Carbonyl-Scavenging Drugs & Protection Against Carbonyl Stress-Associated Cell Injury

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Abstract: This mini-review highlights the chemical and cytoprotective properties of various hydralazine analogues that block the induction of cell death by acrolein, a highly toxic contributor to "carbonyl stress" during a diverse range of human diseases. Recent work on the action of hydralazine against various carbonyl-mediated pathologies is also reviewed.

Key Words: Carbonyl stress, acrolein, protein adducts, hydralazine.

1. CARBONYL STRESS AND HUMAN DISEASE

"Carbonyl stress" is an umbrella term used to describe a situation of heightened cellular exposure to toxic carbonyl compounds that are produced during oxidative stress, lipid peroxidation and other deleterious cellular processes. Key contributors to carbonyl stress include such lipid-derived species as the 1,3-dicarbonyl malondialdehyde, 4-hydroxy-alkenals, 2-alkenals (e.g. acrolein, crotonaldehyde), γ-keto-aldehydes (e.g. levuglandins, isoketals), 4-oxonon-2-enal, and also various sugar-derived 1,2-dicarbonyls such as glyoxal, methylglyoxal, glucosones and deoxyglucosones [1-3]. As electrophiles, these carbonyl compounds typically deplete cellular stores of glutathione and also attack cell macromolecules, forming a range of adducts within cell proteins and DNA [4-6]. Increased carbonyl modifications of DNA and protein have been detected in the diseased tissues of a large number of degenerative conditions [2, 3]. Such damage is relevant to the pathogenesis of these diseases since it potentially disrupts a wide array of cell processes, perturbing signalling and effector proteins involved in cell death regulation, antioxidant response pathways, MAP kinase signalling, endoplasmic reticulum stress responses and p53-dependent DNA damage response signalling [7].

Due to increasing knowledge of the noxious effects of carbonyl compounds, a number of researchers have initiated a search for compounds that counteract "carbonyl stress" during human disease. The most common approach involves seeking compounds with either antioxidant efficacy or metal chelating activity that act "upstream" of carbonyl formation to minimise oxidative processes that generate these noxious electrophiles [8]. A contrasting "downstream" strategy involves administering drugs that possess electron dense centres in the expectation they will act as sacrificial nucleophiles within tissues, scavenging toxic carbonyls and attenuating macromolecular damage and perturbation of cell signalling pathways. At present, only a handful of reviews discuss the chemico-pharmacological properties of carbonyl-scavengers. One early review by Shapiro focussed upon drugs known to trap sugar-derived carbonyls [9]. New reviews by Aldini and associates comprehensively cover a broad range of reactive carbonyls and a diverse set of carbonyl trapping reagents [2, 3]. To minimise overlap with these recent reviews, the present mini-review focuses on efforts within our laboratory to optimise a class of hydrazinophthalazine drugs for activity towards acrolein, one of the most reactive lipid-derived carbonyls. A growing body of literature that explores the efficacy of hydrazinophthalazine compounds against various carbonyl-mediated disease states is also discussed.

One limitation to knowledge in this area is that few carbonyl-trapping compounds have been evaluated from a structure-activity standpoint to clarify structural and chemical features that facilitate the suppression of carbonyl stress *in vivo*. One exception includes work by Wondrach and associates to define structure-activity relationships for D-penicillamine and related cysteine derivatives in terms of their activity as dicarbonyl scavengers [10, 11]. Much less information is available concerning structure-activity relationships for compounds active towards α,β-unsaturated aldehydes and other lipid-derived electrophiles. As highlighted by Shapiro [9], one desirable feature for nucleophilic carbonyl scavengers is that they lack significant pharmacological or physiological actions other than their carbonyl-trapping reactivities. Thus a major reason for conducting structure-activity assessment of these compounds is to identify analogues that are inert in terms of their effects on physiological systems or drug receptors and yet exhibit strong reactivity towards noxious carbonyl compounds. Such a chemico-pharmacological profile would allow the administration of sufficient doses to suppress carbonyl stress without eliciting unwanted changes within the respiratory or cardiovascular systems, the CNS, the GI-tract or other major body systems.

Even if biologically-inert yet chemically reactive carbonyl-sequestering molecules are identifiable, clarifying the role of carbonyl-scavenging in any protective effect these compounds might exhibit in animal models of disease can be complicated by other issues related to the chemistry of these substances. For example, the same chemical properties that

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confer carbonyl-trapping reactivity on a molecule may also bestow antioxidant and metal-chelating activities [12]. Preferably, the status of competing cytoprotective mechanisms should be taken into account during the testing of carbonyl scavengers in animal models, ideally by monitoring oxidative-stress biomarkers at the same time as assessing carbonyl-mediated macromolecular damage.

2. ACROLEIN AS A CARBONYL STRESS MEDIATOR

Acrolein is a volatile 3-carbon α,β-unsaturated aldehyde that exhibits two striking properties, namely pronounced electrophilicity on the one hand and strong toxicity toward living tissues on the other. This chemico-toxicological profile ensures acrolein is very damaging to the epithelial lining of the lung, and on this account it received considerable attention as a chemical warfare agent during World War 1. In addition, since it is a major by-product of the combustion of a wide range of organic matter, exogenous acrolein plays a key role in various smoke-related toxicological syndromes including pulmonary smoke inhalation injury [13]. New findings also strongly incriminate acrolein in lung tumourigenesis in cigarette smokers [14]. Acrolein also forms during the metabolism of widely used oxazaphosphorine anticancer drugs, mediating the bladder cystitis seen in patients receiving cyclophosphamide and other members of this class [15]. Finally, in addition to these foreign and environmental sources, acrolein forms endogenously during a range of biochemical processes, including polyamine metabolism by amine oxidases; L-threonine oxidation by activated neutrophils; and free radical-catalysed membrane peroxidation [16-18]. The various sources of acrolein and the mechanisms underlying its contribution to human disease are summarised in schematic form in Fig. (**1**). Such endogenous sources are a likely explanation for the increased levels of acrolein-adducted proteins in the affected tissues of such neurodegenerative conditions as Alzheimer's disease and traumatic spi-

