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Isolevuglandins as mediators of disease and the development of dicarbonyl scavengers as pharmaceutical interventions.

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Abstract

Products of lipid peroxidation include a number of reactive lipid aldehydes such as malondialdehyde, 4-hydroxy-nonenal, 4-oxo-nonenal, and isolevuglandins (IsoLGs). Although these all contribute to disease processes, the most reactive are the IsoLGs, which rapidly adduct to lysine and other cellular primary amines, leading to changes in protein function, cross-linking and immunogenicity. Their rapid reactivity means that only IsoLG adducts, and not the unreacted aldehyde, can be readily measured. This high reactivity also makes it challenging for standard cellular defense mechanisms such as aldehyde reductases and oxidases to dispose of them before they react with proteins and other cellular amines. This led us to seek small molecule primary amines that might trap and inactivate IsoLGs before they could modify cellular proteins or other endogenous cellular amines such as phosphatidylethanolamines to cause disease. Our studies identified 2-aminomethylphenols including 2-hydroxybenzylamine as IsoLG scavengers. Subsequent studies showed that they also trap other lipid dicarbonyls that react with primary amines such as 4-oxo-nonenal and malondialdehyde, but not hydroxyalkenals like 4-hydroxy-nonenal that preferentially react with soft nucleophiles. This review describes the use of these 2-aminomethylphenols as dicarbonyl scavengers to assess the contribution of IsoLGs and other amine-reactive lipid dicarbonyls to disease and as therapeutic agents.

Keywords

lipid peroxidation; reactive lipid species; isolevuglandins; malondialdehyde; 4-oxo-nonenal; aldehyde scavengers; oxidative injury

1. Introduction

Products of lipid peroxidation play important roles in physiology and pathophysiology. This review will focus on the role of reactive lipid aldehyde species produced by lipid peroxidation, particularly isolevuglandins, in disease processes and the use of small molecule aldehyde scavengers as therapeutics for these diseases. The pharmacological mechanism of action for these aldehyde scavengers differs substantially from typical

therapeutic compounds (e.g. receptor agonist and antagonists). Therefore, their effective deployment as therapeutics (and accurate interpretation of the results of interventional studies) requires a sound understanding of the lipid aldehydes they scavenge and the mechanisms whereby these various aldehydes exert their effects. We therefore review key aspects of lipid aldehyde formation in section 2 and how individual classes of aldehydes differ in their targeting of cellular nucleophiles to form biologically active adduct in section 3. Section 4 then details the development of small molecules intended to scavenge isolevuglandins and the current knowledge about the selectivity of individual 2-aminomethylphenol compounds such as 2-hydroxybenzylamine for scavenging various classes of aldehydes. We believe this section will be of great value to those who seek to employ these scavengers in various cell and in vivo experiments and to appropriately interpret their results. Readers whose primary interest is in the accumulating evidence for lipid aldehydes driving disease and that aldehyde scavengers are effective therapeutics are encouraged to skip forward to section 5 and then return to earlier sections as needed. Section 6 discusses the current status of early clinical trials for 2-hydroxybenzylamine.

Polyunsaturated fatty acids (PUFAs) like arachidonic acid are highly vulnerable to undergo hydrogen abstract and then addition of oxygen to form peroxy radicals (Yin, Xu, & Porter, 2011). These peroxy radicals then undergo numerous secondary reactions, several of which form reactive lipid aldehydes including malondialdehyde (MDA), 4-hydroxy-nonenal (HNE), 4-oxo-nonenal (ONE), methylglyoxal (MGO) and isolevuglandins (IsoLGs) (Fig. 1).

MDA and HNE were the first reactive lipid aldehyde species to be identified and have been reviewed extensively (Ayala, Munoz, & Arguelles, 2014; Parola, Bellomo, Robino, Barrera, & Dianzani, 1999). In hindsight, this earlier identification compared to other reactive lipid aldehydes may have in part been due to their relatively slow reactivity which allowed their unreacted forms to be measured in biological samples. In contrast, the high reactivity of 4-ketoaldehydes like IsoLGs for cellular amines like lysine (Brame, Salomon, Morrow, & Roberts, 1999) means that only the IsoLG adduct, and not the unreacted aldehyde, can be readily measured. This high reactivity also makes it challenging for standard cellular defense mechanisms such as aldehyde reductases and oxidases to dispose of IsoLGs before they react with proteins and other cellular amines such as phosphatidylethanolamines (PE) and nuclei acids and thereby inflict damage. A class of small molecules, 2-aminomethylphenols, that effectively scavenge IsoLGs, as well as other lipid aldehydes, have been developed as therapeutics in order to prevent modification of critical cellular amines by these reactive lipid aldehydes and thereby prevent or treat disease.

2. Formation of reactive lipid aldehyde species by peroxidation of polyunsaturated fatty acids

Three classes of reactive lipid aldehydes are relevant to understanding the therapeutic efficacy of the 2-aminomethylphenol aldehyde scavengers that are the focus of this review. These classes are 1,4-dicarbonyls (e.g. isolevuglandins), 1,3-dicarbonyls (e.g. malondialdehyde), and α -alkenals (e.g. 4-hydroxy-nonenal). In the subsections below, we provide key details related to the mechanism of formation. This detailed information is

provided for those seeking to understand which lipid aldehydes might be relevant in the disease conditions they wish to study.

2.1 Formation of isolevuglandins and related 1,4-dicarbonyls.

Enzymatic peroxidation of arachidonic acid by prostaglandin H₂ synthases (commonly called cyclooxygenases) specifically generates the bicyclo-endoperoxide product prostaglandin H₂ (PGH₂). Non-enzymatic peroxidation of arachidonic acid by free radicals generates four regioisomers of PGH₂ (H₂-isoprostanes). Both enzymatically formed PGH₂ and non-enzymatically formed H₂-isoprostanes undergo non-enzymatic rearrangement and ring opening to form 4-ketoaldehydes (Salomon & Miller, 1985; Salomon, et al., 1999; Salomon, Subbanagounder, Singh, O'Neil, & Hoff, 1997) (Fig 2).

All isomers of this 4-ketoaldehyde family are collectively given the trivial name of isolevuglandins (IsoLGs) (Brame, et al., 1999). Previously, IsoLGs were often referred to as isoketals as a short-hand term for isomers of 4-keto-aldehydes (Brame, et al., 2004). However, this term had caused confusion because the term “ketal” properly refers to a different chemical functional group. For this reason, the use of the term isoketal has been discontinued and probably should be avoided. Non-enzymatic rearrangement of any regioisomer of H₂-isoprostane or PGH₂ gives rise to two distinct IsoLG regioisomers (the D₂- and E₂- regioisomers) (Salomon & Miller, 1985), so that a total of 8 theoretical IsoLG regioisomers are formed (Fig. 3). Because the non-enzymatic reactions are racemic at each of the chiral carbons, eight stereoisomers of each regioisomer are possible, thus leading to a total of 64 possible regio- and stereoisomers of IsoLG. Studies following non-enzymatic rearrangement of PGH₂ in aqueous buffer found that about 20% became IsoLGs, while in DMSO nearly all became IsoLGs (Boutaud, Brame, Salomon, Roberts, & Oates, 1999; Lund, et al., 1984). Although several nomenclatures have been proposed for naming individual IsoLG regioisomers, we have chosen to name them by the position of the hydroxyl group and the ketone relative to the carboxylate group (Fig. 3).

Peroxidation of any PUFA with a least three appropriately spaced double bonds (e.g. linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid) gives rise to analogous 4-ketoaldehydes as IsoLGs (Bernoud-Hubac, Davies, Boutaud, Montine, & Roberts, 2001) (Fig. 4). In addition to 4-ketoaldehydes, recent work has suggested that peroxidation of PUFAs generates significant amounts of the 1,4-dialdehyde, succinaldehyde (butanedial) (Miyashita, et al., 2014).

2.2 Formation of MDA and related 1,3- and 1,2-dicarbonyls.

MDA is a 1,3-dialdehyde that can form as a non-enzymatic fragmentation of lipid peroxides, with multiple molecules of MDA potentially formed by peroxidation of a single molecule of arachidonic acid (Del Rio, Stewart, & Pellegrini, 2005). MDA is also generated enzymatically by thromboxane synthase, which converts PGH₂ to three products in equal quantities: MDA, 12-hydroxy-heptadecatrienoic acid and thromboxane A₂ (Haurand & Ullrich, 1985). Not surprisingly, MDA is one of the most abundant reactive lipid species found in tissues and plasma. Related 1,3-dicarbonyls can form from peroxidation of nucleic acids (Plastaras, Riggins, Otteneider, & Marnett, 2000).

