REVIEW

Protein modification by acrolein: Relevance to pathological conditions and inhibition by aldehyde sequestering agents

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Acrolein (ACR) is a toxic and highly reactive α,β-unsaturated aldehyde widely distributed in the environment as a common pollutant and generated endogenously mainly by lipoxidation reactions. Its biological effects are due to its ability to react with the nucleophilic sites of proteins, to form covalently modified biomolecules which are thought to be involved as pathogenic factors in the onset and progression of many pathological conditions such as cardiovascular and neurodegenerative diseases. Functional impairment of structural proteins and enzymes by covalent modification (crosslinking) and triggering of key cell signalling systems are now well-recognized signs of cell and tissue damage induced by reactive carbonyl species (RCS). In this review, we mainly focus on the in vitro and in vivo evidence demonstrating the ability of ACR to covalently modify protein structures, in order to gain a deeper insight into the dysregulation of cellular and metabolic pathways caused by such modifications. In addition, by considering RCS and RCS-modified proteins as drug targets, this survey will provide an overview on the newly developed molecules specifically tested for direct or indirect ACR scavenging, and the more significant studies performed in the last years attesting the efficacy of compounds already recognized as promising aldehyde-sequestering agents.

Keywords:
Acrolein / Aldehyde-sequestering agents / Neurodegenerative diseases / Protein adducts / Reactive carbonyl species

1 Introduction

Protein modification by reactive carbonyl species (RCS), especially those generated by peroxidation of polyunsaturated fatty acids (PUFAs), in the last few years has gained an even greater importance, in view of the emerging deleterious role of the RCS–protein adducts in the aetiology and/or progression of several human diseases, such as cardiovascular (atherosclerosis, long-term complications of diabetes) and neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease [PD], and cerebral ischemia). Although protein carbonylation and the chemistry of the reactions that give rise to carbonyl groups are quite well characterized [1], the overall biology of oxidative protein modifications remains complex and incompletely defined.

Among the variety of RCS (three to nine carbons in length) generated by oxidative decomposition of PUFAs, the α,β-unsaturated aldehyde acrolein (ACR) is by far the strongest electrophile, showing the highest reactivity toward nucleophilic sites on proteins [2, 3]. Although lipid peroxidation generates far more 4-hydroxy-*trans*-2-nonenal (HNE) than ACR [4], it has been reported, based on the urinary daily excretion, that production of ACR metabolites in humans is 10^2-fold greater than that of HNE metabolites [4].

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Abbreviations: ACR, acrolein; **AGE,** advanced glycoxidation end product; **ALE,** advanced lipoxidation end product; **apoA-1,** apolipoprotein A-1; **CAR,** carnosine; **FDP-Lys,** N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine; **GSH,** glutathione; **GST,** GSH-*S*-transferase; **HGF,** human gingival fibroblast; **HNE,** 4-hydroxy-*trans*-2-nonenal; **HY,** hydralazine; **MP-Lys,** N^{ε}-(3-methylpyridinium) lysine; **NAC,** *N*-acetylcysteine; **NO,** nitric oxide; **NQO1,** NAD(P)H:-quinone oxidoreductase 1; **PC-ACR,** protein-conjugated acrolein; **PD,** Parkinson's disease; **RCS,** reactive carbonyl species

This clearly indicates that sources other than lipid peroxidation, such as amino acids and polyamines (see below), are mainly involved in ACR production.

ACR reacts preferentially with Cys, Lys, and His residues (the Lys adducts being the more stable products) via Michael-type addition reactions preserving the aldehyde functionality on the modified protein, rather than by Schiff's base formation. The order of ACR reactivity for protein nucleophiles (Cys>His>Lys) is the same as that established for HNE [3, 5–7].

The reaction of ACR with Lys may result in β-substituted propanals (R-NH-CH_2-CH_2-CHO), but the major adduct formed on reaction with protein is the N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine adduct (FDP-Lys) [8] (Fig. 1). This compound is a reactive intermediate that can covalently bind to thiols, such as glutathione (GSH) [9], through the retained electrophilic carbonyl moiety. Another ACR-Lys adduct, N^{ε}-(3-methylpyridinium)lysine (MP-Lys), was identified later and found to be a highly stable end product [10].

Much of the literature is available on the cytotoxic effects of ACR, evaluated both in vitro, in several cell lines, and in vivo in different experimental animal models. Major pathways dysregulated by ACR and related to the cytotoxic effect include those involved in apoptosis, cell cycle control, transcription, cell signaling, and protein biosynthesis [11]. By contrast, studies on the ability of ACR to covalently modify proteins are less widespread, and among them, the majority has been carried out in vitro, often forced by exposing a target protein to supra-physiological concentrations of the reactive aldehyde.

Identifying targets for ACR modification is worthwhile since such knowledge can provide mechanistic insight into cellular or metabolic pathways that are dysregulated by electrophiles, and recent proteomics studies have identified various cytoskeleton-associated proteins as ACR targets [12, 13].

Hence, the first aim of this review was to examine the effects of ACR modification on protein structure and function, in order to better understand the consequences of such modifications at cell level, and to gain a deeper insight into the occurrence of ACR-modified peptides/proteins in vivo, mainly focusing on those publications where unambiguous characterization was performed. As the toxic role of exogenously/endogenously produced ACR, several efforts have been addressed to find the molecules that are able to counteract/prevent ACR-mediated injury. This represents the second aim of this survey, dedicated to discussing the recently developed pharmacological approaches to the management of pathological situations and chronic diseases in which oxidative stress and ACR formation is massively involved.

2 ACR sources

As previously outlined, ACR can be produced from peroxidation of membrane phospholipids, although the major

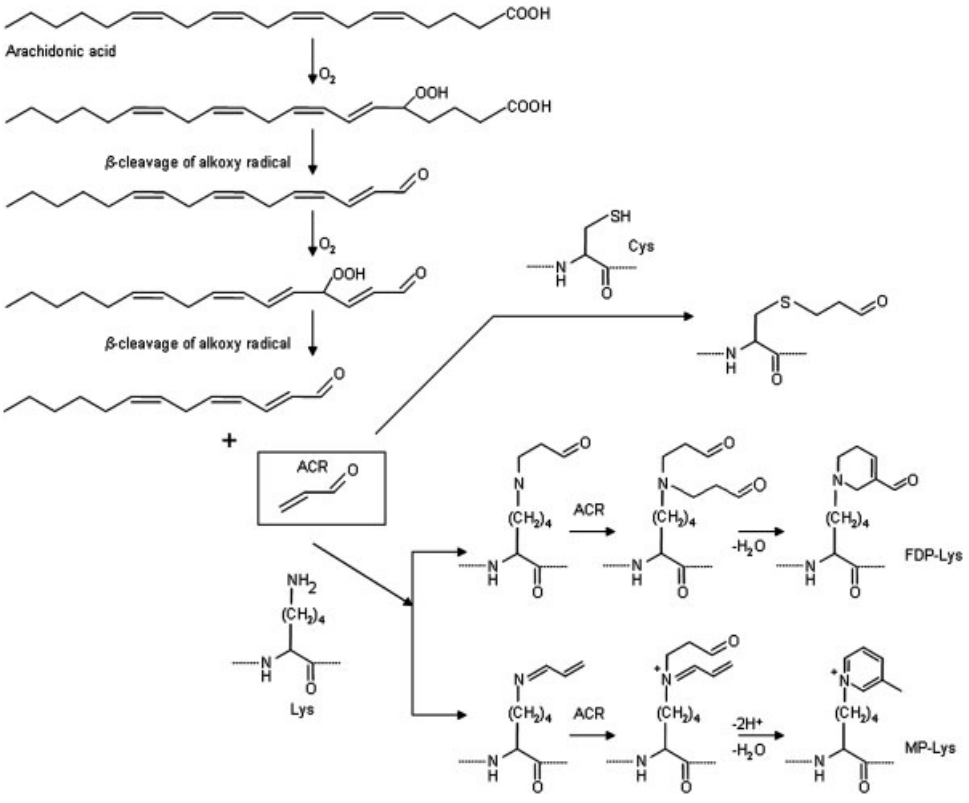

Figure 1. Generation of ACR from lipid peroxidation pathway and consequential reaction with Lys (forming FDP-Lys, *N*-(epsilon)-3(formyl-3,4-dehydropiperidino)Lys and MP-Lys, N(epsilon)-(3-methylpyridium) Lys) and cysteine.

aldehydes produced during lipid peroxidation are malondialdehyde and HNE [14], but it is also formed endogenously through metabolism of amino acids and polyamines. In this context, it is worthwhile to emphasize the correlation found between ACR produced from polyamines by polyamine oxidases, and chronic renal failure or stroke [15]. ACR has been recognized as the major toxic compound produced from spermine and spermidine by amine oxidase in uremic subjects [16, 17]: ACR (and protein-conjugated ACR), by accumulating in the blood of patients with chronic renal failure, may function as uremic toxin which accelerates the progression of uremia [17].