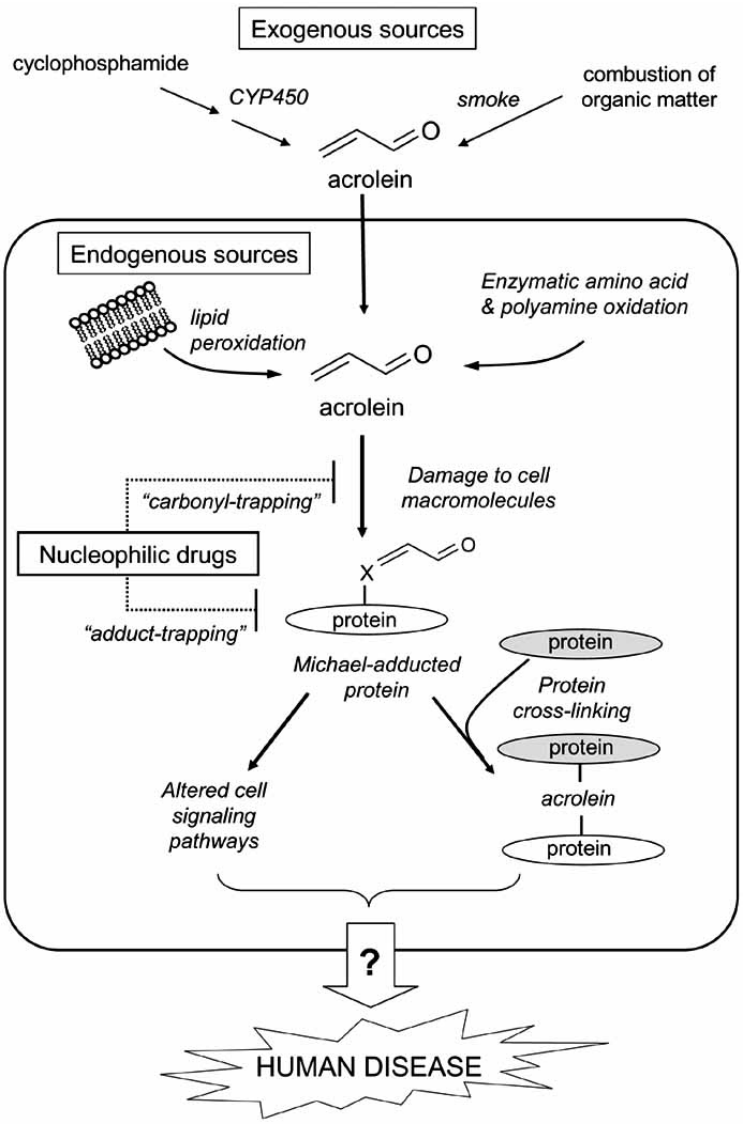


Fig. (1). Sources of exposure to acrolein and the mechanistic basis for its role in diverse human health disorders. Nucleophilic drugs such as hydralazine are hypothesised to act as either scavengers of free, unadducted aldehyde or *via* an "adduct-trapping" reaction involving neutralisation of electrophilic carbonyl groups in Michael-adducted proteins.

nal cord injury, as well as various cardiovascular disorders including atherosclerosis. A list of health disorders in which acrolein has been incriminated is shown in Table **1**, together with an indication of the presumed source of the acrolein exposure as well as the analytical methodologies used to confirm a role for acrolein in each condition. At present, many questions surround the detection of acrolein or acrolein-modified macromolecules in most of the diseases listed in Table **1**, including the fundamental issue of whether such damage is simply associative in nature or whether acrolein-mediated cell injury indeed plays a causal role in disease pathogenesis. Such concerns seem particularly pertinent to health conditions in which acrolein exposure predominantly involves endogenous sources (e.g. the various neurodegenerative conditions listed in Table **1**), but are less pressing for those diseases involving exogenous sources where the role for acrolein is comparatively unambiguous (e.g. smoke inhalation injury, oxazaphosphorine side-effects).

3. PROTECTING AGAINST ACROLEIN TOXICITY USING DRUGS

Given its role in such a range of pathological conditions, the development of compounds that suppress acrolein toxicity could be beneficial in a number of clinical settings. Compounds with documented acrolein-scavenging properties include various thiol-containing nucleophiles such as 2-mercaptoethane sulfonate (MESNA), 2,6-dithiopurine and lipoic acid [19-21]. Such reagents typically add directly to the "soft" β–carbon possessed by acrolein. Since these rapid reactions compete effectively with the reaction of acrolein with biological nucleophiles including thiol groups in proteins, they can be considered true carbonyl scavengers in the sense specified above. As efficient scavengers of free acrolein, S-containing nucleophiles seem suitable candidates for use in applications involving ongoing exposure to acrolein, an expectation that is exemplified in the long-standing and successful use of MESNA in cancer patients receiving oxazaphosphorine chemotherapy drugs [19, 20]. However, the highly hydrophilic character of MESNA ensures it is not suitable for use in conditions in which cell and tissue penetration by a carbonyl scavenger is required.

In terms of its mechanism of action, a particularly interesting acrolein-ablating compound is hydralazine, a nitrogen-containing "hard" nucleophile that has been in clinical use for over 50 years as a vasodilatory antihypertensive [22]. Hydralazine is one of only a small number of nucleophilic compounds that is a confirmed scavenger of biogenic carbonyls within the human body (e.g. a hydrazone formed during reactions with the glycolysis intermediate pyruvate represents a major fate of hydralazine within the systemic circulation) [23]. Its ability to suppress acrolein toxicity was first described inadvertently in rats, where hydralazine administration attenuated the cardiotoxicity of allylamine, a metabolic precursor to acrolein [24]. To explore the chemical basis for such cytoprotection, we examined the reaction of acrolein with hydralazine, isolating 2 isomeric hydrazones formed during reaction of the terminal hydrazino nitrogen of hydralazine with the carbonyl group of acrolein [25]. Low levels of the predominant isomer formed during such reactions, (1E)-acrylaldehyde phthalazin-1-ylhydrazone, were detected in the culture media of isolated liver cells exposed to hydralazine concentrations that suppressed acrolein-mediated cell killing [25]. Use of ESI-MS to analyse products formed during reactions between hydralazine and acrolein revealed the formation of higher mass oligomeric species comprising multiple acrolein and hydralazine molecules,

Table 1. Health Conditions in which Acrolein is Incriminated. Presumed Sources of Acrolein are Highlighted Along with an Indication of the Methodology Used to Demonstrate its Involvement

Condition	How Acrolein Detected	Likely Source	Reference
Acute atopic dermatitis (*S. Aureus*)	immunodetection of urinary acrolein-lysine (ELISA)	endogenous	[80]
Age-related senescence (mice)	immunodetection of urinary acrolein-lysine (ELISA)	endogenous	[81]
Alzheimer's lesions	immunodetection of adducted protein	endogenous	[15]
Atherosclerotic plaques	immunodetection of adducted protein	endogenous	[65]
Childhood bacterial meningitis	immunodetection of urinary acrolein-lysine	endogenous	[82]
Cyclophosphamide urotoxicity	"trial & error" toxicity testing of drug metabolites in rats	exogenous	[15]
Diabetic nephropathy	immunodetection of adducted protein	endogenous	[83]
Inhalational smoke injury	composition analysis & use of synthetic smoke	exogenous	[13]
Neoplastic cell transformation	immunodetection of adducted protein	endogenous	[84]
Photodamaged skin	immunodetection of adducted protein	UV-induced lipid peroxidation	[85]
Sick premature neonates	immunodetection of urinary acrolein-lysine	endogenous	[86]
Type 1 diabetes (juvenile)	immunodetection of urinary acrolein-lysine	endogenous	[87]
Retinitis pigmentosum	immunodetection of adducted protein	endogenous	[88]
Tobacco-related lung cancer	p53 mutational spectra	exogenous	[14]

although these species proved difficult to isolate and so were not fully characterised.

