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Scavenging Reactive Lipids to Prevent Oxidative Injury

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Abstract

Oxidative injury due to elevated levels of reactive oxygen species is implicated in cardiovascular diseases, Alzheimer's disease, lung and liver diseases, and many cancers. Antioxidant therapies have generally been ineffective at treating these diseases, potentially due to ineffective doses but also due to interference with critical host defense and signaling processes. Therefore, alternative strategies to prevent oxidative injury are needed. Elevated levels of reactive oxygen species induce lipid peroxidation, generating reactive lipid dicarbonyls. These lipid oxidation products may be the most salient mediators of oxidative injury, as they cause cellular and organ dysfunction by adducting to proteins, lipids, and DNA. Small-molecule compounds have been developed in the past decade to selectively and effectively scavenge these reactive lipid dicarbonyls. This review outlines evidence supporting the role of lipid dicarbonyls in disease pathogenesis, as well as preclinical data supporting the efficacy of novel dicarbonyl scavengers in treating or preventing disease.

1. INTRODUCTION

ROS: reactive oxygen species

IsoLG: isolevuglandin

2-HOBA: 2-hydroxybenzylamine

PUFA: polyunsaturated fatty acid

ONE: 4-oxo-nonenal

MDA: malondialdehyde

HNE: 4-hydroxy-nonenal

Oxidative stress, or an overabundance of reactive oxygen species (ROS), including $O_2^{\cdot -}$ and hydroxyl radicals, is a pathophysiologic process critical to many diseases. ROS play key roles in normal signaling pathways, and their production by immune cells during inflammation is an important component of host defense. However, an overabundance of ROS produces radical-catalyzed lipid peroxidation, which ultimately leads to modification of proteins, DNA, and other macromolecules, causing cellular injury. Unfortunately, treatments with dietary antioxidants such as vitamins C and E have been ineffective in clinical trials for numerous diseases (1–5). This failure results in part from the inability of these treatments to actually reduce oxidative injury in humans (based on biomarkers) and also because these treatments interfere with normal ROS signaling. Other antioxidants, such as *N*-acetyl cysteine (6), epigallocatechin gallate (7), and curcumin (8), have shown some promise in vitro and in vivo, yet whether their benefits are attributed to direct antioxidant activity or to another mechanism is unclear. For example, although *N*-acetyl cysteine is a scavenger of ROS, its pharmacologic actions include increasing glutathione levels and being a mucolytic to possessing anti-inflammatory properties (6).

Although lipid peroxidation generates multiple reactive products that might contribute to disease processes, perhaps the most important compose a class of dicarbonyl compounds termed isolevuglandins (IsoLGs) that react rapidly with downstream biological targets. Small-molecule scavengers of these highly reactive lipid mediators have been developed, and their use in preclinical models has implicated IsoLGs in the pathophysiology of multiple diseases linked to oxidative injury. In this review, we focus on conditions for which evidence of a beneficial effect of dicarbonyl scavengers is strongest. For the most extensively studied agent, 2-hydroxybenzylamine (2-HOBA), clinical trials began in the summer of 2020.

2. REACTIVE LIPID OXIDATION PRODUCTS AND THEIR CHEMISTRY

One of the sites most susceptible to ROS damage is polyunsaturated fatty acids (PUFAs) in cell membranes and the circulation. The peroxidation of PUFAs, including arachidonic acid and linoleic acid, generates lipid peroxides that nonenzymatically degrade to many reactive secondary products, some of which have reactive groups (aldehydes and ketones). Lipid dicarbonyls are secondary products that have two closely spaced reactive groups, making them extremely reactive with proteins and other macromolecules and therefore highly damaging. Important lipid dicarbonyls include IsoLGs (also called isoketals), 4-oxo-nonenal (ONE), and malondialdehyde (MDA) (**Figure 1**). Another group of reactive lipids are α,β-unsaturated aldehydes such as acrolein and 4-hydroxy-nonenal (HNE). In this section, we briefly describe the chemistries and biological targets of these two classes of reactive lipids. For a more detailed review of their formation, chemistries, and mechanisms of action, the readers are referred to a review by Davies et al. (9).

2.1. Lipid Dicarbonyls

IsoLGs are a family of 4-ketoaldehyde regioisomers formed from the peroxidation of arachidonic acid. They react with their biological targets within seconds and are considered the most reactive lipid peroxidation product identified to date. IsoLGs react exclusively with primary amines (lysyl residues of proteins, phosphatidylethanolamine, and nucleic acids) to form stable amine adducts, such as pyrrole, lactam, and hydroxylactam monoadducts or pyrrole-pyrrole cross-links (**Figure 1**) (10–12). Although MDA (a 1,3-dialdehyde) is less reactive than IsoLG, it also preferentially reacts with primary amines, including lysines, to form *N*-propenal (13) and dihydropyridine (14) monoadducts and also a dilysyl-cross-linked adduct (14). Unlike IsoLGs, which react only with primary amines, MDA also reacts to some extent with thiols (14), arginines (14), and histidines

