


Figure 6.8 Western blot of hydralazine trapped aldehyde-protein adducts from the hepatocytes incubated with low concentration allyl alcohol (10 μ M), nitrofurantoin (600 μ M) and tBuOOH (250 μ M). Cells were treated with either the toxic species alone or co-incubated with hydralazine (100 μ M) for 30 minutes and 60 μ g protein loaded into each lane. The lanes correspond to: allyl alcohol alone (lane 1), allyl alcohol plus hydralazine (lane 2), nitrofurantoin alone (lane 3), nitrofurantoin plus hydralazine (lane 4), tBuOOH alone (lane 5) and tBuOOH plus hydralazine (lane 6).

7.4 DISCUSSION

As was highlighted in the Introduction to this Chapter, allyl alcohol causes cellular injury via its bioactivation to acrolein, followed by the induction of oxidative stress and lipid peroxidation (Jaeschke *et al*, 1992). This Chapter thus addressed the possibility that hydralazine possessed antioxidant actions that interfere with oxidative cell death in allyl alcohol treated cells. The protection afforded by hydralazine was compared to that afforded by a number of antioxidants against allyl alcohol, tBuOOH and nitrofurantoin toxicity. Allyl alcohol is believed to generate oxidative stress and lipid peroxidation by promoting iron release from ferritin and other iron complexing proteins and by depleting glutathione stores (Miccadei *et al*, 1988; Pompella *et al*, 1988; Jaeschke *et al*, 1992). tBuOOH is a hydroperoxide which fragments into peroxy and alkoxy radicals, whereas nitrofurantoin generates nitrofurantoin radicals in cells, which then initiate a redox cycling pathway that results in the generation of superoxide radicals (Lyng *et al*, 1988; Biel *et al*, 1993; Martinez *et al*, 1995).

The protection against tBuOOH and nitrofurantoin toxicity by hydralazine and dihydralazine was not as efficient as with allyl alcohol toxicity. For tBuOOH toxicity, this is possibly the result of an intense oxidative insult ensuing from the fragmentation of the hydroperoxide into alkoxy and peroxy radicals, whereas allyl alcohol induced oxidative stress might be less severe due to the preponderance of non-radical species (acrolein). The more intense burst of oxidants by tBuOOH is consistent with the observation that toxicity mediated by tBuOOH was more rapid than for allyl alcohol. In contrast, nitrofurantoin toxicity was expressed more slowly, resulting in approximately 80% LDH leakage after 3 hours. This is possibly due to the production of a less reactive nitrofurantoin radical, which is then responsible for the production of superoxide radicals due to the cycling of xenobiotic radical and neutral species (Carbonera *et al*, 1988). Alternatively, the better cytoprotection afforded by hydralazine and dihydralazine against allyl alcohol toxicity may be due to a greater contribution of adduct-trapping reactions to protection against the alcohol compared with tBuOOH and nitrofurantoin toxicity. This may be supported by the observation that only a low level of hydralazine-stabilised aldehyde-protein adducts were observed following tBuOOH and nitrofurantoin. Hence, it is likely that protein adduction by α,β -unsaturated carbonyls is less extensive in tBuOOH and nitrofurantoin toxicity than in allyl alcohol toxicity. However, the fact that the same approximately 55 kDa protein was the primary target in cells exposed to these diverse oxidants is highly intriguing and worthy of future attention. It would be interesting to establish whether this 55 kDa protein is the same one that was heavily carbonylated in cells treated with acrolein or MVK in Chapter 4. This finding of

limited adduct-trapping suggests the protection mediated by hydralazine and dihydralazine against tBuOOH and nitrofurantoin toxicity is more likely due to the radical scavenging properties of the hydrazines.

It was interesting to note that whereas hydralazine and dihydralazine were poorer at protecting against nitrofurantoin toxicity than against the other toxins, BHT and Cu-DIPS provided some protection against nitrofurantoin toxicity but not against allyl alcohol or tBuOOH toxicity. This supports the role of superoxide radicals in the toxicity of nitrofurantoin. The fact that BHT and Cu-DIPS did not protect against allyl alcohol and tBuOOH toxicity reflects either the relative unimportance of superoxide radicals in the generation of toxicity by these compounds or that simply the extent of oxidative stress and lipid peroxidation overwhelmed the antioxidants. Also, BHT may not protect against allyl alcohol toxicity if this toxicity is not critically dependent on lipid peroxidation. The nonessential role of lipid peroxidation in allyl alcohol mediated cell death was also proposed by Hormann *et al* (1989), who reported that BHT, Trolox and desferrioxamine blocked MDA production but not the loss of GSH and cell death in rat hepatocytes. In contrast, the sulfhydryl compounds N-acetyl cysteine and dithiothreitol blocked the loss of protein-sulfhydryl groups and cell death (Hormann *et al*, 1989). They therefore concluded that lipid peroxidation is not essential to allyl alcohol mediated cell death but that the loss of protein-sulfhydryl groups is, supporting a role for protein modification in allyl alcohol mediated cell death.

Surprisingly, the antioxidant diphenylamine was the most efficient compound at protecting against allyl alcohol, tBuOOH and nitrofurantoin toxicity, with protective EC₅₀'s in the low micromolar range. Its ability to protect against toxicity was presumably not due to an ability to trap acrolein adducts, since as a secondary amine it is expected to trap adducts less readily than hydrazines. Also, diphenylamine displayed little acrolein scavenging capability in the assay described in Chapter 2 (data not shown). Diphenylamine also did not scavenge galvinoxyl and DPPH radicals, although it has been suggested to be an alkoxy radical scavenger (Hu and Shih, 1997). The protection mediated by diphenylamine may therefore represent another protective mechanism unrelated to radical scavenging. The nature of diphenylamine's antioxidant properties are not well understood, although one group reported that while diphenylamine inhibits malondialdehyde generation and cell death induced by oxidative toxins, it in fact promotes lipid hydroperoxide generation (Sugihara *et al*, 1993). Thus, it is possible that diphenylamine acts as both a redox cyclor and a stabiliser of lipid hydroperoxides (the latter is similar to BHT).

Interestingly, cytoprotection against allyl alcohol, tBuOOH and nitrofurantoin by Trolox (a good radical scavenger) was generally poor, whereas cytoprotection by the weak radical scavenger Tempo was generally good during the first hour of toxicity. Although a number of investigators have reported that water soluble compounds are relatively ineffective at preventing lipid peroxidation whereas lipid soluble compounds are highly effective (Kaneko *et al*, 1991; Fraicco *et al*, 1993; Massaeli *et al*, 1999), this explanation does not explain the discrepancy between cytoprotection and radical scavenging effects for Trolox and Tempo shown here. This is because the experimental log P reported for Tempo is 1.81 (Rosen *et al*, 1982) and 2.9 for Trolox (Ancerewicz *et al*, 1998). However, Massaeli *et al* (1999) reported that hydrophilic and hydrophobic antioxidants are both more effective at scavenging free radicals generated in an aqueous environment compared to those generated in a lipid environment. Furthermore, Nilsson *et al* (1989) suggested that although lipid solubility is important for penetration of antioxidants through lipid membranes, some water solubility is required to allow antioxidants to access the hydrophilic sites of radical generation, such as the redox active sites of P450's (Nilsson *et al*, 1989). This may suggest that Tempo is better able to scavenge radicals generated in an aqueous environment than Trolox, hence providing better protection against oxidative mediated cell death. This highlights the necessary compromise between the lipid solubility of a radical scavenger and its ability to access aqueous compartments of the cell where the radicals are being generated. This is therefore one physicochemical property that may be considered when developing cytoprotective hydrazines directed against α,β -unsaturated aldehyde toxicity.

The importance of lipid solubility of antioxidants was further investigated by Ancerewicz *et al* (1998) who discovered that although there was no correlation found between lipophilicity and DPPH radical scavenging properties, antioxidant lipophilicity was important for preventing lipid peroxidation. For instance, they determined that compounds with log P's below 5 were only weakly effective at preventing against lipid peroxidation although were good scavengers of DPPH. The authors derived a number of calculations to predict the ability of radical scavengers to prevent lipid peroxidation. They determined that the best way to predict the ability of antioxidants to prevent against lipid peroxidation was to take into account lipophilicity, log Z (calculated as the slope of the line between the DPPH reaction rate constant, k, and the ratio of [antioxidant]/[DPPH] producing 50% loss of DPPH), oxidation enthalpy and enthalpy of hydrogen abstraction (from the antioxidant). This indicates that the ability of radical scavengers to prevent lipid peroxidation is more complex than simply an association between radical scavenging capacity and lipophilicity.

In spite of the poor correlation between cytoprotection and DPPH scavenging by the antioxidants, the correlation between cytoprotection and DPPH scavenging for the hydralazine analogues was good. This is intriguing given that a number of studies have reported that hydralazine is a redox cycler that promotes mild oxidative stress (Sinha and Patterson, 1983; Weglarz and Bartosz, 1991; Runge-Morris, 1994; Goodwin *et al*, 1996). Also, given the existing correlation between cytoprotection and log P this also indicates that DPPH scavenging was correlated with lipophilicity. The strong correlation between cytoprotection and radical scavenging by the hydralazine analogues is due to their structural similarity, as previous investigators have noted similar correlations between the radical scavenging and lipid peroxidation blocking effects of structurally related compounds. This correlation is generally lost, however, by the addition of structurally diverse compounds (Nilsson *et al*, 1989; Ancerewicz *et al*, 1998).

The existing correlation between DPPH radical scavenging and cytoprotection also indicates that hydralazine and its analogues may provide some protection against allyl alcohol toxicity by blocking lipid peroxidation. However, one group of investigators have suggested that although lipid peroxidation occurs during allyl alcohol toxicity, this process is not essential to toxicity (Hormann *et al*, 1989). Therefore, the ability of the hydralazine analogues to block lipid peroxidation needs to be investigated to evaluate the significance of this mechanism in the protective effects of the analogues. For instance, an investigation is required to evaluate whether hydralazine blocks lipid peroxidation in allyl alcohol exposed cells. A preliminary study was conducted during this project to address this question using the TBARS assay for malondialdehyde production (which is used as an indicator of lipid peroxidation), however hydralazine interfered with the assay as it appeared to scavenge malondialdehyde (data not shown). Another assay is therefore required in order to address this issue, although this was considered beyond the scope of the current thesis. Specifically, an alternative assay that does not involve detection of electrophilic species that could possibly react with hydralazine is required to address this issue.

The present Chapter aimed to briefly examine the possible role of radical scavenging in the cytoprotection against allyl alcohol toxicity afforded by hydralazine and related aromatic hydrazines. This was achieved by examining the correlation between the cytoprotective effects of the hydrazines and their radical scavenging properties. The results suggested that some of the protection afforded by these hydrazines may be related to their radical scavenging effects,

although thus far no clear evidence has been presented that this mechanism is significant in hepatocytes. However, given the clear correlation between the adduct-trapping properties of aromatic hydrazines and cytoprotection (Chapter 3), it seems that protein adduction by acrolein is an important step in the induction of cellular toxicity by allyl alcohol. Given that acrolein has been shown to release iron from iron binding proteins and that this process is likely involved in the induction of lipid peroxidation, trapping reactions may serve to block this process (Ferrali *et al*, 1990; Jaeschke *et al*, 1992). Evidence of this mechanism is therefore required to confirm the pharmacological role of adduct trapping in blocking oxidative stress and lipid peroxidation by allyl alcohol. Also, an investigation is required to evaluate how trapping reactions block this process. For instance, protein adduct-trapping by hydrazine containing compounds might block conformational changes in iron binding proteins, perhaps preventing the release of iron. Alternatively, trapping of acrolein adducts in other antioxidant proteins may block functional changes, possibly preventing inhibition of the protein by acrolein. Further work should endeavour to identify which intracellular proteins are targets for acrolein and also which residues are important for maintaining the integrity of target proteins. The radical scavenging properties of the hydrazines may then serve to trap any radicals produced by ensuing oxidative stress that escapes the primary trapping mechanism of protection. This would imply that the radical scavenging properties of arylhydrazines serve as a secondary protective mechanism.

Chapter 7:

General discussion and conclusion

7.1 2-ALKENAL-TRAPPING REACTIONS

Given the role acrolein and related α,β -unsaturated aldehydes play in exacerbating and mediating various disease processes, our group has focussed on developing drugs to counteract this toxicity. One treatment method that was explored was the administration of nucleophilic compounds that would effectively act as sacrificial molecules for acrolein adduction. The theory was that by administering a compound that was a stronger nucleophile than the amino acids typically adducted by acrolein, the administered compound would be preferentially adducted by the aldehyde, sparing proteins and DNA. The compounds that were initially screened were the amines carnosine, pyridoxamine and methoxyamine and the hydrazines aminoguanidine, hydralazine and dihydralazine (Burcham *et al*, 2000). Hydralazine and dihydralazine were chosen on the basis that hydralazine, a potent antihypertensive due to its $C=N=N-C=NH-NH_2$ functional group, is one of the most nucleophilic drugs in clinical use (Reece, 1981). This initial study showed that the amines, as well as aminoguanidine, are poor scavengers of acrolein, whereas hydralazine and dihydralazine are highly efficient at removing free acrolein from buffered solution at physiological pH and temperature (Burcham *et al*, 2000). Also, hydralazine and dihydralazine were shown to protect isolated mouse hepatocytes against allyl alcohol toxicity. Due to the fact that the addition of acrolein directly to cells represents a somewhat non-physiological way of assessing its toxicity, allyl alcohol was used as a means to generate acrolein by oxidative biotransformation within cells. Carnosine, pyridoxamine and aminoguanidine did not protect against allyl alcohol toxicity, even at concentrations up to 10 mM (Burcham *et al*, 2002). Methoxyamine, which had a mild acrolein scavenging capability, showed some cytoprotection, but only at concentrations that were an order of magnitude higher than the concentrations of hydralazine and dihydralazine required to elicit cytoprotection. However, Burcham and co-workers (2004) also noted that hydralazine protected against allyl alcohol toxicity even when hydralazine and dihydralazine were added to the culture dishes after a 25

minutes treatment with allyl alcohol followed by allyl alcohol washout. Allyl alcohol toxicity was still observed to occur after this washout. This raised questions about the mechanism of action of hydralazine, as the results showed that protection could be afforded even after acrolein toxicity had been initiated.

The general aim of the current study was therefore twofold; to determine the mechanism of hydralazine mediated protection against acrolein toxicity and to develop or identify better cytoprotective compounds. The former aim required an initial investigation of the significance of acrolein scavenging in the cytoprotection against allyl alcohol toxicity. The latter aim was based upon removing the antihypertensive effect of hydralazine while still retaining cytoprotective efficacy. It was expected that this would ultimately be achieved by disrupting the $=N-N=C-NH-NH_2$ moiety which is responsible for the antihypertensive effects of the drug (Reece, 1981), while retaining the hydrazine group, which appears to mediate its cytoprotective capacity. A working hypothesis that hydralazine mediated protection against acrolein toxicity by scavenging free aldehydes guided initial experiments in this study.

