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3D molecular modeling, free radical modulating and immune cells signaling activities of the novel peptidomimetic L-glutamyl-histamine: possible immunostimulating role

Mark A. Babizhayev[a,b,*], Yuri A. Semiletov[a,c], Yuri A. Lul'kin[a], Natalia L. Sakina[d], Ekaterina L. Savel'yeva[a], Ludmila M. Alimbarova[c], Igor Ph. Barinskii[c]

[a] *Innovative Vision Products Inc., Delaware, USA*
[b] *Moscow Helmholtz Research Institute of Eye Diseases, Moscow, Russian Federation*
[c] *D.I. Ivanovskii Institute of Virology, Moscow, Russian Federation*
[d] *N.M. Emanuel Institute of Biochemical Physics, Moscow, Russian Federation*

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Abstract

An original representative of the patented by author family of histamine-containing peptidomimetics L-glutamyl-histamine (L-Glu-Hist) was synthesized and characterized as a biologically active compound with a role of cytokine mimic leading to cellular responses of improved specificity. The study assesses the ability of L-Glu-Hist to affect molecular modeling, modulate free radical activity and influence immune cell signaling. The energy-minimized 3D conformations of L-Glu-Hist derived from its chemical structure resulted in stabilization for Fe^{2+} chelating complexes. L-Glu-Hist accelerated the decrease of ferrous iron in the ferrous sulfate solution in a concentration-dependent mode and showed the ferroxidase-like activity at concentrations less than 3 mM in the phenanthroline assay, whereas in the concentration range 3–20 mM L-Glu-Hist restricted the availability of Fe^{2+} to phenanthroline due to binding of ferrous ions in chelating complexes. L-Glu-Hist showed stimulatory effect on phosphatidylcholine liposomal peroxidation (LPO) catalyzed by the superoxide anion radical ($O_2^{\bullet -}$)-generating system (Fe^{2+} + ascorbate) at low (less or about 1 mM) L-Glu-Hist concentrations and both revealed the inhibitory effect on LPO in this system of high (∼10 mM) L-Glu-Hist concentration. The stimulation of LPO by L-Glu-Hist was related to the ability of peptidomimetic in small (∼0.05 mM) concentrations to release $O_2^{\bullet -}$ free radicals as determined by the superoxide dismutase-inhibitable cytochrome *c* reduction assay. $O_2^{\bullet -}$ release by L-Glu-Hist might result from its ferroxidase-like activity, while inhibition of LPO by L-Glu-Hist was caused by its chelating activity to Fe^{2+} ions, prevention of free radical generation and lipid hydroperoxide-degrading ability of 5–20 mM L-Glu-Hist. L-Glu-Hist released $O_2^{\bullet -}$ in concentrations which stimulated [^{3}H]-thymidine incorporation into DNA and proliferation of mouse spleen lymphocytes and mononuclear cells from human blood. L-Glu-Hist modulates the ability of oxygen free radicals to act as signaling agents at low concentrations, influencing gene expression. The structural peptide-like analogues of L-Glu-Hist such as L-Glu-Trp, carcinine (β-alanylhistamine), but not L-Pro-Glu-Trp were active in stimulating thymidine incorporation and in inducing proliferation of mononuclear cells as compared to mitogen concanavalin A at doses 2.5–25.0 μg/ml. Our data provide evidence that L-Glu-Hist may act as a very fast, specific and sensitive trigger for lymphocyte proliferation and immunoregulation. The cited abilities and further obtained in vivo results make Immudilin® ((INCI: glutamylamidoethyl imidazole, aqueous solution), L-Glu-Hist) a useful immunoregulatory agent.

