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Therapeutic potential of carnosine and its derivatives in the treatment of human diseases

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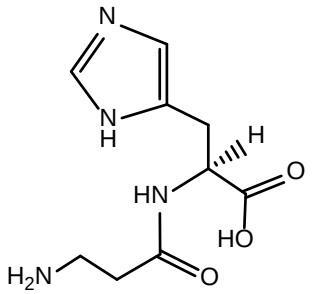
KEYWORDS: cancer, neurodegenerative diseases, carnosine, β-alanine, L-histidine

ABSTRACT: Despite significant progress in the pathogenesis, diagnosis, treatment and prevention of cancer and neurodegenerative diseases, their occurence and mortality is still high around the world. The resistance of cancer cells to the drugs remains a significant problem in oncology today, while in the case of neuro-degenerative diseases, therapies reversing the process are still yet to be found. Furthermore, it is im-portant to seek new chemotherapeutics reversing side effects of currently used drugs or helping them perform their function in order to inhibit progression of the disease. Carnosine, dipeptide constisting of β-alanine and L-histidine has a variety of functions, to mention anti-oxidant, anti-glycation and reducing the toxicity of metal ions. It has therefore been proposed to act as a therapeutic agent for many patho-logical states. The aim of this paper was to find if carnosine and its derivatives can be helpful in treating various diseases. Literature search presented in this review includes review and original papers found in SciFinder, PubMed and Google Scholar. Searches were based on substantial keywords concerning ther-apeutical usage of carnosine and its derivatives in several diseases, including neurodegenerative disor-ders and cancer. In this paper, we review articles finding carnosine and its derivatives are potential therapeutic agents in many diseases, to mention cancer, neurodegenerative diseases, diabetes, schizophrenia. Carnosine and its derivatives can be used in treating neurodegenerative diseases, cancer, diabetes or schizophrenia, although their usage is limited. Therefore, there's an urge to synthesize and analyse new substances, overcoming the limitation of carnosine itself.



Carnosine (β-Ala-His)

1. INTRODUCTION

Carnosine is one of the most common dipeptides found in humans. Discovered by a Russian chemist [1], carnosine was extracted from the Liebig's meat extract in 1900 [2]. Although many researchers studied this substance over the last century, many aspects of its usage and mechanisms of action are still to be elucidated. Carnosine's presence in mammalian tissues is wide and although it is most abundant in

humans, its methylated analogues are found in different types of animals as well. While anserine (β-alanine-N^{π}-methylhistidine) is found mostly in birds and rodents, ophidine (β-alanine-N^{τ}-methylhistidine) is present in whales, dolphins and snakes [3].

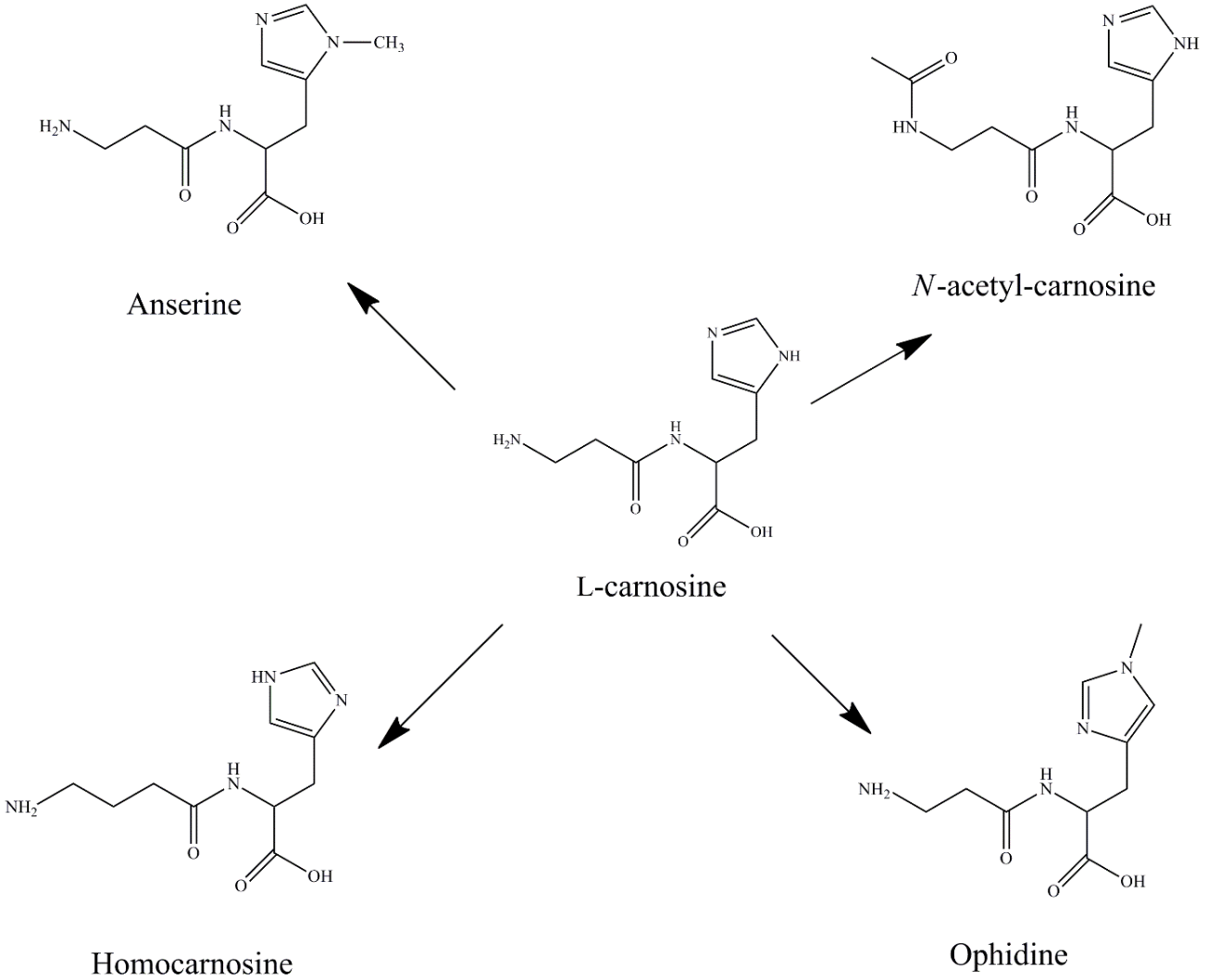


Fig. 1. Structure and chemical names of carnosine and related dipeptides.

Those β-alanyl containing peptides are mostly found in skeletal muscles, but when it comes to the central nervous system, mostly found there are γ-aminobutyryl derivatives of carnosine, like homocarnosine (γ-aminobutyryl-L-histidine). This difference is probably due to the bioavailability of these components in

the nearby tissues, for example γ-aminobutyryl containing peptides are typical for the central nervous system, probably due to the availability of its precursor γ-aminobutyric acid in this tissue [4].

Over the last century, researchers had found that carnosine possesses many activities presented in Table 1, to mention preventing brain cells from oxidative stress as an antioxidant and oxygen free-radical scavenger, wound healing promoter, anti-glycating agent, metal ion (zinc and cooper) chelator and stimulator of heat shock proteins expression [2,3,5,6]. It also has the capacity to modulate immune response in many different ways. For example, carnosine can suppress apoptosis of human neutrophils and increase the phagocytic activity of macrophages coupled to oxygen free-radicals production [7]. In astrocytes carnosine leads to axonal regrowth of neurons under ischemic conditions, working as a therapeutic agent for brain-related conditions [8].

Due to possessing many functions, it has been speculated that carnosine could prevent various diseases. Research has confirmed that idea, showing the positive effect of carnosine on diabetes, cancer and neurodegenerative diseases, for example Alzheimer's disease [32,33]. Carnosine seems to have an ability to activate the brain function by overcoming the blood-brain barrier, reaching the brain and activating glial cells. Those secrete neurothrophins activating neuronal cells indirectly in response to carnosine [34]. This ability might be important in AD treatment due to the fact that neurotoxic beta-amyloid species accumulating in the brain of the patients can result in the dysregulation of pro-inflammatory cytokines production [35]. Glial cells activated by carnosine could support the clearance of beta-amyloid species and trigger the repair of neurons damaged throughout the Alzheimer's disease.

Though carnosine is not degraded by regular peptidases, it is quickly cleaved to β-alanine and histidine by hydrolytic enzymes – carnosinases (half-life of carnosine in human serum < 5 min) [3]. Due to that fact carnosine therapeutic uses are limited. To overcome this limitation, researchers try to find a derivative or

the structural analog possessing the same function as carnosine itself but being immune to carnosinases in the same time. One of the possibilities is conjugating carnosine with several types of organic molecules, which could deliver the whole molecule to a specific target [2].

Table 1. List of carnosine functions together with their year of first citation.

