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Lipoxidation-Derived Reactive Carbonyl Species as Potential Drug Targets in Preventing Protein Carbonylation and Related Cellular Dysfunction

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1. Introduction

Reactive oxygen species (ROS) normally exist in cells and tissues at low concentrations and the fact that ligand-stimulated ROS generation plays a role in signal transduction suggests that ROS are involved in the redox regulation of many physiological functions.[1] Nevertheless, they can also cause extensive damage. Oxidative stress can occur when the mechanisms involved in maintaining the normal reductive cellular milieu are impaired, when ROS production is accelerated, or both, and can lead to the destruction of cellular components, which results in the deterioration of cellular structure and signalling, and ultimately death by apoptosis or necrosis.

ROS-induced modifications of cell and tissue components do not necessarily translate to a pathogenic phenotype, because processes can be activated to sustain physiological functions.

Cells and tissues have antioxidant compounds, including antioxidant enzymes and nonenzymatic proteins, to scavenge or otherwise eliminate ROS, and enzymes for reparation of some protein oxidative modifications, that is, intra- and inter-chain protein disulphides, most oxidative modifications of Cys (for example, glutathionylation and cysteinylation), Met sulfoxidation, and Tyr nitration.[2–6]

ROS and secondary low-molecular-weight reactive carbonyl species (RCS) derived from carbohydrates, lipids, or amino acid oxidation may interact with proteins to cause oxidation of their polypeptide backbone, peptide bond cleavage, protein–protein cross-linking, and a range of amino acid side-chain modifications.[7] Carbonyl moieties are produced on protein side chains of Lys, Arg, Pro, and Thr, when these amino acids are oxidised into ketone or aldehyde derivatives. In parallel, protein carbonyls (PCOs) can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the *N*-terminal amino acid is blocked by an α-ketoacyl derivative. Reactive aldehydes derived from peroxidation of lipids, such as 4-hydroxynonenal (HNE), 2-propenal (acrolein, ACR), malondialdehyde (MDA), and glyoxal (GO) covalently modify proteins through carbonylation and may contribute to oxidative tissue damage. These carbonylated compounds are known as advanced lipoxidation end products (ALEs), formed by Michael-addition of these reactive aldehydes to the nucleophilic side chain of Cys, His, or Lys residues. Reducing sugars and the corresponding oxidation products (GO and methylglyoxal, MGO) can also modify proteins through carbonylation by reacting at the primary amino group of Lys residues, forming advanced glycation end products (AGEs).[8]

Since carbonylation can alter protein structure and function, and cause the formation of protein aggregates, the "carbonyl stress" hypothesis emphasises the role of RCS, derived from different sources through both oxidative and nonoxidative reactions, and resulting from decreased renal detoxification, excretion of reactive carbonyl precursors of AGEs/ALEs from plasma, or both, in the induction of pathogenic protein modifications.[9–11]

2. Protein Carbonylation in Disease

Studies on protein carbonylation have been greatly facilitated by the availability of a number of robust and accurate methods, including redox proteomics and mass spectrometry technologies, to detect and quantify protein-bound carbonyls in cells, tissues, and body fluids.[12–16] Therefore, protein carbonylation constitutes one of the best characterised biomarkers of oxidative stress and oxidative damage in several conditions and diseases.[15,17–19]

Elevated protein carbonylation and related accumulation of oxidised proteins have been found during aging and in various diseases such as Alzheimer's disease (AD), chronic renal failure, diabetes, rheumatoid arthritis, and chronic lung disease.[8,12,15,17,19]

For instance, the plasma PCO content of children with juvenile rheumatoid arthritis is much higher than in healthy children, and increases with inflammatory process activity.[20] Thus,

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it would appear to be a good marker of inflammatory process activity and disease progression, and may be of use in monitoring possible pharmacological treatments. Elevated PCO concentrations, which correlated well with ALE measurements and indices of neutrophilia and neutrophil activation, have also been reported in critically ill patients following major trauma or sepsis.[21] Patients with acute pancreatitis had a significantly increased concentration of PCOs in plasma, which related to disease severity, thus confirming that this protein modification could be a useful biomarker of oxidative injury.[22] Carbonylated proteins were increased in bronchoalveolar lavage (BAL) fluid of patients with sarcoidosis, idiopathic pulmonary fibrosis, and systemic sclerosis, relative to healthy controls, with a significant difference for those with sarcoidosis and idiopathic pulmonary fibrosis. The proteomic approach to the analysis of BAL fluid revealed that protein carbonylation is a process involving specific carbonylation-sensitive proteins and that in idiopathic pulmonary fibrosis a greater number of proteins are the target of oxidation.[23]

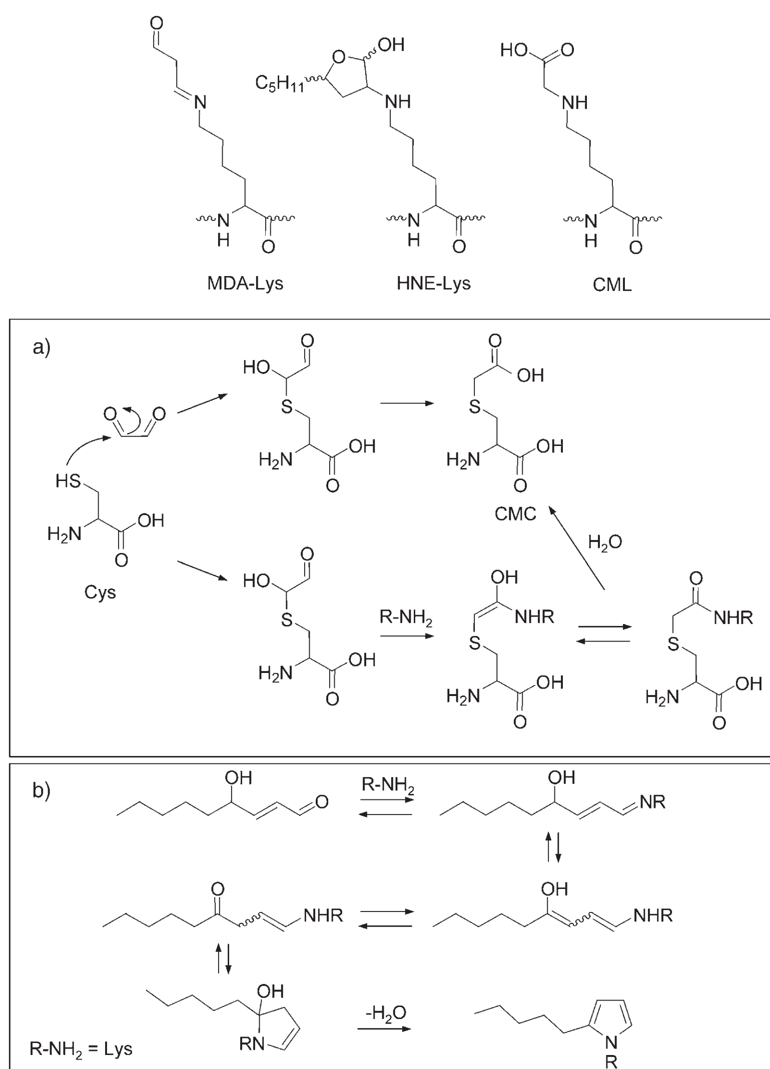

Figure 1. Structures of the main ALEs and mechanisms of their formation. a) Adapted from Zeng and Davies (2005)[29] and b) adapted from Sayre et al. (1993)[25] with permission of the American Chemical Society.

