Research article

Drug Testing and Analysis

Received: 24 November 2010 Revised: 10 January 2011 Accepted: 10 January 2011 Published online in Wiley Online Library: 18 March 2011

(www.drugtestinganalysis.com) DOI 10.1002/dta.265

Bioactivation antioxidant and transglycating properties of N-acetylcarnosine autoinduction prodrug of a dipeptide L-carnosine in mucoadhesive drug delivery eye-drop formulation: powerful eye health application technique and therapeutic platform

Mark A. Babizhayev*

A considerable interest in N-acetylcarnosine ocular drug design for eye health is based on clinical strategies to improve ocular drug delivery through metabolic enzymatic activation. Human biology aspects of ocular N-acetylcarnosine deacetylation during its pass through the cornea to the aqueous humor and dipeptide hydrolyzing enzymes are characterized. Novel approaches to ocular drug delivery increasing intraocular bioavailability of N-acetylcarnosine biologically activated metabolite carnosine become an integral development ensuring prolonged retention of the medication in the mucoadhesive precorneal area and facilitating transcorneal penetration of the natural dipeptide with the corneal promoters.

A comprehensive list of techniques for peptide drug design, synthesis, purification, and biological analyses was considered: liquid chromatography (LC), high performance liquid chromatography (HPLC), ^{1}H and ^{13}C nuclear magnetic resonance (NMR), electrospray ionization (ESI) mass spectroscopy, and spectrophotometry. The antioxidant activity of therapeutics-targeted molecules was studied in aqueous solution and in a lipid membrane environment. A deglycation therapeutic system was developed involving removal, by transglycation of sugar or aldehyde moieties from Schiff bases by histidyl-hydrazide compounds or aldehyde scavenger L-carnosine. Clinical studies included ophthalmoscopy, visual acuity (VA), halometer disability glare tests, slit-image, and retro-illumination photography.

N-acetylcarnosine 1% lubricant eye drops are considered as an auto-induction prodrug and natural ocular redox state balance therapies with implications in prevention and treatment of serious eye diseases that involve pathways of continuous oxidative damage to ocular tissues(cataracts, primary open-angle glaucoma, age-related macular degeneration) and sight-threatening glycosylation processes (diabetic retinopathy and consequent visual impairment) important for public health. The results of the study document that the therapeutic benefit in clinical trials is associated with the bioactivation universal antioxidant and transglycating properties of N-acetylcarnosine acting as the ophthalmic prodrug of L-carnosine, and depends on the nature of the specific drug delivery lubricant eye-drop formulation applied as the topical solution.

The research highlights findings in N-acetylcarnosine prodrug activation, transport mechanisms, drug-to-drug interactions, and formulations in order to unlock the optimization of complicated ocular pharmacology of N-acetylcarnosine. Patented N-acetylcarnosine lubricant eye-drop formula was marketed as numerous human biological brands reaching important distribution networks on over 550 000 bottles sold.

Nature Does Nothing Uselessly.
- Aristotle

Keywords: N-acetylcarnosine eye drops; prodrug formulations; hydrazide carnosine derivatives; age-related ophthalmic diseases; natural medicine; human biology

Introduction

L-carnosine (β-alanyl-L-histidine), first identified in beef extract [1] has been found to be one of the most abundant (1–20 mmol/l) nitrogenous compounds present in the non-protein fraction of vertebrate skeletal muscle [2–4] and certain other tissues, including olfactory epithelium, bulbs (0.3–5.0 mmol/l)[5] and also the crystalline lens.[6,7] Some related compounds, for example, N-acetylcarnosine (N-acetyl-β-alanyl-L-histidine), anserine (β-alanyl-3-methyl-L-histidine), homocarnosine (γ-amino-butyryl-L-histidine) and carcinine (β-alanylhistamine) have been reported[8–10] to be present at millimolar concentrations in several mammalian tissues, including

* *Correspondence to: Dr Mark A. Babizhayev, Innovative Vision Products, Inc., Moscow Division, Ivanovskaya 20, Suite 74 Moscow 127434 Russian Federation. E-mail: markbabizhayev@mail.ru*

Innovative Vision Products, Inc., 3511 Silverside Road, Suite 105, County of New Castle, Delaware 19810, USA

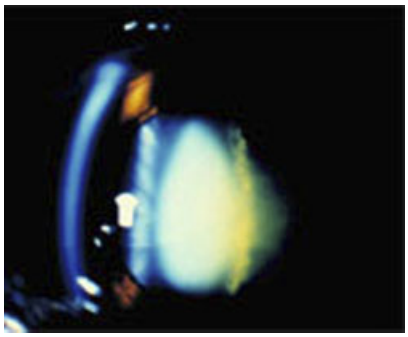


Figure 1. Cataract. Slit lamp photograph reveals marked lens opacity of the eye. The elderly patient complained of a slowly progressive, painless loss of vision.

skeletal muscles, cardiac tissue, and brain, although there are interesting differences in their tissue distribution, activities, and metabolic transformation.[8,11] The most common causes of vision loss among the elderly are age-related macular degeneration, glaucoma, cataract, and diabetic retinopathy. We know from numerous population-based surveys that a significant number of cataract operations (Figure1) may have poor outcomes (defined as presenting VA of less than 6/60).[12] Poor outcomes are distressing or disappointing for patients. They reflect badly on the health or surgical facility and on the surgical team. Poor outcomes may also affect the sustainability of services; they discourage other patients from coming for surgery and make patients even more reluctant to contribute towards the cost of cataract operations. More than 17 million people are blind because of cataract and 28 000 new cases are reported daily worldwide.[13]

The role of free radical-induced lipid oxidation in the development of cataracts has been established.[14–17] Glycation reactions, especially Maillard reactions, occur *in vivo* as well as *in vitro* and are associated with the chronic complications of diabetes mellitus and aging diseases by increases in oxidative chemical modification of lipids, DNA, and proteins. In particular, long-lived proteins such as lens crystallines, collagens, and hemoglobin may react with reducing sugars and their byproducts to form advanced glycation end-products (AGEs).

Scientists at Innovative Vision Products, Inc. (IVP) studied the effects of 1% N-acetylcarnosine lubricant eye drops (Can-C™) designed as a prodrug of L-carnosine containing a mucoadhesive cellulose-based compound combined with corneal absorption promoters in a drug delivery system. There exists empirically successful reasoning that N-acetylcarnosine eye drops are reputed to cure cataracts and other eye diseases. With available data from our own studies and from analyses of repurchase behaviour, researchers judged the patients' status of visual functions that affect the quality of life, counting the persistence and compliance rates per period to self-administered eye drops.

Materials and methods

L-carnosine and N-acetylcarnosine were synthesized by Hamari Chemicals Ltd (Osaka, Japan) per specifications proposed by IVP. The other carnosine derivatives described (Figure 2) were synthesized at IVP-connected laboratories and patented by IVP for health care and ophthalmic applications.[18–20] The standard peptide chemistry procedures were employed for the synthesis of carnosine derivatives (Figure 2) and the obtained compounds were purified by liquid chromatography (LC) or high performance liquid chromatography (HPLC) to obtain pure specimens as confirmed by nuclear magnetic resonance (NMR) and mass spectroscopy (MS).[18]

N-acetylcarnosine ocular drug delivery study design

Formulations and animals Grey Chinchilla rabbits (male) aged 3–4 months weighing 2–3 kg were used. Animal experiments conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research. Thirty minutes prior to the ocular incision, the right eyes of the rabbits were instilled with 80 µl of formulation A (Can-C™) containing 1% N-acetylcarnosine (NAC) and the control right eyes of separate rabbits were similarly instilled with their vehicles (placebo) solutions. Formulation A was presented in the final ophthalmic tubes (per volume of 2.5 ml) and in the moiety of the plastic bottles. Placebo solution contained the same ingredients without NAC.

Surgical procedure

Topical anaesthesia of the rabbits' eyes was performed after 25 min of instillation of the formula ophthalmic solutions with instillations of 4% lidocaine hydrochloride solution eye drops (three times with 1 drop at 1.5–2.0 min intervals). The eye drops of 4% lidocaine hydrochloride contained benzaltonium chloride preservative. When ocular anaesthesia was achieved, the lids were extended and fixed with the lid-holder and the ocular bulb was fixed by tweezers in the area of the inferior rectus muscle. A stab incision was performed transcorneally 1.0–2.0 mm from the limbus in the temporal superior quadrant. Aqueous humor (0.1–0.2 ml) was aspirated from the anterior chamber of a rabbit eye with 25-gauge needle connected to an insulin syringe and immediately introduced into an Eppendorf tube with addition of thanol (0.2 ml), keeping the sample on ice before extraction.

Extraction of imidazoles from aqueous humor

Extractions of imidazole-containing compounds from the aqueous humor aliquots were performed according to Babizhayev *et al*.[21] The published data showed that all the desired imidazole-containing compounds in the aqueous humor thus obtained could be of good purity and recovery.[21] Portions of aqueous humor were added to ethanol as above and thoroughly mixed (20 °C, 15 min). Extracts were centrifuged (2000 × g, 15 min) and the supernatants removed. Samples were frozen in the gradient of temperatures to −70 °C and lyophilized using the apparatus JOAN (Lyon, France). The lyophilized residue was dissolved in 1 ml of 0.1 M Na_2HPO_4 (pH 2.1 adjusted with 85% phosphoric acid) and filtrated through the membrane filter with the dimensions of pores 0.22 µm directly prior the analysis.

Analytical HPLC for detection of L-carnosine and N-acetylcarnosine

Reverse phase analytical HPLC was performed using a Breeze chromatography system (Taunton, Massachusetts, USA), detector Waters 2487 Dual λ Absorbance Detector, column (250 × 4.6 mm) Symmetry 300 C_{18} 5 µm (Waters Corporation, Taunton, Massachusetts,USA), loop 20 µl. The column was eluted isocratically at 30 °C with the cited phosphate buffer 0.1 M Na_2 HPO4 (pH 2.1) over 25 min at a flow rate of 1.0 ml/min. Eluates were monitored for absorbance at 210 nm. The standards of L-carnosine and N-acetylcarnosine were prepared by weighing of the dry material

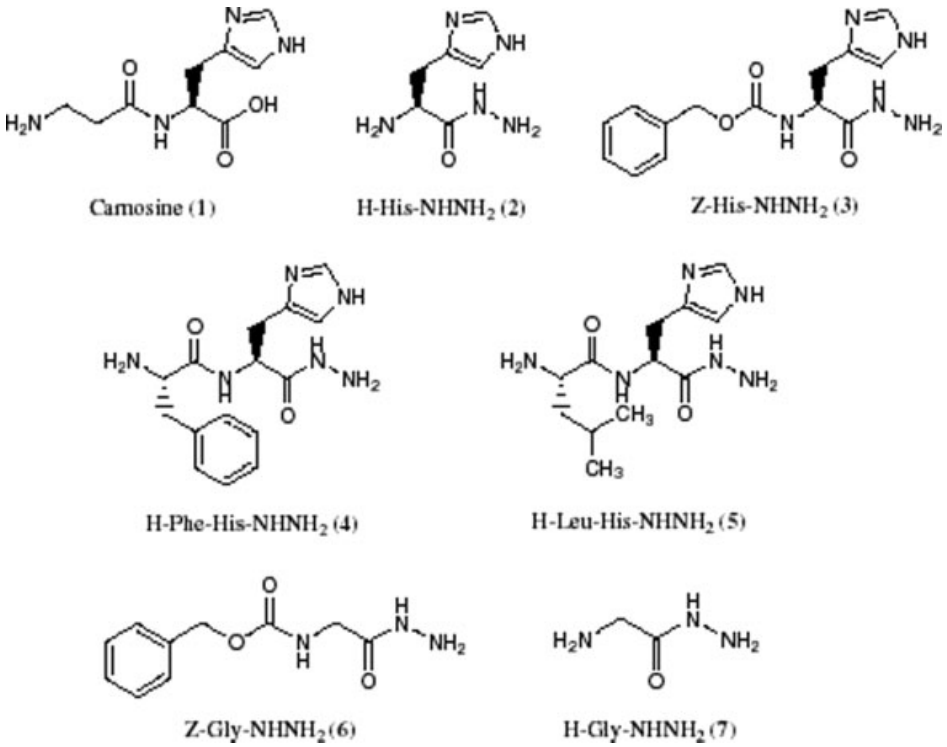


Figure 2. Synthesized at IVP-connected laboratories and patented by IVP for health care and ophthalmic applications carnosine derivatives [18–20]. The standard peptide chemistry procedures were employed for the synthesis of carnosine derivatives and the obtained compounds were purified by LC or HPLC to obtain pure specimens as confirmed by NMR and mass spectroscopy.[18].