Keywords: L-Glutamyl-histamine; L-Glutamyl-tryptophan; Peptidomimetics; Free radical oxygen species; Superoxide radical; Iron chelating and ferroxidase activities; Immune cells signaling; Spleen lymphocytes; Blood mononuclear cells; Immunostimulating activities; Lymphocyte proliferation

* Corresponding author. Present address: Innovative Vision Products Inc., Moscow Division, Ivanovskaya 20, Suite 74, Moscow 127434, Russian Federation. Tel.: +7 095 977 2387; fax: +7 095 977 2387.
E-mail address: markbabizhayev2004@yahoo.com (M.A. Babizhayev).

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1. Introduction

Reactive species derived from oxygen and nitrogen serve as intracellular messengers that drive signal transduction [43,44]. Oxidation–reduction or redox-dependent reactions have proven to be important in regulating numerous processes that determine the physiological and pathophysiological function of cells and tissues. For example, lymphocytes and human fibroblasts constantly generate small amounts of superoxide radical as growth regulators [44,37,38,14]. Superoxide release has been also reported from endothelial cells [36] and smooth muscle cells [29] albeit at levels of one to two orders of magnitude less than that encountered in the case of stimulated macrophages or neutrophils. The engulfed foreign particles in the plasma membrane vesicle are exposed to a high flux of superoxide radicals in the phagocyte cytoplasm which form H_2O_2 via the dismutation reaction and some of the activated oxygen species are also released extracellularly. Once the phagocytic vacuole is formed, fusion with other granules in the neutrophil cytoplasm releases myeloperoxidase which utilizes H_2O_2 as a co-substrate with chloride producing the reactive oxidant hypochlorous acid.

Most of the proteins that play key roles in proliferative signal transduction function in a membrane environment, or in close association with membranes, and it is established that the activity of integral membrane proteins are modulated by the lipids of the bilayer [31]. Thus, protein kinase C has a very specific lipid requirement for its activity namely phosphatidyl serine [15]. In the presence of lipid hydroperoxides within cell membranes the perturbation of membrane organization occurs due to their polarity. The plasma membrane-bound enzyme Na^+/K^+-ATPase important for cell viability can be inactivated by radicals produced during lipid peroxidation (LPO) [52]. However, at low 'non-toxic' concentrations of LPO products some effects have been observed which have considerable relevance to cell proliferation. These include the stimulation of adenylate cyclase and phosholipase C activity in cell membranes [19,42], an inhibition of ornithine decarboxylase activity [11] and the expression of early growth regulating genes [49,16]. Consistently, strategies to modulate intracellular redox status by antioxidants and other redox enhancing agents show remarkable therapeutic potential.

Recently the original family of histamine-containing peptidomimetic compounds has been synthesized [9,10]. Its first-representative natural pseudodipeptide carcinine (β-alanylhistamine) has been described as universal antioxidant in both lipid and aqueous domains with repairing activity to cell membrane damages physiologically linked to L-carnosine (β-alanyl-L-histidine) in the carnosine-histidine-histamine metabolic pathway and endowed with higher resistance to enzymatic hydrolysis comparatively to L-carnosine. Because the receptor systems of histamine are thought to play a central role in growth, wound healing, various types of shock [24,22,32,30], as well as having influences on mammalian cardiac, renal, pulmonary, gastric, neurological, ocular and immunological physiology [2,27,34,26], we decided to examine the immune cells related cytokine-like activity of the novel histamine-containing peptidomimetic L-glutamyl-histamine (L-Glu-Hist) using the proliferation test of lymphocytes in vitro. We present evidence in this study that L-Glu-Hist peptidomimetic compound can both stimulate and inhibit the metal-dependent generation of lipid-derived peroxide intermediates as well as concomitantly release low concentrations of oxygen free radicals and scavenge a number of free radicals such as superoxide ($O_2^{\bullet -}$) free radicals. The limited release of oxygen free radicals by L-Glu-Hist occurs in concentrations of this peptidomimetic which stimulate proliferation of the cultured immune cells indicating that this step can be a sensitive trigger for immunomodulation and lymphocyte proliferation.