2.1. ALE formation: reaction mechanisms

Most of the biological effects of intermediate lipid-derived RCS are attributed to their capacity to react with the nucleophilic sites of proteins to form advanced lipoxidation end products (ALEs), such as MDA–Lys (Schiff base adduct), HNE–Lys (Michael adduct),[24] HNE–Lys (pyrrole derivative),[25] FDP–Lys [N^ε-(3-formyl-3,4-dehydropiperidino)lysine],[26] MP–Lys [N^ε-(3-methylpyridinium)lysine,[27] levuglandin adducts (pyrrole derivatives),[28] CMC [*S*-(carboxymethyl)cysteine],[29] CML [N^ε-(carboxymethyl)lysine],[30] and GOLD (GO–Lys dimer).[31,32] In particular, CML is now considered to be a general marker of carbonylic stress and long term damage to proteins in aging, AD, atherosclerosis, and diabetes.[33] Figures 1 and 2 show the structures of the most important ALEs and the intermediate RCS involved in their formation. Although the mechanism for some of them is still uncertain, it is now established that CML and CMC are formed by reaction of GO with Lys and Cys residues respectively (reaction of GO with a sulfhydryl group sets the stage for a Cannizzaro reaction that leads to formation of CMC) (Figure 1 a). The mechanism of carboxymethylation of Lys by GO to give CML could involve the formation of a Schiff base adduct, followed by either classical enolization, dehydration, and elimination reactions[34] or a Cannizzaro-type rearrangement.[30,35] The condensation between HNE and Lys residues which leads to pyrrole is multistep (Figure 1 b). Studies on the Paal-Knorr condensation of amines with 1,4-dicarbonyl compounds[25] showed that initial Schiff base reactivity of HNE with amines could lead to a common intermediate in the pathway for pyrrole formation (see below). Levuglandin (isoketals) adducts (Figure 2 a) are formed by reaction of highly reactive γ-ketoaldehydes (generated from arachidonic acid oxidation by the H_2-isoprostane pathway) with Lys residues, with a mechanism similar to that described for α,β-unsaturated aldehydes.[28] This involves formation of a hemiaminal adduct that, after dehydration, forms the reversible imine (Schiff base). Unlike the α,β-unsaturated aldehydes, the remaining carbonyl function in the γ-dicarbonyl-derived hemiaminal can undergo intramolecu-

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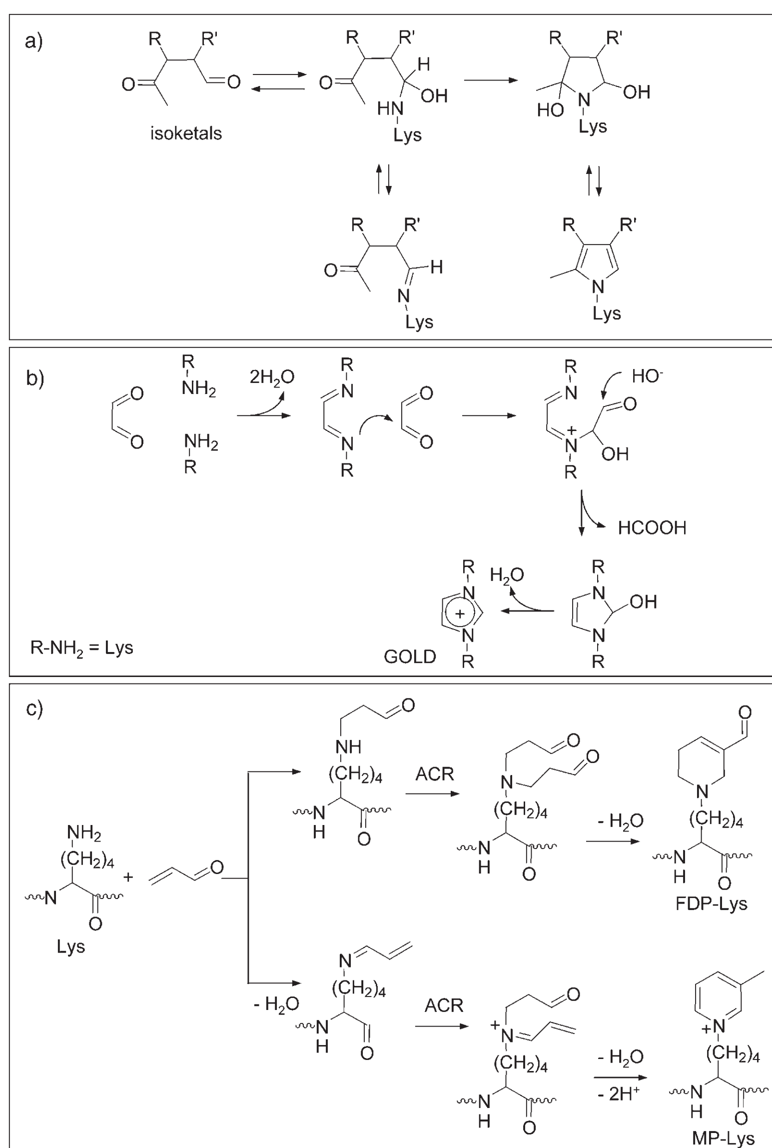

Figure 2. Structures of the main ALEs and mechanisms of their formation. a) Adapted from Davies et al. (2004)[28] with permission from Elsevier; b) adapted from Wells-Knecht et al. (1995)[31] with permission from the American Chemical Society.

lar nucleophilic attack by the amine, the unstable intermediate pyrrolidine adduct then undergoes dehydration to give the irreversible pyrrole adduct.

The dicarbonyl structure of GO makes it able to react with two Lys residues to form protein imidazolium cross-links (that is the GO–Lys dimer, GOLD). GOLD was originally isolated from reactions of GO with the model peptide N^{α}-hippuryllysine:[31,32] the proposed mechanism for imidazolium cross-link formation includes the initial reaction of the dicarbonyl with two lysine molecules forming a labile Schiff base, diimine cross-link, and then recruitment of a second molecule of GO for the cyclisation reaction (Figure 2 b). This intermediate undergoes a Cannizzaro-type rearrangement after nucleophilic attack by hydroxide, yielding a five-membered ring structure which loses a hydroxyl group to form GOLD. The mechanism proposed by Uchida[26] for FDP–Lys formation (Figure 2 c) involves nucleophilic addition of the amino group to the ACR double bond (C3) to give a secondary amine with retention of the aldehyde group. This intermediate reacts with another ACR molecule by a Michael addition forming a derivative that, after aldol condensation and dehydration, gives the FDP–Lys adduct. The formation of MP–Lys may be reasonably explained by the mechanism involving the formation of a Schiff base derivative as the first intermediate.[27] The Schiff base further reacts with a second ACR molecule by a Michael addition to generate an imine derivative. The subsequent conversion of this imine derivative to the final product (MP–lysine) requires two oxidation steps and intramolecular cyclisation, but its detailed mechanism has not yet been elucidated.