Carnosine's function	**Year of discovery**	**Citations**
Anti-oxidant properties	1984	[9–13]
Chelating agent	1960	[11,14,15]
Anti-glycation	1960	[14,16,17]
Anti-aging	1999	[18,19]
Wound healing	1975	[20–23]
pH buffer	1960	[14]
Anti-cross-linking property	1995	[24]
Anti-inflammatory activity	1971	[20]
Senescence delay	1994	[25]
Reactive Carbonyl Species Detoxification	2013	[29,30]
Anti-neoplastic	1986	[31]

The aim of this review is to resume the most recent scientific data on the topic of carnosine, its metabolism and action; and its derivatives and their therapeutical potential in various diseases, such as neurological disorders, ischemia damage and cancer.

1.1 SYNTHESIS AND SUPPLEMENTATION OF CARNOSINE

Carnosine synthesis (Fig. 2) isn't due to the protein catabolism and so it requires the activity of an enzyme synthesizing this dipeptide from its component amino acids. Carnosine synthase (ATPGD1) belongs to the ATP-dependent enzymes family [36] and has a highly conserved *C*-terminal domain with the catalytic site. Thanks to that, this enzyme has an ability to ligate β-alanine

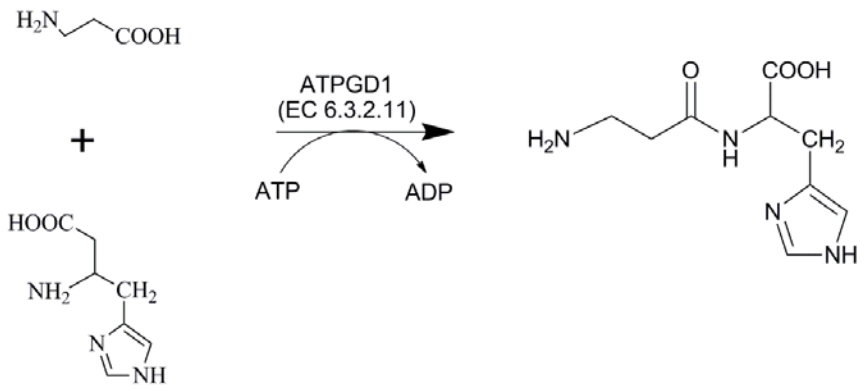


Fig. 2. An illustration of L-carnosine synthesis from its constituents – β-alanine and L-histidine; using ATP-consuming carnosine synthase.

to L-histidine and other, related amino acids. The process is accompanied by a stoichiometric formation of ADP [4], not AMP as belived in the past [37].

Interestingly, carnosine synthase is not very specific to whether it is an acceptor of β-alanine or γ-aminobutyryl [4], which enables local carnosine synthesis in olfactory neurons and glial cells of the brain. Carnosine itself could've been transported through blood-brain barrier but most of the brain carnosine content is thought to be a result of *de novo* synthesis [38]. Still, efficacy of the synthesis concerning carnosine is reported to have 15-25-fold higher catalytic efficiency than for homocarnosine [4].

In the case regarding methyl derivatives of carnosine it's worth mentioning that anserine and ophidine are synthesized through *N*-methylation of carnosine performed by *N*-methyltransferase, catalyzing the transfer of methylgroup from S-adenosyltransferase (SAM) onto carnosine [4,39].

Carnosine synthase uses two amino acids forming carnosine – β-alanine and L-histidine, but the origin of those substrates is very much different. β-alanine is synthesized in liver, through uracil and thymine degradation, while L-histidine has to be supplemented as it is not synthesized *de novo* in humans [37]. Interestingly in 2006, Harris et al. was the first to acknowledge that not L-histidine but β-alanine is the rate-limiting precursor of carnosine in human skeletal muscle [40], which was later confirmed by Blancquaert et al. in 2017 [41].

Orally ingested β-alanine can be used for carnosine synthesis as well. Supplements containing β-alanine are widely distributed and used by atheletes to increase the amino acids level in muscles and to result in beneficial effect during exercises. Studies have found that such elevations of carnosine levels in the muscles could actually benefit exercise but when performance is limited by intramuscular pH decrease [42], due to the fact that carnosine can act as an intracellular pH buffer, neutralizing lactic acid produced in a working muscle [4,43]. Chronic oral supplementation of 4-6g of β-alanine each day for 4-10 weeks increases carnosine levels in muscles by up to 80% [40,43,44]. Recently, Furst et al. has shown that β-alanine supplementation of 2.4g/day can increase physical performance and improve endurance exercise in the

elderly [45]. However only a small percentage of β-alanine is used after digestion (3-6%). This might be due to the fact that ingested β-alanine is probably used for other metabolic routes, such as transamination [46].

In 2010 Drozak et al. performed kinetic analysis of recombinant carnosine synthase and determined the value of the Michaelis constant (K_m), i.e. the concentration of the substrate at which the reaction runs at half the maximum speed, for each of the amino acids in carnosine. It turned out that K_m for β-alanine is smaller (0.09 mM) than for L-histidine (0.37 mM), which may indicate greater significance of histidine in the synthesis process [4]. However, Blancquaert et al. has shown L-histidine concentration declines in both muscle and plasma for about 30% after supplementing β-alanine. It might be important due to the fact that low histidine content in the plasma is associated with obesity and diabetes [47]. Positively, this decline in L-histidine levels can be prevented by co-supplementing it alongside with β-alanine [41].

Blancquaert et al. also found that upon dietary β-alanine exposure, transaminases can degrade all excess exogenous β-alanine to maintain tissue homeostasis [46]. That is why to improve β-alanine supplementation, inhibiting transaminases might be useful, although it's difficult because of the side-effects the inhibitors possess. Nevertheless, regulation of carnosine levels in muscles is poorly understood and many enzymes and transporters involved in carnosine metabolism have just been molecularly identified or still are yet to be discovered.

Research mentioned above is mostly limited to muscles and unfortunately, there is still a lack of studies considering other tissues. As mentioned, carnosine can be found in skeletal muscle, in a concentration up to 20mM. Quite high concentrations of carnosine can also be found in the vertebrate brain (0.7-2.0mM) [3,48,49]. Carnosine is observed to be in the stomach, kidneys, and olfactory bulb as well, but still 99% of all carnosine present in an organism is found in muscles [3,44,50]. Carnosine can also be detected within erythrocytes, at a level ten times higher than in plasma [51]. Studies show that the carnosine

concentration differs due to several factors, like gender (males tend to have higher levels of carnosine), age (the older the person the less carnosine they have) and diet (due to the fact that meat is one of its sources, vegetarians have less carnosine in skeletal muscles) [52,53]. However, herbivores can still have a high amount of carnosine and its derivatives in muscles, even though they are nowhere to be found in plants. Interestingly, there is an endogenous pathway to get β-alanine for carnosine synthesis by uracil degradation [46].

Considering the fact that carnosine synthase activity is mostly cytosolic (98%), the process needs presence of a membrane transporter for the uptake of β-alanine. In human skeletal muscles, the transporter responsible for *trans*-sarcolemmal β-alanine uptake is taurine transporter, TauT [52]. In the brain however, carnosine transport is possible *via* PepT2, proton-coupled oligopeptide transporter, exhibiting broad substrate specificity for dipeptides and controlling peptide trafficking, leading to brain homeostasis [54]. The same transport system was found in lungs and the kidneys [55]. Due to indifference between carnosine synthesis in muscles and brain, point might be taken that β-alanine oral intake could lead to other remarks. In 2015, Solis et al. conducted a study to check this hypothesis, analyzing the effect of supplementation of 6.4g of β-alanine each day for a month in vegetarians and omnivores [56]. Contrary to findings of muscle carnosine levels, the team found no influence of supplementation on the brain carnosine levels. This discovery might lead to an assumption that carnosine synthesis is not limited by the β-alanine uptake from the bloodstream. However, it should be noted that the study of Solis and his coworkers was done on a small group of participants and there is a need of research expanding that topic.

1.2 CARNOSINASES

As already mentioned, carnosine half-life in serum is less than 5 minutes, which limits carnosine therapeutic application, leading to a lower bioavailability of this dipeptide in body fluids. Moreover the activity of those enzymes is getting higher with age, leading to smaller accumulation of carnosine in tissues [57].

Human carnosinases were first described by Perry et al. in 1968 [58], but back then they were considered to be a single enzyme. Ten years later it was then found to exist in two isoforms, named human serum carnosinase (CN1) and tissue carnosinase (CN2) [59]. Both of them are members of the M20 family of metalloproteases and their genes (CNDP1 and CNDP2, respectively) show 53% sequence identity in humans, yet CN1 and CN2 characterize with different properties [39]. While CN1 has a narrow substrate spectrum, hydrolyzing only the histidine-containing dipeptides, CN2 is nonspecific. CN1 remains the only known human enzyme able to hydrolyse both L-carnosine and homocarnosine [60]. Interestingly researchers found another enzyme, homocarnosinase, cleaving homocarnosine, yet detection of this enzyme was found only in rats and hogs [59,61]. It is also worth mentioning that rodents, with the exception of the Syrian hamster, don't have active CN1, so the homocarnosinase can be a replacement for CN1 [62]. Due to this lack of CN1, it is also much harder to design an *in vivo* study concerning carnosinases and carnosine metabolism in humans. Still, CN2 is active in rodents and all other mammals and in mice CNDP2 gene shows 91% identity with the human version of this gene [63].

