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Human carnosinases: A brief history, medicinal relevance, and *in silico* analyses

Klaudia Chmielewska [a], Serena Vittorio [b], Silvia Gervasoni [b,d], Krystyna Dzierzbicka [a], Iwona Inkielewicz-Stepniak [c], Giulio Vistoli [b,*]

[a] Department of Organic Chemistry, Faculty of Chemistry, Gdansk University of Technology, Gdansk, Poland
[b] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, Via Mangiagalli 25, 20133, Milan, Italy
[c] Department of Medical Chemistry, Faculty of Medicine, Medical University of Gdansk, Gdansk, Poland
[d] Physics Department, University of Cagliari, Cittadella Universitaria, SP 8 km 0.700, 09042, Monserrato (CA), Italy

Carnosine, an endogenous dipeptide, has been found to have a plethora of medicinal properties, such as antioxidant, antiageing, and chelating effects, but with one downside: a short half-life. Carnosinases and two hydrolytic enzymes, which remain enigmatic, are responsible for these features. Hence, here we emphasize why research is valuable for better understanding crucial concepts like ageing, neurodegradation, and cancerogenesis, given that inhibition of carnosinases might significantly prolong carnosine bioavailability and allow its further use in medicine. Herein, we explore the literature regarding carnosinases and present a short *in silico* analysis aimed at elucidating the possible recognition pattern between CN1 and its ligands.

Keywords: Carnosine; Peptide; Carnosinase; Hydrolysis; Enzyme inhibition

Klaudia Chmielewska MSc in Biotechnology (joint Faculty of Biotechnology, University of Gdansk & Medical University in Gdansk), PhD student in Organic Chemistry in Gdansk University of Technology and research-and-teaching fellow in Chemoinformatics in University of Gdansk. Fascinated about biochemistry and drug design, Klaudia creates complex research projects including both computational and experimental research and believes such interdisciplinary approach is the future of science.

Serena Vittorio Received her PhD in Applied Biology and Experimental Medicine at University of Messina in 2021, working under the supervision of Prof. L. De Luca. During her doctoral studies, she spent six months as visiting scientist at University of Vienna, under the supervision of Prof. Thierry Langer, and at "Fondazione Ri.MED" of Palermo under the supervision of Dr. Ugo Perricone. In 2021, she joined the research group of Prof. Giulio Vistoli at University of Milan as postdoctoral researcher. Her research is mainly focused on the application of computational approaches for the rational design of new therapeutic agents.

Giulio Vistoli Integrally associated with University of Milan since 1989, receiving a PhD under supervision of Prof. L. Villa. Further, in 1996 he spent a period in Lausanne, under the supervision of Prof. B. Testa, with whom he collaborates till today. In 1999, he became assistant professor in medicinal chemistry at Universityof Milan, associate professor in 2010 and full professor in 2019. His recent research topics focus on the development of novel approaches for performing efficient virtual screening campaigns and for predicting the metabolism of xenobiotics with special focus on the computational techniques based on machine learning and big data.

* *Corresponding author. Vistoli, G. (giulio.vistoli@unimi.it)*

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Introduction

Peptides have a broad spectrum of biological activities, show high selectivity to their targets, and are relatively cheap to produce, which makes them interesting drug candidates. Hence, it is no wonder that peptide drugs have gained a lot of attention in recent years and already represent 5% of the global pharmaceutical market.[p1] However, peptides are poorly absorbed and quickly hydrolyzed, which deeply impacts their efficacy and further usage in therapy. Carnosine, a short β-Ala-L-His dipeptide, is no exception in these respects. Though immune to regular peptidases, it is quickly hydrolyzed by dedicated carnosinases, meaning that it mostly serves as a source of precursor residues rather than a bioavailable ligand.

Carnosine is synthesized through the enzymatic condensation of β-Ala and L-His catalyzed by carnosine synthase 1 (CARNS1), a cytosolic ligase highly expressed in brain and skeletal muscle tissue.

Experimental studies showed that the overall absence of CARNS1 in gene knockout (KO) mice disrupts skeletal muscle metabolism[p2] and impairs cognitive function,[p3] thus providing evidence of the importance of this enzyme and its products in human development. Instead, contradictory results were found in the literature concerning the role of endogenous carnosine production in diabetes. Patients with diabetes possess lower levels of carnosine in muscle.[p4] Over the years, several reports highlighted that carnosine administration enhances insulin secretion and decreases fasting blood glucose in diabetic mouse models. On the contrary, low serum concentrations of the dipeptide are associated with an increment of fasting blood glucose and a reduction of insulin levels.[p5],[p6] In contrast, a study conducted by Wang-Eckhardt and coworkers showed that CARNS1 KO in a type II diabetic mouse model did not result in any difference in insulin or fasting blood glucose concentrations relative to a conventional diabetic mouse model expressing CARNS1.[p7] These outcomes suggested that carnosine is not directly implicated in insulin secretion.

To accomplish its function, carnosine synthase requires tissue-specific transporters to deliver β-Ala and histidine, like TauT (muscles), PepT1 (intestines), and PepT2 (lungs, kidneys, brain).[p8],[p9] This finding further shows how carnosine synthesis can be impacted by a variety of different proteins. For example, PepT2 is additionally responsible for the overall uptake of carnosine by the cells,[p10] whereas increased taurine intake delays β-Ala transport by TauT.[p11] Furthermore, overactivation of CARNS1 protects against ischemia–reperfusion by regulating intracellular pH homeostasis and hampering the accumulation of lipid peroxidation products.

Carnosine could potentially become an attractive drug if its stability issues were to be resolved. It is attractive for its functionality as well as for its safety and sustainability, in line with the European Commission's recommended framework on drug design. Carnosine has less adverse effects than many drugs on the market and is not toxic to humans or the environment.

The complexity of carnosine metabolism, which is not yet fully understood, suggests that an increase in carnosine concentration could most easily be obtained by regulating carnosinase activity. Hence, understanding carnosinases, including their function, preference toward particular ligands, and impact on processes like inflammation, neurodegeneration, or ageing is a crucial step for further discussion of carnosine supplementation and therapeutic administration.

Hence, not only can carnosine be a drug but also a gateway to new research on cognitive decline and ageing. During our lifespan, the activity rate of carnosinases increases with age, and this is correlated with faster cognitive decline.[p9],[p12] Indeed, carnosine modulates several neuronal activities. In more detail, carnosine is able to cross the blood–brain barrier, triggering glial cells to release neutrophins such as the nerve growth factor (NGF),[p13] which is known to exert a neuroprotective effect.[p14] Furthermore, carnosine interferes with the formation of Aβ fibrils, whose accumulation is associated with neurodegeneration in Alzheimer's disease (AD). Additionally, the dipeptide is involved in serotonin and dopamine metabolism, provides support for neurons, and attenuates neurotoxicity through many different mechanisms, like increasing TGF-β production.[p9],[p15] Interestingly, TGF-β production slows down with age and as such is one of the first symptoms of cognitive decline, as well as different forms of dementia such as AD.[p13],[p15]

The ability of carnosine to preserve verbal episodic memory and improve cognitive function in the elderly[p12] additionally confirms that research on carnosinases might not only deeply impact the healthcare industry but also the daily life of people worldwide.