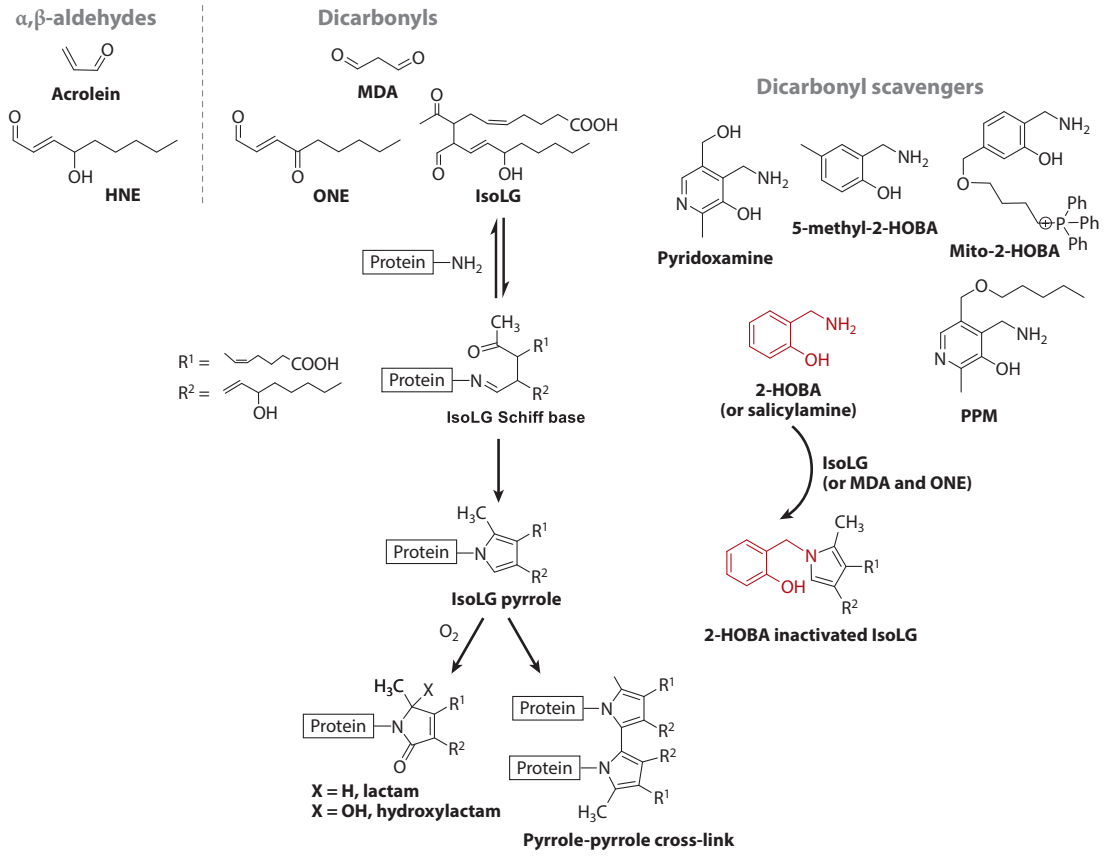

Figure 1

Lipid peroxidation forms highly reactive dicarbonyls that can be selectively scavenged by small-molecule compounds containing 2-AMP moieties. Lipid peroxidation generates many products including α,β-unsaturated lipid aldehydes and lipid dicarbonyls. IsoLG is the most reactive of the lipid dicarbonyls, reacting exclusively with primary amines, such as lysyl residues of proteins (shown), but also with phosphatidylethanolamine or nucleic acids. Initially, a reversible Schiff base adduct forms, but further reaction with the ketone of IsoLG forms irreversible pyrrole adducts. In the presence of molecular oxygen, the IsoLG pyrrole oxidizes to form lactam and hydroxylactam monoadducts, as well as pyrrole-pyrrole cross-links. Small-molecule compounds with a 2-AMP moiety such as 2-HOBA (shown in *red*) react selectively and efficiently with dicarbonyls like IsoLG, which inactivates their ability to adduct to biological targets. Abbreviations: 2-AMP, 2-aminomethylphenol; 2-HOBA, 2-hydroxybenzylamine; HNE, 4-hydroxy-nonenal; IsoLG, isolevuglandin; mito-2-HOBA, mitochondria-targeted 2-hydroxybenzylamine; MDA, malondialdehyde; ONE, 4-oxo-nonenal; PPM, 5′-*O*-pentyl-pyridoxamine.

(15, 16). Both IsoLG and MDA amine adducts are significantly elevated in several diseases (9). Formation of these amine adducts has detrimental effects; for example, they alter the activity of enzymes or create ligands for pattern recognition receptors and thereby directly participate in disease pathogenesis.

2.2. Lipid α,β-Unsaturated Aldehydes

Unlike lipid dicarbonyls, which preferentially react with hard nucleophiles (e.g., primary amines), α,β-unsaturated aldehydes like HNE and acrolein preferentially react with soft nucleophiles such as thiols (e.g., cysteine and glutathione) and imidazoles (e.g., histidine) by Michael addition to form adducts (17). Because ONE is both a lipid dicarbonyl and an α,β-unsaturated aldehyde, it shows

characteristics of both classes. For instance, ONE can react by Michael addition with thiols, but this 4-ketoaldehyde also rapidly reacts with primary amines to form pyrroles (18), cross-links (18), or the stable ketoamide adduct (19).

Because lipid peroxidation typically generates a variety of reactive lipids and many nonreactive lipid products, simply finding elevated levels of a particular lipid adduct in a diseased tissue does not definitively demonstrate a specific contribution of that reactive lipid class to the disease state. Small-molecule scavengers that selectively intercept lipid dicarbonyls (but not α,β-unsaturated aldehydes like HNE and acrolein) before they can react with cellular amines have been developed in order to elucidate the contribution of IsoLGs and other lipid dicarbonyls to disease and for use as therapeutics.

2-AMP: 2-aminomethylphenol

PPM: 5′-*O*-pentyl-pyridoxamine

Mito-2-HOBA: mitochondria-targeted 2-hydroxybenzylamine

3. DICARBONYL SCAVENGERS

We identified a group of small molecules that react rapidly with IsoLGs and thereby preemptively scavenge these and other lipid dicarbonyl mediators to prevent downstream modification of biological targets (**Figure 1**). Our initial screen tested several commercially available compounds with primary amine moieties and with known in vivo bioavailability for their ability to block the reaction of synthetic IsoLG with radiolabeled lysine (20). This screen identified pyridoxamine as an effective IsoLG scavenger. Subsequent structure-activity relationship studies identified that the 2-aminomethylphenol (2-AMP) moiety of pyridoxamine is essential (20) and that various 2-AMP analogs reacted ∼2,000 times faster with IsoLG than does lysine. This reactivity of 2-AMP analogs compared with that of lysine appears to be driven by the phenolic hydrogen's role in stabilizing the imine adduct formed by the initial reaction of the aldehyde with the amine in a manner that then enhances its reaction with the ketone (21). 2-AMPs react only very slowly with lipid α,β-unsaturated aldehydes that lack a second carbonyl such as HNE (22), so that 2-AMPs preferentially scavenge dicarbonyls such as IsoLGs, MDA, and ONE.