In vivo, ACR is also known to be formed from threonine by neutrophil myeloperoxidase at sites of inflammation [18]. This implies that phagocyte-mediated formation of these products may be of central importance in promoting tissue injury at sites of inflammation.

ACR could originate also from another amino acid, methionine, both as a volatile product from the reaction with ninhydrin and as a product of the reaction with dehydroascorbic acid/ascorbic acid, by heating in aqueous solutions in the presence of atmospheric oxygen [4].

Drug metabolism is another source of ACR. It is well known, in fact, that the oxazaphosphorines (cyclophosphamide, ifosfamide, and trofosfamide), although widely used in clinical practice for their antitumor and immunomodulatory activities, are responsible for urotoxicity, neurotoxicity, and nephrotoxicity. This is because their metabolism, involving cytochrome P450 biotransformations, leads to the formation of ACR [19] which has been associated with the development of hemorrhagic cystitis or diffuse inflammation of the bladder resulting in dysuria, hematuria, and hemorrhage. Between 2 and 40% of cyclophosphamide-treated patients develop hemorrhagic cystitis, which is thought to result from the generation of ACR in the kidney or the bladder [20]. Involvement of ACR in urotoxicity is confirmed by the ability of thiols such as *N*-acetylcysteine (NAC) and GSH [21], which readily form Michael adducts with unsaturated aldehydes, to prevent cyclophosphamide toxicity in animals. In this context, it has also been demonstrated that pretreatment with dietary or nutrient inducers of glutathione-*S*-transferase (GST) may be of use in minimizing bladder injury in patients undergoing cyclophosphamide therapy [22].

ACR and many other type-2 alkenes constitute a large family of environmental and food contaminants that include acrylamide, methyl vinyl ketone, methyl acrylate, and HNE, all characterized by electrophilic properties [23]. ACR is indeed a common air pollutant, to which humans are continuously exposed and is present in high concentrations in wood, cotton, and tobacco smoke, automobile exhaust and industrial waste and emissions. Population living or working in areas with heavy automotive traffic may be exposed to a higher level of ACR via inhalation of smoke or automotive exhaust, and the effects induced by ACR at respiratory, ocular, and gastrointestinal level have been recently reviewed [24].

ACR, as one of the major toxic by-products of smoke developed during pyrolysis or combustion of plastic materials, has been linked to severe pulmonary damage and recognized as one of the main causes of death following smoke inhalation [25]. As ACR is found in all types of smoke (cigarette smoke, the exhaust from internal combustion engines, the vapors of overheated cooking oil), it is not surprising that environmental smoke can act as a cytotoxic, mutagenic, and carcinogenic factor.

Very recently ACR and other α,β unsaturated aldehydes have been identified as sources of toxicity to activated sludge biomass in polyester manufacturing wastewater [26].

Food substances are an additional source of ACR exposure. Relatively high levels of ACR have been detected in beer, wine, rum, bread, and other foods [27]. The formation of ACR in foods, especially cooking oils, is further increased by cooking, frying, and reheating [28]. A very recent study demonstrated increased exposure to the volatile toxicants and carcinogens ACR, crotonaldehyde, and benzene in Chinese women who regularly cook, providing a plausible lead for further investigating the role of volatile compounds generated during high-temperature cooking with oils as causes of lung cancer. Compared with controls, women who engaged in regular home cooking had significantly higher levels of mercapturic acids of ACR, the final metabolites arising by conjugation of ACR with GSH [29]. Metabolic removal of ACR involves multiple pathways, which include reduction, oxidation, and conjugation with GSH. The detailed metabolic fate of ACR has been recently discussed by Stevens and Maier [4], whose review also gave a comprehensive picture of the ACR sources and an insight into the ACR–biomolecule interactions relevant to human health and disease. The environmental and endogenous origins of ACR are summarized in Supporting Information Table 1S.

3 ACR involvement in diseases

Several lines of evidence indicate that soft electrophiles such as ACR produce toxicity by a common mechanism involving the formation of Michael-type adducts with nucleophilic sulphydrilic groups. In this context, LoPachin et al. [30], in reviewing the adduct chemistry of the α,β-unsaturated carbonyls and possible protein targets, suggested that protein adduct chemistry of the conjugated type-2 alkenes might provide insight into the pathogenesis of certain human neurodegenerative diseases. Type-2 alkenes share in fact a common mechanism of action at nerve terminals in the brain, and the combined effects of these substances could contribute to some neurologic disorders [30].

They also hypothesized that ACR, HNE, and other α,β-unsaturated carbonyls can induce neurotoxicity by inhibiting nitric oxide (NO) signaling at the nerve terminal. As

many of the Cys residues involved in RCS adduction are also NO acceptors, irreversible adduction of the corresponding thiolate groups will inhibit NO signaling, with the consequent toxic response of the nerve terminal.

In an attempt to discern the toxicological significance of ACR-modified proteins, LoPachin et al. [30] studied in detail the molecular mechanisms of ACR (and HNE) toxicity and recognized the nucleophilic targets and adduct formation types. Through a combined proteomic, quantum mechanical, and kinetic approach, they propose a unified mechanistic hypothesis of α,β-unsaturated aldehyde toxicity, involving the formation of 1,4 Michael-type adducts with highly nucleophilic sulfhydrilic thiolate groups on cysteine residues of functionally critical proteins. The quantum mechanical parameter softness (σ), used as an index of π-electron mobility, indicated that ACR (and HNE) are relatively soft electrophiles that rapidly form adducts with sulfhydrilic groups, thus confirming what had already been demonstrated by applying different proteomic approaches to isolated protein adducts (reviewed in [30]).

ACR has recently been recognized as an important contributing factor to the prothrombotic risk in human exposure to pollutants such as tobacco smoke or automobile exhaust fumes, or through dietary consumption, due to the increased platelet activation [31]. It is a central player in slow and progressive ''secondary injury'' cascades. Indeed, ACR–biomolecule complexes formed by crosslinking with proteins and DNA are associated with a number of pathologies, especially central nervous system (CNS) trauma and neurodegenerative diseases.

Growing evidence indicates that ACR may play an important role in the pathogenesis of neurodegenerative disorders. In a very recent review focused on the ACR involvement in neurodisorders [32], the authors pointed out that one of the emerging suspected culprits in their development is ACR, which tends to be significantly elevated in the brains or spinal cords of people who have Alzheimer's disease, PD, amyotrophic lateral sclerosis, and other neurologic disorders [30, 33–36]. Wood et al. [37] developed the concept of ''aldehyde load'' in neurodegenerative mechanisms and underlined the role of polyamine metabolism in generating a number of reactive aldehydes that participate in the death of compromised tissue.

The role of by-products of lipid oxidation in Alzheimer's-diseased brains, with a particular focus on ACR has also been recently reviewed [33]. The review by Hamann and Shi [38] summarizes the cellular and biochemical mechanisms of ACR-induced membrane damage, mitochondrial injury, oxidative stress, cell death, and functional loss in spinal cord injury. The effects of ACR and other lipid-derived carbonyls on the function of brain mitochondria as well as the role of mitochondrial aldehyde detoxification pathways and their potential role in the development of Alzheimer's disease have also been reviewed [39].

The concept of ''carbonyl overload'' on metabolic pathways involved in detoxification of RCS and the subsequent protein carbonylation has also been associated with uremic patients, and a pioneer in this field was the group of Miyata which first defined as ''carbonyl stress'' the common feature underlying long-term uremic complications [40].

4 ACR-protein adducts formation

The group of Uchida was a pioneer in the field of protein modified by ACR. In 1998, he provided evidence for the first time that the formation of ACR and its conjugate with Lys residues is involved in the oxidative modification of human low-density lipoprotein (LDL) [41]. The adduct was identified as FDP-Lys, a novel ACR-Lys adduct, whose importance became even greater, because it allowed the development of specific antibodies which recognize in vivo formation of ACR-modified proteins, by constituting an epitope for the antibody against the aldehyde [42]. Immunohistochemical studies carried out with a monoclonal antibody were used later also to detect colocalization of apolipoprotein A-1 (apoA-1) with ACR adducts in human atherosclerotic lesions and to reveal the possible interference of ACR with normal reverse cholesterol transport by high-density lipoproteins (HDLs) [43]. However, the techniques used by Uchida et al. [41] in studying the modification of apolipoprotein B-100 (apoB-100) in oxidized LDL did not allow the determination of the modification sites. This problem was solved only later by using LC-MS/MS analysis in combination with a newly developed sample preparation procedure (PVDF membrane as an alternative to the commonly used in-gel digestion protocol) to investigate oxidative modifications of apoB-100 isolated from LDL of healthy subjects exposed to ACR [44]. This approach, leading to detection and identification of a number of ACR-modified Lys residues, namely 87 FDP-Lys and 13 MP-Lys, would be useful for studying atherosclerosis development by monitoring oxLDL generation and accumulation in atherosclerotic lesions.