Carnosinases: a brief history

The first description of an enzyme able to hydrolyze carnosine was provided in 1949 by Hanson and Smith through experiments on porcine kidneys.[p16] Eleven years later, the occurrence in humans was reported by Shikata, who proposed that it might be implicated in the postmortem release of histidine.[p17] In the same year, Rosenberg demonstrated that carnosinase is activated by divalent metal ions, which led to its classification as a metalloenzyme.[p18]

Another 8 years later, Thomas Perry studied the urine of two patients with an odd, unknown neurological disorder. One of the substances he found in his probes was carnosine. This piqued his interest sufficiently to test another 37 samples from a group of healthy patients (13 adults and 24 children).[p19] As suspected, Perry did not find a single sample without carnosine present, and he concluded that his patients must lack the enzyme responsible for carnosine hydrolysis. Such carnosinase deficiency (carnosinemia) was also linked to the absence of urea cycle enzymes,[p20] leading to profound mental retardation and sensory peripheral neuropathy.[p21] However, that disorder is so rare that only 13 cases were reported worldwide up to 1985,[p22] and since then the number had risen to 30 individuals according to the National Organization for Rare Disorders (NORD). Nevertheless, those cases allowed scientists to further connect carnosinemia with deficit disorder, neurofibromatosis, seizures, and neurosensory hearing loss.[p22] Given the endogenous system of carnosine synthesis, simple exclusion from the diet is not sufficient to silence the disease, which makes it extremely hard to treat. Recently, a groundbreaking study linked carnosinase deficiency with the terminal deletion of chromosome 18 with a breakpoint at

q21.3,[p23] which might be a turning point in the development of treatments for carnosinemia.

The revelation that carnosinase is present in two forms rather than one came in 1972, when researchers at the University of New York performed starch block electrophoresis from human serum, kidney, liver, and spleen extracts.[p24] Another team later obtained the same results from swine kidneys and described differences in mass and the hydrolysis rate.[p25] A spark of curiosity occurred in other scientists, and various enzymatic tests were conducted to find an enzyme hydrolyzing carnosine in the olfactory bulb of mice and rats,[p26],[p27] as well as in the kidneys of a goose[p28] and a dog.[p29] Finally, 15 years later, the presence of carnosinase in the human nasal mucosa was reported.[p30]

Later, the first derivative of carnosinase was found, called homocarnosinase,[p31] with its deficiency named homocamosinosis.[p32] However, its occurrence is still so scarce that the clinical significance has yet to be elucidated.[p33]

Further, in 1985, carnosinase was confirmed to serve as a mechanism for acquiring histidine, which the diet fails to provide,[p34] exerting a beneficial role in diseases related to histidine deficiency like Parkinson's disease and multiple sclerosis.[p35]

One way to fool an enzyme is to slightly meddle with the chemical structure of its substrates. Therefore a study was designed to determine how the hydrolysis rate in the kidneys of rats would change if carnosine was modified through methylation, decarboxylation, or acetylation; the results revealed that these derivatives have a longer half-life than carnosine.[p36]

A trailblazing work in the field of carnosinases was published by Teufel and his colleagues; they characterized two human carnosinases as members of the M20 metalloprotease family, CN1 (EC 3.4.13.20) and CN2 (EC 3.4.13.18), with a mass of 56.8 and 52.7 kDa, respectively.[p37] While the first member was found mostly in the central nervous system, the latter preferred central and peripheral tissues. The most groundbreaking finding was the clear difference between the hydrolysis rates of CN1 and CN2. Interestingly, CN2 was not found to degrade homocarnosine or carnosine at physiological pH. When the pH was increased to 9.5, carnosine was eventually fit for hydrolysis, but as most human tissues maintain lower values of pH, the possibility of this occurring is quite low.

CN1 was quickly defined as a 'true Xaa-His dipeptidase', with the best activity for carnosine followed by four similar structures: *N*-methyl-carnosine, Ala-His, Gly-His, and γ-aminobutyryl-His (homocarnosine). Additionally, the enzyme was discovered not to degrade tripeptides containing histidine residue in the central or C-terminal position.[p37]

In 2003, Teufel and his colleagues were the first to determine the sequence of genes encoding both CN1 and CN2, which they named *CNDP1* and *CNDP2*, respectively.[p37] Sequence identity between them was estimated to be 49% and, 2 years later, an additional comparison between human carnosinases (CN1 and CN2) and the mouse CN2 gene showed 53% (CN1_human: CN2_mouse) and 91% (CN2_human:CN2_mouse) similarity, respectively.[p38] Those two studies suggested that this might be a very well-conserved region. Another study attempted to explore gene difference respect to mice CN1 and revealed that mice lack the signal peptide in the CNDP1 gene, and thus do not express this enzyme.[p39] The key properties of CN1 and CN2 are summarized in Table 1.

CN1

Serum carnosinase (EC 3.4.13.20) is the rate-limiting enzyme responsible for the hydrolysis of carnosine as well as anserine and homocarnosine. In humans, the reaction takes place mostly in the serum but its occurrence was also found in cerebrospinal fluid, which might suggest its importance in brain metabolism.

While not observed in newborn infants,[p31],[p37] CN1 was detected in the plasma of 2-month-old children at a concentration of 24 ng/ml. However, activity of the enzyme was not observed, probably because it was in its less active monomeric form.[p40]

The *CNDP1* gene, which leads to the synthesis of human serum carnosinase, is located on exon 2 of chromosome 18 (18q22.3). It encodes for 508 amino acids, three *N*-glycosylation sites, and a signal peptide composed of 29 amino acids with leucine repeats. However, its expression can be due to the occurrence of polymorphism.

The variant with the shortest trinucleotide repeat $(CTG)_5$ polymorphism in *CNDP1* gene, named *CNDP1 Mannheim* (D18S880; homozygosity for the five-leucine allele), is associated with a low likelihood of nephropathy as well as the lowest CN1 expression levels.[p41],[p42]

CNDP1 gene polymorphism has a higher prevalence in men than women.[p43] However, it should be noted that the latter was found inconsistently in African American and Mexican American populations.[p44],[p45] It must be noted that patients suffering from diabetes associated with the $(CTG)_5$ polymorphism might accumulate higher concentrations of carnosine.

Interestingly, the polymorphism in specific alleles of *CNDP1* can be correlated with athletic abilities,[p46] although physical training does not change the concentration of CN1 in the serum.[p47] It must be emphasized that this polymorphism might not always be favorable. Patients with diabetes with the $(CTG)_5$ polymorphism and mild cognitive impairment (MCI) or AD are characterized by an excessive amount of carnosine in their bloodstream, which might deeply affect their clinical response to carnosine treatment. Hence, caution in such situations is advised, and more investigations are needed to better understand

TABLE 1

Chemical properties and distribution of human carnosinases.