There's not much information about CN2 function, yet it's been established that it might be important for glutathione (GSH), one of the most common tripeptides in human cells. As a result of extracellular cleavage of GSH, cysteinyl glycine dipeptide (Cys-Gly) is formed, which is then autooxidized, leading to

the formation of pro-oxidative compounds such as thiol or oxygen radicals. This means that Cys-Gly degradation has a protective effect on cells and protects them from oxidative stress [64]. Also, it's been suggested that histidine produced through CN2 activity is then transformed to histamine via histidine decarboxylase, leading to the activation of histaminergic neurons, therefore regulating the autonomic nervous system [63].

Inhibiting the activity of carnosinases can serve as a strategy to strengthen the effects of carnosine supplementation, especially in the case of conditions characterized with higher carnosinases activity. Qiu et al. identified a selective serum CN1 inhibitor, SAN9812, also known as carnostatin [65]. It was analyzed *in vivo* and shown its effectiveness both in the mouse model and in healthy people. Yet there is a need to conduct a series of experiments analyzing the influence of SAN9812 on the organism of a patient with a high level of CN1 or activity.

2. THE ACTIVITY OF CARNOSINE AND ITS DERIVATIVES AGAINST SELECTED DISEASES AND DISORDERS

2.1 NEUROLOGICAL DISORDERS

Neurons are not normally reproduced nor replaced by themselves. This means that after neurodegeneration, which is the loss of structure or function of neurons, they cannot be replaced. Diseases considering neurodegeneration are a huge medical and economical problem worldwide. Huge amount of neurodegenerative disorders is due to dementia. The number of cases is constituently increasing and it has been estimated that the global cost of care for dementia patients in 2015 was 818 billion US dollars [66]. Also neurodegenerative and neuropsychiatric disorders are mostly treated symptomatically, with drugs showing low efficacy. Due to that fact, millions of patients worldwide and huge therapy costs there is a need of intense studies considering new therapeutics for neurodegenerative diseases.

Neurodegenerative disorders share several physiological and biochemical processes and are associated with aggresome inclusion bodies, perturbing cellular homeostasis [67]. Protein misfolding and fibrillation cause the formation of β-amyloid aggregates (Aβ) and tau proteins (Alzheimer's disase), Lewy bodies (Parkinson's disease) or inclusion bodies (Huntington's disease) [2]. The accumulation of these misfolded proteins is in the center of etiology of these diseases, triggering a cascade of events, often resulting in fiber aggregates of these proteins with amyloid structure rich in β-sheet [68]. All mentioned aggregates and many more are linked to metal ion binding and changes in metal homeostasis [69,70]. The use of carnosine in that matter will be discussed further in this review.

Other aspect disturbed in neurodegenerative disorders is protein ubiquitination. Ubiquitin is an amino acid required for the degradation of 80% of intracellular proteins in eukaryotes [71] and is involved in so called Ubiquitin-Proteosome system (UPS). Its abnormal function has been observed not only in neurological disorders, but in cancer as well.

In the late nineties, the role of carnosine in neurodegeneration has been investigated in *in vitro* studies. Preston et al. presented a study where the negative effects of β-amyloid peptide on rats brain could have been prevented by adding carnosine [48]. Later, other group of researchers found that in transgenic animal model of Alzheimer's disease, carnosine supplementation promotes a strong reduction in the hippocampal intraneuronal accumulation of Aβ and slowed down the progression of the disease [32]. Aloisi et al. had shown that such supplementation has an inhibitory effect on the formation of aggregates of Aβ [72], which is dependent on the ability of carnosine to perturb the hydrogen bond network near the residues with the key roles in fibrillogenesis [73]. It is also worth mentioning that in triple-trangenic Alzheimer's disease model mice carnosine has shown to be involved in restoring mitochondrial function and counteracting amyloid pathology [74].

A double-blind randomized controlled trial by Hisatsune et al. shows that supplementation of carnosine also leads to preservation of cognitive function in the elderly [75]. This suggests that carnosine activates brain function, but due to the fact that it is quickly cleaved by carnosinases in serum, it can be proposed that carnosine function would be elicited not directly through direct delivery to the brain, but most likely by brain-gut interaction [76]. That statement is supported by an observation made by Kadooka et al. that carnosine augments the expression of brain-derived neutrophic factor (BDNF), a protein secreted by neurons in order to support the survival of other neurons and to stimulate the growth of new ones [77]. Interestingly, BDNF can cross blood-brain barrier, which means it may activate brain-gut interaction [78], which supports the idea that carnosine, by increasing the expression of BDNF, works through brain-gut interaction. What is more, carnosine activates CREB (cAMP response element-binding protein) and its relative pathways, which triggers the synthesis of secretory factors related in brain-gut interaction [76]. The other aspect present in neurodegenerative diseases is the oxidative and nitrosative stress. RNS (Reactive Nitrogen Species) and ROS (Reactive Oxygen Species) are occurring naturally in aerobic metabolism of a cell, but during an inflammation process they are over activated in immune cells [79]. It leads to the higher production pro-inflammatory cytokines and aggregating Aβ, which results in neuronal cell death [80].

In 2019 Caruso proved that carnosine can decrease the expression of inducible nitric oxide synthase and the concentration of RNS. He also showed that in an model of Aβ-induced inflammation carnosine decreases the secretion of pro-inflammatory cytokines, therefore reducing the inflammation itself [79].

The other important ability of carnosine is its anti-protein-cross-linking properties, associated with anti-glycation and inhibition of cataract formation. Glycation is a non-enzymatic, post-synthetic protein modification, in which sugars are covalently linked to proteins in a process first described by Maillard [81] then leading to a formation of advanced glycation products (AGEs). AGEs are synthesized in Maillard reaction between carbohydrates and proteins [80]. Glycation is first initiated by the covalent attachment of

reducing sugars to amino groups of proteins, lipids or nucleic acids to produce reversible and an unstable Schiff base. Then the Shiff base may undergo Amadori rearrangement and change to a more stable Amadori product. It means that AGEs can incorporate proteins via cross-linking, then interfering with tissue function and leading to aggregation [82].

Although glycation occurs in normal metabolism, it progresses in some conditions, like oxidative stress [83]. It is widely accepted that AGEs have a negative effect on health and cognition in humans [84]. Neurodegenerative diseases characterize with oxidative stress as mentioned before and so, more AGEs are forming. Studies show that AGEs cause neurotoxicity [80,85,86] by changing the function of proteins, promoting mitochondrial dysfunction and leading to the increase of reactive oxygen species (ROS) and finally to cell death [83,87]. Still complete mechanism of AGEs remains unclear [84]. Carbonyl compounds then can react with macromolecules with nucleophilic groups, like DNA, proteins or aminophospholipids, nonenzymatically, forming a variety of adducts and crosslinks by an irreversible reaction. Such compounds are named advanced lipoxidation end products (ALEs) [88]. ALEs work similarly to AGEs, resulting in oxidative stress and inflammation. Lowering down the number of AGEs and ALEs in a tissue reduces the inflammation and cellular dysfunction, therefore improving the health of an organism [89].

Table 2. Examples of advanced glycation/lipoxidation end products.

Type of glycation product	Examples
AGEs	glucosepane, carboxymethyl-hydroxy-lysine, carboxyethyl-lysine (CEL), fructose-lysine
ALEs	MDA-Lys; HNE-Lys; FDP-Lys; carboxymethyl-lysine (CML); *S*-carboxymethyl-cysteine

Table 3. Classification of reactive carbonyl species (RCS) with examples.

Type of RCS	Examples
α,β-unsaturated aldehydes	4-hydroxy-*trans*-2-nonenal (4-HNE); acrolein
ketoaldehydes	methylglyoxal
dialdehydes	glyoxal, malondialdehyde

Amadori products formed in result to glycation, undergo dehydration and rearrangement to form highly reactive carbonyl species (RCS) [90]. AGEs and ALEs are RCSs related compounds as well. Those cytotoxic substances damage proteins, lipids and nucleid acids, causing cytotoxicity and mutagenicity [91]. Formation of both AGEs and ALEs are produced in neurodegenerative diseases due to the excess level of free radicals and reactive carbonyl compounds. In a healthy organism RCS are detoxified through transformation with aldehyde dehydrogenases (ALDHs) in phase I and with glutathione conjugating enzymes in phase II [30].

Carnosine can act as a phase II substrate, scavenging intracellular RCS by forming covalent adducts [3] then excreted in the urine [30], therefore, performing RCS detoxification. Interestingly, Orioli has found that this scavenging also decreases urinary markers of protein carbonylation, like AGE and ALE [92]. Carnosine also significantly inhibited the formation of both AGEs and ALEs, *in vitro* and *in vivo* [93,94]. It has also been found that MDA, one of the ALEs, cultured with neurons causes cytotoxicity, reversible by carnosine [95]. Interestingly, methylation of amino groups of β-alanyl or histidine residue abolishes the ability of carnosine derivatives to react with RCS, like HNE [36]. Due to that fact balenine isn't able to perform the reaction.