In addition to 1,3-dicarbonyls, fragmentation reactions of lipid peroxides also generate 1,2-dicarbonyls. Some of the best-studied of these are methylglyoxal and glyoxal, although these are most commonly thought of as degradation products of carbohydrates (Thornalley, 2007).

2.3 Formation of α,β -unsaturated alkenals.

Lipid hydroperoxides undergo further modification and cleavage to form α,β -unsaturated aldehydes. The presence of a hydroxyl or oxo group at the 4-position increases the usual reactivity of the 2-alkenal. Depending upon the oxidative pathway, the resulting alkenal can have an alkyl group or carboxyl at the other end (Fig. 5). HNE and ONE, the most extensively studied in this class of compounds, are formed by peroxidation of both arachidonic acid and linoleic acid. Multiple mechanisms have been proposed for their formation that are not exclusionary and likely account for the abundance of these products (Liu, Porter, Schneider, Brash, & Yin, 2011; Schneider, Porter, & Brash, 2004; Schneider, Tallman, Porter, & Brash, 2001). Analogous 4-hydroxy-alkenals and 4-oxo-alkenal products can form from peroxidation of any PUFA with a bis-allylic hydrogen including linolenic acid, eicosapentanoic acid, and docosahexaenoic acid (Fig. 5). Although the carboxylic acid species have received less attention than the alkyl analogs, phosphatidylcholines esterified with the carboxylate species may play a role in tissue injury (Gu, et al., 2011).

3. Reactive lipid aldehydes exert their effect by modifying cellular nucleophiles.

Reactive lipid aldehydes like IsoLGs appear to primarily exert their biological effects not as the parent molecule per se, but via their reaction with cellular nucleophiles (e.g. proteins, nucleic acids, and aminophospholipids) to form stable adducts that alter cellular function. This contrasts with another unstable bioactive lipid, thromboxane A_2 , which exerts its effect directly as a receptor agonist. Like IsoLG, thromboxane A_2 also has an extremely short half-life and it is virtually impossible to detect in vivo; but unlike IsoLG, the stable product of thromboxane A_2 that is readily detected in vivo, thromboxane B_2 , is biologically inactive. That IsoLGs exert their effects by forming adducts with cellular nucleophiles, rather than by unreacted IsoLGs acting as agonists for receptors is based on findings that preparations containing only stable adducts of IsoLGs (such as PE modified by IsoLG, amyloid modified by IsoLG, or HDL modified by IsoLG) recapitulate most, if not all, of the effects induced by adding similar concentrations of synthetic IsoLG in these systems (Kirabo, et al., 2014; May-Zhang, et al., 2018; Sidorova, et al., 2015; Sullivan, Matafonova, Roberts, Amarnath, & Davies, 2010; Wu, et al., 2014).

Demonstrating a role for IsoLGs and other amine reactive lipid aldehyde species in disease processes broadly requires three types of experiments: measure, mimic, and minimize. First, measurements in tissues must show elevated adduct levels at an early enough stage of the disease process so that the aldehydes plausibly drive the disease rather than simply resulting from the process. Second, administration of IsoLGs (or other reactive lipid aldehydes) or their stable adducts to cells or animal models should mimic key elements of the disease. Third, interventions that selectively minimize adduct formation (i.e. aldehyde scavengers) should reduce disease progression and severity.

In order to appropriately measure aldehyde adducts and to identify likely biological effects, as well as to understand potential effects of their scavenging by 2-aminomethylphenols, it is critical to understand the biochemistry of individual lipid aldehydes in terms of their preferences for specific cellular nucleophiles, their reaction rates with these targets, and the chemical structure of their adducts. For this reason, we describe the key details of these reactions in the subsections below. Then in section 4 we describe the development of the 2-aminomethylphenol class of aldehyde scavengers, a key development that has made possible intervention studies to assess the effect of selectively minimizing adduct formation on disease.

3.1 Reaction of IsoLGs.

In cells and tissues, it has proved nearly impossible to detect unreacted IsoLGs, as they are extremely short-lived (half-life ~2 min) (Brame, et al., 1999) due to their reactivity with primary amines (e.g. lysine and phosphatidylethanolamine) to form reversible Schiff base adducts that then react with the second carbonyl of IsoLG to form irreversible pyrrole adducts (Fig. 6) (Salomon, Jirousek, Ghosh, & Sharma, 1987). In the presence of molecular oxygen, the IsoLG pyrrole adducts readily oxidize to form lactam and hydroxylactam adducts as well as pyrrole-pyrrole crosslinks (Fig. 6) (Boutaud, et al., 1999; Iyer, Ghosh, & Salomon, 1989; Jirousek, Murthi, & Salomon, 1990). On a mole per mole basis, crosslinking of proteins by IsoLG greatly exceeds that for other reactive lipid aldehydes, so that this crosslinking likely underlies many of the adverse effects of IsoLGs on cells and tissues. Unlike most reactive lipid aldehyde species, IsoLGs do not react with soft nucleophiles such as thiols (e.g. cysteine) or imidazoles (e.g. histidine). IsoLGs also react with deoxyadenine, deoxyguanine and deoxycytidine, with the deoxycytidine adduct being the most abundant in DNA (Carrier, Amarnath, Oates, & Boutaud, 2009). IsoLGs also react with the primary amine within the guanidino group of arginine, but this reaction is at least an order of magnitude slower than for the ϵ -amine of lysine. Furthermore, the reaction with arginine results in a bis-urea adduct that separates from the protein, leaving behind an ornithine residue in place of the original arginine (Zagol-Ikapitte, et al., 2004).

Aside from the reaction rates of IsoLG with various primary amines, additional factors including proximity to the source of IsoLG, the concentrations of specific primary amines relative to others, and adjacent groups that alter the pKa of the primary amine influence which targets are modified in cells or tissues (Abbat, Dhaked, Arfeen, & Bharatam, 2015; Amarnath, Amarnath, Valentine, Eng, & Graham, 1995). When exogenous synthetic IsoLG is added to cells, phosphatidylethanolamine adducts are two to three-fold more abundant than protein adducts, and only small amounts of nucleic acid adducts are formed (Sullivan, et al., 2010).

Compared to the extensive literature reporting increases in MDA and HNE and their adducts during various disease processes (Ayala, et al., 2014; Del Rio, et al., 2005; K. Uchida, 2000), the literature reporting on changes in IsoLG adduct levels remains relatively sparse. This is primarily because assay kits or antibodies to measure IsoLG-protein adducts are not commercially available. The most accurate method for measuring IsoLG proteins adducts is by proteolysis of samples down to the IsoLG-lysyl adducts (IsoLG-Lys) and then analyzing

IsoLG-Lys by HPLC coupled with stable isotope dilution mass spectrometry (LC/MS) (Boutaud, et al., 2001; Brame, et al., 2004). We have recently published a highly simplified and optimized version of this LC/MS analysis (IsoLG-Lys) (Yermalitsky, et al., 2019). In addition to the LC/MS method, polyclonal antibodies (Poliakov, Meer, Roy, Mesaros, & Salomon, 2004; Salomon, et al., 2000; Salomon, et al., 1999; Salomon, Subbanagounder, O'Neil, et al., 1997) or single chain phage display antibodies (ScFv) (Davies, et al., 2004) that recognize IsoLG-protein adducts have been used for semi-quantitative methods like immunoblotting, immunohistochemistry, and flow cytometry analysis (Kirabo, et al., 2014; Mont, et al., 2016). Using these methodologies, evidence for the formation of IsoLG adducts in a number of disease states has slowly emerged. These studies will be summarized in Section 5 of this review.

3.2 Reactions of 1,3- and 1,2-dicarbonyls.

Like IsoLG, MDA almost exclusively reacts with primary amines including lysine and arginine, as well as with nucleic acid bases such as adenosine and guanosine (Hartley, Kroll, & Petersen, 1997; Marnett, Riggins, & West, 2003). The reaction rate of MDA is relatively slow (Slatter, Murray, & Bailey, 1998), at least several orders of magnitude slower than IsoLG, so that unreacted MDA can be readily measured in tissues using the thiobarbituric acid substances assay. Despite its slower reactivity, protein adducts of MDA can be readily detected in tissue because it appears to be produced in higher abundance than other dicarbonyls during peroxidation of polyunsaturated fatty acids. The most abundant lysine adduct of MDA is the reversible Schiff base propenal adduct (Fig. 7) (Esterbauer, Schaur, & Zollner, 1991). If a second lysine or other primary amine is in appropriate proximity to the propenal adduct, it can form a crosslink. Other MDA adducts include dihydropyridine adducts that form from the condensation of multiple MDA molecules (Esterbauer, et al., 1991; Slatter, et al., 1998).