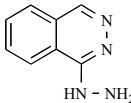
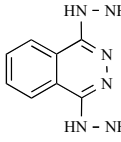
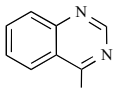
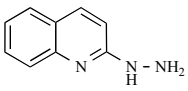
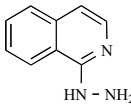
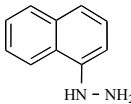
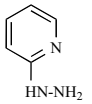
4. ACROLEIN SCAVENGING AND CYTOPROTECTION BY HYDRALAZINE ANALOGUES

The vasodilatory properties of hydralazine ensure that use of the drug as an acrolein-ablating therapy will be accompanied by unwanted cardiovascular actions. We thus sought to identify structural analogues that could block acrolein toxicity without exerting cardiovascular effects. This goal seemed feasible, since in classic pharmacophore characterisation studies an intact –C=N–N=C–NH–NH_2 moiety was essential for the hypotensive action of hydralazine [26]. Since each of the four nitrogen atoms as well as their position within the drug is required for vasoactivity, it seemed possible to identify structural analogues in which the configuration of the heteroatoms is disrupted and yet acrolein-sequestering and cytoprotective actions are retained.

To explore this possibility, a series of hydralazine analogues were assessed for their ability to trap equimolar concentrations of acrolein within buffered solutions at neutral pH and 37 °C, using methods used previously to compare the acrolein-sequestering activity of hydralazine to that of various amine compounds [25]. The compound structures are shown in Table **2** and include close structural analogues of hydralazine (e.g. compounds II and III) as well as compounds lacking one or both of the ring nitrogen atoms (Compounds V and VI) and also a variant of Compound III in which the hydrazine group and ring nitrogen are switched (Compound IV). A monocyclic nitrogen-containing hydrazinoaromatic was also tested, namely hydrazinopyridine (Compound VII). All compounds were obtained from commercial suppliers with the exception of hydrazinoisoquinoline (Compound V) which was synthesised from 1-chloroisoquinoline [27]. The structure and purity of synthetic Compound V was confirmed by ^{1}H NMR and mass spectrometry.

The acrolein-sequestering capability varied considerably among the various hydrazinoaromatic compounds (Fig. (**2**)). A slow decline in acrolein concentrations occurred in controls, presumably reflecting acrolein hydrolysis or headspace

Table 2. Acrolein-Scavenging and Cytoprotective Efficiencies of Hydralazine Analogues. The EC_{50} (Mean ± S.E. of 3 Independent Determinations) Afforded Half-Maximal Protection Against Cell Death in Hepatocytes During a 60 min Incubation with 100 μM Allyl Alcohol [29]. Different from Hydralazine, * = p < 0.05, ** = p < 0.005 (Unpaired T-test). Log P Values were Calculated using *SciFinder Scholar*

Drug Analogue	Structure	Acrolein Scavenging Rate (mmol.min^{-1})	EC_{50} (μM)	Calculated Log P
Hydralazine (I)		32	18 ± 0.81	1.00
Dihydralazine (II)		39	13 ± 0.29**	0.32
Hydrazinoquinazoline (III)		33	10 ± 0.05**	1.42
Hydrazinoquinoline (IV)		21	11 ± 0.44**	1.26
Hydrazinoisoquinoline (V)		20	6.0 ± 0.14**	1.13
Naphthylhydrazine (VI)		11	7.0 ± 0.19**	2.48
Hydrazinopyridine (VII)		29	40 ± 6.1*	-0.03

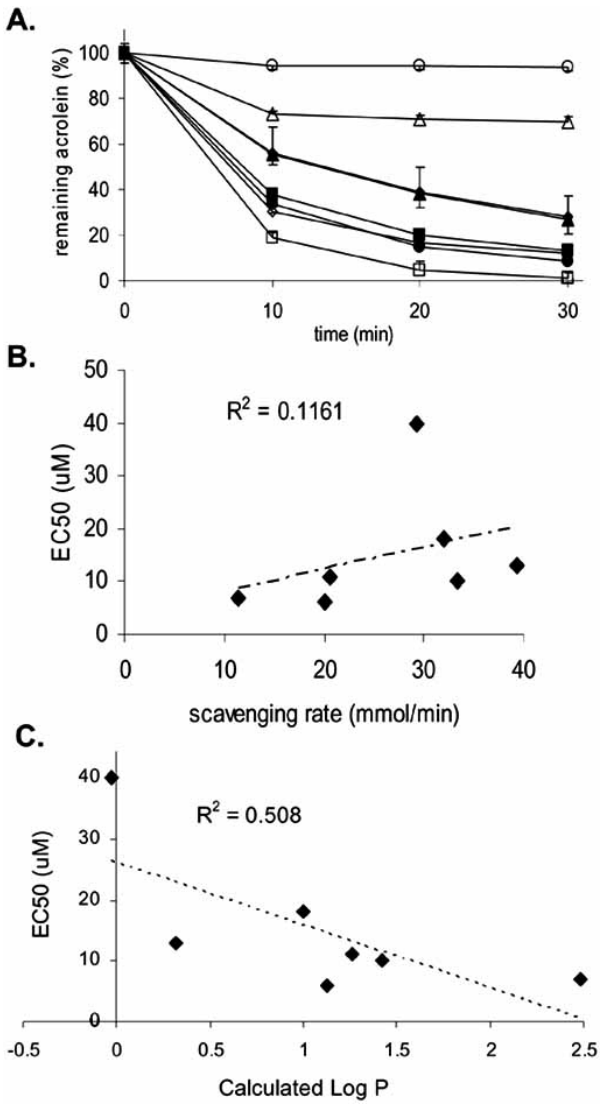


Fig. (2). *Panel A)* The kinetics of acrolein disappearance in equimolar mixtures of acrolein and various hydrazinoaromatic compounds during 30 min incubations at neutral pH and 37 °C. At each time point, free acrolein concentrations were measured in reaction mixture aliquots using UV-HPLC. The starting concentration of acrolein and nucleophilic compounds was 0.5 mM, and acrolein levels at each time point are expressed as a percentage of the starting concentration. The various treatments are: (o) control, buffer only; (•) hydralazine; (□), dihydralazine; (■), hydrazinopyridine; (Δ), naphthylhydrazine; (▲), hydrazinoquinoline; (◊), hydrazinoquinazoline; (◆), hydrazinoisoquinoline. *Panel B)* Correlation between scavenging rate (mmol acrolein consumed per min) and the EC_{50} for cytoprotection for the seven hydrazine compounds shown in Panel A. Each data point represents the mean ± S.E of 3 independent observations. *Panel C)* Correlation between cytoprotective efficacy (EC_{50}) and calculated Log P.

loss (Fig. (**2A**)). Dihydralazine was the most efficient scavenger tested, reducing free acrolein concentrations by approx. 80% within 10 minutes. The three analogues exhibiting the highest rates of acrolein scavenging, hydralazine, dihydralazine and hydrazinoquinazoline, all possess two ring nitrogen atoms, suggesting this structural feature facilitates reactivity as a carbonyl scavenger, although the precise location of the ring heteroatoms does not appear to be critical. The bicyclic analogues possessing a single ring nitrogen, namely hydrazinoquinoline (Compound IV) and hydrazinoisoquinoline (Compound V) displayed intermediate efficiency with respect to acrolein trapping, although the monocyclic analogue hydrazinopyridine was more active in this regard (Table **2**, Fig. (**2A**)). The least efficient scavenger was the analogue lacking any ring heteroatoms, naphthylhydrazine, which reduced acrolein concentrations by just 20% over the first 10 minutes of the reaction (Fig. (**2A**)).

