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Carbonyl Scavengers as Pharmacotherapies in Degenerative Disease: Hydralazine Repurposing and Challenges in Clinical Translation

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ALD, alcoholic liver disease; *E*-APH, (1E)-acrylaldehyde phthalazin-1-ylhydrazone; CTT, carbonyl-trapping threshold; 3-HPMA, 3-hydroxypropyl mercapturic acid; HSAB, hard-soft acid-base; LDE(s), lipid-derived electrophile(s); LPO, lipid peroxidation; MEC, minimum effective concentration; MTC, minimum toxic concentration; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SCI, spinal cord injury.

Abstract

During cellular metabolism, spontaneous oxidative damage to unsaturated lipids generates many electrophilic carbonyl compounds that readily attack cell macromolecules, forming adducts that are potential drivers of tissue dysfunction. Since such damage is heightened in many degenerative conditions, researchers have assessed the efficacy of nucleophilic carbonyl-trapping drugs in animal models of such disorders, anticipating that they will protect tissues by intercepting toxic lipid-derived electrophiles (LDEs) within cells. This Commentary explores recent animal evidence for carbonyl scavenger efficacy in two disparate yet significant conditions known to involve LDE production, namely spinal cord injury (SCI) and alcoholic liver disease (ALD). Primary emphasis is placed on studies that utilised hydralazine, a clinically-approved "broad-spectrum" scavenger known to trap multiple LDEs. In addition to reviewing recent studies of hydralazine efficacy in animal SCI and ALD models, the Commentary reviews new insights concerning novel lifespan- and healthspan-extending properties of hydralazine obtained during studies in model invertebrate organisms, since the mechanisms involved seem of likely benefit during the treatment of degenerative disease. Finally, noting that human translation of the histoprotective properties of hydralazine have been limited, the final section of the Commentary will address two obstacles that hamper clinical translation of LDE-trapping therapies while also suggesting potential strategies for overcoming these problems.

Contents

1. Introduction

Awareness of the health impact of toxic substances within the exposome to which one is exposed over a lifetime expanded over recent decades to include molecules of endogenous origin as well as those entering the body from environmental sources [1]. Notable among the toxicants arising internally are the various α,β-unsaturated aldehydes such as 4-hydroxynonenal and acrolein that form during lipid peroxidation (LPO) as well as other cellular processes. Whereas toxic xenobiotics often acquire electrophilicity during bioactivation by drug-metabolising enzymes, many endogenous toxicants inherently possess such reactivity due to their intrinsic structural features. Hence, the basic paradigm guiding investigation of the pathological roles of endogenous lipid-derived electrophiles (LDE) in particular diseases resembles the classic scenario applying to the role of reactive metabolites in drug-induced toxicities: "electron deficient" endogenous LDEs are attacked by nucleophilic ("electron rich") centres in cell proteins, forming adducts that disrupt associated macromolecular functions, thereby posing a fundamental mechanistic insult to living cells.

Due to its presumed pathogenetic significance, much effort has been devoted to such goals as clarifying the chemistry of protein adduction by LDEs; identifying the target proteins incurring adduction within cells; assessing the dose-dependence of damage to specific targets; mapping the spatial distribution of damaged proteins within tissues; or establishing the timing of protein damage in relation to the onset of cell death or other pathological outcomes [2-4]. While such investigations were once conducted using antibody-based methods, improvements in the enrichment, identification and quantitation of LDE-adducted proteins using proteomic strategies have invigorated the field [4-6]. The list of disorders that involve protein damage by electrophilic endogenous toxicants is now extensive, and includes high prevalence degenerative disorders such

as those affecting the cardiovascular and nervous systems as well as cancer. The fact that LDE-mediated protein damage is usually concentrated within anatomical regions showing overt signs of disease pathology is a key factor implicating endogenous LDEs in the onset and progression of human disease [7].

Such knowledge also underpins expectations that drug interventions to suppress protein damage by LDEs might slow the progression of degenerative diseases involving these toxicants. While many strategies have been explored, a common approach has involved administering small-molecule antioxidants to suppress LDE production at its source by quenching radical-mediated peroxidation of polyunsaturated fatty acids (PUFAs). Unfortunately, many evaluations of such molecules within randomised clinical trials have either failed to demonstrate human efficacy or displayed evidence of increased mortality [8-11]. The poor clinical performance of antioxidants is partly attributable to their nonspecific suppression of the essential physiological roles played by reactive oxygen species, thereby eliciting unanticipated side-effects and tissue dysfunction [12, 13]. As suggested by Schmidt and associates, these disappointments may reflect the influence of an overly reductionist epistemology in which free radicals represent the “axis of evil” and antioxidants “the Holy Grail” [14]. Growing knowledge of the normal signalling functions of free radicals in the regulation of cell metabolism rendered such binary conceptions untenable [15].

One alternative to antioxidant suppression of LDE production involves using carbonyl-trapping molecules to directly intercept reactive LDEs within cells and tissues [16-19]. Since LDEs are less likely to play the essential cell signalling functions attributed to reactive oxygen species, this strategy seems less susceptible to the side-effects that hamper clinical translation of radical-quenching antioxidants. This Commentary briefly surveys this experimental strategy and reviews recent encouraging findings concerning the efficacy of carbonyl-scavenging molecules in preclinical

models of two significant diseases, namely spinal cord injury (SCI) and alcoholic liver disease (ALD). The focus is primarily upon hydralazine, a classic antihypertensive that is a compelling candidate for repurposing given its excellent LDE-trapping profile and drug-like pharmacokinetic properties.

2. Carbonyl Scavenging as a Therapeutic Strategy.

2.1. LDE-Trapping Activity

An ideal LDE-scavenger will possess a combination of at least three core characteristics, namely ready reactivity with noxious LDEs; drug-like physicochemical properties that confer good tissue-penetrating capabilities; and a limited pharmacological profile due to minimal activity at "off-target" receptors [20]. The possession of a nucleophilic group that confers reactivity with LDEs is especially important, and typical cytoprotective scavengers possess one or more nucleophilic heteroatoms such as an O, N, or S [18].