4.1 In vitro evidence

Employing recombinant human protein and Western blot with ACR-Lys-specific antibodies, other authors [45] demonstrated that ACR severely compromises the functional integrity of apolipoprotein E (apoE, an exchangeable antiatherogenic apolipoprotein), in terms of heparin binding, lipid binding, and the LDLr-binding interaction. A combined analytical approach based on circular dichroism, infrared spectroscopy, and fluorescence indicated significant tertiary structural changes in ACR-modified apoE3-NT, although the chemical nature of ACR crosslinking or adduct formation with apoE3 is not known at present.

ACR (but not aliphatic aldehydes) has been found to be a potent inactivator of protein tyrosine phosphatase 1B, a component of an important class of cysteine-dependent enzymes that work in tandem with protein tyrosine kinases

to regulate a number of critically important mammalian signal transduction pathways [46]. MS analysis of the modified enzyme indicated that inactivation occurs via conjugation addition of ACR to the catalytic Cys residue at the active site of the enzyme.

Although modification of Cys residue leading to inactivation of enzymes has been reported for other enzymes such as aldose reductase [47], the reactivity of ACR with protein thiols has remained largely unexplored and the in vivo formation of the corresponding adducts has not been unequivocally demonstrated.

Hence, the reaction of ACR with Cys residues as well as the stability of ACR-Cys adducts has been thoroughly investigated by Cai et al. [48], by using model peptides containing different nucleophilic centers (Cys, His, and Lys residues as well as the free amino terminus of proteins). By MS analysis, it was demonstrated that ACR reacts avidly with Cys residues through Michael addition to form M+56 Da adducts that, however, are unstable and undergo conversion to M+38 adducts, due to intramolecular Schiff base formation. These findings, suggesting an apparent loss of protein–ACR Michael adducts over time, may explain the reason why other studies have failed to identify ACR–Cys adducts.

Although intramolecular crosslinks by ACR may be important in the initial stages of protein aggregation, the evidence for their formation in proteins has been sparse, due to methodological difficulties. The group of Uchida [49] characterized the protein crosslinks formed upon ACR treatment of a model peptide, chain B from bovine insulin (insulin B chain), by MS techniques after digestion of the modified peptide. The authors used as a model peptide the insulin B chain from sequence FVNQHLC*GSHLVEALYLVC*GERGFFYTPKA (C*: Cys-SO_3H) which contains four main possible modification sites of ACR: the *N*-terminal amino acid residue (Phe1), two His residues (His5 and His10), and one Lys residue (Lys29). The amino groups (*N*-terminus [Phe1] or Lys29) and the His residues (His5 or His10) were identified as the main sites involved in the formation of inter and intramolecular crosslinking adducts. These results allowed the proposal of a mechanism of protein crosslinking by ACR, involving inter and intramolecular crosslinking adducts between amino groups and the side chain of His through Michael addition and Schiff base formation.

In the last few years, several in vitro studies were carried out in conjunction with computational studies, aimed at defining the effects of carbonyl modification on protein structure and with functional studies (carried out with suitably prepared modified proteins) with the final aim of providing key information to the biologist in explaining the effects of modification by RCS on protein function, and understanding the consequences of such modifications at cell level.

For example, Lambert et al. [50] demonstrated that aldehydes in cigarette smoke can regulate gene expression by direct modification of a transcription factor. In particular, by the use of MALDI-TOF-MS and nanospray LC-ESI-MS/MS, they showed that ACR inhibits cytokine gene expression in human T lymphocytes by alkylating two amino acids (Cys61 and Arg307) in the DNA-binding domain of NF-κB1 (p50 subunit), resulting in inhibition of p50 DNA binding. They also found that crotonaldehyde reacted with Cys61, but not Arg307, whereas the saturated aldehydes in cigarette smoke did not react with p50.

ACR has been shown to selectively modify thioredoxin-1, a protein involved in the regulation of the antioxidant function in endothelial cells, by adduction at the nonactive site Cys-73 [51]. A series of additional in vitro studies also demonstrated that such modification was accompanied by inhibition of enzymatic activity and associated with increased production of reactive oxygen species and stimulation of monocyte adhesion to endothelial cells, an early event of atherosclerosis.

The pathogenetic role of protein carbonylation was clearly elucidated by the articles of Shao et al. [43, 52], who studied the impact of ACR modification on apoA-1. The major site modified by ACR, leading to the formation of a FDP-Lys adduct, was found to be Lys226. As this residue is located near the center of helix 10 in apoA-1, a region involved in the ability of apoA-1 to transport lipid, modification of Lys226 by ACR results in a decreased cholesterol efflux from cells via the ATP-binding cassette transporter A1 pathway. Hence, modification of specific sites on apoA-1 might interfere with cholesterol transport by HDL and contribute to atherogenesis by impairing cholesterol removal from the artery wall. As another function of HDL is as a vehicle of paraoxonase-1, an enzyme system that possesses peroxidase-like activity that can contribute to prevent lipoprotein oxidation, as well as a homocysteine-thiolactonase activity that may be linked with its anti-atherogenic properties, Gugliucci et al. [53] studied the effects of ACR on the enzyme activity following incubation with HDL. SDS-PAGE demonstrated the formation of high-molecular-weight modified proteins, but ACR–protein crosslinks formation has been only postulated, and not confirmed. Similarly, it was postulated that enzyme inhibition involves modification of Cys residues critical for the catalytic site, but no definitive structure characterization was provided. Coincubation of HDL with cysteine, but not aminoguanidine or carnosine (CAR) prevented ACR inhibition of paraoxonase activity.

Using different methodologies (intrinsic fluorescence, functionality, heparin affinity, and conformational features), Martínez-Martínez et al. [54] studied the effect of ACR on antithrombin, a key anticoagulant serpin, both in vitro and in vivo (mice). ACR, even at low dose in vitro, was found to impair the anticoagulant function of purified antithrombin by affecting its heparin affinity. The loss of activity was believed to be due to the modifications of some exposed Lys residues, particularly Lys11, Lys114, and Lys125, occurring on the serpin and affecting the heparin-binding domain, but

no unequivocal characterization of the modification sites on the protein has been carried out. The same mechanism of antithrombin inhibition by ACR and the same hypothesis on the modification sites were previously postulated by Gugliucci [55]. In addition, because high concentrations of ACR are required to cause mild functional effects in vivo, although increased levels of ACR may contribute to the risk of thrombosis, it can be concluded that the clinical relevance in thrombosis is to be considered as minor.

ACR selectively modifies synaptosomes proteins related to vital neuronal functions [13]. Proteomic analysis (2-D electrophoresis followed by MS) identified, in synaptosomes isolated from Mongolian gerbils forebrains, tropomyosin-3-γ isoform 2, tropomyosin-5, β-actin, mitochondrial Tu translation elongation factor, and voltage-dependent anion channel as the main carbonylated proteins. As all these proteins are involved in a wide variety of cellular functions including energy production and metabolism, neurotransmission, protein synthesis, cytoskeletal integrity, and neuronal plasticity, it can be argued that ACR may significantly contribute to oxidative damage in Alzheimer's disease brain.

By a combined methodological approach, based on MS, conformational, and functional analyses, we studied in detail the effect of α,β-unsaturated aldehydes on polymerization of actin, a protein relatively abundant in mammalian cells and found to be highly carbonylated in vivo, leading to disruption of the cytoskeleton and loss of the monolayer barrier function [56, 57]. The significant accessible surface and the thiol acidity of Cys 374 make this residue the primary target site for both ACR and HNE. However, modification of this site did not result in actin polymerization impairment or in protein-folding alteration. However, ACR also reacts with actin His87 and His173 (and minimally with His40), and this kind of modification inhibits polymerization in a dose-dependent manner. Molecular modeling analyses indicated that structural distortions of the ATP-binding site induced by ACR (Michael adducts) on His residues could explain the changes in the polymerization process.