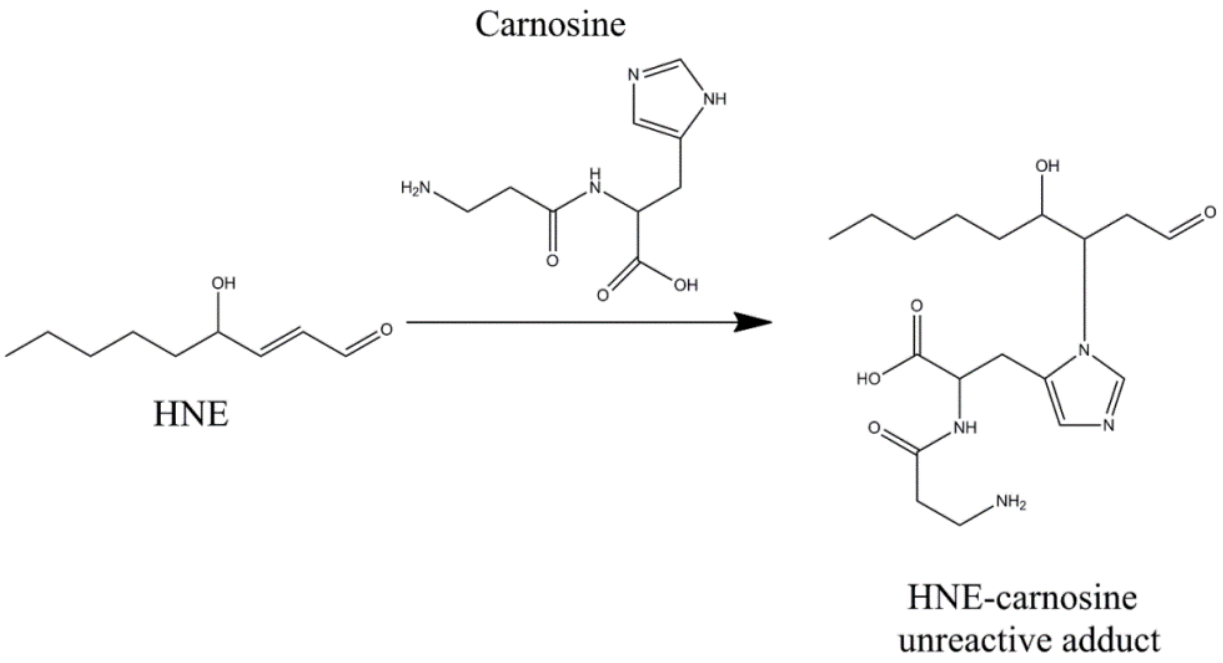


Fig. 3. An illustration of carnosine-induced sequestration of HNE, which results in forming HNE-canosine nonreactive adducts. (based on [96]).

Parkinson's disease (PD) characterizes with the accumulation of Lewy bodies, protein aggregates composed of alpha-synuclein (ASN). ASN is being glycated in a post-synthetic modification, which can contribute to its misfolding [97]. ASN is widely distributed in the body, including erythrocytes. Given the fact that carnosine is also present in erythrocytes it could be said that it can decrease alpha-synuclein misfolding due to its anti-glycation properties. That statement is supported by Kang and Kim study, proving ASN aggregation can be inhibited by carnosine in a model system [98]. Interestingly, more recent data based on a yeast model of PD confirms that decreasing ASN aggregation reduces its cytotoxicity [99].

Modifications of carnosine regarding activity against RCS concern the modification of the amino group of β-alanine with hydrazide and 1,2-diol moieties and substitution of β-alanine with aliphatic residues [100].

In 2011, Grasso produced a glycoconjugate named CDDCar (D-carnosine with β-cyclodextrin) [101]. D-Carnosine (DCar) is the enantiomer of naturally occurring L-carnosine (LCar), with the ability to avoid hydrolysis by carnosinases. It also shows the same quenching activity as LCar *in vitro* [102] and has been suggested for treatment for oxidative stress disorders, yet it still has a smaller bioavailability than L-Car, due to not being recognized by hPepT1, a transporter responsible for the uptake of small peptides in the colon [74]. Cyclodextrins (CDs) are cyclic chiral oligomers of D-(β)-glucopyranosyl units linked by (*R*)-1,4-glycosidic bonds. CDs are mostly used as drug delivery systems, favoring metal-ion complexation, which might be useful in the case of carnosine, as it can act as ion-chelating agents, especially for cooper (II) and zinc (II) [101,103]. This is important due to the fact that both cooper and zinc ions can induce the aggregation and oligomerization of Aβ *in vitro* and promote amyloid plaque formation *in vivo* [70,104]. Carnosine has the ability to chelate Cu^{2+} and Zn^{2+}, protecting neurons from lipid peroxidation [105].

CDDCar has an improved bioavailability in comparison with DCar alone, due to the improved site-specific transport to several tissues. The researchers also stated that cooper (II) binding properties of CDDCar differ from L-Car analouges [101]. The conjugation resulted in the higher antioxidant activity as well [74].

Vistoli has synthesized stable D-carnosine (β-alanyl-D-histidine) derivatives, resistant to carnosinase and with increased quenching efficacy. Conversion from L- to D-carnosine resulted in higher serum stability, but lowered the reactivity. Modifying D-carnosine with aryl groups by solid-phase synthesis, allowed to form derivatives highly selective to RCS with a high serum stability due to D-histidine conformation [102].

Another research group has analyzed 16 D-carnosine derivatives designed in such a way two promoieties having similar lipophilicity to avoid hydrolytic enzymes focusing on the more lipophilic one [106]. Some of the analyzed prodrugs had both terminal groups modified, which resulted in *N*-acetyl derivatives, ethoxy and benzyloxy derivatives, while other compounds had only one terminus modified with alkyl esters, amidic or carbamate moieties. While acetyl derivatives were prepared by acetylation of D-Car by acetic anhydride, ethoxy derivatives were coupled by adding ethoxy-β-Ala-OH with D-histidine ester. The last compounds, benzyloxy's were coupled analogically to ethoxy groups, with the exception of using benzyloxy-β-alanine. Finally, tested derivatices hydrolysis rate happened to be propotional to their lipophilicity, indicating that they are mostly hydrolyzed by carboxylesterases. Study found that *N*-acetylation wasn't suitable for designing D-carnosine prodrugs. The most promising compound was D-carnosine octyl ester hydrochloride, used for *in vivo* studies. The oral ingestion of this derivative in Zucker *fa/fa* obese rats resulted in higher bioavailability of a compound with comparison to D-carnosine alone. It was also stated that D-carnosine octyl ester hydrochloride decreases the dyslipidemia and reduced the amount of oxidative stress. The protective effect of this derivative was found to be due to forming adducts with HNE, emerging in higher amounts of these substances in the urine of tested animals. This

can lead to an assumption that same situation could occur in the case of neurodegenerative diseases, also concerning higher oxidation and glycation rate.

In 2012, Menini and his co-workers used D-carnosine octylester again, on ApoE mice to analyse its effect on inflammation [107]. They found that not only has the compound reduced carbonylation of proteins and generation of AGEs and ALEs, but also inhbited the oxidative and endoplasmic reticulum stress, reducing the inflammation. A couple of years later Menini studied the D-carnosine octylester once more as an extra to the diabieties treatment, to analyze whether the reduction of AGEs formation by early (first 11 weeks), late (week 9 to 19) or extended (20 weeks) treatment has a different impact in the same animal model [108]. The study has found that D-carnosine octylester there are no significant differences between early and late treatment although the usage of this derivative itself resulted in the inhibition of AGEs formation, which prompted attenuated inflammation and blunted oxidative stress.

A different study by Vistoli, focusing carnosine derivatives, to mention: D-carnosine, homocarnosine, carnosinamide, anserine, carcinine and *N*-acetylcarnosine; focusing their quenching activity towards two RCS species – α-(methylglyoxal) and β-(malondialdehyde) dicarbonyls [109]. Results show that analyzed derivatives were moderately reactive to malondialdehyde, due to forming adduct through Michael addition. The highest quenching activities for mentioned derivatives was possessed by D-carnosine, followed by anserine and carcinine in the case of malondialdehyde. For this aldehyde, it has been stated imidazole ring of histidine is not involved in the quenching mechanism. Quenching activity towards methylglyoxal has shown a different pattern, showing carnosine derivatives provide a rather poor activity. The highest percentage of activity was shown for carnosinamide, 3.8 times higher than observed with L-carnosine, but still the percentage was comparable to the ones obtained for malondialdehyde. In the case of a mechanism, the carboxyl group can be replaced with non-ionizable H-bonding groups, but not removed.

In 2018, Kulikova presented research on nanomicellar complex of carnosine with α-lipoic acid in a model of early-stage Parkinson's disease [110]. Acute intraperitoneal administration of the complex not only normalized the total antioxidant activity in the brain tissue of tested rats but revised the metabolism of serotonin and dopamine as well. This let diseased rats to have a physiological level of monoamines, same as in the case of observed in rats without induced early-stage Parkinson's disease. Importantly, this normalization was observed to be more efficient when rats were treated with carnosine-α-lipoic acid complex, although antioxidant activity was marked as the same for both complex and its components alone.

