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REVIEW ARTICLE

Transforming dietary peptides in promising lead compounds: the case of bioavailable carnosine analogs

Giulio Vistoli · Marina Carini · Giancarlo Aldini

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Abstract The ability of carnosine to prevent advanced glycoxidation end products (AGEs) and advanced lipoxidation end products (ALEs) formation, on the one hand, and the convincing evidence that these compounds act as pathogenetic factors, on the other hand, strongly support carnosine as a promising therapeutic agent for oxidative-based diseases. The mechanism/s by which carnosine inhibits AGEs and ALEs is still under investigation but an emerging hypothesis is that carnosine acts by deactivating the AGEs and ALEs precursors and in particular the reactive carbonyl species (RCS) generated by both lipid and sugar oxidation. The ability of carnosine to inhibit AGEs and ALEs formation and the corresponding biological effects has been demonstrated in several in vitro studies and in some animal models. However, such effects are in line of principle, limited in humans, due to the effect of serum carnosinase (absent in rodents), which catalyzes the carnosine hydrolysis to its constitutive amino acids. Such a limitation has prompted a great interest in the design of carnosine derivatives, which maintaining (or improving) the reactivity with RCS, are more resistant to carnosinase. The present paper intends to critically review the most recent studies oriented to obtaining carnosine derivatives, optimized in terms of reactivity with RCS, selectivity (no reaction with physiological aldehydes) and the pharmacokinetic profile (mainly through an enhanced resistance to carnosinase hydrolysis). The review also includes a brief description of AGEs and ALEs as drug targets and the evidence so far reported regarding the ability of carnosine as inhibitor of AGEs and ALEs formation and the proposed reaction mechanisms.

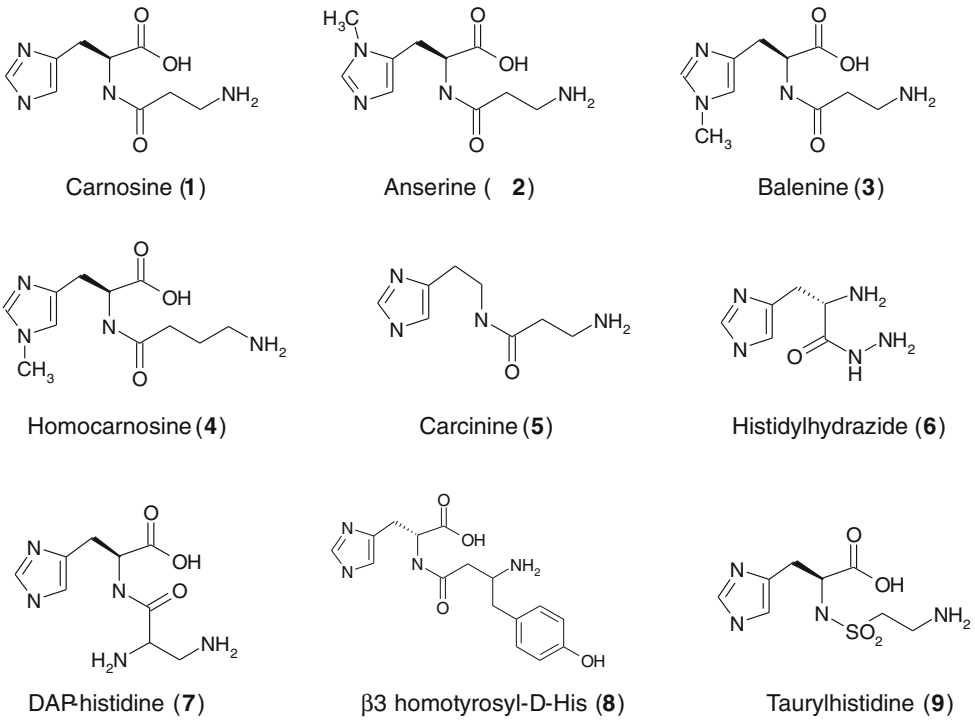
Keywords Carnosine and derivatives thereof · Advanced glycoxidation end products (AGEs) · Advanced lipoxidation end products (ALEs) · Reactive carbonyl species · Carbonyl quenching

G. Vistoli · M. Carini · G. Aldini (✉)
Department of Pharmaceutical Sciences "Pietro Pratesi", Università degli Studi di Milano, via Mangiagalli 25, 20133 Milan, Italy
e-mail: giancarlo.aldini@unimi.it

Introduction

Although carnosine (**1**, Scheme 1) was discovered more than a century ago by the Russian scientists Gulewitsch and Amiradzibi (1900), its biological role is still far from clear. Of course, several hypotheses have been proposed, such as that carnosine acts as a buffering agent in the skeletal muscle and as a neurotransmitter and antioxidant, as recently reviewed by Hipkiss (2009). Moreover, in vitro and animal studies strongly suggest that carnosine is able to restrain some oxidative-based diseases such as diabetes (Riedl et al. 2011; Lee et al. 2005; Pfister et al. 2011), atherosclerosis (Rashid et al. 2007) and metabolic distress syndrome (Aldini et al. 2011), and also ischemia–reperfusion damage in different organs, including the brain (Pekcetin et al. 2009; Dobrota et al. 2005) and the heart (Lee et al. 1999). Such a protective effect has been initially put down to the antioxidant and metal ion chelating ability of carnosine (Klebanov et al. 1998), as well to a pro-histaminic effect, this latter mainly taken into account to explain the anti-ischemic effect (Kurata et al. 2006). More recently, the ability of carnosine to prevent, inhibit and reverse the formation of oxidative-based non-enzymatic modifications and in particular the advanced glycoxidation (AGEs) and lipid-oxidation end products (ALEs) has also been considered (Hipkiss et al. 1998; Aldini et al. 2005).

Scheme 1 Naturally occurring histidine-containing dipeptides (**1**–**5**) and some relevant synthetic derivatives (**6**–**9**)



AGEs and ALEs are a diverse group of compounds formed by the reaction of nucleophilic amino acids such as Lys, Arg, His and Cys with reducing sugars and reactive carbonyl species (RCS) arising from sugar oxidation and degradation (AGEs) or from lipid oxidation (ALEs, see below).

Carnosine was found to significantly inhibit AGEs and ALEs formation as demonstrated in both in vitro and animal studies. The mechanism/s by which carnosine inhibits AGEs and ALEs is still under investigation but an emerging hypothesis is that carnosine acts by deactivating RCS (quenching activity), the precursor of the non-enzymatic oxidative-based protein modifications. In particular, this mechanism has already been fully clarified for the quenching effect of carnosine on α,β-unsaturated aldehydes, including 4-hydroxy-2-nonenal (HNE) and acrolein, a reactive class of RCS arising from the lipid peroxidation cascade and leading to the ALEs formation (Zhou and Decker 1999; Aldini et al. 2002; Carini et al. 2003; Liu et al. 2003).

The ability of carnosine to prevent AGEs and ALEs formation, on the one hand, and the convincing evidence that these compounds act as pathogenetic factors, on the other hand, lead us to strongly consider carnosine as a promising therapeutic agent. However, it should be underlined that the ability of carnosine to detoxify RCS, and in turn to inhibit AGEs and ALEs formation thus to restrain oxidative-based diseases, has only been demonstrated in animal models, while only limited intervention studies in humans exist so far (Aldini et al. 2005).

The ability of carnosine to inhibit AGEs and ALEs formation and hence its beneficial effect is limited in humans, due to the presence of human carnosinase (absent in rodents), which catalyzes the carnosine hydrolysis to its constitutive amino acids (see below). Such a limitation has prompted a great interest in the design of carnosine derivatives, which maintaining (or improving) the reactivity with RCS, are more resistant to carnosinase.

The present paper intends to critically review the most recent studies oriented to obtain carnosine derivatives, optimized in terms of reactivity on RCS, selectivity (no reaction with physiological aldehydes) and pharmacokinetic profile (mainly through an enhanced resistance to carnosinase hydrolysis). The review also includes a brief description of AGEs and ALEs as drug target and the evidence so far reported regarding the ability of carnosine as inhibitor of AGEs and ALEs formation and the proposed reaction mechanisms. This information is necessary to make the reader more comfortable with the different approaches proposed for carnosine optimization.