It was shown that the ability of the amine compounds, as well as aminoguanidine, to scavenge acrolein from solution was very poor. In contrast, hydralazine and dihydralazine were highly efficient scavengers of both acrolein and crotonaldehyde, although the sulfur containing compound examined, MESNA, was more efficient. In addition, hydralazine and dihydralazine effectively protected hepatocytes against allyl alcohol, crotyl alcohol, pentenal, propargyl alcohol and malondialdehyde toxicity. The current study also confirmed that hydralazine protects against allyl alcohol induced liver injury in whole mice in a dose dependent manner. *E*-APH (the main isolable product of reactions of free acrolein and hydralazine) was further shown to be a detoxification product of co-incubation of hepatocytes with allyl alcohol and hydralazine, which provided support for the role of acrolein scavenging in cytoprotection mediated by the antihypertensive drug. This product, however, was not detected in the plasma of whole mice treated with allyl alcohol (90 mg/kg) and hydralazine (300 μ mol/kg) for 1 hour, possibly due to the rapid removal of free *E*-APH by hepatocytes as shown *in vitro*.

Following this initial investigation of hydralazine's ability to scavenge acrolein and protect against its toxicity, a number of analogues of hydralazine as well as various hydrazines were examined for their ability to scavenge free acrolein and protect against allyl alcohol toxicity. A number of aryl hydrazino compounds were identified as being up to six times more effective than dihydralazine at protecting cultured mouse hepatocytes against allyl alcohol toxicity. It

appeared from the compounds tested that hydrazine groups attached directly to conjugated ring systems were required for cytoprotective activity, since addition of a $-\text{CH}_2-$ spacer between the hydrazine and the phenyl rings decreased cytoprotective potency. Unexpectedly, however, a correlation analysis between the acrolein scavenging effects of the compounds and their cytoprotective EC_{50} 's revealed an inverse correlation between scavenging capacity and cytoprotection, indicating that the mechanism of cytoprotection by hydralazine was not due to aldehyde scavenging per se.

7.2 ADDUCT-TRAPPING REACTIONS

Following results showing that aldehyde scavenging reactions do not fully explain the mechanism of hydralazine induced cytoprotection against aldehyde toxicity, an alternative hypothesis was therefore examined; that hydralazine mediated protection is due to an ability to "trap" acrolein-protein adducts formed from allyl alcohol within cells. This view was based upon work performed concurrently in the laboratory which was recently published (Burcham *et al*, 2004). To explore this mechanism, acrolein-KLH antiserum was raised in rabbits to detect acrolein modified proteins in BSA and cells (Burcham *et al*, 2004). It was initially determined by these authors that hydralazine abolished immunorecognition of acrolein-BSA adducts when added 30 minutes after acrolein in a concentration dependent manner. In a similar manner, co-treatment of primary cultured hepatocytes with acrolein (500 μM) and hydralazine (300 to 3000 μM) abolished the immunorecognition of acrolein-protein adducts generated during a 15 minute exposure of cells to acrolein alone. However, data obtained using the acrolein-KLH antibody did not definitively reveal how hydralazine was blocking the detection of acrolein adducts. To directly determine whether adduct-trapping was responsible, an antibody was similarly raised in rabbits against hydralazine stabilised acrolein-KLH adducts. The use of this antibody allowed for not only the detection of hydralazine bound acrolein-protein adducts, but also for the tentative detection of acrolein-protein adducts. Using this antibody, it was determined that hydralazines ability to block immunorecognition by the acrolein-KLH antibody was due to a concentration dependent formation of hydralazine stabilised acrolein-protein adducts in BSA (Burcham *et al*, 2004). With longer incubations of acrolein with BSA prior to the addition of hydralazine, the ability of hydralazine to block immunorecognition of acrolein-BSA adducts decreased. The site of these adducts were suggested to be on lysine and histidine residues, as pre-incubation of either antibody with the corresponding modified polyaminoacyl derivatives inhibited immunorecognition. These experiments did not address the issue of whether

hydralazine traps acrolein adducts at cysteine residues. It is expected, however, that modification of cysteine residues in proteins accompanies lysine and histidine modifications, as the cysteine containing tripeptide GSH is rapidly adducted by acrolein even in the presence of hydralazine. These results suggested that the ability of hydralazine to trap acrolein-protein adducts was crucial in its ability to protect against allyl alcohol toxicity. The results also indicated that although the current study showed that scavenging of acrolein occurs in cells exposed to allyl alcohol and hydralazine, evident via the formation of *E*-APH, trapping of acrolein-protein adducts by hydralazine also occurs on proteins that have escaped the removal of free acrolein by hydralazine.

Although the abovementioned immunochemical data indicated that hydralazine readily traps acrolein-adducted proteins, the identity of the trapped adducts needed to be determined by mass spectrometry. In particular, the identity of the acrolein adducts likely to form during short incubations of hepatocytes with allyl alcohol needed to be indirectly determined to identify the toxic species. To address this in a simple cell-free model, the modification of a model lysine containing peptide (PPE) by acrolein was investigated. The results of mass spectrometry investigations suggest that the drug-trapped adducts detected by the acrolein-KLH antibody are likely to include mono-, bis-, FDP-lysine and FDP-glycine adducts on the N-terminus. Given that the acyclic adducts of acrolein form on lysine and N-terminal glycine residues within the time that acute toxicity to the aldehyde occurs, it may be that 2-alkenal toxicity is mediated by these acyclic products and not by the cyclised FDP-lysine and EMP-lysine identified by Uchida (1999). Given that these cyclic adducts form after longer incubations of the aldehydes with proteins and that they are highly immunogenic, they may contribute to toxicity expressed over longer time frames. In particular, they may trigger inflammatory responses *in vivo*, perhaps eliciting lipid peroxidation and cell death due to the production of inflammatory cytokines and oxygen centred radicals.

However, given that acrolein was still capable of forming 6-membered cyclic products during incubation with PPE, the possibility that these products contribute to short term allyl alcohol toxicity could not be fully dismissed. The toxicity and alkylating capacity of methylvinyl ketone was therefore examined, in the expectation that it is more sterically hindered at the carbonyl carbon than acrolein, thereby resulting in a lesser ability to form cyclised products on lysine. This ketone was found to rapidly kill isolated hepatocytes with the toxicity prevented by co-incubation with hydralazine. It did not, however, result in a decrease in cellular GSH, possibly due to a slower reactivity with thiol compounds than aldehydes (Esterbauer *et al*, 1975).

Methylvinyl ketone was further shown to form mono- and bis-adducts of PPE and hydralazine was able to trap these products. Importantly, however, in keeping with expectations, methylvinyl ketone did not produce a FDP-type adduct. Furthermore, methylvinyl ketone exhibited a similar ability to carbonylate both BSA and hepatocytes as acrolein. This suggested that methylvinyl ketone induces cell death by adducting cellular macromolecules and that this death is mediated by the mono- and bis- adducts of the ketone. Hydralazine was therefore likely to have protected against this toxicity by trapping the methylvinyl ketone adducts formed on cells, although the rabbit antiserum (anti hydralazine/acrolein-KLH) was unable to detect these species.

The fact that the reaction of nucleophiles at the carbonyl group of methylvinyl ketone is hindered due to the presence of an additional methyl group on the carbonyl carbon, may ensure this carbonyl would have a lowered capacity to form crosslinks. In contrast, comparison of the toxic potency of acrolein and methylvinyl ketone in cells showed that methylvinyl ketone kills cells more efficiently than the aldehyde. Also, since methylvinyl ketone induced cell death in the absence of a significant loss of GSH, this may suggest oxidative stress and lipid peroxidation makes a lesser contribution to cell death by the ketone relative to acrolein. This might suggest methylvinyl ketone toxicity is due to adduction of cellular macromolecules, rather than the induction of oxidative stress as is suggested for allyl alcohol. Contrary to this deduction, however, is the fact that the rate of PPE adduction by methylvinyl ketone appeared to be much slower than for the reaction of acrolein at high concentrations of carbonyl. Whether the same differences in adduction kinetics are evident between acrolein and methylvinyl ketone with cysteine and histidine containing peptides should be explored.

In vivo toxicity studies performed in the current study using allyl alcohol as a classic hepatotoxicant supported the *in vitro* results of Burcham *et al* (2004) suggesting that adduct trapping is an important contributor to the time dependent hepatoprotection by hydralazine. When administered 20 minutes after allyl alcohol, 200 $\mu\text{mol/kg}$ hydralazine protected against hepatic injury to the same extent as with co-administration. However, when hydralazine was administered 30 minutes after allyl alcohol no significant protection was observed. Co-administration of hydralazine also resulted in intense dose dependent formation of hydralazine-acrolein-protein adducts as assessed by both Western blotting and immunohistochemistry. This corresponded with hepatoprotection by the hydrazine. An explanation for this time dependent protection may lie in the formation of crosslinked species via acyclic Michael acrolein adducts, although given the similarities between the kinetics of allyl alcohol and methylvinyl ketone

toxicity this seems unlikely, since methylvinyl ketone is expected to be less efficient as a crosslinking reagent. However, it does appear that the presence of carbonylated proteins is important in the toxicity of acrolein and allyl alcohol alike, suggesting that these species undergo further reactions within 30 minutes that render the cell incapable of recovery. It is also possible that crosslinking is not involved in the acute toxicity of carbonylating agents, or rather, that the number or identity of adducted species is more important. For instance, overwhelming cellular proteins with multiple carbonyl groups may sufficiently corrupt the function of a number of proteins important for cell survival. Alternatively, accumulation of FDP-lysine adducts may ultimately determine the fate of the cell, where trapping the carbonyl groups of mono- and bis-adducts by cytoprotective drugs blocks the formation of these cyclic species.

The unexpected finding that acrolein adduction was not localised in the periportal zones raises intriguing questions about the zonal heterogeneity of allyl alcohol hepatotoxicity *in vivo*. It does appear, however, that the cells themselves do not have altered susceptibilities to allyl alcohol toxicity, as previous investigators have not observed any differences in the toxicity of allyl alcohol in cultured or perfused periportal and pericentral cells (Penttila, 1988; Przybocki *et al*, 1992). The cellular localisation of hydralazine-acrolein-protein adducts also suggests that DNA-protein crosslinks in the nucleus do not contribute to short term allyl alcohol toxicity, since this organelle was not labelled by the antibody. Hence, based on the distribution of trapped adducts it appeared that the key site of acrolein damage was the cytosol and cell membrane. Acrolein may trigger cell death by compromising the integrity of the cell membrane or by adducting crucial proteins in the cytosol. Sites of acrolein adduction thought to contribute to this toxicity include iron binding proteins, such as ferritin, and the mitochondrial membrane (Ferrali *et al*, 1990; Jaeschke *et al*, 1992; Tirmenstein *et al*, 2000). Adduction of these targets apparently results in an altered redox status in the cell, due to the ensuing overproduction of free radicals from the Fenton reaction or interruptions in the respiratory electron transport chain (Miccadei *et al*, 1988; Jaeschke *et al*, 1992; Tirmenstein *et al*, 2000).

7.3 ANTIOXIDANT PROPERTIES OF HYDRALAZINE

Given that allyl alcohol also induces lipid peroxidation in exposed cells, many researchers have suggested that its toxicity is mediated by the induction of oxidative stress secondary to the depletion of cellular GSH. Also, given that antioxidants such as α -tocopherol protect against allyl alcohol toxicity and that some researchers have suggested that hydralazine may possess

antioxidant properties, the ability of antioxidants to protect against allyl alcohol toxicity was investigated. An effort was thus made to characterise the antioxidant properties of hydralazine using the galvinoxyl and DPPH assays. Through screening of various hydralazine analogues and hydrazines, a strong correlation was found between the DPPH radical scavenging capacity of the hydrazines and their ability to prevent allyl alcohol toxicity, while the correlation for galvinoxyl was much weaker. This provides support for the view that allyl alcohol toxicity is due to oxidative stress and lipid peroxidation and raises intriguing questions about the relationship of antioxidant actions versus adduct-trapping to the cytoprotection. However, since the toxicity of allyl alcohol is due to its biotransformation to acrolein and given that acrolein is not itself a pro-oxidant, it is unlikely that it forms radicals without first adducting proteins. It is possible that acrolein induces oxidative stress by both releasing iron from iron binding proteins in cells, such as ferritin, and by inhibiting detoxifying enzymes such as catalase, superoxide dismutase and glutathione peroxidase. These would have the effect of increasing the production of reactive oxygen species and inhibiting the detoxification of hydrogen peroxide.

Given that acrolein adduction may underlie the induction of oxidative stress following allyl alcohol exposure, the role of adduct trapping by hydralazine becomes important to potentially block iron release and the functional changes induced in proteins by acrolein. Hence, one group of investigators recently examined possible molecular mechanisms underlying allyl alcohol toxicity in hepatocytes. Maddox *et al* (2003) reported that up to 100 μ M allyl alcohol activates protein kinase C δ (PKC) after a 90 minute incubation in rat hepatocytes. Specific inhibition of this protein by rottlerin abolished allyl alcohol toxicity. This effect may also reflect functional consequences of protein crosslinking reactions by acrolein. A similar effect was not seen for other PKC isoforms (Maddox *et al*, 2003). This indicates that there appears to be multiple pathways by which allyl alcohol induces cell death, which includes effects on signal transduction. Hydralazine, however, does not affect PKC, eliminating PKC inhibition as a potential mechanism of cytoprotection (Sun and Robinson, 1995). Again, however, hydralazine may interact with acrolein adducted cysteine residues on the protein, thereby blocking downstream reactions that may underlie the functional consequences of protein activation by acrolein. It is likely, however, that whereas allyl alcohol toxicity appears to be mediated by a wide variety of mechanisms, hydralazine may only act on a select few of these processes.

7.4 CONCLUSION

The results of this work indicate that hydralazine and other aryl-hydrazines provide protection against toxicity induced by unsaturated carbonyl compounds when administered soon after commencing exposure to their alcohol precursors. The precise mechanisms involved in this protection require additional investigation, although results obtained thus far indicate that 2-alkenal sequestering reactions do not mediate protection by nucleophilic compounds. Alternatively, adduct-trapping reactions at lysine and N-terminal residues might prevent functional consequences in proteins that ultimately lead to cell death. The mechanism of this prevention may lie in the ability of adduct-trapping compounds to block crosslinking reactions. The identity of these critical cellular proteins and their subcellular localisations are still the subject of some speculation, although iron-binding proteins such as ferritin are likely contributors to carbonyl toxicity. In such proteins, adduct trapping reactions may block the release of iron from these proteins, thereby blocking downstream oxidative stress induced by the free transition metal. Such a mechanism would also explain the observations made by a number of investigators showing that antioxidants also protect against allyl alcohol toxicity. In addition to adduct-trapping reactions, the results of this study also suggest that hydralazine and other cytoprotective aryl-hydrazines possess radical scavenging properties that likely also contribute to the protection seen by these nucleophiles. This mechanism of protection is likely to be important in trapping any radicals generated as a consequence of functional changes in proteins that escape blockade by the initial trapping reactions.

7.5 FUTURE DIRECTIONS

This work has raised a number of questions regarding the mechanism of cytoprotection by hydralazine. However, this work is still in its infancy and a lot of work needs to be done to confirm the mechanism of protection proposed in this thesis. For instance, the protection of allyl alcohol toxicity needs to be investigated over a longer period of time (ie. 24 hours) to assess whether specific types of intervention completely abolish toxicity. It would also be of interest to examine the type of cell death occurring after extended incubations of hepatocytes with high (ie. 100 μ M) and low (ie. 10 μ M) concentrations of allyl alcohol and hydralazine. For instance, does hydralazine merely delay oncotic cell death or does it prevent oncotic cell death and allow for slower onset apoptotic cell death? This would be of interest in chronic diseases, which are accompanied by local necrosis and massive inflammation. The high doses of hydralazine used in

this study would be inappropriate for use in humans, particularly given its vasoactive properties. Such antihypertensive effects and other side effects of hydralazine, however, may be overcome through the use of one of the analogues identified. Therefore, further investigation into the toxicity and antihypertensive effects associated with these analogues is warranted.