2.2. Advanced lipoxidation end products (ALEs) in chronic human diseases

Age-related diseases are characterised by an increase in chemical damage to proteins in specific organ systems. This is most obvious for chronic diseases such as atherosclerosis and diabetes. Diseases such as atherosclerosis,[36,37] vascular injury,[9] diabetes, diabetic complications (neuropathy, nephropathy, retinopathy, and lens disorders), and hyperlipidaemia, [9,11,18,38–40] are associated with increased chemical modification, by both ALEs and AGEs, of plasma and tissue proteins at the sites of pathology. MDA plasma concentration is increased in diabetes mellitus and MDA is found in the atherosclerotic plaques promoted by diabetes.[41] Adducts of apolipoprotein B-100 Lys residues with MDA and HNE have been characterised extensively in human atherosclerotic lesions.[36,37,42] ACR reacts with Lys residues of apolipoprotein A-I (apoA-I), the major protein of the high-density lipoprotein that plays a critical role in mobilising cholesterol from artery wall macrophages. ACR adducts are co-localised with apoA-I in human atherosclerotic lesions. Moreover, the ability of ACR-modified apoA-I to remove cholesterol from cultured cells is impaired, suggesting that carbonylation might interfere with the normal function of apoA-I (promoting cholesterol removal

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from artery wall cells), thus playing a critical role in atherogenesis.[43]

The accumulation of RCS is recognised as a common feature of the aging of tissue proteins, and levels of these compounds are increased either systemically or locally in a broad range of diseases, including renal, hepatic, neurodegenerative diseases, diabetes, and atherosclerosis.[14,44–46] Proteins modified by ALEs accumulate in patients with diabetic nephropathy,[47] in haemodialysis patients,[48] type 2 diabetes outpatients,[49] dialysis-related amyloidosis,[50] human alcoholic liver diseases,[51] osteoporosis,[52] AD, and other neurodegenerative diseases including progressive supranuclear palsy, Pick's disease, Lewy bodies related diseases, amyotrophic lateral sclerosis (ALS), and Huntington's disease.[53,54]

The rate of ALEs (and AGEs) accumulation in diabetes is related to the severity of complications, rises linearly with age, and correlates with the severity of microvascular disease.[9]

ALEs are a major factor in the pathogenesis of atherosclerosis. The peroxidation of lipids in lipoproteins in the vascular wall leads to local production of RCS that mediate recruitment of macrophages, cellular activation and proliferation, and chemical modification of vascular proteins by ALEs. ALEs and their precursors affect the structure and function of the vascular wall, setting the stage for atherogenesis. ALEs affect not only the structure and recognition of tissue proteins, including lipoproteins, but also modify the charge, hydrophobicity, and elasticity (cross-linking) of the extracellular matrix of the vascular wall.[42] The increased risk for atherosclerosis in diabetes may result from additional carbonyl production from carbohydrates and additional chemical modification of proteins by AGEs.[42] However, it should be noted that ALEs are present at only trace levels in proteins. For example, CML, MDA–Lys, and HNE–Lys have been detected in atherosclerotic plaques, but the total extent of lysine modification by all of these compounds is less than 1% of the Lys residues in plaque proteins, even at advanced age or advanced stages of disease.

Products of auto-oxidation or metabolism of amino acids are also sources of ALEs, such as CML,[55] so that it is difficult to determine their actual source in tissues by chemical analysis alone, even during hyperglycaemia in diabetes. Furthermore, formation of ALEs and AGEs is not an isolated process, but part of a range of oxidative chemical modifications of tissue proteins that increase in aging and disease. AGEs, ALEs, and amino acid oxidation products, including tyrosine oxidation products (chloro-, nitro-, and dityrosine) and methionine sulfoxide, appear together at sites of tissue injury and inflammation, such as atherosclerotic plaques, and in protein deposits in neurodegenerative diseases such as AD and PD.[56]

Increased concentrations of HNE–protein adducts have been reported in the lungs of smokers with and without chronic obstructive pulmonary disease (COPD). Notably, HNE concentration in the pulmonary epithelium, airway endothelium, and, particularly, in neutrophils of COPD patients was found to be inversely associated with lung function.[57] Diaphragms of severe COPD patients showed both higher PCOs and HNE–protein adducts than controls. Furthermore, negative correlations were found between carbonyl groups and airway obstruction (that is, reactive carbonyl levels correlated with the disease severity), and between HNE–protein adducts and respiratory muscle strength (that is, HNE–protein adduct formation correlated with respiratory muscle function).[58] In addition, elevated MDA levels were found in both plasma and breath condensate in asthmatics.[55]

HNE-derived epitopes have been detected in a variety of animal models of oxidative stress, and in tissues prepared from humans having clinically diverse diseases associated with oxidative stress.[59] Collectively, the results of these studies document the association of HNE-derived epitopes with diseases linked, directly or indirectly, with chronic inflammation. The involvement of HNE in the pathogenesis of several human diseases is likely related to its reactivity towards cellular nucleophiles. Low, basal levels of HNE are present in cells ($<1\,\mu\text{M}$), and may act as a signalling molecule at these concentrations. However, under conditions of oxidative stress, uncontrolled production of HNE may saturate pathways for metabolism, yielding unwanted modifications of biological molecules and initiation of a disease process.[60]

HNE and MDA adducts of protein Lys residues have been detected under a number of experimental and disease conditions, such as human renal carcinoma,[61] nigral neurons of Parkinson's patients,[62] hepatitis C, haemochromatosis, and other chronic liver diseases.[63]

Increased levels of ACR– and HNE–protein adducts have also been found in both cardiovascular and neurodegenerative diseases.[45,59,64] Numerous studies have demonstrated increased lipid peroxidation (as assessed by increased levels of both free and protein-bound HNE and ACR), isoprostanes, and neuroprostanes in the brains of subjects who died of AD relative to age-matched controls, whereas lipid peroxidation is not a significant feature of usual aging.[65–68] ACR-modified proteins accumulated in vulnerable regions and specifically, in the paired helical filaments in brains of patients with AD.[69,70] HNE–protein adducts are demonstrable in senile plaques and tangles in AD, in tissue, including cerebrospinal fluid, from amyotrophic lateral sclerosis (ALS) patients, and Lewy bodies in Parkinson's disease (PD).[59,62,68,71] In particular, the glial glutamate transporter GLT-1 (EAAT2) has increased binding of HNE in the brain of subjects afflicted with AD.[65]

Neurofilament proteins are major targets of HNE modification.[72] The phosphorylation-dependent carbonylation of tau by HNE contributes to the generation of the major conformational changes associated with neurofibrillary tangles.[73] Individual proteins (dihydropyrimidinase-related protein 2, heat-shock protein 70, and α-enolase) modified by HNE have been identified in the spinal cord tissue of the G93A-SOD1 transgenic mouse model of familial ALS.[74]

Recent mass spectrometric data demonstrate significant, although heterogeneous, increases in the direct carbonylation of amino acids and an increase in glycoxidative and lipid peroxidation-derived protein damage in brain samples from AD patients compared to aged individuals.[75] In addition, it has been shown that HNE is covalently bound in excess to the α class of glutathione *S*-transferase, which can detoxify HNE, and the multidrug resistance protein-1 in the brain of AD-affected indi-

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viduals. Collectively, the data suggest that HNE may be an important mediator of oxidative stress-induced impairment of this detoxifying system and may therefore play a role in promoting neuronal cell death.[76]

3. Intervention Strategy Aimed to Prevent Protein Carbonylation

RCS generated from oxidised polyunsaturated fatty acids (PUFAs) and the corresponding adducts with proteins (that is, carbonylated proteins) are widely used as biomarkers of lipid-peroxidation and, in general, of oxidative stress. A strict correlation between carbonyl stress and certain human diseases is well established (see previous section). Whether RCS represent a cause or an effect is still to be fully clarified, although, for some diseases, we believe convincing evidence support a RCS pathogenic role, such as in the case of diabetic-related diseases, age-dependent tissue dysfunction, and metabolic distress-syndrome. Consequently, RCS, in addition to being a predictive biomarker, also represents a biological target for drug discovery.

3.1. Molecular targets for preventing RCS-mediated diseases

Taking RCS and carbonylation damage as drug-targets, different molecular strategies can be considered as summarised below (Figure 3):

1. Inhibition of the lipid-peroxidation chain-reaction, the primary source of RCS, by a direct (radical-scavenging), indirect (metal-ion chelating) mechanism, or both. Regarding the radical-scavenging approach, different target radicals should be considered: 1) ROS, such as $^{\bullet}OH$ and its radical precursor $O_2^{\bullet-}$ (through the formation of H_2O_2), which are responsible for initiating the lipid-peroxidation process through an H-abstraction mechanism; 2) lipid alkoxyl, peroxyl, and dienyl radicals which propagate the chain-reaction. The indirect antioxidant mechanism is based on the deactivation of the pro-oxidant effect of transition metal-ions through a chelating mechanism.
2. Inactivation of the unstable intermediate products of lipid-peroxidation, whose breakdown leads to the formation of RCS.
3. Scavenging of RCS, forming nonreactive and noncytotoxic reaction products.
4. Induction of the enzymatic or nonenzymatic degradation of accumulated ALEs, and generally of undigested oxidation reaction products.