Cataracts are insoluble aggregates forming due to dysfunctional protein interactions, leading to increased lens opacity [111]. In other words, cataract is the clouding of the eye lens, preventing clear vision. Goldstein et al. have reported that β-amyloid can deposit in human lens in Alzheimer's disease, forming supranuclear cataracts [112]. Although cataracts can be surgically removed, it's a risky procedure in many cases, so the need of new pharmaceuticals emerge. Several small molecules are able to counteract the formation of cataracts and so carnosine brought the attention of researchers. In 2009, a study found that carnosine interferes with fibrils of aggregates growth and inhibits the formation of protein aggregates in lens [28]. Interestingly not only carnosine but also its derivatives were tested for such cause. *N*-Acetylcarnosine (*N*-acetyl-β-alanyl-L-histidine) and anserine were found to act like mini-chaperones and to suppress aggregation of crystallin, protein associated to the lens *in vitro* [113]. Importantly, *N*-acetylcarnosine is more resistant to hydrolysis by carnosinase than carnosine itself, which means it is more efficient in that matter [114].

Scientists from Innovative Vision Products, Inc. produced lubricant eyedrops containing a *N*-acetylcarnosine and in 2009 Babizhayev reported that this medicament had an antioxidant, AGEs and ALEs scavenging and transglycation activity, with a great usage against cataracts [115]. Study concerned

50,5 thousands of human subjects, most from which shown improvement after therapy with the lubricant. Overall research suggests that *N*-acetylcarnosine can reverse and prevent forming of cataracts.

Grasso synthesized a derivative produced to overcome the limitation of carnosine being cleaved by carnosinases, in 2017 [116]. They linked carnosine with trehalose, disaccharide composed of two glucose molecules linked by an *O*-glycosidic bond. The function of trehalose is to prevent denaturation and the formation of conformational changes in proteins, which could be helpful in preventing accumulation of Aβ. Actually, Ruitian has shown for a fact that trehalose inhibits β-amyloid aggregation and reduces its toxicity [117]. Grasso has synthesized a derivative, TrCar2, in which trehalose is linked to the dipeptide through the carboxylic group, leaving the amino terminal group unaltered [116]. This combination lead to the protection of the compound against carnosinases and increased the activity of the derivative in complexes with copper relative to carnosine alone. These complexes are also important due to their antioxidant properties, potentially preventing brain oxidative damage. The trechalose-carnosine derivative has also demonstrated the ability to inhibit the formation of amyloid aggregates and to promote cellular clearance of pathogenic proteins. TrCar2 was synthesized by an amide condensation reaction starting from the respective 6-amino-6-deoxy-α,α'-trehalose (TrNH2) and *tert*-butoxycarbonyl-(*N*-β-alanyl-L-histidine) (BocCar) in the presence of routinely employed activating agents [116].

Lanza and his coworkers created derivatives of carnosine and its amide functionalized with glucose and lactose [118]. The synthesis was carried out almost identically for all chemicals, by adding carnosine methyl ester and 2-bromoethyl-(2,3,4,6-tetraacetyl)-β-D-glucopyranoside in anhydrous DMF. The product was then evaporated from DMF, purified on a Sephadex column and concentrated under vacuum. The specific synthesis description is available at [118].

All derivatives of carnosine presented in this study were found to be less potent for hydrolysis by carnosinases in human serum than carnosine alone, potentially able to act as chelating agents [118].

Recently, Zhang and his coworkers proposed a novel conjugate of carnosine and Ac-LVFFARK-NH_2, CarLK7 [105]. Ac-LVFFARK-NH_2 is a heptapeptide derived from the central hydrophobic Aβ sequence, displaying effective inhibition against amyloidogenesis [119]. Researchers tested this conjugate *in vitro* and shown that Car-LK7 exhibited enhanced inhibition capability on Aβ aggregation, as compared to LK7 alone. Car-LK7 has also shown protection activity against cooper (II) mediated Aβ cytotoxicity and higher activity on arresting Aβ-Cu^{2+}-catalyzed ROS than carnosine alone. Interestingly, the compound isn't cytotoxic *in vitro* and almost eliminates the Aβ cytotoxicity at equimolar dose [105]. Still, this conjugate needs further analysis *in vivo*.

Carnosine seems to be a versatile compound, with the ability to prevent amyloid aggregation and deposition but also inhibition of protein cross-linking and free radical generation, as well as possessing the anti-inflammatory activity. Such multimodal profile contributes to explain carnosine neuroprotective effect and show its huge therapeutic potential. Due to the fact that L-carnosine is quickly cleaved by carnosinases, the urge of designing new derivatives emerge. Hopefully more research on that topic will appear in the future and we'll be able to use the therapeutic potential of carnosine.

2.2 CANCER ACTIVITY

Cancer has become a disease with one of the highest rates of mortality in the world. It's been estimated that in 2012 alone, 14.1 million new cases of cancer occurred and in the same year 8.2 millions of patients were lost to this disease [120]. It's been calculated that about 15.5 million people will die each year starting by 2020 [121].

Although cancer is a group of diseases with more than 200 different types of pathologies and etiology, there are some common features in cancer cells. In 2000 Hanahan and Weinberg published a paper concerning the hallmarks of cancer [122]. They extended their work later in 2011 and overall presented 6 basic features of cancer cells, enabling to distinguish them from healthy cells [123].

The first one concerns one of the most essential aspect of cancer cells - the possibility to sustain proliferative signaling, which means they deregulate the production and release of substances responsible for homeostasis therefore escaping the fate normal cells share. Doing so, cancer cells are able to persist in a continuous signal leading to cell growth or division.

Another cancer cell characteristic is its ability to evade growth suppressors. Suppressors are a group of compounds limiting cell division and growth, activated after detecting damage in the genetic material. Its function is to lead to an apoptosis of a dysfunctional cell. Apoptosis, induced cell death may occur through the intrinsic pathway, whereas the p53 protein is activated or extrinsic pathway where the signal about cell death is extracellular and is transmitted to death receptors located on the cell membrane. Cancer cells often mutate and inactivate the p53 and inhibit the intrinsic signals for apoptosis, like the production of Bax/Bak pro-apoptotic factors.

Due to the fact that both somatic and cancer cells need access to oxygen and nutrients in the tissue, they need the presence of blood vessels. This need is met by the ability of cancer cells to induce angiogenesis, the process of forming new capillaries. This very same process is used by normal cells, for example in the case of embryogenesis or wound healing but is activated only temporarily [124]. In the case of cancer, angiogenesis is almost always active, which enables the disease to progress to different tissues.

Another common feature of cancer cells, related to angiogenesis is their ability to activate invasion and metastasis. The key factor determining metastasis is oxygen deficiency in the tumor area. Then cancer

activates HIF-1α (*Hypoxia-Inducible Factor-1α*) a transcription factor regulating the expression of proangiogenic and prometastatic factors. The primary tumor, surrounded by new blood vessels, passes from the epithelial to mesenchymal phenotype, due to which it acquires the ability to migrate through the lumen of the blood vessel to other tissues [125].

Cancer cells also characterize with enabling replicative immortality. This involves the continuous reproduction of telomeres, complexes consisting of non-coding DNA fragments and associated proteins called shelterin, located at the ends of chromosomes. In the case of healthy cells, after each cell division, the telomere shortens. This aspect is related to the so called Hayflick Limit [126], which mean each cell has a finite number of cell divisions and after the maximum amount of divisions, cell should go into the state of rest where it retains metabolic activity and later undergoes apoptosis. This helps in maintaining homeostasis, eliminating cells potentially accumulating errors and mutations over its lifetime. However more than 90% of cancer cells has active telomerase enzyme, which doesn't let telomeres shorten after cell division. This means cancer cells can escape their fate, therefore becoming immortal.

Currently used cancer treatments usually result in toxic effects on normal cells. That’s why researchers focus on finding new therapies, focusing on the treatment of cancerous cells with little or no toxicity on the other cells. Peptides naturally synthesized in human bodies are interesting to study for such purpose, due to their safeness. They can also easily penetrate tissues due to their small size. Carnosine, due to its functionality, is a good candidate for testing in cancer treatments.

The first publication covering anti-neoplastic effect of carnosine was shown in 1986 by Nagai and Suda *in vivo* [31]. In their work, researchers implanted Sacroma-180 tumour cells in ddY mice and a day after implantation, applied carnosine subcutaneously 2 cm from the implantation site starting on the next day. In comparison to the control, it was clear that carnosine not only inhibited tumour growth but also reduced

mortality rate of tested mice. 10 years later, another work appeared, by Holliday and McFarland, testing carnosine *in vitro*. Researchers found carnosine selectively inhibited transformed and neoplastic human and rodent cell lines. Interestingly, they also analyzed anserine, D-carnosine and homocarnosine, on HeLa cells. Nevertheless while anserine has shown similar effect as carnosine, the other derivatives has shown none effect whatsoever [127]. Only 3 years later the same researchers also shown that in the absence of pyruvate, L-carnosine was able to inhibit the growth of embryonic teratocarcinoma cells [128].