It has previously been suggested in this thesis that illnesses that involve carbonyl stress and which are prevalent during old age (ie. Alzheimer's disease, atherosclerosis and arthritis) may be alleviated using nucleophilic drugs. This was initially suggested with the view to trapping free aldehydes generated during lipid peroxidation. It is now proposed that adduct-trapping reactions at reactive carbonyl-protein adducts formed from such free aldehydes also deserves attention as a way of protecting against the toxic effects of the aldehydes, hence alleviating the presenting illness. As hydralazine is a clinically used antihypertensive drug (most likely used predominantly in the elderly), an investigation may be undertaken to determine whether the drug provides relief against illnesses associated with oxidative damage. For instance, a determination may be made of whether the drug (by trapping reactive carbonyl-protein adducts) slows the formation of crosslinked proteins in Alzheimer's disease. One possible mechanism by which hydralazine may provide relief against Alzheimer's disease could be via blocking the formation of β -amyloid crosslinks, a major mediator of neuronal cell death (Perry *et al*, 1991; Pike *et al*, 1991; Roher *et al*, 1996; Harper *et al*, 1997). Alternatively, blocking reactive carbonyl groups on adducted low density lipoproteins may prevent their accumulation in macrophages, hence blocking differentiation of macrophages into foam cells (the predominant cell type in atherosclerotic lesions) (Fogelman *et al*, 1980; Henriksen *et al*, 1981; Esterbauer *et al*, 1993). These mechanisms may be investigated using cell based and mass spectrometry experiments to determine whether hydralazine is capable of preventing the formation of β -amyloid crosslinks and whether trapping carbonyl-LDL adducts prevents the uptake of modified LDL into macrophages.

The identity of proteins vital to the onset of 2-alkenal toxicity is also required, as thus far their identity has been subject to speculation. An investigation into the identity of such protein targets was beyond the scope of this thesis, which was aimed specifically at identifying the mechanism of 2-alkenal cytoprotection mediated by hydralazine and to identify/develop novel analogues with lesser vasoactivity. However, the focus of future work should be aimed at identifying major protein targets for acrolein following administration of allyl alcohol to whole Swiss mice as well as their subcellular localisation. This could be achieved through 2D gel electrophoresis of the livers of allyl alcohol treated and control mice, followed by Western blotting and identification

of acrolein adducted proteins using the acrolein/KLH antiserum raised in this laboratory. Digestion and tandem mass spectrometry of corresponding proteins from control mice would serve to identify these proteins using peptide mass fingerprinting and information gained from the 2D gel, including the size of the adducted proteins and their pI's. A similar second experiment would be aimed at identifying adducted proteins trapped by hydralazine. Following identification of these proteins *in vitro* and *ex vitro* experiments may be conducted to determine if: 1) using specific inhibitors or inducers of target proteins influences the toxicity of allyl alcohol to isolated hepatocytes, 2) if adduction by acrolein affects the function of the proteins and 3) if subsequent trapping by hydralazine restores the loss of protein function mediated by acrolein adduction. Further mass spectrometry experiments on peptide fragments of adducted proteins may serve to identify the residues which are important for the functional changes induced by acrolein adduction. For instance, the importance of cysteine adduction has not been addressed here, although adduction of such residues may have important functional consequences, possibly by interfering with the formation of disulfide bonds within proteins.

The antioxidant role of hydralazine also needs to be addressed further by examining if 1) it possesses iron or copper chelating properties and 2) if it blocks lipid peroxidation in cells. The second point cannot be addressed by analysing the MDA content in allyl alcohol and hydralazine exposed cells, as hydralazine might trap free MDA, thereby confounding the results. However, analysis of other less reactive lipid peroxidation products may address this problem. For instance, analysis of isoprostanes formed during this process might provide insight into the relevance of hydralazines antioxidant actions in biological systems. These are important questions, as the role of antioxidant properties of hydralazine is still not clear, particularly its relationship to adduct-trapping reactions.

The role of crosslinking reactions in 2-alkenal toxicity also needs to be explored further. This is the subject of current investigations by Dr Phil Burcham who is investigating the ability of hydralazine to trap carbonyl-retaining acrolein adducts in model proteins and its subsequent ability to prevent crosslinking between proteins. However, although such experiments may address the question as to whether hydralazine blocks protein crosslinking, it does not provide any information pertaining to whether such reactions underlie toxicity to unsaturated carbonyls. Although experiments with methylvinyl ketone suggested that acyclic adducts were important mediators of 2-alkenal toxicity, the ketone group might slow subsequent crosslinking reactions, thereby raising questions about the role of such reactions in acrolein toxicity.

It is also important to determine the fate of trapped adducts. For instance, are these adducts stable or do they target proteins for digestion by proteases? If they are removed by proteases, how soon does this process occur once trapped adducts are formed? These questions may be addressed by inhibiting various cellular proteases and monitoring the intensity of trapped adducts using the hydralazine-acrolein/KLH antiserum. It would also be interesting to determine the fate of digestion products following expulsion from the cell. For instance, whether their removal by the immune system induces massive localised oxidative stress due to the possible strong immunogenicity of the hydrazone groups, or whether their removal occurs in the absence of such a response.

Appendix 1

Chemical characterisations and HPLC assay validation.

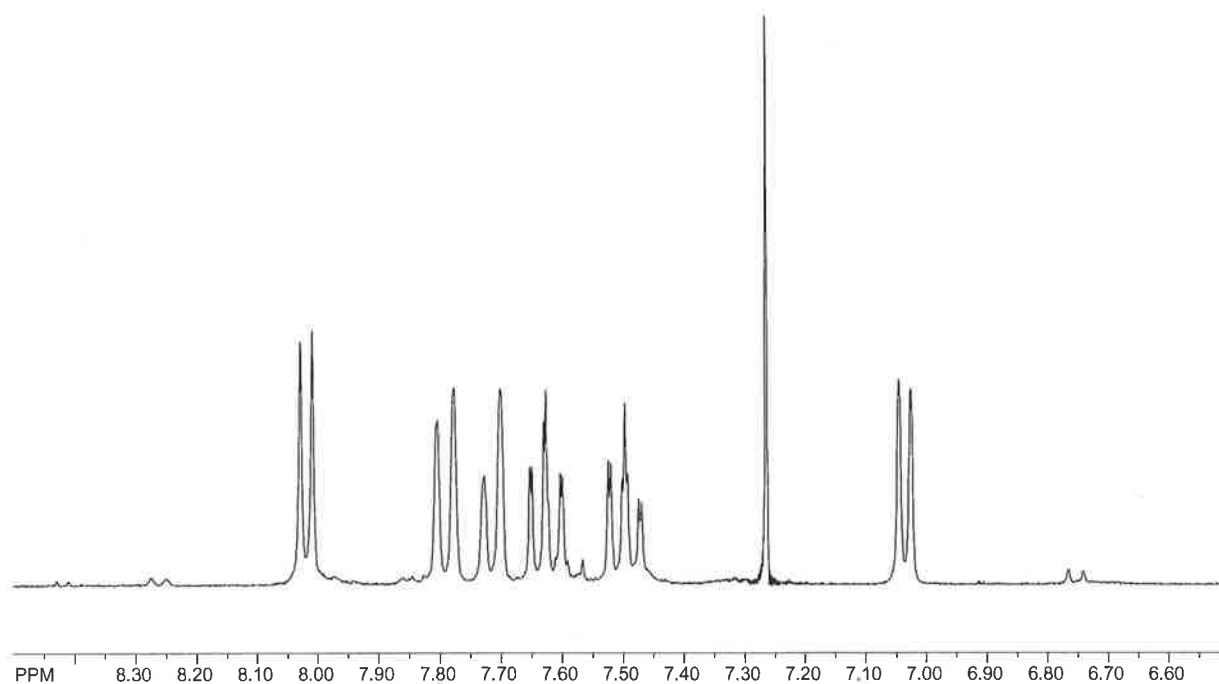
A1.1 Confirmation of 1-Hydrazinoisoquinoline

MP of synthesised 1-hydrazinoisoquinoline= 174-175°C

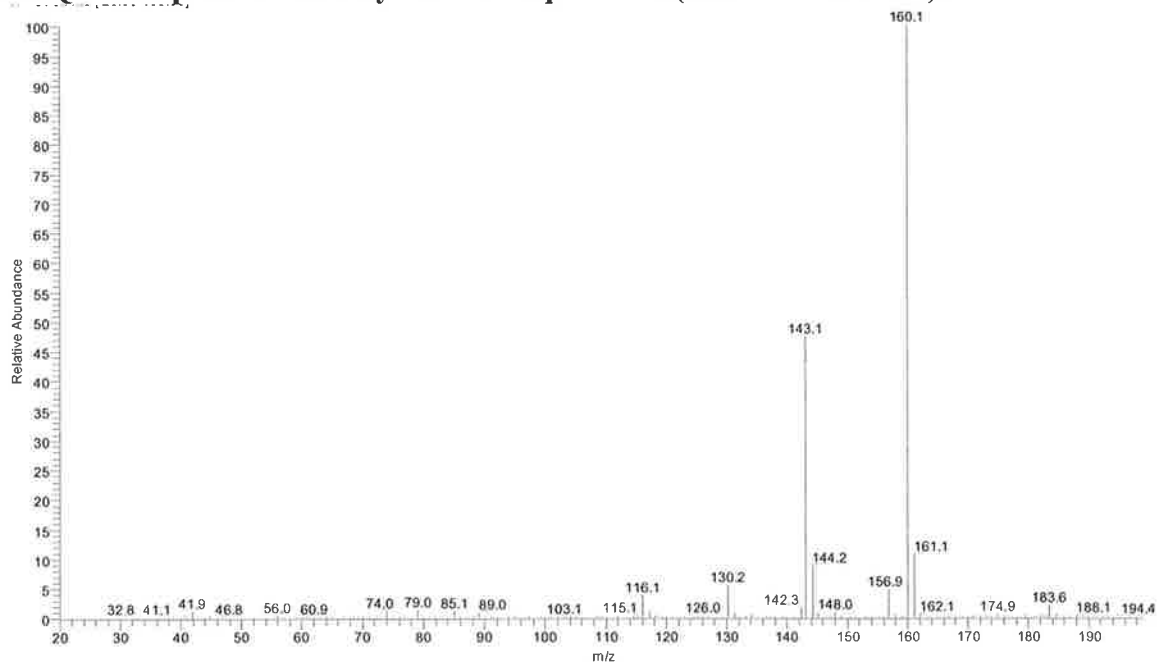
MP of 1-hydrazinoisoquinoline (Albert and Catterall, 1967) = 172-173°C

IR spectrum (cm^{-1} , nujol): 3333, 3266, 3187, 2923, 1625, 1597, 1562, 1524

Expanded ^1H NMR of 1-hydrazinoisoquinoline (CDCl_3):



LCQ mass spectrum of 1-hydrazinoisoquinoline (acetonitrile:water):



A1.2 HPLC Assay Validation for Acrolein and Crotonaldehyde

Values of acrolein and crotonaldehyde close to the limit of quantification (LOQ, 0.5 μM) are not vital to the interpretation of the data as it indicates that only 1-2% of remaining aldehyde in solution is present. For this reason, % coefficient of variation (%CV, which shows the reproducibility of aldehyde concentrations) and % Inaccuracy (% Inacc, which represents the accuracy of aldehyde concentrations) limits have been set at 50%, providing the QCM (middle quality control concentration, 5 μM) and QCH (high quality control concentration, 17.5 μM) are within 20% variance. An acceptable slope (determined as the area under the peak over aldehyde concentration in μM) %CV was determined to be 20% based on the number of validation replicates.

Inter-day validation of acrolein and crotonaldehyde:

	Acrolein		Crotonaldehyde	
	Slope	r^2	Slope	r^2
Mean	511.33	0.9982	446.73	0.9998
SD	21.41	0.0008	26.35	0.0002
% CV	4.19		5.90	
N	10		10	

QC (μM)	Acrolein			Crotonaldehyde		
	H (17.5)	M (5.0)	LOQ (0.5)	H (17.5)	M (5.0)	LOQ (0.5)
Mean	17.61	4.84	0.69	17.42	5.02	0.56
SD	0.17	0.10	0.05	0.10	0.03	0.05
% CV	0.98	2.14	7.97	0.57	0.66	8.2
% Inacc	0.63	-3.26	38.08	-0.45	0.0	11.15
N	10	10	10	10	10	10

Intra-day validation of acrolein and crotonaldehyde:

	Acrolein		Crotonaldehyde	
	Slope	r^2	Slope	r^2
Mean	475.2	0.9983	446.14	0.9996
SD	-86.2	0.0007	5.94	0.0007
% CV	5.06		1.33	
n	6		6	

QC (μ M)	Acrolein			Crotonaldehyde		
	H (17.5)	M (5.0)	LOQ (0.5)	H (17.5)	M (5.0)	LOQ (0.5)
Mean	17.47	4.93	0.66	17.48	4.99	0.57
SD	0.04	0.03	0.04	0.04	0.05	0.08
% CV	0.23	0.67	5.73	0.25	1.02	13.59
% Inacc	-0.17	-1.5	33	-0.11	-0.20	13.0
N	6	6	6	6	6	6

Reproducibility of Acrolein Dilutions and Multiple Injections:

Multiple solutions of acrolein (10 μ M) in buffer were made up to check the reproducibility between acrolein dilutions and of manual injections onto the HPLC from one solution of acrolein (100 μ l). The area under the acrolein peak was used to assess reproducibility.

	Repeat injections	Multiple dilutions
Mean area	5467.1	5518.8
SD	123.5	101.0
%CV	2.3	1.8
N	6	6

A1.3 Validation of the *E*-APH Assay on the ODS Hypersil (150 X 4.6 mm) column

Area under the APH peak was used to assess APH content. The % coefficient of variation (%CV) limits have been set at 30 % variance provided the % Inaccuracy (%Inacc) is within 20%.

Inter-day validation of the APH assay:

	Slope	r ²
Mean	1226.1	0.9994
SD	35.6	0.0012
%CV	2.9	
N	6	

QC (μM)	H (10.0)	M (3.0)	LOQ (0.5)
Mean	9.92	2.98	0.53
SD	0.13	0.06	0.06
%CV	1.35	1.93	10.46
%Inacc	-0.85	-0.50	6.91
N	6	6	6

Intra-day validation of the APH assay:

	Slope	r²
Mean	1257.9	0.9999
SD	15.8	0.0000*
%CV	1.26	
N	6	

* value to 6 decimal places is 0.000049

QC (μM)	H (10.0)	M (3.0)	LOQ (0.5)
Mean	9.94	3.01	0.51
SD	0.02	0.02	0.02
%CV	0.20	0.62	3.51
%Inacc	-0.57	0.20	2.52
N	5	6	6

Reproducibility of Multiple Stock Solutions, Dilutions and Injections of APH:

Stock solutions of APH were made and used for up to 7 days (5 mM). The reproducibility in making these stock solutions was therefore assessed along with the reproducibility of making up multiple dilutions from this stock and of multiple manual injections from one solution. A volume of 100 μl of 10 μM solutions were injected onto the column to assess this where area under the APH peak was used.