The same combined LC-ESI-MS/MS and computational approach was applied to demonstrate in vitro the high reactivity of human serum albumin (HSA) toward α,β aldehydes, to study the stoichiometry of reaction with HNE and to identify the amino acid residues more susceptible to carbonyl attack [58]. On the basis of these findings, we proposed [59] a new MS approach, based on LC-MS/MS analysis of tag HNE/ACR-modified peptides of carbonylated albumin and actin that can be applied to the digested proteins isolated from the biological samples or directly to the digested sample (one-shot procedure). By using 2-D SDS-PAGE followed by immunoblot analysis and MALDI-TOF mass fingerprint analysis of the isolated and digested protein, we demonstrated that HSA is the major nucleophilic plasma target of HNE [60] and that Cys 34 is the target site on HSA for α,β-unsaturated aldehydes adduction. Application of a newly developed MS/MS approach [61] to study the Cys34 modification of human plasma following incubation with mildly oxidized LDL allowed easy identification of adducts with ACR (and HNE) and the use of this method to recognize changes of HSA in human diseases involving systemic oxidation/carbonylation.

As albumin is the major carbonylated protein in the bronchoalveolar lavage fluid of older smokers, we recently demonstrated that exposure of HSA to cigarette smoke [62] induces depletion of Cys34 free thiol and marked decrease of free Lys residues. Nanoscale capillary LC-ESI-MS/MS analysis detected ACR (and crotonaldehyde) Michael adducts at Cys34, Lys525, Lys351, and His39, and ACR Schiff base at Lys541 and Lys545. The aldehyde-sequestering agents, hydralazine (HY) and pyridoxamine, partially prevented HSA carbonylation.

We also recently studied the effect of the ACR content in cigarette smoke on human gingival fibroblasts (HGF) and in particular on GSH levels (Colombo, G. et al., submitted for publication). Exposure to cigarette smoke induced a rapid and lethal effect on HGF as well as aberrations in their cellular morphology, which was strictly linked to a dramatic decrease of total GSH (GSH+2GSSG+PSSG). By LC-ESI-MS/MS analysis, it was demonstrated that GSH consumption in HGF cells exposed to cigarette smoke is mainly, but not exclusively due to the formation and export of GSH-ACR (and GSH-crotonaldehyde) adducts. Hence, the results suggest a reaction of GSH with some of the many other cigarette smoke-reactive components.

HSA was also used as a model for studying the effects of inhaled anaesthetics on the ability of ACR to chemically modify human serum albumin [63], with the final aim of understanding the mechanisms associated with (or causing) postoperative cognitive dysfunction, a side effect of surgery and anaesthesia particularly in the older population. This is because aldehyde-mediated modification is dependent on the folded state of the protein, and protein conformation may be influenced by inhaled anaesthetics. Trifluoroethanol, used as a model aesthetic, increases the formation of fluorescent ACR-HSA adducts, probably due to formation of MP-Lys adducts, which, however, were not definitively identified. Interaction of the compound with HSA alters protein conformation, allowing previously cryptic target residues to be more susceptible to ACR modification.

Very recently, the effect of ACR on the most important function of insulin, on the hypoglycaemic activity in vivo and the consequences of glucose transportation in adypocites, has also been investigated [64]. This effect seems to involve insulin carbonylation rather than intermolecular crosslinks formation or aggregates, as demonstrated by characterization of a ACR–insulin adduct following gel filtration chromatography, SDS-PAGE, and carbonyl determination [65].

Finally, in an attempt to gain a deeper insight into the protein targets for ACR within respiratory epithelium, Burcham et al. [66] demonstrated in A549 lung cells that

ACR elicits particular reactivity with intermediate filaments. This is a family of proteins components of the cytoskeleton that differ from actin microfilaments and tubulin microtubules in terms of the large number of genes that encode them, their broad cellular distribution, relative insolubility, and diverse cellular functions. In particular, the multifunctional intermediate filament proteins vimentin, keratin-18, keratin-7, and keratin-8, involved in diverse cellular functions, were found to be the main targets. In particular, adduction of keratins-8 and -18, the two most abundant intermediate filaments in A549 cells, was linked to a loss of cellular adhesive strength.

4.2 Ex vivo–in vivo studies

Formation of ACR protein adducts has been associated with a variety of pathological conditions. For example, Arikketh et al. [67] investigated the role of ACR adducts in the development of atherosclerosis or atherogenesis. In particular, ACR–Lys adducts have been detected in plasma LDL and in the aorta of cyclophosphamide-treated animals by a combined analytical approach (agarose gel electrophoresis, immunoblot, and immunohistochemical methods). ACR-positive material was also clearly detected in the thickening intima (aortic tissues) of apoE-KO mice at the very early stage of atherogenesis [68] and in postmortem eyes from patients with age-related macular degeneration, a neurodegenerative disease of the retina [69].

The same ACR–Lys adduct was used to measure the status of oxidative stress and its relationship to the degree of prematurity and clinical condition in neonates [70], by studying three groups of subjects: healthy term neonates, clinically stable preterm neonates requiring no supplemental oxygen, and clinically sick preterm neonates requiring supplemental oxygen and ventilator support. The results clearly indicated that in the sick preterm group, neonates developing active retinopathy showed significantly higher urinary levels of ACR–Lys adduct than the other neonates without retinopathy.

Increased levels of hemoglobin FDP–Lys have been associated with the severity of diabetic retinopathy in type 1 and type 2 diabetic patients, and its formation is involved in the pathogenesis of this sight-threatening complication of diabetes [71].

In an attempt to find a link between advanced lipoxidation end products (ALEs) accumulation and neuroretinal changes in diabetic retinopathy, Yong et al. [72], using antibodies against several ALEs, did not find significant differences in the intensity of staining for HNE, hydroxy-hexenal (HHE), and malondialdehye (MDA)-modified proteins between retinas from the nondiabetic and diabetic rats. By contrast, they demonstrated a selective accumulation of the ACR-derived ALE adduct FDP-Lys in Müller glia from diabetic rats, thus suggesting a massive involvement of this adduct in determining abnormalities in the early stages of diabetic retinopathy (Fig. 2). The pathogenic potential of FDP-Lys in Müller glia was also confirmed in in vitro studies, by demonstrating that FDP-Lys is not a stable end product but acts as a reactive intermediate capable of mediating protein–protein crosslinking reactions [39].

By testing the role of aldose reductase in regulating formation of ALEs and advanced glycoxidation end products (AGEs) in murine models of diet-induced obesity and

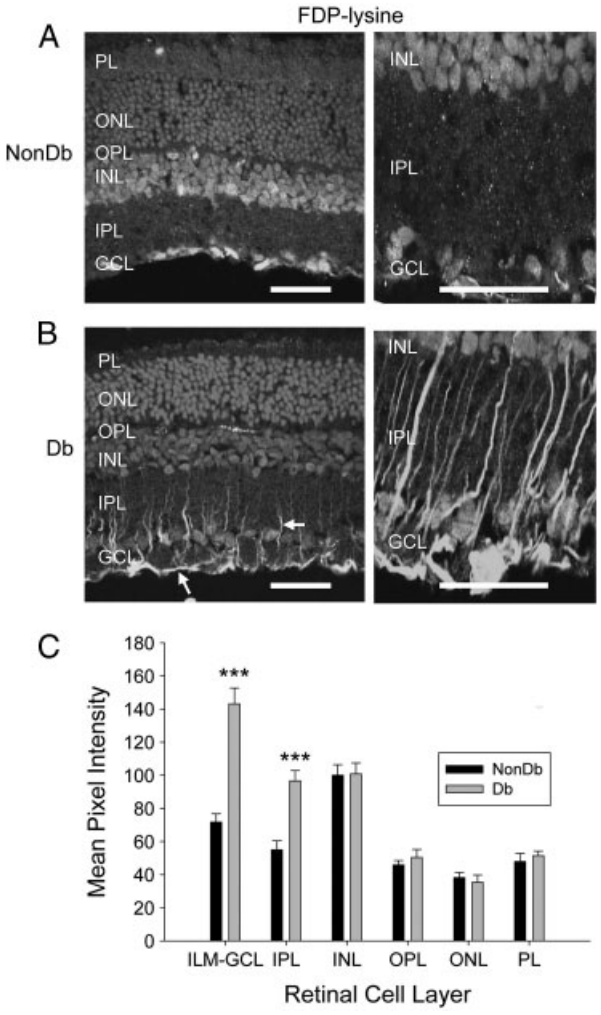

Figure 2. FDP-Lys immunoreactivity in vertical sections of retina from nondiabetic (NonDb) and diabetic (Db) rats of 4 months disease duration. Nuclei were counterstained with propidium iodide. (A) Low and high magnification images of FDP-Lys immunoreactivity in the normal rat retina. Strong immunolabeling was detected in the retinal ganglion cell layer (GCL) and nuclei in the inner nuclear layer (INL). (B) Low- and high-power confocal micrographs of FDP-Lys immunoreactivity in diabetic rat retina. Prominent immunolabeling appeared at the ILM and in radial processes in the inner retina (arrows). (C) Summary data showing that diabetes caused a statistically significant increase in FDP-Lys immunolabeling limited the innermost retinal layers. Reproduced with permission from [72].

streptozotocin-induced diabetes, Baba et al. [73] demonstrated that mice fed a high-fat diet showed increased accumulation of AGEs and protein–ACR adducts in plasma compared with wild-type (WT) and aldose-reductase (AR)-null mice fed normal chow (Supporting Information Fig. 1S). AGEs and ACR adducts were also increased in the epididymal fat of wild-type and aldose-reductase-null mice fed a high-fat diet.