Mechanisms of AGEs and ALEs formation and their biological implications

Various excellent reviews have recently been published regarding the mechanism of formation of AGEs and ALEs as well as on their pathological implications to which the reader can refer for an in-depth reading. This paragraph is intended to schematically summarize the main reaction

mechanisms of AGEs and ALEs formation with particular emphasis on the main RCS species involved in their formation since these represent the target of carnosine and derivatives. Moreover, the main molecular and cellular mechanisms by which AGEs and ALEs act as damaging compounds are briefly reported.

The carbonyl groups of glucose and other reducing sugars react with amino groups in proteins, leading to the formation of Schiff bases which undergo rearrangements involving ene-diol or eneaminol intermediates, to yield more stable glycated products, referred to as Amadori products (Baynes and Thorpe 2000). Amadori adducts, produced under anaerobic conditions, rearrange to form more reactive dicarbonyl compounds such as 1- and 3-deoxyglucosone (DGs), which may react further to yield glyoxal (GO) or methylglyoxal (MGO) by reverse aldol reactions. Phosphorylated intermediates in anaerobic metabolism of carbohydrates, such as triose phosphates and fructose 3-phosphate, also eliminate phosphate to form methylglyoxal (Thornalley 1996) and 3-deoxyglucosone (Lal et al. 1997), respectively. These dicarbonyl compounds can react with arginine residues, with the amino terminal amino group and also with the nucleophilic amino acids including lysine, histidine, tryptophan and cysteine. The structures of different AGEs have been determined in tissue proteins (see the review by Baynes 2003), the most abundant including Nε-(carboxymethyl)-lysine (CML) or the ethyl derivative (CEL), Nε-(carboxymethyl)-arginine (CMA), hydroimidazolones, pyrraline, argpyrimidine and a variety of cross-link structures involving Lys–Lys or Lys–Arg residues such as cross-line, glucosepane and pentosidine.

Decomposition of lipid hydroperoxides initiates chain reactions that produce a large variety of reactive carbonyl compounds, including linear and cyclic aliphatic aldehydes and ketones, and their hydroxy-, keto- and α,β-unsaturated derivatives (Guéraud et al. 2010. Among them, the most studied carbonyl intermediates are α,β-unsaturated aldehydes [4-hydroxynonenal (HNE) and acrolein (ACR)], di-aldehydes [malondialdehyde (MDA) and glyoxal], keto-aldehydes [4-oxo-trans-2-nonenal (ONE) and isoketals (IsoK)]. 2-Hydroxyheptanal is another major aldehydic product of lipid peroxidation of ϖ-6 PUFAs (linoleic acid, arachidonic acid), while 2-hydroxyhexanal is generated in much lower yield. Lipo-peroxidation-derived RCS easily react with cysteine, histidine, and lysine residues in protein, generating characteristic ALEs such as MDA-Lys (Schiff base adduct), HNE-Lys, HNE-Cys, HNE-His (Michael adduct), HNE-Lys (pyrrole derivative), FDP-Lys [N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine], levuglandin adducts (pyrrole derivatives), and N^{ε}-(hexanoyl)lysine (hexanoic acid amides) and N^{ε}-(carboxymethyl)lysine (CML) (Pamplona 2011; Aldini et al. 2007).

It is now well established that AGEs and ALEs are involved in oxidative cellular damage through different mechanisms including direct protein dysfunction, protein oligomerization and deposition, signal transduction, immune response and activation of the receptor for AGEs (RAGEs), as briefly described here below.

The covalent modifications of AGEs and ALEs can induce a function derangement (e.g., structural or enzymatic) of the protein itself, due to the protein conformational change or as a consequence of the catalytic site distortion or impairment caused by the covalent modification. For instance, protein modification of collagen by AGEs and in particular cross-links formation impairs its mechanical properties. The modified collagen becomes structurally inadequate, more resistant to normal hydrolytic turnover, leading to a thicker and less distensible vascular wall matrix (Ciulla et al. 2011).

AGEs and ALEs protein modifications can also induce signal transduction causing a damaging response. As an example, HNE binding to type II collagen and the consequent ALE product results in signal transduction, inducing multiple abnormalities of chondrocytes phenotype and function, suggesting its contribution in osteoarthritis development (El-Bikai et al. 2010). AGEs can also induce vascular smooth muscle proliferation via a prolonged agonist-induced Ca^{2+} increase leading to increased activation of calcineurin and subsequently nuclear factor of activated T cells. This mechanism was suggested to contribute to the pathogenesis of vascular disease in diabetes mellitus (David et al. 2008).

Another mechanism with which AGEs and ALEs induce the damaging effect is represented by protein oligomerization and fibrillogenesis. 4-oxo-2-nonenal and 4-hydroxy-2-nonenal bind α-synuclein and promote the formation of α-synuclein oligomers, which were found cytotoxic when added exogenously to a neuroblastoma cell line (Näsström et al. 2011). Several pieces of evidence have been so far accumulated revealing the ability of a variety of RCS to bind β-amyloid peptide, supporting the involvement of endogenous aldehydes and AGEs and ALEs in amyloid deposition related to Alzheimer disease. In particular, formaldehyde, methylglyoxal, malondialdehyde and 4-hydroxy-2-nonenal were reported not only to be capable of enhancing the rate of formation of beta-amyloid β-sheets, oligomers and protofibrils but also of increasing the size of the aggregates (Chen et al. 2006; Ellis et al. 2010; Siegel et al. 2007).

AGEs and ALEs have antigenic properties and their damaging pro-inflammatory effect may be due to an autoimmune response, as already demonstrated in a variety of inflammatory-based diseases including diabetes, atherosclerosis and rheumatoid arthritis (Turk et al. 2001; Virella et al. 2003; Drinda et al. 2002).

Most of the biological and damaging effects of AGEs have been ascribed to a receptor-mediated mechanism, the most extensively studied receptor being RAGE (namely, Receptor for AGEs) which is a type I trans-membrane glycoprotein of the immunoglobulin superfamily of cell surface receptors. The AGE–RAGE interaction alters cellular signaling, promotes gene expression and enhances the release of pro-inflammatory molecules. It elicits the generation of oxidative stress in numerous cell types. More in details, AGEs–RAGE interaction activates the transcription factor NFk-B via Ras and redox-sensitive signaling pathways, leading to the transcription of genes coding for inducible nitric oxide synthase and a variety of cytokines including IL-1, IL-6 and TNF-α. The importance of the AGEs–RAGE interaction and downstream pathways leading to injurious effects has been amply demonstrated in different animal studies and it is believed to be involved in oxidative-based diseases including diabetic complications, atherosclerosis and some neurological disorders (Yamagishi 2011; Fleming et al. 2011; Stitt 2010; Barlovic et al. 2011). Moreover, the deleterious link of AGEs with diabetic vascular complications has been suggested in many human studies (Barlovic et al. 2011). Therefore, the inhibition of AGES formation, blockade of AGEs–RAGE interaction and the suppression of RAGE expression or its downstream pathways are now promising targets for therapeutic interventions against diabetic vascular complications and in general for inflammatory-oxidative-based diseases (Takeuchi et al. 2010).