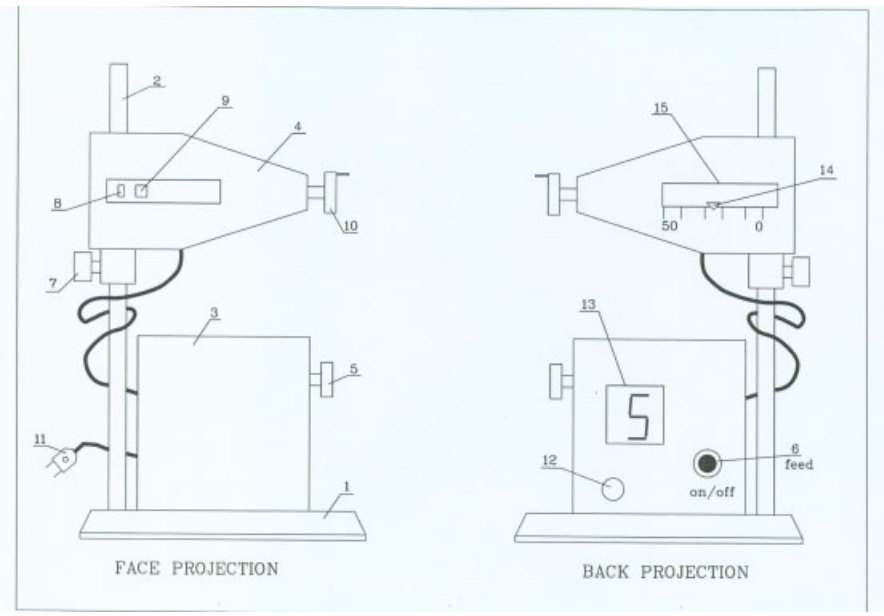


Figure 3A. Halometer DG (face and rear) projection.

using the analytical balance Mettler Toledo Columbus, OH, USA (accuracy 0.00004) and were further dissolved in the phosphate buffer 0.1 M Na_2 HPO4 (pH 2.1). The quantitative determination of l-carnosine and N-acetylcarnosine in the samples was undertaken using the technique of external standard according to the area of the peak and linear extrapolation. The standards of eye drops were prepared by dissolution of initial solutions of eye drops by 100-fold using the phosphate buffer 0.1 M Na_2 HPO4 (pH 2.1). Statistical significance was evaluated by the unpaired Student's *t*-test and P=0.05 was taken as the upper limit of significance.

Peroxidation reaction system of liposomes

The techniques for phospholipid extraction, purification, preparation of phosphatidylcholine (PC, derived from egg yolks) liposomes

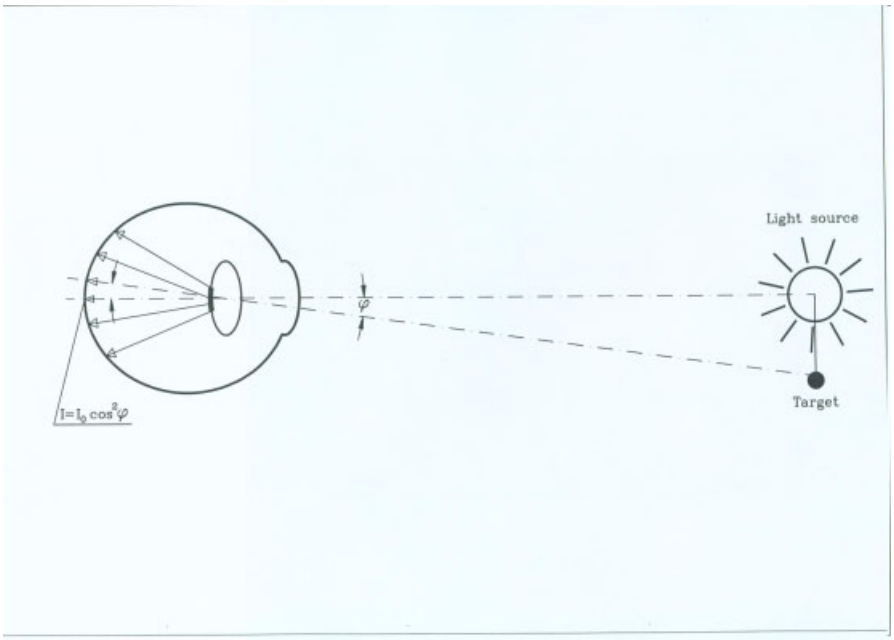


Figure 3B. Principle of the glare test based on the measurement of the glare radius (r, mm) a new metric for glare sensitivity. I_0 = Indicatrix of light scatter; φ = angle. The technique utilizes a self-illuminating red or green optotype target and tangential 2-mm 'point light source' seen from a distance of 30 cm. The patient's task is to move the optotype closer to the glare source until it disappears due to the veiling glare from the glare source. A halometer score is determined as follows. The target is approached from the source so that the patient becomes unable to distinguish the target from the source and then, the target is slowly taken away until the exact moment when the patient distinguishes the target; at this time, the incident light angle φ between the source and the target is measured. The target is always fixated with the foveal vision. The target and the 'point light source' are viewed in the same vertical plane, tangential to the plane of emitted light. In this case, to measure the angle φ of the incident light between the source and the target, it is necessary only to measure its projection on this vertical plane, which means to measure the distance between the source and the target. The measured glare radius is defined as a target image projection for the vector of light scatter (indicatrix of light scatter $I = I_0\cos^2\varphi$) when the glare source is activated and the patient is asked to recognize the target during illumination of the eye with a glare source.

(reverse-phase evaporation) and peroxidation of liposomes techniques have been described previously in detail.[22–24]

Electrospray ionization-mass spectrometry (ESI-MS) spectra were acquired with a Mariner (PerSeptive Biosystems, Inc. Framingham, MA 01701 United States) mass spectrometer instrument using a mixture of neurotensin, angiotensin, and bradykinin at concentration of 1 pmol/IL as external standard. Samples were prepared by dissolving the compound (10^{-5} M) in acetonitrile/water 1 : 1 mixture with 1% acetic acid.

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance DRX 400 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent ($CDCl_3$ or DMSO-d_6) as internal standard. Reaction courses and product mixtures were routinely monitored by thin layer chromatrography (TLC) on silica gel (precoated Polygram Sil G/UV 254 from Macherey-Nagel, Duren, Germany) and visualized with UV lamp (254 nm) or iodine vapors. Reagents and solvents were of high-purity grade and were purchased from Sigma–Aldrich, St. Louis, MO, USA, J.T. Baker, Phillipsburg NJ, USA, and Carlo Erba, Val de Reuil, France.

^{13}C NMR experiments

Glucose–ethylamine (G–E) was synthesized by incubating 500 mM ^{13}C-glucose and ^{15}N-ethylamine at pH 12 and 37 °C for 3 h.[18] At the end of the incubation period, about 75% of the starting material was converted to glucose–ethylamine in equilibrium with the starting materials. NMR experiments were conducted under conditions which stabilized Schiff base enough to be able to observe them by NMR over several hours. The reaction mixture (0.5 ml in a 5-mm NMR tube) included 250 mM Hepes, pH 8.5, 10% D2O, and 20 mM concentration of carnosine or one histidyl-hydrazide derivative. The reaction was performed at room temperature and it was initiated by adding an aliquot of G–E to produce a final concentration of 20 mM. At that time, consecutive NMR spectra of 20 min duration were acquired using 580 scans, 60° pulses, and an interpulse delay of 2.05 s. Spectra were analyzed using information from model compounds and chemical shifts from literature. The area of the G–E doublet at 90.00 ppm was calculated and plotted against time after subtraction of the natural G–E Schiff base decay measured in a blank experiment. Transglycation efficiency of L-carnosine and carnosine derivatives 2–7 (Figure 2) was assessed following Szwergold protocol,[25] using the Schiff base glucosyl–ethylamine (G–E) as a model of the first intermediate in the glycation process of side chain primary amines of proteins. ^{15}N labelled ethylamine was used to minimize electric quadrupole moment and obtain a C-1 peak of glucose as a sharp doublet centred at 90.00 ppm. The kinetics of the transglycation reaction for the control reaction, for carnosine, and for compounds 2–7 are illustrated in Figure 6A. For a better evaluation of the transglycation kinetics of the compounds, for each ^{13}C spectrum the integral of the buffer Hepes signals (50–55 ppm range) was set as = 1, then the integral of the C-1 glucose peak at 90.00 ppm was measured and integration values, normalized and corrected for the natural decay of the G–E Schiff base (control curve), were plotted against time in Figure 6B.

Clinical studies

The first enrolled cohort of examined subjects consisted of 75 older adults with age-related uncomplicated cataracts in one or

Figure 3C. Photograph of working prototype of the Halometer DG tester. The instrument can be used in the pre-testing examination room of optomemtrist and ophthalmologists offices and also for self testing.

Table 1. Computer user questionnaire Do you notice any of these visual symptoms? Please rate the following symptoms by underlining the appropriate description

Symptom	Severity		
Headaches during or after working at the computer	Mild	Moderate	Severe
Overall bodily fatigue or tiredness	Mild	Moderate	Severe
Burning eyes	Mild	Moderate	Severe
Distance vision is blurry when looking up from the computer	Mild	Moderate	Severe
Dry, tired, or sore eyes	Mild	Moderate	Severe
Squinting helps when looking at the computer	Mild	Moderate	Severe
Neck, shoulder, or back pain	Mild	Moderate	Severe
Double vision	Mild	Moderate	Severe
Letters on the screen run together	Mild	Moderate	Severe
Driving/night vision is worse after computer use	Mild	Moderate	Severe
'Halos' appear around objects on the screen	Mild	Moderate	Severe
Need to interrupt work frequently to rest eyes	Mild	Moderate	Severe

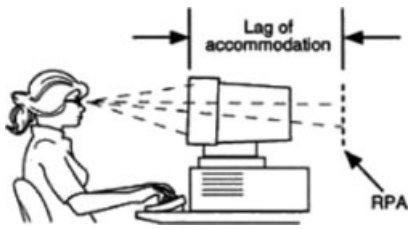


Figure 3E. Vision problems during computer works. The eyes have a very hard time focusing on the pixel characters. They focus on the plane of the computer screen, but cannot sustain that focus. They focus on the screen and relax to a point behind the screen, called the 'resting point of accommodation' (RPA) or dark focus. The RPA is different for every individual, but for almost everyone, it is further away than the working distance to the computer. The working distance is the distance from the computer user's eyes to the front of the screen. So, the eyes are constantly relaxing to the RPA, and then straining to refocus on the screen. This constant flexing of the focusing (ciliary body) muscles is what creates fatigue, and generates burning and tired eyes. In clinical studies, it has been found that there is a significant difference in the glasses prescription required for focusing on a standard printed near card (called a Snellen card) and focusing on the image of a typical computer screen, both at a viewing distance of 20 inches. Many patients needed a different correction in each eye.

both eyes, and 72 adult subjects who did not have cataract in either eye. Patients in these subsamples suffered from different degrees of glare problems. Those with cataract ranged in age from 53 years to 83 years (mean± SD, 69 ± 8 years), with 48% female, 100% white and of non-Hispanic origin. The non-cataract subjects ranged in age from 54 to 78 years (mean ± SD, 66 ± 8 years); 53% were female, with 100% being white. Subjects who were cataract-free had to meet the same inclusion criteria as the subjects with cataract described previously.[26–31] All subjects with cataracts were required to meet the following inclusion criteria: (1) cataract in one or both eyes with best-corrected VA of 20/40 or worse in one or both eyes as indicated by the medical record; (2) no previous cataract surgery in either eye; (3) a primary diagnosis of cataract in the medical record; and (4) living independently in the community. Specific items needed to be addressed if appropriate: (5) driving skills: legally licensed to drive and drove during the five years prior to enrollment; (6) related general or eye health problems experienced during computer use – this was assessed by asking subjects if they had any symptom problems in the specific areas listed in Table 1, Figure 3E.

Among participants, bilateral cataracts were present in 95% of subjects according to the medical records from the most recent eye examination (within one month of enrollment). In the right eye, 46% had nuclear sclerotic cataract, 8% had cortical cataract, 9% had posterior subcapsular cataract, and 38% had a combination of at least two types. The breakdown was similar in the left eye, with 49% nuclear sclerotic, 10% cortical, 7% posterior subcapsular, and 35% combination. Seventy-four percent of subjects with cataract had no additional ocular conditions other than refractive error; 6% had early nonexudative age-related maculopathy, 9% had primary open-angle glaucoma (POAG) associated with cataract, 3% had diabetic retinopathy, 1% had a combination of two of these problems, and 7% had another ocular condition. Subjects who were cataract-free had to meet the same inclusion criteria as the subjects with cataract, except that they were required to be free of cataracts and to have a best-corrected VA of 20/25 in each eye, according to medical record review. No cataract-free subjects had secondary eye conditions other than refractive error. Patients with known or presumed hypersensitivity to any component of the ophthalmic preparations (active substances or excipients), and those treated with drugs that could interfere with

Table 2. Demographic and ergonomic occupational characteristics of cataract and non-cataract adult subjects enrolled in the study

	Cataract		Non-cataract	
	n	%	n	%
Total	75		72	
Age groups				
50–59 years	18	24	18	25
60–69 years	43	57	40	56
70–85 years	14	19	14	19
Sex				
Female	36	48	38	53
Male	39	52	34	48
Race	White 75	100	White 72	100
Driving exposure *				
Total	40	53	42	58
<150 km/wk	23	58	17	41
>=150 km/wk	17	42	25	59
Computer users				
Total	47	63	51	71
Occasional computer users**	17	36	21	41
Moderate computer users**	18	38	16	31
Intensive computer users**	12	26	14	28

Data are presented as numbers and percentages.