Since identifying molecules that satisfy all three requirements has proved difficult, some of our most promising LDE-trapping agents such as hydralazine are existing pharmaceutical agents that possess good drug-like physicochemical properties and exhibit excellent reactivity towards LDEs, but also elicit conventional pharmacological actions via transient interactions with target receptors. Nevertheless, providing they satisfy the first two requirements, such "pharmacologically active" carbonyl scavengers can be of value if they exert their electrophile-trapping effects at drug concentrations that are lower than those eliciting classic receptor-mediated pharmacological responses [21]. As depicted in Figure 1, an ideal carbonyl scavenger would be delivered at doses that achieve plasma concentrations that exceed an "Carbonyl-Trapping Threshold" (CTT) and yet fall below the Minimum Effective Concentration applying to any receptor-mediated

pharmacological responses, while also being distinct from doses that elicit toxicity (i.e. << MTC, Figure 1A). In short, an ideal LDE scavenger will suppress LDE-mediated macromolecular adduction at doses causing neither receptor-mediated side effects nor overt tissue injury (Figure 1B).

2.2. Chemical Complementarity of Scavengers and Electrophiles

The fundamental chemical properties that govern electrophile-trapping selectivity need careful consideration when selecting carbonyl scavengers. To ensure efficient trapping of electrophiles and protection of vulnerable macromolecules, the chemical properties of nucleophilic scavengers should complement those of target LDEs known to play pathogenetic roles in the specific disease of concern. According to Pearson's Hard and Soft Acids and Bases (HSAB) theory , the interactions between LDEs and nucleophilic amino acids within protein targets – and also with low mass carbonyl scavengers – are non-arbitrary and subject to "hardness" and "softness" considerations on the part of the participating reagents [22, 23]. On this understanding, both electrophiles and nucleophiles can be classified as either "hard" (non-polarisable) or "soft" (polarisable) according to their relative electron mobility or "polarizability." Hence "soft" LDEs such as acrolein and 4-hydroxynonenal favour reactions with the soft nucleophilic thiolate anion possessed by cysteine residues, typically via Michael addition reactions involving thiolate addition to the β-carbon to form a carbonyl-retaining adduct [23]. While acrolein and 4-HNE also attack harder amine groups, in kinetic terms these reactions are less favoured than reactions with cysteine [24]. Correspondingly, on this view "soft" carbonyl scavengers would be best suited to conditions in which LPO features prominently since this pathway yields numerous "soft" LDEs such as acrolein and 4-HNE.

Alternatively, endogenous electrophiles such as methyl glyoxal and glyoxal that form during carbohydrate autoxidation are comparatively hard species that display minor reactivity with

cysteine and instead favour interactions with hard primary amine groups in proteins via classic Schiff base chemistry. Hence a carbonyl scavenger intended for use in conditions that afflict diabetics and involve overproduction of sugar-derived “hard” electrophiles should possess hard nucleophilic centres, usually an N or O. Since this Commentary is focussed on scavengers that target “soft” LPO-derived electrophiles, readers with interests in the issues surrounding scavengers used against “hard” carbohydrate-derived glycating electrophiles are directed to other excellent publications [25].

2.3. Subcellular Distribution of Macromolecular Targets

The selection of carbonyl scavengers is further complicated by the heterogeneity within the cellular adductome with respect to the subcellular localization of protein targets. Typically, individual proteins differ not just in the number and accessibility of nucleophilic residues that they contain, but also the subcellular microenvironment in which they are normally present. Since such factors influence their vulnerability to electrophiles, patterns of protein adduction by LDEs and other reactive species must be clarified on a protein target by target basis [26]. For example, early studies of protein adduction by LDEs in hepatocytes suggested that adduction of “soft” cysteine groups within abundant cytosolic proteins was very conspicuous, suggesting it might serve a protective function by “mopping up” or “scrubbing” reactive LDEs that might otherwise attack harder nucleophilic sites in critical lower abundance proteins [27]. The latter category likely includes many mitochondrial proteins which are known critical targets for endogenous electrophiles [5, 6]. Clearly, it would be important that administered carbonyl scavengers possess physicochemical properties allowing them to protect critical targets of the “toxicologically significant” variety than the abundant “cytosolic electrophile scrubber” category which likely sustain survivable damage (Figure 2) [5]. Yet “hard” versus “soft” scavengers could conceivably

differ in the extent to which they protected these distinct protein targets. For example, since its direct reactivity with soft LDEs is strongly favoured in kinetic terms, a soft nucleophilic scavenger might fully scavenge all intracellular LDEs, thereby protecting abundant non-essential “soft” targets in cytosol as well as low abundance “hard” targets that are of high toxicological relevance. A hard nucleophilic scavenger on the other hand, may not trap LDEs sufficiently quickly to protect “soft” protein targets, yet it might prevent damage to critical “hard” targets within mitochondria or other subcellular settings, thereby affording cytoprotection against cell death.

Due to the preceding considerations, identifying carbonyl scavengers that protect every intracellular target against all endogenous electrophiles seems an unduly ambitious goal. It is more likely that certain narrow spectrum scavengers will prove useful in distinct health conditions in which particular electrophiles play dominant roles, while other broad spectrum scavengers may be useful in conditions involving many diverse electrophiles [28].