Carnosine prevents ALEs and AGEs formation

Hipikiss first reported the ability of carnosine to inhibit AGEs and ALEs formation. In particular, he found that when carnosine was co-incubated with different RCS acting as AGEs and ALEs precursor and including MDA, GO, MGO, acetaldehyde, formaldehyde and glucosone, the formation of protein cross-link, dimerization and oxidative modifications were significantly and dose-dependent inhibited (see the review by Hipkiss and Brownson 2000; Hipkiss 2009). The in vitro ability of carnosine to inhibit AGEs and ALEs induced by a variety of RCS and including reducing sugars was then confirmed by several other studies. More recently, Pietkiewicz et al. (2011) used human and pig muscle-specific enolase as a protein model for in vitro modification by HNE, nonenal, acrolein, GO and MGO and found that carnosine counteracted AGEs formation and protected enolase against total loss of catalytic activity. Carnosine and related peptides were found effective in reducing AGEs as determined fluorometrically and formed by incubating albumin and collagen with peritoneal dialysis fluids from uremic patients (Alhamdani et al. 2007a). Beside in vitro and mechanistic studies, the ability of carnosine to prevent AGEs and ALEs has also been demonstrated in cellular and animal studies. Pepper et al. (2010), by using a prokaryotic model, provided further evidence for carnosine's ability to suppress the formation of AGEs in vivo. In particular, carnosine was found to suppress MG- and glucose-mediated toxicity in E.coli as well as the formation of AGEs such as CML as determined by ELISA. Cheng et al. (2011) demonstrated in cultured neurons that MDA caused protein cross-linking and cytotoxicity, and that both these effects were reversed by carnosine treatment. Carnosine was found to enhance HPMC (human peritoneal mesothelial cells) viability against the toxic effect of RCS originated from glucose oxidation through the protection of cellular protein from modification and from ROS-mediated oxidative damage (Alhamdani et al. 2007b). Regarding the in vivo evidence of carnosine to inhibit AGEs and ALEs formation, we recently found that carnosine treatment of Zucker rats for 24 weeks significantly reduced the urinary AGEs, protein carbonylation in the kidney and improved collagen solubility as an indicator of the extent of collagen cross-linking (Aldini et al. 2011). Moreover, the Michael adduct between carnosine and HNE and the corresponding metabolites were identified in the urine, suggesting that the detoxifying reaction of carnosine occurs in vivo. However, more studies regarding the in vivo effect of carnosine on AGEs and ALEs formation should be carried out, also considering the recent studies by the group of Mannheim who demonstrated that oral carnosine treatment, beside protecting retinal capillary cells and kidneys from apoptosis and podocyte loss in experimental diabetic retinopathy/nephropathy, was ineffective in reducing CML-, MG- and *N*-acetylglucosamine-protein levels (Riedl et al. 2011).

The mechanism by which carnosine prevents AGEs and ALEs formation is still under investigation and probably we should consider not only one but more molecular mechanisms, also considering that AGEs and ALEs formation is a quite complicated pathway, involving different reaction mechanisms and several catalyzing agents including transition metals. Formation of AGEs and ALEs involves oxidation, metal ions and a direct reaction of carbonyls toward the nucleophilic sites of protein. Since carnosine is an antioxidant, metal-ion chelator and a trapping agent of RCS, it is quite difficult to dissect the different mechanisms as also pointed out by Onorato et al. (2000) for other AGEs inhibitors such as pyridoxamine and aminoguanidine. Moreover, Hipkiss et al. (2001) have proposed an additional mechanism: carbonylated proteins may become "carnosinylated" at carbonyl groups and that this may protect them from degradation and/or cross-linking.

However, we have enough evidence to indicate that at least for ALEs generated by α,β-unsaturated aldehydes, carnosine acts by a direct RCS quenching mechanism. We firstly demonstrated the ability of carnosine to covalently react with HNE by measuring by HPLC the disappearance of the RCS when incubated in the presence of the peptide. The reaction mechanism was then elucidated by studying the quenching effect of carnosine derivatives and by characterizing the chemical structures of the reaction products as determined by MS and NMR studies. In particular, we found that the *N*-acetylation of the β-alanine amine group precluded the quenching activity and that the two constitutive amino acids were less efficient than the dipeptide, thus indicating that the two amino acids acts synergistically as dipeptide and that the reaction involves the β-alanine amine group (vide infra, Aldini et al. 2002).

As depicted in Fig. 1 and better detailed in "A rational drug design approach for carnosine derivatives as RCS detoxifying agents," the multistep reaction mechanism, which was reported by two independent groups (Aldini et al. 2002; Liu et al. 2003), involves both reactive groups of carnosine (i.e., amino group and imidazole ring). Indeed, it begins with the formation of a Schiff base intermediate to yield the macrocyclic adduct through an intramolecular Michael addition, which finally hydrolyzes to form the stable hemiacetal derivative (**4a**). The ability of carnosine to quench HNE has been then demonstrated in biological matrices and in particular in oxidized skeletal muscle by measuring the reaction product **4a** by using LC–ESI–MS (Orioli et al. 2005). Furthermore, carnosine was found able to quench HNE in vivo as demonstrated by identifying the HNE-carnosine Michael adduct as well as metabolites in the urine of Zucker obese rats, which represent a well-established animal model of oxidative stress leading to protein oxidation and carbonylation (Orioli et al. 2007).

Carnosine bioavailability and metabolic fate in humans

In humans, diet represents the main source of carnosine (CAR) and histidine derivatives (HD), which are contained in significant amount in red and white meats. Dietary carnosine is readily absorbed intact, primarily in the jejunum by a carrier-mediated transport system (Bauchart et al. 2007). The enzymatic hydrolysis of the peptide bond represents the main metabolic fate of carnosine, and in humans, it mainly occurs in the plasma by a specific serum hydrolase (carnosinase) that cleaves the β-alanine–histidine peptidic bond. The two hydrolyzed amino acids are then delivered to tissues and then again synthetized to carnosine

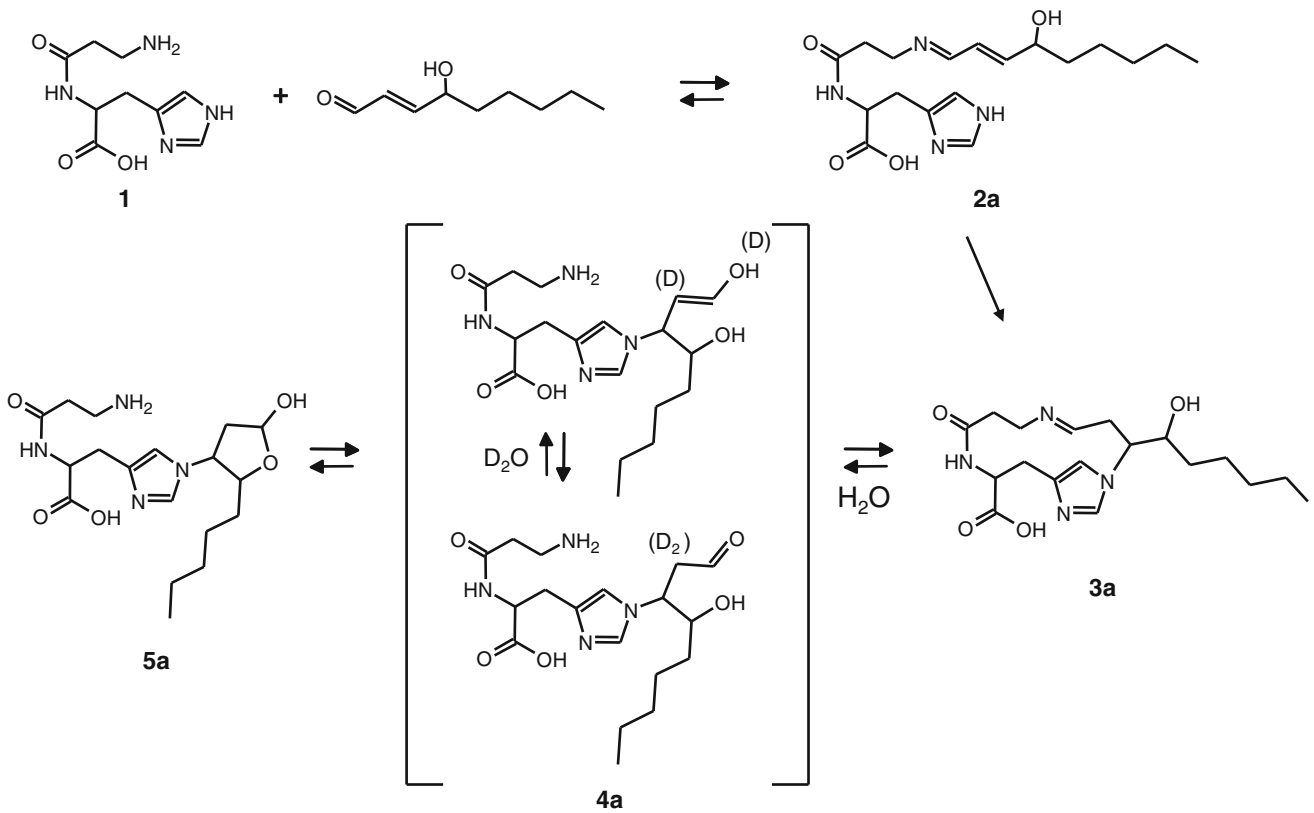


Fig. 1 Three-step mechanism by which carnosine reacts with HNE. Figure reproduced from Aldini et al. (2002) with kind permission from Elsevier

in those tissues characterized by the presence of carnosine synthetase, such as skeletal muscle, heart and in some regions of the CNS (Aldini et al. 2005).