* Driving subjects were classified into two categories according to whether they drove more or less than the median number of km (150 km) driven per week based on the distribution of all subjects. Although this was a self-report measure, prior studies indicate that older adults can provide valid estimates of driving exposure.[42]

** ***Occasional computer user***

This individual typically uses the computer for less than three hours per day. This user tends to have an extensive variety of different tasks (computer and other) and they are unlikely to regularly spend extended amounts of time sitting and working at the computer.

** ***Moderate computer user***

This individual typically uses the computer between three and five hours per day. This user tends to have some variety in the daily work tasks but they regularly may spend up to half the workday at the computer.

** ***Intensive computer user***

This individual typically spends more than five hours per day on the computer. This user may have a limited number of non-computer tasks or none at all. These individuals are considered to be at high risk of developing computer-related injuries if precautions such as appropriate workstation design, layout, and work practices are not addressed.

this trial, were also excluded from the study. The subjects were recruited and examined by IVP's ophthalmology practices. The study protocol was approved by the Corporative Review Board for Human Use. After the purpose of the study had been explained, each subject was asked to sign a document of informed consent before enrolling. Demographic data, driving status during the prior five years and computer use at work were confirmed by interview (Table 2).

Procedures

After enrolment, subjects were computer-randomized into two groups assigned according to the double-blind method: to receive treatment with *N*-acetylcarnosine 1% eye drops (Table 3) (Can-C™), or to a control group who received placebo eye drops. The blinded

Table 3. Specification of cGMP manufactured N-Acetyl-L-Carnosine used in IVP drug Can-C ™ development and clinical studies

No.	Test Name	Specification
1	Appearance	White powder
2	Identification	Positive
3	Optical rotation	$[\alpha_D^{20}] + 25.2^\circ - +27.5^\circ$
4	pH	4.5–5.5
5	Heavy metals	NMT 10 ppm
6	Related substances	L-Carnosine: NMT 0.3% Others: NMT 0.2%
7	Residual solvent	2-propanol: NMT 500 ppm
8	Water	NMT 5.0%
9	Residue on ignition	NMT 0.10%
10	Assay	NLT 99.5% (HPLC area)

NMT: not more than; NLT: not less than.

testing was carried out by an independent medical worker who handed out the NAC versus placebo eye drops (control group) to the randomized subject members of the clinical groups. The enrolled subjects underwent follow-up examinations at baseline and nine months after enrolment. Test examiners were masked to the driving histories of all subjects. Two types of visual functions were assessed: VA and glare sensitivity (disability glare). All acuity measurements were made while subjects wore the lens correction they typically used during the performance of everyday distance activities, including driving. Each eye was assessed separately. Distance acuity was measured as described before using the letter chart and its standard protocol, and was expressed as log minimum angle resolvable.[26–33] For each eye, VA measurements were grouped into four categories: 20/25 or better, 20/25–20/30, 20/35–20/50, and worse than 20/50. These cut points were chosen because they were the approximate quartiles of the acuity distribution and included the practically significant cut point for driving licensure in many countries (20/40 to 20/50). Some of the vision problems from this course will include computer-related work and the circumstances under which that work is performed.

Slitlamp biomicroscopic examination or exampled photographic registration was performed after pupil dilation to a minimum of 6 mm with tropicamide.

Disability glare was defined with an optical instrument and method for measuring susceptibility to glare of a human vision system as described [26–29,30,31,34,35] and schematically presented in Figures 3A–3C. A constant 'point'–like a bright glare source was used to create the glare condition (Figure 3C). The examining room was dark (less than 20-foot candles) as typical when working with the glare testers to assure maximum contrast of the projected target. Tests were performed with the best correction in place. The indicator of optotypes on the front or back panels of the instrument indicated the tested optotype to the patient or clinician, respectively.The diagnostic block of a device contained source light window (glare source) and the moving indicator of the optotypes illuminated with red or green light (Figure 3C). The back panel of the Halometer device facing the clinician was equipped with a chart/scale and with a moving indicator of the optotype transition. According to a special embodiment of the invention,[26–29,30,34,35] for the clinical testing of glare sensitivity of a patient we used an illuminated target with red or green colour, which enabled the assessment of the effect of wavelength on the scattered light.

Treatments with N-acetylcarnosine 1% lubricant eye drops

NAC eye drops (Can-C ™) contained a 1% solution of NAC[19,36–38] with a lubricant 0.3% carboxymethylcellulose in the isotonic ophthalmic formulation in borate buffer with preservative benzyl alcohol (corneal absorption promoter) and showed the increased intraocular absorption of the active principle (L-carnosine) in the aqueous humor compared to topical administration of a pure 1% NAC solution:

Deionized water	970 g
Glycerine, 1.0%	13 g
N-Acetylcarnosine, 1.0%	10 g
Carboxymethylcellulose, 0.3%	3 g
Benzyl alcohol, 0.3%	3 g
Potassium borate	7.91 g*
Potassium bicarbonate	3.47 g*

*Or what is necessary to bring the solution up to a pH of ~6.3–6.8.

The ophthalmic formulation thus creates a facility to examine the efficacy of treatment for improvements of vision during the short-term periods of administration of *N*-acetylcarnosine 1% eye drops (nine months in the present study).

The administration schedule was two drops instilled twice daily, for patients assigned to NAC and those assigned to placebo (the same formulation without *N*-acetylcarnosine, 1%) alone for nine months. The use of other topical or nutritional antioxidants was not measured or evaluated between the two groups. The control groups and the treated group did not take any prescribed antioxidant vitamins that might have added to the antioxidant level. Neither the investigators nor the patients knew who was receiving NAC.

Statistical analyses

Statistical analysis was performed by Student's *t* test; $p = 0.05$ was taken as the upper limit of significance. To assess associations, correlation and linear regression analyses were used.

Patient compliance in the clinical group described in this study to self-administer N-acetylcarnosine lubricant eye drops (Can-C ™) was considered fine.

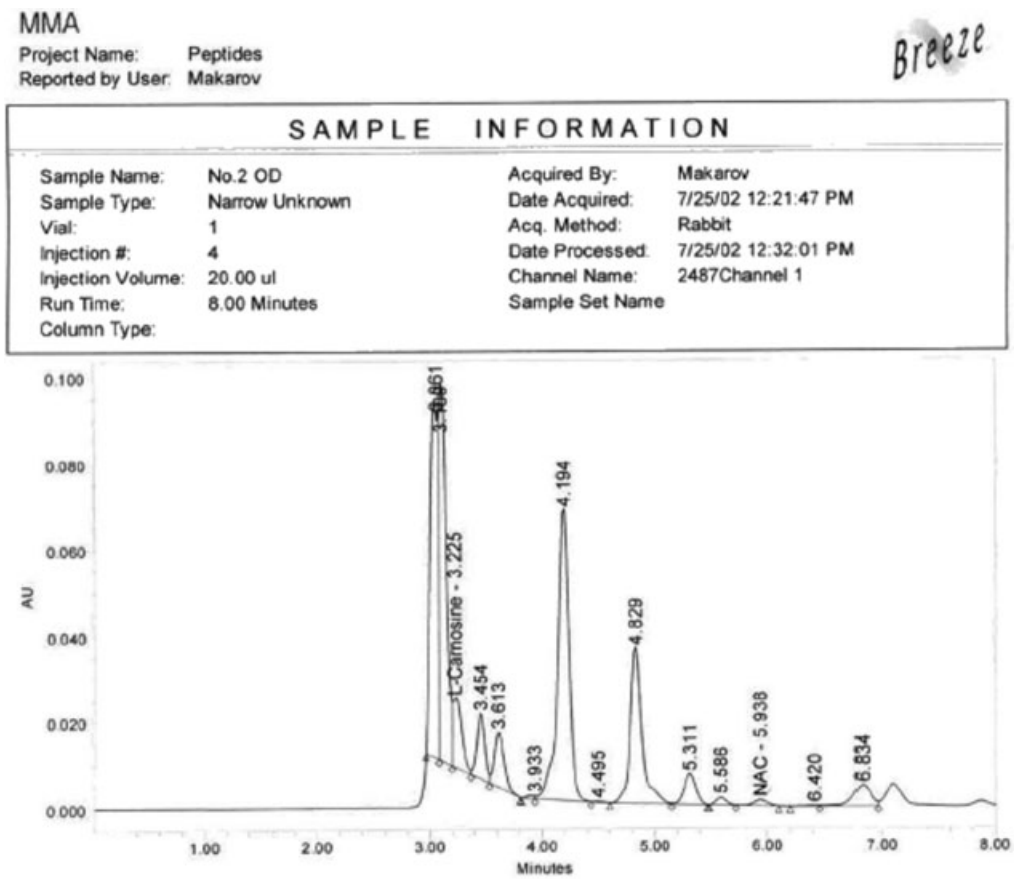
Results

N-acetylcarnosine ocular drug delivery in ophthalmic formulation with mucoadhesive polymer–macromolecule cellulose compound (carboxymethylcellulose), synergistic lubricant(s), and corneal absorption promoters enhances deacetylation of N-acetylcarnosine and carnosine intraocular bioavailability by facilitating transcorneal penetration of peptide.

Due to its relative hydrophobicity compared to L-carnosine, NAC might cross the cornea of the treated eye gradually and maintain longer the concentration of the active principle (L-carnosine) reaching the aqueous humor. In the present section of the study, we considered whether NAC acts in the ophthalmic formulation with lubricants and preservatives when topically administered to the eye as a time release carrier (prodrug) of L-carnosine. The HPLC pattern of an extract of the aqueous humor obtained 30 min after instillation to the rabbit eye of ophthalmic formulation containing 1% NAC, lubricants carboxymethylcellulose, glycerine and preservative benzyl alcohol in the borate buffer confirms that the peak characteristic of L-carnosine has a concentration and a retention time (3.225 min) clearly distinct from N-acetylcarnosine (6.0 min) and basically different from the dead time of the column (3.0 min) (Figure 4). This identified peak of L-carnosine quantified and integrated by the data processor showed that virtually all N-acetylcarnosine after the overall extraction efficiency is converted into the L-carnosine compound with a retention time of 3.225 min (Figure 4). The data on the L-carnosine-related product were blanked with the control placebo data applied to the paired eyes of the animals. The mean ratio of L-carnosine (C)/(NAC) relevant to the L-carnosine release in the aqueous humor 30 min after instillation of Formulation A (Can-C ™) with 1% Nacetylcarnosine into the rabbit eye corresponded to C/NAC= 6.64 ± 0.06 ($n = 8$, where n = number of the examined treated rabbit eyes; only right eyes were treated). In the control placebo formulation-treated eyes, the same indices could not be quantified at statistically significant rate. Concentrations of imidazole products in the aqueous humor corresponded to those of intact rabbit eyes and refer to baseline values of L-carnosine 0.19 ± 0.10 μg/ml related products variously detected in extracts from normal animals. Our data demonstrate that topical administration of pure L-carnosine (1% solution) to the rabbit eye (instillation, subconjunctival injection) does not lead to accumulation of this natural compound in the aqueous humor over 30 min in concentration exceeding that in the placebo-treated matched eyes, and its effective concentration is exhausted more rapidly.[21,29] In another aspect, the data presented in the Results section demonstrate the prospects of applications of an ophthalmic composition comprising NAC, or its pharmacologically acceptable salt in combination with a cellulose compound or its pharmaceutically acceptable salt which are effective to treat the eye complex of symptoms. This complex of symptoms may have an oxidative component in their genesis, such as senile cataract, glaucoma, inflammation or diabetic ocular complications. The topical administration of N-acetylcarnosine in the developed and patented lubricant eye-drop formulation delivers pure L-carnosine and allows its increased intraocular absorption into the aqueous humor surrounding the lens, thus enabling significant improvements in anticataract efficacy.[39,40] This formulation also optimizes beneficial effects in a number of ocular degenerative age-dependent disorders.[39,40]

Antioxidant activity of N-acetylcarnosine versus L-carnosine in the liposome peroxidation system. Metabolic bioactivating antioxidant activity of N-acetylcarnosine. Scavenging activity of L-carnosine towards dialdehyde products of lipid peroxidation.