3. Criteria for Proving LDE-Sequestration In Vivo

Identifying scavenger compounds that readily trap biogenic electrophiles under “test tube” conditions within the laboratory is important for screening purposes, but extra proof is needed to ensure such reactions contribute to any desirable actions the scavengers might exhibit within animals or disease-affected humans. Many bioactive low-mass xenobiotics are pleiotropic entities that can disrupt multiple biological processes, hence care is needed to ensure that any presumed electrophile-trapping mechanism is causally involved in any observed therapeutic response to carbonyl scavengers in vivo. To maximise the scientific rigour of claims concerning the role of carbonyl-scavenging in the efficacy of administered nucleophiles in animals or humans, experimental studies should ideally address four key criteria:

First, quantitative analysis should be performed on biofluids collected from test subjects to confirm that administered scavengers actually decrease free concentrations of target LDEs. Ideally the biofluids should be as directly relevant to the diseased tissue as possible, but in many cases blood or urine samples are acceptable surrogates. Generally, dose-dependency should be demonstrated, such that increasing doses of scavengers elicit proportional reductions in circulating LDE concentrations. Obtaining such information can be challenging if the electrophile(s) of concern react readily with nucleophilic blood or tissue constituents, but generally the high sensitivity of modern analytical instrumentation allows reliable quantitation of the very low free concentrations of endogenous LDEs found in blood or plasma. If the noxious LDE is detoxicated by glutathione and gives rise to urinary mercapturic acid conjugates, then these species can also be used to confirm the efficacy of carbonyl-trapping drugs, a strategy that assisted the evaluation of hydralazine as a treatment for spinal cord injury in rodents (see below).

A second criterion involves direct proof for the formation of scavenger-electrophile conjugates within the body. Once again, dose-dependency should be demonstrable such that the circulating concentrations of drug-LDE conjugates should rise in proportion to the magnitude of the administered scavenger dose. While a propensity to undergo further metabolism may complicate efforts to measure some drug-LDE conjugates in vivo [29], for most scavengers the quantitation of stable trapping products in blood or urine would strongly reinforce the in vivo relevance of this putative mechanism of drug action.

The third criterion is that scavenger administration is shown to decrease levels of electrophile-adducted macromolecules within body tissues. Ideally, the suppression of adduction should be demonstrated within the diseased tissues most relevant to the respective medical condition, a criterion that is usually achievable within animal-based studies. Since this goal may be unachievable in humans, researchers might resort to quantifying electrophile-modified proteins

within accessible biofluids such as blood. For example, adduction of the Cys34 of serum albumin is a potential "off-target" surrogate for protein adduction within extravascular tissues of ischaemic rats [30].

The fourth criterion is that scavenger administration demonstrably elicits dose-dependent reductions in disease- or toxicity-relevant biomarkers. These can involve a range of molecular, biochemical or histological indicators that, when taken together, confirm that the scavenger affords histoprotection extending from the fundamental molecular level to the whole organ or system levels of biological organisation. An ability to correlate these disease endpoints with conventional pharmacological biomarkers such as plasma drug concentrations would help confirm the significance of electrophile-trapping to biological responses to administered scavengers.

Ideally, each of these four criteria should be satisfied during evaluations of nucleophilic scavenger drugs in cellular, animal or human studies. These considerations will be kept in mind while exploring recent studies of the carbonyl-sequestrating properties of hydralazine.

4. Hydralazine as a Model LDE Scavenger

Among the strongest N-containing nucleophiles in clinical use, hydralazine was developed by Swiss researchers in the early 1950s [31]. The first orally available peripheral vasodilator, it remains a second line therapy for patients with essential hypertension. Due to its potential to induce angina and tachycardia when used alone, hydralazine is often combined with β–blockers and diuretics. The precise mechanisms underlying the vasorelaxant properties of hydralazine remain unclear, although interference with inositol triphosphate-induced calcium release in arterial smooth muscle cells is a commonly-invoked explanation [32].

Hydralazine belongs to a select set of approved medicines that are documented carbonyl-scavengers in humans, a consequence of its strongly nucleophilic hydrazine group [20]. Formation of a hydrazone via a nonenzymatic reaction with pyruvate is a major fate of any orally administered hydralazine that reaches the systemic circulation [33]. Awareness that hydralazine might also scavenge LDEs in vivo is traced to longstanding observations concerning its protective efficacy against allylamine vasculotoxicity, a syndrome that involves enzymatic formation of acrolein, a highly electrophilic 3-carbon electrophile [34, 35]. Long recognised as a key contributor to environmental pollution, acrolein also forms endogenously via the degradation of lipids, amino acids and polyamines [36].

The possibility that hydralazine suppresses allylamine toxicity by scavenging acrolein was reinforced during cell-based scavenger screening experiments in which it showed far greater protective efficacy against acrolein-mediated cytotoxicity than the other N-centred nucleophiles tested [37]. The conjugate (1E)-acrylaldehyde phthalazin-1-ylhydrazone (E-APH) was isolated as the major product of reactions between acrolein and hydralazine and also detected within hepatocyte media during incubations with hydralazine and the acrolein precursor allyl alcohol (Figure 3) [29]. In comparisons of the reactivity of several classic carbonyl scavengers toward multiple biogenic electrophiles, hydralazine effectively sequestered a wider range of sugar- and lipid-derived electrophiles than the other compounds tested [38]. The carbonyls trapped by hydralazine included “soft” LDEs such as malondialdehyde and 4-hydroxynonenal as well as the “hard” sugar-derived electrophiles methylglyoxal and glyoxal [38]. Such knowledge of its “broad-spectrum” LDE-trapping activity have motivated the evaluation of hydralazine within animal models of various degenerative diseases. We will now review recent studies of hydralazine efficacy in animal models of two very different health conditions in which LDEs play substantive roles, spinal cord injury (SCI) and alcoholic liver disease (ALD). SCI and ALD seem worthy of attention because hypertension

frequently occurs in patients with these conditions, a factor that renders the potential blood pressure-lowering effects of hydralazine less problematic in these cohorts.