	Full name	Occurrence	Specificity	Mass (kDa)	Activating ion	Optimal pH	Isoelectric point (p*I*)	K_m[a] value
CN1	Serum carnosinase	Brain, liver, serum	Narrow	167	Zn^{2+}	7.5	4.4	1.27
CN2	Cytosolic tissue carnosinase	Ubiquitous	Broad	90	Mn^{2+}	9.5	5.6	15

[a] Michaelis constant (concentration of a carnosine required to achieve half the maximum rate of reaction).[p37]

the clinical efficacy of carnosine in patients with diabetes with MCI or AD.[p13],[p48]

The polymorphism of leucine repeats in the signal sequence of the *CNDP1* gene also affects CN1 transport from the liver into the serum. The shortest allelic form of this polymorphism, with the lowest reported activity of CN1, gives the highest carnosine concentrations; this probably mostly works against tissue damage.[p5] An *in vivo* study on *db/db* mice showed that renal *CNDP1* activity is upregulated by post-translational modifications induced by reactive oxygen and carbonyl species, leading to higher carbonyl stress when overexpressed.

Interestingly, KO of the CNDP1 gene results in doubling of the renal concentration of carnosine in the kidneys, which is believed to protect renal cells from high glucose levels.[p49] Additionally, it leads to significantly lower renal asparagine, glutamine, serine, and arginine concentrations.[p50]

It is noteworthy that, although most carnosine is found in muscles, the polymorphism of *CNDP1* was not found to affect muscle carnosine levels of healthy individuals[p51] despite affecting the serum concentration.[p52] Moreover, athletes seem to have lower CN1 activity,[p53] which might be of interest considering that CN1 activity rises proportionally to age and correlates with cognitive decline.

Considering that mice do not naturally express CN1, researchers have sought an appropriate animal model to replace them. Fish seem to be suitable for this purpose, with zebrafish being proposed for carnosinase-related research.[p54] However, the specificity of the enzyme differs from the human isoform as zebrafish CN1 do not seem to hydrolyze anserine.

It must be emphasized that understanding the nature of CN1 through basic research projects could lead to better understanding of which animal models might be best to further study CN1.

The first report on a possible molecular mechanism of allosteric activation of CN1 was presented in 2006,[p55] with a homology model developed on the basis of β-alanine synthetase and PepV dipeptidase bring used due to the lack of available similar structures. Molecular docking of carnosine and the generated CN1 polarized the carbonyl group, proving interaction with zinc ions of the enzyme. Additionally, imidazole was found to interact with Thr424 and Leu254, while the N-terminal amine group interacts with Gly115, Asp116, and Glu451.

Several years later, the crystal structure of human serum carnosinase was deposited in Protein Data Bank (code: 3DLJ), enabling researchers to investigate the binding modes of carnosine and its possible mechanism of degradation. In the active site of CN1 the two zinc ions are coordinated by His106, Asp139, Glu173, Glu174, Asp202, and His452, as shown in Figure 1. His235 in dimeric CN1 was proposed to form a H-bond with the ligand's carbonyl oxygen atom, thereby enhancing its polarization to trigger enzyme activity. This contact was not observed for the monomeric structure and was therefore considered as a possible reason why the dimer shows higher activity overall.[p56]

In silico analysis showed that the carbonyl group of carnosine interacts with a zinc ion, while carboxylate has a crucial interaction with Arg350. Moreover, the protonated amino group was found to contact Asp139 and Asp202, all of which is in line with earlier research.[p56],[p57] Additionally, a nitrogen atom from the

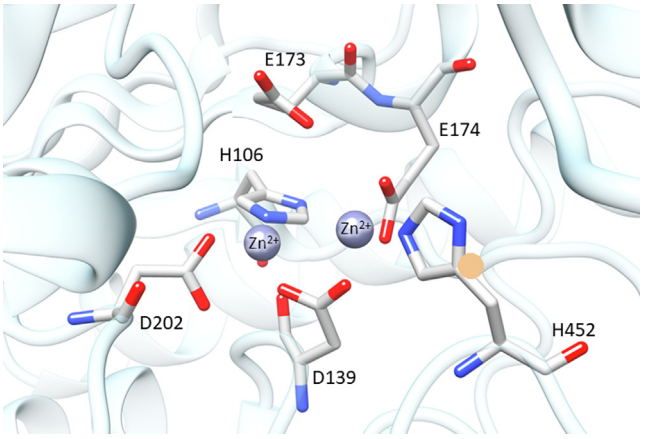

FIGURE 1
A closer look at the active site of CN1, including the Zn^{2+} ions and amino acids involved in their coordination.

amide bond was found to interact with Ser423, and the imidazole ring was located within a hydrophobic subpocket where one of the nitrogen atoms stabilizes an H-bond with Glu173[p49] (see Figure 5a).

Another important residue for the overall efficacy of hydrolysis of CN1 is Cys102, which when carbonylated led to an increase of CN1 activity. Interestingly, when the same residue underwent *S*-nitrosylation, CN1 efficacy dropped. The latter modification may be of importance for dysregulated NO metabolism, which usually contributes to the early stage of cancer pathogenesis. On this basis, it might be that enhanced *S*-nitrosylation of enzymes during cancerogenesis can also affect CN1 activity. Modifications of Cys102 residue also influence a nearby amino acid, His106, which, as already mentioned, is important for the zinc coordination in the catalytic site. Therefore, it was proposed that thiol-containing compounds can serve as a new and interesting class of inhibitors, acting via allosteric interaction and preventing the binding of carnosine to the orthosteric site of CN1.[p57]

Despite several theoretical findings regarding CN1 ligand recognition have been reported, no mechanism of action has been confirmed in the literature. However, as a metallopeptidase from the M20 family coordinated by zinc ions, CN1 might mimic the mechanism described for aminopeptidases from *Aeromonas proteolytica*.[p56],[p58] As schematized in Figure 2, the reaction would start with the formation of a hydroxyl ion after the activation of a water molecule in the active site of CN1, which transfers a proton to the carboxyl group of the nearby residue, Glu173. The resulting hydroxyl ion exerts a nucleophilic attack on the carbonyl oxygen from the peptide bond of the ligand, forming a gem–diolate intermediate. To behave as a leaving group, the amide nitrogen should be stabilized by an H-bond, triggering the donation of a proton from Glu173 and thus resulting in the release of the free histidine. In the last step, the carboxylic acid of the N-terminal amino acid (Xaa) of the peptide would form, restoring Glu173 to the deprotonated state.