The comparative antioxidant activity of NAC and L-carnosine was assessed in the liposome peroxidation system (acting as oxidative lipid membrane substrate) catalyzed by Fe^{2+} + ascorbate (Figures 5A and 5B). The accumulation kinetics of molecular lipid peroxidation (LPO) products such as malondialdehyde (MDA) and liposomal conjugated dienes and trienes are shown in Figures 5A–5C. The results demonstrate that the LPO reactions in the model system of lipid membranes are markedly inhibited by L-carnosine. The effective concentrations of L-carnosine are 10 and 20 mM. Data on the biological effectiveness of L-carnosine as antioxidant preventing phosphatidyl-choline (PC) liposomal or linoleic acid peroxidation in physiological concentrations ranges of 5–25 mM have already been published.[21,37,41] Figure 5A shows that the level of thiobarbituric acid reactive substances (TBARS, dialdehydes) reached at 5-min incubation decreases in the presence of L-carnosine (10 or 20 mM) at 10 min and at later time points (20 mM), which must be due to a loss of existing TBARS or peroxide precursors of MDA and not due to a decreased formation of peroxide compounds. From the data published by Babizhayev,[22]



	Peak Name	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height	Amount	Units
1		3.061	402670	21.41	83190	24.86		
2		3.100	389886	20.73	87965	26.28		
3	L-Carnosine	3.225	89479	4.76	16173	4.83	3.392	mkg/ml
4		3.454	76173	4.05	15081	4.51		
5		3.613	76279	4.06	12690	3.79		
6		3.933	2892	0.15	625	0.19		
7		4.194	463529	24.65	67930	20.30		
8		4.495	1150	0.06	174	0.05		
9		4.829	257552	13.69	36153	10.80		

Figure 4. HPLC of extract of aqueous humor aspirated 30 min after the instillation of ophthalmic formulation with 1% NAC and lubricants into the rabbit eye. The integrated concentration of the carnosine related product (3.392 µg/ml, 3.225 min) is attributed to accumulation of carnosine in the ophthalmic formulation-treated eye. Chromatograms of solutions of L-carnosine and its putative N-acetyl derivative show that these compounds are well separated. The elution order of the compounds was compared to a predicted order based upon their relative hydrophobicities and the chromatographic system was shown to be suitable to monitor the behaviour of other histidine containing derivatives of L-carnosine. The calibrating chromatograms showed the predicted elution order and the average elution times for each standard of L-carnosine and N-acetylcarnosine in mixtures. Peaks were unequivocally identified by comparison of their retention times to those of the authentic standard compounds or of putative acetylated compound run singly. Tests for specific chemical reactivity provided additional evidence for the identification of L-carnosine and N-acetyl-carnosine[21].

it follows that the addition of carnosine against a background of accumulated peroxide products (dialdehydes), determinable according to MDA, leads to a decrease in their concentration. Most of data obtained indicate that carnosine, in contrast with other 'gold standard' antioxidants, at a concentration 15–50 mM, interacts directly with the already formed LPO products (dialdehydes) in the membrane structures, providing for neutralization of their injurious action. The β-alanine and L-histidine contained in carnosine, imidazole (chemically the most active part of the histidine molecule) as well as reduced glutathione (endogeneous antioxidative substrate of the lens) have no eliminating effect on ascorbate-dependent LPO product accumulation.[22] Thus, in histidine and imidazole medium, the final content of LPO products (dialdehydes) does not differ from that observed in Tris-HCl buffer (control), while the initial rate of accumulation of peroxides (dialdehydes) was even higher than in the control.

The ability of the histidine-containing compound NAC to inhibit the (Fe^{2+} + ascorbate)-induced oxidation of PC liposomes was

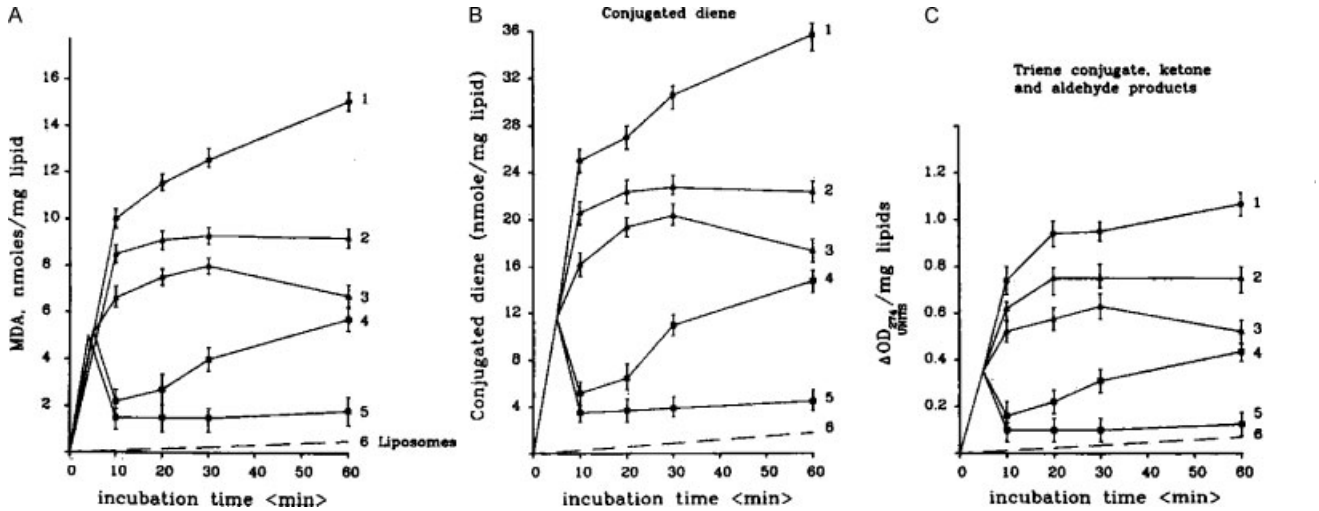


Figure 5. Accumulation of lipid peroxidation products (TBARS, measured as MDA) (A), diene conjugates (B), triene conjugates and ketone and aldehyde products (274 nm absorbing material) (C)) in liposomes (1 mg/ml) incubated for 60 min alone (6, dotted line) and with addition of the peroxidation-inducing system of Fe^{2+} + ascorbate (1). Antioxidants N-acetylcarnosine (NAC) (10 or 20 mM) (2,3) or L-carnosine (10 or 20 mM) (4,5) were added at the fifth minute of the incubation period to the system containing the peroxidation inducers. Samples were taken at zero time and at time intervals indicated in the figures and were used immediately for measurement of TBARS (see Materials and methods. Peroxidation reaction system). A similar amount of sample was partitioned through chloroform and used for detection of conjugated dienes and trienes dissolved in 2–3 ml of methanol-heptane mixture (5:1 *v*/*v*).

compared with that of equimolar concentrations of L-carnosine. The antioxidant activity of 10 and 20 mM NAC corresponded to 38% and 55% inhibition of LPO for the two concentrations after 60-min incubation. NAC exhibited less antioxidant protection than L-carnosine, corresponding to 60% and 87% of the equimolar (10 or 20 mM) L-carnosine inhibition percentage. However, because NAC can act as a time release version metabolized into L-carnosine during its cross through the cornea to the aqueous humor (but not oral use), the bioactivating antioxidant activity of NAC converted to

L-carnosine *in vivo* application is significantly increased. Once released from NAC, L-carnosine in the aqueous humor might act against peroxidation of the lens during its target pharmaceutical use.

The bioactivating prodrug N-acetylcarnosine approach has been utilized to enhance the ocular delivery of L-carnosine. The N-acetylcarnosine prodrug approach is one of the most promising in ophthalmology and viable strategies currently being investigated in this study for ocular drug delivery. Careful consideration and understanding of ocular tissue metabolic processes within the eye and site-specific cornea/conjunctiva tissues has important implications for controlling the activity of considered therapeutic peptide agents, and for providing the potential for intraocular antioxidant bio-activation of certain N-acetylcarnosine prodrug formulations and designed codrugs, thus enabling significant improvements in efficacy and the minimization of local and systemic side-effects.

Transglycation activities of L-carnosine derivatives

The data presented in Figures 6A–6C show that the transglycating efficiency of the tested carnosine derivatives is generally lower than that of carnosine, with the exception of **leucyl-histidylhydrazide (5)** which transglycation activity is markedly higher than of carnosine in the tested objective G-E Schiff base decay system. logP value and transglycating efficiency of the derivatives show a good correlation ($R^2 = 0.38$) (Figure 6C). The hydrazide moiety of **leucyl-histidylhydrazide (5)** boosts the aldehyde scavenging efficiency of compound,[18] and in combination with a free N^{α}-amino group, concurs in the disruption of the Schiff base adduct G–E as a model of protein glycation. Further structure/activity relationship details the synergistic efficacy of **leucyl-histidylhydrazide (5)** in therapeutic applications.[20] The data are related to sample supporting the IVP invention of the worldwide patented codrug formulation including N-acetylcarnosine (a bioactivating ophthalmic prodrug of L-carnosine) and a revealed tripeptide peptidomimetic reversing the glycosylation (glucose-derived intermolecular) crosslinks in proteins (Advanced Glycation End Products (AGEs)) and the Schiff bases for the next-generation treatment of ophthalmic complications of Diabetes Mellitus (DM), such as the development of visual impairment or blindness consequent to cataract formation, retinopathy or glaucoma.[19,38,20] Diabetes affects the (outer) lens, middle (vitreous), and inner (retina) areas of the eye.

Randomized, double-masked Phase II clinical trial of cataract patients treated with N-acetylcarnosine lubricant eye drops (Can-C™) compared with placebo treatment. Clinical study to evaluate the safety and efficacy of treatment.Sample characteristics.

Table 2 lists the demographic and ergonomic occupational characteristics of the cataract (n = 75) and non-cataract groups (n = 72). Those with cataracts were similarly older on average with the non-cataract group of subjects. Both groups were split evenly between male and female, and had similar racial composition, with the 100% white population.

Table 4 lists the visual function for both groups enrolled in the study and the distribution of VA and disability glare scores for subjects with cataracts and those without. As would be expected by the case definition for cataract group membership, those in the cataract group had impairments in visual function as compared to the non-cataract group. This was true for both the 'worse' and 'better' eyes. In addition, VA in the range of 20/35 to 20/50 and disability glare readings in the range of glare radius more than 12 mm was associated with driving difficulties (such as crash involvement) and computer-related injuries (Table 1). Although not statistically significant, there was also a possible relationship between VA worse than 20/50 and crashing during

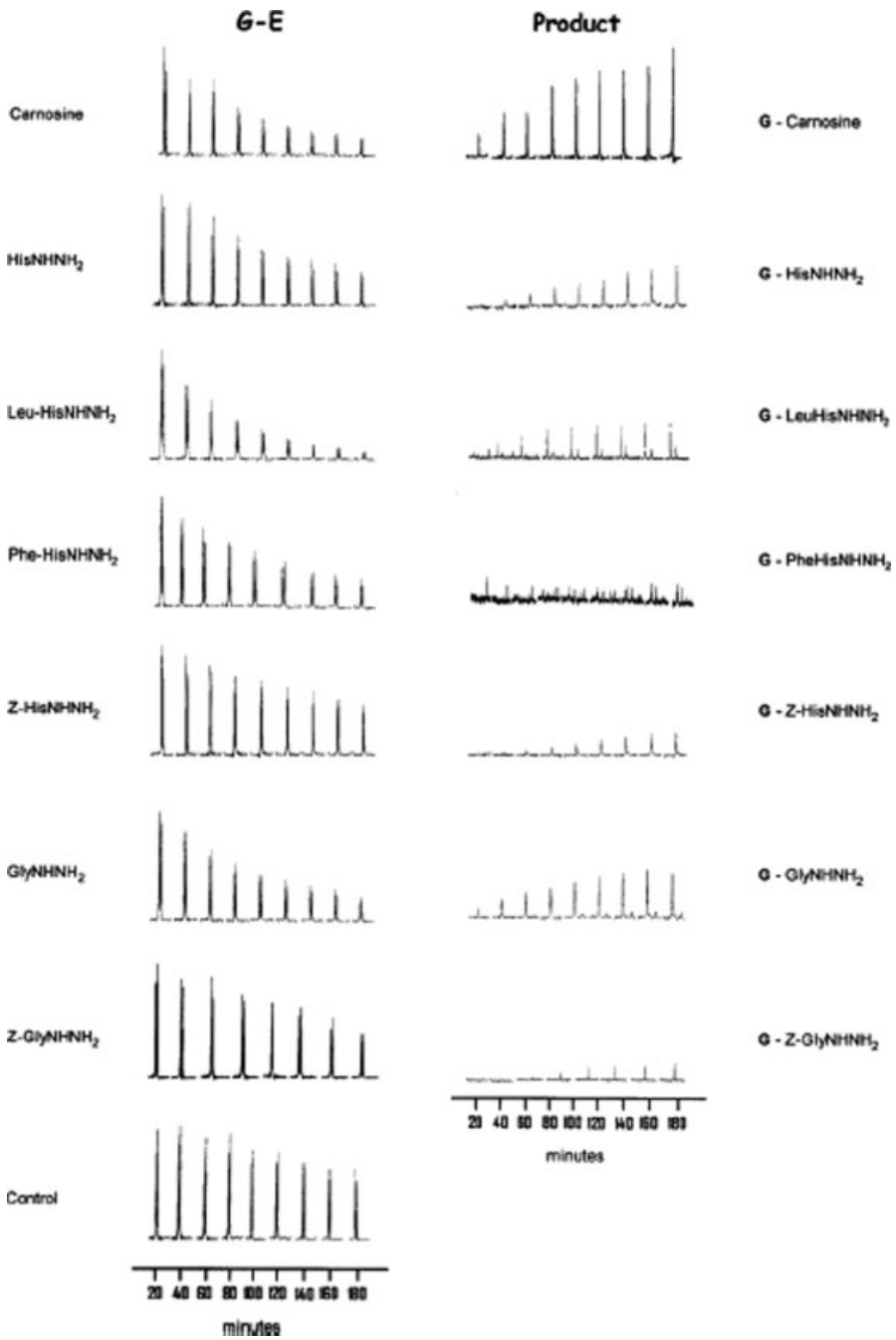


Figure 6A. Kinetics of transglycation of G–E by carnosine and related compounds (left column: serial spectra of G–E as a function of time; right column: serial spectra of the respective transglycation products).

a driving experience.[42] Disability glare (glare radius measured in millimeters) was correlated for statistical significance with VA at red and green targets at baseline and a 9-month examination interval in the total samples of older subjects with cataract and non-cataract older adult subjects (Table 5).