4.1. Role of LDE in Spinal Cord Injury

In terms of its impact on affected individuals and their families, traumatic injury to the spinal cord ranks among the most devastating misfortunes to befall human beings. Since affected individuals are often young adults, the long term social impact of SCI is enormous. Although physical trauma is the initiating event, the final extent of functional loss in SCI is governed by secondary pathophysiological processes which propagate damage radially along the cord from the lesion epicentre [39]. Many deleterious mechanisms are involved in the secondary phase, including neuronal calcium dyshomeostasis, glutamate excitotoxicity, oxidative stress, ischemia/reperfusion, inflammatory cell recruitment and activation, cytoskeletal proteolysis, and both apoptotic and necrotic cell death [40, 41]. Tissue deterioration during the secondary phase can take weeks or months to peak, finally conferring a dysfunctional tissue environment featuring apoptosis, demyelination, glial scarring and Wallerian degeneration. Awareness that secondary processes strongly influence overall impairment in SCI has inspired a broad effort to evaluate various pharmaceutical and biotechnology-based interventions that attempt to attenuate these pathophysiological events [42].

Oxidative stress features strongly in SCI due to the many pathways by which reactive oxygen and nitrogen species form within damaged cords, including superoxide production via several routes such as mitochondrial uncoupling; redox-cycling of oxidised neurotransmitters; NADPH oxidase activation within recruited immune cells; and release of redox-active transition metals [43-45]. Lipid concentrations within white matter are high, and phospholipase A2 activation during SCI

causes a sustained release of arachidonic acid and other PUFAs [46]. Liberated lipids can then undergo either enzymatic conversion to bioactive eicosanoids or nonenzymatic peroxidation to release various noxious LDEs. Experimental evidence for early, sustained release of malondialdehyde, acrolein and 4-hydroxynonenal within animal models of SCI is long standing [47-52]. Since noxious migratory LDEs can diffuse laterally from the primary lesion, the likelihood that they act as "toxicological second messengers" to amplify neuronal damage during SCI is high.

A comprehensive body of experimental work by Shi and associates suggests a clear causal role for the short-chain LDE acrolein in both the neuronal loss and neuropathic pain that accompany SCI [47, 53]. In their early work, an immunochemical approach was used to confirm early formation of acrolein-adducted proteins in rat spinal cords just 4 hours after compression injury, with damage peaking one day after the injury and remaining above controls 1 week later [47]. Consistent with the proposal that acrolein diffuses along the cord from the primary lesion, acrolein-adducted proteins were detected at the primary injury site and in neighbouring spinal cord zones [47]. 4-Hydroxynonenal, another diffusible LDE, also contributed to protein adduction in adjacent cord segments [47]. In related work, exogenously added acrolein induced changes in axonal conduction in guinea pig spinal cord segments resembling those seen in rodents subjected to SCI [54]. Exogenous acrolein also elicited morphological changes in chick dorsal root ganglion cells that were typical of those seen in SCI [55].

Direct LDE involvement in SCI pathogenesis was reinforced during an in vivo study in which acrolein microinjection into the spinal cords of anaesthetised rats reproduced many of the behavioural and histological changes seen in SCI [56]. Behavioural monitoring and locomotor testing was performed for 7 weeks after acrolein administration, while spinal cord tissues were evaluated for histological changes either 24 h or 7 weeks after acrolein exposure. Intriguingly, a

single dose of acrolein induced time- and dose-dependent declines in locomotor performance as well as persistent histological alterations including reactive gliosis, macrophage infiltration, neuronal degeneration and demyelination [56]. Consistent with the idea that acrolein acts as a diffusible toxicant within injured spinal cords, the tissue volume occupied by the lesion at the injection site expanded during the study [56]. With the proviso that a single bolus dose of acrolein cannot fully reproduce the in vivo setting within damaged cords in which perpetual LPO likely generates LDE over extended timeframes, these results suggest a substantive pathogenetic role for acrolein in SCI.

In addition to mediating neuronal loss in SCI, short-chain LDEs such as acrolein are further implicated in the sensory hypersensitivity that accompanies the neuropathic pain beneath the spinal lesion site that plagues SCI survivors [57]. Typically, neuropathic pain after SCI manifests as either hyperalgesia, a heightened sensitivity to pain-inducing stimuli, or allodynia, a painful perception of normally non-painful stimuli. The biological basis for neuropathic pain in SCI is complex since it involves spinothalamic interplay between events within the brain and injured cord, but on the basis of animal studies is known to involve changes in glial activation, upregulated expression of chemokines and their receptors, neurotrophic factor release, altered ion channel expression, and also changes in multiple receptor systems including those for cannabinoids, vanilloids, GABA, dopamine and eicosanoids [58]. Intriguingly, in addition to suggesting a role for endogenous acrolein in post-SCI sensory hypersensitivity in rats [53, 59], inhalational exposure to exogenous acrolein appears to further sensitise SCI rats to neuropathic pain behaviours [60]. Although extrapolation to humans is challenging due to the complexities with which individual patients perceive and experience neuropathic pain, these factors indicate that pharmacological suppression of LDE availability within damaged cords may provide benefits beyond preservation of neural functions alone.

4.2. Hydralazine Efficacy in Animal SCI Models

A body of work from the Shi group confirms protective efficacy for hydralazine against SCI-associated neurodegeneration in multiple experimental systems. In early in vitro work, hydralazine suppressed acrolein-induced cell death in rat PC12 pheochromocytoma cells, a popular tool in SCI research [61]. Subsequently, hydralazine was shown to inhibit acrolein-mediated protein damage in isolated guinea pig spinal cord segments subjected to contusion injury ex vivo [62]. A similar experimental system allowed confirmation that acrolein could serve as a diffusible mediator of tissue injury following SCI, with acrolein-adducted proteins detected in healthy spinal cord segments that were co-cultured with damaged spinal segments [63]. The presence of hydralazine suppressed such secondary damage in healthy co-incubated tissues [62].