2. Materials and methods

2.1. Chemicals and biological reagents

All chemicals were of reagent grade. L-Carnosine was obtained from Neosystems Laboratories (France), carcinine·2HCl was provided by Exsymol SAM Laboratories (Principaute de Monaco). L-Histidine and histamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA); SOD was derived from Serva (Germany). Synthetic peptides L-Glu-Trp and L-Pro-Glu-Trp were synthesized according to specifications owned by Dr. Yu. A. Semiletov (Institute of Virology, AMS, Russian Federation). All peptides were purified by preparative reverse phase HPLC with the demonstrated level of purity >98% for peptides.

2.2. Analytical data for L-Glu-Hist

L-Glu-Hist was synthesized according to the specification owned by Exsymol SAM (Monaco, Principaute de Monaco) [9]. To ascertain the physicochemical characteristics of L-Glu-Hist as a pure compound, NMR spectra were recorded in D_2O on an impulsed Fourier transformed spectrometer (Bruker AC 200–200 MHz). ^{13}C NMR spectra were recorded in D_2O solution on a Bruker AC 200 spectrometer at 50 MHz. Mass spectra of the compound were obtained on a FINNIGAN INCOS-500 E mass spectrometer using the solid probe and electron impact at 70 eV, chemical ionization with isobutane at recording temperature 200 °C. Analytical t.l.c. was performed on Silica Gel 60 F254 plates (Merck) using as solvent isopropyl alcohol/25% ammonia/H_2O (l4:1:5) by volume. The plates were developed with a solution containing 0.2% ninhydrin, 0.5% cadmium acetate and 2% acetic acid in ethanol. IR spectra were monitored on a Nicolet 5-PC spectrophotometer. Reverse phase analytical HPLC was performed using a Philips PU 4811 chromatography system equipped with Waters 486 Tuneable Absorbance Detector. Twenty microliters of a solution containing 1 mg of the sample dissolved in 20 ml of eluent was injected onto a column (C18 Waters Symmetry® 4.6 mm × 250 mm) packed

with 5 μm silica beads). The column was eluted isocratically at 25 °C with 0.1% aqueous solution of trifluoroacetic acid (pH 2.5) over a period of 6 min at a flow rate of 1 ml/min. Eluates were monitored for absorbance at 211 nm. The melting point of the compound was determined on an Electrothermal 9100 melting point apparatus. The product was collected to give mp = 174–177 °C, t.1.c. R_f = 0. 5, HPLC retention time = 3.5 min, calculated for $C_{10}H_{16}N_4O_3$ C, 50.0; H, 6.7; N, 23.3. Found C, 49.75; H, 6.89; N, 23.65.

2.3. Molecular modeling

Low-energy 3D conformations of L-Glu-Hist were derived using the PM_3 method of the MOPAC 6.0 program [50]. The precise energy minima conformations were determined by semiempirical quantum mechanics. This technique is structuring a pool of energetically accessible shapes especially suitable for dipeptides comparatively to big protein molecules. The program is supplemented with ZINDO/1 computer software for estimation of chelating properties of the dipeptides and related compounds. The conformational geometry optimization was carried out using the revised computer program [51].

2.4. Fe^{2+} chelating and ferroxidase-like activity

The ability of L-Glu-Hist to decrease the concentration of free ferrous ions in Tris–HC1 buffer (100 mM, pH 7.4) was monitored by the 1,10-*o*-phenanthroline chelating assay modified from Ref. [56]. The reaction was started by the addition of 12.5 μM $FeSO_4$ to the reaction mixture which contained 3–20 mM L-Glu-Hist. Sixty minutes after incubation at 37 °C, the reactions were stopped by the addition 100 μM 1,10-*o*-phenanthroline (Serva), and A_{515} was immediately read. The ferroxidase activity was also discriminated from the rate of ferrous iron oxidation in the presence of 5 mM ascorbic acid using EDTA as a chelating standard. The concentration of (Fe^{2+}–1,10-*o*-phenanthroline) chelating complex was determined using the molar extinction ε_{515} =10 931 M^{-1} cm^{-1}.