Older subjects enrolled in the study were divided into two groups: treated with NAC and control group (Tables 6 and 7). Table 6 lists the analogous and adjusted analyses for the worse eye, which generated results in the eyes with cataracts upon treatment with NAC prodrug ophthalmic formulation are qualitatively similar to those for the better eye. None of the baseline differences between the different groups were significant. The two groups were similar in smoking history, sunlight exposure, and alcohol use. There were no substantial differences in the use of sunglasses, where the patients lived, or occupational hazard exposure between the two groups.

Ophthalmic examinations indicated that the methodological variances of measurements were approximately equal. Correlations of glare sensitivity at red versus green targets were significant (Table 5). Intra-operator correlation coefficients obtained as repeated measurements for each combination of operator, eye (right or left), and glare radius (at red and green targets) were statistically significant and presented earlier.[26–31] Overall, the reproducibility for the one operator was good. Tables 4–8 sum-

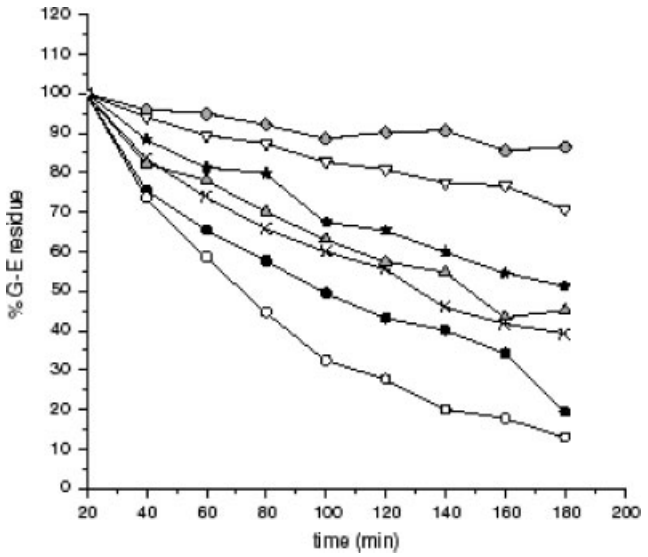


Figure 6B. Transglycation efficiency of tested compounds: carnosine **1** (●); His-$NHNH_2$ **2** (*); Z-His-$NHNH_2$ **3** (◉); Phe-His-$NHNH_2$ **4** (△); Leu-His-$NHNH_2$ **5** (○); Z-Gly-$NHNH_2$ **6** (▽); Gly-$NHNH_2$ **7** (×). The area of the G–E doublet at 90 ppm was plotted against time and corrected for the G–E Schiff base decay measured in the control experiment.

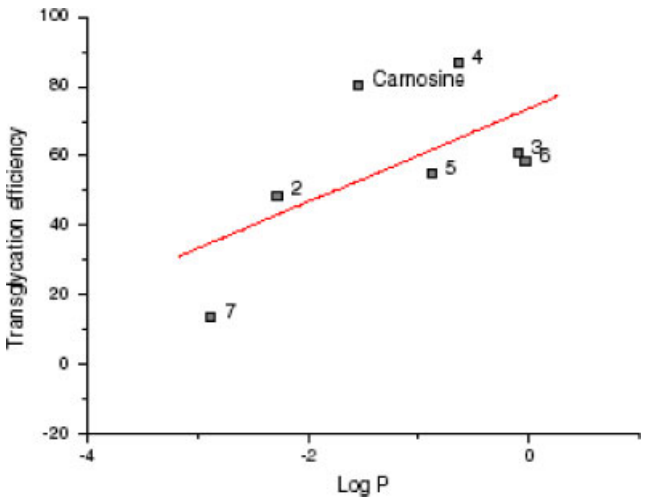


Figure 6C. logP/Transglycation efficiency (% of G–E removal after 180 min incubation) correlation for carnosine and compounds 2–7. $R^2 = 0.38$.

marize the effects of study treatment on VA and glare sensitivity over nine months in older subjects with cataracts and without. In the control placebo-treated group, comparison with baseline values showed some variability of data in a gradual worsening of glare sensitivity at red and green targets and minimal VA changes over nine months (Table 7). Glare sensitivity mostly indicated changes in lens clarity and confirmed the tiny changes in the optical media of the eye at the short-term follow-up examinations when slit lamp associated image analysis data and densitometric readings did not differ significantly with baseline.[26–31]

In the NAC-treated group, the nine-month follow-up generally showed an improvement in VA (according to the distribution score of distance acuities in worse and better eyes) and a significant improvement in glare sensitivity at red and green targets was documented in worse and better eyes using a critical cut point

Table 4. Distribution of visual acuity and disability glare in the cataract and No-cataract groups of the adult subjects enrolled in the study at baseline examination

	Adult subjects			
	Cataract group		Non-cataract group	
	n	%	n	%
Total	75		72	
Worse eye				
Visual acuity				
20/25 or better	9	12	50	70
20/25 to 20/30	14	19	13	18
20/35 to 20/50	48	64	6	8
Worse than 20/50	4	5	3	4
Disability glare readings (glare radius)				
At red target				
<12 mm	10	14	27	38
>=12 mm	65	86	45	62
At green target				
<12 mm	7	9	20	28
>=12 mm	68	91	52	72
Better eye				
Visual acuity				
20/25 or better	12	16	53	74
20/25 to 20/30	30	40	9	12
20/35 to 20/50	25	34	7	10
Worse than 20/50	8	10	3	4
Disability glare readings (glare radius)				
At red target				
<12 mm	29	38	40	55
>=12 mm	46	62	32	45
At green target				
<12 mm	18	24	24	34
>=12 mm	57	76	48	66

Normal measures of glare sensitivity of young subjects (20–30 years) with best correction without cataracts are 3 ± 2 mm (mean SD) of at least four measurements at red and green targets in the daytime[30].

halometer score (Tables 6 and 8). VA was mostly improved in older subjects with cataracts in a worse and better eye and an improvement in glare sensitivity was found both in older subjects with cataracts and without in worse and better eyes after nine months of treatment with NAC. The exampled images of cataract reversal in older subjects are presented on the slit lamp images just to note (Figures 7A–7C).

The NAC-treated eyes had statistically significant difference in VA, glare sensitivity compared with the control group ($p < 0.001$) at the nine-month timepoint of treatment, as supported by the overall *t*-test results of the ratio of the follow-up data to the baseline values (Table 8). The previously published data illustrate examinations over 24 months of the eyes treated with NAC to show that the effect of treatment is sustainable over more prolonged periods.[27–31] In the eyes of older subjects with different localization and grade of cataract and in non-cataract older adult subjects, short-term and prolonged treatment with NAC did not seem to result in a worsening of the visual outcome in this study and elsewhere.[27–31] Topical short- or long-term administration of 1% NAC to the eye was very well tolerated, with no ocular or systemic

Table 5. Linear correlation coefficients (R) between the characteristics of older subjects with cataracts and non-cataract older adult subjects measured by visual acuities (VA) and glare radius (GR at red and green targets) at baseline and at nine-month follow-up ophthalmic examinations

	Baseline study			Nine months		
Parameter	VA	GR red target	GR green target	VA	GR red target	GR green target
Older subjects with cataract[+]						
VA	X	−0.63*	−0.65*	X	−0.47	−0.45
GRred target		X	+0.83*		X	+0.94*
GRgreen target			X			X
Older adult no-cataract subjects[++]						
VA	X	−0.61*	−0.66*	X	−0.43	−0.46
GRred target		X	+0.81*		X	+0.91*
GRgreen target			X			X

[+] Number of eyes examined 75; * $p < 0.01$.
[++] Number of eyes examined 72.

adverse effects, no hyperaemia of conjunctival vessels, and no signs of allergy or other toxic manifestations being reported. The presence of the glycerin ingredient in the Can-C ™ formulation of eye drops moisturizes the entire ocular surface with a lubricating effect. In general, glycerin reduces edema and clears corneal haze by attracting water through semi-permeable corneal epithelium. Patients experienced an improvement in distressing symptoms of pain and haziness, besides recovery to a variable degree in VA. No clinically significant changes from baseline, and no statistically significant differences between the treatment and control groups, were observed regarding ocular comfort and ocular signs and symptoms (lack of burning and stinging, blurred vision, ocular dryness, superficial punctate keratitis, foreign body sensation, itching, ocular discharge, ocular pain, tearing, ocular inflammation, photophobia). All patients completed the study without any problems related to their allocated treatment.

According to the records of repurchase behaviour, the unique and patented N-acetylcarnosine lubricant all-in-one eye drops formula Can-C ™ can also provide beneficial results with the following eye-disorders:

- Presbyopia.[19,38]
- Open-angle primary glaucoma (in combination with beta-blockers).
- Corneal disorders.[19,38,40]
- Computer vision syndrome.
- Eyestrain.
- Ocular inflammation.
- Blurred vision.
- Dry eye syndrome.
- Retinal diseases.
- Vitreous opacities and lesions.
- Complications of diabetes mellitus and other systemic diseases.
- Contact lens difficulties, particularly with soft contact lenses. (Not only do the lubricants in the Can-C™ N-acetylcarnosine eye-drop help to make wearing contact lenses more comfortable, but n-acetylcarnosine is also believed to reduce the build-up of lactic acid in the eye, thus enabling the lens to be left safely in the eye for longer.)

N-acetylcarnosine lubricant eye drops have been successfully used for medically oriented home healthcare usually helping seniors recover or exercise with aid in recovery from visual impairment or illness including cataracts. It is important to note that most work for home health agencies, hospitals, or public health departments is licensed by the state.

Discussion

Prodrugs are pharmacologically inactive molecules that require an enzymatic and/or chemical transformation before release of a pharmacologically active parent drug *in vivo*. In our clinical and development case, N-acetylcarnosine prodrug may offer a way to overcome poor drug-like properties of a very potent but susceptible to enzymatic hydrolysis carnosine lead and provide the opportunity to convert a non-developable natural dipeptide molecule into a worldwide patented potent candidate for clinical use. In the present study, several different carnosine analog structures were synthesized to achieve the bioactivation of N-acetylcarnosine prodrug for improved intraocular drug delivery of L-carnosine and a strong deglycation therapeutic system, including **leucyl-histidylhydrazide** or other histidyl-hydrazide derivatives resistant to enzymatic hydrolysis by carnosinase and acting as competitive inhibitors of this enzyme (non-hydrolyzable substrate analogs). These screens could shorten the time for a drug candidate to reach the market by combining the discovery and development sections.

The present studies cumulate an extension of the report on the 5-year experience with the new vision-saving drug N-acetylcarnosine lubricant eye drops and further the additional prospective data analyses including visual monitoring, glare testing, and the application of N-acetylcarnosine eye drops in the randomized double-masked, placebo-controlled crossover study. To determine whether glare (Halos) was significantly responsible for the change in visual functions in older subjects with cataract, we proposed a new halometer diagnostic process, which is a form of disability glare test. We evaluated NAC 1% eye drops in the short-term nine-month therapy of cataracts and for improvement of visual functions in older subjects with no cataracts. The NAC 1% eyedrops mostly improved the vision of the older adult subjects regardless of whether they had cataracts or not, but the improvement of visual acuity was significantly better in the group of cataract subjects versus older adult subjects in the non-cataract matched older adult group.