Luo et al. [74] studying the functional and cellular effects of ACR in murine myocardium, found for the first time that ACR induces left ventricular dilatation and dysfunction. By using a conventional proteomic approach (2-D gel electrophoresis and MALDI-TOF MS), they demonstrated that these effects are related, at least in part, to modification of select proteins regulating myocardial contraction (sarcomeric/cytoskeletal proteins: cardiac α-actin, desmin, myosin light polypeptide 3) and energy metabolism (mitochondrial creatine kinase-2, ATP synthase), Site-specific protein modification was confirmed by immunohistochemical co-localization.

ADP/ATP translocase 1 was found as an in vivo target of ACR in rat cardiac mitochondria [75]. By using a newly developed strategy for the covalent labeling and quantification of oxylipid-modified proteins and peptides in complex samples, based on hydrazide-functionalized isotope-coded affinity tag (HICAT), Cys-256 was identified as the target site for ACR conjugation.

Elevated levels of albumin modified by ACR were found during brain infarction in an experimental animal model of thrombosis [76]. The same authors also demonstrated that the measurement of protein-conjugated acrolein (PC-ACR), together other indexes, can be a useful biomarker of small infarction and that ACR prevalently originates from spermine metabolism rather than from PUFAs peroxidation [76, 77]. The levels of PC-ACR were significantly higher in subjects with silent brain infarction than in normal subjects [78]. Hence, according to these authors, the induction of brain infarction is well correlated with the increase in PC-ACR at the locus of infarction and in plasma, and ACR is more strongly involved than reactive oxygen species (ROS) in cell damage. By using MS/MS, these authors reported for the first time in vivo that Lys-557 and Lys 560 are the critical sites on albumin for ACR adduction [77]. At the locus of brain infarction, ACR was found to be mainly conjugated to albumin, as also observed by Yoshida et al. [79] who demonstrated that these Lys residues located at the surface of domain III conjugate with ACR more effectively than with HNE. Indeed, the levels of ACR-conjugated albumin were increased 25- to 30-fold compared with levels at the corresponding locus in normal mice, whereas the levels of HNE-conjugated albumin at the same locus of infarction were only two to fourfold greater than levels at the corresponding locus in normal mice.

Modification of synaptic proteins in hippocampus after traumatic brain injury, through modifications by ACR (and HNE or nitrotyrosine) has been demonstrated in Sprague–Dawley rats subjected to a unilateral moderate cortical contusion [80].

By Western blot analyses of samples precipitated with anti-ACR antibody, Shamoto-Nagai et al. [81] demonstrated that α-synuclein, a key protein in the development of neural degeneration in PD through its conformational change, undergoes modification by ACR in the dopamine neurons of the substantia nigra containing neuromelanin from PD patients (Supporting Information Fig. 2S). Through a series of in vitro studies carried out using recombinant α-synuclein modified by ACR and dopaminergic SH-SY5Ycells, increased protein oligomerization was also demonstrated, as was the generation of polymerized ACR-modified proteins, which are able to inhibit proteasome activity in vitro. It is well known in fact that the initial ACR–protein adduct, containing an electrophilic center, induces severe conformational changes in itself and other proteins through intra and intercrosslinking [9, 66]. Hence, ACR may start a series of events leading initially to modification and aggregation of proteins and ultimately to neuronal death in PD via impairment of the proteasome.

Significant elevation of ACR protein adduct levels has been demonstrated in an animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE) mice [82] The key role of ACR as pathogenic factor in multiple sclerosis, by perpetuating oxidative stress, causing progressive myelin damage and functional loss, was confirmed by the ability of HY treatment to significantly decrease spinal cord injury.

ACR has been assumed to play a key role also in prostate carcinogenesis acting as a toxic, environmental pollutant and as a toxic oxidation product of spermine and spermidine. These ubiquitous cationic amines are in fact abundant in the prostate and are involved in the regulation of cellular proliferation and differentiation, even inducing cell death by oncosis [83]. In fact, ACR–protein adducts were identified as predictive markers of primary prostate carcinomas, and in particular of tumor recurrence in patients after radical prostatectomy [84]. Immunohistochemistry of samples obtained by radical prostatectomy of 70 patients revealed the association of ACR–protein adducts formation with progression of carcinoma, and allowed the demonstration of that the relapse might be predicted with 90% accuracy if clinical parameters and the intensity of ACR presence in tumor stroma are considered together. ACR has also been associated with transition from benign to malignant colon tumors, as indicated by the evaluation of the immunohistochemical distribution of ACR-protein adducts in 113 human colon tumors, revealing their presence even in nonmalignant colon tissue [85].

ACR (and HNE) has also been associated with actinic elastosis, which in photodamaged skin of aged individuals is characterized by the accumulation of fragmented elastic fibers in the sun-exposed areas. Ten years ago, Tanaka et al. [86] demonstrated in specimens of sun-damaged human skin that antibodies against both the aldehydes react with

the accumulations of elastic material. Double immunofluorescence labeling demonstrated that ACR/elastin and HNE/elastin were colocalized in the actinic elastosis.

The influence of ACR–protein adducts formation on the water-holding capacity of stratum corneum was investigated in vitro and in vivo by measuring the surface conductance. The results indicated that exposure of stratum corneum to ACR impairs such function through the modification of protein–water interaction [87]. Attenuated total reflection-infrared spectroscopy applied to reconstructed human epidermis exposed to ACR indicated alterations in the secondary structure of protein, which were accompanied by diminished fibrous keratin structure, as determined by transmission electron microscopy [88].

ACR was also found to be involved in the primary Sjögren's syndrome, a systemic autoimmune disorder mainly affecting the salivary and lacrimal glands characterized by dry mouth and eyes as a result of decreased salivary and lacrimal secretion due to the destruction of salivary glands [89]. The levels of PC-ACR in saliva of patients affected by the syndrome were found to be strictly correlated with the severity of the disease, presumably reflecting the extent of salivary gland destruction. Supporting Information Fig. 3S shows the measurement of PC-ACR in saliva from control subjects and in patients. As previously observed at the locus of brain infarction [77], albumin in saliva (and other 28 kDa proteins) was the main protein target for modification by ACR.

5 ACR-sequestering agents

5.1 Direct agents

As previously outlined by us [3, 90] and others [40], the concept of oxidative stress as such cannot fully describe the sequela of events leading to degenerative diseases, and the new concept of carbonyl stress or ''carbonyl overload'' emerged recently as more fitting from a mechanistic point of view. RCS, derived mainly but not only from oxidative stress, activates a cascade of intracellular signals that ultimately lead to cell and tissue damage by covalently modifying biological macromolecules (proteins, nucleic acids). Hence, RCS and RCS-modified proteins must be considered not only as important mediators of toxic events, but must be regarded as new drug targets. For this reason, the efforts of several research groups in the last few years have been directed in this way, to find and develop new chemical agents able to trap and neutralize these toxic mediators before their impact on macromolecules.

In the last few years, we reported that different pharmacological approaches aimed at preventing or inhibiting formation of ALEs deserve interest, and among them some promising compounds able to trap α,β-unsaturated aldehydes have been identified [3, 90]. These endogenous and exogenous compounds can deactivate or degrade cytotoxic carbonyls behaving as sacrificial nucleophiles, thus to spare cell macromolecules thereby slowing disease progression [91, 92]. Although this strategy will not completely abolish the oxidative stress, it can reduce the toxicological consequences of RCS attack and slow down the progression of pathological events. Figure 3 shows the structures of the so far identified aldehyde-sequestering agents NAC, HY, pyridoxamine, aminoguanidine, CAR, whose properties, demonstrated both in vitro and in vivo, have been extensively considered and discussed in our previous reviews, collecting articles published until 2005.

Hence, in this survey, we report only the advancements in this field (newly developed molecules specifically tested for ACR scavenging) and the more significant studies performed in the last few years attesting the efficacy of compounds already recognized as promising candidates.