Assays in cultured cells and platelets revealed that more hydrophobic analogs of pyridoxamine, including 2-HOBA (also known as salicylamine) and 5′-*O*-pentyl-pyridoxamine (PPM), are far more effective dicarbonyl scavengers than pyridoxamine, presumably because of their greater hydrophobicity (23), which allows for entry into cellular membranes where IsoLGs and other lipid dicarbonyls are formed (24). Importantly, despite being phenolic compounds, 2-HOBA, PPM, and their other alkyl analogs do not have significant antioxidant capacity or the ability to inhibit cyclooxygenase activity (25). Because mitochondria may be prominent sites of lipid peroxidation and lipid dicarbonyl formation, a 2-AMP analog with a triphenylphosphine moiety, which targets the compound to mitochondria, has been generated (mito-2-HOBA) (26). Several 2-AMPs, including 2-HOBA and PPM, have reasonable absorption, distribution, metabolism, excretion, and toxicity properties (27–30) and can be administered in drinking water (30, 31), which has allowed for their use in animal models (**Figure 2**). 2-HOBA is a natural product with an excellent preclinical and clinical safety profile. In 2019, it was approved as a dietary additive, and phase II clinical trials for 2-HOBA have begun. 2-HOBA and related lipid dicarbonyl scavengers represent a paradigm shift in pharmacologic strategy to prevent injurious cellular modification by oxidative stress.

4. EFFICACY IN DISEASE STATES: CELLS, ANIMAL MODELS, AND HUMANS

4.1. Cardiac Arrhythmias

Cardiac arrhythmias are a major source of morbidity and mortality in the United States and throughout the Western world. Both ventricular and atrial arrhythmias have been linked to

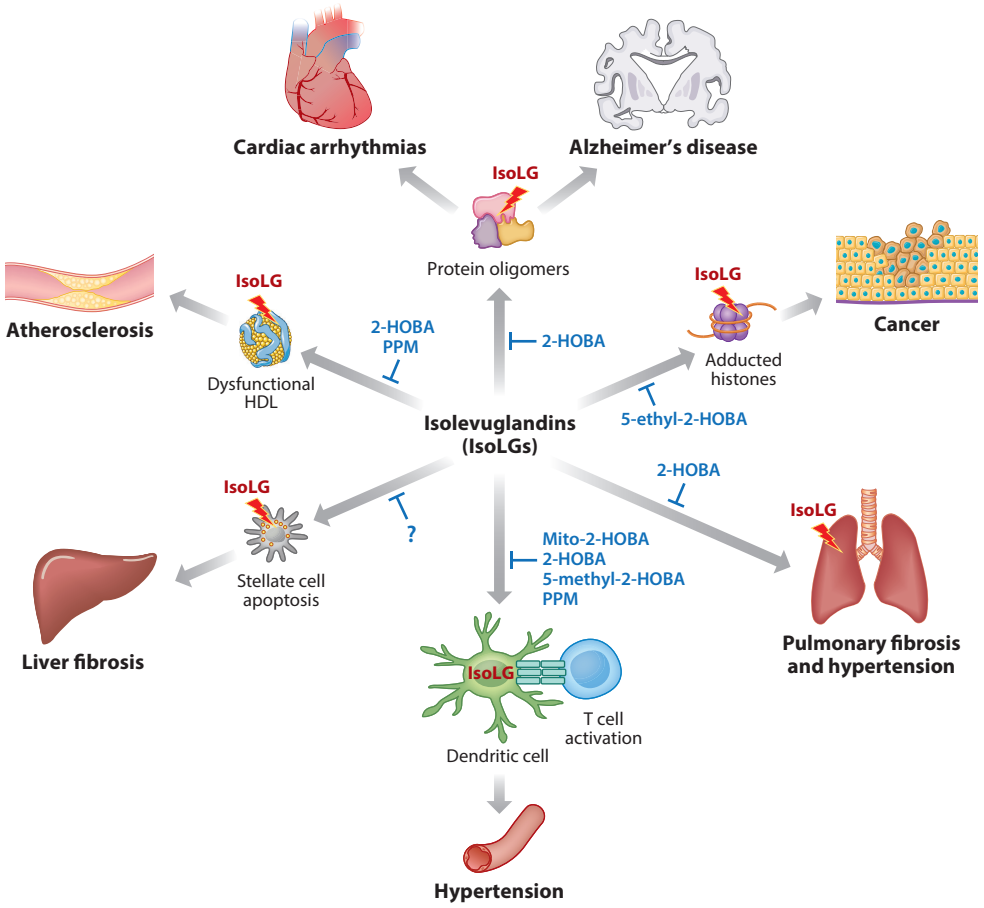

Figure 2

IsoLGs can participate in the pathophysiology of multiple diseases through their adduction (shown in *red*) to proteins, lipids, and DNA. Preclinical studies indicate the potential of dicarbonyl scavengers (shown in *blue*) to prevent IsoLG adduction and disease progression. Some figure elements adapted from Servier Medical Art (CC BY 3.0). Abbreviations: 2-HOBA, 2-hydroxybenzylamine; HDL, high-density lipoprotein; IsoLG, isolevuglandin; mito-2-HOBA, mitochondria-targeted 2-hydroxybenzylamine; PPM, 5′-*O*-pentyl-pyridoxamine.

oxidative stress, although the precise mechanisms have been unclear. Increasing evidence indicates a role for IsoLGs in the pathogenesis of these arrhythmias.