Intact Sprague-Dawley rats subjected to either moderate or severe trauma to the T-10 level of the spinal cord also exhibited less pronounced neurodegeneration after hydralazine treatment [64]. Concurring with the abovementioned criteria concerning proof of scavenging actions in vivo, the authors confirmed that lower urinary levels of the acrolein metabolite 3-hyroxypropyl mercapturic acid (3-HPMA) accompanied the neuroprotection afforded by hydralazine (i.e. Criterion 1), and that the drug suppressed levels of acrolein-adducted proteins within spinal cord lesions (i.e. Criterion 3) [64]. The same group further established that hydralazine suppressed the pro-nociceptive changes accompanying sensory hypersensitivity that occur in rodent SCI models, thereby meeting the evidentiary expectations relevant to Criterion 4 applied during testing of carbonyl-sequestering therapeutics [59, 60]. Although direct electrophile scavenging by hydralazine was not confirmed by quantifying urinary drug-LDE conjugates (Criterion 2), the work by Zheng and associates helpfully adds to a sizeable body of animal-based research that supports a

pathogenetic role for LDEs in SCI, and further confirms that electrophile-sequestering drugs such as hydralazine can provide functional benefits in these conditions. (redundant sentence deleted from here)

4.3. Role of LDE in Alcoholic Liver Disease (ALD)

Damage to multiple body systems accompanies alcohol abuse, with the liver showing particular vulnerability due to its proximity to the gut and high capacity for ethanol bioactivation [65]. Around 50% of deaths among alcoholics are attributed to alcoholic liver disease (ALD), a serious disorder against which minimal progress has been made in terms of improved pharmacological interventions, with the best available treatments providing only temporary benefit to many patients [66].

ALD comprises several distinct phases, beginning with the steatotic phase which commences within a week or two of individuals beginning to drink heavily (>60 g alcohol/day). The centrilobular appearance of large fatty droplets is an early sign of steatosis onset, an outcome that was long attributed to boosted lipogenesis and suppression of lipid catabolism secondary to shifts in the redox state of the hepatic NAD/NADH pool [67]. Newer theories tend to attribute steatosis to transcriptional upregulation of various lipogenic genes driven by such ligand-responsive transcription factors as SREBP, $PPAR_{\alpha}$ and PXR [68, 69], or a suppression of the inhibitory effects of AMP-activated protein kinase upon acetyl CoA carboxylase activity, the rate-limiting enzyme in fatty acid synthesis [70].

A novel alternative pro-steatotic mechanism emerged with growing knowledge of the role of toxic LDEs in ALD. Several factors increase the vulnerability of hepatocytes to LPO during ethanol exposure, including formation of reactive oxygen species via such routes as CYP2E1-catalysed ethanol oxidation; a shift in the mitochondrial NADH redox state; and the activation of NADPH

oxidases within macrophages. Since membrane PUFAs are fragmented during this barrage of endogenous oxidants, the hepatic proteome of heavy drinkers readily accumulates damage by multiple LDEs including acrolein and other α,β-unsaturated aldehydes [71]. Improved proteomic tools enabling the detection of LDE-modified proteins have supplied new insight into the pro-steatotic mechanisms occurring in animal ALD models. Since electrophilic α,β-unsaturated aldehydes are characteristically attacked by protein nucleophiles to form Michael adducts that retain a carbonyl group, LDE-adducted proteins can be captured using carbonyl-reactive biotin hydrazide-based probes, thus allowing the selective extraction and identification of carbonylated proteins from pathological samples.

By using such technology to identify hepatic proteins that are damaged by LDEs within a murine ALD model, Petersen and associates gained new knowledge concerning the metabolic pathways that are disrupted during heavy alcohol exposure [72]. Their animal model attempted to reproduce heavy drinking exposures seen in humans, with mice subjected to a 6-week treatment during which the alcohol content of their Lieber-DiCarli liquid diet increased from 2 to 6%. Biotin hydrazide labelling/streptavidin purification then allowed recovery of LDE-adducted proteins from several pooled liver fractions before the damaged proteins were identified via collision induced dissociation LC-MS/MS [72]. Although the study was complicated by technical challenges due to the low yield and instability of protein adducts, four LDEs were found to mediate protein carbonylation in ALD mice, namely 4-hydroxynonenal, malondialdehyde, 4-oxononenal, and acrolein [72]. Intriguingly, target proteins for these LDEs were clustered within metabolic pathways that mediate hepatic lipid metabolism, and conspicuously included various acyl-CoA dehydrogenases that catalyse the β-oxidation of lipids. By implicating key members of this catabolic pathway as LDE targets, this work provided novel clues concerning the onset of steatosis during ALD.

In a subsequent study, this group extended the scope of their investigations by adopting the use of electron transfer dissociation (ETD) during LC-MS/MS to improve the detection of adducted peptides, enabling identification of proteins that were damaged by some ten or so LDEs, including the four electrophiles detected in the earlier study (malondialdehyde, acrolein, 4-hydroxynonenal and 4-oxononenal) as well as 4-hydroxyhexenal and a series of 5- to 9-carbon 2-*trans*-alkenals [73]. The investigators also discontinued their practice of pooling livers from multiple mice to better observe inter-individual differences in protein adduction patterns. These improvements allowed identification of some 829 LDE-adducted proteins in murine ALD livers, with the mitochondrial proteome showing particular vulnerability to damage [73]. Proteins involved in maintaining fatty acid homeostasis again proved susceptible, with critical targets such as Acyl CoA Synthetase Long Chain Family Member 1, 3-ketoacyl-CoA thiolase B, microsomal triglyceride transferase protein and Acyl CoA Oxidase 2 all sustaining damage by various LDEs [73]. By showing that reactive LDEs targeted several fundamental processes involved in maintaining lipid homeostasis within the liver, including β-oxidation, lipid synthesis and lipid transport, these observations greatly strengthened the causal association of noxious LDEs with the onset of alcoholic steatosis in rodents.