	Repeat injections	multiple dilutions	multiple stock soln^s
Mean	11567.9	11836.0	12003.4
SD	122.2	199.5	503.0
%CV	1.06	1.69	4.19
N	6	6	3

A1.4 Validation of *E*-APH and (1*E*,2*E*)-BPH Assays on the Zorbax Eclipse XDB C18 (250 X 4.6 mm) column

Inter-day assay validation for APH:

	Slope	r^2
Mean	1335.4	0.9979
SD	36.7	0.0015
%CV	2.75	
N	6	

QC (μ M)	H (11.25)	M (4.5)	LOQ (0.9)
Mean	11.27	4.41	1.05
SD	0.20	0.11	0.08
%CV	1.73	2.46	7.60
%Inacc	0.22	-2.10	16.6
N	6	6	6

Intra-day assay validation for APH:

	Slope	r^2
Mean	1407	0.9975
SD	33.7	0.0033
%CV	2.39	
N	3	

QC (μ M)	H (11.25)	M (4.5)	LOQ (0.9)
Mean	11.00	4.53	1.03
SD	0.41	0.09	0.04
%CV	3.78	1.93	4.10
%Inacc	-2.35	0.76	16.00
N	3	3	3

Inter-day assay validation for BPH:

	Slope	r²
Mean	1774.28	0.9965
SD	396.75	0.0028
%CV	22.32	
N	3	

QC (μM)	H (12.0)	M (5.0)	LOQ (1.0)
Mean	12.03	6.09	0.97
SD	0.45	0.21	0.19
%CV	3.74	3.4	19.59
%Inacc	0.17	1.50	-3.00
N	3	3	3

Intra-day assay validation for BPH:

	Slope	r²
Mean	1778.37	0.9982
SD	57.24	0.0013
%CV	3.21	
N	3	

QC (μM)	H (12.0)	M (5.0)	LOQ (1.0)
Mean	11.82	6.05	0.95
SD	0.13	0.27	0.08
%CV	1.10	4.38	7.97
%Inacc	-1.58	0.83	-5.00
N	3	3	3

A1.5 Validation of the HPLC Assay for *E*-APH in Cells

Inter-day assay validation for APH:

	Slope	r ²
Mean	861.89	0.9960
SD	203.48	0.0023
%CV	23.00	
N	3	

QC (μM)	H (3.0)	M (0.6)	LOQ (0.1)
Mean	2.98	0.55	0.09
SD	0.22	0.14	0.03
%CV	7.05	25.45	28.89
%Inacc	-0.67	-8.33	-10
N	3	3	3

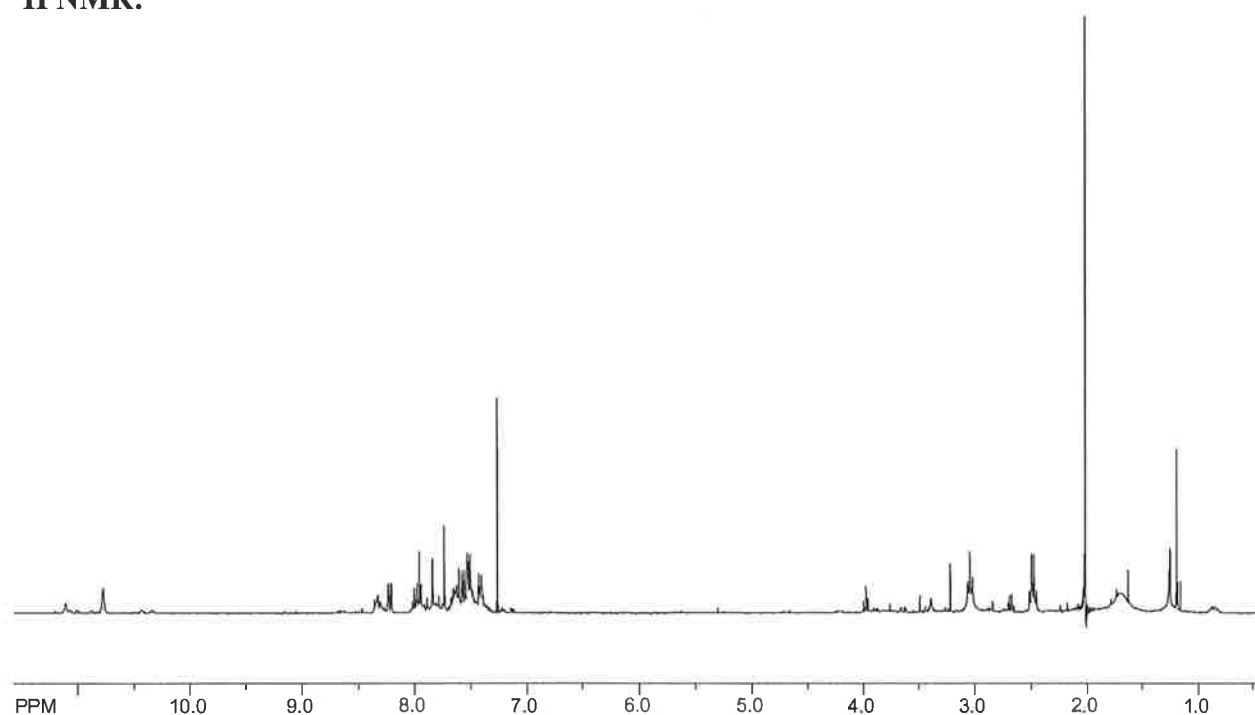
Intra-day assay validation for APH:

	Slope	r ²
Mean	887.32	0.9974
SD	175.09	0.0017
%CV	19.73	
N	3	

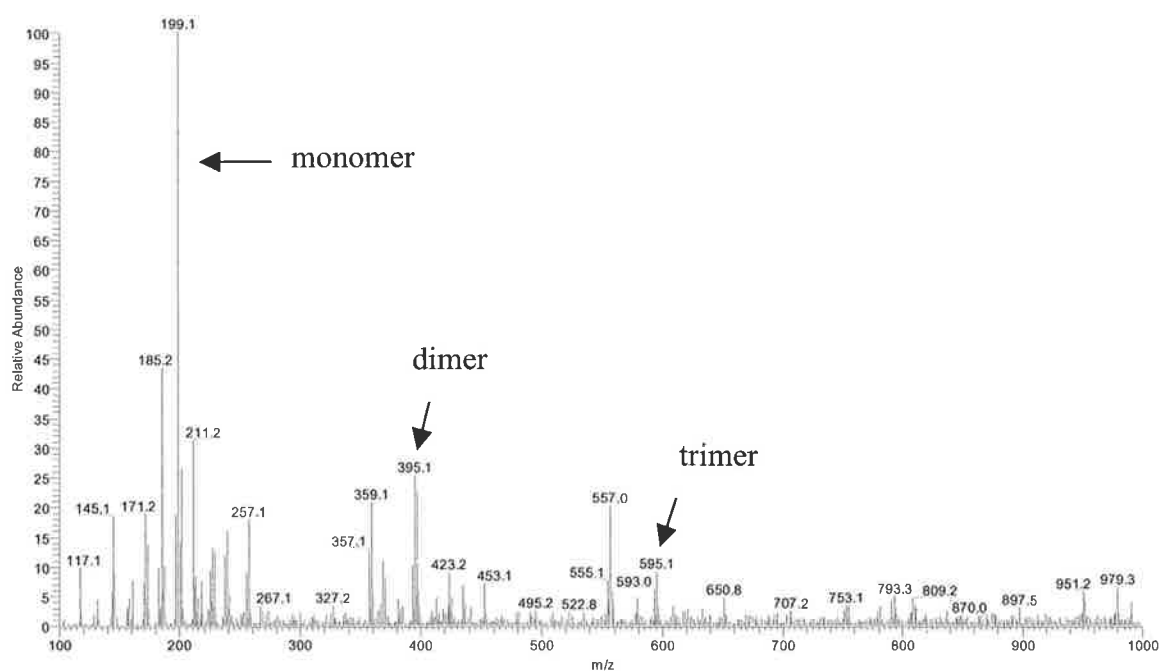
QC (μM)	H (3.0)	M (0.6)	LOQ (0.1)
Mean	2.99	0.62	0.11
SD	0.10	0.06	0.04
%CV	3.34	9.52	27.27
%Inacc	-0.33	3.33	10
N	3	3	3

A1.6 ^1H NMR and LCQ Mass Spectra of Oligomeric APH

^1H NMR:



LCQ mass spectrum:



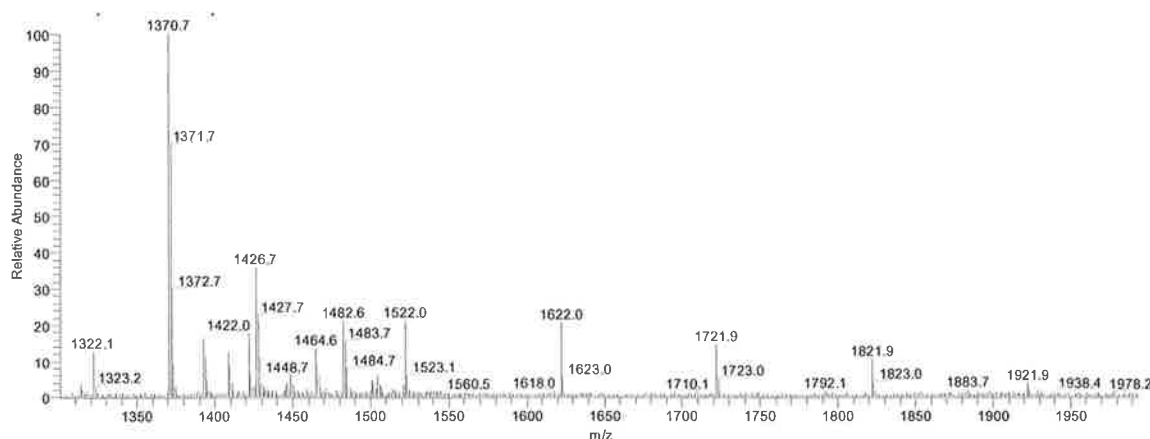
Appendix 2

Time course for acrolein-PPE and hydralazine/acrolein-PPE adduct formation.

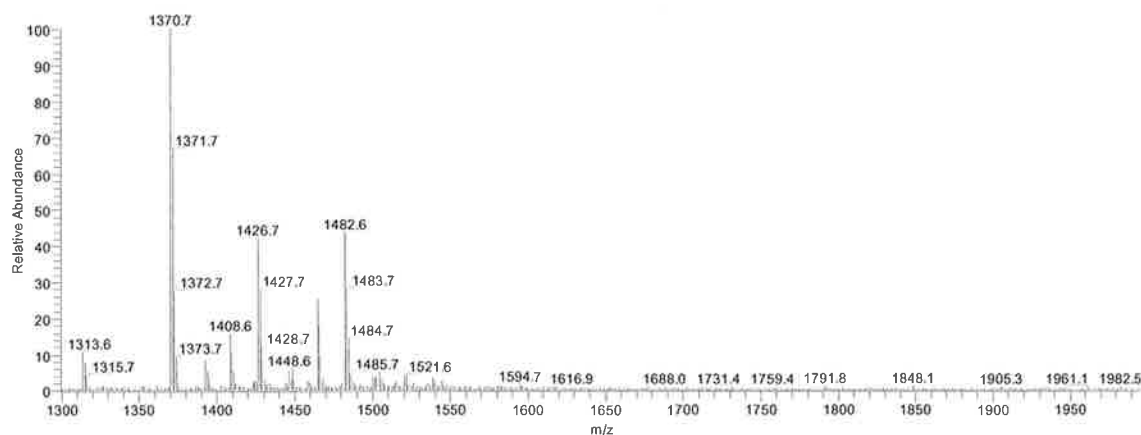
A2.1 Time Course for Acrolein-PPE Adduct Formation

PPE was incubated with a 100 fold excess of acrolein and incubated at 37°C for 3 hours. 25 μ l aliquots of PPE were diluted in an acetonitrile:glacial acetic acid mixture such that 25 μ M PPE was injected onto the LCQ at a flow rate of 8 μ l/minute in 25% acetonitrile and 1% glacial acetic acid in water. Ions were monitored in positive ion mode.

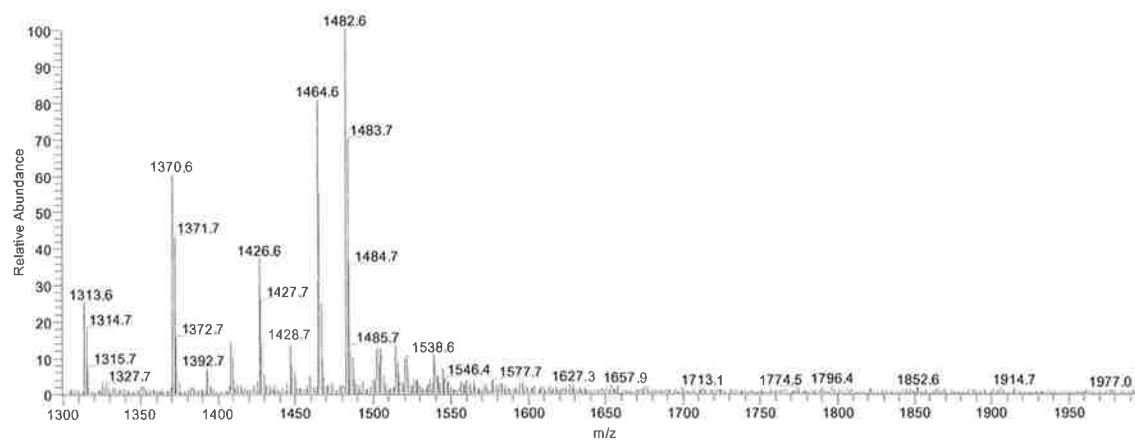
1 hour reaction:



1.5 hour reaction:



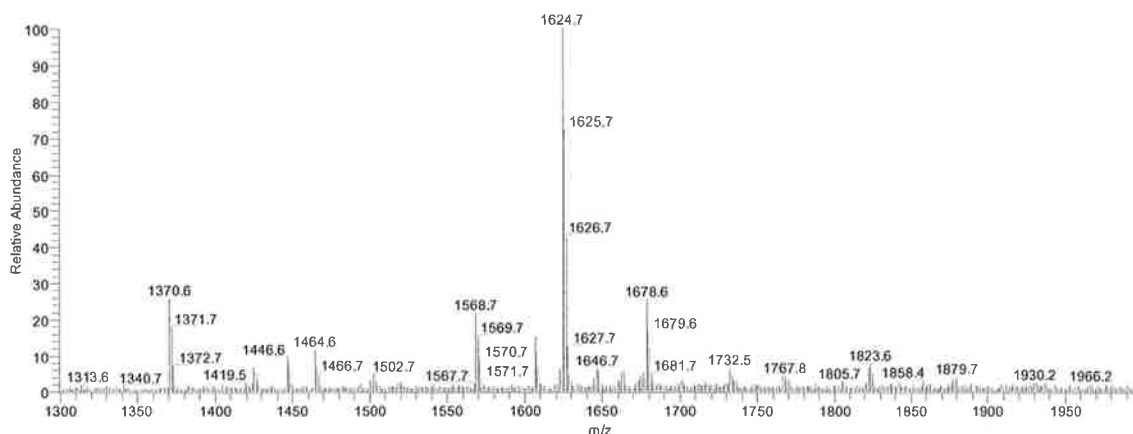
3 hour reaction:



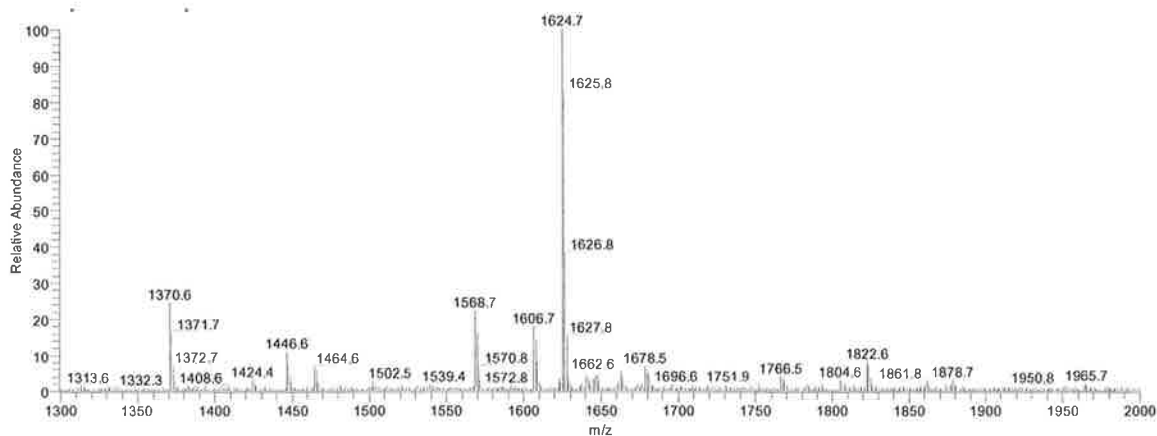
A2.2 Time Course for Trapping of Acrolein-PPE adducts by Hydralazine.