4.1.1. Ion channel dysfunction and ventricular arrhythmias. Inward Na^+ current through $Na_v1.5$ channels initiates the cardiac action potential and is critical for normal conduction in the heart. Both acquired and genetic conditions that cause dysfunction and/or reduced expression of cardiac Na^+ channels are linked to life-threatening ventricular arrhythmias. Ventricular tachycardia (VT) and ventricular fibrillation (VF) that cause sudden cardiac death are most commonly precipitated by myocardial ischemia, which depletes antioxidant cellular defenses to cause oxidative stress. Patients with underlying structural heart disease are also predisposed to such arrhythmias, as depressed Na^+ current and slowed conduction at the border between scar and normal myocardium promote abnormal reentrant circuits causing tachyarrhythmias. In the

AF: atrial fibrillation

PAO: preamyloid oligomer

ANP: atrial natriuretic peptide

Cardiac Arrhythmia Suppression Trial, treatment with potent class Ic Na^+ channel blockers increased mortality in patients following a recent myocardial infarction (32). Moreover, mortality was greatest in patients at highest risk for recurrent ischemia (i.e., after a non-Q wave myocardial infarction) (33). In Brugada syndrome, loss-of-function mutations in *SCN5A*, which encodes $Na_v1.5$, are associated with VT/VF despite a structurally normal heart.

In a canine model of recent myocardial infarction, IsoLG adducts accumulated in the ischemic epicardial border zone of the infarct, where conduction was slowed (34). The potential mechanisms linking ischemia and IsoLG to Na^+ channel dysfunction were investigated with heterologously expressed human $Na_v1.5$ channels and a cultured cardiac cell line, atrial HL-1 cells (34). Treatment with either the oxidant *tert*-butyl-hydroperoxide or IsoLG caused a leftward shift in Na^+ channel availability to promote inactivation, with a reduced Na^+ current amplitude in cardiomyocytes. There was also a synergistic effect of oxidants with the class Ic drug flecainide to slow recovery from inactivation (34). Click chemistry was employed to demonstrate that *tert*-butyl-hydroperoxide treatment leads to lipoxidative modification of the Na^+ channel (35). For both $Na_v1.5$ and cardiomyocyte Na^+ currents, the dicarbonyl scavengers 2-HOBA and PPM prevented oxidant-mediated shifts in channel availability and blunted suppression of Na^+ current (35). Taken together, these data provide strong evidence that IsoLGs play a causative role in oxidant-mediated Na^+ channel dysfunction that promotes VT/VF, and that inhibition of this pathway could be protective.

Genetic or pharmacologic suppression of rapid cardiac delayed rectifier K^+ current, or I_{Kr}, excessively prolongs the ventricular action potential and QT interval on the electrocardiogram (ECG), leading to the polymorphic VT torsades de pointes that causes syncope or sudden cardiac death. Although IsoLG exposure caused concentration-dependent inhibition of I_{Kr} in a cardiomyocyte cell line (24), the clinical relevance of this effect remains unclear.

4.1.2. Atrial fibrillation. As the most common sustained cardiac arrhythmia in the Western world, atrial fibrillation (AF) often results in devastating clinical outcomes, including stroke and death. Abundant evidence links oxidative stress and ROS directly to the pathogenesis and progression of AF. However, upstream therapy targeting ROS levels directly has been ineffective in clinical trials (36), highlighting the limited understanding of appropriate molecular targets.

Although the biological targets for IsoLGs have not been fully identified, it is clear that IsoLGs markedly accelerate misfolding/aggregation of amyloid-forming proteins, including amyloid-β and natriuretic peptides (37, 38). Aggregation generates preamyloid oligomers (PAOs), which are now recognized to be the primary cytotoxic species in amyloid (39). Using a novel, imaging-based method for quantitation, Sidorova et al. (40) detected PAOs containing atrial natriuretic peptide (ANP) in the atria of most patients without AF undergoing elective cardiac surgery and determined that oligomer burden was independently associated with hypertension. In a cellular model simulating AF, rapid stimulation of atrial HL-1 cells generated both IsoLG adducts and PAOs (41), composed at least in part of ANP. Direct exposure of atrial cells to IsoLGs also caused PAO production. In the presence of 2-HOBA, pacing-induced PAO formation was virtually eliminated and the myocyte stress response was blunted, indicating a cytoprotective effect. Unlike 2-HOBA, curcumin was ineffective.

In a murine model of hypertension (chronic angiotensin II infusion), IsoLG adducts and PAOs developed in the atria of hypertensive mice prior to significant structural or histologic abnormalities (42). By mass spectrometry, IsoLG adducts were greater in the left atrium, from which AF typically originates, than in the right atrium, with an approximately 16-fold increase similar to that seen in brains affected by Alzheimer's disease (AD) (43). These effects were prevented by

2-HOBA but not by 4-hydroxybenzylamine (4-HOBA), a 2-HOBA analog previously shown to be an ineffective scavenger in vitro. Hypertensive mice demonstrated inducible AF, and this was suppressed by 2-HOBA but not by 4-HOBA. Normalizing blood pressure also prevented AF, and mechanically stretched atrial cells generated cytosolic IsoLG adducts and PAOs that were prevented by 2-HOBA. Both ANP and BNP (B-type natriuretic peptide) generated cytotoxic oligomers, which contributed to the formation of PAOs in atria.

DC: dendritic cell

Obesity is also linked to inflammation and oxidative stress. A mouse model of diet-induced obesity provided similar preliminary results: Obese mice demonstrated inducible AF and elevated atrial IsoLG adducts that were suppressed by 2-HOBA but not by 4-HOBA (44). Collectively, these findings support the concept of scavenging IsoLGs, rather than IsoLGs targeting the generation of ROS per se, as a novel therapeutic approach to prevent AF.