The intervention strategy based on a direct radical-scavenging (antioxidant) approach that provides a "first line of defence" against free radicals, such as vitamin E and vitamin C, has failed to show beneficial effects in RCS-dependent diseases. There is limited evidence, from humans and animal models of atherosclerosis, that antioxidant therapy alone or diet supplementation can provide protection.[77–79] Several clinical trials have failed to provide clear evidence for the efficacy of the antioxidant treatment in diabetic subjects,[80] although growing evidence shows that ROS and RCS production is increased in diabetes and that oxidative stress is associated with the long-term complications of diabetes. Some of these trials have been criticised in terms of insufficient dosing regimens or duration of antioxidant therapy, harmful interactions between the antioxidant compounds, or flaws in enrolling or excluding subjects. Neurodegenerative disorders, including PD, AD, and ALS, clearly display increased indices of ROS and RCS (especially ACR and HNE) in affected brain regions. Antioxidant treatments have displayed different effects in human patients: for example, whereas vitamin E has displayed some efficacy for the

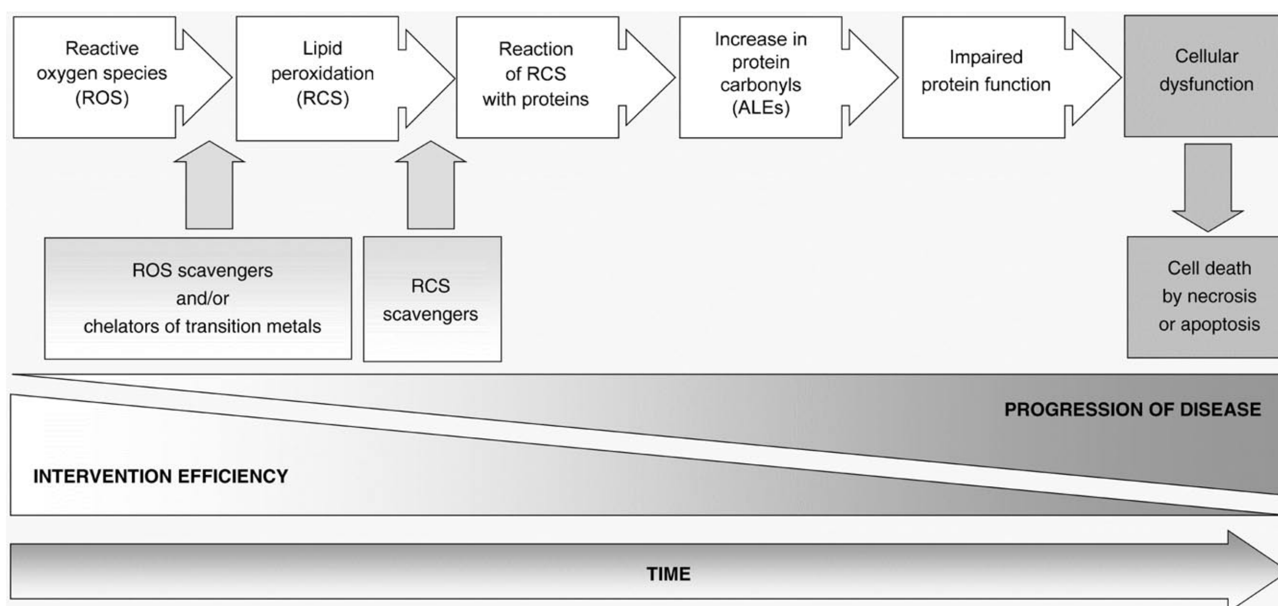

Figure 3. Different molecular strategies and targets aimed at preventing RCS-mediated diseases.

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treatment of AD, it has not proved useful so far for treatment of PD.[81] One possible limitation of antioxidant therapy is that significant damage to macromolecules and tissue injury, which ultimately lead to cell death, will already have occurred by the time overt symptomatology of the disease is observed. Hence, antioxidant therapy can only, at best, rescue undamaged macromolecules and surviving cells, an effect that might not be sufficient to attenuate symptomatology.[17]

Among the molecular approaches summarised above, the most promising in terms of preventing or inhibiting carbonyl stress-related diseases is based on those compounds able to deactivate the intermediate products of lipid peroxidation (pyridoxamine) or to scavenge and thus, neutralise RCS. Although several studies have already been conducted to develop AGE breakers, no study on compounds inducing enzymatic or nonenzymatic degradation of ALEs has been reported, although this is an emerging field.

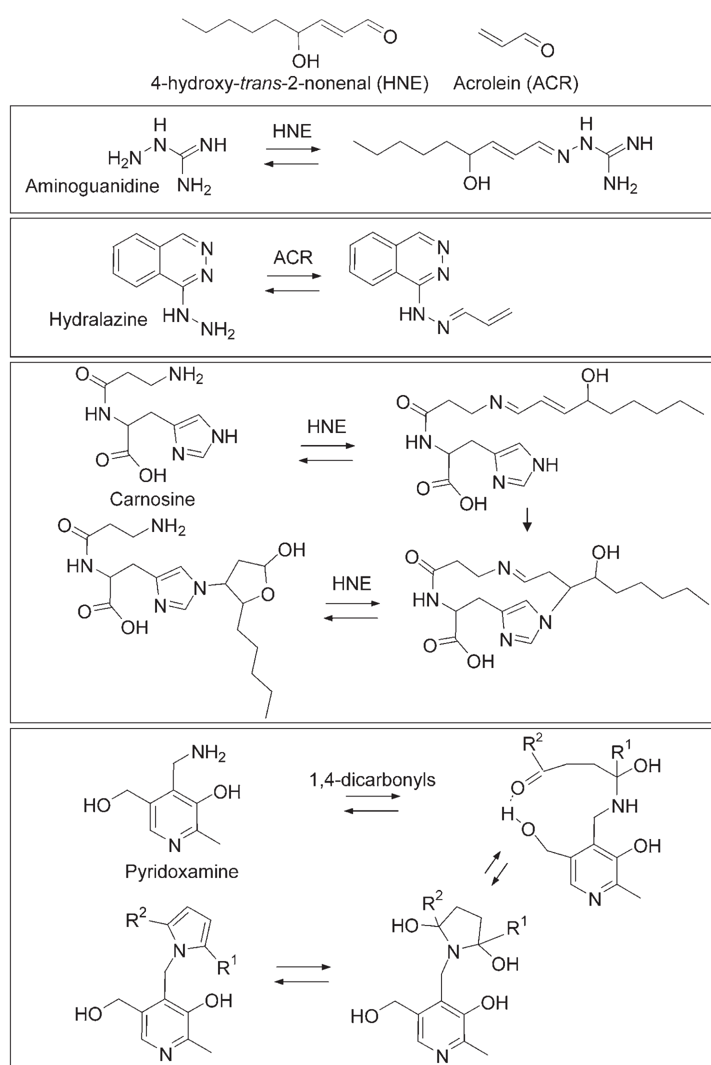

Figure 4. RCS scavengers: structures and reaction mechanisms.