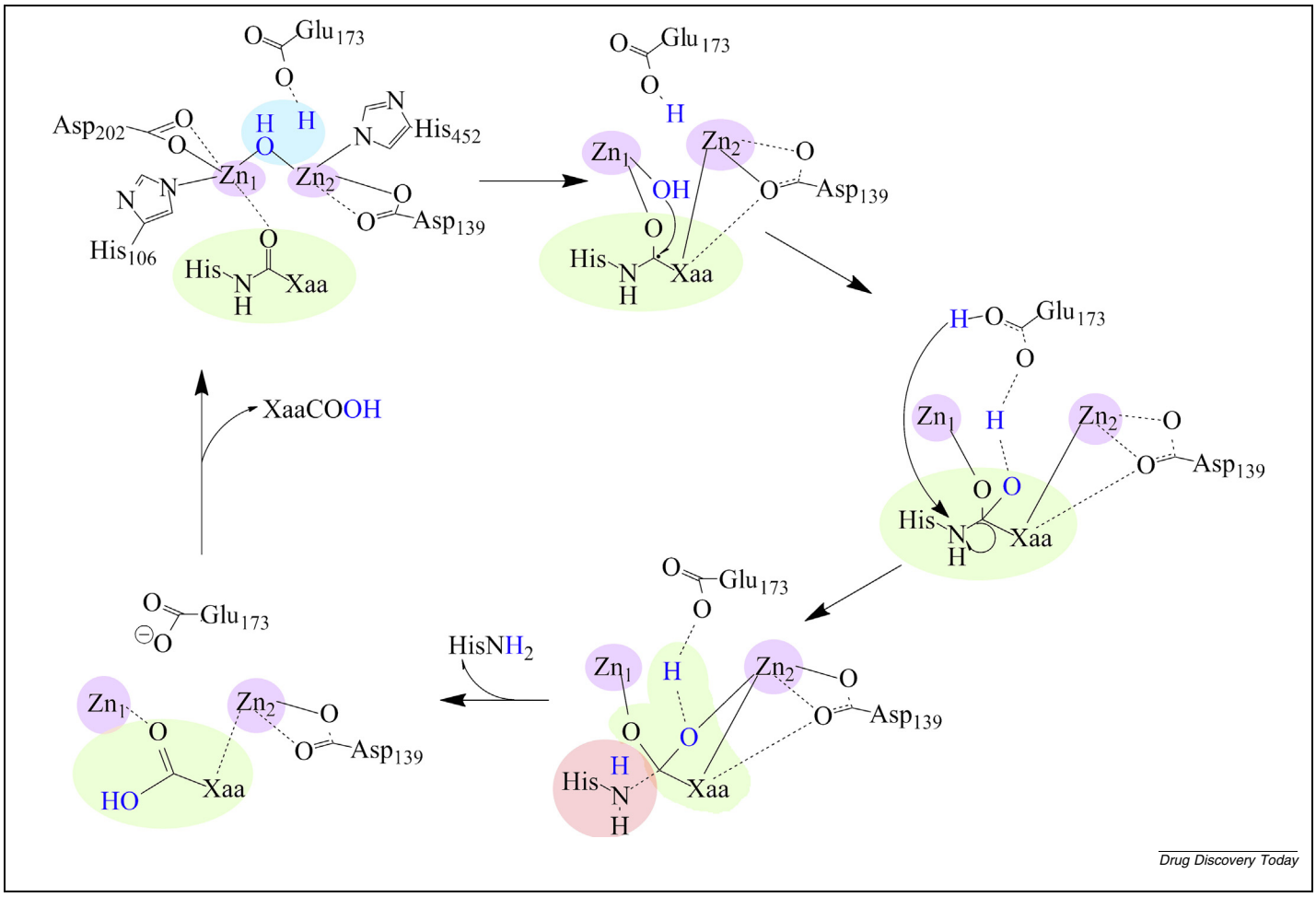

FIGURE 2

Proposed mechanism of CN1 hydrolytic activity. Blue color denotes products of water hydrolysis, zinc ions are circled in violet, and the ligand is circled in green. To highlight the site of hydrolysis, the histidine residue before hydrolysis is circled in red.

If this mechanism is indeed adopted by carnosinase, ligands bearing groups able to mimic the gem-diolate transition state (e.g. hydroxyl function near to the carbonyl group) could severely perturb the activity of the enzyme, lowering its hydrolysis rate. Although research on this topic is still scarce, the hypothesis seems to be supported by the first reported inhibitor of CN1, carnostatine {SAN9812; (*S*)-2-[(*S*)-4-amino-2-hydroxybutanamido]-3-(1*H*-imidazol-5-yl)propanoic acid}, a hydroxyl analogue of homocarnosine. Structural differences, although small, were sufficient to change the CN1 activity rate by 10% compared to carnosine, both in transgenic mice expressing CN1 and in human serum.[p48]

Additionally, three CN1-resistant compounds were found, D-carnosine, carnosinol (FL-926-16),[p59] and salicyl-carnosine,[p60] entities characterized by the modification of carnosine at C- and N-termini. Whereas salicyl-carnosine escapes hydrolysis for up to 3 hours, the same amount of carnosine is 86% cleaved after the first hour.[p60] This might be explained by the structural similarity of salicyl-carnosine to tripeptides.[p37] Interestingly, when glycoconjugated to sugars, carnosine also evades hydrolysis, presumably due to the large size of the conjugated metabolite that cannot fit the relatively small active pocket of CN1.[p61]

Taken together, it seems that knowledge on human serum carnosinase remains scant. Subsequent to the first inhibitor and some resistant structures (Figure 3), we can only assume that more findings will emerge in the next few years, increasing knowledge of the mechanism of action of CN1 or presenting new derivatives, thus enabling us to overcome the problems that occur when *CNDP1* expression is disturbed.

CN2

CN2 (EC 3.4.13.18), the cytosolic dipeptidase, is much less familiar to researchers due to its lack of specificity. Although it has a plethora of recognized ligands, carnosine is not its favorite, as it can only be hydrolyzed at pH = 9.5. Therefore, it is questionable whether CN2 truly affects carnosine metabolism, and some researchers speculate that it might be more reasonable to focus solely on the role of CN1. However, it must be emphasized that this enzyme is a highly conserved entity in many species whereas CN1 might have a broader role.