The ADME (adsorption, distribution, metabolism, excretion) profile of carnosine in humans is not fully elucidated, and no studies are available for the other food-derived HD. This is mainly due to the lack of specific and sensitive methods for their measurement in biological matrices as well as the scarcity of ADME studies in humans. Gardner et al. (1991) first described the urinary and plasma profile of carnosine and β-alanine in healthy volunteers, after ingesting 4 g of carnosine, and using an ion-exchange amino acid analyzer with ninhydrin detection for β-alanine and carnosine quantitation. The amount of carnosine recovered in urine was found to range from 1.2 to 14% of the ingested dose, while only small amounts of carnosine were detected in the blood. More recently, Park et al. (2005) monitored by HPLC the plasma levels of carnosine in healthy volunteers following consumption of 200 g of ground beef (equivalent to a carnosine dose of almost 250 mg). Carnosine was detected in plasma 15 min after beef consumption, and maximal concentration was recorded 2.5 h after consumption (Cmax 150 μM). More recently, the profile of histidine dipeptides in plasma and urine after ingesting beef, chicken, chicken broth or pure peptides in humans has been reported. A significant excretion of HD and in particular of anserine was found in urine, despite the low and in many cases undetectable content of HD peptides in the serum (Yeum et al. 2010).

A rational drug design approach for carnosine derivatives as RCS detoxifying agents

RCSs are now considered promising drug targets since they are the precursors of AGEs and ALEs which in turn are involved in the onset and propagation of different oxidative-based diseases. Considering RCS as target, a promising drug discovery approach is based on the design of compounds acting by forming a covalent adduct with the reactive moiety (carbonyl function) of RCS thus to be detoxified. Different compounds have been proposed and acting through this mechanism (carbonyl quenching or sequestering compounds) among which aminoguanidine, hydralazine, metformin and pyridoxamine (see reviews by Aldini et al. 2006, 2007). For most of these compounds, the reaction mechanism has been fully clarified and the reaction products identified in both in vitro and in vivo conditions. Furthermore, their efficacy was demonstrated in different animal models, including obese (Zucker), diabetic and atherosclerotic rats (Aldini et al. 2007). However, for most of the proposed compounds, the clinical use is limited due to two main issues: the promiscuous activity and the lack of selectivity. Aminoguanidine is a well-known inhibitor of NOS, and hydralazine is a potent vasodilating agent. Moreover, both of these compounds act through a nucleophilic amino group, which stably condenses with the carbonyl functions. Hence, they are not selective toward cytotoxic compounds since they also form stable Schiff bases with physiological carbonyl compounds, such as pyridoxal phosphate, thus inducing their depletion and liver damage. Aminoguanidine reached diabetic nephropathy clinical trials, but the external safety monitoring committee recommended the early termination of the studies due to safety concerns. To our knowledge, pyridoxamine is the only RCS scavenger and AGEs inhibitor to be in clinical phase for the treatment for diabetic kidney disease (Williams et al. 2007) as searched in http://clinicaltrials.gov.

As described in the previous part, carnosine is a very efficient inhibitor of AGEs and ALEs and its beneficial effect has been demonstrated in different animal models by independent research groups. Moreover, carnosine is a safe dietary compound, selective as RCS quencher since it does not cross-react with physiological aldehydes such as pyridoxal phosphate, due to its peculiar quenching mechanism (see below). However, the clinical application of carnosine is, in line of principle, greatly limited due to the presence of serum carnosinase (CN1, EC 3.4.13.20), which greatly reduces the bioavailability of carnosine in human circulation and kidney, which represent two districts where protein oxidation occurs in several oxidative-based diseases (atherosclerosis, diabetes and metabolic syndrome). Such limited bioavailability has stimulated the study of carnosine derivatives resistant to carnosinase. Moreover, the search for carnosine derivatives is currently extending toward derivatives, which are more reactive as RCS quenchers and also able to detoxify different chemical classes of RCS, beside α,β-unsaturated aldehydes.

Carnosine derivatives: improving quenching activity and selectivity

The rational design of improved and selective carnosine analogs cannot ignore the precise three-step mechanism by which carnosine reacts with HNE and related α,β-unsaturated carbonyl derivatives (Aldini et al. 2002; Liu et al. 2003). As illustrated by Fig. 1, the reaction starts with the condensation between the carbonyl function and the carnosine primary amine, resulting in the formation of a reversible α,β-unsaturated imine intermediate (**2a**). The second step involves the key intramolecular Michael addition between the imidazole $N\tau$ atom and the acceptor β carbon atom to yield the corresponding macrocyclic system (**3a**). The quenching reaction ends with the hydrolysis of the instable imine function followed by an intramolecular condensation to give the final hemiacetal derivative (**5a**).

Since the physical mixture of the two amino acids (βAla + His) is devoid of significant quenching activity, one may argue that the first imine intermediate acts as an intramolecular catalyzer constraining the imidazole ring to approach the β carbon atom in an arrangement constantly conducive to the Michael addition, which would be further favored due to the slightly more electrophilic character of the imine derivative compared to the free carbonyl as recently analyzed (Appel and Mayr 2011).

Accordingly, the quenching activity of carnosine derivatives could be improved by optimizing either the first intermediate formation or the Michael addition. The former can be promoted by increasing the reactivity of the amino group or by replacing it with other moieties (e.g., alcohols, diols, hydrazines, hydrazides and diamines) able to condense with the carbonyl group. The latter could involve two different strategies: (a) enhancing the reactivity of the Michael donor group by replacing the imidazole ring with more nucleophilic systems or (b) favoring the approach of the imidazole ring to the reactive β carbon atom by modulating the conformational profile of the imine intermediate through suitable modifications principally focused on the β-alanine portion. The second approach should pay attention also to the stability of the macrocyclic intermediate since too large (or too narrow) rings are unstable and may hamper the overall quenching reaction. In this section, the mentioned strategies will be discussed in depth, and the corresponding derivatives will be described.

Promoting the imine formation sets important challenges since there are several factors that may hamper the beneficial effects of an increase in amine reactivity/nucleophilicity. Indeed, although the relation between nucleophilicity and basicity is, as a rule, quite complex and not always directly proportional, several studies evidenced a good correlation between proton affinity and nucleophilicity for amine derivatives (Jaramillo et al. 2007). Hence, a nucleophilicity increase is unavoidably paralleled by a corresponding increase in the fraction of protonated forms at physiological pH. On the other hand, the amine protonation precludes the imine formation since the protonated species cannot attack the carbonyl carbon atom to yield the corresponding carbinolamine (Hall and Smith 1998). This means that the remarkable quenching activity of carnosine can also be due to its moderate basicity (pK = 9.32, Vistoli et al. 2009), whereas more nucleophilic amino groups should result in a reduced fraction of reactive neutral forms with an overall worsening of the quenching activity.