3.2. Reactive carbonyl species scavengers

Compounds described in the literature as RCS scavengers (Figure 4) can be divided into two groups: 1) endogenous or naturally occurring compounds, such as the endogenous dipeptide carnosine (CAR) and the vitamer pyridoxamine (PYR); 2) synthetic compounds initially described by their pharmacological or biological activities, such as the vasodilating antihyperthensive drugs hydralazine (HY) and dihydralazine (di-HY), the iNOS inhibitor aminoguanidine (AG), and the oral hypoglycemic agent metformin (MF). All these compounds have been then identified, using different approaches, as RCS scavengers. PYR,[82,83] which was introduced as a novel and effective post-Amadori inhibitor, was later described as a RCS trapping agent,[84] able to inhibit the chemical modification of proteins (mainly Lys residues) during lipid peroxidation reactions in vitro (in a protein–lipid model system and during copper-catalysed oxidation of low-density lipoproteins).

AG is considered to be a prototype scavenging agent of α,β-dicarbonyls, able to prevent the formation of AGEs from α,β-dicarbonyl precursors such as glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone, and α,β-dicarbonyl moieties of glycated proteins, to form 3-amino-1,2,4-triazine derivatives.[47,85]

CAR was first identified as a scavenger of dialdehydes such as MDA and GO,[86] and later in our laboratory as a scavenger of α,β-unsaturated aldehydes such as HNE and ACR.[87,88] In particular, we demonstrated the CAR scavenging effect towards cytotoxic aldehydes in biological matrices by using a peptidomic approach; with the aim of identifying the main detoxification pathways of unsaturated aldehydes in skeletal muscle, rat gastrocnemius homogenate was spiked with HNE and, after 1 h incubation, peptides and peptide adducts were identified by LC–ESIMS–MS analysis.[87,89] Figure 5 shows the two-dimensional plot of native (upper panel) and HNE spiked (lower panel) sample, the latter characterised by the presence of two adducts at *m/z* 383 and 397, which were identified by MS–MS analysis as Michael adducts of HNE with CAR, and anserine (ANS), respectively.

In addition, CAR–HNE has been shown to be an early, stable, and specific biomarker of oxidative stress in rat skeletal muscle exposed to oxidative damage.[90] These results demonstrate that CAR and related dipeptides (ANS) react in tissues with HNE generated from the lipid peroxidation: this indicates the existence in skeletal muscle, highly susceptible to peroxidative attack, of a histidine dipeptide-dependent detoxification pathway against cytotoxic HNE, which is alternative or concomitant to that involving thiol-containing peptides.

3.3. Reaction mechanism of RCS scavenging

RCS scavengers, although belonging to different chemical classes, are all characterised by at least one nucleophilic centre such as thiol, histidine, or primary amine group, responsible

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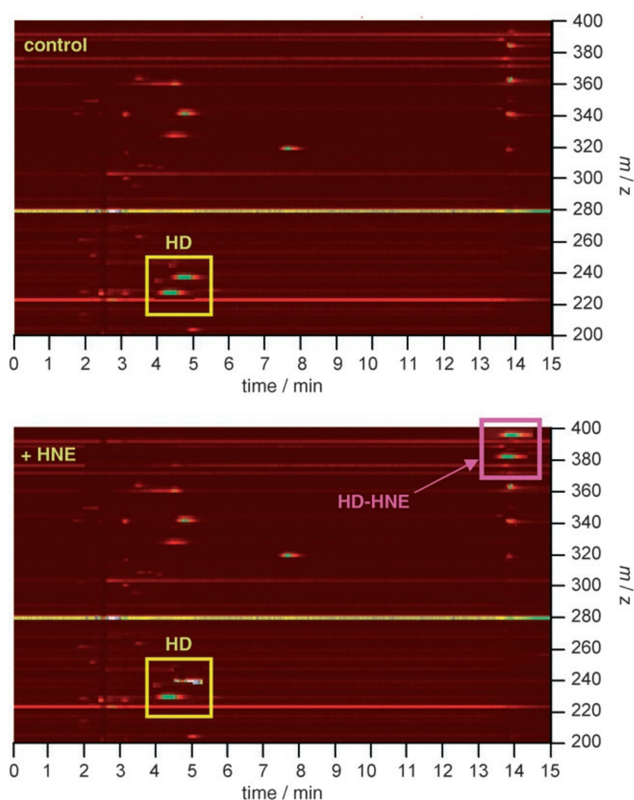

Figure 5. LC–ESIMS–MS analysis (two-dimensional plot *m/z* values against retention times) for identification of endogenous peptides as HNE-sequestering agents in rat skeletal muscle. HD = histidine-containing dipeptides (carnosine and anserine); HD–HNE = HNE Michael adducts with histidine-containing dipeptides.

for the scavenging effect. Although the scavenging mechanism depends on the target aldehyde and on the nucleophilic group of the scavenger, generally, the following reactions are usually involved: 1) Michael adduction between the electrophilic centre of the target aldehyde (such as C3 of HNE) and the nucleophilic group of the scavenger, and 2) resonance-stabilised Schiff base formation between the aldehyde and the primary group of the scavenger compound. Some of the reaction mechanisms are summarised in Figure 4: GSH is a typical example of a RCS scavenger acting through a Michael adduction, while AG and HY scavenge RCS through the formation of a stabilised Schiff base.[91–93] CAR reacts with unsaturated aldehydes by a mixed mechanism involving both the Schiff base formation between the β-alanine amino group and the aldehydic function, which then catalyses the Michael adduction between C3 of the aldehyde and the N^{τ} of the histidine group.[87] The catalytic role of the amino group of β-alanine is well demonstrated by the poor scavenging effect of the *N*-acetyl derivative with respect to CAR, and is explained by the conformation assumed by the imine derivative that favours the Michael adduction by nearing the N^{τ} to C3 (G. Vistoli, personal communication). PYR is characterised by a 2-methyl-3-hydroxyl-5-hydroxymethyl pyridinic ring substituted in position 4 by a nucleophilic aminomethyl moiety. In addition to trapping different classes of RCS such as dialdehydes (MDA and GO)[94,95] and 1,4-dicarbonyls such as the recently discovered 4-ketoaldehydes (levuglandins, isoketals, and neuroketals),[96] PYR is also able to neutralise the intermediate reaction products of lipid-peroxidation such as KODE (keto-octadecadienoic acid), a product of both nonenzymatic and enzymatic peroxidation of linoleic acid, by a mechanism involving the formation of a carbinolamine adduct of PM to the KODE, followed by metal-catalysed, oxidative cleavage of the carbon–carbon bond adjacent to a carbonyl group, yielding the hexanoic amide.[84]

Interestingly, some compounds are characterised by a broad spectrum scavenging ability towards different classes of reactive carbonyls, as in the case of AG, which is an active scavenger of different α,β-dicarbonyls such as GO, MGO, 3-deoxyglucosone, and MDA, and α,β-unsaturated aldehydes. This broad activity is in part due to the strong basic nucleophilic amino group that forms a stable Schiff base with the aldehyde function. However, this mechanism greatly limits the specificity of AG, since it is not only active towards cytotoxic aldehydes, but also towards biogenic and physiological aldehydic compounds such as pyridoxal phosphate.[97] HY, which is characterised by a strong nucleophilic hydrazine group, has been found to react nonspecifically towards physiological carbonyls such as circulating α-keto acids to form hydrazones.[98]

Most of the lipid-peroxidation-derived RCS contain at least two reactive centres, for example, an electrophilic C3 and an aldehyde function (α,β-unsaturated aldehydes), or two aldehyde groups (GO and MDA). Consequently, an efficient and selective carbonyl scavenger should be designed to react with a target RCS through a specific mechanism involving both the reactive centres of RCS, and not to simply interact with a carbonyl function. This type of mechanism avoids cross-reactivity with unreactive and physiologically relevant carbonyl compounds.