The compounds were also evaluated for an ability to suppress allyl alcohol toxicity in isolated mouse hepatocytes (Table **2**). Allyl alcohol is rapidly oxidised to acrolein by alcohol dehydrogenase within liver cells, and is a useful model of acrolein-mediated cell injury [28]. This model previously allowed identification of hydralazine as a powerful inhibitor of acrolein-mediated cell killing, with the drug showing activity at concentrations that were 2 to 3 orders of magnitude lower than those required for other amine compounds with putative carbonyl-trapping activity [25, 29]. Results obtained during screening of the hydralazine analogues against acrolein-mediated cell death are reported as EC_{50} values in Table **2**, with the EC_{50} defined as the compound concentration affording half-maximal protection against cell death during a 60 min incubation with allyl alcohol. The onset of cell death was assessed *via* measurement of lactate dehydrogenase leakage. Each compound was tested at 1, 3, 10, 30 and 100 μM concentrations. With the exception of hydrazinopyridine (VII), each of the compounds had improved cytoprotective efficacy relative to hydralazine (Table **2**). However, contrary to expectations, acrolein scavenging and cytoprotective potency were poorly correlated (Fig. (**2B**)), with the comparatively poor scavenger naphthylhydrazine (VI) showing strong cytoprotective potency. This outcome suggests that trapping of free acrolein is not the primary mechanism whereby these nucleophilic compounds afford cytoprotection against acrolein-mediated cell death. Possible alternative cytoprotective mechanisms are discussed below. A stronger correlation was evident between the cytoprotective EC_{50} and calculated Log P for the various compounds (Fig. (**2C**), indicating that membrane permeability and access to intracellular targets facilitates blockade of cell death in this experimental system.

Significantly, compounds in which the –C=N–N=C–NH–NH_2 pharmacophore essential for hydralazine vasoactivity was disrupted exhibited modestly improved cytoprotective activity relative to the parent drug (Table **2**). Thus hydrazinoquinazoline (III), hydrazinoquinoline (IV), and hydrazinoisoquinoline (V) are all expected to lack vasodilatory actions on account of the disrupted pharmacophore, yet each of these compounds exhibited lower EC_{50} values than hydralazine in the cytoprotection assay (Table **2**). These observations strengthen the expectation that the cytoprotective efficacy of hydralazine can be dissociated from its other pharmacological actions that are unwelcome in a carbonyl trapping agent.

5. MECHANISM OF CYTOPROTECTIVE ACTION OF HYDRALAZINE

Consistent with the poor correlation between acrolein-scavenging activity and cytoprotective potency manifest in Fig. (**2B**), findings from a recent study of hydralazine protection against acrolein-mediated toxicity in hepatocytes also implied that acrolein-trapping was not the primary cytoprotective mechanism [30]. Thus not only were levels of extracellular acrolein unchanged in the culture media of allyl alcohol-treated hepatocytes in the presence of protective hydralazine concentrations, but the levels of (1E)-acrylaldehyde phthalazin-1-ylhydrazone, the major species formed during reactions between free acrolein and hydralazine, were very low relative to free acrolein concentrations [30]. In contrast, the drug reacted readily with protein-bound acrolein, a reaction we termed "adduct-trapping" based on work in which reactivity with acrolein-adducted protein distinguished hydralazine from other, less efficacious carbonyl scavengers [31]. Using antiserum raised against hydralazine-labelled, acrolein-modified protein, such adduct-trapping reactions were detected in cell proteins extracted from allyl alcohol-treated cells that were co-exposed to 2 to 50 μM concentrations of hydralazine [31]. Intense protein adduct-trapping also occurred in the livers of allyl intoxicated mice that received hepatoprotective doses of hydralazine [32].

Taken together, the finding that hydralazine failed to alter levels of free acrolein in the cell culture model and also reacted readily with protein-bound acrolein under these conditions suggests it is very difficult to trap free acrolein within an intracellular environment using a nitrogen-based nucleophile. This most likely reflects the intracellular abundance of free thiol groups on glutathione and proteins, which ensure nitrogen-containing nucleophiles such as hydralazine compete poorly with the rapid reaction between acrolein and thiol groups. Such a scenario is consistent with the recognition that analytical efforts to measure free acrolein levels in cells and tissues are complicated by the rapid reaction with cell macromolecules [33]. The realities confronting efforts to scavenge free acrolein with a nitrogen-containing scavenger are exemplified in the scavenger data shown in Fig. (**3**), which depicts free acrolein concentrations in buffered solutions during incubation of acrolein with equimolar concentrations of various biological nucleophiles, including the nucleophilic amino acids lysine and cysteine, as well as the various nucleosides present in DNA, deoxyadenosine (dA), deoxyguanosine (dG), deoxythymidine (dT) and deoxycytosine (dC). These reactions were performed under the same conditions as those used to compare the acrolein-scavenging efficacies of the hydralazine analogues shown in Fig. (**2A**). Under these conditions, the rate with which acrolein reacted with the thiol-containing amino acid cysteine greatly exceeds its reactivity with the other biological nucleophiles, but also its reaction with hydralazine shown in Fig. (**2A**). Thus, within the first 10 min of reaction at 37 °C, cysteine had consumed essentially all of the available acrolein (Fig. (**3**), yet in the presence of hydralazine approx. 35 % of initial acrolein concentrations remained at this time (Fig. (**2A**)). Although extrapolation from this simple experimental system to the cellular setting involves many assumptions, these results suggest that least in the case of acrolein-mediated cell injury, even an excellent nitrogen-containing nucleophile such as hydralazine cannot act in the manner of a classical carbonyl scavenger highlighted above and thereby block macromolecular adduction. Thus, for a compound to act as a true carbonyl scavenger within biological systems, the kinetics of the reaction between the drug and the carbonyl must exceed the rate of the reaction of the carbonyl with its major intracellular target.

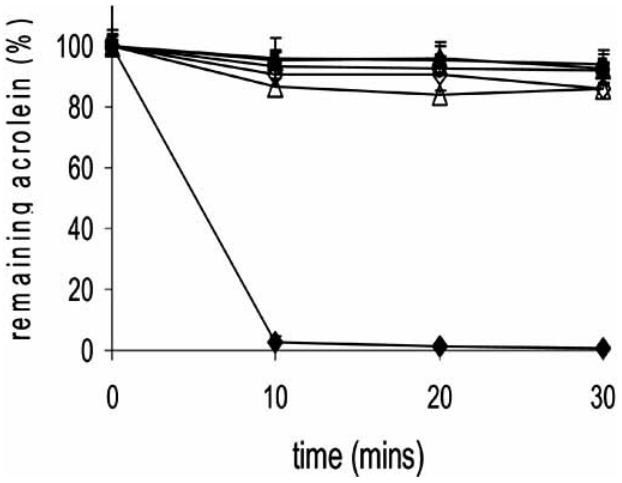


Fig. (3). Kinetics of acrolein trapping by various endogenous nucleophiles (amino acids [Lys, Cys] and deoxynucleotides [dA, dG, dT, dC]). Reactions involving equimolar mixtures of acrolein and each nucleophile were carried out over 30 min at neutral pH and 37 °C. At each time point, free acrolein concentrations were measured in reaction mixture aliquots using UV-HPLC. The starting concentration of acrolein and nucleophilic compounds was 0.5 mM, and acrolein levels at each time point are expressed as a percentage of the starting concentration. The various treatments are: (o), control, + buffer only (□), deoxyguanosine (■) deoxyadenosine (Δ) deoxythymidine (▲) deoxycytosine (◊) N_{α}-acetyl-L-lysine (◆) N_{α}-acetyl-L-cysteine. Each data point represents the mean ±S.E of 3 independent observations.