2.5. Superoxide anion radical ($O_2^{\bullet -}$) generation assay

$O_2^{\bullet -}$ concentration was determined by the superoxide dismutase (SOD) inhibitable cytochrome *c* reduction assay [12]. Xanthine oxidase (grade I from buttermilk, Sigma, Oxidoreductase, EC 1.1.3.22) (0.02 U/ml) and xanthine–Na-salt (0.025 mM) (Serva) were used to generate $O_2^{\bullet -}$ in the absence of iron ions (+0.1 mM EDTA.) or in the presence of iron ions (-EDTA). Reaction mixture (final volume 2.0 ml) contained 50 μM cytochrome *c* (Serva), 50 mM KH_2PO_4/KOH buffer, pH 7.4 ± 0.1 mM EDTA, with or without 4.94 U/ml SOD and added tested compound (L-Glu-Hist). The reaction rate of cytochrome *c* reduction was monitored spectrophotometrically using the optical density at 550 nm and at 25 °C. The concentration of $O_2^{\bullet -}$ radicals was calculated from the SOD-inhibitable cytochrome *c* reduced using $\Delta\varepsilon_{550\,nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

2.6. Peroxidation of liposomes

The techniques for phospholipid extraction, purification and preparation of liposomes (reverse-phase evaporation technique) have been described previously [4,6]. Peroxidation of phosphatidylcholine (PC, derived from egg yolks) was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbic acid to the suspension of liposomes (1 mg/ml) in 0.1 M Tris–HC1 buffer (pH 7.4). The incubations were performed at 37° C. The ability of L-Glu-Hist or related compounds (see Table 2) to affect LPO in liposomes was controlled. The concentration of LPO products in the oxidized lipid substrates was measured by reaction with 0.125% (w/v) thiobarbituric acid (TBA) (malonyl dialdehyde (MDA) products) and by accumulation of net diene conjugates corresponding to the level of lipid hydroperoxides [6]. An average MW of phospholipid was assumed to be ≈730 Da.

2.7. Cell studies

2.7.1. Preparation of mouse spleen lymphocyte

Ma1e intact BALb/c mice, approximately 5–6-weeks old, housed in a room with controlled, illumination (LD 12:12) and temperature (23 ± 2° C), were used as spleen donors. Spleens were aseptically removed and transferred to Medium 199 with penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (50 μg/ml). A suspension of the spleen cells was obtained from the washed spleens introduced into the flasks where the wedges of organs were slightly cut and the contained cells were carefully squeezed out mechanically by the pair of curved ocular forceps from the fixated and praparated organ. Suspension of the spleen cells was then filtered via the sterilized cotton filter and the medium was added for centrifugation containing Medium 199 + 10% fetal calf serum. The obtained cells were washed twice during the centrifugation procedure at 1000 rpm (100–200 × *g*) for 10 min. This procedure yielded a population containing more than 90% of viable cells, as estimated by staining with a solution of 0.25% trypan blue. The cell suspension was dissolved to the concentration 5×10^6 cells/ml. The isolated cel1s were cultured in a 96-well containing plate at 37° C in a humidified atmosphere of air CO_2 (95.5%) in the growth culture medium RPMI-1640 (Sigma) with 20% fetal calf serum, 0.2 M L-glutamine, 10 mM Hepes buffer (pH 7.35), 5.10^{-5} M 2-mercaptoethanol and gentamycin (50 μg/ml).

2.7.2. Separation of mononuclear cells from human blood

Peripheral venous blood was collected by venipuncture from healthy blood donors and introduced into the tubes with heparin (10 U/ml). The freshly prepared blood was mixed in equal volumes with 0.9% NaCl. After incubation for 15 min

at room temperature, 6–8 ml of the obtained mixture was carefully layered onto the Ficoll–Paque (Pharmacia) 3.0 ml solution with a density gradient of 1.077 g/ml. The mononuclear cel1s were collected at the interface after centrifugation at 2000 rpm (380–400 × *g*) for 30 min at room temperature. The lymphocyte ring was transferred into the sterilized tube and washed twice in PBS (without divalent cations, Ca^{2+} and Mg^{2+}) by the repeated centrifugation at 1000 rpm (100–200 × *g*) for 10 min. The viable cells were counted. The cells were suspended in Medium 199. During all separation of cells, the cell suspensions were kept in siliconized test tubes.