Study of the adverse effects of foreign chemicals on human health is complicated by toxicodynamic interactions occurring during "real-world" exposures involving simultaneous exposure to multiple toxicants [74]. The abovementioned findings implicating ten or more LDEs in proteome adduction during ALD highlight the need to consider any synergistic interactions that might occur between multiple endogenous toxicants within alcohol-intoxicated livers. Since the ten LDE identified by Petersen and associates all possess an α,β-unsaturated carbonyl system, they likely exert toxicity via a shared tendency to form Michael adducts on cysteine groups in target proteins. During study of toxicodynamic interactions within binary and ternary mixtures of α,β-unsaturated electrophiles, LoPachin *et al.* noted a potential for significant synergistic interactions

between malondialdehyde and acrolein, two LDE that are implicated in ALD but are also known targets for scavenging by hydralazine [75]. The possibility that hydralazine might suppress the onset of steatosis during chronic alcohol intoxication by mitigating the toxicological consequences of interactions between structurally related endogenous LDEs is a question to which we now turn.

4.4. Hydralazine Efficacy in Animal ALD Models

In a recent ground-breaking study by Chen and associates, strong protective efficacy against hepatosteatosis was demonstrated for hydralazine within murine ALD [78]. The researchers employed the NIAAA "chronic + binge" ethanol feeding model which achieves elevations in serum markers of liver injury, inflammation and hepatic steatosis that approximate outcomes seen in heavy drinking humans [77]. Best performed on the C57BL/6N background, this murine model features a 10 day chronic exposure to a Lieber-DeCarli liquid diet containing 5% ethanol followed by a single oral gavage with ethanol (5 g/kg) early on the morning of Day 11, with the animals euthanized 9 h later to allow collection of tissue samples [77]. Consistent with the induction of damage typical of ALD, the ethanol treatment increased serum transaminase levels, elicited micro- and macrovesicular hepatic steatosis and raised levels of TUNEL-positive cells in liver lobules, a reliable marker of hepatic apoptosis [78].

Using an immunochemical method to detect protein damage, a strong increase in levels of acrolein-lysine adducts were noted in mouse livers following "chronic + binge" ethanol exposures [78]. The adducts were diffusely distributed, but slightly more pronounced in periportal cells. Comparable increases in acrolein-lysine adduct levels were noted in a rodent hepatocyte-derived cell line during incubations with the primary metabolite of ethanol, acetaldehyde, suggesting a role for metabolism in the induction of acrolein-mediated protein damage [78]. Within the in vivo

model, formation of acrolein-adducted proteins was closely associated with the induction of ER stress, revealed by elevated levels of the activating transcription factors ATF3 and ATF4 [78]. A strong induction of apoptosis accompanied the ER stress, as shown by the phosphoactivation of JNK1 and JNK2, proteolytic activation of caspase-12 and upregulation of CHOP [78]. Interestingly, upregulation of the UPR-protective response did not accompany ER stress induction in this murine ALD model [78].

Consistent with its ability to limit acrolein availability, daily hydralazine administration (5 mg/kg, i.p.) during the alcohol exposure period strongly suppressed the onset of hepatic injury in the NIAAA ALD mouse model, blocking the formation of acrolein-adducted proteins and diminishing levels of ER stress and hepatic apoptosis biomarkers [78]. Hydralazine also suppressed circulating plasma transaminase levels and abolished histological signs of hepatic steatosis [78].

These interesting findings provide new knowledge concerning the protective effects of hydralazine in ALD, with the experimental approach designed to ensure that evidence was obtained to meet Criteria 3 and 4, although the question of whether hydralazine administration reduced the tissue concentrations of free LDEs was not addressed (Criterion 1), nor was the formation of scavenger-electrophile conjugates confirmed (Criterion 2). Despite these limitations, by providing clear evidence for a strongly histoprotective effect of hydralazine in a robust murine ADH model, the work by Chen and associates makes a key contribution to the pharmacological evaluation of electrophile-scavenging therapeutic agents.

Although carbonyl-trapping likely accounts for the hydralazine efficacy during ALD, since recent work in novel non-mammalian systems has revealed unexpected “lifespan-extending” effects of hydralazine that could conceivably contribute to histoprotection in vivo, we will briefly turn our attention to these new findings.

4.5. Novel Cytoprotective Properties of Hydralazine

While LDEs are known contributors to spontaneous proteome damage during organismal ageing, any investigation of the potential for carbonyl scavengers to afford health benefits by suppressing such damage are complicated by the complexities and cost of performing lifespan extension studies in mammalian species. In recent times, the emergence of experimental approaches involving “simple” model organisms with short lifespans have allowed novel investigations of the effects of carbonyl-scavengers on the functional deficits that accompany ageing [79, 80].

In a study led by Snell and associates, hydralazine was one of three FDA-approved drugs identified as lifespan and healthspan extenders in the rotifer species *B. manjavacas* [81]. Bracionid rotifers are small aquatic herbivores (approx. 400 microns in length) that exhibit a lifespan of about 2 weeks at room temperature (22 °C), an ideal timeframe for experimental interventions. In the recent study, hatched animals were grown on 24-well plates in the presence of various drugs as well as 5-fluoro-2-deoxyuridine to suppress asexual division, with swimming speed, survival and mitochondrial activity monitored as experimental endpoints [81]. Strikingly, although the swimming speed of female rotifers declined during the final week of their lives, the presence of a low concentration of hydralazine (1 µM) attenuated this declension while simultaneously preserving the falling reproductive potential of the animals [81]. Hydralazine also extended the rotifer lifespan by one-third while also preserving overall mitochondrial function in ageing animals. Although possible mechanistic explanations for hydralazine’s anti-ageing effects were not explored in this innovative study, the possibility that they involve scavenging of toxic LDEs seems likely.

In other work, Dehghan and associates used a *C. elegans* nematode bioassay to explore hydralazine's lifespan-extending properties [82]. The presence of hydralazine (100 μM concentration) enhanced worm survival by 25% while also suppressing the ageing-related loss of locomotor activity and the formation of the ageing pigment lipofuscin [82]. Hydralazine exerted comparable effects within a transgenic worm strain that expressed an amyloidogenic fragment of human *tau* protein, a key participant in neurodegenerative diseases [82]. To identify protein contributors to the protective phenotype, the researchers used global proteomic screening based on the incorporation of isotopically-labelled amino acids to identify nematode proteins that were upregulated in the presence of hydralazine. One strongly drug-responsive pathway was controlled by the transcription factor SKN-1, the worm ortholog of the cytoprotective Nrf2 pathway that regulates antioxidant responses in human cells [82]. This unexpected finding confers considerable additional interest upon the cytoprotective properties of hydralazine.