This is the case of CAR, which reacts with both the aldehyde and C3 of HNE and ACR through two functional groups as described above. Also PYR is characterised by a scavenging mechanism, which involves two functional groups and makes the compound a specific scavenger towards intermediate hydroperoxides and dicarbonyls (Figure 4). Thus, the optimal scavenger should possess a specific trapping activity towards cytotoxic lipid-derived aldehydes to avoid cross-reactivity with functional and endogenous aldehydes. This specificity can be achieved by the presence of at least two functional groups able to react with the reactive centres of the target RCS.

In addition to specificity, an important feature to be considered is the metabolic stability and the pharmacokinetic profile of the scavenger. To be effective, the RCS scavenger should be present at the site of RCS formation at a certain concentration to compete with the endogenous nucleophilic compounds such as proteins for the carbonylation reaction. Most of the RCS scavengers that have been reported are hydrophilic compounds, easily absorbed from the gastrointestinal tract, and with a plasma half-life not longer than 2 h.[98–102] PYR and AG are excreted mainly in urine, while CAR is hydrolysed by carnosinases to the constitutive amino acids, β-alanine and histidine. The pharmacokinetic properties of PYR and AG explain their role in preventing kidney diseases in diabetic and in obese

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rats, two pathologies where carbonyl stress is supposed to play a causative role. The pharmacological efficacy of some RCS scavengers (AG, PYR, and CAR), extensively studied in several carbonyl stress-related diseases such as diabetes and aging, have been recently reviewed.[103–106] Table 1 summarises the fundamental findings in this field, obtained in different models of diabetic complications and aging.[39,40,56,107–12]

The hydrophilic or lipophilic character of the scavenger as well as its plasma stability are fundamental molecular properties that need to be considered to selectively deliver the scavenger to the target tissue affected by the carbonylation damage. In this context, no lipophilic derivative able to reach the CNS has been reported and this could be an interesting future perspective, as carbonylation damage seems to be involved in several neurodegenerative pathologies including PD, AD, and ALS (see previous section).

No new drug has been designed and developed as a specific RCS scavenger, and only a few examples of structure optimisation of the already known trapping agents are reported. The first is the aminoguanidine-pyridoxal Schiff base adduct (PL–AG), developed as a safer alternative to AG, that has been shown to be more effective than AG in preventing diabetic nephropathy in mice,[122] and diabetic neuropathy and cataracts in STZ-diabetic rats, without decreasing pyridoxal phosphate levels in tissues.[123,124] The second example is the 6-dimethylamino derivative of pyridoxamine (dmaPM), designed to act as both a carbonyl- and a radical-trapping agent.[125]

This compound showed excellent inhibition of AGE formation in vitro[126] and seems to be extremely promising as an ALE inhibitor, but further studies are required to demonstrate its efficacy in vivo. More recently, some hydrazide or 1,2-diol analogues of carnosine, where the His residue was placed at the C terminus instead of the N terminus to avoid recognition by carnosinase, have been synthesised and proposed as neuroprotective agents.[127] Compounds bearing the histidine residue and the hydrazide moiety show the highest scavenging ability against *trans*-2-nonenal, greater than that of carnosine, but only one of them, Z-L-histidyl hydrazide, was efficient in protecting neuroblastoma cells and rat hippocampal neurons from HNE-induced death (carnosine had no significant effect). These results clearly indicate that Z-L-histidyl hydrazide is a candidate for further in vivo tests in animal models of neurodegenerative disorders.

3.4. Screening techniques in the development of RCS scavengers

Due to the growing interest in RCS sequestering agents, an integrated approach aimed to screen, select, and optimise RCS scavengers is required. As summarised in Scheme 1, the ap-

Table 1. Pharmacological efficacy of RCS scavengers in different experimental models.

Experimental model	Pharmacological effects	Reference
PYRIDOXAMINE		
STZ-induced diabetic rat	↓ retinopathy	[39]
(Type I diabetes)	↓ AGE, ALE accumulation in retina	
	↓ renal disease development	[113]
	↓ AGE, ALE, MDA–Lys in skin collagen and ↓ dyslipidaemia	
	↓ peripheral neuropathy development	[56]
Zucker obese rats	↓ albuminuria	[40]
(Type-2 diabetes)	↓ creatinemia	
	↓ systolic blood pressure	
	↓ dyslipidaemia	
ApoE KO mice + STZ	↓ total plaque area	[114]
(Type-1diabetes-accelerated atherosclerosis)		
AMINOGUANIDINE		
STZ-induced diabetic rats	↓ neuropathy, retinopathy, nephropathy	[107]
	↓ early cardiac hypertrophy	[115]
Aged Fisher 344 rats	↓ aortic stiffening	[116]
	↑ arterial and ventricular function	[117]
	↓ AGEs, ALEs in serum, aorta, and heart	[118]
	↓ HNE-modified proteins	
ApoE KO mice + STZ	↓ total plaque area	[119]
OLEF rats	↓ albuminuria, mesangial expansion	[108]
(Type-2 diabetes)		
Diabetic dogs	Prevention of retinopathy development	[112]
CARNOSINE		
Senescence Accelerated Mice	↑ mean lifespan	[110]
Global ischaemia in rat brain	↓ ischaemic injury and mortality	[109]
	↑ heart contractility	[111]
Ischaemia/reperfusion-induced acute renal failure in rats	↓ renal dysfunction	[120]
Diabetic Balb/cA mice	↓ triglycerides and cholesterol in heart and liver	[121]
	↓ lipid oxidation levels in kidney and liver	
	↑ glutathione peroxidase activity	

STZ: streptozotocin; AGEs: advanced glycation end products; ALEs: advanced lipoxidation end products; OLETF: Otsuka Long-Evans Tokushima Fatty.

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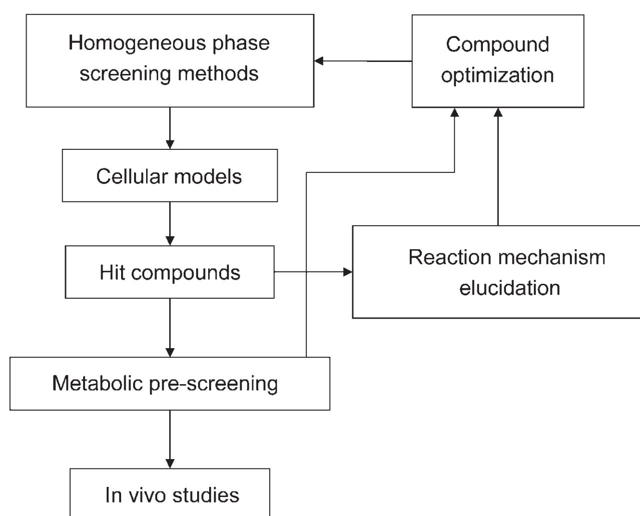

Scheme 1. Methodological approach in the development of RCS scavengers.

proach should consider an initial step based on homogeneous phase screening methods, aimed at selecting the most reactive and specific compound towards the different classes of RCS and the physiological aldehydes (pyridoxal phosphate, retinaldehyde). The second step is based on cellular models that can evaluate the cytoprotective effect of RCS scavengers towards cytotoxic RCS. Once the most promising compounds have been selected, the reaction mechanism should then be fully clarified to optimise suitable derivatives in terms of reactivity as well as specificity. Moreover, conventional metabolic prescreening tests should be carried out to optimise the bioavailability, pharmacokinetic, and metabolic properties before performing in vivo studies. The efficacy of the sequestering compounds is then evaluated in in vivo models of genetically or chemically induced RCS-related diseases.