2.7.3. Proliferation of lymphocytes in vitro

Concanavalin A (Con A) (Sigma, type III containing 15% protein) was used as the mitogen activator of T/B-lymphocytes with preferential activation of T-lymphocytes. The stimulation of cells was performed by the simultaneous addition of cell suspension (100 μl) and 100 μl Con A solution to the well of the 96-well plate (5×10^5 cells/well) incubated at 37° C for 72 h in a humidified incubator containing 5% CO_2. Optimal mitogenic concentrations of the control or tested stimuli were determined as essential to cause maximum cell response at preserved cellular viability. The final concentrations of Con A were 2.5, 5, 10 and 25 μg/ml. Suboptimal stimuli of Con A for the cultured cells were 5–10 μg/ml. In the test of blank cell cultures Con A was substituted for test peptide or 0.9% NaCl, respectively. Sixteen hours prior the end of cell incubation, 0.5 μCi of [^{3}H]-thymidine (Amersham Centre, UK) was added to the cell samples (10 μ1/well). Upon the termination of incubation with 1abel, the 96-well plate with cells was removed onto the ice and the precipitation of radioactive cell-containing material was performed onto the filters with the pore diameter of 0.45 μm using the automatic cell-collecting equipment Titertek Cell Harvester, Flow (UK). The filters were dried at 37° C for 40 min and then replaced into the quartz flasks containing the scintillating liquid (5 ml) (4 g POPOP + 0.1 g PPO per l toluene). The radioactivity of incorporated [^{3}H]-thymidine was detected using β-spectrometer. Incorporation of [^{3}H]-thymidine into lymphocytes determined by this procedure was shown to be correlated with cell growth [33]. The index of cell stimulation (SI) detectable as a stimulation of [^{3}H]-thymidine incorporation was determined by the ratio:

counts per minute in cultures with tested compound (mitogen)/counts per minute in control cultures (addition of 0.9% NaC1)

Student's *t*-test was used to determine the statistical significance. When comparing each dose of compound with a control (the number of groups each time of comparison equals two), an ANOVA and *t*-test will give similar results with $t^2_{crit} = F_{crit}$ and $t^2_{obs} = F_{obs}$

3. Results

3.1. 3D chemical structuring of L-Glu-Hist

Fig. 1 shows the energy-minimized 3D conformations of L-Glu-Hist derived from its chemical structure (Fig. 1a) using stick (Fig. 1b), ball-and-stick (Fig. 1c) and space filling (Fig. 1d) models. Due to energy differences determined by molecular mechanics, PM_3 semiempirical quantum mechanics among different peptidomimetic conformations, a dynamic equilibrium of energetically permissible C- and N-linked analogues of rotamers exists in an aqueous solution. The resulting minimized structure provides that a common characteristic for all the calculated conformations is that the claw-like structure of compound results in a proper stabilization for the metal chelating complexes such as when the complex of iron: L-Glu-Hist is obtained (Fe^{2+} ion is tightly enveloped into the intra-space of L-Glu-Hist molecule) (see, stick (Fig. 2a) and space filling (Fig. 2b) models of the ferrous ion chelating complex with L-Glu-Hist peptidomimetic). The calculated lowest energy conformations for metal-bound rotamers are present at neutral pH and result in any apparent significant adverse steric interactions during changes of pH, temperature or competition with high-affinity metal chelator (such as EDTA). One (L-Glu) or both (L-Glu + Hist) of the peptidomimetic components provide specificity for metal ion binding, quenching or release of a number of free radicals, binding of hydroperoxide in an imidazole-peroxide adduct [46]. Its structure can be related to histamine and both L-Glu residues for a structure–activity exploration. The structural coincidence of L-Glu-Hist moiety with histamine may have effects on regulatory proteins in the cell(s) and on DNA/genes involved in normal growth and metabolism.