Although it is impossible to directly correlate quenching activity with amine's basicity, probably because the imine formation is only the first step in the carbonyl scavenging mechanism, the detrimental effect of a basicity increase can be documented by the reduced quenching activity of homocarnosine (**4,** Scheme 1) as demonstrated by mass spectrometry analyses (Carini et al. 2003; Aldini et al. 2004). Specifically, the quenching activity of homocarnosine is about one half that of carnosine, and the difference can be explained by an increase in the amine basicity due to a weaker electron-drawing effect of the peptide group in γ (pK = 9.89 for homocarnosine, unpublished data).

Consequently, the imine formation may be enhanced by lowering the basicity of the amino group so as to increase the fraction of neutral species able to yield the imine derivative. Nonetheless, such a strategy should be cautiously pursued since the stability of imine function increases when nucleophilicity decreases due to the stabilizing effect of electropositive substituents on the C=N double bond (Tahmassebi 2001). This implies that carnosine derivatives endowed with relatively basic amines will lose their selectivity by trapping even physiological carbonyls. Altogether, these considerations emphasize that the reactivity of the amino group should be carefully balanced to maximize the quenching activity without undermining the selectivity. The basicity of the carnosine amino group may represent a good balance to assure both reactivity and selectivity suggesting that the basicity of carnosine analogs should remain close to that of carnosine itself.

Despite the discussed concerns in promoting the first intermediate formation, several compounds were designed pursuing this strategy probably because of a wide synthetic accessibility. In addition, the mechanism of some important carbonyl quenchers (not related to carnosine structure, e.g., aminoguanidine, hydralazine and dihydralazine) is restricted to the ability of condensing with the carbonyl function without having Michael donor moieties. In detail, the quenching activity of these compounds is completely due to a hydrazine moiety, which is consistently able to condense with carbonyl compounds irrespectively of the environmental pH. Indeed, all hydrazine derivatives share quite similar quenching activities almost independently of the remaining portion of the molecule (Galvani et al. 2008). This characteristic renders the compounds also able to trap the dicarbonyl derivatives (e.g., GO, MGO, MDA) but undermines their specificity thus depleting physiological carbonyl compounds. Furthermore, hydrazine and hydrazide moieties can induce hepatotoxicity through a mechanism, which goes beyond the depletion of physiological carbonyl compounds, and which seems to be initiated by a common metabolite, acetyl hydrazide, which evolves to yield the extremely reactive and toxic diazohydroxide derivative, as investigated for antituberculosis isoniazid (Metushi et al. 2011).

Nevertheless, several carnosine derivatives have been designed to improve the imine formation through the introduction of a hydrazine (or hydrazide) function. These compounds can be subdivided into two classes: the first includes compounds that can be related to retrocarnosine

(i.e., His-βAla) and the hydrazine moiety is linked to the carboxyl group thus yielding a reactive hydrazide, while the derivatives of the second group lack the β-alanine residue and can be related to the histidyl-hydrazide structure (Guiotto et al. 2005). Both groups comprise very active molecules and, in particular, histidyl-glycyl-hydrazide and histidyl-hydrazide (**6,** Scheme 1) showed a quenching activity significantly greater than that of carnosine. In addition, glycyl hydrazides without the histidine residue were far less active than the parent compound, thus suggesting that the combined presence of both reactive groups plays a key role in the scavenging activity which is not restricted to the sole imine formation. In the same study, the authors also proposed carnosine analogs in which the amino group is replaced by 1–2 diol moiety but they were less active than carnosine probably because the acetalic intermediates lose the characteristic electrophilicity of α,β-unsaturated carbonyl derivatives. The promising activity of histidyl-hydrazide prompted Guiotto and co-workers (Guiotto et al. 2007) to investigate a set of analogs obtained by coupling the free amino group with different aminoacyl moieties. Among them, the leucyl-histidyl-hydrazide showed a remarkable quenching activity against both dicarbonyls and α,β-unsaturated carbonyls. The prepared compounds demonstrated also that the imidazole ring is required to quench α,β-unsaturated carbonyls, while the hydrazide moiety is enough to trap dicarbonyls.

Another moiety that can replace the amine group of β-alanine to promote the imine formation is the *N*-terminal 2,3 diamiopropionic (Dap) residue, as proposed by Sasaki and co-workers (Sasaki et al. 2009) who reported a set of Dap analogs endowed with a marked quenching activity toward dicarbonyl species. Among these derivatives, D-Dap-D-Leu and L-Dap-L-Val showed a significant activity in quenching methylglyoxal with the formation (as studied by NMR for D-Dap-D-Leu derivative) of two possible adducts characterized by pyrazine rings. Similarly, Cacciatore and co-workers (Cacciatore et al. 2005) prepared a set of carnosine analogs containing the Dap residue instead of β-alanine and with different degree of *N*-acetylation (see for example derivative **7,** Scheme 1). The compounds were found to be protective agents against oxidative stress but their carbonyl quenching was never assayed.

A second strategy to obtain improved carnosine derivatives may promote the Michael addition and this approach should be more effective since it favors the formation of covalent and stable adducts specific for α,β-unsaturated carbonyls thus avoiding interference with physiological carbonyl species. This second strategy can be pursued by (a) increasing the nucleophilicity of the Michael donor group and/or (b) modulating the conformational profile of the imine intermediate to favor close and reactive conformations.

With regard the nucleophilicity of Michael donor moieties, this approach is simply based on the consideration that Michael addition, like most polar organic reactions, can be seen as a combination between electrophile and nucleophile reactants and, although general scales for electronic properties are not yet available, electrophilicity and nucleophilicity can be conveniently parameterized by quantum–mechanical indices (Mayr 2005). For example, LoPachin et al. (2009) exploited HOMO/LUMO energies to rationalize the extreme reactivity of the thiolate ion and the moderate reactivity of the imidazole ring and amino group toward α,β-unsaturated aldehydes. Despite the possibility of parameterizing the electronic effects on the Michael addition, very few carnosine derivatives modified on the imidazole ring were published. Only recently, D'Arrigo et al. (2009) reported the synthesis of carnosine derivatives modified at the imidazole ring, but their quenching activity was not described. Although of natural origin, anserine (**2**, βAla-1-methylHis) and balenine (**3**, βAla-3-methylHis) can be seen as carnosine derivatives modified at the imidazole and characterized by a *N*-methylated ring (Aldini et al. 2005). Orioli et al. (2005) showed that anserine reacts with α,β-unsaturated aldehydes with a mechanism completely superimposable to that of carnosine yielding to the formation of a *N*-methyl imidazolium adduct. Moreover, the quenching activity of anserine is very similar to that of carnosine, thus suggesting that the *N*-methylation does not strongly influence the imidazole nucleophilicity. Unfortunately, the reactivity of balenine toward reactive carbonyl species was never reported. With a view to enhancing the whole nucleophilicity, histidine peptides containing BIMA (bis(imidazol-2-yl)methylamine) and BIP (bis(imidazol-2-yl)propionic acid) residues were also reported as potent metal chelators (Osz et al. 2004).

To better understand the possible role for the conformational profile of the carnosine imine intermediate, Fig. 2 shows the equilibrium (as derived by a clustered Monte-Carlo procedure) between folded conformations in which the imidazole ring and the β carbon atom are close enough to yield the corresponding adduct and extended structures in which the reactive moieties are too distant to give the Michael addition. A deeper analysis of the conformational profile for this intermediate emphasizes the critical role of the torsion angles, which pertain to the β-alanine residue, while those corresponding to the histidine residue show a greater flexibility and have a minor influence on the whole imine conformation. As a trend, synclinal conformations in the monitored torsions favor folded geometries, which can be stabilized by polar interactions between the hydroxyl function and the carboxyl group or the imidazole nitrogen atom, although frequent stabilizing interactions also involve π–π stacking between the imidazole ring and the

imine or peptide bonds. While avoiding a systematic analysis of all rotatable torsions, the folding of the imine intermediate might be evaluated by considering the distance between the imidazole $N\tau$ atom and the acceptor β carbon atom. A threshold of 9.0 Å can be taken as an useful (albeit arbitrary) cut-off to discriminate between folded and extended geometries of carnosine and its derivatives. Considering this distance threshold, Fig. 2 evidences that only one half of the computed conformers for the carnosine intermediate assumes a folded geometry, thus suggesting that much can be done to enhance the quenching activity of carnosine derivatives by modulating their conformational properties.