Similar conclusions were drawn during a recent study of the efficacy of pyridoxamine as a scavenger of 1,4-dicarbonyl compounds, since pyridoxamine was judged an efficacious carbonyl scavenger since it reacted more rapidly with model 1,4-dicarbonyls than these substances attacked the ε–amine group of lysine, the preferred cellular target for these electrophiles [34]. In contrast, the slower rate of reaction of acrolein with hydralazine compared to its reactivity with its preferred cellular targets explains why hydralazine failed to block glutathione depletion or protein carbonylation in allyl alcohol-intoxicated cells [30]. Thus, judged by the criteria of a carbonyl scavenger attenuating intracellular levels of reactive carbonyls and minimising macromolecular modification, hydralazine does not classify as a true member of this class and other mechanisms must be sought to explain its cytoprotective actions.

Since pronounced, concentration-dependent "adduct-trapping" accompanied hydralazine cytoprotection both *in vitro* and *in vivo*, it could be that its ability to readily attack protein-bound acrolein contributes to its protective efficacy. This possibility was suggested during use of mass spec-

trometry to monitor reaction products formed upon exposure of an acrolein-modified model peptide to hydralazine, with ions corresponding to hydrazones formed by hydralazine at several types of carbonyl adducts readily detected [31, 35]. Precisely how formation of hydrazones with acrolein-adducted proteins might attenuate acrolein toxicity is unclear; however one possibility is that this reactivity blocks the formation of macromolecular cross-links which may be deleterious to cells [30]. During *in vitro* experiments, formation of hydrazones at acrolein-modified residues correlated with the inhibitory effects of hydralazine on acrolein-induced oligomerisation of a model protein, ribonuclease A [30]. Experiments are currently underway to determine whether protein cross-linking accompanies acrolein exposure in cellular models, and to assess the degree to which carbonyl-scavenging/adduct-trapping drugs interfere with these processes. A recent study explored similar issues in brain synaptosomes following exposure to acrolein, with the results indicating that hydralazine had no effect on the formation of high-mass species (the latter were detected using antibodies against a range of synaptosomal proteins) [36]. However, very high concentrations of acrolein were required to generate high mass proteins in this system (25 to 100 mM) and a 1 mM concentration of hydralazine was employed (i.e. acrolein: drug ratio = 100). In our recent work, the inhibitory effect of hydralazine on acrolein-induced oligomerisation of ribonuclease A was manifest over an acrolein:drug ratio of approx. 0.1 to 1 [30].

Among the analogues tested for cytoprotective efficacy, hydrazinoisoquinoline (V) inhibited cell death most strongly (Table **2**). This compound was less efficient than hydralazine as a scavenger of free acrolein (Fig. (**2A**)). To explore its efficacy as an inhibitor of acrolein-induced protein cross-linking, we examined the ability of hydrazinoisoquinoline to disrupt the oligomerisation of ribonuclease A by acrolein. These experiments were conducted using a protein oligomerisation assay we recently described elsewhere [30]. Fig. (**4**) shows the effects of hydralazine and hydrazinoisoquinazoline (0.3, 1 and 3 mM concentrations) on protein dimer, trimer and tetramer abundance when the compounds were added to acrolein-premodified ribonuclease A followed by an overnight incubation at 37 °C. In contrast to its lesser reactivity with free acrolein compared to hydralazine, hydrazinoisoquinoline showed very similar activity to hydralazine in its inhibitory effects on the acrolein-induced protein oligomerisation, reducing levels of dimeric and trimeric species while restoring levels of monomeric starting material (Fig. (**4**)). Thus, reactivity with protein-bound acrolein may better predict the cytoprotective efficacy of hydrazinoisoquinoline than its acrolein-scavenging activity. Again, experiments are planned to clarify the extent to which interference with protein cross-linking contributes to the protective efficacy of hydrazinoisoquinoline in a cellular model.

6. EFFICACY OF HYDRALAZINE AGAINST ACROLEIN-MEDIATED CELL INJURY

The list of health conditions in which acrolein is known to participate (Table **1**) includes disorders in which its role is direct and unambiguous as well as several in which the mechanistic significance of acrolein involvement is less clear. In general, cytoprotective compounds such as hydralazine seem most likely to benefit conditions in the former category, since the strength of acrolein involvement in a given condition seems predictive of the likelihood of positive therapeutic outcomes elicited by acrolein-ablating compounds. To date, the efficacy of hydralazine or related compounds has only been examined in a subset of the conditions highlighted in Table **1**. In the next section, a number of recent studies from various labs that explored hydralazine efficacy in several disease settings are discussed, along with a discussion of the rationale underlying the use of hydralazine in each condition.