This represents additional mode of evidence suggesting that carnosine applied in the form of NAC prodrug reverses lens opacities in humans.[19,27–31,36–39]

Table 6. Visual function in the better and worse eyes after nine months of treatment with N-acetylcarnosine 1% eye drops (Can-C™) versus baseline examination

	Adult subjects			
	Cataract group		Non-cataract group	
	n	%	n	%
Total	39		37	
Baseline examination				
Worse eye				
Visual acuity				
20/25 or better	5	13	26	70
20/25–20/30	8	21	5	14
20/35–20/50	22	56	3	8
Worse than 20/50	4	10	3	8
Disability glare readings (glare radius)				
At red target				
<12 mm	6	15	15	41
>=12 mm	33	85	22	59
At green target				
<12 mm	4	10	11	30
>=12 mm	35	90	26	70
Better eye				
Visual acuity				
20/25 or better	5	13	25	68
20/25–20/30	12	31	8	22
20/35–20/50	17	43	2	5
Worse than 20/50	5	13	2	5
Disability glare readings (glare radius)				
At red target				
<12 mm	10	26	21	57
>=12 mm	29	74	16	43
At green target				
<12 mm	8	21	12	32
>=12 mm	31	79	25	68
After nine months of treatment with N-acetylcarnosine 1% eye drops				
Worse eye				
Visual acuity				
20/25 or better	9	23	27	73
20/25–20/30	16	41	7	19
20/35–20/50	13	33	2	5
Worse than 20/50	1	3	1	3
Disability glare readings (glare radius)				
At red target				
<12 mm	12	30	25	67
>=12 mm	27	70	12	33
At green target				
<12 mm	10	25	21	56
>=12 mm	29	75	16	44
Better eye				
Visual acuity				
20/25 or better	15	38	30	80
20/25–20/30	18	47	5	14
20/35–20/50	4	10	1	3
Worse than 20/50	2	5	1	3
Disability glare readings (glare radius)				
At red target				
<12 mm	18	45	30	81
>=12 mm	21	55	7	19
At green target				
<12 mm	19	46	21	57
>=12 mm	21	54	16	43

Table 7. Visual function in the better and worse eyes after nine months of treatment with placebo (control group) versus baseline examination

	Adult subjects			
	Cataract group		No cataract group	
	n	%	n	%
Total	36		35	
Baseline examination				
Worse eye				
Visual acuity				
20/25 or better	3	8	21	60
20/25–20/30	7	19	7	20
20/35–20/50	23	64	5	14
Worse than 20/50	3	8	2	6
Disability glare readings (glare radius)				
At red target				
<12 mm	9	25	17	49
>=12 mm	27	75	18	51
At green target				
<12 mm	6	17	11	31
>=12 mm	30	83	24	69
Better eye				
Visual acuity				
20/25 or better	11	31	25	72
20/25–20/30	17	47	5	14
20/35–20/50	6	17	5	14
Worse than 20/50	2	5	0	0
Disability glare readings (glare radius)				
At red target				
<12 mm	19	53	23	66
>=12 mm	17	47	12	34
At green target				
<12 mm	13	36	16	46
>=12 mm	23	64	19	54
After nine months of treatment with placebo				
Worse eye				
Visual acuity				
20/25 or better	2	6	19	54
20/25–20/30	6	17	9	26
20/35–20/50	25	69	5	14
Worse than 20/50	3	8	2	6
Disability glare readings (glare radius)				
At red target				
<12 mm	8	22	16	46

Table 7. (*Continued*)

	Adult subjects			
	Cataract group		No cataract group	
	n	%	n	%
>=12 mm	28	78	19	54
At green target				
<12 mm	5	14	8	23
>=12 mm	31	86	27	77
Better eye				
Visual acuity				
20/25 or better	9	25	25	71
20/25–20/30	17	47	5	14
20/35–20/50	8	22	5	14
Worse than 20/50	2	6	0	0
Disability glare readings (glare radius)				
At red target				
<12 mm	16	44	21	60
>=12 mm	20	56	14	40
At green target				
<12 mm	10	28	14	40
>=12 mm	26	72	21	79

The present study has addressed ocular nutritional activity of natural non-racemized (L) form of synthetic N-acetylcarnosine and its utility in metabolism-focused, ophthalmic-specific drug design.[19,27–30,36–39] When cataracts were accompanied with primary open-angle glaucoma (POAG), NAC was prescribed 15 min prior the topical application of beta-blocker, specifically used to decrease the intraocular pressure.[19,38] The improvement of visual functions in patients with cataracts associated with POAG was accompanied with significant decrease of intraocular pressure and increase in the outflow facility in the eyes of patients with POAG treated with the indicated combined therapy.[19,38]

An oxidative stress induced by accumulation of LPO products in the lens membranes during cataract progression is considered to be a primary cause of reduced glutathione (GSH) deficiency and disturbance of the redox balance in the lens. It seems essential to improve the capacity of the lens to withstand oxidative stress

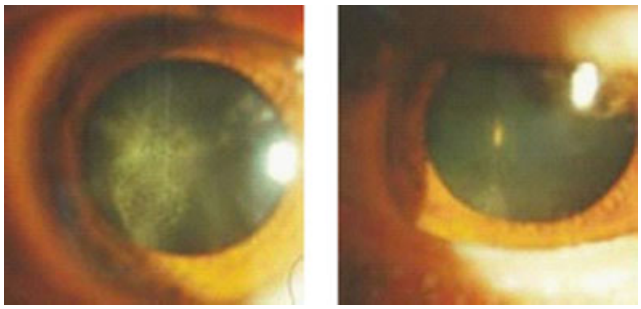


Figure 7A. The pictures show the treatment of human cataract in the older subject with the eye drops of 1% Can-C ™ for the period of five months. The left image shows the appearance of cataract which resembles a bat in its form and the right image shows that this opacity has disappeared after the cited period after treatment with N-acetylcarnosine is completed. The lens has become clearer.

induced by lipid peroxides since the opacified lenses have reduced activities of lipid peroxidases that would use glutathione as a substrate.[22,41,43] In our study carnosine has been proposed to act as universal antioxidant both in lipid membrane and aqueous environments and its activity in the crystalline lens can be related to the prevention of the free-radical-induced inactivation of activity of the proprietary antioxidant enzymes, such as superoxide dismutase.[44,45] Carnosine exhibits an ability to inhibit LPO catalysts besides inhibiting free metals, scavenging OH· and lipid peroxyl (RO2·) radicals or donating hydrogen ions. In addition to inhibiting the generation of lipid peroxyl radicals, carnosine catabolyzes lipid hydroperoxides to their alcohols both in aqueous medium and in a phospholipid system.[11] A possibility exists from our study that carnosine is reacting directly with MDA and other aldehydes/ketones. Indeed, carnosine has been shown to protect against MDA-induced crosslinking and toxicity, and a hydroxynonenal-carnosine adduct has recently been characterized, providing further evidence for carnosine's potential as an aldehyde scavenger.[18,46–50] The ability of L-carnosine to inhibit LPO reactions as well as to diminish the content of LPO-derived products (including aldehydes) makes N-acetylcarnosine applied with lubricant carboxymethylcellulose a prominent tool in the ocular environmental therapy especially, of the posterior subcapsular and cortical cataracts, whose mechanism can be related with the toxic effects of LPO products.[16] The production

Table 8. Mean ± SD of changes (improvement) in visual functions

Treatment group	Visual acuity	Glare radius
9-month follow-up of older subjects with cataract		
Control group	0.90 ± 0.03 ($n = 36$)	1.53 ± 0.07 ($n = 36$)
NAC-treated group	$1.54 \pm 0.05^{*+}$ ($n = 39$)	$0.41 \pm 0.05^{*}$ ($n = 39$)
9-month follow-up of older adult no-cataract subjects		
Control group	0.96 ± 0.03 ($n = 35$)	1.27 ± 0.05 ($n = 35$)
NAC-treated group	$1.20 \pm 0.04^{*}$ ($n = 37$)	$0.38 \pm 0.05^{*}$ ($n = 37$)

The measure of visual acuity readings after nine months of treatment was divided by the clinical baseline measure of visual acuity for each eye individually to get ratios, and then the average of those ratios through each clinical group of eyes was calculated. Similarly with glare, the calculating of the ratio of glare sensitivity at red and green target after nine months of treatment to the baseline reading of glare sensitivity for each eye was undertaken, and then the ratios were averaged through the whole groups of eyes.
NAC, N-acetylcarnosine (Can-C™).
* $p < 0.001$ compared to control group who received placebo eye drops.
$^{+}$ $p < 0.001$, where an improvement of visual acuity is statistically significantly better in the group of older subjects with cataract than an improvement of visual acuity in the group of older adult non-cataract subjects.

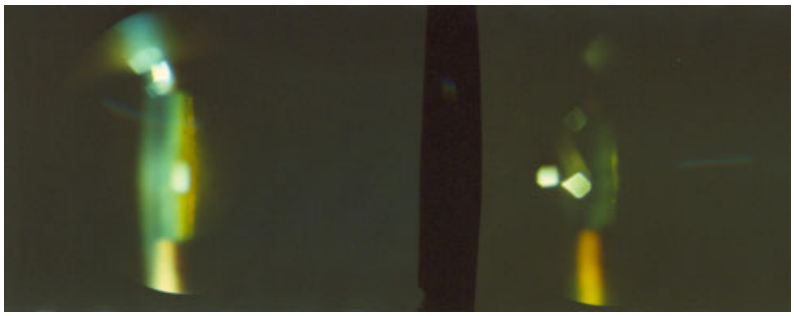


Figure 7B. Treatment of posterior subcapsular cataract in the older subject for the period of nine months with Can-C ™. Left image before treatment; right image after nine months of treatment. The opalescence lens opacities areas are reversed and the lens becomes clearer (look like dark greenish zones) at the right image.

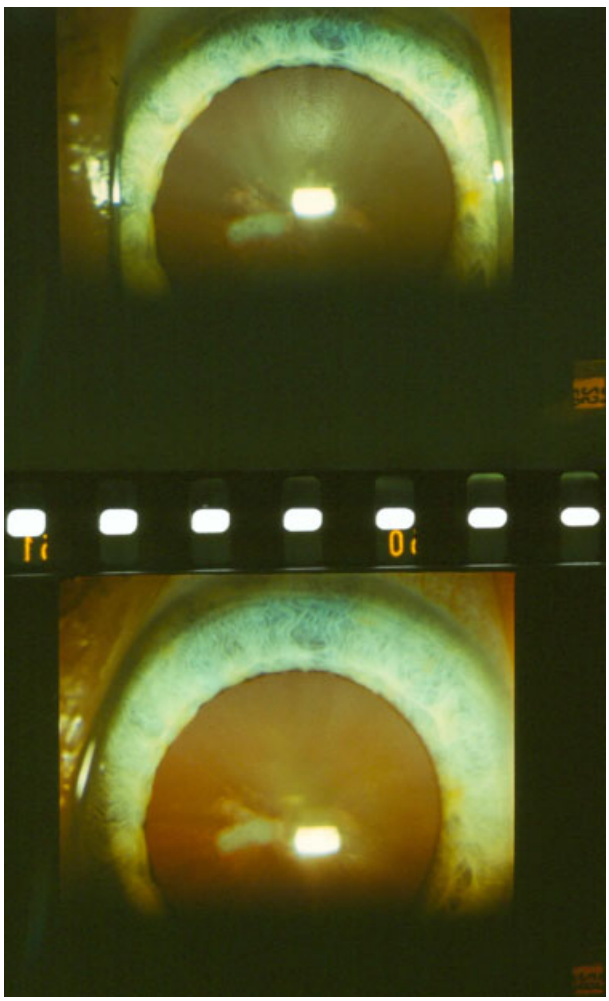


Figure 7C. Treatment of cortical cataract in the upper segment of the pupil image of the older woman for the period of nine months. Upper image: the lens before treatment. Lower image: after nine months of treatment with the Can-C ™. The appearance of rose reflex at the lower image demonstrates that the lens becomes clearer.

of high molecular weight protein complexes by disulfide bridges and covalent links with dialdehydes has been implicated in the formation of senile and other cataracts in humans. Both types of cross-linking may be caused by depletion of the lens's reduced glutathione and accumulation of LPO products in the lens tissue. The results of our studies suggest that L-carnosine is able to prevent the loss of reduced glutathione and to remove the secondary LPO products in biological systems. This, in turn, may lead to dissociation of the intermolecular protein cross-links due to glutathione-protein thiol-disulfide exchange mechanism and utilization of lipid peroxides and dialdehydes derived from LPO process, anchoring protein-lipid complexes in the lens.[22]

There is growing evidence that carnosine prevents oxidation and glycation, both of which contribute to the crosslinking of proteins.[51–56] Protein glycation, which promotes aggregation, involves the unwanted reaction of carbohydrate oxidation products with proteins.[57] Glycation of lens alpha-crystallin occurs *in vivo* and may contribute to cataractogenesis. Antiglycation compounds, such as carnosine may be preventive, but interestingly carnosine reverses lens opacity in human trials. The mechanism for this observation may involve carnosine's ability to disaggregate glycated protein. Seidler *et al*.[58] recently investigated this hypothesis using glycated alpha-crystallin as the *in vitro* model. The data support the hypothesis that carnosine disaggregates glycated alpha-crystallin. In the studies of Price *et al*.,[59] L-carnosine exhibited the antiglycating activity with the estimated IC_{50} 4 μM for inhibition of copper catalyzed oxidation of ascorbic acid that proposes this natural dipeptide as a potent inhibitor of glycation reactions in the lens proteins mediated by metal-catalyzed oxidation of ascorbate present in the aqueous humor. Actually, carnosine may prevent accumulation of Amadori products (forming after re-arrangements of products of primary glycation) within lens cells and tissues as well as cross-linking of biomolecules.