A 10 fold excess of hydralazine (compared to acrolein) was reacted for 1 hour at 37°C with a reacted mixture of acrolein and PPE (3 hours). Hydralazine trapped acrolein-PPE was monitored by positive ion mass spectrometry (25 μ M PPE in 25% acetonitrile/1% glacial acetic acid).

30 minute reaction:



1 hour reaction:



Appendix 3

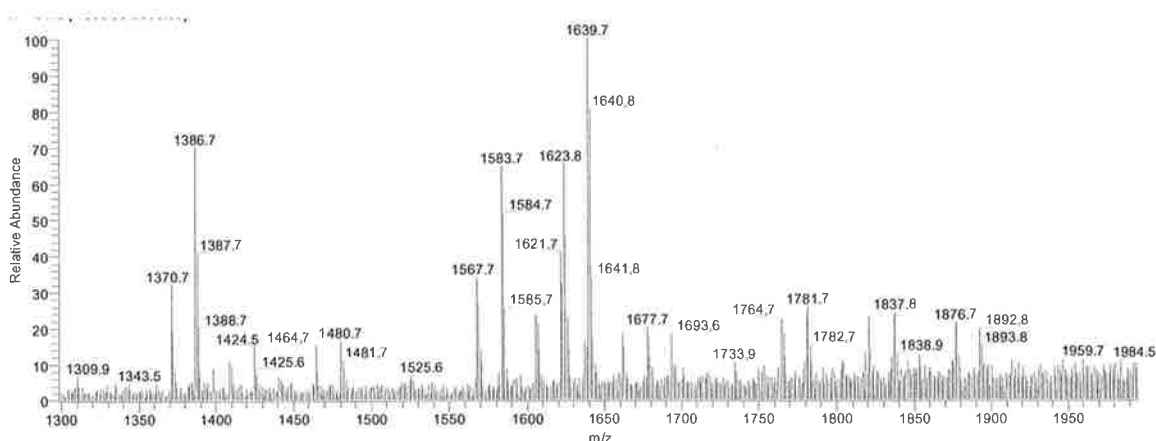
Formation of hydrazine/acrolein-PPE adducts.

A3.1 Time Course for Acrolein-PPE Trapping by Hydrazinoisoquinoline and Adduct Structure Characterisation by MS/MS.

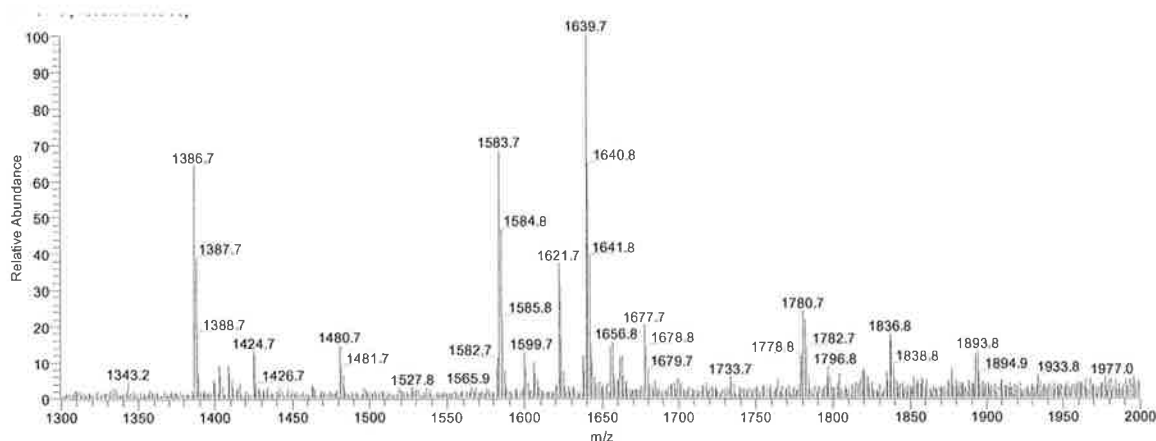
The identification of trapped adducts by MS/MS was via the method reported in Chapter 3 of this thesis. This was by identifying which residue (lysine or N-terminal glycine as indicated by the location of acrolein adducts of PPE in Chapter 3) lost a higher mass than that of its own molecular weight. For instance, cleavage of a lysine residue containing one Michael adducted acrolein was shown by a loss of 184 mass units from this residue (ie molecular weight of lysine plus the molecular weight of Michael adducted acrolein) instead of 128 mass units (the molecular weight of lysine). Below, the mass of each fragment from B cleavages (lower brackets) or Y+2 cleavages (upper brackets) are shown.

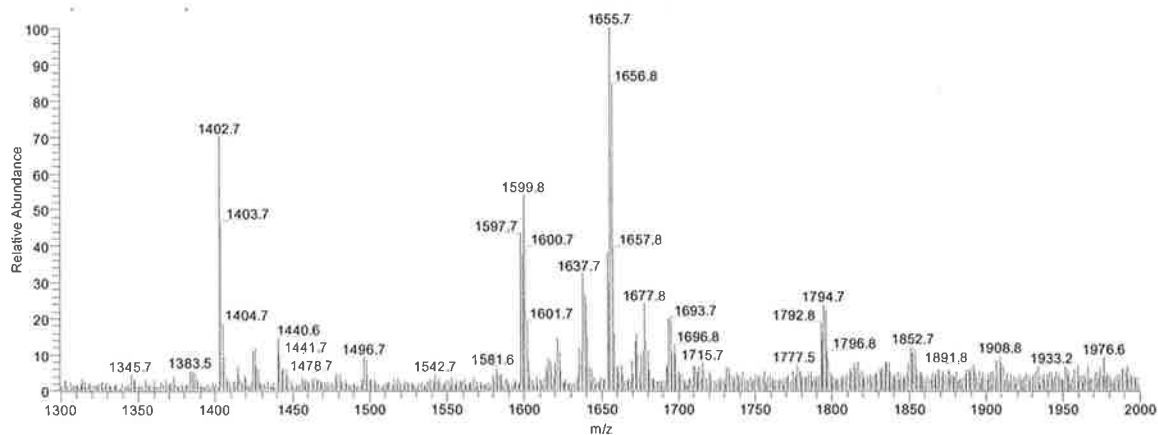
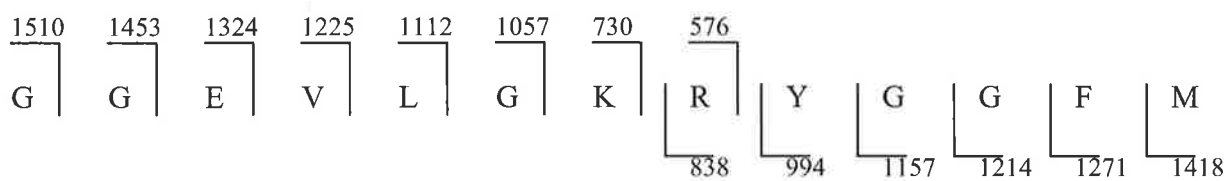
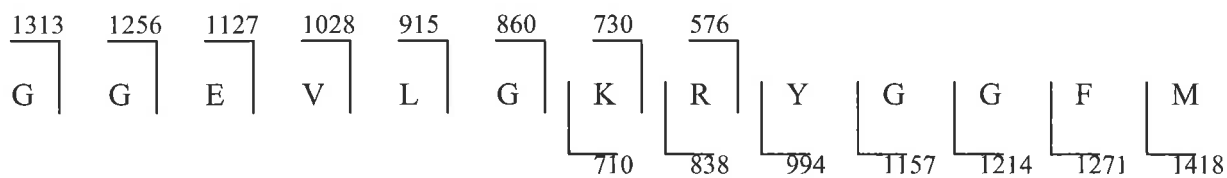
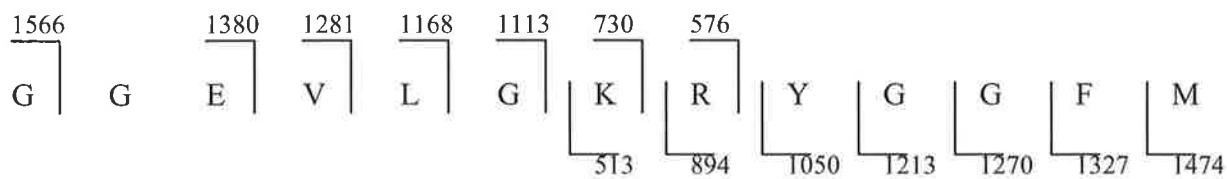
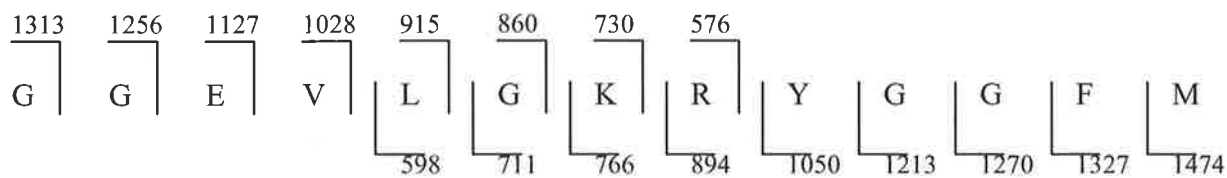
A 10 fold excess of hydrazinoisoquinoline (compared to acrolein) was reacted for 1 hour at 37°C with a reacted mixture of acrolein and PPE (3 hours). Hydrazinoisoquinoline trapped acrolein-PPE was monitored by positive ion mass spectrometry (25µM PPE in 25% acetonitrile/1% glacial acetic acid).

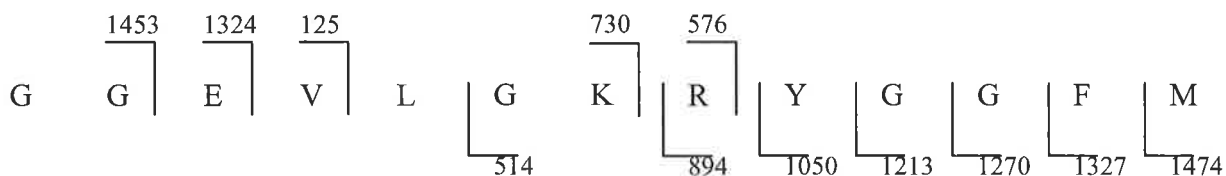
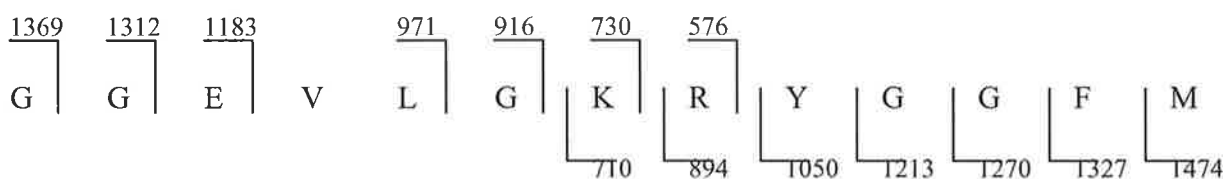
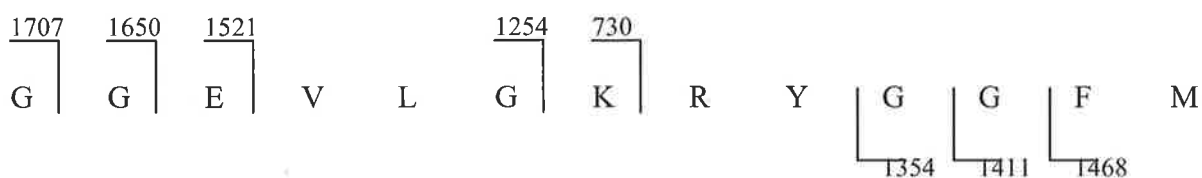
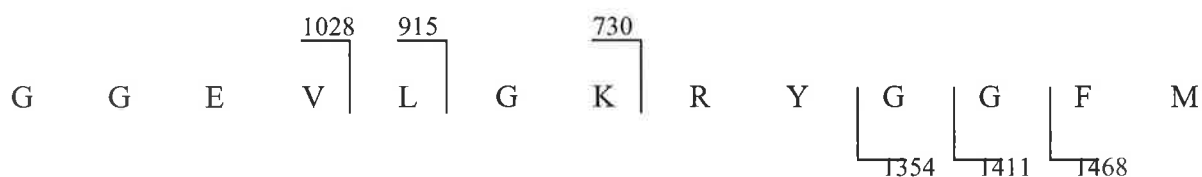
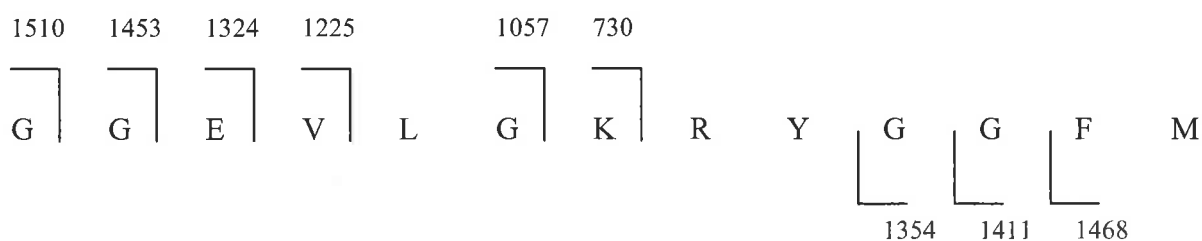
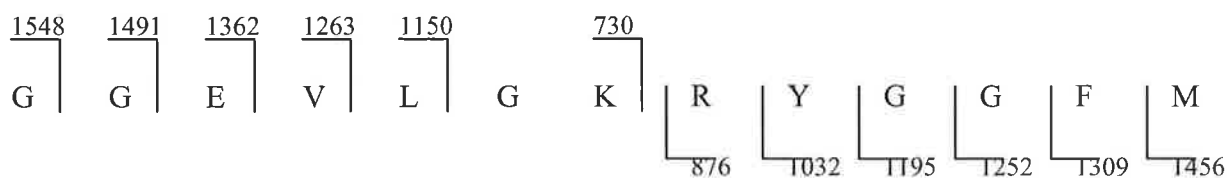
30 minute reaction:

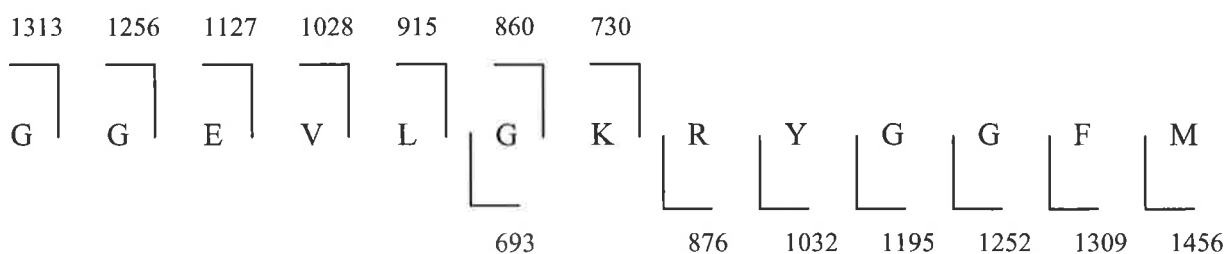
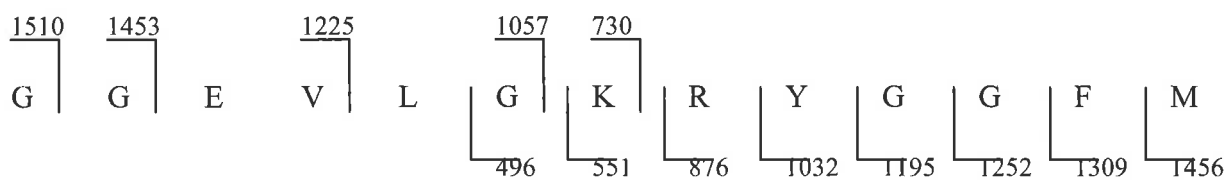
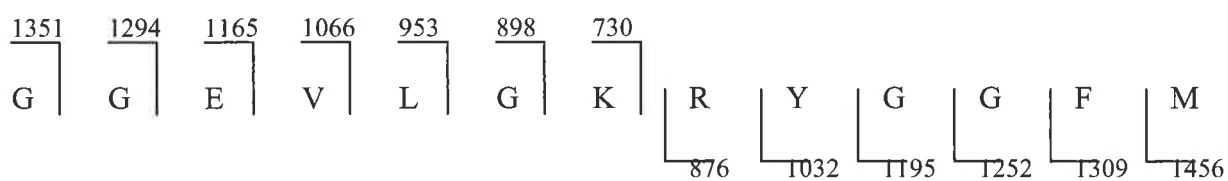


1 hour reaction:



2 hour reaction:**MS/MS of Hydralazinoisoquinoline (HIQ) Trapped Acrolein-PPE Adducts:*****m/z* 1567. mono HIQ-mono acrolein (K)*****m/z* 1567. mono HIQ-mono acrolein (G)*****m/z* 1623. mono HIQ-bis acrolein (K)*****m/z* 1623. mono HIQ-bis acrolein (G)**

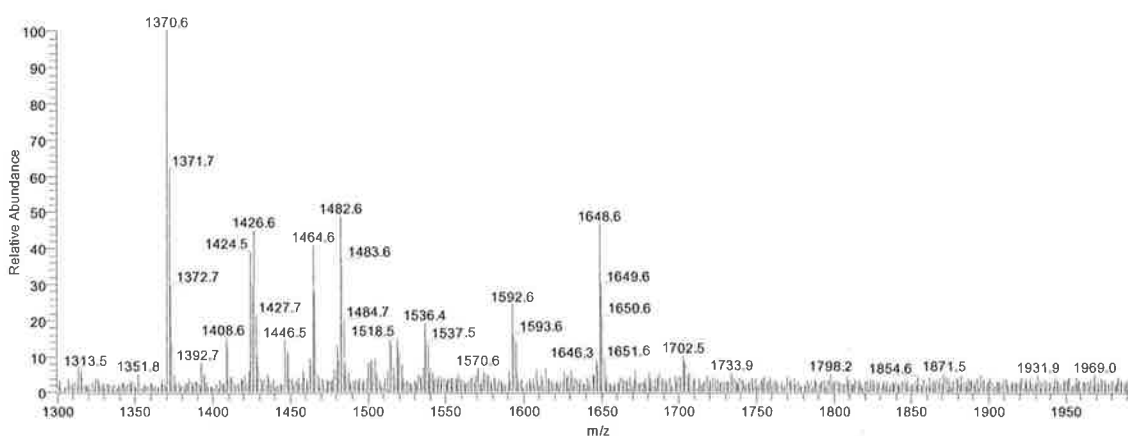
m/z* 1623. mono HIQ (K)-bis acrolein ($1 \times G/1 \times K$)**m/z* 1623. mono HIQ (G)-bis acrolein ($1 \times G/1 \times K$)*****m/z* 1764. bis HIQ-bis acrolein (K)*****m/z* 1764. bis HIQ-bis acrolein (G)*****m/z* 1764. bis HIQ-bis acrolein ($1 \times G/1 \times K$)*****m/z* 1605. mono HIQ-FDP (K)**

m/z* 1605. mono HIQ-FDP (G)**m/z* 1605. mono HIQ (K)-1 × Michael (K)/1 × Schiff (G)*****m/z* 1605. mono HIQ (G)-1 × Michael (G)/1 × Schiff (K)**

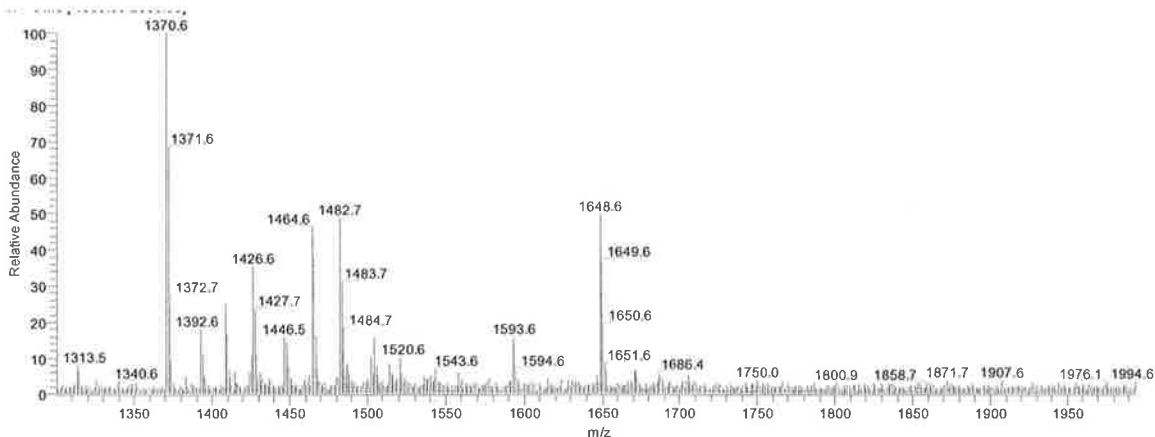
A3.2 Time Course for Acrolein-PPE Trapping by 1,1-Diphenylhydrazine and Adduct Structure Characterisation by MS/MS

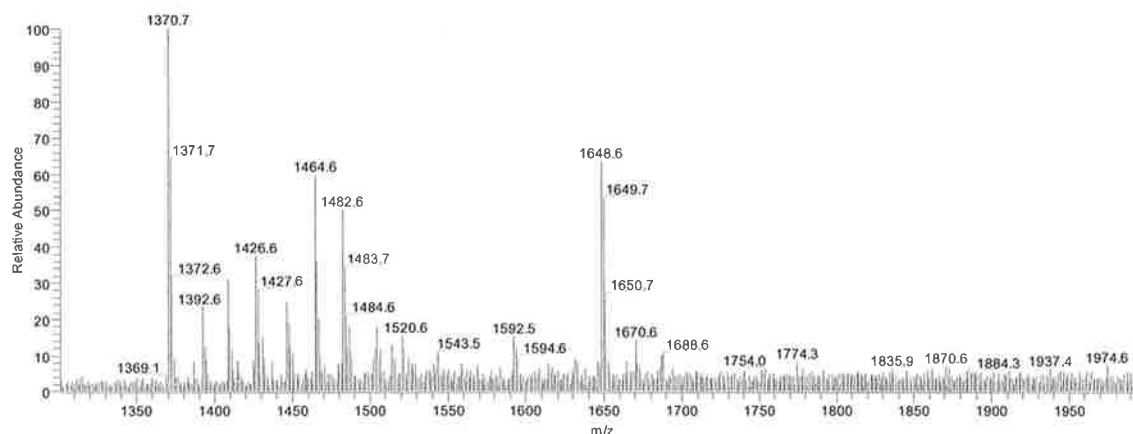
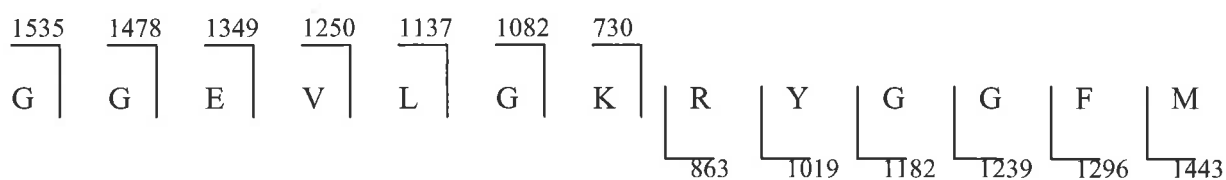
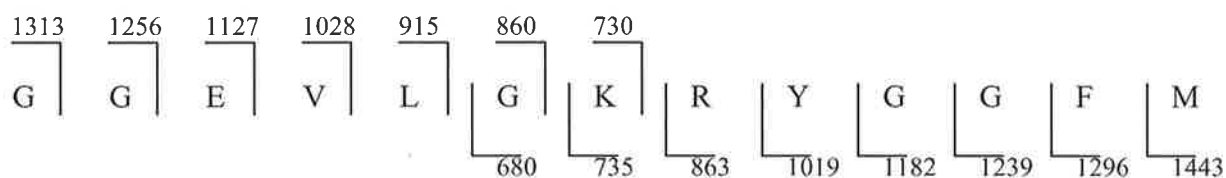
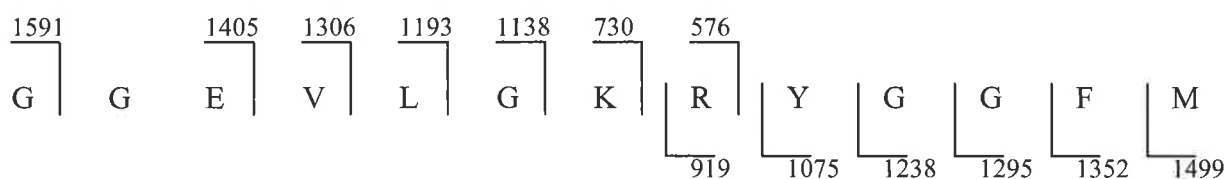
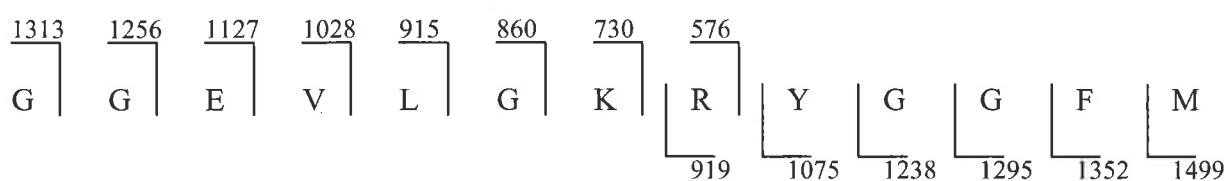
A 10 fold excess of 1,1-diphenylhydrazine (compared to acrolein) was reacted for 1 hour at 37°C with a reacted mixture of acrolein and PPE (3 hours). 1,1-Diphenylhydrazine trapped acrolein-PPE was monitored by positive ion mass spectrometry (25µM PPE in 25% acetonitrile/1% glacial acetic acid).

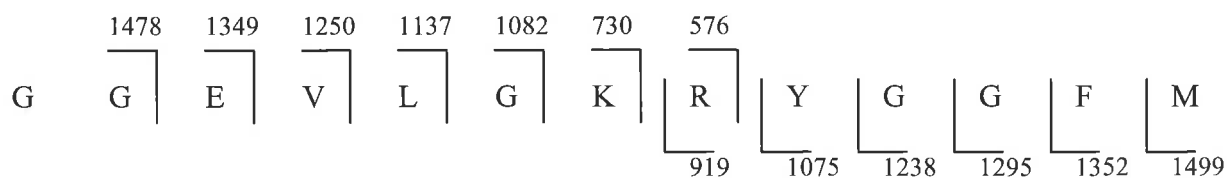
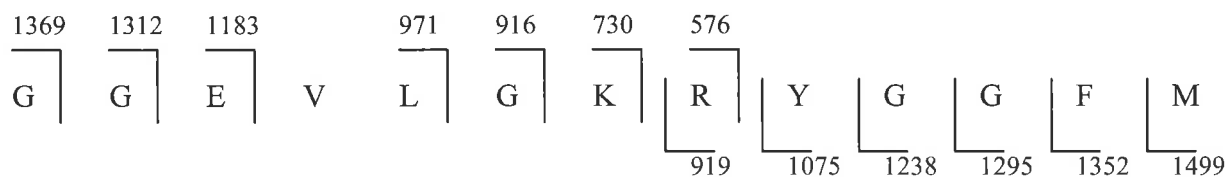
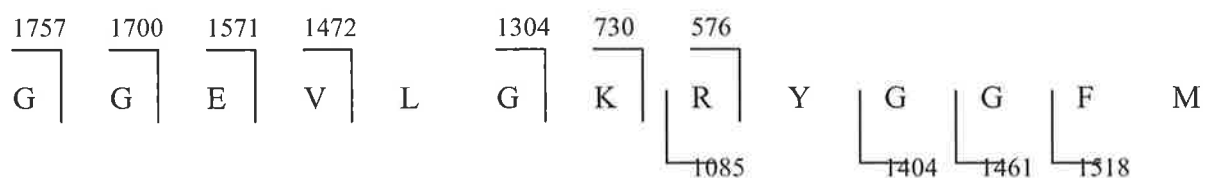
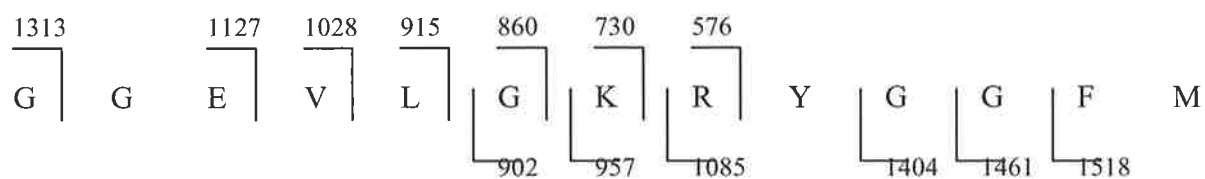
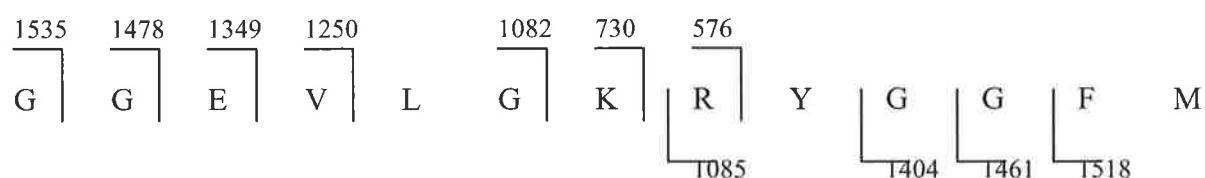
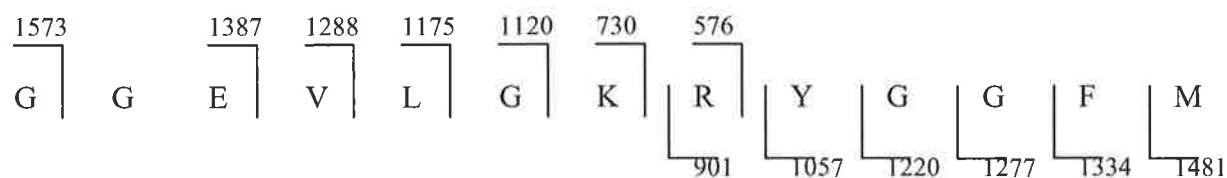
30 minute reaction:

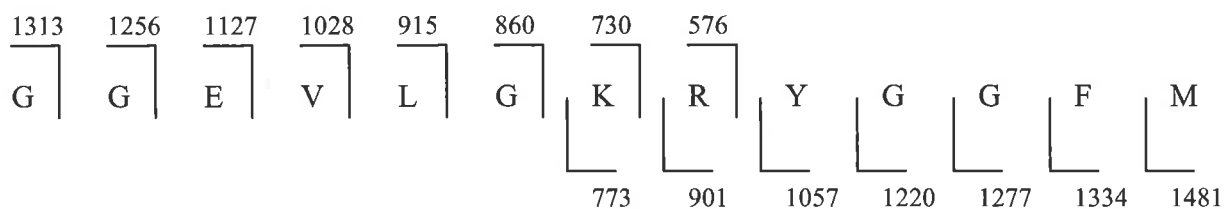
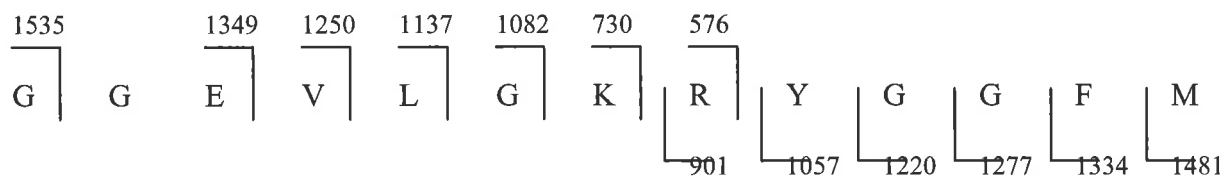
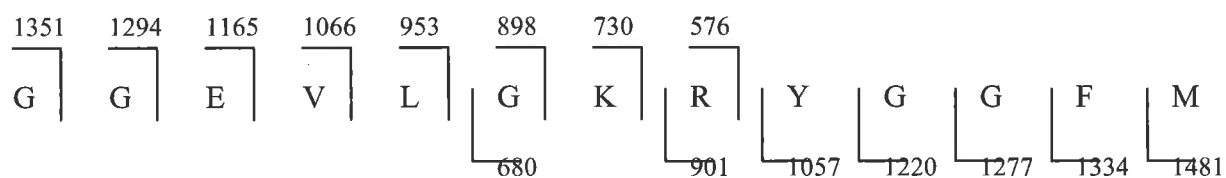


1 hour reaction:



2 hour reaction:**MS/MS of 1,1-Diphenylhydrazine (DPH) Trapped Acrolein-PPE Adducts:** **m/z 1592. mono DPH-mono acrolein (K)** **m/z 1592. mono DPH-mono acrolein (G)** **m/z 1648. mono DPH-bis acrolein (K)** **m/z 1648. mono DPH-bis acrolein (G)**

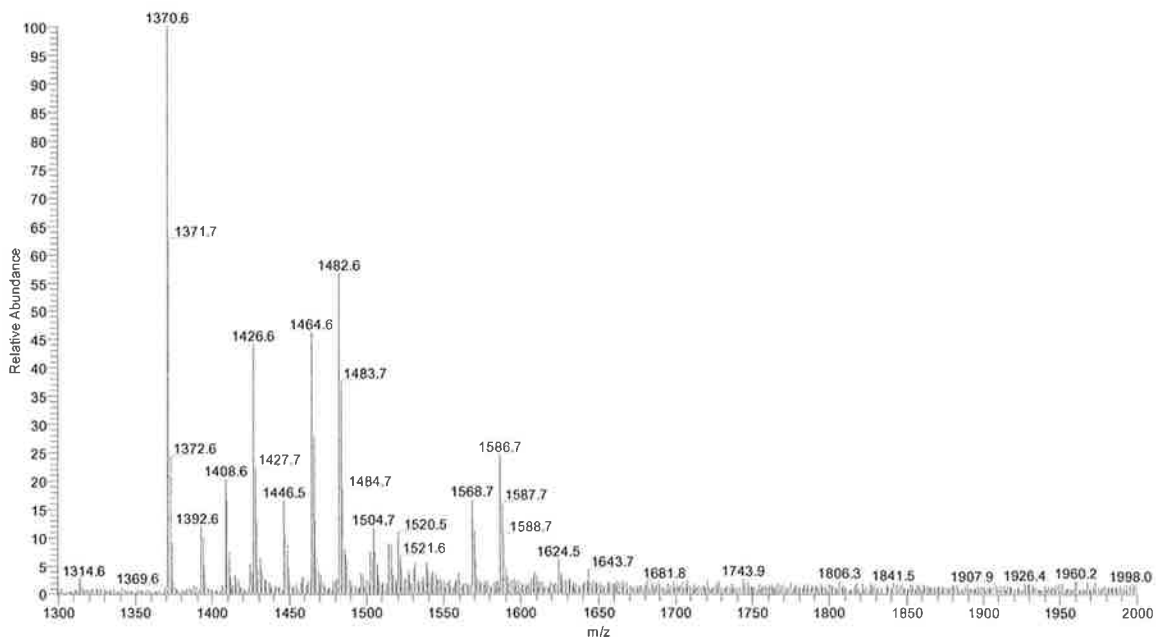
m/z* 1648. mono DPH (K)-bis acrolein ($1 \times G/1 \times K$)**m/z* 1648. mono DPH (G)-bis acrolein ($1 \times G/1 \times K$)*****m/z* 1814. bis DPH-bis acrolein (K)*****m/z* 1814. bis DPH-bis acrolein (G)*****m/z* 1814. bis DPH-bis acrolein ($1 \times G/1 \times K$)*****m/z* 1630. mono DPH-FDP (K)**

m/z* 1630. mono DPH-FDP (G)**m/z* 1630. mono DPH (K)-1 × Michael (K)/1 × Schiff (G)*****m/z* 1630. mono DPH (G)-1 × Michael (G)/1 × Schiff (K)**

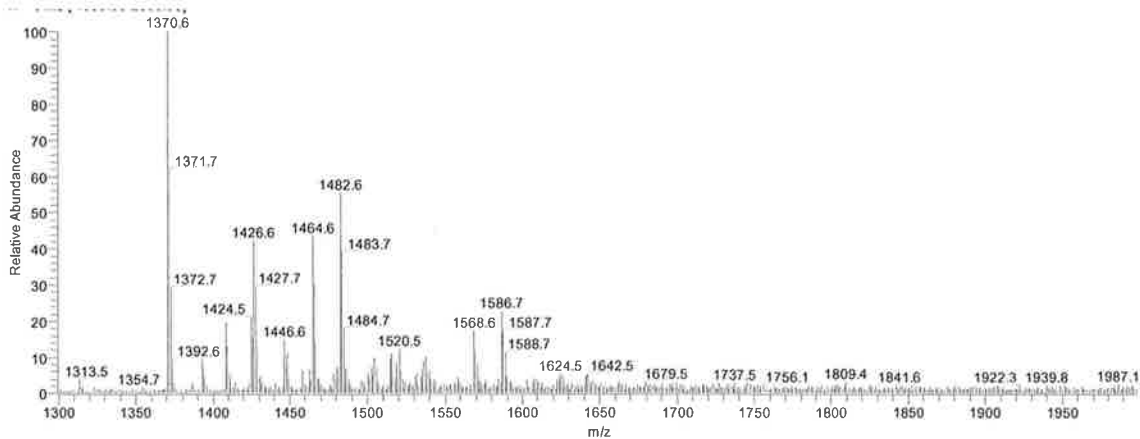
A3.3 Time Course for Acrolein-PPE Trapping by Benzylhydrazine and Adduct Structure Characterisation by MS/MS

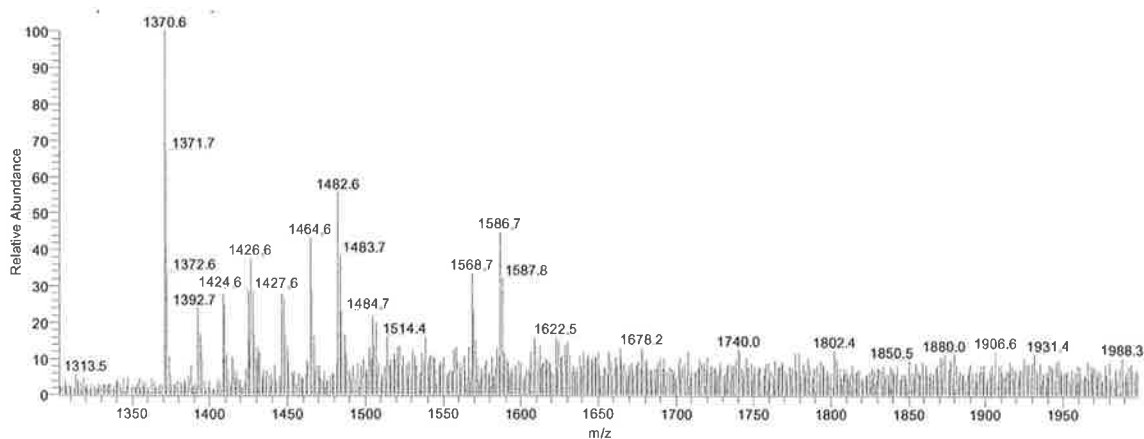
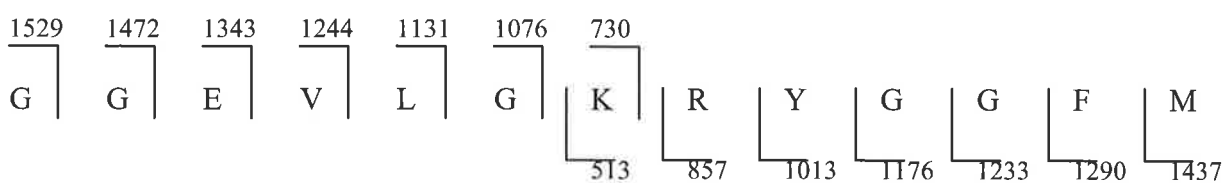
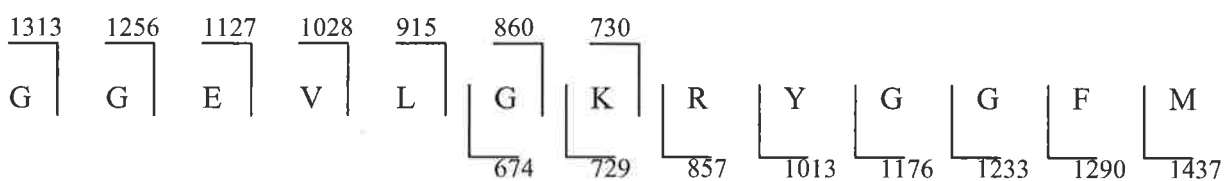
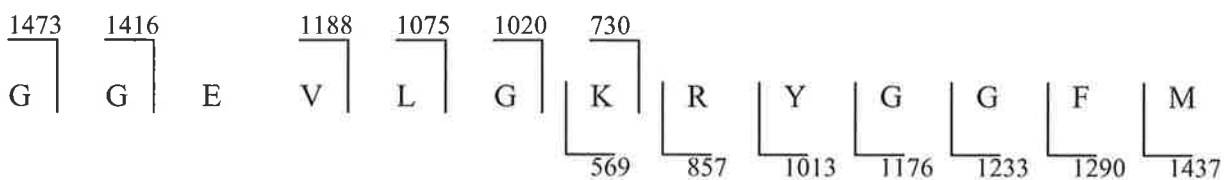
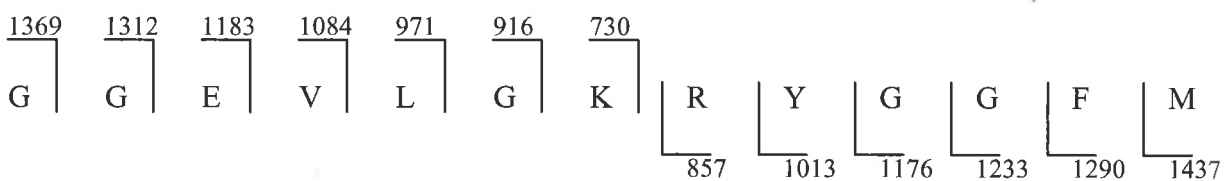
A 10 fold excess of benzylhydrazine (compared to acrolein) was reacted for 1 hour at 37°C with a reacted mixture of acrolein and PPE (3 hours). Benzylhydrazine trapped acrolein-PPE was monitored by positive ion mass spectrometry (25 μ M PPE in 25% acetonitrile/1% glacial acetic acid).