NAC, which has been considered for a long time only as a reactive oxygen species scavenger, is now recognized as a strong scavenger of α,β-unsaturated aldehydes, being able to inhibit ACR-induced apoptosis [93], and to reduce the size of brain infarction in animal models [77]. Yoshida et al. [78]

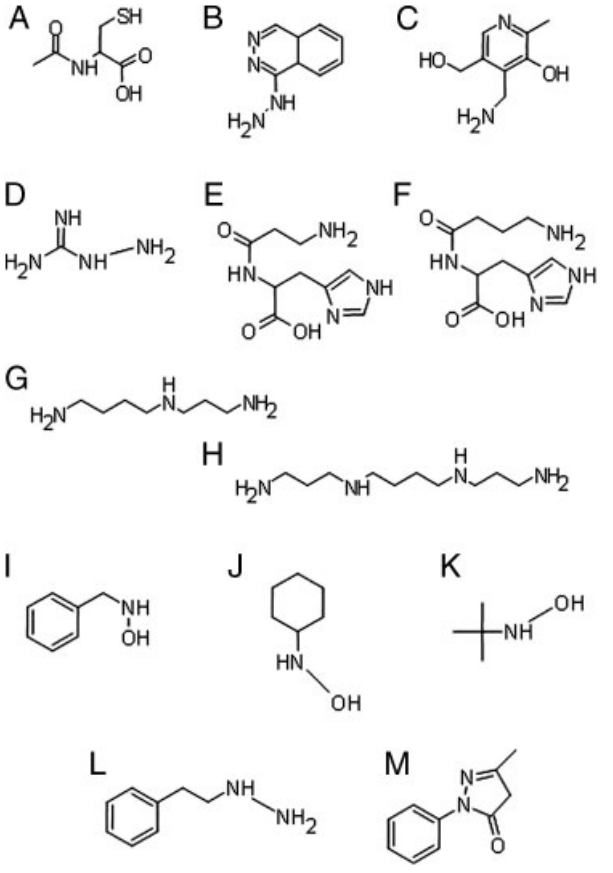

Figure 3. Structures of some effective sequestering agents. (A) *N*-acetyl-cysteine, (B) HY, (C) pyridoxamine, (D) aminoguanidine, (E) CAR, (F) homocarnosine, (G) spermidine, (H) spermine, (I) *N*-benzylhydroxylamine, (J) cyclohexyl hydroxylamine, (K) *t*-butyl hydroxylamine, (L) phenelzine, and (M) edaravone.

confirmed such data, providing evidence that the effect of NAC in brain infarction is mainly due to removal of ACR. This is not surprising, due to the high reactivity of thiols with α,β-unsaturated aldehydes, making Cys GSH the main detoxification system for them [3].

Under the conditions in which ACR induced 100% cell death in SN56 cholinergic neurons, several thiols including cysteine, NAC, 2-mercaptoethanesulfonic acid, mercaptopropionyglycine, and cysteamine have been shown to provide dramatic protection by acting as direct sequestering agents against cytotoxic aldehydes [94]. In addition, all these thiols were found to increase the intracellular cysteine levels via disulfide interchange reactions.

HY has been recognized for many years as an efficient ACR scavenger neutralizing ACR toxicity by forming a hydrazone derivative [95] and the group of Burcham extensively studied this compound in preventing ACR-induced toxicity in different experimental animal models (reviewed in [3]). HY has also been shown to target carbonyl-containing ACR adducts, forming hydrazones that may prevent participation by modified proteins in nucleophilic additions that generate inter and intramolecular crosslinks [96]. With the final aim of gaining a deeper insight into the chemistry underlying the ability of the compound to trap ACR–protein adducts, Kaminskas et al. [97] used MS/MS analysis to investigate the reactivity of HY with ACR-modified preproenkephalin (fragments 128–140), by monitoring the loss of adducted peptide in the presence of the scavenger and detecting the reaction products.

ACR scavenging has been recognized as a potential novel mechanism of attenuating oxidative stress following spinal cord injury [38]. A novel target to attenuate oxidative stress is highly warranted, in view of the fact that free radical scavengers have been largely ineffective in clinical trials and that ACR, having a significantly longer half-life than the transient free radicals, may represent a potentially better target of therapeutic intervention. HY has been shown to significantly improve behavioral outcomes and to attenuate myelin damage in spinal cord of experimental autoimmune encephalomyelitis mouse, an animal model of multiple sclerosis [82]. Hence, treatment with anti-ACR compounds may be a novel therapeutic approach to attenuate ACR-mediated pathology in multiple sclerosis, also taking into account that multiple ACR-trapping agents, including HY and phenelzine, are FDA approved medications [92, 95, 98, 99]. Other neurodegenerative diseases and trauma where carbonyl stress has been implicated, such as amyotrophic lateral sclerosis [100], Alzheimer's disease [101, 102], and traumatic spinal cord injury [38, 103, 104] may also benefit from such treatment.

The role of ACR and the potential of ACR scavenging in spinal cord injury have been recently reviewed [38]. The same authors [105] also confirmed the critical role of ACR in secondary injury following ex vivo spinal cord trauma, by demonstrating that ACR–Lys adducts are capable of diffusing from compressed tissue to adjacent, otherwise uninjured tissue and that injury is significantly attenuated by HY. As HY treatment resulted in significantly less membrane damage 2 h following compression injury, but not immediately after, it was suggested that ACR, increased to pathologic concentrations following spinal cord injury, may contribute significantly to secondary injury.

In parallel, several HY analogues have been developed and their chemical and cytoprotective properties evaluated. The ability of these compounds to block the induction of cell death induced by ACR under different pathological conditions has been reviewed by Burcham et al. [106].

Despite these positive findings, HY being a common anti-hypertension drug cannot be used chronically to restrain ACR-induced damage. In addition, the metabolic fate of HY-trapped ACR-protein adducts is still unknown, as is their potential to induce adverse reactions such as autoimmune disease (the lupus syndrome being the most common immunological reaction to HY treatment) [107].

Hence, a chitosan nanoparticle-based therapeutic system has been developed as an alternative approach to overcome the problems related to blood pressure management, and its efficacy proven in vitro [108]. Although the promising results, indicating that systemic exposure of drug can be significantly reduced, since it will be delivered to the intended site of action, the efficacy of this new system remains to be demonstrated in animal models of spinal cord and brain injury.

As compounds bearing primary or secondary amino group may be useful for detoxification of ACR, Yoshida et al. [79] also determined whether polyamines can attenuate or prevent toxicity caused by ACR. Spermidine (and putrescine), but not spermine (Fig. 3), was found to interact with ACR and to partially reduce ACR-induced cell toxicity, giving rise to the formation of FDP–spermidine and MP–spermidine, similar to the products of ACR-conjugated Lys [42, 109].

In an attempt to demonstrate for aldehyde scavengers a neuroprotective effect superior to those of free radicals scavengers, Wood et al. [110] evaluated a series of hydroxylamines (*N*-benzylhydroxylamine, cyclohexylhydroxylamine, and *t*-buthylhydroxylamine) (Fig. 3) in an in vitro model of neurodegeneration induced by the reactive aldehyde, 3-aminopropanal (3-AP), a product of polyamine oxidase metabolism of spermine and spermidine. Unlike free radical scavengers (TEMPO and TEMPONE) and the antioxidant ascorbic acid, which were ineffective in this model, a dose-dependent protective effect was elicited by all the tested compounds. The same authors extended these findings in vivo, by studying the effects of *N*-benzylhydroxylamine in a rat model of hippocampal neurodegeneration, a model involving an increased polyamine metabolism and hence an increased generation of reactive aldehydes. The compound, given at a daily subcutaneously dose of 50 mg/kg for 17 days, provided 100% protection against neurodegeneration, thus further supporting the potential clinical investigation of effective sequestering agents for RCS.

A series of in vitro experiments were also undertaken to determine if the hydrazine function of the monoamine oxidase inhibitor phenelzine (Fig. 3) could also neutralize reactive aldehydes and block their cytotoxicity in ischemia-reperfusion brain injury [99]. The results indicate that phenelzine like HY avidly neutralizes ACR (and 3-aminopropanal), but its sequestering ability is not limited to cytotoxic aldehydes, because many other endogenously generated aldehydes may aspecifically react to give hydrazones (i.e. poor selectivity). Studies are in progress to evaluate the aldehyde-sequestering profile of newly synthesized analogues of phenelzine bearing a substituted hydrazine function that are weak inhibitors of monoamine oxidase but retain neuroprotective properties in ischemia and neurotoxicity animal models.

The Burcham group [111] recognized bisulfite as another highly efficient ACR scavenger, able to suppress, unlike HY, protein adduction and crosslinking during ACR toxicity. Several carbonyl scavengers (sodium bisulfite, d-penicillamine, HY and its structural nonvasoactive analogue 1-hydrazinoisoquinoline) were tested [112] for their ability to attenuate toxicity in A549 human lung cells induced by exposure of a smoke extract (polyethylene combustion products). Bisulfite, but not HYs suppressed both the adduction and the crosslinking of intermediate filament targets [113]. For this reason, other authors suggested that the cytoprotective actions of HY against electrophilic carbonyls may involve antioxidant actions [114].