3.4.1. Cell-free screening methods

The simplest in vitro approach involves the incubation of the scavenger with each target RCS in phosphate buffer pH 7.4, accompanied by HPLC monitoring of the time-dependent consumption of the scavenger[127] or of the aldehyde[87,92,128]. The choice to monitor the aldehyde or the sequestering agent depends on the response of the analyte to conventional HPLC detectors (UV-DAD or fluorimetric). For instance, when the scavenging activity towards unsaturated aldehydes (such as HNE, ACR, crotonaldehyde, and nonenal as these compounds are characterised by a conjugated chromophore) is studied, the time course of the scavenging reaction is determined by measuring their disappearance by HPLC–UV analysis. This approach has recently been applied to study the scavenging efficacy of His-dipeptides, carnosine, and derivatives towards HNE[87] and to compare the HNE scavenging efficacy of N^{α}-acetyl-L-cysteine (NAC) and some lipophilic congeners (N^{α}-acetyl-L-cysteine methylester and N^{α}-pentanoyl-L-cysteine) to that of AG. Furthermore, HPLC monitoring of unreacted ACR revealed the following ranking of potency as ACR-sequestering agents: MESNA (sodium 2-mercaptoethanesulfonate) $>$ dihydralazine $>$ hydralazine $\gg$ methoxamine $>$ carnosine $\cong$ pyridoxamine $>$ aminoguanidine.[93]

When the target RCS to be tested is an unconjugated aldehyde with poor absorption properties such as GO, the efficacy of the scavenger is usually measured by UV monitoring of RCS consumption following derivatization. The concentration of GO can be conveniently determined using Girard's reagent T at 326 nm, as described before for the reaction kinetics of GO–pyridoxamine.[94] Alternatively, when a chromophore is developing during the reaction of GO with the scavenger, the potency of the scavenger can be established by UV monitoring of the reaction product: for example the reaction of GO with AG in sodium phosphate buffer was followed by measuring the absorbance of the triazine product, 3-amino-1,2,4-triazine, at 320 nm.[129]

One additional advantage of screening the RCS scavenging activity by monitoring consumption of one of the reagents, is to obtain information regarding the reaction kinetics. The second-order rate constants (k_{2nd}) can be calculated from the pseudo first-order rate constants obtained from the slope of the curves indicating the time course of the disappearance of the target RCS/scavenger as measured by the AUC during the reaction with the trapping reagent.[128]

Since most aldehydes covalently adduct the nucleophilic sites of proteins and induce cross-links, a series of methods aimed at evaluating the ability of the sequestering agent to inhibit protein structure modifications have been developed. Among them, the RNase A oligomerization assay has been widely employed. The method is based on bovine pancreas ribonuclease A (RNase A) as model protein because its low mass (12.4 kDa) permits ready detection of oligomeric forms induced by HNE, ACR, or *trans*-4-oxo-2-nonenal, a more reactive cross-linking agent than HNE, by SDS-polyacrylamide gel electrophoresis or immunochemical analysis.[130–133] Hence, once the conditions for the induction of protein cross-links by RCS are established, the effect of the scavenger on protein oligomerisation can be explored using SDS-PAGE or Western blotting to assess the role of adduct-trapping in any inhibitory outcome. For example, ACR causes rapid time- and concentration-dependent cross-linking, with dimerized protein detectable within 45 min of commencing protein modification; by assessing as endpoints oligomer abundance, ACR-lysine adduction, and protein carbonylation, it is possible to establish the potency of a scavenger in inhibiting cross-linking. Such an approach has been recently applied to demonstrate that a) hydralazine targets carbonyl-retaining Michael adducted-proteins, forming hydrazones that may prevent participation by modified proteins in nucleophilic additions that generate inter- and intramolecular cross-links;[134] and b) carnosine inhibits HNE-induced RNase cross-linking by direct covalent trapping, either of free HNE or of intermediates in the reaction pathway leading to cross-linking, with a potency greater than that of AG.[130]

The main limitation of most of the above reported methods, although establishing the order of reactivity of a series of potential sequestering agents, is that medium or high throughput screening may not be performed, and more importantly, they require an isolated or pure testing compound. To over-

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come these limits, we recently developed a mass spectrometric direct infusion tool, a medium throughput screening method, which permits evaluation of the sequestering efficacy of compounds in a mixture (chemical libraries), and the detection of the presence of reactive compounds towards RCS in crude extracts. At the same time the reaction products are identified and characterised by tandem MS. Moreover, by using endogenous aldehydes such as pyridoxal phosphate as target carbonyls, the method gives information on scavenging selectivity.[135] The method involves the following steps: a) incubation of the target RCS with a mixture of derivatives at a 1:1 molar ratio for different time periods; b) sample spiking with Tyr–Hys as internal standard; c) ESIMS analysis of the mixture using the infusion tool. The scavenging efficiency (reactivity) is determined by calculating the relative consumption for each derivative with respect to the internal standard (ion response of the respective protonated or deprotonated molecular ions). When the derivative consumption is over 10%, the reaction products are searched and characterised by tandem MS.

3.4.2. Cell models

Although essential in the first screening phase, these in vitro assays are not sufficient to draw conclusions on the efficacy of the scavenging compounds in biological matrices, and further tests must be performed on cell models. This is exemplified by the work of Neely et al.:[92] in simple in vitro systems, without other nucleophiles present, both NAC and AG had comparable chemical reactivity with HNE, but in more complex models AG did not compete efficiently with protein-bound nucleophiles for HNE. Only NAC and its lipophilic congeners were able to block HNE-protein adduct formation in vitro and in neuronal cultures.

Hence the scavenging efficiency can be determined in cell systems by evaluating the cytoprotective profiles of the compounds (inhibition of cell death induced by exposure to RCS) and by identifying the RCS and scavenger reaction products in the biological matrix. This approach, using SH-SY5Y neuroblastoma cells and rat hippocampal neurons exposed to HNE,[127] has been recently applied to investigate the cytoprotective efficacy of some histidyl-containing carnosine analogues, containing hydrazide or 1,2-diol moieties. The HNE–UVB-mediated death of keratinocytes has been used by us to demonstrate the protective effect of carnosine in a model that mimics the skin damage induced by UV radiation (sequential exposure to UVB and HNE).[136] The LC–MS–MS analysis of cell supernatants clearly shows formation of the HNE–carnosine Michael adduct, confirming that the cytoprotective mechanism is due to the capacity of the dipeptide to trap the cytotoxic aldehyde and to inhibit its intracellular diffusion. D-penicillamine has been shown to protect human skin keratinocytes and fibroblasts (CF3 cells) against GO-induced carbonyl toxicity.[128] HY has been demonstrated to be an efficient ACR scavenger and a powerful inhibitor of ACR-mediated toxicity in hepatocytes (forming hydrazone derivatives in a rapid Schiff-type reaction)[93,137] affording strong cytoprotection at concentrations several orders of magnitude lower than those of other scavengers, including AG, CAR, and PY. Hydralazine not only scavenges free ACR directly, decreasing intracellular ACR availability and thereby suppressing macromolecular adduction, but also, in a second "adduct-trapping" mechanism, the drug forms hydrazones with ACR-derived Michael adducts in cell proteins, preventing secondary reactions of adducted proteins that may trigger cell death.

3.4.3. Reaction mechanism elucidation for compound optimization

Elucidation of the scavenging reaction mechanism is an important step in the discovery process of new sequestering agents. In particular, the identification of the reactive moiety responsible for the scavenging effect allows application of several strategies aimed at increasing the reactivity and optimizing specificity. Reaction products of RCS and sequestering agents have been elucidated by different analytical techniques and in particular ^{1}H- and ^{13}C NMR, mass spectrometry (MALDI–TOF–MS or ESIMS), or by a combined approach. NMR has been used to elucidate the structures of AG–HNE, AG–MDA, and NAC–HNE adducts.[91,92] In this way it was possible to establish that NAC and AG react differently with HNE; specifically, NAC forms a Michael adduct while AG forms a resonance-stabilised imine.