AGEs are a class of complex, often unstable, reactive compounds formed in excess during aging and diabetes mellitus. According to the 'glycation hypothesis', accumulation of AGEs alters the structural properties of tissue proteins and reduces their susceptibility to catabolism. Diabetic vascular complication is a leading cause of acquired blindness, end-stage renal failure, a variety of neuropathies and accelerated atherosclerosis, which could account for disabilities and high mortality rates in patients with diabetes. Chronic hyperglycemia is essentially involved in the development and progression of diabetic micro- and macroangiopathy. In this section, we discuss the molecular mechanisms of diabetic vascular complication, specially focusing on AGEs and their receptor system. Retinopathy may be associated with an upregulation of the receptor for AGEs (RAGE) in a proinflammatory axis, concomitant with increases in AGEs.[60] Several types of drugs including patented transglycation AGE inhibitors, such as prodrug and codrug formulations of carnosine and histidine-hydrazide analogs, and their therapeutic implications in the devastating diabetes complications is a

promising strategy for the prolonged relief and survival of patients. The role of histidine in transglycation by carnosine and leucyl-histidylhydrazide is important, and may determine the rate of intermolecular transfer of glycosyl residue on/from glucosyl-carnosine or glucosyl-histidylhydrazide compounds. It is possible that such adducts could either be transported out of cells or metabolized further, providing thereby an irreversible set of reactions for removal of aldosamines from proteins and phospholipids such as phosphatidylethanolamine and phosphatidylserine.

Both carnosine and the carnosine derivative N-acetyl carnosine have been shown to be effective in inhibiting the UV-induced aggregation of beta L-crystallin at 20 mM concentration. The molecular chaperon-like properties similar to those of alpha-crystallin underlie the anticataract mechanism of action of carnosine and of the acetyl derivative of carnosine (prodrug).[39,61]

Carnosine, when present at surprisingly high levels (about 20 mM or over), can delay senescence of cells and reverse the senescent phenotype, restoring a more juvenile appearance. As better antioxidants/free-radical scavengers than carnosine do not demonstrate these antisenescent effects,[62] additional properties of carnosine must contribute to its antisenescent activity.

The concentration of carnosine in transparent crystalline lenses detected was about 25 μM. At different stages of cataract development, the level of carnosine fell, reaching about 5 μM.[6] The L-carnosine liberated in aqueous humor can provide antioxidant protection around the lens, and penetrate and accumulate in the lens tissue.[21,63] Recently, the pharmacokinetic properties of the extra-doses of L-carnosine, applied topically to the rabbit eyes were tested.[64] Their goal was to evaluate the ocular pharmacokinetics of carnosine 5% eye drops following topical application. Carnosine 5% eye drops were topically applied repeatedly (50 muL × 4) at an interval of 5 min. Aqueous humor and lens were collected after 5, 15, 30, 45, 60, 90, 120, 150, and 180 min. Carnosine concentration was determined by high performance liquid chromatography-tandem quadrupole mass spectrometer (HPLC-MS/MS). Carnosine concentration in treated eyes was significantly higher than control eyes. Peak concentration (C (max)) of carnosine in treated aqueous humor occurred 60 min following topical administration, with the administrated concentration (total-endogenous concentration) of 40.9 + / − 18.9 mug/ml. The area under the concentration-time curve between 0 and 180 min (AUC (0–180)) was 3276.8 (mug/ml) × min. Carnosine concentration in treated lens rose rapidly to an effective level and changed slightly with time after topical administration. The administrated concentration of carnosine in lens at the last time point (180 min, 1.92 + / − 1.65 mug/ml) was not significantly different with the highest value (15 min, 2.11 + / − 1.83 mug/ml). The reason for these observations of L-carnosine presence in the aqueous humor (which should be considered as a rather artificial ophthalmic experience), was that the repetitive (multiple) topical administration of the extra doses of 5% carnosine to the eye inhibits (as an excessive substrate compound) the tissue carnosinase enzymatic activity present in the corneal and conjunctival tissues. The multiple topically applied instillations of carnosine to the eye (50 mul × 4) are not practical, what is more, this mode of instillations of 5% carnosine to the eye can be allergic during long-term administration, because the inflammatory mediator histamine can be released at the ocular surface during the breakdown of carnosine repeatedly instilled into the conjuntival sac of an eye in the increased doses.

Another publication of the Australian scientists demonstrates further misunderstanding of N-acetylcarnosine ophthalmology concept.[65] The authors applied radical probe-mass spectrometry (RP-MS) based on protein footprinting studies to investigate the effectiveness of the antioxidant N-acetylcarnosine in preventing oxidative damage to lens crystallins present in the eye of mammals. No evidence was found to suggest that the antioxidant had any significant direct effect on reducing the levels of oxidation within the most abundant lens crystallins, α and β-crystallin, at the molecular level at increasing concentrations of N-acetylcarnosine. The results of this laboratory study suggest that the therapeutic benefit demonstrated in clinical trials is associated with the nature or formulation of the topical solution and/or that the mode of action of NAC as an antioxidant is not a direct one. Our study indicates that N-acetylcarnosine *in vitro* behaves as a weak antioxidant. The therapeutic benefit demonstrated in clinical trials is associated with the **bioactivation** universal antioxidant and transglycation properties of N-acetylcarnosine acting as the ophthalmic prodrug of L-carnosine in a specific drug-delivery lubricant eye-drop formulation enhancing the intraocular uptake of L-carnosine during the topical administration of an ophthalmic solution.

In the previous studies, the author has determined through histologic measurements that carcinine, a decarboxylated carnosine natural derivative is also effective in reducing retinal light damage in mice.[19,38,66–68] The further clinical analysis of the protective effects of lubricant carcinine eye drops against retinal degeneration revealed that the observed carcinine protection of photoreceptor cells from oxidative stress is morphometric and functional. This is supported by the fact that carcinine is only slightly affected by the enzymatic hydrolysis with natural peptidases.[19,20,38,66–68] The lack of the acute toxicity of carcinine introduces the experimental proof for this regular ocular topical therapeutic application modality. The therapeutic administration of carcinine with lubricant eye drops and its bioavailability in patented by IVP eye-drop lubricant formulations thereof have been supported in the matched studies.[19,38,66–68]

Conclusion

Currently there is considerable interest in N-acetylcarnosine based on strategies to improve ophthalmic drug delivery through metabolic activation. The prevalent causes of visual impairment that involve the pathways of continuous oxidative damage to ocular tissues are cataracts, glaucoma, and age-related macular degeneration, which are responsible for 69% of blindness globally. The majority of studies report that sight-threatening diabetic retinopathy and consequent visual impairment are prevalent and are therefore an important public health problem.

In this paper, the advantages of N-acetylcarnosine prodrug and molecular mechanisms of its implication in prevention and treatment of serious eye diseases and their complications are discussed. The research highlights the latest findings in N-acetylcarnosine prodrug activation, transport mechanisms, transglycation with carnosine, histidyl-hidrazide analogs, drug-to-drug interactions to study drug formulations in order to unlock some of complicated ophthalmic pharmacology aspects of N-acetylcarnosine and to provide ideas for relevant future studies into optimization of this promising agent.

Acknowledgements

This work was planned, organized, and supported by Innovative Vision Products, Inc. (County of New Castle, DE, USA). Innovative Vision Products, Inc., holds the worldwide patent (including

PCT International Publication No. WO 2004/028536 A1) for the application of N-acetylcarnosine for the treatment of ophthalmic disorders, including cataracts as well as (PCT International Publication No. WO 2004/064866 PCT/JP2004/000351) protecting the therapeutic applications and formulations of carnosine and amino acid derivatives stabilizing carnosine from enzymatic hydrolysis by carnosinase (inhibited by a nonhydrolyzable substrate analog). Innovative Vision Products Inc., is a pharmaceutical and nanotechnology development company with a focus on innovative chemical entities, drug delivery systems, and unique medical devices to target specific biomedical applications. Over the last decade it has developed a track record in developing these technologies to effectively address the unmet needs of specific diseased populations.

Conflict of interest

Declaration of interest: The author reports an interest in the intellectual property of the described modalities protected with the patents. The author bears primary responsibility for accuracy of statements made and employment of the described products and for the content and writing of the paper.