4.2. Hypertension

Hypertension affects nearly half of the American population and is the leading cause of mortality due to myocardial infarction, stroke, heart failure, and chronic kidney disease (45). Multiple studies have demonstrated a causative role of immune cell activation in the pathogenesis of hypertension (46–52). Dendritic cells (DCs) play a pivotal role in initiating the adaptive immune responses that contribute to hypertension (53). In mouse models, hypertension was associated with increased formation of ROS by DCs, leading to the generation of immunogenic IsoLGs, which activated immune cells (31). In response to angiotensin II, the increase in $O_2^{\cdot -}$ production by DCs was almost entirely dependent on NADPH oxidase (31). IsoLG-mediated protein modification activated DCs to produce Th17-polarized T cell cytokines interleukin (IL)-6, IL-1β, and IL-23. When these activated DCs were cocultured with primed T cells, they drove T cell proliferation and production of cytokines IL-17A, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), which contributed to hypertension. Adoptive transfer of activated DCs predisposed naïve recipient mice to develop hypertension in response to a subpressor dose of angiotensin II. In addition, IsoLGs in renal DCs mediated activation of memory T cells to produce cytokines, including IL-17A and IFN-γ, which contribute to exacerbation of hypertension in response to repeated hypertensive stimuli (54). These studies suggest that IsoLGs play a role in activating the immune system, leading to hypertension.

Upon exposure to oxidative stress, IsoLG-protein adducts can be formed not only intracellularly within DCs but also in the kidneys and vasculature (31, 55). These extracellular IsoLG-adducted peptides can be phagocytosed by DCs and presented to T cells, possibly through cross-presentation. Exposure of DCs to IsoLG-modified renal homogenates enhanced their ability to drive T cell activation and proliferation (31). In addition, DCs pulsed with aortic homogenates from transgenic mice with vascular specific overexpression of the NADPH oxidase subunit $p22^{phox}$ exhibited increased production of cytokines, including IL-1β, IL-6, transforming growth factor-β1 (TGF-β1), and granulocyte-macrophage colony-stimulating factor (GM-CSF), and activated T cells to proliferate and produce cytokines, including IL-17A, IL-17F, TNF-α, and IFN-γ (55). These studies indicate that increased oxidative stress leads to the formation of IsoLG-protein adducts that are presented to T cells by DCs, leading to hypertension. The mechanisms by which both endogenous and exogenous IsoLG-modified peptides are processed and presented to T cells in hypertension are still not known.

Compelling evidence suggests that scavenging IsoLGs can ameliorate inflammation and hypertension. Coadministration of 2-HOBA during angiotensin II–induced hypertension prevented the accumulation of IsoLGs in the heart, vasculature, and kidneys, which attenuated

inflammation, renal damage, and hypertension. Other scavengers, such as 5-methyl-2-HOBA and PPM, also attenuated angiotensin II–induced hypertension, whereas control compounds *N*-methyl-2-HOBA and 4-HOBA did not (31).

LDL: low-density lipoprotein

HDL: high-density lipoprotein

In addition to NADPH oxidase–derived ROS, mitochondria-derived ROS lead to the formation of IsoLG, which induces mitochondrial dysfunction by opening the mitochondrial permeability transition pore regulatory subunit cyclophilin D (56). Depletion of cyclophilin D attenuated hypertension and the associated endothelial dysfunction by preventing mitochondrial ROS production; targeting mitochondrial IsoLG by mito-2-HOBA also attenuated hypertension (26, 57). Specifically, mito-2-HOBA reduced angiotensin II–induced accumulation of IsoLG adducts in heart mitochondria, reduced vascular oxidative stress, and preserved endothelial nitric oxide. Collectively, these observations suggest that IsoLGs mediate hypertension and provide a potential therapeutic target for this disease.

4.3. Atherosclerosis

The accumulation of low-density lipoprotein (LDL) in the intima of the artery wall plays an essential role in the initiation and pathogenesis of atherosclerosis, which causes myocardial infarction, stroke, and peripheral vascular disease (58). Given that cardiovascular morbidity and mortality still occur in a significant percentage of subjects receiving LDL-lowering therapy (59), new strategies and therapies are needed to further ameliorate this residual risk.

High-density lipoprotein (HDL) cholesterol levels are inversely associated with risk of atherosclerotic cardiovascular events. An important function of HDL is to mediate reverse cholesterol transport, by which cholesterol is transported from peripheral tissues to the liver for clearance. Mounting evidence shows that HDL can become dysfunctional under certain pathological conditions. The failure of HDL-increasing drugs to reduce cardiovascular risks in clinical trials (60, 61) suggests that HDL function may be a better therapeutic target than levels of HDL cholesterol (62–64).

In addition to its antiatherogenic functions, HDL serves as a sink for plasma lipid hydroperoxides (65) and secondary products, including F_2-isoprostanes (66), that are also generated from peroxidation of arachidonic acid in parallel with IsoLGs (67). Levels of F_2-isoprostanes are higher in HDL than in LDL of human plasma (66, 68), raising the possibility that IsoLGs may also target HDL. In subjects with familial hypercholesterolemia, an autosomal dominant disorder characterized by high levels of LDL-C in serum and premature atherosclerosis, HDL had higher levels of IsoLGs (69) and other reactive dicarbonyls, including ONE (70) and MDA (16), compared with HDL from healthy control subjects. Dicarbonyl modification of HDL cross-linked its main scaffold protein apolipoprotein A-I (apoA-I) and impaired its cholesterol efflux capacity (69) and HDL–apoA-I exchange ability (69, 70). IsoLGs also reacted with lipids in HDL, specifically phosphatidylethanolamines (71), and the consequences of this lipid modification remain to be investigated.

In vitro data demonstrated that the potent dicarbonyl scavengers 2-HOBA and PPM protected HDL from oxidative modification and improved HDL function. Treatment of *Ldlr*-deficient mice fed a Western diet with 2-HOBA dramatically reduced the extent of atherosclerosis in the absence of changes in plasma lipid levels (72). In addition, treatment of *Apoe*-deficient mice on a Western diet for 16 weeks with PPM significantly reduced the extent of atherosclerotic lesions and enhanced features of plaque stability (73). Therefore, these results support the therapeutic potential of reactive dicarbonyl scavenging in the treatment of atherosclerotic cardiovascular disease.