Among many 1,2-dicarbonyls, methylglyoxal (MGO) has attracted the most attention, since it is a major toxic product formed when glucose levels are high. The primary target of MGO in proteins is the guanidine moiety of arginine. Model studies showed that the initially-formed imidazoline adducts of arginine and MGO undergo further modifications to form the stable argpyrimidine (Klopfer, Spanneberg, & Glomb, 2011) (Fig. 8).

3.3 Reactions of α,β -unsaturated aldehydes.

HNE and other hydroxyalkenals primarily react with soft nucleophiles such as cysteine and histidine to form reversible Michael adducts (Nadkarni & Sayre, 1995; K. Uchida, et al., 1994) (Fig. 9). HNE also reacts with lysine to form pyrroles in very small amounts (Sayre, Arora, Iyer, & Salomon, 1993). The rate of reaction of HNE with proteins is more than 10 times slower than that of IsoLG (Brame, et al., 1999). Both free and adducted HNE are detected in various tissues during disease conditions (Hardas, et al., 2013; Yoshida, et al., 2000).

ONE only differs from HNE by substitution of the ketone for the hydroxy group, but this unsaturated 4-ketoaldehyde is far more reactive than HNE because ONE reacts with cellular nucleophiles in a variety of ways. Reaction of ONE with primary amines forms a highly

stable 4-ketoamide adduct (Zhu & Sayre, 2007). ONE is also a powerful Michael acceptor of thiols and imidazoles. The resulting substituted 4-oxononanal is even more reactive towards lysines and other primary amines than the original ONE (Aluise, et al., 2015; Zhu, Gallogly, Mieyal, Anderson, & Sayre, 2009) (Fig. 10).

4. Characterization of 2-aminomethylphenols as effective scavengers of IsoLGs and other amine-reactive lipids aldehydes

The profound adverse effects of IsoLG modifications in cultured cells suggest that the formation of IsoLG adducts *in vivo* could lead to disease (Guo, et al., 2015; Longato, et al., 2017; Sidorova, et al., 2015). Thus, we sought therapeutic strategies that would prevent such modifications *in vivo* and thereby mitigate development of disease. One potential strategy would be to stop the initial formation of IsoLGs by suppressing lipid peroxidation using robust antioxidants. A major drawback of this strategy is that any chemical antioxidants powerful enough to suppress lipid peroxidation will also suppress production of other reactive oxygen species (ROS) like hydrogen peroxide that are essential secondary messengers that control key cellular processes. For this reason, robust suppression of ROS formation may create adverse effects that counterbalance the beneficial effects of suppressing lipid peroxidation. A potentially better and more selective strategy is suggested by the fact that IsoLGs and related dicarbonyls primarily exert their effects through modification of cellular amines. Therefore, it may be possible to selectively block their modification of cellular amines by trapping and sequestering these highly reactive aldehydes using small molecules possessing a primary amine moiety. By rapidly scavenging and sequestering IsoLGs, proteins and PE could be protected from modification without simultaneously inhibiting critical cell signaling by hydrogen peroxide and other ROS. For this strategy to be effective, these small molecules would need to have good bioavailability and react with IsoLGs and other lipid dicarbonyls far more rapidly (at least two orders of magnitude) than cellular amines like lysine or phosphatidylethanolamine.

4.1 Identification and characterization of 2-aminomethylphenols as IsoLG scavengers.

We screened a number of commercially available compounds with primary amine moieties that were reported to have good bioavailability and low toxicity *in vivo* including pyridoxamine, aminoguanidine, and glycine (Amarnath, Amarnath, Amarnath, Davies, & Roberts, 2004). To our surprise, pyridoxamine (PM), a vitamin B6 vitamer, almost completely blocked the reaction of IsoLG with radiolabeled lysine or with serum albumin (Amarnath, et al., 2004). PM seemed a particularly attractive scaffold for further investigation because it had shown efficacy in animal models of diabetic nephropathy and was advancing to clinical trials (Metz, Alderson, Thorpe, & Baynes, 2003). Although subsequent results from diabetic nephropathy trials with PM showed its effect were extremely modest, it was safe for use in humans (Lewis, et al., 2012; Williams, et al., 2007).

To investigate the mechanism underlying the profound protection afforded by PM against IsoLG modification of lysine, we performed structure-activity relationships for the rate of pyrrole formation with various PM analogs (Amarnath, et al., 2004) using the model 4-ketoaldehyde, 4-oxopentanal (OPA). Our structure-activity relationship studies were guided

by previous studies on the role of PM in transamination reactions (Metzler, Ikawa, & Snell, 1954) that showed that the Schiff base is stabilized by hydrogen-bonding with the adjacent phenolic group and the facilitative participation of the phenolic group in Schiff base formation as a strong donor hydrogen bond (Adrover, et al., 2010; Adrover, Vilanova, Munoz, & Donoso, 2009; Caldes, Vilanova, Adrover, Munoz, & Donoso, 2011).

Structure-activity relationship studies using a variety of PM analogs (Fig. 11) showed that stabilization of the initial Schiff base by the phenolic hydrogen and hydrogen bond donation play a similar role in the reaction of PM with 4-ketoaldehydes as it does in transamination (Fig. 12). The second-order rate constant for pyrrole formation of the OPA reaction with PM was more than 100 times greater than that for the OPA reaction with 4-picolinylamine (which lacks the phenolic group) (Table 1) (Amarnath, et al., 2004). Enhanced reactivity extended to the benzene analog of PM, 2-hydroxybenzylamine (2-HOBA, salicylamine), while the significantly lower rates found for 4-hydroxybenzylamine (4-HOBA) and 2-methoxybenzylamine confirmed the helpful participation of the phenolic group hydrogen of 2-aminomethylphenols (2-AMPs) in cyclization of the pyrrole (Amarnath, et al., 2004). Because 4-HOBA shows only trivially enhanced reactivity compared to *N*-acetyl-lysine for 4-ketoaldehyde, it serves as a useful negative control (non-scavenger) in cellular and in vivo studies testing the effects of dicarbonyl scavenging by 2-HOBA.

The key observations of the structure activity relationship studies above led to the proposed mechanism for the reaction of 2-AMPs with 4-ketoaldehydes including IsoLGs: (i) initial formation of the hemiaminal between the amine and the aldehyde, (ii) the acidic phenol protonates the keto group thus accelerating the nucleophilic attack on the latter during the rate-limiting step, and (iii) the rapid loss of 2 molecules of water (Fig. 12).

Although 2-HOBA and PM showed similar reactivity with 4-ketoaldehydes in aqueous buffers, in actual physiological conditions IsoLGs are produced in a lipophilic environment not readily penetrated by the zwitterionic ionic form of PM that predominates at neutral pH. Indeed, we found that 2-HOBA, which is much more hydrophobic than PM, was also much more effective than PM at blocking the formation of IsoLG protein adducts in platelets and other cells (Davies, et al., 2006). We therefore synthesized 5'-*O*-alkyl derivatives of PM with medium chain ethers (pentyl, hexyl, and heptyl) (Fig. 11). These alkylated PMs shared similar reactivity with OPA as PM in aqueous buffers, but their greater hydrophobicity (Table 3) greatly enhanced their efficacy. For instance, 5'-*O*-pentyl-pyridoxamine (PPM) showed significantly enhanced ability to suppress IsoLG modification of proteins in cells and to protect against hydrogen peroxide induced cytotoxicity (Davies, et al., 2006). 5'-*O*-pentyl-pyridoxine (PPO), which lacks the methylamine moiety of PPM, serves as a useful negative control compound (non-scavenger) in cellular and in vivo studies testing the effects of dicarbonyl scavenging by PPM. It is also important to note that the vast majority of 2-aminomethylphenol analogs that are excellent IsoLG scavengers do not exert any inhibitory effect on cyclooxygenases, despite sharing similar salicylate structure to acetyl salicylic acid (I. Zagol-Ikapitte, et al., 2010).