3.2. Effect of L-Glu-Hist on the decrease of ferrous iron

L-Glu-Hist accelerated the decrease of ferrous iron in the ferrous sulfate solution in a concentration-dependent mode of 3–20 mM L-Glu-Hist pronounced by the 10–60 min of incubation (Fig. 3a). The kinetic curves presented in Fig. 3a demonstrate that there is a dose-dependent increase in the rate of ferrous iron disappearance. A ferrous iron chelator 330 μM EDTA showed a complete decrease of the accessible to 1,10-*o*-phenanthroline ferrous ions by the second minute after EDTA addition to the ferrous sulfate solution (Fig. 3, curve 8). The rates of decrease of ferrous iron accessible to 1,10-*o*-phenanthroline in the presence of L-Glu-Hist are indicative on the interference of iron binding (pure ferrous iron chelating) properties at higher >5–20 mM L-Glu-Hist concentrations (Fig. 3a, curves 3–5) and the acceleration of autooxidation of ferrous iron (ferroxidase-like activity) of L-Glu-Hist at lower less or equal to 3 mM concentrations (Fig. 3a, curves 1 and 2). L-Glu-Hist chelating activity appears weaker than that of EDTA but it is com-

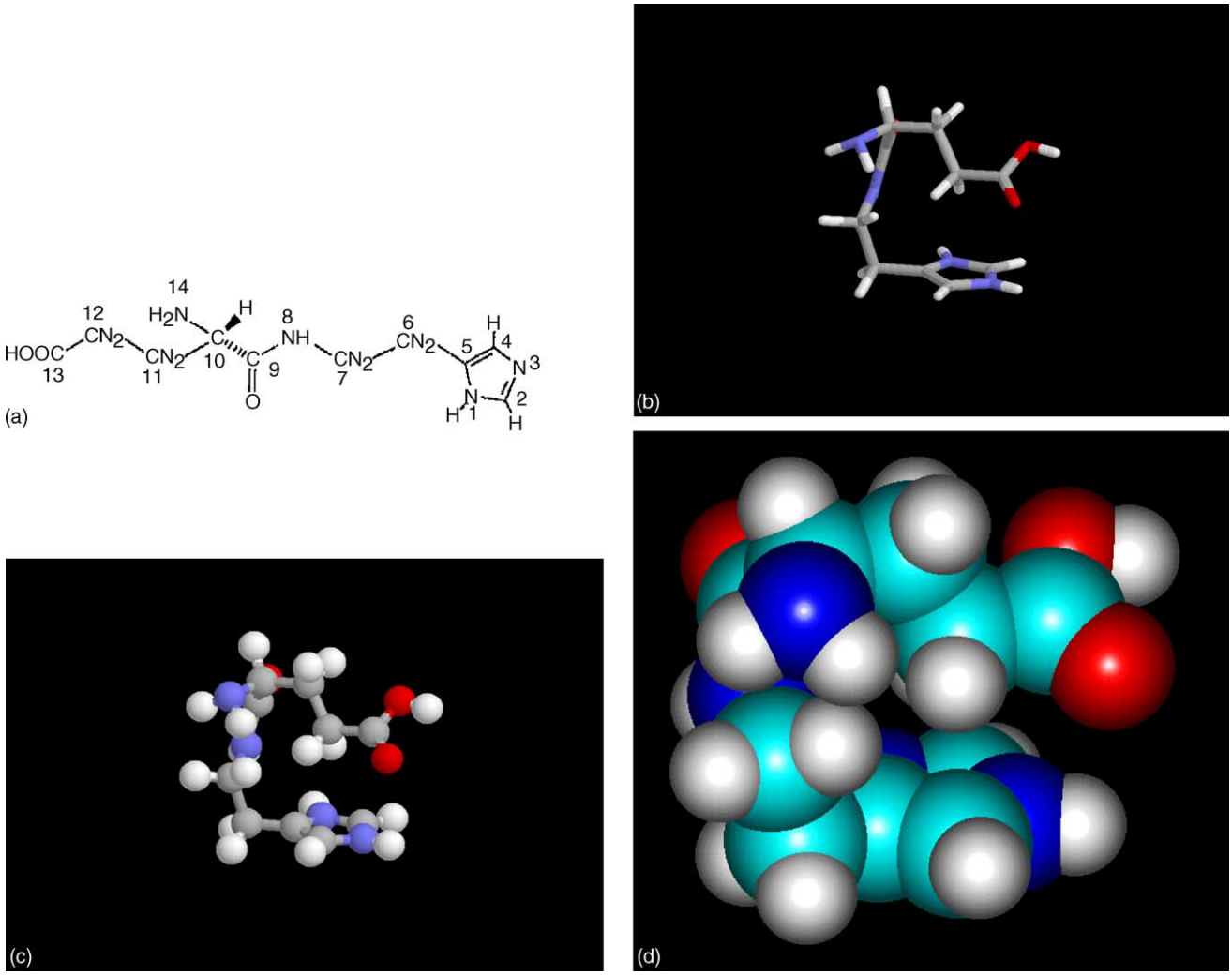

Fig. 1. Structure of L-glutamyl-histamine, shown as (a) chemical structure and energy-minimized 3D structure: (b) stick model; (c) ball-and-stick model; (d) space filling model.

petitive with ferrous iron chelating activity shown by 1,10-*o*-phenanthroline. Based on the high affinity properties of 1,10-*o*-phenanthroline to bind preferably ferrous but not Fe^{3+} ions, there is a potential preference for Fe^{2+} chelating by L-Glu-Hist over Fe^{3+} that is important for the rationale of later experiments. The reference curves (6, 7) in the presence of EDTA (3 and 33 μM) and curve (1) of autooxidation of