Over the years, more and more works were published covering this topic, confirming the results of Holiday and McFarland on other types of cancer cells [127]. Up to this day, 182 publications on the topic of carnosine's use in cancer have been shown (SciFinder® for Academics). But it was at the beginning of 21st century when more publications started to emerge. Overall the usage of carnosine in cancer is well described in a review by Gaunitz and Hipkiss from 2012 [6]. Still, since then new derivatives of carnosine were created and tested.

In 2002, Kang published a study proving L-carnosine prevents DNA fragmentation caused by ROS (Reactive Oxygen Species) and protects against apoptosis through mitochondrial pathways [129]. Six years later, another paper suggested an ability of carnosine due to its antioxidant function. Chuang and Hu analyzed L-carnosine effect on metalloproteinases (MMPs), main extracellular matrix (ECM) enzymes [130]. MMPs are involved, among others, in embryogenesis, wound healing and most importantly in the case of cancer – in angiogenesis [131]. Although MMPs are important to normal cells, they also play an important role in tumor growth, altering the processes of invasion like proteolytic degradation of ECM, down-regulating angiogenesis or decreasing the immunological response of NK cells to cancer cells, therefore promoting tumor progression [132]. Therefore Chuang and Hu tested the use of L-carnosine on MMP-9 inhibition in a highly invasive hepatocarcinoma, SK-Hep-1 cells. They found that L-carnosine significantly inhibited cell migration and invasion, down-regulating MMP-9 expression [130].

In the same year, Fouad treated cisplatin-induced acute renal damaged mice with intraperitoneal injection of carnosine (10mg/kg/day) for 6 days. After 3 days of treatment they started to administer cisplatin (20mg/kg/day). Results have shown that carnosine treatment reduced the concentrations of serum creatinine, elevated by cisplatin. Interestingly, carnosine has also reduced the increase in malondialdehyde and decreased superoxide dismutase activity in renal cortical homogenates. What is more, carnosine also reduced cisplatin-inducedrenal tubular necrosis. Altogether, findings of Fouad show that carnosine can protect against nephrotoxicity usually found in patients treated with cisplatin [133].

Due to that function and presence of imidazole ring in carnosine structure, enabling a stable substitution of iodine radionuclide, Maurin and Garnuszek decided to create complexes of carnosine with radioactive platinum as potential candidates for radio-chemotherapeutics [134]. The compounds were synthesized in a multi-step reaction, starting from modifying carnosine with SATA (*N*-succinimidyl *S*-acetylthioacetate) linker or 2-IT (2-iminothiolane) linker, followed by deacylation of the conjugate and mixing the product with platinum complex. In the case of complexes with iodine radionuclide (Pt(II)(IT-[^{125}I]Carnosine) and Pt(IV)(IT-[^{125}I]Carnosine) carnosine was first labeled with ^{125}I. Newly synthesized complexes were then studied *in vivo* on Wistar rats and Lewis rats with subcutaneously implanted rat's pancreatic tumor cells (AR42J). Analysis have shown that compounds were characterized with low accumulation in thyroid gland, meaning carnosine bound effectively to iodine. Modification of platinum complexes with carnosine changed biodistribution of the compounds, resulting even in 5 times higher amounts in blood and lower excretion with the urine, leading to higher complex accumulation in tumor cells. Nevertheless study doesn't analyze the complexes cytotoxicity [134].

In 2013, Moustafa presented complexes of carnosine, anserine and *N*-acetylcarnosine with oxaliplatin, one of the most commonly used, cytotoxic anti-cancer drug containing platinum [135]. Complex's cytotoxicity was analyzed on hepatocellular carcinoma HepG2 and the results suggest that carnosine can inhibit the

cytotoxic effect of oxaliplatin, changing the IC_{50} of that anti-cancer drug from 18.7µg/ml to 24µg/ml. This effect is probably not due to reduced drug accumulation but due to the chelation of platinum by carnosine.

Another study finds that carnosine can protect colorectal cancer patient from oxaliplatin-induced peripheral neuropathy by targeting Nrf-2 and NF-κB [53]. Elevated level of Nrf-2 results in an anti-oxidant and anti-inflammatory effect. Also Nrf-2 can inhibit NF-κB and TNF-α, therefore preventing neuroinflammation. Interestingly Nrf-2 can also regulate expression of many genes encoding cytoprotection in response to chemical and radiation stress, leading to reduced apoptosis and enhanced cell survival, through an anti-apoptotic protein, Bcl-2 [136].

In 2010, Renner found carnosine inhibits ATP production in malignant glioma cells [137]. The data was confirmed by Iovine [138], expanding it to the knowledge of carnosine affecting ROS production. Such anti-proliferative effect of carnosine was also found to correlate with the inhibition of expression of HIF-1α in human colon cancer cells [139]. HIF-1α is a protein often over-expressed in many types of cancer and is an obstacle for the usage of many drugs. The ability of carnosine to reduce HIF-1α levels is also correlating with its involvement in HIF-1α degradation via ubiquitin-proteasome system [139]. HIF promotes the expression of carbonic anhydrase IX, a transmembrane enzyme maintaining intracellular pH under hypoxic conditions, which results in acidification of tumor microenvironment. Carnosine was found to inhibit carbonic anhydrase IX-mediated acidosis by changing HIF-1α expression and increasing the extracellular pH [140]. Suppressing HIF-1 also results in the decrease of IGF binding protein 1 (IGFBP1), followed by a decrease in the blood glucose levels [141]. This is important for glucose homeostasis, for example in diabetes patients.

Another interesting study reports carnosine has an ability to inhibit the proliferation of human gastric cancer. Treatment didn't induce necrosis nor apoptosis, nevertheless it reduced maximal oxygen

consumption, reducing mitochondrial function of a tumor [126]. Rybakova also reported for glioblastoma cells that together with the decrease in cellular ROS concentration, an increase in cyclin B1 was observed [142]. This resulted in blocking the cell cycle in G2 phase.

Pandurangan studied the efficacy of carnosine against human cervical carcinoma cells and presented that *in vitro*, adding carnosine to cancer cells inhibits their growth up to 23% alone [143].

Although these results are promising, it might not be as bright *in vivo*, for example due to the presence of pyruvate. Carnosine inhibits glycolytic ATP production, but the reaction can be reversed due to oxidative phosphorylation fueled by pyruvate. Unfortunately after cultivating cells with pyruvate, glioblastoma cells didn't show any effect of carnosine on viability, although carnosine attenuated tricarboxylic acid cycle [144].

Interesting approach was presented by Garofalo et al., proposing to use oncolytic adenovirus with carnosine absorbed on the viral capsid, via electrostatic interaction [145]. The idea was that due to the fact that concentration of carnosine necessary to arrest tumor progression exceeds the one currently used in clinical settings and so the usage of viruses could lead to overcoming this limitation. Interestingly, complexing carnosine with oncolytic virus led to a synergistic cytotoxic effect both *in vitro* and *in vivo*. The complex was inducing apoptosis in cell lines and reduced tumor growth in lung and colon cancer xenograft models [145].

Another idea was to create complexes of liposomes containing carnosine derivative and antigen binding fragment (Fab') of Trastuzumab, monoclonal antibody inducing an immune-mediated response, downregulating HER2 gene, which plays a huge role in the progression of breast cancer. Such therapeutical complex would be a type of antibody-drug conjugate (ADCs). Accardo et al. presented such conjugate using carnosine amphiphilic derivative, $(C18)_2$-L2-Car, synthesized by solid-phase methods using oxoethylene linkers and two C18 alkyl chains on *N*-terminus part of the dipeptide [146].

Liposomes were then prepared by mixing the carnosine derivative with different amounts of phosphatidylcholine and cholesterol forming Lip/Car conjugates. The size of obtained immunoliposomes were about 100nm and were then analyzed *in vitro* in human breast, human colorectal adenocarcinoma and human prostate cancer cell lines. Although maximum used concentration of carnosine (50μM) didn't affect cell viability, the IC_{50} values for Lip/Car with no Fab' functionalization were higher than the ones used in control. Liposome with Anti-HER2-Fab' were found to have a antiproliferative effect greater than for Lip/Car and lower IC_{50} values. Those immunoliposomes happened to decrease cell viability. Binding with HER2, carnosine was able to act as an ionophore for copper and zinc ion, altering metallostasis of tested cancer cells, leading to cell death [146].

As mentioned before, carnosine has an inhibitory effect on senescence of somatic cells. It has been estimated that carnosine prevents pro-cancerogenic activity of senescent peritoneal mesothelium (HPMCs) towards ovarian cancer cells, therefore inhibiting the progression of a tumor [147].

Fouad analyzed therapeutic effect of carnosine versus doxorubicin *in vivo*, finding that carnosine had significantly higher impact on ROS, RNS and inflammation biomarkers [148]. The study also showed that carnosine treatment results in higher concentration of apoptotic biomarkers, like Bax, cytosolic cytochrome C and caspase-3. Carnosine also inhibited the expression of AFP, glycoprotein which is a tumor biomarker of hepatocellular carcinoma.

Recently, carnosine has been demonstrated to inhibit the migration of glioblastoma cells and prevention of colony formation, selectively eliminating tumor cells [149].