Although 2-HOBA, PPM, and other 2-AMPs were originally developed to scavenge IsoLGs, the same properties that make them excellent scavengers of the highly reactive IsoLGs also

make them reasonably good scavengers of less reactive lipid dicarbonyls like MDA and MGO that also preferentially modify primary amines. 2-AMPs are therefore properly considered as dicarbonyl scavengers rather than IsoLG scavengers. The scavenging properties of 2-AMPs for other classes of reactive lipid aldehydes are outlined below.

4.2 Scavenging reactions of 2-AMPs for MDA and other 1,3-dicarbonyls

The reactions of MDA with PPM or 2-HOBA are only somewhat faster reactions with 4-HOBA or *N*-methyl-2-HOBA (Table 2). Their enhanced reactivity likely stems from stabilization of the resulting amine by the phenolic group, further inhibiting reaction of this MDA-propenal adduct with lysines and cross-link formation (Zagol-Ikapite, et al., 2015) (Fig. 13). That this mechanism still confers reduced formation of MDA protein adducts in cells is shown in studies with human platelets incubated with arachidonic acid to generate MDA via thromboxane synthase. While vehicle-treated platelets had high levels of Lys-MDA-Lys crosslinks, these crosslinks were lowered to control values by preincubation with 2-HOBA (Zagol-Ikapite, et al., 2015).

4.3 Scavenging reactions of 2-AMPs for MGO and other 1,2-dicarbonyls

The effectiveness of PM as a scavenger for MGO has been well established (Chetyrkin, et al., 2011). Alkylation of PM, resulting in greater access to hydrophobic regions of proteins, dramatically increases MGO scavenging potential with the increased reaction rates being proportional to the chain length. Based on these observations, the key step is the addition of the phenolic group to the imine leading to highly reactive pyrido-1,3-oxazine (Fig. 14). Since 2-HOBA lacks the strongly electron-attracting pyridine group, the formation of the reactive Schiff base is not favored and 2-HOBA is much less effective in scavenging MGO. Hence, HxPM was able to protect arginine residues in the lysozyme from MGO attack, while 2-HOBA was not (Table 3) (Amarnath, Amarnath, Avance, Stec, & Voziyan, 2015).

4.4 Scavenging reactions of 2-AMPs for ONE but not HNE

Complete scavenging of ONE requires reaction with both dicarbonyl and the double bond. 2-AMPs meet this task remarkably well (Amarnath & Amarnath, 2015). Similar to the reaction with MGO, the key step in the reaction with ONE is the formation of the pyrido-1,3-oxazine from the imine. Subsequent to isomerization of the double bond to the *cis*-orientation, it is poised to undergo one more ring closure. Dehydration to the multicyclic pyrrole is irreversible and completes the reaction (Fig. 15). Again, the intermediacy of pyrido-1,3-oxazine means PM has an advantage over 2-HOBA (Table 4). When ONE does form a Michael adduct with thiol groups, it is rapidly consumed by 2-AMPs before lysyl residues can react with it to form intra-protein crosslinks.

2-AMPs do not serve as effective scavengers for 4-hydroxyalkenals such as HNE that preferentially react with soft nucleophiles such as cysteines and histidines. This is because in the reaction between HNE and 2-AMPs, the Schiff base that is in equilibrium with the starting compounds only undergoes very slow tautomerization to the enamine that can then cyclize to the pyrrole (Fig. 16) (Sayre, et al., 1993). Since the presence of phenolic group of 2-AMPs does not seem to influence the shifting of protons needed to form the enamine, this pathway cannot compete with the Michael addition reaction of cysteine or histidine residues.

5. Evidence for IsoLG and other lipid dicarbonyls as contributors to disease processes.

As previously stated, establishing a role for IsoLG adducts as drivers of disease processes and the value for 2-AMPs as therapeutic compounds in these disease requires three critical pieces of evidence: that adduct levels are increased early in the disease process, that addition of IsoLG or IsoLG adducts recapitulate key aspects of the disease processes, and that reduction of adduct levels using 2-AMPs reduce disease progression. The current status of such evidence for a number of diseases is described below.

5.1 Atherosclerosis.

Atherosclerosis remains the leading cause of death worldwide. The formation and progression of atherosclerotic plaques to vulnerable plaque rupture involves complicated mechanisms related to endothelial dysfunction, vascular proliferation, cell death, inflammation, and oxidative stress. This process evolves from accumulation of vascular lipids underlying a fibrous cap to thinning of this cap and ultimately plaque rupture. Growing evidence suggests a significant role of IsoLGs in the pathogenesis of atherosclerosis through its modification of lipoproteins.

The first evidence for formation of IsoLG-protein adducts *in vivo* came from pilot ELISA studies using antibodies against IsoLG-protein adducts showing that these were elevated in the plasma of patients diagnosed with atherosclerosis (Salomon, Subbanagounder, O'Neil, et al., 1997). These pilot studies were later confirmed with additional anti-IsoLG antibodies and more samples for each disease (Poliakov, et al., 2004; Salomon, et al., 2000). The importance of the IsoLG adducts was demonstrated by studies showing that low-density lipoprotein (LDL) stably modified by IsoLG is readily taken up by macrophages through scavenger receptors (Hoppe, Subbanagounder, O'Neil, Salomon, & Hoff, 1997), transforming the macrophages into atherogenic foam cells. This process is similar to that observed following LDL modification by MDA (Fogelman, et al., 1980) or HNE (Hoff, et al., 1989) but in the case of IsoLG occurs at much lower concentrations due to the highly reactive nature of IsoLGs. Interestingly however, only about 20% of IsoLG protein adducts isolated from plasma were found associated with LDL (Salomon, et al., 2000), suggesting the possibility that IsoLG contribute to atherogenesis through modification of other proteins and by other mechanisms.

We have recently found a significant amount of IsoLG-protein adducts in high-density lipoprotein (HDL) of patients with familial hypercholesterolemia (233.4 ± 158.3 pg/mg protein) compared to age-matched healthy control volunteers (90.1 ± 33.4 pg/mg protein) (May-Zhang, et al., 2018). We also found that the oxidative enzyme myeloperoxidase (MPO), which is increased in atherosclerosis (Nicholls & Hazen, 2005) forms IsoLG adducts with both protein (May-Zhang, et al., 2018) and phosphatidylethanolamine (PE) (Guo, et al., 2015) on HDL. ApoAI is the major apolipoprotein of HDL (accounting for ~70% of its protein mass) and is responsible for many of the anti-atherogenic functions of HDL (Ma, Liao, Lou, & Wu, 2004; Zhang, et al., 2003). MPO-mediated oxidation as well as *ex vivo* modification of HDL by IsoLGs potently crosslinks apoAI (May-Zhang, et al.,

2018). Using cellular models of atherosclerosis to determine the functional consequences of IsoLG modification of HDL, we found that HDL stably modified with IsoLG loses its ability to exchange apoAI (a critical step in the ability of HDL to carry out reverse cholesterol transport), promote macrophage cholesterol efflux, and protect against endotoxin-induced macrophage inflammation (May-Zhang, et al., 2018). Furthermore, low levels of IsoLG adducted to HDL also enhances IL-1 β and IL-6 expression by macrophages beyond endotoxin-stimulation alone. It is important to note that in these experiments, no unreacted IsoLG was present, so that these effects are the result of the IsoLG adducts on HDL, not unreacted IsoLG acting on receptors. These studies demonstrate that IsoLG modification not only renders HDL dysfunctional but also contributes to a pro-inflammatory process.

Although it is not known whether the IsoLG modification of apoAI or PE drives HDL dysfunction, there is significant evidence indicating that IsoLG-modified PE may promote inflammation and contribute to the atherogenic process. We have previously found that IsoLG modification of PE converts PE from a relatively inert molecule to a biologically active molecule that induces cytokine expression and adhesion molecule surface expression in endothelial cells (Guo, et al., 2011). IsoLG modified PE also induces an ER stress signaling response, which is reduced by addition of chemical inhibitors of ER stress (Guo, et al., 2011). These responses are mediated by relative low levels of IsoLG-modified PE (1–3 μ M). These findings illustrate a link between oxidized phospholipids and atherogenesis since ER stress response proteins are markedly increased in atherosclerotic lesions (Tabas, 2009). Our subsequent studies show the relevance of IsoLG-PE in human atherosclerosis, where we found elevated IsoLG-PE levels in plasma isolated from patients with familial hypercholesterolemia (Guo, et al., 2015). In cellular studies, we found that IsoLG-PE potently stimulated NF κ B activation and expression of inflammatory cytokines in macrophages, which requires activation of the RAGE receptor, suggesting that the stable IsoLG-PE adduct is a RAGE receptor agonist (Guo, et al., 2015). Thus, our investigations suggest that IsoLG-PE activation of RAGE in macrophages and potentially other cell types may contribute to increased inflammation in hypercholesterolemia and atherosclerosis.