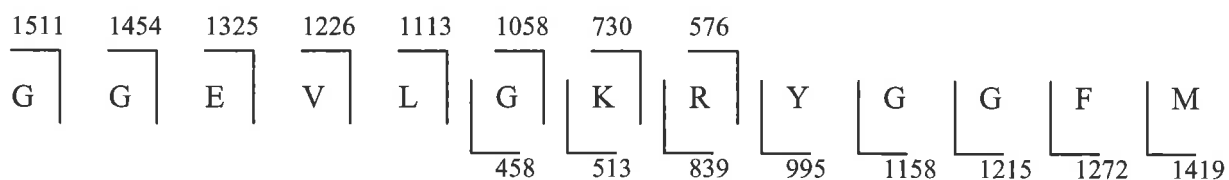
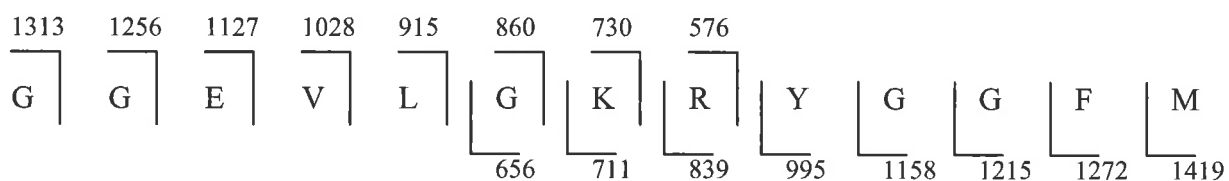
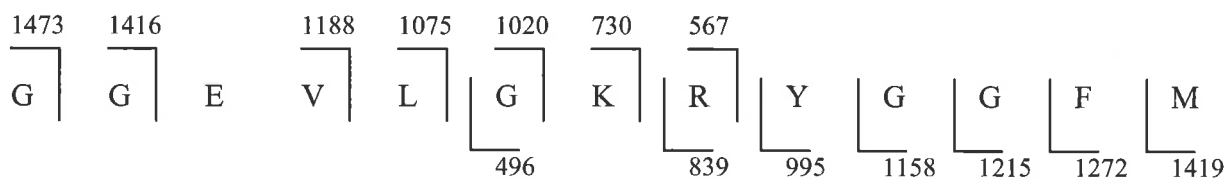
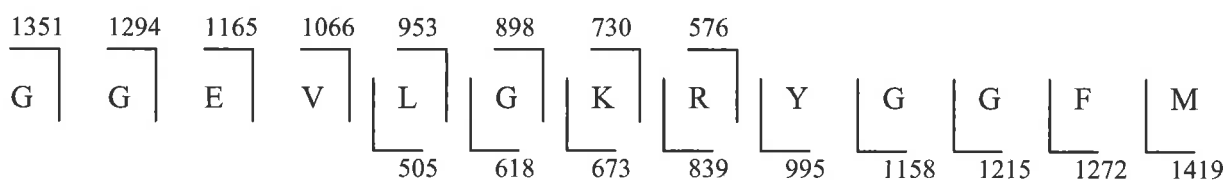
30 minute reaction:



1 hour reaction:



2 hour reaction:**MS/MS of benzylhydrazine (BH) trapped acrolein-PPE adducts:** **m/z 1586. mono BH-bis acrolein (K)** **m/z 1586. mono BH-bis acrolein (G)** **m/z 1586. mono BH (K)-bis acrolein ($1 \times G/1 \times K$)** **m/z 1586. mono BH (G)-bis acrolein ($1 \times G/1 \times K$)**

m/z* 1568. mono BH-FDP (K)**m/z* 1568. mono BH-FDP (G)*****m/z* 1568. mono BH (K)-1 × Michael (K)/1 × Schiff (G)*****m/z* 1568 mono BH (G)-1 × Michael (G)/1 × Schiff (K)**

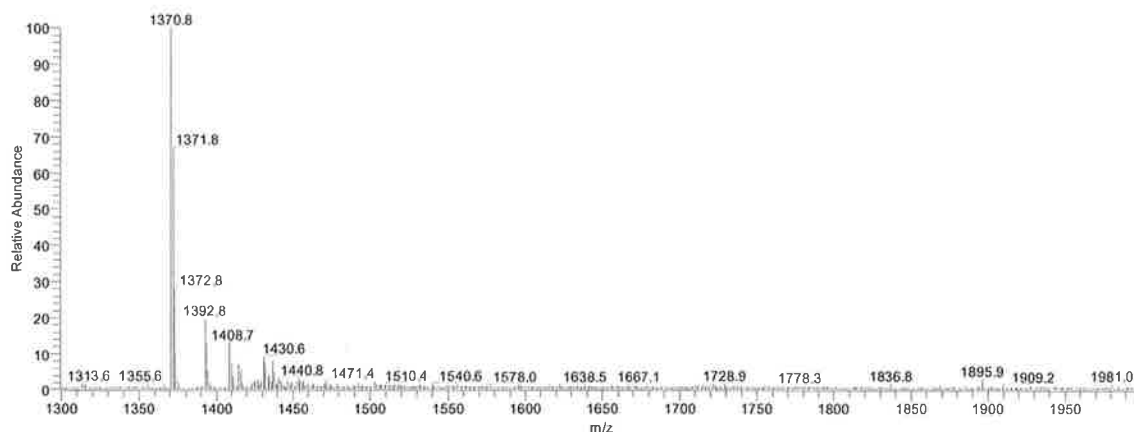
Appendix 4

**Time course for methylvinyl ketone-PPE and
hydralazine/methylvinyl ketone-PPE adduct formation.**

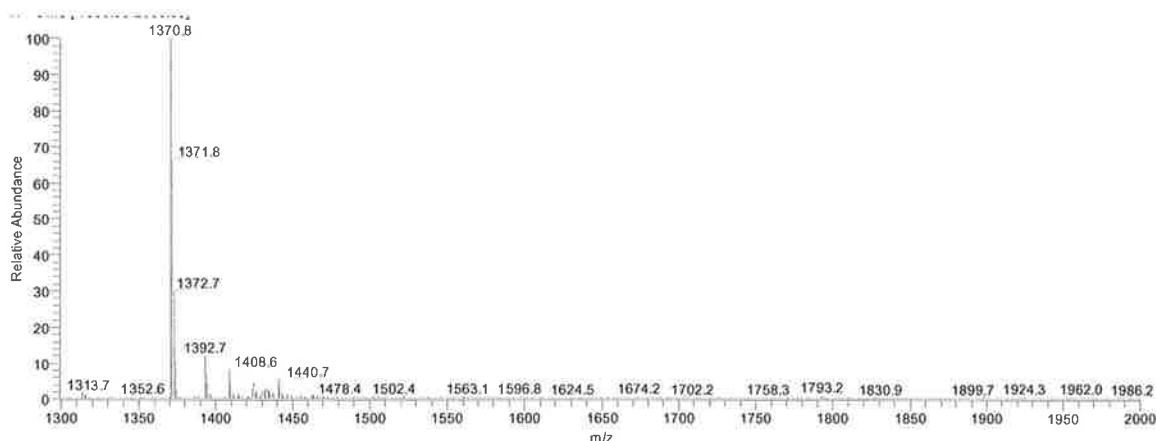
A4.1 Time Course for Methylvinyl Ketone-PPE Adduct Formation.

PPE was incubated with a 100 fold excess of MVK and incubated at 37°C for 22 hours. 25 μ l aliquots of PPE were diluted in an acetonitrile:glacial acetic acid mixture such that 25 μ M PPE was injected onto the LCQ at a flow rate of 8 μ l/minute in 25% acetonitrile and 1% glacial acetic acid in water. Ions were monitored in positive ion mode.

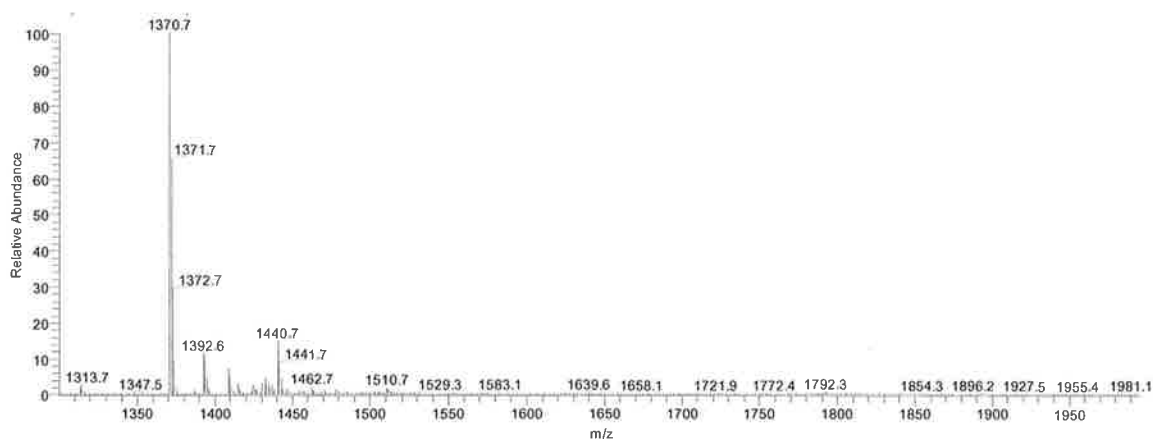
30 minute reaction:



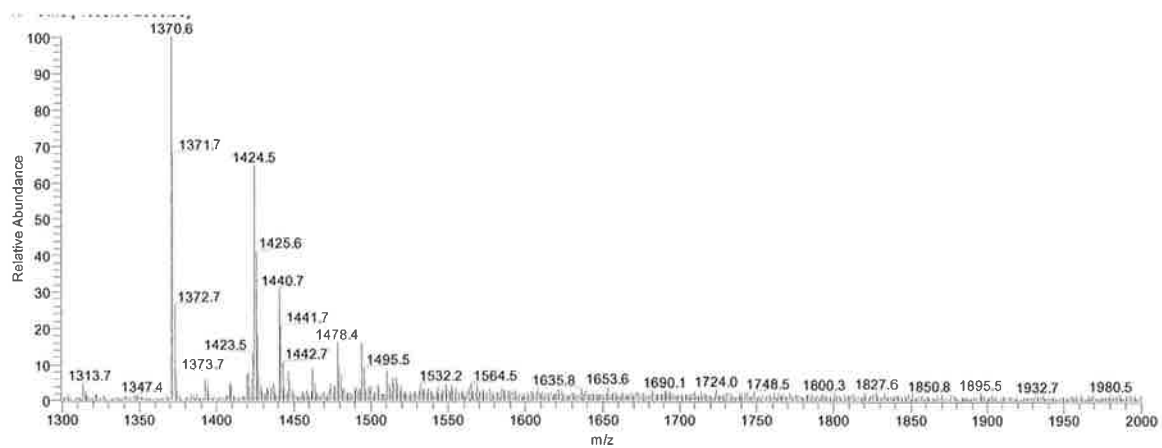
1 hour reaction:



3 hour reaction:



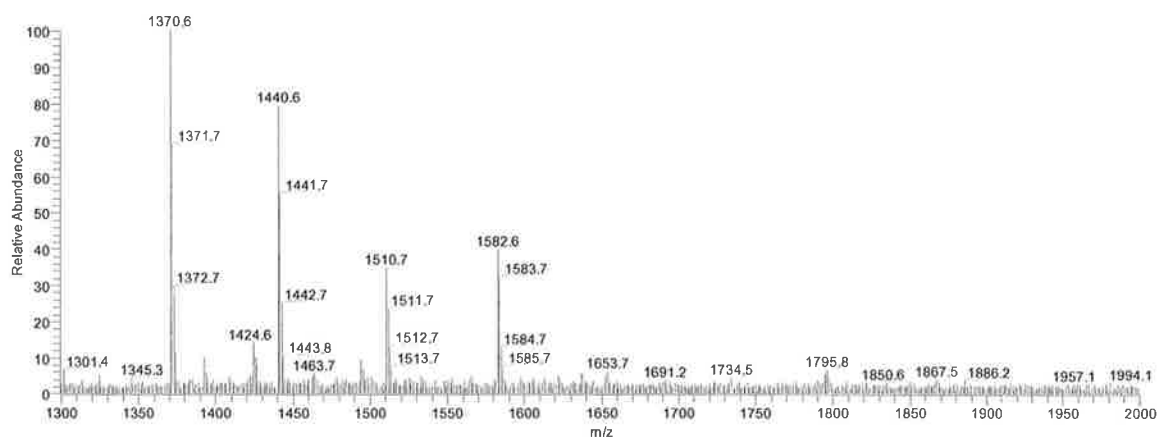
6 hour reaction:



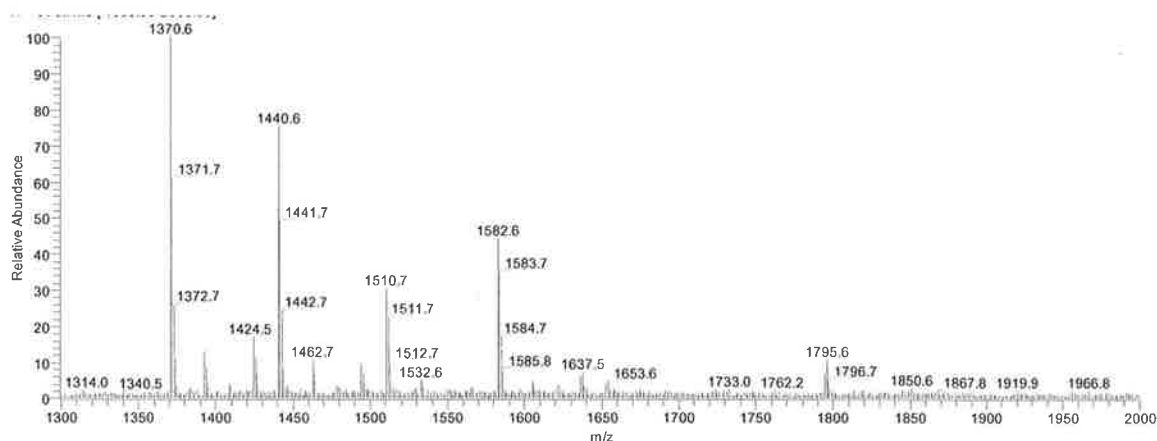
A4.2 Time Course for Trapping of Methylvinyl Ketone-PPE Adducts by Hydralazine.

A 10 fold excess of hydralazine (compared to MVK) was incubated for 1 hour at 37°C with a reacted mixture of MVK and PPE (3 hours). Hydralazine trapped MVK-PPE was monitored by positive ion mass spectrometry (25µM PPE in 25% acetonitrile/1% glacial acetic acid).

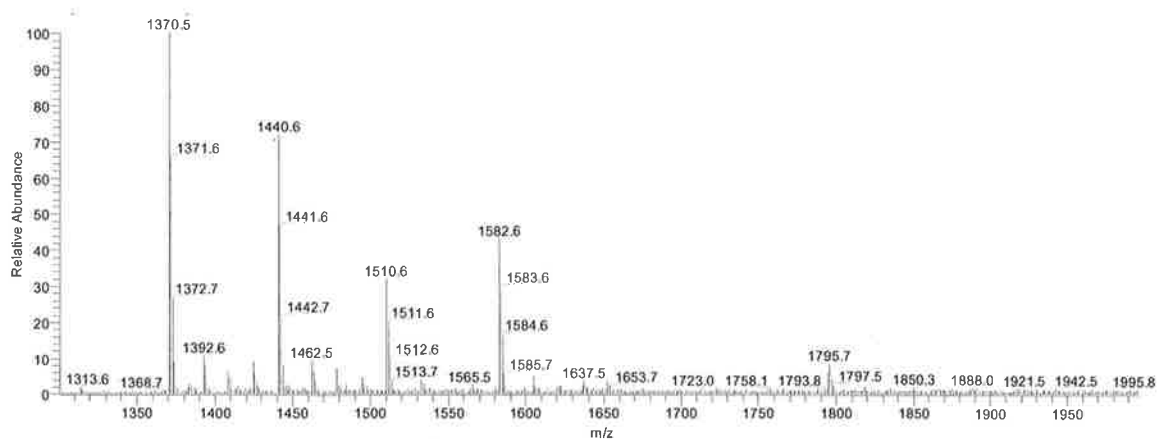
1 hour reaction:



1.5 hour reaction:



3 hour reaction:



Appendix 5

First author publications arising from this thesis.

Kaminskas, L.M., Pyke, S.M., and Burcham, P.C. (2004) Strong protein adduct trapping accompanies abolition of acrolein-mediated hepatotoxicity by hydralazine in mice

Journal of pharmacology and experimental therapeutics, v. 310 (3), pp. 1003-1010

NOTE:

This publication is included in the print copy of the thesis held
in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1124/jpet.104.067330>

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PhD project, or can of worms?
You be the judge!