4.4. Alzheimer's Disease

AD: Alzheimer's disease

The brain, an organ that contains high levels of PUFAs, consumes approximately 20–30% of inhaled oxygen. This makes the brain particularly sensitive to oxidative stress and free radical attack (74). Oxidative damage has long been implicated in the pathogenesis of neurodegenerative disorders (75). The most common neurodegenerative disease in the elderly is Alzheimer's disease (AD), which is characterized by neuronal degeneration in select brain regions involved in cognition and emotion. Markers of lipid peroxidation are increased in the AD brain along with the deposition of extracellular senile amyloid plaques, intracellular neurofibrillary tangles, and the loss of synapses (75). Normally, the proteasome complex maintains protein quality control and contains essential proteolytic enzymes for the processing of amyloid-β and tau proteins, but these functions are inhibited in AD (76). Evidence that formation of IsoLG adducts contributes to these pathological processes began with early in vitro studies showing that IsoLG modification of amyloid-β peptides cross-linked these peptides (77) and increased their rate of oligomerization (37) and that these IsoLG-modified peptides inhibited the proteolytic activity of the 20S proteasome (77). The modified amyloid-β peptides formed PAOs that were neurotoxic in studies using primary murine neuron cultures (78).

An analysis comparing human postmortem brains from AD patients and from age-matched control subjects showed that IsoLG-lysine adducts were dramatically elevated (~12-fold) in the hippocampus and that the level of adducts correlated with disease severity (43). A follow-up study confirmed that IsoLG-protein adducts were increased in the hippocampus but not in the cerebellum of postmortem brains from AD patients (79). Immunostaining of the brain sections with a commonly employed D11 single-chain fragment variable (ScFv) anti-IsoLG single-chain antibody (79) revealed that these adducts were localized to pyramidal neurons in the hippocampus (79), which potentially could disrupt hippocampus-dependent memory formation.

Studies of animal models of AD also showed elevation of IsoLG-protein adducts in the brain. A common animal model of AD is the double transgenic mouse expressing a chimeric mouse/human amyloid precursor protein and mutant human presenilin 1 (APP-PS1). One study generated an APP-PS1 mouse line with a conditional deletion of microglial prostaglandin E_2 receptor-4 (APP-PS1;EP4-cKO). Microglial EP4 receptors exerted potentially anti-inflammatory effects (80) and may serve to protect against AD. In contrast, EP1, EP2, and EP3 exerted proinflammatory and/or proamyloidogenic effects in AD mouse models (81–83). APP-PS1;EP4-cKO mice had increased IsoLG-lysine adducts in the posterior cortex at 5 months of age compared with APP-PS1 or nontransgenic mice, which correlated with enhanced inflammation and amyloid deposition (84). This finding suggested that EP4 signaling may have a beneficial effect of suppressing IsoLG formation in the APP-PS1 model.

Currently, therapeutic options for AD only temporarily improve symptoms of memory loss and help regain behavioral control but do not inhibit the progression of disease. Animal studies using IsoLG scavengers such as 2-HOBA show some promise in preventing memory deficits in AD. A study administered 2-HOBA via drinking water to transgenic mice expressing human *ApoE4*, another mouse model of AD, beginning at 4 months of age and continuing through the life of the animal. Although 2-HOBA did not affect the growth, physical activity, and survival of the animals, it protected mice from spatial working memory loss (79). Compared with untreated mice, mice that received 2-HOBA had significantly fewer errors and shorter latency times in a water radial arm maze task, which stringently tests spatial working memory. This study did not define specific mechanisms by which 2-HOBA prevented the loss of working memory, but scavenging IsoLGs might prevent the induction of neuronal dysfunction and inhibition of the proteasome by IsoLG-adducted amyloid-β proteins. 2-HOBA has not been tested in human clinical trials for AD, but

because of its oral bioavailability and safety in healthy subjects, it holds great therapeutic potential for AD and other neurodegenerative diseases.

4.5. Pulmonary Disease

With growing industrialization, the worldwide burden of respiratory diseases is rising rapidly and is a major cause of death following cardiovascular diseases. The lung is the initial organ in the human body that encounters oxygen, and the adult lung inhales an average of 10,000 to 15,000 liters of air every day. To defend against chronic oxidative insult, the lungs are endowed with powerful extracellular antioxidant defense systems. However, prolonged exposure to hyperoxia (e.g., during mechanical ventilation) or environmental toxins, such as cigarette smoke or industrial emissions, induces oxidative stress and triggers respiratory diseases, including chronic obstructive pulmonary disease, asthma, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis.

4.5.1. Hyperoxia. Hyperoxia has been shown to cause time-dependent lipid oxidation and damage in the lungs within hours. In mice breathing room air (21% oxygen), few airway cells showed D11 ScFv anti-IsoLG immunostaining, whereas exposure of animals to ~100% oxygen for 7 h substantially increased immunostaining, specifically in large and small airway epithelial cells as well as in alveoli.

4.5.2. Redox homeostasis and pulmonary fibrosis. In healthy mice under normal conditions, IsoLG adducts were detected at low levels in different cell types in the lung, suggesting that they are a by-product of normal lung metabolism (85). Low levels were also detected in human lung tissue from normal donors. In mice deficient in NADPH oxidase, IsoLG adducts were substantially reduced, indicating that this enzyme is a major source for IsoLGs (85). The transcription factor Nrf-2 drives a major pathway for ROS suppression, and Nrf-2 deficiency in mice increased IsoLG adducts. Direct exposure of human endothelial cells to IsoLGs was cytotoxic and promoted apoptosis. To identify the protein targets of IsoLG in the lung, researchers performed immunoprecipitation experiments using D11 ScFv in cultured lung endothelial cells. Histones were a prominent target of IsoLGs, with more than 160 proteins captured by D11 ScFv immunoprecipitation.