CN2 in humans is present in the form of a homodimer[p37] and is most abundant in the tuberomammillary nucleus of the hypothalamus, a region in the brain where histaminergic neu-

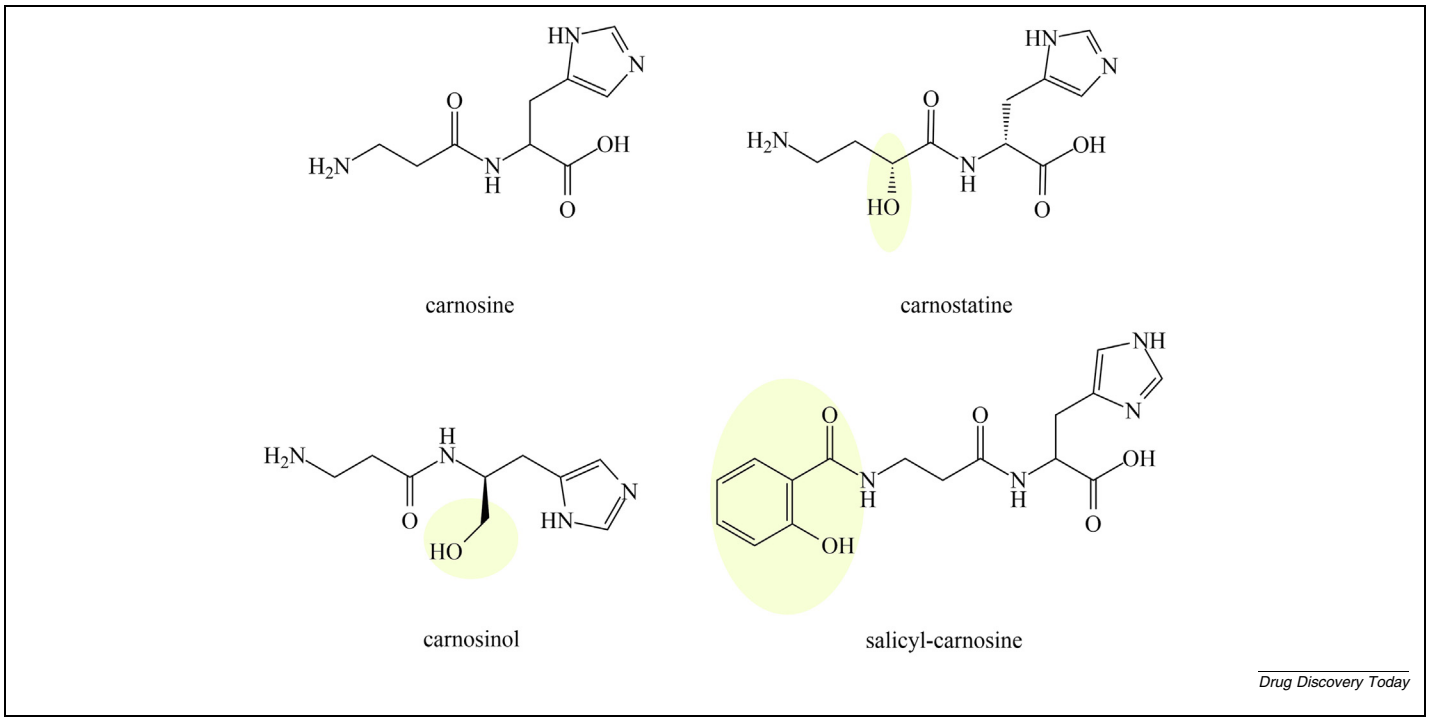

FIGURE 3
Chemical structures of carnosine and its derivatives capable of disrupting CN1 activity, with their modifications highlighted in yellow.

rons are highly concentrated.[p38] However, CN2 does not degrade homocarnosine.[p57] The main difference between the structures of CN1 and CN2 involves the different ions in its active site required for proper catalytic activity. While CN1 has zinc (II) ions, CN2 has manganese (II) ions.

An interesting study was performed on *Drosophila melanogaster*, one of the best animal models due to its high genetic similarity to humans. The gene *CDNP1* has never been detected in *Drosophila*, but an *CNDP* orthologous gene, named *CG17337,* produces a ubiquitous enzyme that can be classified as a probable orthologue of *CNDP2*. CN2 might also play a crucial role in glutathione metabolism, since it is active against peptides having a Cys-Gly motif.[p62] Additionally, loss of the *CG17337* region was found to result in a significantly shorter lifespan of *D. melanogaster*. Transgenic *D. melanogaster* with *CNDP2* KO, however, showed unaltered viability.[p63]

In humans, renal cell carcinoma and breast cancer cells were found to express higher CN2 concentrations, whereas knockdown of *CNDP2* resulted in the inhibition of cell proliferation, arrest of the cell cycle and, in the end, slower progression of the disease.[p64] This prompted further investigations of the role of this cytosolic nonspecific metalloprotease in cancer. Further studies on the overexpression of *CNDP2* in gastric[p65] and ovarian cancer reported that active CN2 promotes metastasis via the PI3K/AKT signaling pathway.[p66] Based on these findings, one might speculate that CN2 works as a functional tumor suppressor. In fact, researches showed that inhibiting the expression of *CNDP2* reduces gastric cancer progression.[p65]

Another interesting aspect of CN2 is that it mediates the reverse proteolysis of lactic acid with amino acids, leading to the synthesis of *N*-lactoyl-amino acids, although their metabolic role is yet to be clarified.[p67]

In contrast to studies concerning human serum carnosinase, the experimental structure of CN2 was resolved in complex with its known inhibitor, bestatin {[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-(*S*)-leucine} (PDB code: 4RUH).[p68] This molecule is known to inhibit many different dipeptidases, like aminopeptidase B (EC3.4.11.6) and leucine aminopeptidase (EC3.4.11.1).[p68],[p69] Interestingly, bestatin is marketed as an antineoplastic drug in Japan, which might partly confirm the involvement of CNDP2 in cancerogenesis. Additionally, it has been established that inhibition of CN2 with bestatin *in vitro* does not diminish the anticancer effect of carnosine.[p70]

A short analysis of the bestatin–CN2 complex showed that, as already reported in the literature,[p71] one manganese ion is coordinated by the carboxyl groups of Asp132 and Glu167, the imidazole nitrogen atom of His445, and the amide oxygen atom from bestatin. The other manganese ion coordinates the carboxylate oxygens of Asp132 and Asp195, the imidazole nitrogen atom of His99, and the N-terminus of bestatin (Figure 4). Additionally, it was found that the His228 residue is crucial for enzymatic activity of CN2. The same researchers further claimed that the mutant version of CNDP2, without Asp132, also loses its activity, a finding that is easily explainable given that this residue is involved in the coordination of both manganese ions within the active center of CN2.[p72]

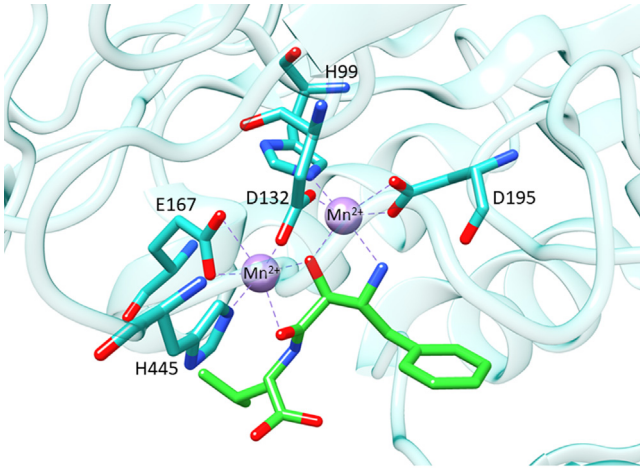

FIGURE 4
Visualization of CN2 active site and residues crucial for metal ion binding in complex with bestatin.