Other authors [115], by investigating the effect of different RCS-trapping agents (HY, methoxylamine, aminoguanidine, pyridoxamine, CAR, taurine, and z-histidine hydrazide) on the formation of protein carbonyls during depletion of brain GSH, came to the same conclusion about HY. HY only (and none of the other scavengers tested) prevented protein carbonylation (thus to suggest that the majority of protein carbonyls in this oxidative stress paradigm do not derive from stable lipid peroxidation products) and its efficacy is mostly due to its antioxidant properties rather than to a RCS-trapping intervention.

1,3-Dicarbonyl enol substructures represent a new class of neuroprotective agents able to scavenge electrophilic unsaturated aldehydes through their nucleophilic enolate forms [116]. The acidic hydrogen atoms of enolic hydroxyl groups, as well as those from the α-carbon of 1,3-diketo tautomers, are ionized under physiological conditions, and the conjugate bases formed (resonance-stabilized structures; Supporting Information Fig. 4S) provide key nucleophilic sites for reactions with electrophiles such as unsaturated aldehydes. These compounds, by sequestering ACR, prevent the loss of sulfhydryl groups, which in turn preserved synaptosomal membrane transport and promoted neuronal cell survival. The observed rank order of potency 2-acetylcyclopentanone>acetylacetone, 1,1,1-trifluoro-2,4-pentanedione>>diethylmalonate, 2,5-hexanedione was found to be related to differences in the reaction rates of individual 1,3-dicarbonyl compounds with ACR, which are determined by the acidity (pK_a) of the parent compound and by the inherent nucleophilicity of the corresponding enolate. Hence, these enols might be considered rational candidates for the treatment of acute or chronic neurodegenerative conditions that have oxidative stress as a common molecular etiology, and deserve a deeper investigation on their in vivo potentialities.

We previously demonstrated that histidine-containing dipeptides CAR (β-alanyl-L-histidine, L-CAR) and homocarnosine (HCAR) are selective quenchers of α,β-unsaturated aldehydes and the reaction mechanism with ACR was fully elucidated by a MS approach [117]. The mechanism involves a sequential addition of 3 mol of ACR/mole dipeptide to both the β-alanine and histidine residues, with the formation of several intermediates and final products (Supporting Information Fig. 5S) which well explain the selectivity of these compounds to quench ACR. However, the serum instability of CAR in humans, leading to the rapid hydrolysis to inactive histidine and β-alanine by the specific dipeptidase carnosinase, prompted us to design and develop new CAR aryl derivatives as sequestering agents of RCS [118]. Stability to carnosinase was achieved by isomerization of the histidine residue, leading to D-CAR (D-CAR, β-alanyl-D-histidine) which maintains the same quenching activity of L-CAR. A molecular modeling approach was used to select the most promising candidates characterized by an increased quenching efficacy, up to threefold greater than D-CAR. D-CAR, owing to its carbonyl scavenger properties, has been shown to ameliorate dyslipidemia and renal function in Zucker obese rats, a nondiabetic animal model of nephropathy where carbonyl stress is massively involved in renal damage [119].

In parallel, with the aim of increasing the oral bioavailability of D-CAR, we designed, synthesized, and evaluated the metabolic stability and the pharmacokinetic properties of a set of ester, amide, and carbamate-based prodrugs [120]. The octyl-ester of D-CAR was chosen as candidate for pharmacological testing and we found that the compound is effective in reducing obesity-related diseases in the same animal model used for D-CAR (Zucker rats) through a RCS quenching mechanism.

Another endogenous tripeptide glycyl-histidyl-lysine (GHK), a liver cell growth factor isolated from human plasma never considered before as an ACR quencher, was found to react with ACR giving rise to the formation of several products, among which the FDP-Lys adduct [121].

The growing need to find new molecules sharing both antioxidant and carbonyl scavenger properties to restrain lipid-derived oxidative/carbonyl stress associated with a variety of pathologic conditions, prompted us to focus our attention on a compound already known as a potent radical scavenger, having protective effects against cerebral ischemia/reperfusion injuries, but poorly investigated for its carbonyl-sequestering ability, edaravone [122]. Edaravone (Fig. 3) is a potent quencher of ACR (and HNE), and computational studies allowed us to elucidate the mechanism

of interaction with RCS and to explain the lack of selectivity as an RCS scavenger, being able to react also with nontoxic, physiologically relevant aldehydes, such as pyridoxal. These findings shed light on a new mechanism by which the compound can exert its efficacy in vivo (since 2001 it has been approved by the Japanese health authorities as a neuroprotective agent for the treatment of acute cerebral infarction), and constitute the molecular basis to start a discovery process of more selective edaravone analogues.

Several natural polyphenols have been shown to act as direct trapping agents of lipid peroxidation derived-ACR and HNE [123]. In particular, flavan-3-ols, theaflavins, cyanomaclurin, and dihydrochalcones effectively trapped both the α,β-unsaturated aldehydes working as sacrificial nucleophiles, the most effective being phloretin. LC-MS/MS and NMR were used to characterize diACR-conjugated phloretin as the main reaction product, and to elucidate the mechanism of interaction. This involves the Michael addition of phloretin to the C═C double bond of ACR, followed by the formation of hemiacetal between the hydroxyl group in the A ring of phloretin and the C═O carbonyl group in ACR (Fig. 4). As flavones, flavanones, flavanonols, flavonols, and other phenolics were ineffective, it was possible to identify the phloroglucinol moiety as the structural key feature shared by the effective scavengers. Hence, very likely ACR/HNE undergo electrophilic substitution on the A ring of these polyphenols, as previously demonstrated for green tea polyphenols and in particular epigallocatechin gallate [124, 125].

While the function of ascorbic acid as biological antioxidant is well known for many years, its ability to participate in Michael addition reactions has been only recently recognized [126]. The protective effects of ascorbic acid against ACR-induced toxicity have been demonstrated in different cell and animal models, as in cultured human bronchial epithelial cells [127], or in spinal cord white matter isolated from guinea pigs [128], or in human very LDL in vitro [129]. However, the effect has been generally attributed to an antioxidant mechanism and none of these studies considered the hypothesis of a direct interaction between ACR and ascorbic acid as a new detoxification mechanism. Through a combined NMR and LC-MS analytical approach, the group of Kesinger [126] demonstrated that ACR reacts with ascorbate to give a Michael adduct in human monocytic THP-1 cells exposed to ACR. They also provided evidence for its subsequent metabolic conversion to 5,6,7,8-tetrahydroxy-4-oxooctanal (THO), in a reaction catalyzed by lactonase activity of recombinant human paraoxonase 1 and paraoxonase 2 (Supporting Information Fig. 6S). This new biotransformation pathway represents a complementary pathway to GSH for ACR detoxification and these results shed light on new mechanism of intervention of an old molecule.

5.2 Indirect agents

Pharmacological manipulation of intracellular thiol pools might offer a new approach to the design of neuroprotective drug candidates. Upregulation of cellular aldehyde-detoxification factors has been recognized as a promising strategy for protecting against ACR-induced cytotoxicity [130] and the role of chemically induced GSH and GST in protecting against α,β-unsaturated aldehydes-mediated cytotoxicity has already been described in vascular smooth muscle cells [131]. The same authors demonstrated that the nutraceutical compound 3H-1,2-dithiole-3-thione, found in cruciferous vegetables, affords protection against ACR-induced toxicity in rat aortic smooth muscle cells [132], and in human primary astrocytes [133]. The mechanism of protection involves induction of cellular antioxidants (reduced GSH and GSH-reductase) and upregulation of phase 2 enzymes, GST, and the NAD(P)H:quinone oxidoreductase 1 (NQO1). The protective effect of the compound on ACR-mediated