Oxidative stress is a prominent component of radiation-induced lung injury. For cultured human endothelial cells, exposure to 5 Gy of cesium-137 gamma rays increased IsoLG adducts over fourfold (85). Similarly, for normal mice irradiated with 16 Gy, there was a severalfold increase in IsoLG adducts in the lung at 6 weeks, and the onset and persistence of pulmonary fibrosis correlated with accumulation of IsoLG-modified protein. In human tissue from patients with idiopathic pulmonary fibrosis undergoing lung transplantation, there was a marked increase in D11 ScFv staining compared with tissues from organ donor controls. Specifically, collagen 1α1 was modified and was resistant to normal degradation, consistent with other reports showing that IsoLG modification of a protein can impede its proteolysis. Collectively, these results strongly suggest that IsoLGs contribute to radiation-induced lung injury and pulmonary fibrosis.

4.5.3. Pulmonary hypertension. Pulmonary artery hypertension is a disease characterized by a progressive increase in pulmonary vascular resistance that ultimately leads to right heart failure and death. The inherited form, idiopathic pulmonary hypertension (IPH), is most commonly caused by mutations in the gene that encodes the type 2 receptor of bone morphogenic protein (BMPR2). In vascular cells, transgenic mice, and humans expressing BMPR2 mutations, oxidative

stress was increased as a common consequence (86). Accumulating evidence suggests that these mutations cause systemic alterations in multiple metabolic pathways, including increased glucose utilization and altered fatty acid metabolism, changes similar to those in cancer cells. Using human and murine cell culture models, transgenic mice, and samples from patients with IPH, Egnatchik et al. (87) showed that dysfunctional BMPR2 signaling altered the metabolism of glutamine, which became the preferred substrate for energy production. The mechanism of this effect included oxidative injury and IsoLG adduct formation in the mitochondria, leading to inactivation of sirtuin-3 (SIRT-3), a lysine deacetylase critical for mitochondrial energy production and redox balance. SIRT-3 inactivation promoted activation of hypoxia-inducible factor 1α, leading to altered glutamine metabolism. In mice expressing mutant BMPR2, treatment with 2-HOBA lowered pulmonary vascular resistance and prevented the development of pulmonary hypertension, supporting the concept of this novel therapy to interrupt abnormal metabolic pathways in this disease.

COX: cyclooxygenase

GERD: gastroesophageal reflux disease

4.5.4. Asthma. Asthma is a disease of chronic airway inflammation, leading to hyperreactivity and bronchospasm. In a murine model of asthma caused by sensitization to ovalbumin, IsoLG adducts increased in the airway epithelium 24 h following allergen exposure and in macrophages after 5 days (87). Immunoreactivity with D11 ScFv was also evident in the collagen around the airways, blood vessels, and smooth muscle of the airways and vasculature. However, the benefit of scavenging reactive dicarbonyl mediators in this condition has not been examined in animal models or humans.

4.6. Cancer

Cancer cells have increased levels of ROS compared with their normal counterparts. A moderate amount of ROS promotes cell proliferation and differentiation, and an excessive amount causes oxidative damage. Cyclooxygenases (COXs) are a family of enzymes that catalyze formation of prostaglandin H_2, the rate-limiting step of all prostaglandin synthesis. Prostaglandin H_2 can nonenzymatically form IsoLGs. Overexpression of COX-2 has been detected in a number of cancers, and its activity has correlated with a poorer prognosis (88–90). IsoLGs generated by COX-2 modified lysine-rich histones, which are basic proteins that package DNA into chromatin. Histones are critical to chromatin compaction, nucleosome dynamics, and transcription, and dysregulation of these processes in human cancer is frequently observed. IsoLG-histone adducts were observed in multiple cancer cell lines as well as in rat liver, with the highest amounts measured on H4 histones and, to a lesser extent, on H3/H2B histones (91). In macrophage and lung epithelial cell lines in which COX-2 is upregulated, the COX inhibitor indomethacin blocked formation of IsoLG-histone adducts, suggesting that IsoLG-histone adduction under these conditions depended on COX activation (91). 5-Ethyl-2-HOBA, an active scavenging analog of 2-HOBA, also blocked formation of IsoLG adducts on histones without affecting COX-2 activity. IsoLG adduct formation on histone H4 disrupted DNA–histone interaction, which was restored by use of the scavenger (91). These data provide evidence for a role of IsoLGs in the development of cancer by decreasing DNA–histone interactions, which may in turn result in increased DNA transcriptional access to previously silent oncogenes.

Gastroesophageal reflux disease (GERD), a common gastrointestinal disorder in the Western world, occurs in 30% of the population. Chronic GERD increases risk for esophageal cancer, and increased oxidative stress is an important contributor to this elevated risk (92). One study provided evidence that IsoLG adducts generated in gastroesophageal reflux may inactivate tumor suppressor genes (93). When esophageal cells derived from normal esophagus, Barrett's esophagus,

and cancer cell lines were exposed to a concentration of bile salts equivalent to what was measured in patients with GERD, there was a significant increase in IsoLG-protein adducts produced by treated cells compared with untreated control cells. Further, IsoLG-protein adducts were also increased in the esophagus of a mouse model of esophageal reflux injury. A trend toward increased formation of IsoLG-protein adducts was observed in esophageal biopsies derived from patients with GERD compared with those from healthy individuals ($P = 0.07$). In vitro studies showed that bile salt treatment of esophageal cells increased the formation of IsoLG adducts on the tumor suppressor p53, which induced aggregation and inactivation of the p53 protein. The scavenger 2-HOBA efficiently suppressed the formation of these IsoLG adducts and blocked precipitation (93). These data demonstrate a potential link between the formation of IsoLG adducts and the progression of GERD to esophageal cancer.

4.7. Hepatic Disease

Due to the portal circulation, the liver is also subjected to toxin-mediated, oxidative stress–induced injury. ROS are produced by the mitochondria, microsomes, and peroxisomes in liver parenchymal cells. When ROS are excessive, hepatic damage occurs owing to irreversible alterations of lipids and proteins that promote liver diseases, including alcoholic and nonalcoholic steatohepatitis. Increasing evidence suggests a role for IsoLGs in the pathogenesis of these forms of liver injury.