In summary, CN2 does not seem to be of huge importance in carnosine metabolism due to its optimal pH range, but it is involved in oxidative stress management in the cells, which might correlate with cancerogenesis.

Computational studies on CN1

Computational studies of CN1 were hampered for years by the lack of resolved structures. The first CN1 theoretical model was proposed by one of the authors of this paper in 2006, based on the homology with the PepV dipeptidase.[p55] Despite its inaccuracies (especially concerning the interactions elicited by the substrate C-terminus), the model was conveniently employed in molecular dynamics (MD) studies to investigate the allosteric effect of citrate and in docking analyses to rationalize the metabolic stability of D-carnosine.[p73]

In 2008, the Structural Genomics Consortium (SGC) proposed their human CN1 structure, further used to explore the mechanism of carnosine hydrolysis; this involves a nucleophilic attack by a Zn^{2+}-coordinated bridging moiety. The resolved structure was first employed by Carloni and coworkers to better investigate the hydrolytic mechanism of carnosine and anserine via hybrid quantum mechanics/molecular mechanics simulations.[p56] Next, Peters and coworkers rationalized the allosteric inhibitory effect exerted by covalent adducts on Cys102 by MD simulations, which revealed how these adducts induce conformational shifts involving the catalytic pocket.[p57]

Recently, Pongprayoon and coworkers employed MD runs to compare the catalytic pockets of CN1 and CN2. The results revealed that CN1 had a narrower and more electronegative pocket, with increased selectivity as well as stability of the catalytic amino acid triad and zinc ion improving the efficiency of the water-mediated catalysis.[p74] Anserine was found to be more stable inside the pocket of CN1 compared to carnosine, which seems to be due to its bulkier and more hydrophobic imidazole moiety.[p75] Notably, the anserine capacity to bind CN1 suggests its ability to act as a competitive inhibitor, hampering the hydrolysis of carnosine. The study was followed by research on other carnosine derivatives, namely balenine-, omocarnosine-,[p49],[p76] and proline-containing dipeptides,[p77] followed by a study on the metabolic stability of carnosinol in the presence of CN1.[p78]

Original computational studies on CN1

To better understand the hydrolysis of carnosine and some well-known analogues (i.e. anserine, balenine, and homocarnosine), unreported 100 ns MD runs based on already-published docking simulations[p49] are discussed here. Figure 5a shows the putative complex for carnosine, which assumes a pose in line with previous studies in which the carboxylate group of the ligand is engaged in a pivotal interaction with Arg350, while the protonated amino group contacts Asp139 and Asp202. The carbonyl oxygen atom and the NH group of the labile amide function approaches a zinc ion and Ser423, respectively. Finally, the imidazole ring is inserted within a rather apolar subpocket, where the N atom elicits an H-bond with the neutral Glu173, thus explaining the better interaction observed for this tautomer.

The results collected from our MD simulations, including the root-mean-square deviation (RMSD) fluctuations of both protein backbone and ligand, as well as the dynamic behavior of the key interaction between the substrate's carboxylate and Arg350, are presented in Figure 5. Concerning the RSMD profiles (Figure 5b), the substrates revealed similar behaviors, with RMDS values always around 1 Å. Greater fluctuations were observed in backbone RMSD values (Figure 5c) since the homocarnosine complex showed markedly greater mobility compared with the other complexes, which in turn showed comparable stability. In detail, the backbone RMSD of the homocarnosine complex reached values around 6 Å, suggesting that an increase in ligand size can affect the binding pocket architecture.

Figure 5d shows the distance between Arg350 and the carboxyl moiety of the ligand, which reflects dynamic behavior. It is worth noting that carnosine and anserine are closer to Arg350 compared with balenine and homocarnosine. This stronger interaction might be one of the reasons for the measured differences in hydrolytic rate among these substrates.

The MD simulations were also utilized to evaluate the free energy of binding by the molecular mechanics with the generalized Born and surface area solvation (MM-GBSA) approach, as computed for 100 ns trajectories every 5 frames (1000 frames overall), to reduce computational time. The obtained free energies of binding for carnosine and its analogues partially agree with the experimental results: homocarnosine (−49.69 kcal/mol) seems to bind the enzyme stronger than carnosine (−47.43 kcal/mol), which also has a similar binding energy to anserine (−47.45 kcal/mol). Balenine (−39.68 kcal/mol) shows a worse free energy value, which is in line with its greater stability. It should be noted that the interaction energies do not necessarily parallel the hydrolytic rates, which in turn depend on the substrate's capacity to assume a pose conducive to catalysis. The computed free energy values suggest that homocarnosine and, to a minor extent, anserine have a good interaction with the enzyme while failing to properly accommodate the labile group;