Studies are currently underway to determine if 2-AMPs will have significant benefit in attenuating disease in mouse models of atherosclerosis. It is anticipated that the results of these studies will be available within the next year.

5.2 Hypertension.

Hypertension is a major risk factor for atherosclerosis, and IsoLG and other lipid dicarbonyls may contribute to the development of hypertension by mechanisms unrelated to those involved directly in plaque formation. Both innate and adaptive immunity are involved in the pathophysiology of hypertension and recent evidence suggests that IsoLG-protein adducts play a role (Fig. 17) (Kirabo, et al., 2014; McMaster, Kirabo, Madhur, & Harrison, 2015). IsoLG-protein adducts accumulate in antigen presenting dendritic cells (DCs) in several experimental models of hypertension including hypertension induced by angiotensin II, deoxycorticosterone acetate salt, L-Nitroarginine methyl ester/high salt, and smooth muscle specific overexpression of NADPH oxidase subunit p22^{phox} models (Itani, et al., 2016; Kirabo, et al., 2014; Wu, et al., 2014). Using immunohistochemistry analysis with a

single chain antibody that recognizes IsoLGs adducted to lysines on any protein independent of the amino acid backbone, we found that there was an approximately 2-fold increase of IsoLG-protein adducts in the heart and aorta of mice with angiotensin II-induced hypertension. Flow cytometry analysis specifically in antigen presenting dendritic cells revealed a marked increase in accumulation of IsoLG-protein-adducts in hypertensive mice and these results were confirmed by mass spectrometry analysis (Kirabo, et al., 2014).

In addition, IsoLG-protein adducts accumulate in antigen presenting cells of hypertensive humans including CD14⁺ and CD83⁺ cells (Kirabo, et al., 2014). Patients with resistant hypertension exhibited increased levels of plasma F2-isoprostanes, which are co-produced with IsoLGs in the isoprostane pathway of lipid peroxidation, when compared to well-controlled and normotensive subjects (23.2 ± 11.5 vs. 18.3 ± 10.3 vs. 11.6 ± 7.0 pg/mL, $p < 0.05$ respectively; after controlling for body mass index, hemoglobin A1C, low-density lipoprotein, and statin treatment. In addition, humans with hypertension accumulated 3 times more IsoLG-protein adducts in their monocytes, activated CD83⁺ cells and B cells when compared to normotensive individuals (Kirabo, et al., 2014).

DC accumulation of IsoLG-protein adducts correlates with increased surface expression of B7 ligands CD80 and CD86 which are critical for their ability to co-stimulate T cells and for the development of hypertension (Kirabo, et al., 2014; Vinh, et al., 2010). These DCs produce pro-inflammatory cytokines IL-1 β , IL-6 and IL-23 and induce T cell proliferation and production of TNF- α , IFN- γ and IL-17A which contribute to hypertension (Dixon, Davies, & Kirabo, 2017; Kirabo, et al., 2014; McMaster, et al., 2015). Adoptive transfer of these DCs prime T cell activation and promote hypertension in response to sub-pressor doses of angiotensin II (Barbaro, et al., 2017; Kirabo, et al., 2014). Exposure of DCs to the pro-oxidant *tert*-butyl hydroperoxide promotes their ability to support T cell proliferation and hypertension, similar to the effect of angiotensin II and other pro-hypertensive stimuli *in vivo* (Kirabo, et al., 2014). Evidence that IsoLG-protein adducts drive these responses come from studies showing that DCs pulsed with IsoLG-protein adducts from renal and vascular homogenates stimulate T cell proliferation, while DCs pulsed with protein modification by other lipids including HNE, MDA or MGO do not induce this immune response (Kirabo, et al., 2014; Wu, et al., 2014).

There is compelling evidence that scavenging of IsoLGs blunts the development of hypertension. Pre-treatment with 2-HOBA, 5-methyl-2-HOBA and PPM prevents development of hypertension, while related compounds that exhibit low reactivity with IsoLGs such as N-methyl-2-HOBA (N-Me-2-HOBA), and 4-HOBA did not (Kirabo, et al., 2014). Dicarbonyl scavenging also prevents immune cell activation in hypertension. For instance, pre-treatment with 2-HOBA prevents hypertension-induced activation of DCs indicated by increased expression of B7 ligands, cytokine production and their ability to prime hypertension in recipient mice (Kirabo, et al., 2014). These studies suggest that IsoLG scavenging prevents inflammation and hypertension, which is not attributable to inhibition of prostaglandin biosynthesis.

Recent studies have found that one of the most potent hypertensive stimuli contributing to production of IsoLGs in antigen presenting dendritic cells is excess dietary salt and have

defined an intracellular mechanism by which dietary sodium activates immune cells. Sodium (Na^+) enters dendritic cells through amiloride sensitive epithelial sodium channels (ENaC). Intracellular Na^+ is exchanged for calcium (Ca^{2+}) via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Ca^{2+} activates protein kinase C (PKC) which in turn phosphorylates the NADPH oxidase subunit p47^{phox} . This leads to activation of the NADPH oxidase, increased superoxide ($\text{O}_2^{\cdot-}$) and IsoLG-protein adduct formation.(Barbaro, et al., 2017) We found that adoptive transfer of salt-exposed DCs primes hypertension in response to a sub-pressor dose of angiotensin II. (Barbaro, et al., 2017) IsoLG-protein adduct formation is absent in mice lacking the NADPH oxidase and pharmacological scavenging of IsoLGs prevents DC activation, hypertension and end-organ damage.(Barbaro, et al., 2017; Kirabo, et al., 2014) In additional studies, we found that the salt-sensing kinase serum/glucocorticoid kinase 1 (SGK1) in dendritic cells mediates salt-induced expression of ENaC and promotes salt-sensitive hypertension by activation of the NADPH oxidase and formation of IsoLG-protein adducts.(Van Beusecum, et al., 2019) Moreover, we found that in humans and mice, a high salt diet alters the gut microbiome, and this is associated with increased high blood pressure and intestinal inflammation with a marked increase in the B7 ligand CD86 and formation of IsoLG-protein adducts in dendritic cells.(Ferguson, et al., 2019) These studies provide novel insights into the mechanisms by which IsoLGs are formed in inflammation and hypertension.

5.3. Obesity.

Recent studies demonstrate a role of IsoLGs in immune cell activation associated with obesity (McDonnell, et al., 2018). IsoLGs adducts are increased in CD206-expressing macrophages isolated from adipose tissue of high fat-fed mice and *in vitro* M2-polarized macrophages. Co-culture of these macrophages with T cells promotes activation of specifically CD8⁺ T cells. This is associated with a clonal expansion of a specific CD8⁺ TCR repertoire characterized by positively charged and less polar CDR3s in high fat fed mice but not in low fat fed mice (McDonnell, et al., 2018). These studies suggest that IsoLG-protein adducts are immunogenic and may contribute to the inflammatory process leading to hypertension and obesity which may provide insight into mechanisms by which these cardiometabolic conditions lead to an inflammatory state.

5.4 Arrhythmias.

A major sequelae of myocardial ischemia and infarctions is sudden cardiac death due to ventricular arrhythmias and fibrillation. Experimental induction of myocardial ischemia in dogs generates IsoLG-protein adducts (40–70 pg IsoLG adduct/mg protein) in the epicardial border zone of their myocardium (Fukuda, et al., 2005). Treating cultured cardiac myocytes with tert-butylhydroperoxide induces formation of IsoLG-protein adducts and diminishes the functionality of their sodium channels (Nakajima, et al., 2010). Subsequent studies demonstrated that lipid peroxidation of these cells leads to modification of sodium channels by IsoLG and that the dicarbonyl scavengers 2-HOBA and PPM protects cardiac cells from peroxidation-induced loss of function (Nakajima, et al., 2010).

In addition to ventricular fibrillation, a potential role for IsoLGs has been identified in atrial fibrillation that results from rapid atrial activation. Rapid electrical pacing of HL-1 atrial cells results in oxidative stress and formation of preamyloid oligomers that include the

natriuretic peptide ANP (Sidorova, et al., 2015). Treating HL-1 cells with IsoLG was found to facilitate formation of these preamyloid oligomers and treating isolated ANP with IsoLG initiated oligomer formation (Sidorova, et al., 2015). Pre-treatment of HL-1 cells with 2-HOBA partially ablated pacing induced preamyloid oligomer formation (Sidorova, et al., 2015).