4.7.1. Ethanol toxicity. Early studies of ethanol-induced liver injury documented the production of reactive lipid aldehyde species as a consequence of ethanol metabolism, and overwhelming evidence indicates a critical role for ROS in alcohol-induced liver disease (94). In a chronic murine model, IsoLG-protein adducts were detected as early as day 7 of ethanol exposure, coincident with a rise in hepatic enzymes (95). In a model of acute exposure using 6% ethanol, adducts were detected by day 4 of exposure and developed diffusely throughout the liver. Adduct synthesis was not dependent on COX enzymes but rather on TNF-α and the cytochrome P450 enzyme CYP2E1 (95). Subsequent work has identified IsoLGs adducted to phosphatidylethanolamines during chronic liver injury as well (96).

4.7.2. Hepatic fibrosis. A key response of the liver to acute and chronic injury is inactivation of hepatic stellate cells (HSCs), which then transform into myofibroblasts to promote fibrosis and ultimately cirrhosis. An important trigger of HSC activation is oxidative stress. A study examining the dose response of HSCs to IsoLGs has shed light on the mechanisms by which these cells are profibrotic (97). At 0.5–500 nM IsoLG, there was increased production of intercellular adhesion molecule 1 (ICAM-1), as well as cytokines IL-6, IL-8, and IL-1β and the chemokine monocyte chemoattractant protein 1 (MCP1/CCL2). At 5 μM, IsoLGs caused profound cytotoxicity of HSCs characterized by apoptosis, which was not observed at lower concentrations up to 1 μM. IsoLGs also caused the development of intracellular ROS as well as endoplasmic reticulum stress that led to the induction of cellular autophagy (97).

4.7.3. Other toxicity. Not surprisingly, IsoLG adducts have been detected in other forms of oxidative stress–mediated hepatic toxicity. These include carbon tetrachloride–induced liver injury (24) and obesity-related hepatosteatosis in mice receiving a high-fat diet (71).

5. PRECLINICAL TOXICOLOGY AND CLINICAL TRIALS OF 2-HOBA

5.1. Toxicology

Given the wealth of data that demonstrate the beneficial effects of 2-HOBA in multiple model systems, this compound has been developed for commercial therapeutic use. Prior to human administration, the safety of 2-HOBA was investigated in in vitro systems, as well as by acute and chronic oral studies of rodents and rabbits (27, 98–100). There was no evidence of mutagenicity, and 2-HOBA had no effects on major CYP enzymes, including CYP2D6 and CYP3A4. The IC_{50} for inhibition of K^+ current encoded by *HERG* (human I_{Kr}) exceeded 100 μM, indicating that drug-mediated QT prolongation is not anticipated. No toxicity was observed in oral studies using doses up to 1,000 mg/(kg·day) for up to 90 days. In addition, Fuller et al. (100) identified semicarbazide-sensitive amine oxidase, with salicylic acid as a major metabolite, as a primary route of metabolism.

5.2. Phase I Trials

Two phase I trials have assessed the pharmacokinetics and tolerability of 2-HOBA in normal human volunteers. In the initial study, ascending doses of 50 to 825 mg of 2-HOBA were administered as a single dose (28). The time to maximum plasma concentration (C_{max}) was 1–2 h, with an elimination half-life of 2.1 h. There were no serious adverse effects or alterations in vital signs or ECG parameters. Side effects were mild and deemed likely not study related, with the most frequent being increased urination (in two subjects). No side effects were observed in the top two doses administered (550 and 825 mg). The concentrations of salicylic acid in plasma that were found with these two doses were similar to those observed with low to moderate doses of aspirin.

Given that the drug was fully cleared between 8 and 24 h, a chronic dosing study was undertaken during which 500 or 750 mg of 2-HOBA were administered every 8 h for 2 weeks (29). Once again, the drug was well tolerated and no serious adverse effects were observed. Side effects were generally deemed mild in intensity and transient, the most common of which was headache observed in 33% of patients taking placebo and each of the two doses of 2-HOBA. The one exception was a patient who developed a rash of moderate intensity and required removal from the study. The time to C_{max} was 0.8–2 h, and drug exposure (assessed by C_{max} and AUC) was greater following the last dose than the first. A lumbar puncture was performed in three volunteers 90 min after dose administration, and concentrations in cerebrospinal fluid ranged from 34% to 74% of those concentrations observed in plasma, indicating that 2-HOBA crossed the blood-brain barrier during oral therapy. Peak plasma concentrations of salicylic acid averaged 12.8 mg/mL, which was well below the anti-inflammatory therapeutic range (150–300 mg/mL). In addition, there was no evidence that treatment with 2-HOBA inhibited COX enzymes. Thus, repeated oral administration of 2-HOBA is safe and well tolerated, supporting its approval as a new dietary ingredient in 2019.

6. CONCLUSIONS

IsoLGs are highly reactive mediators of lipid peroxidation that have been identified in the early stages of multiple diseases linked to oxidative stress, and preclinical studies have demonstrated their importance in oxidative injury under a wide variety of conditions. IsoLG scavengers represent an attractive alternative therapeutic approach to contemporary antioxidant strategies—that is, one that does not affect the ROS generation that is required for signaling or host defense, but instead rapidly scavenges reactive mediators as they form so that they cannot interact with

biological targets. Collectively, the evidence presented here indicates that IsoLG scavengers represent a promising and novel therapeutic approach to prevent oxidative injury.

DISCLOSURE STATEMENT

Regarding the use of dicarbonyl scavengers: A.K. is a coinventor on a patent related to the treatment of hypertension; S.S.D. is a coinventor on patents related to the treatment of hypertension and Alzheimer's disease; M.F.L. and S.S.D. have applied for a patent regarding the treatment of atherosclerotic cardiovascular disease; and K.T.M. has applied for a patent regarding the treatment of cardiac arrhythmias. All patents are with Metabolic Technologies Inc. and Vanderbilt University. A.K. and L.S.M.-Z. have received funding from the National Institutes of Health (grant F32HL138745 to L.S.M.-Z.). K.T.M. has received funding from the National Institutes of Health and the American Heart Association.

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