References

[1] W. Gulewitsch, S. Amiradzibi. Uber das Carnosin eine neue organische base des fleischextraktes. *Berichte der Deutschen Chemischen Gesellschaft* **1900**, *33*, 1902.
[2] K. G. Crush. Carnosine and related substances in animal tissues. *Comp. Biochem. Phys.* **1970**, *34*, 3.
[3] S. M. Green, F. L. Margolis, M. Grillo, H. Fisher. Enhanced carnosine (β-alanyl-L-histidine) breakdown and histamine metabolism following treatment with compound 48/80. *Eur. J. Pharmacol.* **1984**, *99*, 79.
[4] A. A. Boldyrev, S. E. Severin. The histidine-containing dipeptide carnosine and anserine: distribution properties and biological significance. *Adv. Enzyme Regul.* **1990**, *30*, 175.
[5] F. Margolis. Carnosine in the primary olfactory pathway. *Science* **1974**, *184*, 909.
[6] A. A. Boldyrev, A. M. Dupin, A. Ya. Bunin, M. A. Babizhayev, S. E. Severin. The antioxidative properties of carnosine, a natural histidine containing dipeptide. *Biochem. Int.* **1987**, *15*, 1105.
[7] J. L. Jay, D. J. Miller, J. D. Morrison, J. J. O'Dowd. Histidyl derivatives in rabbit lens and their diminution in human cataract. Meeting Abstracts. *The Journal of Physiology London* **1990**, *420*, 155.
[8] J. J. O'Dowd, D. J. Robins, D. J. Miller. Detection, characterization and quantification of carnosine and other histidyl derivatives in cardiac and skeletal muscle. *Biochim. Biophys. Acta* **1988**, *967*, 241.
[9] P. R. Carnegie, M. Z. Ilic, M. O. Etheridge, M. G. Collins. Improved high-performance liquid chromatographic method for analysis of histidine dipeptides anserine, carnosine and balenine present in fresh meat. *J. Chromatogr.* **1983**, *261*, 153.
[10] L. Flancbaum, D. N. Brotman, J. C. Fitzpatrick, T. Van Es, E. Kasziba, H. Fisher. Existence of carcinine, a histamine-related compound, in mammalian tissues. *Life Sci.* **1990**, *47*, 1587.
[11] M. A. Babizhayev, M.-C. Seguin, J. Gueyne, R. P. Evstigneeva, E. A. Ageyeva, G. A. Zheltukhina. L-Carnosine (β-alanyl-L-histidine) and carcinine (β -alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid peroxidase activities. *Biochem. J.* **1994**, *304*, 509.
[12] D. Yorston. Cataract complications. *Community Eye Health Journal* **2008**, *21*, 1.
[13] C. Kupfer, B. Underwood, T. Gillen, Leading causes of visual impairment worldwide. In *Principles and Practice of Ophthalmology. Basic Sciences*, (Eds: D. M. Albert, F. A. Jakobiec), W. B. Saunders & Co: Philadelphia, **1994**, pp. 1249–1255.
[14] M. A. Babizhayev, A. I. Deyev, L. F. Linberg. Lipid peroxidation as a possible cause of cataract. *Mech. Ageing Dev.* **1988**, *44*(1), 69.
[15] M. A. Babizhayev, A. I. Deyev, V. N. Yermakova, I. V. Brikman, J. Bours. Lipid peroxidation and cataracts: N-acetylcarnosine as a therapeutic tool to manage age-related cataracts in human and in canine eyes. *Drug Res. Dev.* **2004**, *5*(3), 125.
[16] M. A. Babizhayev, A. I. Deyev. Lens opacity induced by lipid peroxidation products as a model of cataract associated with retinal disease. *Biochim. Biophys. Acta* **1989**, *1004*(1), 124.
[17] M. A. Babizhayev, A. I. Deyev. Free radical oxidation of lipids and thiol groups in the formation of a cataract. *Biofizika* **1986**, *31*(1), 109.
[18] A. Guiotto, P. Ruzza, M. A. Babizhayev, A. Calderan. Malondialdehyde scavenging and aldose-derived Schiff bases' transglycation properties of synthetic histidyl-hydrazide carnosine analogs. *Bioorg. Med. Chem.* **2007**, *15*(18), 6158.
[19] M. A. Babizhayev. *Method for Topical Treatment of Eye Disease and Composition and Device for said Treatment*. Patent No. WO 2004/028536 A1. **2004**.
[20] M. A. Babizhayev, K. Meguro. *Combined use of carnosinase Inhibitor with L-Carnosines and Composition.* Patent No. WO 2004/064866 PCT/JP2004/000351. **2004**.
[21] M. A. Babizhayev, V. N. Yermakova, N. L. Sakina, R. P. Rozhkova, G. A. Zheltukhina. *N*-acetylcarnosine is a prodrug of L-carnosine in ophthalmic application as antioxidant. *Clin. Chim. Acta* **1996**, *254*, 1.
[22] M. A. Babizhayev. Antioxidant activity of L-carnosine, a natural histidine-containing dipeptide in crystalline lens. *Biochim. Biophys. Acta* **1989**, *1004*, 363.
[23] M. A. Babizhayev, E. Bozzo Costa. Lipid peroxide and reactive oxygen species generating systems of the crystalline lens. *Biochim. Biophys. Acta* **1994**, *1225*, 326.
[24] M. A. Babizhayev, Y. E. Yegorov. Advanced drug delivery of N-acetylcarnosine (N-acetyl-beta-alanyl-L-histidine), carcinine (beta-alanylhistamine) and L-carnosine (beta-alanyl-L-histidine) in targeting peptide compounds as pharmacological chaperones for use in tissue engineering, human disease management and therapy: from in vitro to the clinic. *Recent Pat. Drug. Deliv. Formul.* **2010**, *4*(3), 198.
[25] B. S. Szwergold. Carnosine and anserine act as effective transglycating agents in decomposition of aldose-derived Schiff bases. *Biochem. Biophys. Res. Commun.* **2005**, *336*(1), 36.
[26] M. A. Babizhayev, A. I. Deyev, V. N. Yermakova, N. G. Davydova, N. I. Krurysheva, V. S. Doroshenko, *et al*. Image analysis and glare sensitivity in human age-related cataracts. *Clin. Exp. Optom.* **2003**, *86*(3), 157.
[27] M. A. Babizhayev, A. I. Deyev, V. N. Yermakova, Y. A. Semiletov, N. G. Davydova, V. S. Doroshenko, *et al*. Efficacy of N-acetylcarnosine in the treatment of cataracts. *Drugs R. D.* **2002**, *3*(2), 87.
[28] M. A. Babizhayev, A. I. Deyev, V. N. Yermakova, Yu A. Semiletov, N. G. Davydova, N. I. Krurysheva, *et al*. N-Acetylcarnosine, a natural histidine-containing dipeptide, as a potent ophthalmic drug in treatment of human cataracts. *Peptides* **2001**, *22*, 979.
[29] M. A. Babizhayev, V. N. Yermakova, A. I. Deyev, M.-C. Seguin. Imidazole-containing peptidomimetic NACA as a potent drug for the medicinal treatment of age-related cataract in humans. *Journal of Anti-Aging Medicine* **2000**, *3*, 43.
[30] M. A. Babizhayev. Rejuvenation of visual functions in older adult drivers and drivers with cataract during a short-term administration of N-acetylcarnosine lubricant eye drops. *Rejuvenation Res.* **2004**, *7*, 186.
[31] M. A. Babizhayev, V. N. Yermakova, Y. A. Semiletov, A. I. Deyev. The natural histidine-containing dipeptide N-acetylcarnosine as an antioxidant for ophthalmic use. *Biochemistry (Moscow)* **2000**, *65*, 588.
[32] F. L. Ferris 3rd, A. Kassoff, G. H. Bresnick, I. Bailey. New visual acuity charts for clinical research. *Am. J. Ophthalmol.* **1982**, *94*, 91.
[33] F. L. Ferris, R. D. Sperduto. Standardized illumination for visual acuity testing in clinical research. *Am. J. Ophthalmol.* **1982**, *94*, 97.
[34] M. A. Babizhayev. Glare disability and driving safety. *Ophthalmic Res.* **2003**, *35*(1), 19.
[35] M. A. Babizhayev, M.-C. Seguin. *Process of Assessment of Ocular Dysfunctions and Implementation Devices of this Process*. US Patent No. 6,007,203. **1999**.

[36] M. A. Babizhayev, E. Bozzo Costa. *Composizioni Farmaceutiche Contenenti N-acetilcarnosina per il Trattamento della Cataratta*. Italian Patent No. A61K gruppo 37/00 20122 MI, **1993**.
[37] M. A. Babizhayev, E. Bozzo Costa. *Pharmaceutical Compositions Containing N-acetylcarnosine for the Treatment of Cataract*. Patent No. PCT/EP 94/03340 SCB 238 PCT, **1994**.
[38] M. A. Babizhayev. *Method for Topical Treatment of Eye Disease and Composition and Device for said Treatment*. US Patent No. US 7,795,203 B2, **2010**, PCT No. PCT/IB03/04259, **2003**.
[39] M. A. Babizhayev, A. I. Deyev, V. N. Yermakova, V. V. Remenschikov, J. Bours. Revival of the lens transparency with N-acetylcarnosine. *Curr. Drug Ther.* **2006**, *1*, 91.
[40] M. A. Babizhayev. Ophthalmic pharmacology of N-acetylcarnosine lubricant eye drops. *J. Pharmacol. Toxicol.* **2006**, *1*(3), 201.
[41] M. A. Babizhayev. Failure to withstand oxidative stress induced by phospholipid hydroperoxides as a possible cause of the lens opacities in systemic diseases and ageing. *Biochim. Biophys. Acta* **1996**, *1315*, 87.
[42] C. Owsley, B. T. Stalvey, J. Wells, M. E. Sloane, G. McGwin Jr. Visual risk factors for crash involvement in older drivers with cataract. *Arch. Ophthalmol.* **2001**, *119*, 881.
[43] N. H. Ansari, L. Wang, S. K. Srivastava. Role of lipid aldehydes in cataractogenesis: 4-hydroxynonenal-induced cataract. *Biochem. Mol. Med.* **1996**, *58*(1), 25.
[44] H. Ukeda, Y. Hasegawa, Y Harada, M. Sawamura. Effect of carnosine and related compounds on the inactivation of human Cu, Zn-superoxide dismutase by modification of fructose and glycolaldehyde. *Biosci. Biotech. Bioch.* **2002**, *66*(1), 36.
[45] J. H. Kang, K. S. Kim, S. Y. Choi, H. Y. Kwon, M. H. Won, T. C. Kang. Protective effects of carnosine, homocarnosine and anserine against peroxyl radical-mediated Cu,Zn-superoxide dismutase modification. *Biochim. Biophys. Acta* **2002**, *1570*, 89.
[46] G. Aldini, M. Carini, G. Beretta, S. Bradamante, R. M. Facino. Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction? *Biochem. Biophys. Res. Commun.* **2002**, *298*(5), 699.
[47] G. Aldini, P. Granata, M. Carini. Detoxification of cytotoxic alpha, beta-unsaturated aldehydes by carnosine: characterization of conjugated adducts by electrospray ionization tandem mass spectrometry and detection by liquid chromatography/mass spectrometry in rat skeletal muscle. *J. Mass Spectrom.* **2002**, *37*(12), 1219.
[48] A. R. Hipkiss, J. E. Preston, D. T. Himswoth, V. C. Worthington, N. J. Abbot. Protective effects of carnosine against malondialdehyde-induced toxicity towards cultured rat brain endothelial cells. *Neurosci. Lett.* **1997**, *238*(3), 135.
[49] A. R. Hipkiss, V. C. Worthington, D. T. Himsworth, W. Herwig. Protective effects of carnosine against protein modification mediated by malondialdehyde and hypochlorite. *Biochim. Biophys. Acta* **1998**, *1380*(1), 46.
[50] S. Zhou, E. A. Decker. Ability of carnosine and other skeletal muscle components to quench unsaturated aldehydic lipid oxidation products. *J. Agric. Food Chem.* **1999**, *47*(1), 51.
[51] L. J. Hobart, I. Seibel, G. S. Yeargans, N. W. Seidler. Anti-crosslinking properties of carnosine: Significance of histidine. *Life Sci* **2004**, *75*, 1379.
[52] C. Brownson, A. R. Hipkiss. Carnosine reacts with a glycated protein. *Free Radical Biol. Med.* **2000**, *28*(10), 1564.
[53] A. R. Hipkiss, H. Chana. Carnosine protects proteins against methylglyoxal-mediated modifications. *Biochem. Biophys. Res. Commun.* **1998**, *248*(1), 28.
[54] A. R. Hipkiss, J. Michaelis, P. Syrris. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. *FEBS Lett.* **1995**, *371*(1), 81.
[55] A. R. Hipkiss, J. Michaelis, P. Syrris, S. Kumar, Y. Lam. Carnosine protects proteins against in vitro glycation and cross-linking. *Biochem. Soc. Trans.* **1994**, *22*(4), 399S.
[56] A. R. Hipkiss. Glycation, ageing and carnosine: are carnivorous diets beneficial? *Mech. Ageing Dev.* **2005**, *126*(10), 1034.
[57] N. H. Ansari, L. Wang, A. A. Erwin, D. F. Church. Glucose-dependent formation of free radical species in lens homogenate. *Biochem. Mol. Med.* **1996**, *59*(1), 68.
[58] N. W. Seidler, G. S. Yeargans, T. G. Morgan. Carnosine disaggregates glycated alpha-crystallin: an *in vitro* study. *Arch. Biochem. Biophys.* **2004**, *427*, 110.
[59] D. I. Price, P. M. Rhett, S. R. Thorpe, J. W. Baynes. Chelating activity of advanced glycation end-product inhibitors. *J. Biol. Chem.* **2001**, *276*, 48967.
[60] S.-I. Yamagishi, K. Nakamura, T. Imaizumi. Advanced glycation end products (AGEs) and diabetic vascular complications. *Current Diabetes Review* **2005**, *1*, 93.
[61] K. O. Muranov, A. K. Dizhevskaia, A. A. Boldyrev, O. E. Karpova, N. L. Sheremet, G. S. Polunin, *et al*. Search for chaperon-like anticataract drugs, the antiaggregants of lens crystallins. Communication. 1. Chaperon-like activity of N-acetyl carnosine dipeptide: in vitro study on a model of ultraviolet-induced aggregation of betaL-crystallin. *Vestn. Oftalmol.* **2008**, *124*(2), 3.
[62] A. R. Hipkiss, C. Brownson, M. F. Bertani, E. Ruiz, A. Ferro. Reaction of carnosine with aged proteins: another protective process? *Ann. N. Y. Acad. Sci.* **2002**, *959*, 285.
[63] M. A. Babizhayev. Ocular drug metabolism of the bioactivating antioxidant N-acetylcarnosine for vision in ophthalmic prodrug and codrug design and delivery. *Drug Dev. Ind. Pharm.* **2008**, *34*(10), 1071.
[64] Z. Tianyang, Z. Ling, W. Liya, Z. Junjie. Ocular pharmacokinetics of carnosine 5% eye drops following topical application in rabbit. *J. Ocul Pharmacol Ther.* **2010**, Jan 19. [Epub ahead of print].
[65] J. W. Ha, A. B. Schwahn, K. M. Downard. Ability of N-acetylcarnosine to protect lens crystallins from oxidation and oxidetive damage by radical probe mass spectrometry (RP-MS). *Rapid. Commun. Mass Spectrom.* **2010**, *24*(19), 2900.
[66] M. A. Babizhayev, M.-C. Seguin. *Pseudodipeptide Product Having an Imidazole Grouping and Applications*. Patent No. WO 95/12581. PCT/FR94/01272. **1994**.
[67] M. A. Babizhayev, M.-C. Seguin. *Coupling Product Obtained from Histamine and an Amino Acid*. Patent No. WO 94/19325. PCT/FR94/00189. **1994**.
[68] M. A. Babizhayev, Y. Courbebaisse, J.-F. Nicolay, Yu A. Semiletov. Design and biological activity of imidazole-containing peptidomimetics with a broad-spectrum antioxidant activity. *Lett. Pept. Sci.* **1998**, *5*, 163.