5.5 Brain Disease.

One of the most common neurodegenerative diseases in the elderly population is Alzheimer's Disease (AD). Risk for AD markedly increases with advancing age, so that rising median lifespan of the U.S. population raises the possibility of a looming AD epidemic. Currently, the best available therapeutic strategies are essentially palliative and have no impact on the progression of AD. An increase in products of lipid peroxidation has long been noted in AD, along with the formation of amyloid plaques and neurofibrillary tangles (Butterfield & Boyd-Kimball, 2018). Evidence that the formation of IsoLG adducts on amyloid might contribute to these pathological processes comes from studies showing that IsoLG modification of amyloid β peptides crosslinks these peptides (Davies, et al., 2002), increases their rate of oligomerization (Boutaud, et al., 2002), and increases their neurotoxicity (Boutaud, Montine, Chang, Klein, & Oates, 2006). Proteins crosslinked by IsoLG, including amyloid, strongly inhibit 20S proteasomal activity (Davies, et al., 2002). Reduced proteasomal activity is a hallmark of AD and may contribute to the accumulation of damaged proteins and loss of neurons (Cao, Zhong, Toro, Zhang, & Cai, 2019).

Levels of IsoLG-Lys measured in hippocampal samples from human post-mortem brain collected after rapid autopsy are ~12 folds higher in AD brain compared to control brain (15 pg IsoLG-Lys adduct/mg protein vs 1.2 pg/mg protein). Importantly, the level of adducts correlates with the severity of AD (Zagol-Ikapitte, et al., 2005). Anti-IsoLG antibody immunoreactivity is markedly increased in the hippocampus but not cerebellum of AD patients compared to age-matched controls (Davies, et al., 2011). The most commonly studied model of Alzheimer's disease is the double transgenic mouse (APP-PS1), expressing the Swedish mutation in the human amyloid precursor protein (APP) and the deletion of exon 9 of the presenilin 1 protein, a component of the γ -secretase (PS1). In these mice, IsoLG-Lys adducts in the posterior cortex increase after 4 months of age and correlate with amyloid deposition and memory impairment (Woodling, et al., 2014). Transgenic mice expressing human *ApoE4*, another mouse model of Alzheimer's Disease, show significant loss of spatial working memory with old age (>12 months) and these mice have elevated levels of IsoLG-Lys adducts in the brain. Long-term treatment with 2-HOBA significantly inhibits the loss of working memory in older *ApoE4* mice (Davies, et al., 2011).

Although there have been relatively few studies on IsoLGs in neurological diseases outside of AD, one recent study demonstrated that there are significant increases in rat models of epilepsy induced by kainic acid and pilocarpine. Levels of adducts were increased in hippocampus and perirhinal cortex after induction of seizures (Pearson, Warren, Liang, Roberts, & Patel, 2017). Treatment with 2-HOBA attenuated development of spatial memory and reference memory deficits, as well as neuronal loss and astrogliosis (Pearson, et al., 2017).

5.6 Lung Injury and Disease.

As a key interface with the external environment, the lung faces significant exposure to potentially harmful agents such as toxicants, pathogens, immunogens, and even simply oxygen, making it potentially vulnerable to lipid peroxidation injury. Several studies demonstrate that various environmental challenges to the lung significantly elevate levels of IsoLG modified proteins. For instance, exposing wild-type C57BL6 mice to 95% oxygen in order to mimic the effects of human patients given oxygen markedly increases anti-IsoLG adduct antibody immunoreactivity in airway epithelial cells (Davies, et al., 2004). Similarly, aerosol challenge with an antigen that mice had previously been sensitized to (ovalbumin) markedly increases anti-IsoLG adduct antibody immunoreactivity in the lung epithelium within hours after challenge. Interestingly, the immunoreactivity moved to surrounding macrophages within 5 days, suggesting modified epithelial proteins may be taken up by the macrophages. Finally, exposure of wild-type C57BL6 mice to a single dose of ionizing radiation (16 Gy) increases anti-IsoLG adduct immunoreactivity in the lung six weeks after irradiation and immunoreactivity is increased even more 16 weeks after irradiation (Mont, et al., 2016). These same investigators found that even without the challenge, there is anti-IsoLG adduct immunoreactivity in the lungs of wild-type mice and that this immunoreactivity is reduced in mice lacking the NADPH oxidase subunit p47phox, but is increased in mice lacking Nrf2, a transcription factor regulating the antioxidant response (Mont, et al., 2016). They also found that anti-IsoLG adduct immunoreactivity is increased in lungs explanted from patients with idiopathic pulmonary fibrosis who underwent lung transplant compared to donor lungs (Mont, et al., 2016). In these samples, anti-IsoLG adduct immunoreactivity co-localizes with collagen 1a1 immunostaining, suggesting that IsoLG modification of collagen might play a role in fibrosis. To the best of our knowledge, no animal studies have been undertaken to examine whether 2-AMPs would benefit these or other models of lung injury. Given that there would be substantial benefit to protect patients against the adverse side effects of otherwise highly efficacious therapies such as radiation or ventilation with oxygen, such studies seem warranted.

In terms of other lung pathologies, 2-HOBA has shown efficacy in a mouse model of pulmonary arterial hypertension (PAH) created by a genetic mutation in the BMPR2 gene (Egnatchik, et al., 2017). Lipid peroxidation is significantly increased in the lungs of human with PAH. In the mouse model of PAH, the activity of histone deacetylase SIRT3 is markedly reduced and this loss of activity can be mimicked by SIRT3 exposure to synthetic IsoLG (Egnatchik, et al., 2017). Treatment of BMPR2 mutant mice with 2-HOBA rescues SIRT3 activity and reduces pulmonary resistance to that of wild-type mice (Egnatchik, et al., 2017). Thus, the formation of IsoLG or related lipid dicarbonyls appears to play a critical role in the development of PAH so that future clinical studies utilizing 2-AMPs are warranted.

5.7 Liver Injury and Disease.

Like the lung, the liver faces significant exposure to xenobiotics, toxicants, and immunogens from the external environment because orally absorbed compounds must pass through the portal system before entering systemic circulation. Detoxification of xenobiotics and toxicants, often via cytochrome P450 enzymes (e.g. CYP2E1), can produce ROS as a

byproduct of the detoxification reaction. Toxicants such as carbon tetrachloride (once commonly used in dry cleaning and as an industrial degreaser) and ethanol are associated with significant oxidative injury to the liver (Albano, Tomasi, Gorla-Gatti, & Dianzani, 1988; Brattin, Glende, & Recknagel, 1985; Rouach, et al., 1997; Sundari, Wilfred, & Ramakrishna, 1997).

Some of the very first evidence for the formation of IsoLG protein adducts in vivo came from rats exposed to carbon tetrachloride. Four hours after oral gavage of a lipid mixture containing carbon tetrachloride, high levels of IsoLG protein adducts (6.4 ± 0.3 pg IsoLG-Lys/mg protein lactam and 21 ± 4 pg/mg protein Schiff base adducts) were detected in the liver (Brame, et al., 2004). Interestingly, almost all of the IsoLG Schiff base forms of these adducts that were found in the liver were esterified to phospholipids, while very little of the lactam form of the adduct was esterified (Brame, et al., 2004). We have recently reported that both liver and plasma contain a phospholipase activity that can convert phospholipid esterified IsoLG-Lys adducts to unesterified IsoLG-Lys adduct (Yermalitsky, et al., 2019). This suggests that most IsoLG initially formed by peroxidation of arachidonic acid is still esterified to phospholipid and can react with the lysine residues of protein to form the Schiff base while still esterified. During the time it takes for the IsoLG adduct to mature into the pyrrole and lactam forms of the adduct, it also undergoes lipolysis by phospholipases to generate non-esterified adducts.

A more common toxicant for humans is ethanol and chronic ethanol consumption markedly increases risk for alcoholic fatty liver disease. Ethanol consumption by female C57BL6 mice for 39 days elevates IsoLG protein adduct levels in a dose dependent manner (Roychowdhury, et al., 2009). Genetic deletion of cyclooxygenases, potential enzymatic sources of IsoLG, had no effect on levels of IsoLG protein adducts, but deletion of either TNFR1 or CYP2E1 significantly reduced IsoLG protein adducts in ethanol consuming mice (Roychowdhury, et al., 2009). Similar to elevations of liver IsoLG protein adducts, chronic ethanol consumption also increases levels of liver IsoLG-PE adducts (Li, et al., 2009). Ethanol consumption is also associated with increased formation of HNE and MDA adducts in the liver (Niemela, 2001).