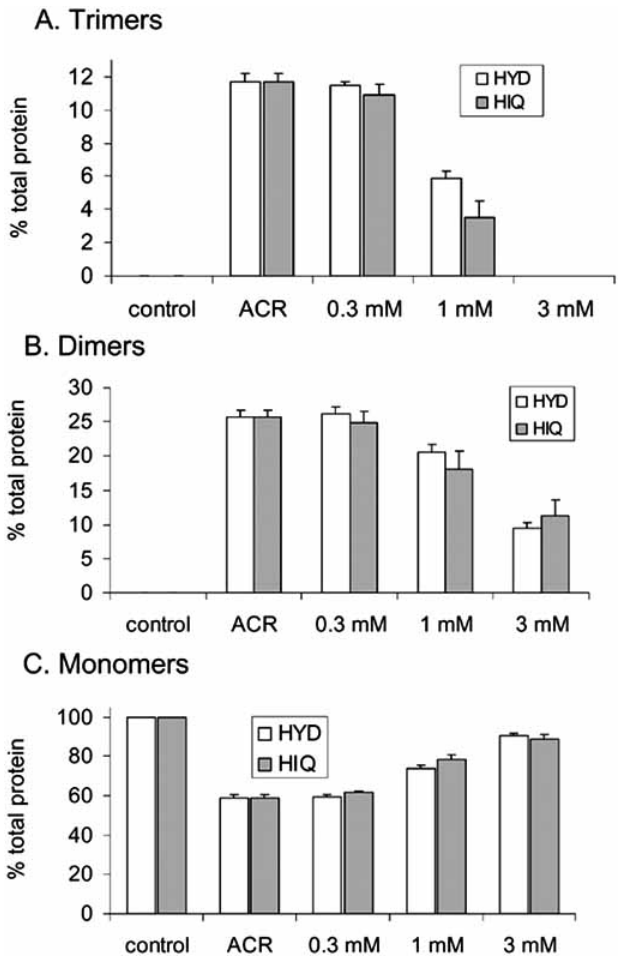


Fig. (4). Comparison of inhibitory efficacies of hydrazinoisoquinoline (HIQ) and hydralazine (HYD) against acrolein-mediated cross-linking of a model protein, ribonuclease A. Assay substrate was prepared by reacting RNase A (2.1 mg/ml) with 3.2 mM acrolein in 2 mL of 50 mM sodium phosphate buffer (pH 7.0) in Eppendorf tubes at 37°C. After 30 min, 190 µL aliquots were removed and added to 0.2 mL tubes containing either 0, 1, 3, or 10 µL volumes of 60 mM hydralazine (diluted to a 10 µL volume with buffer), achieving hydralazine concentrations of 0, 0.3, 1, and 3 mM. Reactions proceeded overnight at 37°C and then the reactions were diluted 3:1 with 5 µL sample buffer. Aliquots containing 30 µg of protein were then resolved on a 14% acrylamide gel for analysis of protein oligomerization *via* Coomassie Blue staining. Each data point represents the mean ±S.E of 3 independent observations.

A. Allyl Alcohol Hepatotoxicity

Allyl alcohol undergoes rapid enzymatic oxidation to acrolein in rodent liver, with toxicity typically expressed within the periportal zone of affected lobules [37]. Although this model has little direct relevance to human health beyond rare instances of allyl alcohol intoxication [38], it nevertheless represents a useful tool for exploring the cellular mechanisms underlying acrolein-mediated liver injury. As indicated above, we used allyl alcohol during *in vitro* screening bioassays for cytoprotective compounds including hydralazine [25, 29]. In an *in vivo* mouse model, hydralazine also provided strong protection against allyl alcohol hepatotoxicity, as assessed *via* measurements of hepatic enzyme markers in plasma [32]. Intense adduct-trapping occurred in a wide range of liver proteins in mice receiving hepatoprotective doses of hydralazine [32]. These experiments confirmed that the protective efficacy of hydralazine against acrolein toxicity is manifest in the whole animal setting.

B. Alzheimer Disease

The combination of high rates of oxygen consumption, a lipid-rich tissue environment, low antioxidant capacity and limited regenerative capacity ensure the brain is particularly vulnerable to oxidative stress and lipid peroxidation [39]. Carbonyl stress contributes to a number of neurodegenerative conditions, with a particularly clear role likely in Alzheimer's disease (AD). Indeed, evidence increasingly suggests that carbonyl stress exacerbates both hallmarks of altered protein processing that are characteristic of the AD phenotype, namely β–amyloid plaque deposition and the formation of neurofibrillary tangles.

An increase in the levels of both free and protein-adducted forms of lipid-derived carbonyls has been detected in the hippocampus and other affected regions of AD brain samples [40]. Aldehyde-adducted proteins have also been detected in hippocampal and inferior parietal lobular tissue from patients exhibiting mild cognitive impairment, a clinical precursor to AD [41]. Multiple pro-oxidative events appear to underlie the accumulation of carbonyl-adducted proteins in AD, including concentration of redox active transition metals in amyloid plaques; direct free radical production by toxic β–amyloid fragments; activation of prooxidant metalloenzymes such as NADPH oxidase; uncoupling of the mitochondrial respiratory chain; and finally, microglial activation and a resulting overproduction of reactive oxygen/nitrogen species [39, 42, 43]. In addition, the compromised activity of enzymatic pathways that detoxicate carbonyl compounds may exacerbate the accumulation of carbonyl-modified proteins in AD [44].

A role for acrolein in AD was first confirmed by Uchida and colleagues who used antibodies to co-localise acrolein- and 4-hydroxynonenal-adducted proteins in the neurofibrillary tangles that are an early feature of AD [45]. Subsequently, although the number of subjects evaluated was low, use of definitive isotope dilution-capillary LC/MS/MS technology allowed detection of increased levels of acrolein-DNA adducts in hippocampal and parahippocampal gyrus samples from AD patients compared to age-matched controls [46]. A further role for acrolein was indicated by the finding that hippocampal neurons, a class of neurons that are lost during AD, display heightened vulnerability to acrolein [47, 48]. In addition, acrolein was found to promote phosphorylation of tau, a microtubule-associated protein found in a hyperphosphorylated state within neurofibrillary tangles [49]. Recently, incubation of tau monomers with acrolein was shown to accelerate the formation of oligomeric tau species that morphologically resemble tangles recovered from AD patients [50].

Since it can alter the solubility of targeted proteins and increase the likelihood of inter- and intramolecular cross-linking, protein damage by carbonyl compounds has been proposed to exacerbate the deposition of neurofibrillary tangles and β-amyloid deposits in the AD brain [50]. Since carbonyl-trapping drugs could conceivably counteract such damage by trapping electrophiles prior to protein adduction and/or cross-linking and aggregation, the potential of such drugs to disrupt formation of protein neurofibrillary tangles and β-amyloid plaques deserves attention. Such possibilities were explored in recent work by Bieschke *et al*. who found that several nitrogen-containing carbonyl scavengers including hydralazine could disrupt fibril formation from β-amyloid fragments (1-40) that was initiated by carbonyl-containing cholesterol oxidation products [51]. The accelerated fibril formation was attributed to a lowering of the critical concentration (K_c) of the peptide secondary to carbonyl adduction on critical nucleophilic residues, promoting conformational changes within the peptide in an effort to bury hydrophobic substituents in the aggregated peptide complex [51]. Whether adducts formed by a low molecular weight carbonyl compound such as acrolein increase the hydrophobicity of β-amyloid fragments comparable to the change in K_c effected by cholesterol derivatives has yet to be established. If so, carbonyl-trapping drugs, especially those possessing moderate hydrophilic character such as hydralazine, might be a novel means of counteracting such deleterious events.

C. Spinal Cord Injury

Physical trauma to the spinal cord induces neurodegenerative changes that are accompanied by a range of molecular and biochemical events including membrane damage, cytoskeletal disruption, altered gene expression, inflammation, glutamate excitotoxicity, Ca^{2+} overload and oxidative stress [52, 53]. The lipid-rich environment within the white and gray matter of the spinal cord is highly vulnerable to lipid peroxidation, hence increased production of carbonyl products including malondialdehyde and 4-hydroxynonenal accompanies the induction of spinal trauma in rats [54-56]. A conspicuous increase in acrolein adducts was also detected in spinal cord proteins extracted from rats 4 hours after subjecting the spinal cord to compression injury, with protein damage peaking at 24 hours and remaining elevated relative to controls 1 week post-injury [57]. Significantly, acrolein appeared to diffuse from its site of formation to induce protein damage at remote loci within the spinal cord, since acrolein-adducted proteins were detected not only in proteins from the injury site (T10 to T11), but also in adjacent spinal cord segments (T8-T9 and T12-T13) [57]. Comparable trends were seen in the levels of 4-hydroxynonenal-modified proteins in the same spinal cord segments [57]. The contribution of these reactive aldehydes to the neurological deteriora-

tion characteristic of spinal cord injury is unknown, although it is noteworthy that exposing isolated guinea-pig spinal cord strips to acrolein produced changes in axonal conduction that resembled those accompanying spinal cord injury *in vivo* [58]. These changes were also consistent with changes to the morphology and cytoskeleton elicited by 100 μM acrolein in chick dorsal root ganglion cells and sympathetic ganglion cells [59].