Thanks to the latest improvements, ESI ionisation mass spectrometry currently represents a very powerful method for exploring RCS-quencher interactions. The introduction of soft ionisation methods made possible the transfer of the intact adduct to the gas phase and therefore determination of its molecular mass with high accuracy and consequently, elucidation of its full structure (molecular characterisation) by MS^{n} experiments. This approach allowed characterisation of the reaction products of CAR–HNE, CAR–ACR, AG–HNE,[87,88,130] and elucidation of the mechanisms of reaction. On the basis of the reaction between CAR and HNE proposed by us,[87] and later confirmed by Liu et al.,[130] and by considering that the formation of the Schiff base is the rate-determining step, Guiotto et al.[127] decided to substitute the primary amine of β-alanine with different nucleophiles. This approach leads to carnosine analogues that form more stable adducts with aldehydes. In the same way, we are now involved in a research project aimed to design, using a molecular modelling approach, carnosine derivatives which are characterised by stable imine intermediates, and the results are currently under patent protection.

3.4.4. In vivo models

Different pathological models where carbonyl stress is involved are available to evaluate the pharmacological efficacy of RCS scavengers. All the experimental models reported in Table 1 (mainly aged, diabetic, obese, and atherosclerotic animals) represent suitable pharmacological tools for this purpose because the carbonylation process has been shown to play a causative role in the development of vascular and organ damage (neuropathy, retinopathy, nephropathy, aortic stiffening, and atherosclerotic plaques).[39,40,56,103–121] In this context, functional parameters (plasma creatinine, proteinuria, albuminuria, and total

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plaque area) and a series of highly stable biochemical markers for carbonylation are used to evaluate the protective effect of long-term administration of the developed carbonyl scavenger, and include:

- plasma and tissue (glomerular and renal tubular) fluorescence as an index of AGEs and ALEs formation;[107]
- protein carbonyls, measured by a spectrophotometric assay following 2,4-dinitrophenylhydrazine derivatisation, or by SDS-PAGE and immunodetection of protein bound 2,4-dinitrophenylhydrazones (Western blotting);[138;139]
- cross-linking of skin collagen, estimated from the kinetics of digestion of the collagen by pepsin;[113]
- Free CML accumulation in plasma, urine, and tissues, determined by immunochemical analysis (competitive indirect ELISA);[39,118]
- CML- or HNE-modified long-lived tissue proteins by Western blotting using monoclonal antibodies.[118]

Antisera against well-defined ALEs such as CML and HNE are a valuable tool for assessing their formation by immunohistochemical techniques. Immunoassays are often used for the quantification of ALEs, but for several reasons the use of antisera for quantitative immunoassays of protein-bound ALEs is questionable. One reason is that the specificity of the antibodies is often difficult to define with certainty and no monospecific antibodies are commercially available. Thus, ALE immunoassays may yield only semiquantitative results. For these reasons, more specific and sensitive analytical approaches have been developed for their quantitative determination in protein hydrolysates, based mainly on LC, GC–MS, and LC–MS–MS methodologies. For example, free CMC or CMC released from proteins by hydrolysis can be easily quantified by reversed-phase HPLC with fluorescence detection after precolumn derivatisation with *o*-phthaldialdehyde.[29] Chemical modifications of skin collagen can be estimated from determination of ALEs (CML, CEL, MDA–Lys, and HNE–Lys) by isotope dilution, selected ion monitoring gas chromatography–mass spectrometry (SIMGC–MS).[40,113] CML (in free and protein-bound form after hydrolysis) is now determined by isotope-dilution LC–MS–MS,[140] a method than can be accurately applied to all the biological matrices (plasma, tissue, and urine). Furthermore, a LC–ESIMS–MS method has been recently set up to measure HNE-modified human serum albumin as a systemic index of lipid-derived carbonylation.[141]

An additional and direct tool to demonstrate the efficacy of the sequestering agent in in vivo conditions, is to identify the reaction products of the testing agent with RCS in urine or plasma. Using this approach, Metz et al.[142] identified twelve PM adducts formed in vitro during incubation of PM with linoleic and arachidonic acid, and six of these compounds were then detected in the urine of diabetic and hyperlipidemic rats treated with PM. This was the first direct demonstration of the ability of PM to trap intermediates of lipid peroxidation reactions in vivo.

Another pharmacological tool, specifically developed in mice to study the protective effect of sequestering agents such as hydralazine towards ACR, is based on the induction of carbonyl stress-mediated liver injury by administration of allyl alcohol, which is converted to ACR by alcohol dehydrogenase.[143] The dose-dependent trapping in liver proteins of the RCS scavenger can be directly determined by Western blotting and densitometric analysis of immunoreactivity in proteins, while the determination of liver enzymes in plasma can be a useful tool to evaluate the overall prevention of hepatic injury.

4. Conclusion

During the past few years, there has been an intensive effort directed at ascertaining the nature of protein carbonylation by reactive aldehydes, and evidence is accumulating that they are causally involved in the pathophysiological effects associated with oxidative stress in cells and tissues in vivo. Different therapeutic strategies have been developed to prevent or restrain the RCS-induced damage. Trapping of lipid-derived reactive aldehydes (identified as the chemical intermediates between hyperglycaemia, hyperlipidaemia and their complications) seems to be the most promising one and represents a new therapeutic approach on which the efforts of the pharmaceutical chemist would have to be focussed in the near future. While there is ample experimental evidence demonstrating the protective effects of ALEs inhibitors in in vitro models, and in some animal models, greater effort is required to confirm the carbonyl trapping capacity of these compounds in humans. The clinical evidence that aldehyde-sequestering agents act as protective drugs is still limited to PYR (now on the FDA "fast track" to phase III clinical trials for prevention of diabetic nephropathy), because the promiscuous activity of some ALE inhibitors, such as AG (inhibitory action on inducible NO-synthase) or HY (vasodilating and antihypertensive effect), has greatly limited their clinical use as carbonyl trapping agents. Therefore, a rational drug design approach aimed at identifying and developing novel aldehyde sequestering agents characterised by high reactivity, specificity, suitable pharmacokinetic profiles, and safety is needed, and represents an emerging field of interest in medicinal chemistry. Thus, taking into account this area of research in medicinal chemistry is in its infancy, several in vitro and in vivo models, and several analytical methodologies are currently available to test to the preclinical level newly developed RCS quenchers.

ABBREVIATIONS

ACR	acrolein
AD	Alzheimer's disease
AG	aminoguanidine
AGEs	advanced glycation end products
ALEs	advanced lipoxidation end products
ALS	amyotrophic lateral sclerosis
ANS	anserine
apoA-I	apolipoprotein A-I
BAL	bronchoalveolar lavage
CAR	carnosine
CEL	N^{ε}-(carboxyethyl)lysine

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CML	N^{ε}-(carboxymethyl)lysine
COPD	chronic obstructive pulmonary disease
di-HY	dihydralazine
ESIMS	Electrospray ionisation mass spectrometry
GO	glyoxal
GSH	glutathione
HNE	4-hydroxy-2-nonenal
HY	hydralazine
KODE	keto-octadecadienoic acid
MALDI-TOF-MS	Matrix-assisted laser desorption time-of-flight mass spectrometry
MDA	malondialdehyde
MF	metformin
MGO	methylglyoxal
MS	mass spectrometry
NAC	N^{α}-acetyl-L-cysteine
PCOs	protein carbonyls
PD	Parkinson's disease
PUFAs	polyunsaturated fatty acids
PYR	pyridoxamine
RCS	reactive carbonyl species
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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Keywords: ALE inhibitors · medicinal chemistry · protein carbonyls · protein modifications · reactive carbonyl species

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