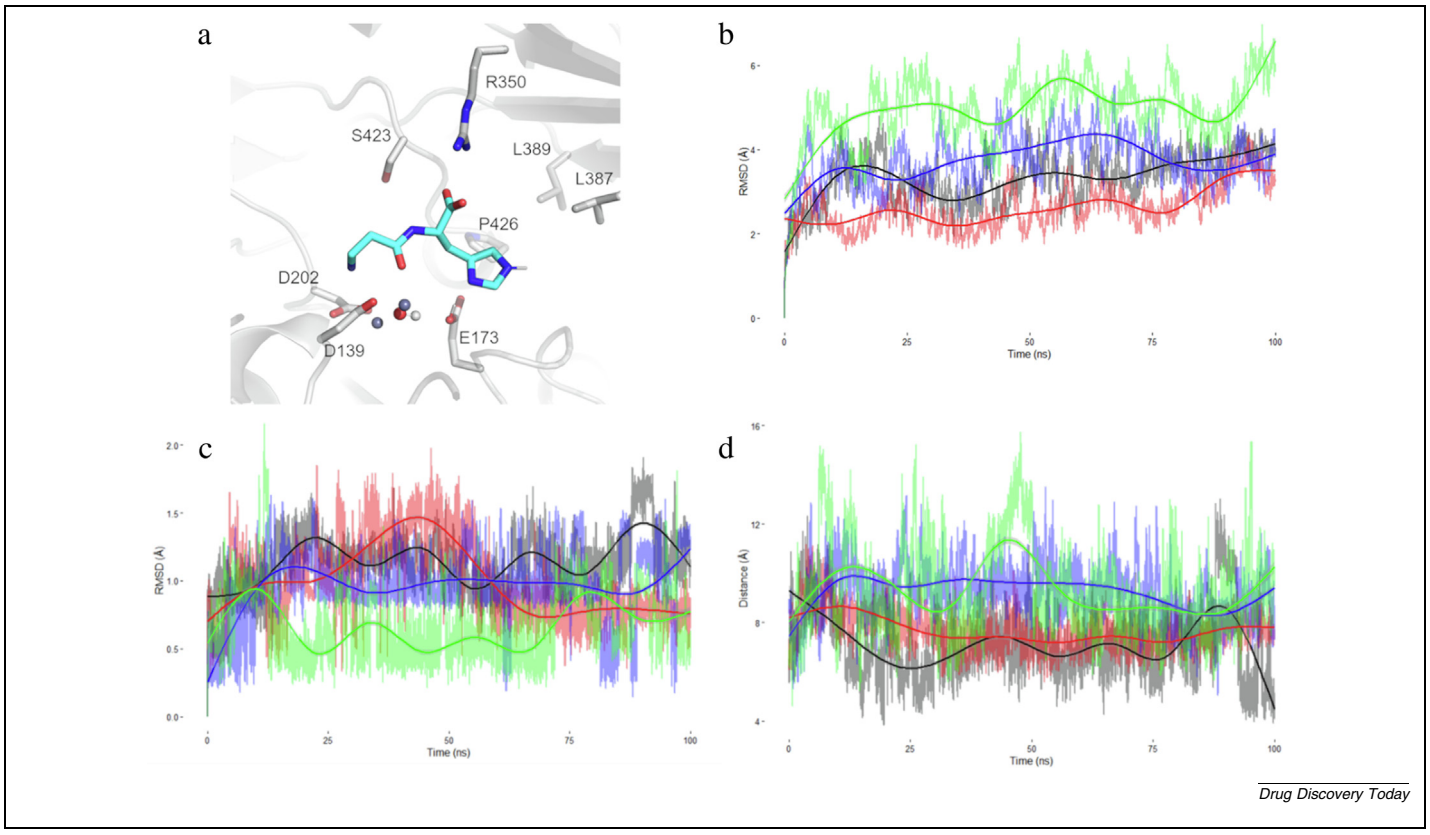

FIGURE 5

Results from the molecular dynamics simulation of carnosine and its derivatives. Panel **(a)** shows the key interactions stabilizing the putative complex with carnosine. Root-mean-square deviation (RMSD) values (expressed in Å) are shown of the protein backbone **(b)** and the ligand along the trajectory **(c)**. The distance between Arg350 and the carboxyl group of the ligand along the trajectory is shown in panel **(d).** In the plots (b–d), carnosine is shown in black, anserine in red, balenine in blue, and homocarnosine in green. Lines are included in the background for every value recorded along the trajectory, and smooth lines showing the trends are shown in the foreground. Panel (a) was adapted from Gilardoni *et al.*[p49] with the permission of Elsevier.

as such, they might behave as enzyme inhibitors, in agreement with what has already been reported.[p75] In contrast, the stability of balenine can be ascribed to a weaker interaction with the enzyme.

As mentioned above, carnostatine is the only specific CN1 inhibitor reported to date in the literature. To the best of our knowledge, no information about its putative binding mode within CN1's active site has been disclosed, and this prompted us to perform an additional *in silico* analysis aimed at probing the recognition process between CN1 and carnostatine and providing new insights to facilitate structure-based design of novel inhibitors.

For this purpose, docking studies were carried out using the crystal structure of human CN1 (PDB ID 3DLJ) by means of two widely used docking programs: PLANTS and GOLD. We postulated that the hydroxyl group of carnostatine might compete with the Zn-coordinating hydroxyl ion in the inhibition mechanism. To test this hypothesis, we performed a docking simulation with and without the OH^- ion in the binding site, as well as without the hydrogen from Glu173, for the reasons explained while discussing the possible mechanism of action of CN1.

Overall, the two docking programs did not show much difference in OH^- occurrence in the binding site, with small RMSD values between the obtained poses. Hence, we decided to further analyze the docking results excluding the hydroxyl ion. The biggest difference in docking results relates to the position of the carbonyl group. In the PLANTS results, the hydroxyl was the only group involved in zinc coordination and the carbonyl oxygen atom assumed a different arrangement. In contrast, the results gained from GOLD highlighted that both the carbonyl and the hydroxyl groups might be involved in Zn coordination (Figure 6).

Furthermore, the distances between the OH of carnostatine and the two zinc ions were greater in the docking pose generated by PLANTS than in those seen in the pose generated by GOLD (Figure 6a,b, respectively). Moreover, in the latter case, the carbonyl's oxygen atom approaches a zinc ion, while in the PLANTS-based complex the carbonyl group is not directed toward the zinc ions. Thus, the binding mode proposed by GOLD mimics that of carnosine, with the carbonyl group of carnostatine coordinating a zinc ion and the hydroxyl group replacing the OH^- ion responsible for the nucleophilic attack and therefore competing with the native substrate for binding to the enzyme.

Interestingly, both docking results show that the hydroxyl group is located close to Glu173 (distance < 4 Å), a residue normally receiving a proton from the bridging water molecule during the catalytic process. This stands as theoretical