Although Dehghan and associates break new ground by identifying Nrf2 (SKN-1) activation as a novel mechanism of hydralazine action, more work will be needed to explain how this strongly nucleophilic drug could activate the KEAP1-regulated Nrf2 pathway, a gene expression system that is classically understood to be activated by cysteine-reactive soft electrophiles [83]. One possibility is that hydralazine belongs to the growing class of non-electrophilic Nrf2 activators that appear to non-covalently disrupt binding interactions between Nrf2 and its regulatory partner KEAP-1 [84]. Another possibility is that hydralazine targets one or more signalling networks known to participate in crosstalk regulation with Nrf2-responsive pathways, such as the GSK-3-β-TrCP, synoviolin, nuclear factor-kappa B (NF-κB), Notch or AMP kinase pathways [85].

Alternatively, it is tempting to speculate concerning an alternative explanation for the Nrf2-upregulating properties of hydralazine that involves the classic electrophile-mediated pathway.

Since KEAP-1 is vulnerable to adduction by low-mass LDEs, the strong "adduct-trapping" properties of hydralazine would ensure the drug attacks any carbonyl-retaining LDE adducts formed on KEAP1 during oxidative stress. We have previously shown that strong protein adduct-trapping occurs in cells exposed to low micromolar hydralazine concentrations [86]. Due to their molecular bulkiness, hydralazine-trapped LDE adducts on KEAP1 might render the protein more prone to CUL3-mediated degradation and thus accelerate Nrf2 liberation, allowing the transcription factor to drive its characteristic transcriptional responses at the promoters of several hundred cytoprotective genes [83].

Collectively, these new observations concerning the cytoprotective, life- and health-span extending properties of hydralazine in two model invertebrate systems may have relevance to its use in carbonyl-mediated pathological syndromes such as those reviewed earlier, SCI and ALD. For example, these novel actions of hydralazine could conceivably supplement its electrophile-trapping actions and thereby confer neuron-preserving effects in damaged spinal cords or help protect hepatocytes against apoptosis in ALD. These possibilities strengthen the rationale for further rigorous testing of hydralazine in animal models of carbonyl-mediated pathologies as a prelude to future human studies.

5. Future Directions

Taken together, accumulative findings from a variety of diverse animal-based experimental systems point to the potential for effective repurposing of a classic antihypertensive drug hydralazine. The final section of this Commentary explores the question of what the future might hold for this drug. What obstacles might prevent effective "off-label" use of hydralazine in novel therapeutic contexts?

5.1. Challenges in Clinical Translation

While the notion that electrophile-trapping drugs might confer benefits in human diseases by protecting critical cell components against damaging LDEs is attractive in theory, this therapeutic strategy is complicated by several issues that will necessitate thoughtful alignment of drug regimens and diseases during clinical evaluations. The commercial, scientific and clinical travails that have plagued human testing of various molecules that counteract tissue damage by toxic glycation end-products display the perils that can accompany using drugs as reagents to disrupt deleterious chemical reactions within the body [25]. We will not explore all possible problems in this regard, but will highlight two factors that seem especially pertinent to hydralazine.

5.1.1. Oral Bioavailability.

Due to the need to facilitate reactions with LDEs, carbonyl scavengers require one or more nucleophilic heteroatoms that confer reactivity with electron deficient centres in target electrophiles (e.g. carbonyl group, conjugated double bond), but unfortunately, these chemical properties often ensure that nucleophilic xenobiotics are excellent substrates for drug metabolising enzymes such as CYP450 isoforms, UDP-glucuronosyltransferases, sulfotransferases or N-acetyl transferases [87]. If it occurs presystemically within the gut wall or liver, such oxidative and conjugative metabolism can greatly undermine the bioavailability of orally-administered medications, a factor that likely contributes to the low efficacy of polyphenolic antioxidants during human testing [88]. These concerns are especially pertinent to hydralazine which, although it has good permeability across intestinal membranes, exhibits low systemic bioavailability (0.16 to 0.3) due to extensive polymorphic N-acetylation within the liver [89]. In addition to forming *N*-acetyl conjugates, the nucleophilic hydrazine substituent also ensures systemic hydralazine readily

undergoes nonenzymatic trapping reactions with pyruvic acid [33]. Due to these chemical conversions, hydralazine exhibits a short plasma half-life (< 1 hour), a factor that may complicate its use as an LDE-sequestering agent in any condition in which there may be a requirement to maintain plasma concentrations above the carbonyl-trapping threshold (CTT) for a significant duration of the dosing interval (Figure 1A). Nonetheless, as has been shown during recent efforts to improve the oral absorption of curcumin using nanofabrication strategies and other innovations, bioavailability can be improved by the adoption of clever oral formulation strategies [90].

Within the context of SCI treatments, however, improving the low oral availability of hydralazine may not be necessary since a more helpful strategy could involve new formulation methods that allow local drug delivery directly to damaged cords. In prior work, Shi and associates successfully developed a novel hydralazine delivery system involving polyethylene glycol-functionalised mesoporous silica nanoparticles which performed well during in vitro efficacy bioassays in PC12 cells [91]. The ongoing development of hydrogels, electrospun fibres and other technologies that permit localised drug delivery to damaged cords may further assist the development of successful localised delivery methods for hydralazine [92, 93].

For conditions such as ALD where oral formulation of LDE scavengers is needed, a better approach might involve the use of pro-drugs which employ a metabolically-labile lipophilic substituent to mask nucleophilic heteroatoms. Many such hydralazine pro-drugs were prepared in the 1970s, and some, such as budralazine, a mesityl oxide hydrazone of hydralazine, have been approved for human use in Japan and other jurisdictions. Budralazine undergoes metabolic conversion to hydralazine in vivo and also exhibits lower potency as an antihypertensive agent, with less propensity to cause reflex tachycardia in humans [94, 95]. In future testing, budralazine could

be a worthy alternative to hydralazine during clinical evaluations of its efficacy in ALD and other LDE-mediated diseases.