In 2018, Tehrani et al., presented a study in which he analyzed 7 linear and 4 cyclic carnosine analogues, prepared by the solid-phase peptide synthesis method [150]. The derivatives were designed as dimer, with direct or inverse sequence of carnosine, with or without proline. Proline was used for cyclization of a

given peptide. All tested analogues exhibited cytotoxic activity *in vitro* in human liver and colon cancer cell lines with IC_{50} comparable to one of the cancer drugs, 5-fluorouracil. In flow cytometry analysis, authors found that compounds cyclo(His-β-Ala-Pro-β-Ala-His), cyclo(β-Ala-His-Pro-His-β-Ala), His-β-Ala and β-Ala-His-Pro-His-β-Ala exhibited apoptotic activities on HepG2 cell line more than carnosine. The same set of compounds with the exception of His-β-Ala (the inverse analogue of carnosine), showed the same activity on HT-29 cell line as well. Overall, the most potent sequence in regards to the authors, was cyclo(His-β-Ala-Pro-β-Ala-His).

One of the most predominatingly studied carnosine derivative in the case of cancer studies is polaprezinc (Z-103), a complex of of L-carnosine and zinc (zinc *N*-(3-aminopropionyl)-L-histidine). Although polaprezinc is mentioned in 528 publications until now, only 28 of them analyse the complex on the topic of cancer (Data collected in SciFinder® for Academics). First of them was prepared by Hayashi, who patented Polaprezinc for improving the appetite and taste function of cancer patients in terminal stage of the disease (Patent No. JP 2002068981). Hayashi wasn't the first to acknowledge the usefulness of zinc-carnosine complex though. Another Japanese researcher, Atsuko Ito published a paper on changes of zinc, copper and iron contents after long-term oral administration of polaprezinc in 1986, the same year Holiday and McFarland shown their study about carnosine [151]. Ito's experiments shown that polaprezinc has a high therapeutic index and inspired other researchers to study this topic. In the years following Ito's publication, Z-103 was tested for chelating and scavenging properties, followed by inhibition of ulcers, protection of erythrocytes against hemolysis, etc. [152–154].

In the following years more than a hundred publications about polaprezinc were published. They focused on antioxidant properties of Z-103, membrane-stabilizing action, gastric mucosal protection, improvement of cell proliferation in bones and wound healing [154–157].

Another study on taste disturbance in malignant tumor patients was published in 2008 by Nakata, although the work was in Japanese only, just like Hayashi's work [158]. Nakata's study shown that polaprezinc improved taste disturbance in 5 of 8 patients with lung cancer. Two years later Watanabe found that zinc-carnosine complex prevents oral mucositis associated with chemotherapy in head and neck cancer patients [159]. The same results were presented by Hayashi in 2014 analyzing patients treated with high-dose of chemotherapy for the same types of cancer [160]. Again, head and neck cancer patients receiving chemotherapy were then analyzed by Doi who acknowledged that polaprezinc also reduces the severity of radiation-induced mucositis and also promotes the recovery of the mucositis, without the reduction of tumor response to radiotherapy [161].

Polaprezinc has also been useful in paclitaxel anti-cancer therapy, reducing the occurrence of peripheral neuropathy in rats during treatment [162]. Importantly, the effect was obtained without affecting the activity of paclitaxel. Oral administration of Z-103 has also reduced another anti-cancer drug-induced mucositis, fluorouracil, in a mouse model [163]. Finally, Fujii et al. shown that polaprezinc can be used to shorten the duration of dysgeusia in patients receiving chemotherapy [149].

Given that data, polaprezinc seems to be a promising substance, useful in mitigating the negative effects of chemotherapy.

Overall, the potential of carnosine and its derivatives in cancer therapy is huge and it gives new possibilities in limiting the negative effects of currently used therapeutics and chemotherapy alone. Carnosine seems to be a good element of cancer therapy and hopefully will be given more attention in the future.

2.3 OTHER DISEASES

Due to the diversity of its function, carnosine can also be considered in therapies for many other diseases. For example, there is a correlation between Alzheimer's disease and type 2 diabeties metabolism, resulting in the fact that these conditions have common causative agents [164]. In fact, many papers emerged confirming that carnosine and its related structures can be used in diabetes treatment. It has been found that carnosine can improve diabetic wound healing *in vivo* [21], prevent apoptosis of glomerular cells [165], inhibit vascular damage [166], ameliorate cognitive deficits [167] and impairs fasting glucose and AGE products as well as TNF-α [168]. It was also found to decrease oxidation and glycation products in serum and liver of diabetic rats [169], including the reduction of IL-6 and TNF-α [170].

A 12-weeks carnosine supplementation on overweight individuals has shown changes in insulin levels, suggesting carnosine can reduce insulin resistance [171]. Also, in rodents, carnosine supplementation decrease food intake and leads to body weight loss [172]. Novel carnosine derivative, carnosinol ((2*S*)-20(3-aminopropanoylamino)-3-(1*H*-imidazol-5-yl)propanol) was presented to lower oxidative stress and harmful effects of obesity [173]. Researchers found that carnosinol has quenching and enhanced lipophilic properties, forming stable adducts with HNE in human serum, therefore acting as an RCS-sequestering agent [96]. What is more, carnosinol was found to be carnosinase-resistant which makes it more potent for treatment than carnosine. Another group of researchers earlier found that carnosinol also inhibits onset and stops progression of diabetic nephropathy in *db/db* mice [174]. Carnosinol also reduced myotube oxidative stress, apoptosis and inflammation. Furthermore, carnosinol was more potent than carnosine and anserine in the preservation of mitochondrial environment, resulting in the reduction of oxidative stress *in vitro* [175]. In addition, carnosinol is characterized with the lack of toxicity, which

combined with the resistance to carnosinases makes it a potent substance with a quite high therapeutic index.

In 2011, Castelletto incorporated a terminal Fmoc (*N*-fluorenyl-9-methoxycarbonyl) unit to carnosine structure. Fmoc-carnosine formed amyloid fibrils containing β sheets above a critical aggregation concentration while still performing a function as a metal ions chelator [95]. This may result in more carnosine biotechnology applications. Another study, presented by Mahapatra, assessed Fmoc-L-carnosine derived tripeptides form pH-sensitive, proteolytically stable hydrogels [176]. Hopefully, in the future there will be more research on attaching small, aliphatic groups to carnosine, for example forming Boc-carnosine.

Interestingly carnosine and homocarnosine brain levels can be raised due to using certain anti-epileptic drugs [177]. Carnosine also inhibits seizures in epileptic model mice and rats [178,179] and can have an anticonvulsant effect on epileptiform activity in rats [180].

Carnosine is also applied in cardiological practice. It has been found that adding carnosine in cardioplegic solution during stopped heart operation allowed the duration of an operation to increase without the signs of necrotic damage of tissues [181]. Carnosine also improved myocardium contractility and functional recovery after ischemia [182]. Zhao also reports carnosine can protect cardiac myocytes against lipid peroxidation products [36]. Carnosine is also beneficial in treating doxorubicin-induced oxidative stress and renal toxicity in rats [183].

Another study shows that carnosine mitigates catecholaldehyde-mediated disruptions in mitochondrial respiration and highlighted the therapeutic potential of this dipeptide in human myocardium [184]. The researchers also report that carnosine can be useful in treating pathological states where there is an

increased MAO activity. It is important to highlight that MAO activity increases with age and can have an impact on neurological disorders.

A double-blind randomized placebo-controlled trial with schizophrenia patients have shown that while carnosine was added to their treatment (4g/day) for 8 weeks, it effectively reduced the primary negative symptoms of the disease [185]. Another such study, but with children with autistic disorder shown that carnosine can improve hyperactivity subscales in patients [186].

Carnosine is also efficient in correction of oxidative stress in different pathologies, which is well described in the review presented by Prokopieva [12]. For example, carnosine can prevent progression of metabolic syndrome [187]. Quite new discovery has been made by Rothan et al., who found that carnosine has a significant antiviral activity *in vitro* against human liver cells infected with Dengue or Zika virus [188]. Moreover, research has shown that carnosine exhibits this function mainly by inhibition of viral genome replication. Different study proved carnosine can also be useful against different kind of virus - influenza A (H1N1). The infection of this virus comes along with higher concentration of nitric oxide in serum [170] which leads to inflammation and free-radicals formation, which explains tissue injuries observed in patients with this influenza virus. However this can be prevented by oral administration of carnosine, leading to inhibition of cytotoxic NO-induced proinflammatory condition and exerts positive effects on inflammatory cells, leading to less inflammation [170].

3. STRUCTURE-ACTIVITY RELATIONSHIP OF CARNOSINE

As pointed out by Boldyrev [3], most functional properties of carnosine come from the fact that it has an imidazole ring in its structure. Comparing the activity of carnosine and L-histidine we might observe that both of them have almost identical functions. Moreover, histidine had more cross-linking activity than carnosine , yet nature decided to store L-histidine in a form of carnosine due to reduction in its toxicity and leading to a stable tissue concentration of L-histidine [3]. Also another advantage of carnosine is that it's able to yield the Michael adduct, enabling reactivity of this dipeptide towards α,β-unsaturated aldehydes, whereas L-histidine needs previous imine formation approaching the imidazole ring to the reactive C3 atom [102]. This means carnosine might have a higher impact on homeostasis than histidine itself. Moreover, carnosine decarboxylation can result in histamine production, important as a neurotransmitter and remaining homeostasis in the gut. Interestingly it has been shown on birds like ducks, that along with the embryogenesis histamine is converted into carnosine and later on the decrease of this peptide is noted, while the increase of its methylated analogue, anserine is observed [3]. It’s reasonable to believe that such enzymatic conversions are due to the fact that carnosine derivatives are more resistant to carnosinases. It can also be assumed by analyzing Table 4.