The mechanisms by which IsoLG and its adducts contribute to fatty liver disease and fibrosis remain largely unexplored. Treatment of cultured hepatic stellate cells (the main cell type that drives hepatic fibrosis) with low doses of synthetic IsoLG (500 nM or less) induces cell activation including expression of α -smooth muscle actin and cytokines, which are hallmarks of fibrosis and inflammation important to fatty liver disease (Longato, et al., 2017). Studies are currently underway to examine whether 2-AMPs can modulate fatty liver disease, but the results of these studies are not yet available.

5.8 Cancer

Lysine-rich proteins such as histones are particularly susceptible to adduction by IsoLGs. We hypothesized that modifications of the positively charged residues by the bulky negatively charged IsoLGs might affect histone interaction with DNA. Using the COX-2 expressing human lung adenocarcinoma cell line A549, we showed that lysine residues in histones are modified by IsoLGs derived from the cyclooxygenase activity (Carrier, Zagol-

Ikapitte, Amarnath, Boutaud, & Oates, 2014). Our data also showed that histone H4 is most abundantly adducted by IsoLGs, suggesting some specificity of adduction based on either lysine accessibility or local reactivity. Importantly, our data indicated that adduction of histones by IsoLGs leads to the disruption of histone-DNA binding, and that this disrupted interaction is restored by treating the cells with 5-ethyl-2-HOBA, an analog of 2-HOBA. These data provide evidence for a role of IsoLGs in the development of cancer by increasing DNA transcriptional accessibility to oncogenes, by modifying the pattern of DNA methylation or affecting DNA repair, processes that are all regulated by histone-DNA interactions. Using the same line of thought, we have recently found that IsoLG-Lys adducts are also elevated in esophageal biopsies of humans diagnosed with gastroesophageal reflux disease (GERD) and in mouse models of GERD (Caspa Gokulan, et al., 2019).

5.9 Platelets and blood disorders.

Platelet activation leads to the formation of both IsoLGs and MDA, via the COX-1 / thromboxane synthase pathway (Hecker, Haurand, Ullrich, Diczfalusy, & Hammarstrom, 1987) (Boutaud, et al., 2003), and both electrophiles form covalent adducts on platelet proteins (Zagol-Ikapitte, et al., 2015; I. Zagol-Ikapitte, et al., 2010). The demonstration that platelet activation leads to IsoLG and MDA protein adducts has led to studies characterizing the functional consequences of these modifications. The demonstration that 2-HOBA protects platelet proteins from modification by isoLGs and MDA (Zagol-Ikapitte, et al., 2015; I. Zagol-Ikapitte, et al., 2010) provides a tool for investigating the functional consequences of the formation of protein adducts in platelets by scavenging the dicarbonyls without inhibiting COX-1 (I. Zagol-Ikapitte, et al., 2010).

5.10 Chronic kidney disease.

The same preliminary report and follow-up study that showed elevated IsoLG-protein adducts in atherosclerosis also showed elevations in IsoLG protein levels in the plasma of patients with chronic kidney disease (2.4 nM) compared to volunteers without disease (1.7 nM) (Salomon, et al., 2000; Salomon, Subbanagounder, O'Neil, et al., 1997). Studies are underway to test the efficacy of 2-AMPs in mouse models of chronic kidney disease, but the results of these studies are not yet available.

5.11 Eye disease.

Glaucoma results from increased ocular pressure that leads to vision loss and a role for lipid peroxidation has been postulated in the pathogenesis of this disease. IsoLG-protein adducts are increased in the trabecular meshwork isolated from cadaver eyes obtained from donors with diagnosed glaucoma compared to normal controls (Govindarajan, Laird, Salomon, & Bhattacharya, 2008). Calpain-1 was identified as a major protein that underwent IsoLG modification in these eyes (Govindarajan, et al., 2008). Exposure of astrocytes isolated from human optic nerves or from rat brain to high pressure to mimic glaucoma results in increased levels of IsoLG protein adducts (C. Charvet, et al., 2011). Treatment with the 2-AMP pyridoxamine reduces IsoLG protein levels in these astrocytes (Govindarajan, Junk, Algeciras, Salomon, & Bhattacharya, 2009).

Macular degeneration is another significant eye disease where lipid peroxidation has been implicated. Exposure of eye tissue to UV light is considered to be a significant driver of this lipid peroxidation. IsoLG protein adducts are increased in retina isolated from patients with age-related macular degeneration (C. Charvet, et al., 2011; C. D. Charvet, Saadane, et al., 2013). IsoLG-PE adducts are also elevated in the plasma of these patients compared to healthy volunteers (Li, et al., 2009). Exposing mice to bright light elevates levels of IsoLG adducts in their retina, but pretreatment of the mice with the 2-AMP pyridoxamine blunts this increase in adducts (C. D. Charvet, Saadane, et al., 2013). Proteomic studies identified the CYP27A1 as a major protein modified by IsoLG in the retina, identified the Lys residues of CYP27A1 that were modified, and showed that these adducts are present in retina isolated from patients with age-related macular degeneration (C. Charvet, et al., 2011). IsoLG modification of CYP27A1 significantly reduced its activity, which could contribute to altered cholesterol homeostasis and retinal injury (C. D. Charvet, Laird, Xu, Salomon, & Pikuleva, 2013).

5.12 Sepsis.

Pathogen challenge with *Candida* results in elevated plasma levels of IsoLG adducts in wild-type mice (107.1 ± 34.6 pmol/mg protein) compared to control mic (28.4 ± 10.7 pmol/mg protein) (Poliakov, et al., 2003). The same challenge in mice lacking myeloperoxidase results in significantly lower levels of IsoLG protein adduct, suggesting that this enzyme which is released from neutrophils and macrophage during inflammatory responses to bacterial pathogens is a major driver of IsoLG formation under these conditions (Poliakov, et al., 2003). To the best of our knowledge, there has not yet been any studies undertaken with 2-AMPs to determine if reducing IsoLG and other dicarbonyl adducts will improve outcomes in sepsis.

5.13 COX induction.

IsoLG is formed by non-enzymatic rearrangement of PGH₂ (Boutaud, et al., 2001; Boutaud, et al., 1999). We have demonstrated that IsoLG-Lys adducts are increased in cells after induction of COX-2 or activation of COX-1 (Boutaud, et al., 2003; Carrier, et al., 2014; I. Zagol-Ikapitte, et al., 2010) These observations were extended to in vivo studies where we showed that overexpression of human COX-2 in neurons lead to ~10 folds increase in IsoLG-Lys adducts formation in the the brain compared to non-transgenic animals (Boutaud, Andreasson, Zagol-Ikapitte, & Oates, 2005) Recently, we showed that IsoLG-Lys adducts were increased in the posterior cortex of the APP-PS1 mouse model of AD and that treatment of these mice with ibuprofen lowered IsoLG-Lys adducts level to those of control non-transgenic mice (Woodling, et al., 2016) Taken together, this evidence suggests that COX activity is a major contributor to IsoLG-Lys adduct formation and that they may contribute to any pathology associated with COX-2 induction or inflammation.

6. Moving 2-AMPs towards the clinic

The importance of protecting proteins from covalent modifications by dicarbonyl scavengers led us to study their pharmacokinetics in animals. Preliminary studies showed that 2-AMPs could be administered in the drinking water, are well-tolerated by mice, and are widely-

distributed in tissues, including the brain (Zagol-Ikapitte, Amarnath, Jadhav, Oates, & Boutaud, 2011; I. A. Zagol-Ikapitte, et al., 2010). In mice, both plasma and tissue half-lives of 2-HOBA are approximately one hour (I. A. Zagol-Ikapitte, et al., 2010). Because of the protective effects shown in animal models of hypertension and AD, the use of 2-AMPs are currently under development to be used in human studies.

6.1 Small animal ADME studies with 2-HOBA

Preclinical studies were performed in mice, rats and rabbit to determine the safety and lack of toxicity of 2-HOBA in animals. 2-HOBA was administered to rodents or rabbits for up to 90 consecutive days. All three studies showed 2-HOBA lacks toxicity when administered orally at doses up to 1,000 mg/kg of body weight (Fuller, Pitchford, Abumrad, & Rathmacher, 2018a, 2018b; Pitchford, Smith, Abumrad, Rathmacher, & Fuller, 2018). The lack of toxicity demonstrated in these studies supported further development of 2-HOBA as a potential intervention for human diseases.