5.1.2. Depletion of Essential Carbonyls

The poor performance of antioxidants in clinical trials is often attributable to side effects resulting from disrupted physiological functions of reactive oxygen species, a conclusion that concurs with observations of biological deficits in knock-out mice lacking specific antioxidant pathways [15]. Since LDEs are unlikely to play comparable signalling roles in human physiology, it is improbable that carbonyl scavengers will be subject to any unexpected toxicity due to LDE depletion per se, yet the possibility of side-effects due to diminished cellular reserves of essential "off target" carbonyl compounds is a known risk accompanying chronic use of nucleophilic drugs. Various CNS pathologies including peripheral neuropathy and paraesthesia are classic toxic outcomes in patients undergoing chronic treatment with arylhydrazino drugs such as isoniazid and phenelzine [96, 97]. The neurotoxicity can have a complex aetiology and likely varies according to the precipitating drug, although interference with pyridoxine-dependent coenzyme functions secondary to depletion of the target aldehyde pyridoxal is a presumed common mechanism. Using a simple cell-free system comprising phosphate-buffered saline (10 mM, pH 7.4), Aldini and associates compared pyridoxal-trapping by hydralazine to that of several classic LDE-scavenging compounds [98]. Although less pronounced than the reactivity shown by Edaravone, an FDA-approved drug used in the treatment of ALS, hydralazine showed significant pyridoxal-trapping activity, consistent with longstanding reports of neuropathy in recipients of this drug [99].

An analogy to the problems accompanying long-term antioxidant ingestion seems obvious, but it is notable that to avoid the latter, researchers have found that better outcomes can be

obtained if antioxidant usage is restricted to the acute intravenous administration of short-term infusions. Efficacy for acute parenteral antioxidant therapy has been shown in such diverse conditions as allergy syndromes, idiopathic hearing loss, pancreatitis, viral neuralgia and advanced cancer [11]. The rationale offered to explain these positive findings is that parenteral antioxidants target massive inflammation-associated ROS production, yet the brief treatment period minimises any side-effects due to disruption of the essential physiological effects of ROS [11]. This line of thinking illuminates possible short-term uses of hydralazine and other carbonyl scavengers in conditions in which LDE formation likely occurs over relatively-short time frames, avoiding the risk of peripheral neuropathy due to pyridoxal depletion that accompanies chronic drug treatment. Clearly, future clinical investigations should assess not just disease biomarkers and clinical efficacy outcomes in recipients of carbonyl scavengers, but should also employ metabolomic or diagnostic techniques to monitor patient biofluids for signs of depletion of essential carbonyl compounds so as to identify patients at risk of untoward drug responses [100].

5.2. Concluding Thoughts

This Commentary has argued that the impressive efficacy shown for hydralazine in two seemingly disparate disease states, SCI and ALD, is a likely consequence of its broad electrophile-scavenging activity, as highlighted in the abovementioned work from the Aldini group which showed that hydralazine trapped a wider range of biogenic electrophiles than other classic carbonyl scavengers [38]. This broad spectrum scavenging activity likely explains why hydralazine was especially efficacious against steatosis in murine ALD, since recent work from the Petersen group showed that the murine hepatic proteome incurs damage by at least 10 toxic LDEs during ALD [73]. Although biotin hydrazide/streptavidin-based methods that provide comprehensive detection of

LDE-modified protein targets have yet to be applied to the study of SCI, existing knowledge of the role of acrolein and 4-HNE in animal models of this condition suggest that numerous LDEs are likely formed in PUFA-rich damaged spinal cords. If so, as is likely the case with ALD, the nonspecific reactivity displayed by hydralazine toward multiple biogenic electrophiles might underlie its clear efficacy in this complex condition. Notwithstanding the limitations accompanying the use of hydralazine and other scavengers, since nonspecific production of noxious electrophiles appears to contribute to the pathogenesis of a wide range of diseases including cancer, chronic inflammatory disease, arthrosclerosis, neurodegeneration, and xenobiotic toxicity, the potential for carbonyl-trapping agents to provide clinical benefits in diverse health conditions seems worthy of direct attention from clinical investigators.

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Figure Legends

Figure 1. An ideal carbonyl scavenger will display reactivity towards noxious lipid-derived electrophiles (LDEs) at plasma concentrations (Panel A) and administered doses (Panel B) that are distinct from those eliciting receptor-mediated pharmacological responses or toxicity.

Figure 2. Selectivity of electrophile-mediated damage to the proteome and implications for the use of carbonyl-scavenging drugs. Experimental studies of the role of electrophile-mediated protein adduction in diverse pathological syndromes have consistently shown that most proteins in cells are not damaged by these reactive species (i.e. blue dots represent unadducted proteins). Instead, noxious electrophiles (e.g. LPO-derived species of endogenous origin) preferentially damage a subset of vulnerable cell proteins known as the adductome (yellow dots = cell proteins containing adducts). Such damage is often survivable, and lethality only occurs when adduction targets a subset of critical proteins, including many of mitochondrial origin (i.e. red dots = adducted critical proteins). In theory, a cytoprotective carbonyl scavenger might protect the latter category of protein targets without necessarily suppressing adduction of abundant "cytosolic electrophile scrubber" targets (i.e. yellow targets).

Figure 3. The strong nucleophilicity of hydralazine ensures it reacts readily with free acrolein to form (1E)-acrylaldehyde phthalazin-1-ylhydrazone (E-APH) while also displaying ready reactivity towards carbonyl-retaining adducts formed by acrolein during reactions with cell proteins. The latter "protein-trapping" reactivity is conspicuous at low drug concentrations [86].