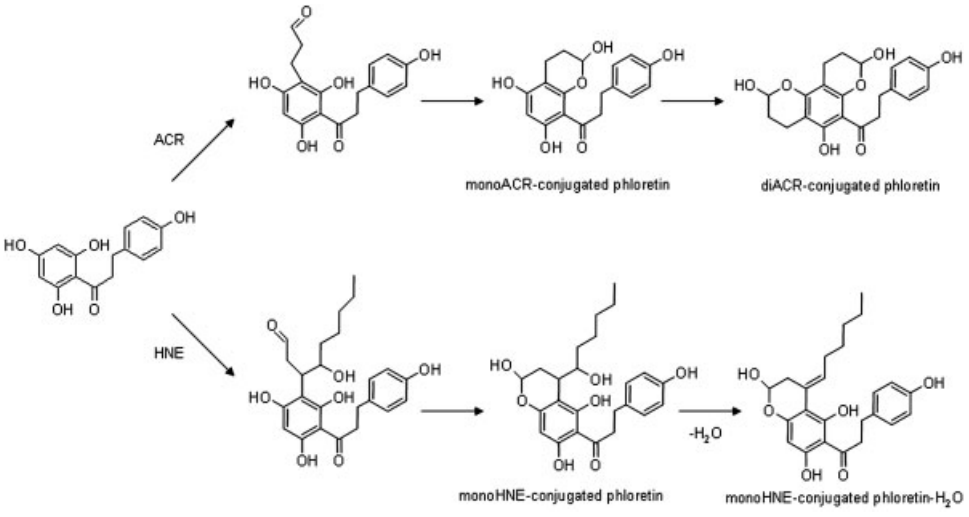

Figure 4. Proposed mechanism of the reaction between phloretin and ACR/HNE. Reproduced with permission from [123].

toxicity has also been demonstrated in human neuroblastoma SH-SY5Y cells, and upregulation of GSH, but not of GST or aldose reductase was found to be the predominant mechanism of protection [130]. A spectrum of cellular antioxidants and phase 2 enzymes including GSH, GSH-peroxidase, GSH-reductase, GST, and NOQ1 was significantly induced in isolated mitochondria of aortic smooth muscle cells by sulforaphane, a cruciferous isothiocyanate compound, which is responsible for an increased resistance of these vascular cells to ACR exposure [134].

Also *Scutellaria baicalensis* Georgi (Labiatae), one of the 50 fundamental herbs of Chinese herbology, has been reported to restrain ACR-induced toxicity in HUVEC cells, an effect which is strictly associated with increased GSH levels and with elevation of mRNA expressions of GSH synthesis enzymes [135].

Polyphenols have been recognized for many years as free radical scavengers and in particular as chain-breaking antioxidants, able to inhibit propagation of the free radical-induced cascade of lipid peroxidation, and among them resveratrol has been identified as one of the most potent and effective. Very recently, the activity of this compound has been extended to the evaluation of its potential protective effects on the ACR-induced oxidative stress in human retinal pigment epithelial cells, and the results indicate that resveratrol might ameliorate ACR-induced or age-related retinal pigment epithelial (RPE) degeneration, such as age-related macular degeneration [136].

Another polyphenol, robinetinidol-(4β→8)-epigallocatechin 3-*O*-gallate (REO), a galloyl dimer isolated from Acacia mearnsii De Wild, could be potentially useful as a protective agent for people exposed to ACR. The compound has been shown to protect human neuroblastoma SH-SY5Y cells from ACR-induced damage via multiple mechanisms of intervention, i.e. attenuation of oxidative stress, NADPH oxidase activity, GSH depletion, protein oxidation/nitration, lipid peroxidation, mitochondrial dysfunction, JNK activation, and caspase activity [137].

The same cell model (neuroblastoma SH-SY5Y cells) was employed to support Pycnogenol®, a patented combination of bioflavonoids extracted from the bark of French maritime pine (Pinus maritima), and already known as a potent free radical scavenger, as a promising approach to the treatment of oxidative stress-related neurodegenerative diseases [138]. The compound significantly and dose-dependently attenuated ACR-induced cytotoxicity, protein damage, lipid peroxidation, and cell death, very likely by modulating oxidative stress and increasing GSH levels.

A similar neuroprotective effect against ACR was observed using standardized extracts of *Bacopa monniera*, a species which has a long history of use in India as a memory-enhancing therapy [139]. Elucidation of the mechanisms underlying this protection is, however, to accomplish.

Also Vitamin E, the main lipophilic chain-breaking antioxidant in humans, has recently been shown to be an indirect antioxidant, by enhancing the cellular antioxidant system and inducing Phase II enzymes [140]. This property was demonstrated in human RPE cell line (ARPE-19), a model of smoking- and age-related macular degeneration, exposed to ACR, where vitamin E afforded a significant cytoprotective effect.

Pretreatment with vitamin E increased in fact the expression and/or activity of glutamate cysteine ligase, NQO1, heme-oxygenase 1, GST and superoxide dismutase and, as a consequence, the GSH levels.

The same cell line (ARPE-19) was used to demonstrate the protective effects of hydroxytyrosol, a natural polyphenol abundant in olive oil, on ACR-induced toxicity and on mitochondrial dysfunction [141]. The same authors [142] later proposed a mechanism for the protective effect afforded by hydroxytyrosol, involving simultaneous activation of two critically important pathways, induction of phase II detoxifying enzymes (including γ-glutamyl-cysteinyl-ligase, NQO1, heme-oxygenase-1, superoxide dismutase, peroxiredoxin, and thioredoxin) and stimulation of mitochondrial biogenesis (through increased protein expression of mitochondrial transcription factor A, uncoupling protein 2, and mitochondrial complexes). Overall, these results indicate that dietary supplementation of hydroxytyrosol may contribute to eye health by preventing the degeneration of retinal pigment epithelial cells induced by oxidative stress.

Finally, another compound known as an antioxidant, α-lipoic acid [143] has been shown for the first time to be a potent inducer of GSH and NQO1 in cultured human neuroblastoma cells challenged by ACR. Hence, also in this case, upregulation of cellular defenses, being responsible for the markedly increased resistance to cytotoxicity induced by ACR, may have important implications for the neuroprotective effects of lipoic acid.

6 Concluding remarks

Overwhelming evidence grown in the last 10 years supports the involvement of protein modification by reactive aldehydes in different pathological situations, and in particular that of ACR in chronic degenerative diseases, especially at central nervous system and renal levels. Substantial research indicates that protein adduction, in addition to the depletion of GSH and other cellular reducing equivalents, is one of the primary mechanisms of ACR toxicity.

The mechanistic relationship between adduct formation and subsequent cellular damage has not been definitively established. However, as emerged from this survey, even more numerous in vitro studies focused on this aspect, and tried to find a specific biological response to protein modification by ACR.

The advancement in proteomic approaches and methodologies had a fundamental role in detection of peptides and proteins covalently modified by RCS, especially for the characterization of sites of modification. In the last few

years, proteomic analysis has gained much more importance, because, in conjunction with biological data, provided a deeper insight into the effects of modification by RCS on protein function, allowing understanding the consequences of such modifications at cell level. Thus, it is reasonable to assume that the next few years will witness a more precise definition of the events that lead from protein oxidative modification to altered cellular function. This represents a critical point to establish whether carbonylation of target proteins is causative, correlative, or consequential to oxidative stress-associated conditions. Understanding the molecular actions of ACR (and other highly reactive α,β-unsaturated aldehydes) could provide insight into many pathogenic conditions that involve initial cellular oxidative stress and could, thereby, not only promote advanced and oriented applications in diagnosis and prevention, but also offer new efficacious avenues of pharmacological defense. Indeed, while the efforts of the researchers have been initially focused on the detection of modified proteins in order to find a causative link with the development/progression of the disease, in the last few years the interest shifted to consider protein carbonylation as a drug target.

This prompted us and many other research groups to study the RCS trapping ability of a variety of nucleophilic compounds. A first conclusion that can be drawn from this survey is that HY is highly effective in preventing ACR-induced toxicity, both in vitro and in vivo, affording strong cytoprotection at concentrations several orders of magnitude lower than those of other RCS scavengers such as aminoguanidine, CAR, or pyridoxamine. However, the hypotensive effect and the poor specificity (HY readily reacts with several biogenic keto compounds due the strong nucleophilic group), make the molecule unsuitable for a clinical use as a direct ACR trapping agent. For this reason, several other hydrazino and nonhydrazino derivatives have been initially tested in vitro as alternative compounds to HY. We believe that an emerging field of interest in medicinal chemistry is indeed represented by a rational drug design approach aimed to identify and develop novel aldehydes sequestering agents characterized by high reactivity, specificity, suitable pharmacokinetic profile, and safety, as that we undertook with CAR derivatives. Results so far obtained are very promising (under patent application) and studies are in progress to further improve the druggability of CAR derivatives.

Another important approach aimed at protecting cells from ACR toxicity is to enhance the cell machinery involved in its detoxification, mainly through upregulation of pathways leading to increased GSH levels and to an increased expression/activity of GSH-related enzymes. This is exemplified by the recently discovered ACR-detoxifying role of ascorbic acid, vitamin E, or polyphenols. Hence, another field on which the efforts of the researchers would have to be focused in the near future could be a detailed reinvestigation on the mechanism of action of those endogenous compounds or micronutrients which are known to be implicated in protection against oxidative stress-induced damage.

The authors have declared no conflict of interest.

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