A recent study (Vistoli et al. 2009) reported a remarkable correlation between quenching activity and folding degree for a set of aryl derivatives of D-carnosine (vide infra). Specifically, this study revealed the possibility to markedly increase the quenching activity by substituting β-alanine with β2 homophenylalanine and β3 homotyrosine (**8**, Scheme 1). As the basicity of the amino group remains very close to that of carnosine and the considered modifications should not modify the imidazole reactivity, the bioactivity enhancement may be ascribed to a beneficial conformational effect exerted by aromatic substituents that should favor folded geometries through intramolecular π–π stacking.

Similarly, the modest quenching activity of endogenous tripeptide Gly–Hys–Lys (GHK) can be rationalized in terms of conformational effects that involve both the closeness between the imidazole and the β carbon atom (as obtained simulating the imine intermediate both at *N*-terminus and at lysine ε-amino group) and the stability of the corresponding macrocyclic intermediates (Beretta et al. 2007).

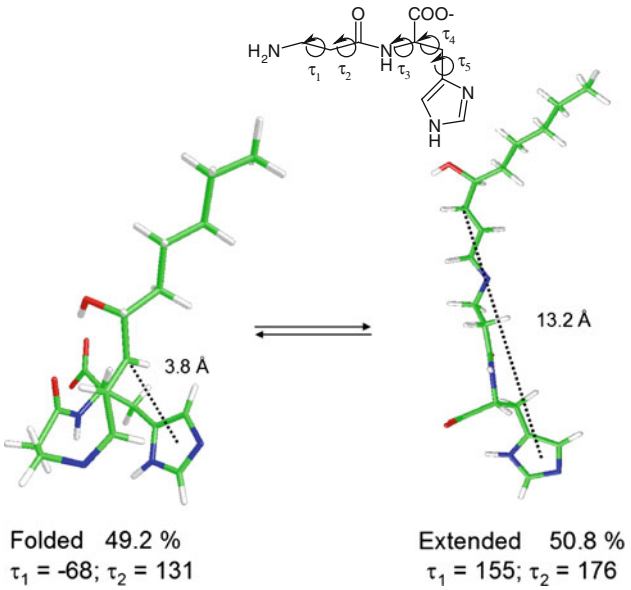


Fig. 2 Conformational profile of carnosine imine intermediate with HNE as a function of torsion angles pertaining β-alanine. The distance between reactive atoms allows the identification of folded and extended conformation whose relative abundance is reported as percentage. Figure reproduced from Vistoli et al. (2009) with kind permission from Wiley

Among the histidine-containing oligopeptides, which can be related to carnosine, it is worth citing the nor-analog in which β-Ala is replaced by a shorter glycine (Gly–His), whose capacity to chelate metals was investigated by several studies (see for example, Rychlewska et al. 2010). Its quenching activity is not yet described but Reis et al. (2011) recently demonstrated that GH peptide (along with other glycine and histidine-containing di- and tripeptides) is highly susceptible to oxidative reactions yielding cyclic endoperoxides and azetidinone derivatives. Other histidine-containing dipeptides have been described in literature with different chemical and/or biological applications, even though their quenching activity has never been assayed. Among these histidine dipeptides, one may mention: (1) Tyr-His, which is characterized by long-range electron-transfer reactions through tyrosyl radicals and represents a model peptide for the regulatory mechanism of many redox enzymes (Moore et al. 2010); (2) Trp-His, a vasodilating dipeptide, which inhibits extracellular Ca^{2+} entry by blocking dihydropyridine-like L-type channels (Wang et al. 2010); (3) γGlu-His, which is produced by *Penicillium roquefortii* and imparts an enhanced taste in blue cheeses (Toelstede et al. 2009); and (4) Ser-His, which catalyzes the DNA cleavage and condensation of peptide fragments thus finding a putative role in primitive chemistry for the origin of biomacromolecules (Gorlero et al. 2009; Ma et al. 2008).

Carnosine derivatives: improving oral bioavailability and disposition

Besides the quest of more active analogs, the main reason that prompted the development of many carnosine derivatives is the scarce oral bioavailability of carnosine, which is actively absorbed but immediately hydrolyzed in human plasma to its constituent amino acids by specific dipeptidases (Pegova et al. 2000) thus preventing its therapeutic applications (see paragraph 4). Specifically, carnosine is hydrolyzed by serum carnosinase (CN1, EC 3.4.13.20), a M20 metalloprotease, which is characterized by its distribution in plasma and brain, its ability to hydrolyze also anserine and homocarnosine, and its absence in non-primate mammals except the Syrian golden hamster (Lenney et al. 1982; Jackson et al. 1991). A second carnosinase isozyme is a cytosolic form (also named tissue carnosinase, CN2, EC 3.4.13.18), which acts as a non-specific dipeptidase having broad substrate specificity and being strongly

inhibited by bestatin (Lenney et al. 1985). Notably, the structure of mouse tissue carnosinase was recently resolved providing structural information for substrate recognition and catalytic mechanism of the M20 metallopeptidases (Unno et al. 2008). In parallel, carnosine is synthesized starting from L-histidine and β-alanine by carnosine synthetase (EC 6.3.2.11), an ATP-dependent enzyme, which is specifically expressed in skeletal muscle, heart and brain and also catalyzes the biosynthesis of homocarnosine (Drozak et al. 2010). Given the rapid hydrolysis of carnosine in plasma, the carnosine effects are tightly confined in the tissues expressing the corresponding synthase. Consequently, the dietary intake of carnosine as well as the supplementation of the constituent amino acids can elevate the carnosine concentration only in those tissues expressing carnosine synthetase as demonstrated by the effect of β-alanine supplementation on muscle carnosine concentrations (Derave et al. 2010).

Carnosine is actively transported by the hPepT1 transporter (Toyobuku et al. 2002; Meredith 2009), a proton-dependent oligopeptide transporter (POT; Newstead 2011), which also transports anserine (Geissler et al. 2010) and is mainly located in the gastrointestinal tract thus assuring a prompt absorption of these dipeptides whose noteworthy hydrophilicity would not allow a detectable passive permeation. Carnosine and anserine are also recognized by the hPepT2 transporter, which principally influences the regional pharmacokinetics in the brain, the reabsorption from renal tubular fluid and pulmonary delivery (Kamal et al. 2008; Kamal et al. 2009). The expression of the human peptide/histidine transporter (hPHT1, SLC15A4) was also observed in the gastrointestinal tract, suggesting that it may participate in the intestinal absorption of all histidine-containing dipeptides (Bhardwaj et al. 2006).

On these grounds, a carnosine derivative with improved bioavailability should (1) be resistant to the carnosinase hydrolysis thus assuring an effective plasma stability; (2) conserve a good affinity for peptide transporters thus assuring an active absorption and an optimal disposition; (3) maintain the moieties that are required for a marked quenching activity combined with a significant selectivity (namely, a moderately basic amino group and a Michael donor moiety). Considering the key role played by serum carnosinase and oligopeptide transporters in determining the pharmacokinetic profile of carnosine derivatives and the lack of experimentally resolved structures, these proteins were modeled by homology techniques and their interaction features were investigated in depth by docking simulations (Vistoli et al. 2006; Pedretti et al. 2008; Pedretti et al. 2011).