Table 4. Comparison of several carnosine derivatives mentioned in this review and their activity.

Name	Carnosine-LVFFARK-NH_2	1-[2-(β-alanyl-L-histidine)ethoxy)]-β-D-glucopyranoside	6-(β-alanyl-L-histidylamino)-6-deoxy-α,α'-trehalose	octyl ester of D-carnosine	β3-methoxyhomophenyl-alanine-D-histidine
Activity	- enhanced inhibition of Aβ aggregation - higher activity against ROS - remarkably increases viability of cells with Cu^{2+}-mediated $Aβ_{40}$ cytotoxicity	-more resistant to carnosinases -increased carnosine bioavailability -controls coordination ability of metal ions (like cooper)	- more resistant to carnosinases - favours β-amyloid anti-aggregating activity of carnosine - promotes clearence of pathogenic proteins from the cell	- derivatization leads to increased bioavailability of a compound by 2.6-fold - decrease development of hypertension and dyslipidemia (restores renal functions) - inhibits carbonylation - RCS sequestration - inhibits oxidative stress, therefore reducing inflammation	- detoxificates reactive carbonyl species - higher serum stability and lower reactivity than D-carnosine
Ref.	(89)	(102)	(100)	(90,91)	(86)

In 2016 Zou and his co-workers enlisted characteristics of peptides related to their antioxidant activity [189]. One of them was molecular weight which, when smaller (<3kDa), lead to the higher anti-oxidant function. Also, amino acid composition of peptides was discussed. It happens to be that hydrophobic residues, like glycine (Gly), proline (Pro) or alanine (Ala) comprise higher activity to scavenge ROS. This means that in the case of carnosine, alanine residue stands for antioxidant properties although as mentioned in [3] this function is also possesed by histidine. It has also been stated that β-alanine regulates histidine metabolic fate and activates the synergistical effect with histidine, in reactive carbonyl species detoxification [3,190].

Modifying carnosine structure in order to increase its activity might be hard due to the fact that to improve the reactivity of nucleophilic center you have to increase its basicity as well [102]. Interestingly it has been found that glycoconjugation protects carnosine moiety from hydrolysis, potentialy improving its bioavailability [118].

In the case concerning anti-cross-linking activity, it has been shown that both β-Alanine and histidine has acted more efficiently than carnosine alone, therefore are both partly responsible for this function. It has been stated that the β-position of alanine might be responsible for the ability of a compound to scavenge reactive carbonyls, although it has been suggested that imidazole ring can provide more protection [82]

.

4. CONCLUSIONS

Carnosine is an endogenous dipeptide widely distributed in human body. Its biggest concentrations are observed in muscles and the brain, two tissues with the most active oxidative metabolism. It has an immense quantity of functions, which is connected to the structure activity relationships in this protein. For example β-alanine residue is responsible for carnosine antioxidant properties, whereas histidine is involved in the ability of carnosine to bind to transition metal ions [95] and to inhibit glycation-induced protein crosslinking [17]. It can also suppress toxicity of a lot of deleterious aldehydic products of lipid peroxidation and glycated proteins, inhibiting advanced glycation end products formation [191,192]. Carnosine also suppress cell senescence in cultured human fibroblasts [25]. It also has an anti-aging activity [18], shown even on senescence-accelerated mice [193].

Due to many abilities of carnosine, the dipeptide finds application in treating many versatile diseases. As mentioned in this paper, carnosine can be used in treating, to mention, neurodegenerative diseases, cancer, diabetes and schizophrenia. Still, despite much research has shown neuroprotective abilities of carnosine, there's no hypothesis for the exact role of carnosine in brain disorders [38].

Unfortunately, carnosine is rapidly hydrolyzed by carnosinases, mostly in human plasma. Due to this fact, carnosine's usage as a potential drug is somehow limited, although it has to be acknowledged that ingestion of β-alanine can promote and increase carnosine muscle levels by up to 80% [40,43,44]. Also, interestingly in order to access the brain, contact with serum carnosinase can be avoided if carnosine is delivered by intranasal delivery route [164] or synthesized *de novo* in olfactory neurons and glial cells of the brain.

Derivatization of carnosine should reduce carnosinases activity while in the same time, enhancing or at least maintaining the positive effects of the compound. The research also implies targeted delivery of the

compound is also beneficial as well as counterbalancing the side effects [74]. Due to the fact that both functional groups in L-Car are essential for the carnosinase recognition, the resistance of carnosine derivatives against carnosinases has been reported for conjugates derivatized both through amine or carboxyl group [74], which gives a brighter perspective in the future research.

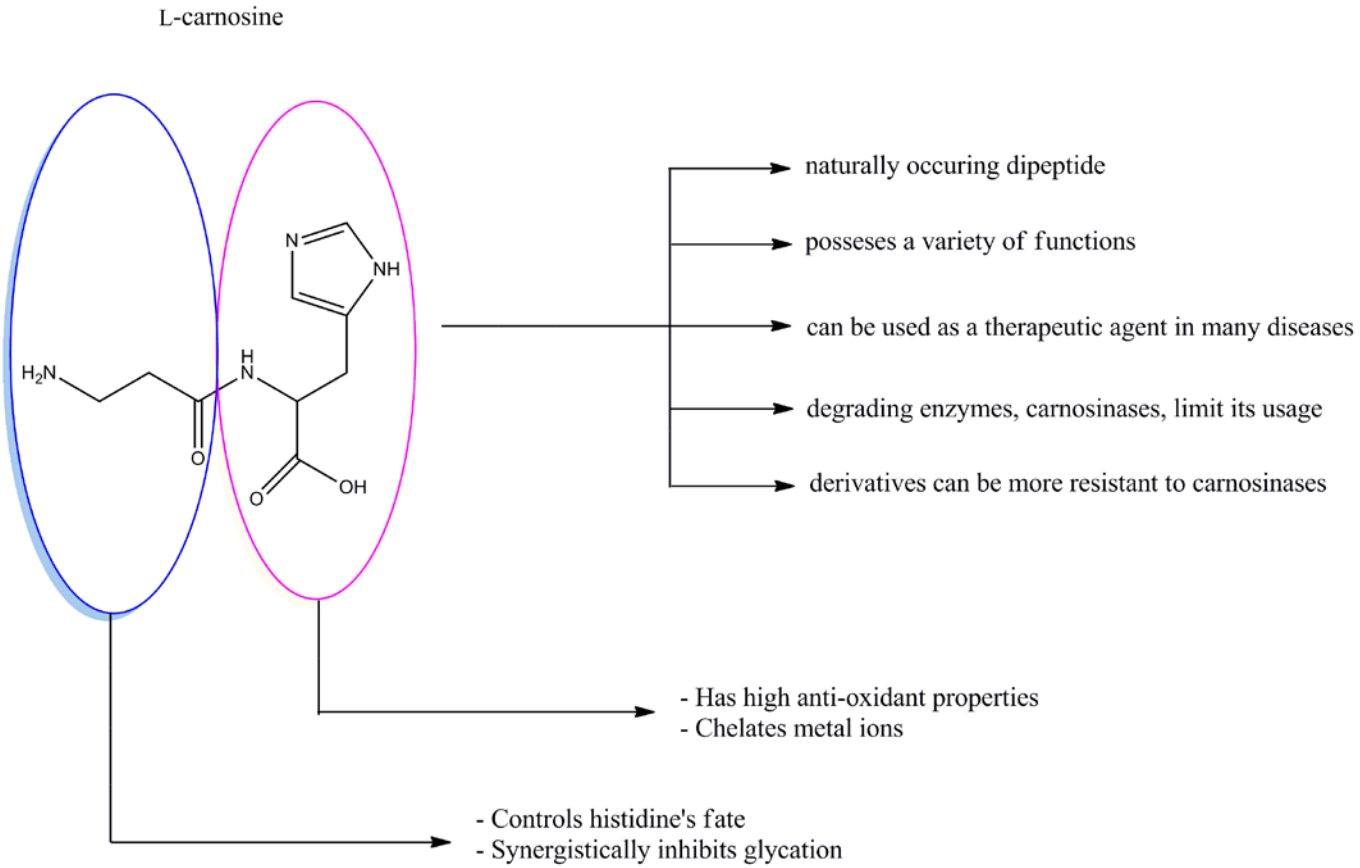


Fig 4. A graphical summary of most important information for carnosine and its constituents.

Still, yet another thing comes as a limitation. As mentioned by Maher and Schubert, due to the fact that carnosine and its derivatives are mostly non-patentable, it can result in discouragement in the commercial research sector [194]. Hopefully, the public sector will take part in the future research on carnosine and its related structures.

5. CONFLICT OF INTEREST

The authors declare there are no known conflicts of interest.

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