6.2 Metabolism of 2-HOBA in humans

Prior to administration in humans, the evaluation of 2-HOBA safety pharmacology was done *in vitro* (Fuller, Pitchford, Morrison, et al., 2018). 2-HOBA was not found to be mutagenic, and did not significantly induce CYP1A2, CYP2B6 or CYP3A4 in human hepatocytes. Also, it had a high unbound fraction in human plasma and had no safety concerns related to QT prolongation. The major hepatic metabolite of 2-HOBA is salicylic acid and is similar in human, rat and rabbit. A minor (less than 5%) metabolite was identified as the glucoside conjugate of 2-HOBA. Altogether, these results and the fact that 2-HOBA is a natural component of buckwheat support the development of 2-HOBA as a nutritional supplement and a first in human safety study was initiated at Vanderbilt University.

The safety, tolerability and pharmacokinetics for a single dose of 2-HOBA were tested in healthy human volunteers (Pitchford, et al., 2019). The results showed that 2-HOBA is safe in single doses up to 560 mg. 2-HOBA is rapidly absorbed and eliminated with a mean half-life of 2.1 h in humans. Using these pharmacokinetics results, we designed a multiple ascending dose study giving 2-HOBA 373 mg and 560 mg 3 times a day for 2 weeks. The main goal of this study was assessing safety and monitoring possible accumulation kinetics of 2-HOBA and its major metabolite, salicylic acid. We also measured the inhibition of all prostaglandin urinary metabolites as a marker of COX inhibition by salicylic acid. The results of this study are currently under review for publication.

One of the limitations of our study in healthy volunteers is the relatively narrow age range of the participants. To palliate to this limitation, we initiated another study at Vanderbilt, assessing the safety of the same two doses of 2-HOBA in an older population. This study is also investigating the effect of 2-HOBA treatment on levels of IsoLG-Lys adducts in HDL and on levels of MDA-Lys adducts in platelets *in vivo*. Results of this study will provide evidence for generalizing our results to a broader population and also should provide efficacy data in humans.

These safety studies are the first step to studying the effects of 2-HOBA in humans for pathologic conditions which may include atrial fibrillation, thrombosis and AD. These proof

of concept studies of efficacy will be the next step toward developing 2-HOBA as a therapeutic agent for treating these diseases.

Abbreviations

2-AMP	2-aminomethyphenol
2-HOBA	2-hydroxybenzylamine
4-HOBA	4-hydroxybenzylamine
AD	Alzheimer's Disease
APC	Antigen Presenting Cells
apoAI	apolipoprotein A-I
COX	cyclooxygenase
CYP	cytochrome P450 enzyme
DC	dendritic cells
GERD	gastroesophageal reflux disease
GSH	glutathione
HDL	high density lipoprotein
HNE	4-hydroxy-nonenal
HxPM	5'- <i>O</i> -hexyl-pyridoxamine
HpPM	5'- <i>O</i> -heptyl-pyridoxamine
IsoP	isoprostane
IsoLG	isolevuglandin
IsoLG-Lys	isolevuglandin-lysyl-adducts
Lys	lysine
LDL	low density lipoprotein
MDA	malondialdehyde
MGO	methylglyoxal
ONE	4-oxo-nonenal
PAH	pulmonary arterial hypertension
PE	phosphatidylethanolamine
PGH₂	prostaglandin H ₂

PM	pyridoxamine
PPM	5'- <i>O</i> -pentyl-pyridoxamine
PPO	5'- <i>O</i> -pentyl-pyridoxine
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
TCR	T cell receptors

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Fig. 1.
Structures of the major reactive lipid aldehydes (MDA, HNE, ONE, MGO, and IsoLG) discussed in this review.

Fig. 2.
Formation of IsoLGs by non-enzymatic rearrangement of bicycloendoperoxides. Each individual IsoLG structure represents 8 potential stereoisomers due to the three racemic bonds (denoted by wavy lines).

Fig. 3.

Formation of 8 IsoLG regioisomers from the 4 H₂-IsoP regioisomers. The specific regioisomer of IsoLG is designated using the same IUPAC nomenclature as for isoprostanes. First by the carbon position of the hydroxyl moiety (5-, 8-, 12-, or 15-), followed by D or E to indicate the relative position of the ketone to the aldehyde, and finally a subscript to designate the number of double bonds in the side chains. D-regioisomers have the aldehyde moiety on the upper side chain with the carboxylate moiety and the ketone on the lower side chain, while E-regioisomers have the ketone on the upper side chain and the aldehyde on the lower side chain. Each regioisomer can have 8 stereoisomers due to the three racemic bonds.

Fig. 4.
Other 1,4-dicarbonyls produced by peroxidation of PUFAs

Fig. 6.
Reaction of IsoLG with primary amine to form pyrrole adducts.

Fig. 7.
Reaction of MDA with lysine.

Fig. 8.
Reaction of MGO with arginine.

Fig. 9.
Reaction of HNE with cellular nucleophiles.

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Fig. 11.
PM analogs used in initial structure activity relationship assays

Fig. 12.
Proposed mechanisms for 1,4-dicarbonyl scavenging by 2-AMPs.

Fig. 13.
Proposed mechanism for reaction of MDA with 2-AMPs

Fig. 14.
Proposed reaction mechanism of MGO with 2-AMPs

Fig. 15.

Proposed mechanism for reaction of ONE with 2-AMPs

Fig. 16.
Proposed mechanism for reaction of HNE with 2-AMPs.

Figure 17: Pathway illustrating how isolevuglandins may activate the immune system in cardiometabolic disease:

Increased oxidative stress in antigen presenting cells (APCs) leads to increased formation of isolevuglandins. This leads to activation APC leading to production of proinflammatory cytokines IL-1B, IL-6 and IL-23. These APC activate T cells to proliferated and produce cytokines that promote hypertension and obesity.

Table 1

Second-Order Rate Constants for Pyrrole Formation with 4-oxo-pentanal

amine	$k \times 10^3 \text{ sec}^{-1}\text{M}^{-1a}$	relative rate
N ^ε -acetyl-lysine	0.180 ± 0.003	1
4-picolinylamine	4.79 ± 0.11	26
PM	493 ± 19	2,740
PPM	513 ± 27	2,850
2-HOBA	359 ± 21	1,994
5-methyl-2-HOBA	495 ± 18	2,750
2-methoxybenzylamine	0.65 ± 0.012	4
4-HOBA	7.7 ± 0.25	43

^a phosphate buffer (pH 7.4); T = 25 °C

Table 2

Rate Constants for Reaction with MDA

amine	$k \times 10^3 \text{ sec}^{-1} \text{M}^{-1}{}^a$
N-acetyl-lysine	Estimated ^b
2-HOBA	28.7 ± 0.33
5-methyl-2-HOBA	18.8 ± 0.10
PPM	93.9 ± 5.9
4-HOBA	12.8 ± 0.28
N-methyl-2-HOBA	11.2 ± 0.16

^a phosphate buffer (0.1 M, pH 7.4) at 37 °C.^b (Koji Uchida, Sakai, Itakura, Osawa, & Toyokuni, 1997)

Table 3

Lipophilicity and second-order rate constant for reaction of amines with MGO

amine	lipophilicity ^a	$k^b \times 10^3 \text{ sec}^{-1}\text{M}^{-1}$
2HOBA	0.18	1.5 ± 0.20
PM	0.08 ± 0.03	3.1 ± 0.16
PPM	2.22 ± 0.14	62.8 ± 7.6
HxPM	10.90 ± 0.83	272 ± 21.6
HpPM	92.6 ± 4.3	948 ± 45

^aThe ratio of concentrations of the amine in ethyl acetate and in water (pH 7.4) after equilibration at ambient temperature (24 °C).^bSecond-order rate constant for the disappearance of the amine, at pH 7.4 and 37 °C

Table 4

Second-Order Rate Constants for Reaction with ONE

amine	$k \times 10^3 \text{ sec}^{-1}\text{M}^{-1}$
2-HOBA	70.2 ± 2.9
5-chloro-2-HOBA	215.6 ± 7.3
PM	86.5 ± 2.8
PPM	347 ± 19.9

In 3:2 phosphate buffer (pH 7.4) and acetonitrile; T = 37 °C