In recent work, cultured undifferentiated PC12 cells were used as a model to reproduce the pathobiological role of acrolein in experimental spinal cord injury. PC12 cells are widely used tools in neurological research since they secrete a range of sympathetic amine neurotransmitters. Using this experimental model, co-incubation with high micromolar concentrations of hydralazine strongly attenuated the loss of PC12 cell viability elicited by acrolein as assessed by a range of cell viability end-points including leakage of lactate dehydrogenase, trypan blue exclusion, cell ATP levels, and MTT reduction [60]. Although these workers did not clarify the mechanisms underlying the protective effect of hydralazine, these observations reinforce findings from our laboratory concerning the effectiveness of hydralazine as an inhibitor of acrolein-mediated cell injury. These findings may also raise the prospect of new therapeutic approaches in the treatment of spinal cord injury, a devastating condition for which few effective therapies presently exist. Although this expectation is tempered by the fact that the literature is replete with examples of experimental therapies that attenuate spinal cord injury in animal models and yet subsequently are found to lack efficacy in humans, the use of carbonyl-trapping drugs represents a novel therapeutic strategy that deserves attention. If reactive carbonyl compounds such as acrolein are indeed key mediators of spinal cord injury, a drug that is directed against these species seems a rational alternative to therapies that target "upstream" events involved in oxidative stress rather than the noxious aldehydes themselves.

D. Atherosclerosis

Reactive carbonyls and related lipid-derived species play a conspicuous role in mediating cell damage in two interrelated diseases that increasingly wreak havoc in industrialised nations, namely atherosclerotic heart disease and diabetes. A hallmark of the former is the deposition of lipid-laden foam cells, reflecting the uncontrolled uptake of modified LDL by macrophages *via* receptor-dependent processes [61]. A number of modifications are known to facilitate lipoprotein accumulation by the various "scavenger" receptors, including oxidation, aggregation and adduction by lipid- and sugar-derived electrophiles [62, 63]. According to the classical view of atherosclerosis pathogenesis, modification of lysine residues in apolipoprotein B-100 increases the overall negative charge of lipoproteins, facilitating uptake by macrophages to produce cholesterol ester-laden foam cells within the subendothelial space of blood vessels [64]. Accumulation of foam cells *via* this route underlies the formation of atherosclerotic plaques which in their advanced stages are prone to rupture and thrombosis, potentially triggering medical emergencies in the form of either heart attacks or stroke. In addition to promoting foam cell formation, oxidised LDL elicit a broad spectrum of other deleterious effects that accompany atherogenesis, including promotion of local inflammation and associated changes in cytokine expression, alterations to the expression of adhesion molecules, growth factors and coagulation proteins, and the migration and proliferation of smooth muscle cells.

Acrolein is one of a number of reactive carbonyl compounds known to modify lysine residues within apolipoproteins. In an early study, Uchida and associates identified acrolein-adducted proteins in fatty streaks within blood vessels from an atherosclerotic subject [65]. Cell-associated staining for acrolein-adducted proteins co-localised with the expression of CD68, an important receptor involved in the uptake of oxidised LDL by human macrophages, suggesting an interrelationship between foam cell formation, lipid peroxidation and acrolein formation. More recently, acrolein was shown to target apolipoprotein A-1, an important component of high-density lipoproteins (HDL) that are involved in recycling cholesterol from arterial wall deposits to the liver [66]. Adduction of a single lysine (lysine-226) located within helix 10 of apoA-1 impaired cholesterol efflux, presumably by interfering with the interaction between two amphipathic α-helices to form a structural element that facilitates cholesterol efflux by the ATP Binding Cassette Transporter A-1[66]. Although its role in atherosclerosis is less clear, acrolein also modifies apolipoprotein E, disrupting the uptake of very low density lipoprotein by hepatocytes [67].

The contribution of acrolein to cardiovascular disease is unlikely to be restricted to the ability to alkylate apolipoproteins and disrupt arterial cholesterol homeostasis. For example, recent work suggests that exposure of human coronary arteries to the acrolein precursor allylamine resulted in hyperconstriction of the vessels in a manner characteristic of vasospasm, a life-threatening complication accompanying atherosclerosis, angina, stroke and other cardiovascular disorders [68]. In other new work, along with 4-HNE, acrolein was found to mediate the inhibitory action of oxidised LDL upon the proliferative responses of rabbit arterial smooth muscle cells to platelet-derived growth factor (PDGF) [69]. This effect was only seen when cells were preincubated for 24 hours with oxidised LDL and was accompanied by adduction of PDGF receptor-β by 4-HNE- and acrolein, reactions that appeared to diminish the affinity of the receptor for PDGF and also strongly inhibited the tyrosine kinase activity of this membrane receptor complex [69]. Receptor internalisation, a classic pathway of down-regulation of the activity of tyrosine kinase receptors, made only a minor contribution to the effect of oxidised LDL on cell responses to PDGF [69]. Given that migration and proliferation of smooth muscle cells are key events in the formation of the fibrous cap of atherosclerotic plaques, these observations suggest that reactive carbonyls may elicit damage that leads to inadequately capped plaques that are vulnerable to rupture.

Given the multifaceted contributions of reactive carbonyls such as acrolein to cardiovascular disease, intervention with an efficient carbonyl-scavenging drug such as hydralazine could potentially disrupt a number of proatherogenic events. Work from a number of labs suggests such a possibility. For example, Brown *et al*. recently demonstrated that hydralazine could block LDL modification by two sugar-derived dicarbonyls, namely methylglyoxal and glycolaldehyde [70]. Hydralazine blocked glycolaldehyde-induced

changes to the electrophoretic mobility of LDL and strongly suppressed the accumulation of free cholesterol and cholesteryl esters by J774A.1 mouse macrophages during incubations with modified LDL [70]. Comparable outcomes were elicited by other hydrazine compounds (e.g. aminoguanidine, Girard's reagents P and T), although typically 5- to 10-fold greater concentrations of these carbonyl scavengers were required to attain a degree of inhibition comparable to that produced by a given concentration of hydralazine.

Promising outcomes were also obtained during study of the effects of hydralazine on the inhibition of PDGFRβ signalling produced by oxidised LDL in rabbit arterial smooth muscle cells [69]. Hydralazine (100 μM) strongly attenuated adduction of PDGFR by 4-HNE and also restored the sensitivity of PDGFRβ to activation by PDGF. Most importantly, these actions of hydralazine were evident in the *in vivo* setting, since hydralazine administration to atherosclerotic cholesterol-fed rabbits abolished 4-HNE adduction within the intima of rabbit aorta and also strongly suppressed the formation of atherosclerotic plaques [69]. These effects were seen in the absence of significant changes in the arterial blood pressure of the rabbits, raising hopes that disruption of plaque growth and instability may be achievable in human subjects using tolerable doses of hydralazine.