Although a stable carnosine derivative can be obtained by replacing the peptide group with non-hydrolyzable moieties according to classic peptidomimetic strategies, a better knowledge of the molecular recognition of carnosine by carnosinase might support the rational design of stable derivatives which conserve the peptide bond. Figure 3 shows the putative complex between carnosine and human serum carnosinase and reveals the key interactions stabilized by the Zinc ions with both the carboxylate and the carbonyl oxygen atom. This last contact polarizes the carbonyl group and has an essential catalytic role common of hydrolases. The ammonium group of carnosine interacts with the carboxylates of Asp116 and Glu451, while the nitrogen atoms of the imidazole ring elicit H-bonds with Leu254 and Thr424, thus providing a rationale for the specificity of serum carnosinase toward histidine-containing dipeptides and for the lower activity of serum carnosinase toward carnosine analogs with a substituted nitrogen in the imidazole ring, even though anserine is, however,

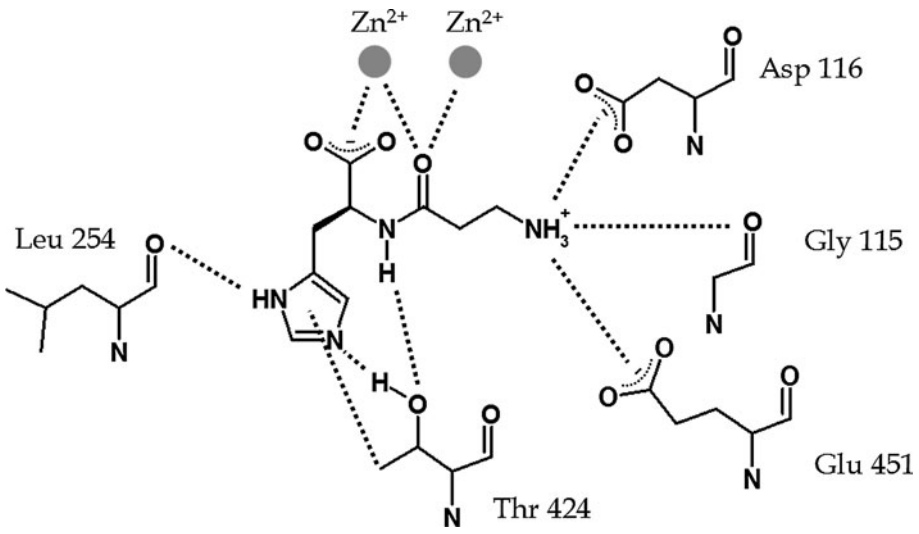


Fig. 3 Main interactions stabilizing the putative complex between carnosine and human serum carnosinase. Figure reprinted from Vistoli et al. (2006) with kind permission from ACS

hydrolyzed by the enzyme. In the light of this complex, a stable carnosine derivative could be obtained by masking the carboxyl or the amino group, even though the latter should be modified in a reversible manner to preserve quenching activity.

Despite the various strategies normally adopted to replace a peptide bond with a non-hydrolyzable moiety, very few carnosine derivatives modified in the peptide group have hitherto been published. In particular, Calcagni and co-workers (Calcagni et al. 1999) described a series of sulphonamido pseudopeptides related to carnosine and designed as carnosinase inhibitors. Among them, Tau-His (**9,** Scheme 1) inhibited the carnosinase activity by 15% at a concentration of 5 mM. However, the quenching activity of these pseudopeptides was not reported.

Very few carnosine derivatives modified at carboxyl group have hitherto been described. The simplest derivative is carcinine (**5**, β-alanylhistamine), which is devoid of the carboxyl group and shows a profile of selective H3 antagonist (Chen et al. 2004). In addition, the radical scavenging and the skin protection of carcinine have repeatedly been described in literature but its carbonyl quenching activity has never been reported (Babizhayev and Yegorov 2010). Bertinaria et al. (2011) reported a set of analogs in which the carboxyl group was transformed in amide by coupling it with primary amines of different size and hydrophobicity. The proposed amides were stable in human plasma, thus confirming the key role of carboxyl group in carnosinase recognition, conserved a good ability to complex cupper ions and showed a carbonyl quenching activity slightly weaker than that of carnosine. More recently, Orioli et al. (2011) described a set of ester prodrugs of D-carnosine (vide infra) and reported that the methyl ester analog is completely stable and has a quenching activity which is superimposable to that of carnosine. Altogether, these few derivatives suggest that the carboxyl group might be modified with modest effects on quenching activity.

The carnosine derivatives modified in the primary amine are more frequent, even though it should be reminded that any modification at the amino group abolishes quenching activity unless it is labile in vivo as in the case of *N*-acetyl carnosine that acts as a prodrug since it is slowly hydrolyzed by non-specific deacetylases and is used in ophthalmic therapies (Babizhayev et al. 2009). The analogs modified in the amino group also include cyclodextrin derivatives (Amorini et al. 2007), glycoside analogs (Lanza et al. 2011) and Trolox modified derivatives (Stvolinsky et al. 2010). These compounds are stable in human plasma, but the large (and stable) modifications in the *N*-terminus prevent the carbonyl scavenging and indeed these compounds have been designed mainly as radical scavengers and/or metal chelators. Moreover, a recent study showed that the *N*-modification with bulky hydrophobic aromatic units (as in the case of Fmoc-carnosine) induces well-defined amyloid fibrils containing β-sheets above a critical aggregation concentration (Castelletto et al. 2011).

A third strategy to obtain stable derivatives involves the inversion of the configuration of histidine residue to yield D-carnosine, namely β-Ala-D-His as proposed by Aldini et al. (2011). Since the inversion of the chiral center prevents a productive pose within the carnosinase catalytic site, D-carnosine is completely stable in plasma. Moreover, it shows a quenching activity identical to that of L-carnosine and this confirms that carbonyl quenchers act by a receptor-independent mechanism, and therefore two enantiomers must have the same chemical reactivity. Unfortunately, D-carnosine is no longer recognized by peptide transporters with a resulting marked decrease in oral bioavailability despite its plasma stability. Indeed, in vivo studies showed that the maximal plasma concentration of L-carnosine was more than twice that of D-carnosine, whose modest passive absorption is clearly interpretable in terms of marked hydrophilicity. The key role of the configuration of transported peptides (as well as of the other pivotal features which govern the recognition, see below) can be clearly illustrated by docking carnosine enantiomers into the hPepT1 homology model as recently generated by us (Pedretti et al. 2008). Figure 4 compares the best poses of L-carnosine and D-carnosine within hPepT1-binding site. Figure 4a depicts the complex of L-carnosine and evidences the key interactions stabilized by ammonium head, which involve a strong H-bond with Tyr588 plus ion-pairs with Glu23 and Glu26. Yet again, the carboxyl terminus is inserted in a niche flanked by backbone atoms of Ala295, Leu296 and Phe297 with which it elicits H-bonds. Finally, imidazole ring stabilizes π–π interactions with Tyr588 and contacts several hydrophobic residues (e.g., Leu296, Ile331, Leu591). By applying the correlative equation developed in the modeling study, L-carnosine is predicted to have a Km for hPepT1 equal to 1.21 mM, which is in encouraging agreement with the experimental value (Km = 2.48 mM). Conversely, Fig. 4b shows that D-carnosine is seen to conserve the interactions elicited by ammonium head, whereas those involving the carboxylate are completely missed. More importantly, the imidazole ring is inserted in a small pocket where it could elicit apolar contacts but it may exert also detrimental steric clashes.