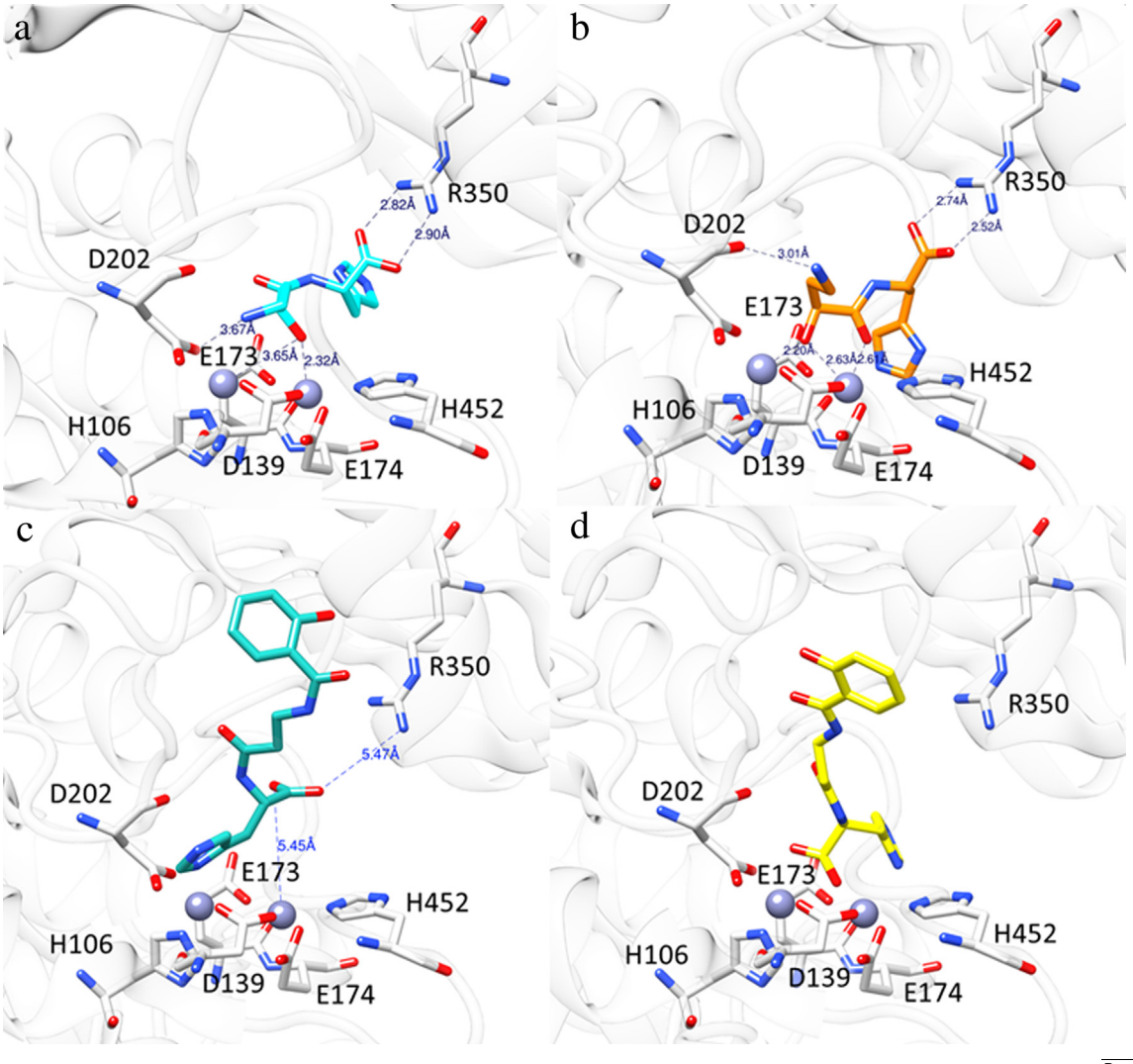

FIGURE 6

Graphical representation of the docking poses of carnostatine **(a,b)** and salicyl-carnosine **(c,d)** and their interactions with some key residues, as computed by PLANTS (a,c) and GOLD (b,d).

confirmation of the mechanism of inhibition we proposed earlier. Overall, the interactions found with the residues of the CN1 active site are similar for both docking studies. Specifically, carnostatine might engage an H-bond between the imidazole and Glu173, as well as ionic interactions between the amino group and Asp202 and Asp139. Furthermore, carnostatine maintains the salt bridge to Arg350.

Thus, carnostatine seems to be an optimal structural compromise, since it possesses the necessary moieties to serve as an inhibitor while having all crucial pharmacophore groups to strongly interact with the enzyme.

Finally, the docking of CN1-resistant molecules was carried out to get more insights into the structural requirements responsible for their greater serum stability compared with carnosine. Whereas carnosinol is modified through the reduction of the carboxylic group into hydroxyl, resulting in a small difference compared with the whole structure, salicyl-carnosine has a vastly different molecular weight, shape and nucleophilicity compared with carnosine, which can affect recognition and stabilization within the active site of CN1.

Given that the putative binding mode of carnosinol has already been reported in the literature,[p78] we focused on the docking of salicyl-carnosine. The results (Figure 6c,d) revealed that salicyl-carnosine is unable to interact with Asp202 due to the lack of a protonated N-terminus. This affects the orientation of the carbonyl group, which is no longer recognized by the zinc ions. Further, salicyl-carnosine fails to approach the carboxyl group of Arg350, which is pivotal for ligand recognition. Therefore, the lack of interactions with the residues crucial for the stabilization of the ligand within the binding pocket might explain why salicyl-carnosine is more resistant to CN1-mediated hydrolysis than carnosine.

Teufel *et al*. showed that CN1 cannot degrade tripeptides containing histidine in the central or C-terminal position,[p37] which might partially explain the ability of salicyl-carnosine to escape hydrolysis for a longer period of time. Finally, salicyl-carnosine

might also behave as an inhibitor of CN1. However, this hypothesis needs additional experimental proof and should be further evaluated by an *in vitro* enzymatic test.

Concluding remarks

Peptides have attracted research interest in the last few years due to their high activity and selectivity. However, their applicability is highly limited due to the activity of hydrolyzing enzymes. Carnosine is not an exception to this problem because, although it is therapeutically useful, it remains active for about 5 min after oral ingestion. Hence, the inhibition of carnosinases might serve as an interesting starting point to improve carnosine therapeutic potency, especially considering that the activity of CN1 rises with each day of our lives. Therefore, such an approach might be useful not only in medicinal pathologies but also in dietary studies, as it could set a course to further studies on the effects of dietary carnosine on cognition and aging.

Considering the presented literature and our preliminary computational study, we strongly suggest that the inhibition of CN1 is connected to the occurrence of an additional strong nucleophile in the ligand structure that is able to catch the zinc ions away from the carbonyl oxygen of the amide bond. Therefore, in the quest to find new potential inhibitors of CN1, chemicals possessing strong nucleophilic groups should be taken into consideration. It must be emphasized that those entities need to keep nucleophilic groups at a distance from carbonyl oxygen, an original recipient of the nucleophilic attack, to ensure stable binding resulting in the occupation of zinc. The structure of such inhibitors must also retain elements crucial for enzymatic recognition, namely the carboxyl group and the imidazole ring.

Although science now understands the usefulness of carnosinases as histidine providers, their higher activity contributes to progressive neurodegeneration. Therefore, although a deficiency of carnosinases is associated with neuroinflammation, their hyperactivity, inevitable with age, is also deleterious for the organism and should not be overlooked.

CN1 inhibition, without additional carnosine supplementation, might not be enough to reach therapeutic concentrations of the dipeptide[(p54)] but can lead to the broader usage of carnosine, which is already recognized to positively affect a plethora of dysfunctions and diseases, with the only limitation being its stability and half-life.[(p9)] Hence, we deeply encourage further research in this area, as it might serve as a huge step toward modulating the metabolism of small peptides for enhancing their therapeutic efficacy and, as a butterfly effect, might even change our perspective on the feasibility of preventing neurodegeneration from progressing with age.

Declarations of interest

No interests are declared.

Authors' contributions

K.C. conceived the review, performed the literature search, and wrote the original draft. In collaboration with S.V., G.V., and S. G., the manuscript was edited and expanded with the addition of original research. K.C. then edited the final draft for submission.

K. Dzierzbicka and I. Inkielewicz-Stepniak are PhD supervisors who were solely responsible for securing the funding for the internship of K Chmielewska at the University of Milan, which enabled the collaboration between facilities.

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CRediT authorship contribution statement

Klaudia Chmielewska: Conceptualization, Writing – original draft, Writing – review & editing. **Serena Vittorio:** Visualization, Writing – original draft, Writing – review & editing. **Silvia Gervasoni:** Data curation, Formal analysis. **Krystyna Dzierzbicka:** Funding acquisition, Supervision. **Iwona Inkielewicz-Stepniak:** Funding acquisition, Supervision. **Giulio Vistoli:** Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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