7. CONCLUSIONS AND FUTURE DIRECTIONS

The past two decades have witnessed strong growth in knowledge concerning the contribution of reactive carbonyls to human disease. Far from "innocent bystanders," the chemical reactivity and biological properties of these endogenous electrophiles ensures they amplify cell damage during oxidative cell injury [71]. Since antioxidant-based interventions have often failed to meet expectations during clinical trials in patients with degenerative conditions [72, 73], a carbonyl-trapping strategy focused on "downstream" mediators of oxidative cell injury deserves greater consideration in light of a growing body of positive experimental findings [74]. Nonetheless, a number of issues seem to require attention if this field is to make genuine progress. First, there is a need to identify new classes of effective carbonyl scavengers that balance excellent carbonyl-trapping chemistry within cells and test-tubes with acceptable pharmacokinetic properties and a tolerable side-effect profile in the body. This review has highlighted work on hydralazine, a promising prototype since it is a known carbonyl-scavenger in humans and has a record of clinical use spanning over 50 years. However, the usefulness of hydralazine as a carbonyl-trapping reagent in the clinical setting will be limited by its cardiovascular properties and side-effect profile. The data presented in this review indicate that problems relating to the cardiovascular actions of hydralazine are surmountable, since hydrazinoisoquinoline and other analogues in which the vasoactive pharmacophore is disrupted retained excellent cytoprotective potency against acrolein toxicity (Table **2**). However, addressing the safety profile of compounds such as hydralazine and hydrazinoisoquinoline may be more difficult, since possession of a hydrazine substituent is a "structural alert" for a number of toxicological outcomes including enzymatic bioactivation, mutagenicity and carcinogenicity [75]. Identification of alternative chemistries to obtain compounds that retain carbonyl-sequestering properties in biological systems and yet avoid noxious functional groups such a hydrazine group would represent a significant advance in this area. A further limitation accompanying reliance upon hydrazine-based chemistry is that the kinetics of carbonyl-sequestration by these compounds is inadequate to compete with the rapid reaction of highly reactive endogenous electrophiles such as acrolein with their preferred thiol targets in cells and tissues. However, given that other important endogenous electrophiles such as 4-HNE exhibit some 2 orders of magnitude lesser reactivity with thiol groups compared to acrolein, hydrazine-based scavengers are likely to more effectively trap 4-HNE within the cellular environment. Indeed, the recent demonstration by Vindis *et al*. (2006) that hydralazine suppressed levels of 4-HNE adducts in the aorta of hypercholesterolemic rabbits is promising in this respect [69]. As recently suggested by Aldini *et al.* (2006), perhaps distinct carbonyl scavengers that are active against particular types of carbonyl-containing electrophiles should be developed for specific disease applications in which different carbonyl compounds are known to predominate. In addition, structural optimisation of carbonyl-scavengers may be required to obtain drugs with physicochemical properties that are appropriate to a given disease application. For instance, different types of compounds may be needed against neurodegenerative disease where diffusion across the blood brain barrier is essential, compared to treatment of respiratory conditions where inhalational delivery of a compound lacking the ability to penetrate systemic tissues may be desirable.

More robust testing of carbonyl-scavengers in appropriate animal models of human disease is another issue requiring greater attention. In particular, confirming that any beneficial outcomes in animal models are truly due to carbonyl-scavenging actions will require careful attention, perhaps by incorporating determination of "biomarkers of carbonyl-scavenging" into the design of such studies. Such a goal will require development of specific analytical assays to detect drug-carbonyl conjugates with sufficient sensitivity to detect the low levels that are likely to be formed under conditions of endogenous carbonyl generation, however such an objective seems increasingly feasible given the improved sensitivity of contemporary analytical instrumentation. Use of these technologies to measure levels of drug-carbonyl conjugates in body fluids (e.g. blood, urine, CSF etc) or tissues collected from drug-treated animals will provide a more sophisticated understanding of the role of carbonyl-scavenging in any beneficial disease outcomes elicited by the compounds. As highlighted in the recent study by Vindis *et al*. (2006), mechanisms other than carbonyl-scavenging might conceivably have contributed to the reduction in plaque size that was effected by hydralazine in cholesterol-fed rabbits (e.g. inhibition of oxidant-generating enzymes such as NADPH oxidase) [69]. Incorporation of measurement of drug-carbonyl conjugates into future animal studies might resolve such fundamental questions relating to mechanism of drug action during animal studies. Such efforts may not be straightforward, however, particularly since we observed that the main conjugate formed by hydralazine during reactions with acrolein, (1E)-acrylaldehyde-phthalazin-1-ylhydrazone, is rapidly metabolised by mouse liver cells [25]. In such an instance, quantification of metabolites formed from these spe-

cies might provide useful insights into the extent of drug-carbonyl scavenging occurring in biological systems.

Development of dosing regimens with carbonyl-trapping drugs that avoid depletion of important endogenous carbonyl compounds is another significant challenge confronting this field. For example, depletion of the aldehydic form of vitamin B (pyridoxal) precipitates a number of neurological conditions (e.g. peripheral neuritis, neuropathy) in patients receiving a range of carbonyl-reactive agents, including hydrazino drugs (e.g. isoniazid, phenelzine, hydralazine, carbidopa), substituted hydroxylamines (e.g. D-cycloserine) and sulfhydryl agents (e.g. D-penicillamine) [76]. The brain is particularly vulnerable to chronic pyridoxal depletion given the importance of this co-factor during the biosynthesis of many important neurotransmitters including dopamine, serotonin, glycine, glutamate and γ–aminobutyrate (GABA). Neurological deficits also occur in individuals with inborn defects promoting accumulation of nucleophilic, pyridoxal-depleting metabolites such as L-Δ^1-pyrroline-5-carboxylic acid [77]. Avoiding neurological side-effects will thus be a challenge during the development of new carbonyl-trapping drugs for use in chronic degenerative conditions. Whether the selectivity of carbonyl-trapping agents can be improved to obtain compounds with attenuated reactivity with pyridoxal seems far from certain. If such a goal is unachievable then pyridoxine supplementation may help minimise neuropathic complications as is the case in isoniazid patients, yet the doses used must be carefully controlled since overdosing with pyridoxine presents its own spectrum of neurological side-effects [76].

Finally, and more positively, one issue that could potentially advance the prospects of the carbonyl-trapping therapeutic approach might involve retrospective epidemiological studies of patients who have received treatment with drugs such as hydralazine. Hydralazine has been subjected to a number of epidemiological trials in the past that were designed to assess its antihypertensive efficacy and safety, the latter studies being motivated by early concerns relating to carcinogenicity risk [78]. Intriguingly, a new wave of clinical studies involving hydralazine have recently been initiated, reflecting the release of a new hydralazine-containing antihypertensive drug combination for use in African-American patients and also a newly discovered ability of hydralazine to reactivate dormant tumour suppressor genes within tumours [79]. Together with historical databases of health indices in recipients of this drug, properly designed investigations to assess the incidence of spontaneous human disease known to involve reactive carbonyls (e.g. see Table **1**) in hydralazine recipients may provide unexpected insights into beneficial or protective effects of this drug.

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