Altogether, these docking results are in agreement with the structural requirements for an optimal transport already evidenced by 3D-QSAR analyses (Biegel et al. 2005) and confirm that a hPepT1 substrate must meet the following conditions: (1) the optimal length corresponds to that of di/tripeptides, while tetrapeptides and single amino acids are not well recognized; (2) the transport is markedly

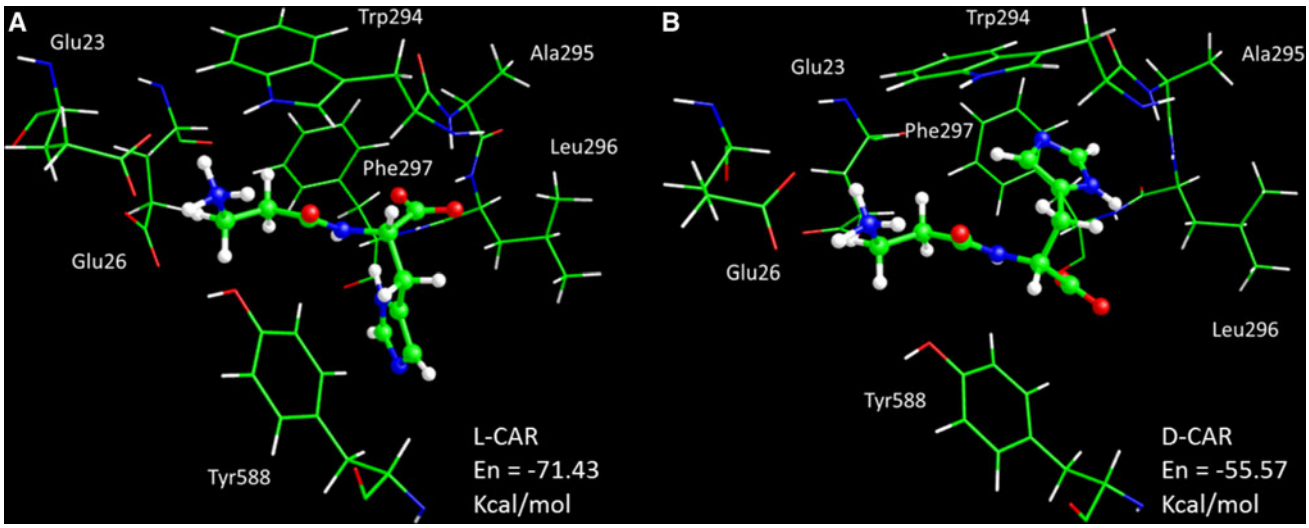


Fig. 4 Comparison of the putative complexes between hPepT1 and L-carnosine (**a**) or D-carnosine (b). One may note the better interactions elicited by L-carnosine as confirmed by electrostatic docking scores. The complexes were computed by exploiting the computational procedure described in Pedretti et al. (2008)

stereospecific: natural amino acids are properly transported, whereas the introduction of D-isomers vastly decreases the affinity as seen for D-carnosine; (3) the terminal groups are not mandatory for the transport, even if modifications of the carboxyl group are better tolerated than those of the amino group and this suggests that carnosine derivatives modified at the carboxy terminus could yet be recognized by hPepT1; (4) central peptide bonds are not required for an optimal bioactivity; (5) hydrophobic side chains are largely preferred. Notably, the same requirements also characterize the substrate recognition of hPepT2 (Pedretti et al. 2011; Biegel et al. 2006), thus suggesting the D-carnosine derivatives should have both a low absorption and a restricted disposition.

With a view to improving the pharmacokinetic profile of D-carnosine, Orioli et al. (2011) prepared a set of ester-, amide- and carbamate-based prodrugs endowed with a suitable lipophilicity to promote a passive absorption. The reported derivatives confirmed that the double protection at both charged termini is unproductive since the two promoieties are not suitably (and simultaneously) hydrolyzed in vivo (Hamman et al. 2005). As a rule, acetyl and carbamate derivatives appeared too polar to be rapidly converted into the parent compound, whereas ester-based prodrugs are promptly hydrolyzed (apart from the too polar methyl ester as previously discussed) since they are good substrates of the major human carboxylesterases as confirmed by in silico studies (Vistoli et al. 2010a, b). Specifically, the octyl-ester of D-carnosine was chosen as the candidate due to its rapid hydrolysis to the bioactive metabolite. Pharmacokinetic studies in rats confirmed the in vitro data and demonstrated that the oral bioavailability of D-carnosine is increased by a factor of 2.6 when given as octyl-ester with respect to D-carnosine. Octyl-ester D-carnosine was also found to dose dependently (3 and 30 mg/kg die equivalent of D-carnosine) restore the development of hypertension, dyslipidemia and renal functions of Zucker fa/fa obese rats and to inhibit carbonylation processes and oxidative stress.

Obtained SARs: a general perspective

As schematized by Fig. 5, the structure–activity relationships derived by the carnosine derivatives until now described can be summarized as follows:

1. The amino group is essential for quenching activity. It could be replaced by other moieties (e.g., alcohols, diols, hydrazines, hydrazides and diamines) able to

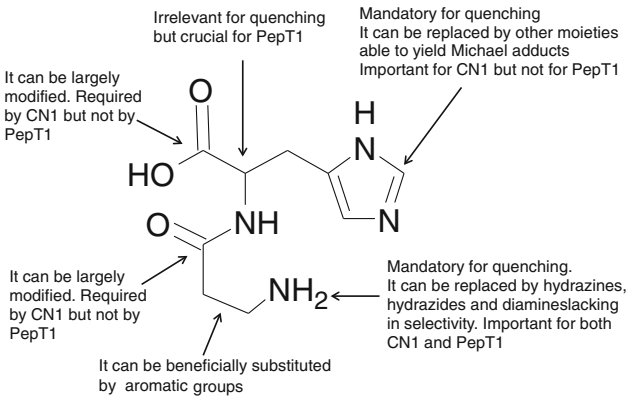


Fig. 5 Principal structure activity relationships (SARs) as derived by analyzing the reported carnosine derivatives. Notice that the considerations described for PepT1 hold reasonably true also for PepT2

condense with the carbonyl group, while emphasizing that an enhancement of imine formation is usually accompanied by a selectivity decrease. Any protection of the amino group should be labile in vivo as in the case of *N*-acetyl carnosine prodrug.
2. The imidazole ring is essential for quenching activity. It could be replaced by more nucleophilic moieties, although no compound was previously published pursuing this strategy.
3. The peptide bond can be replaced to assure plasma stability without affecting hPepT1 recognition and accordingly to well-known peptidomimetic approaches, even though very few carnosine derivatives without peptide group were hitherto described. It can be supposed that peptide bond could have a positive role in quenching activity by modulating the imine conformation and assuring an optimal amine basicity.
4. The carboxyl terminus can be vastly modified, so preventing the interaction with serum carnosinase with minimal effects on the active transport.
5. Inversion of the histidine chiral center does not modify the quenching activity, prevents the carnosinase hydrolysis but, unfortunately, strongly reduces the recognition by peptide transporters as confirmed by in silico models.
6. The alanine skeleton can be variously decorated by aryl substituents with a marked increase in the quenching activity. Conversely, no systematic study analyzed the possibility of replacing β-alanine by proteinogenic α-amino acids.

Conclusions and future perspectives

Without considering carnosine as a general panacea, the data here reported well indicate that carnosine and above all carnosine derivatives, designed to act as potent and selective reactive RCS quenchers, bioavailable and devoid of toxicity, represent a promising new class of therapeutic agents, effective in the treatment for diseases based on protein carbonylation and more generally on RCS-induced cell damage. Moreover, despite the still limited number of reported carnosine derivatives, the available data identify specific roles and relevances of each carnosine substructure (as schematized by Fig. 5), thus fostering the rational design of potent and selective RCS quenchers which are devoid of additional biological properties such as pro-histaminic or metal-ions chelator activity. Improved and truly selective carbonyl quenchers will play a crucial role as therapeutic agents, and, in turn, they will permit a better understanding of the effective pathogenetic roles of the different classes of RCS and, more generally, of protein carbonylation, thus showing the diseases which could be successfully treated by RCS quenchers. Currently, carnosine derivatives have been mainly studied as quenchers of α,β-unsaturated aldehydes but a deeper insight should also be gained on other pathogenetic RCS such as di- and keto-aldehydes and on the corresponding carbonylated proteins. Moreover, future studies should investigate the efficacy of carnosine derivatives on AGE/RAGES axis and their ability not only to prevent but also to reverse protein carbonylation.

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Conflict of interest The Authors are co-inventors of patents related to some of the carnosine derivatives included in the present review and they are partially supported by Flamma S.p.a.

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