ferrous iron are displayed on Fig. 3a. The rate of decrease of ferrous iron below the autooxidation curve indicates that L-Glu-Hist worked as a ferroxidase compound at lower concentrations (<3 mM). Determinations in the presence of 1,10-*o*-phenanthroline/ascorbate (Fig. 3b) revealed that at addition of 5 mM and especially, 3 mM L-Glu-Hist, ferrous iron decreased significantly ($P < 0.05$) by the second minute of the incubation below the autooxidation level of ferrous iron that indicates on the ferroxidase-like activity of low concentrations of peptidomimetic (curves 1–3). After 10 min incubation with a reductant of Fe^{3+} ions ascorbate (Fig. 3b) a corresponding retention of ferrous iron occurred at the baseline level documenting that ascorbate abolished the acceleration of autooxidation of ferrous iron by L-Glu-Hist by 10 min of incubation. Higher concentrations of L-Glu-Hist 10 and 20 mM (Fig. 3b, curves 4 and 5) maintained ferrous iron above the autooxidation curve by the second minute. As compared to high relative affinity of phenanthroline for ferrous ion, this type of kinetics indicates on the rising chelating ability of the L-Glu-Hist peptidomimetic to Fe^{2+} in a dose-dependent order of peptidomimetic. When control incubations of the ferrous sulfate solution were performed with 330 μM EDTA (pure chelator), it was found that 1,10-*o*-phenanthroline-bound ferrous iron increased by the second minute of incubation with ascorbate and further recovered to 25% of the reached level after 10 min during the reduction with ascorbate (Fig. 3b, curve 8). This model system illustrates the competitive binding of ferrous iron ions with the used chelator so removing them from detector (1,10-*o*-phenanthroline) molecule prevented by ascorbate.

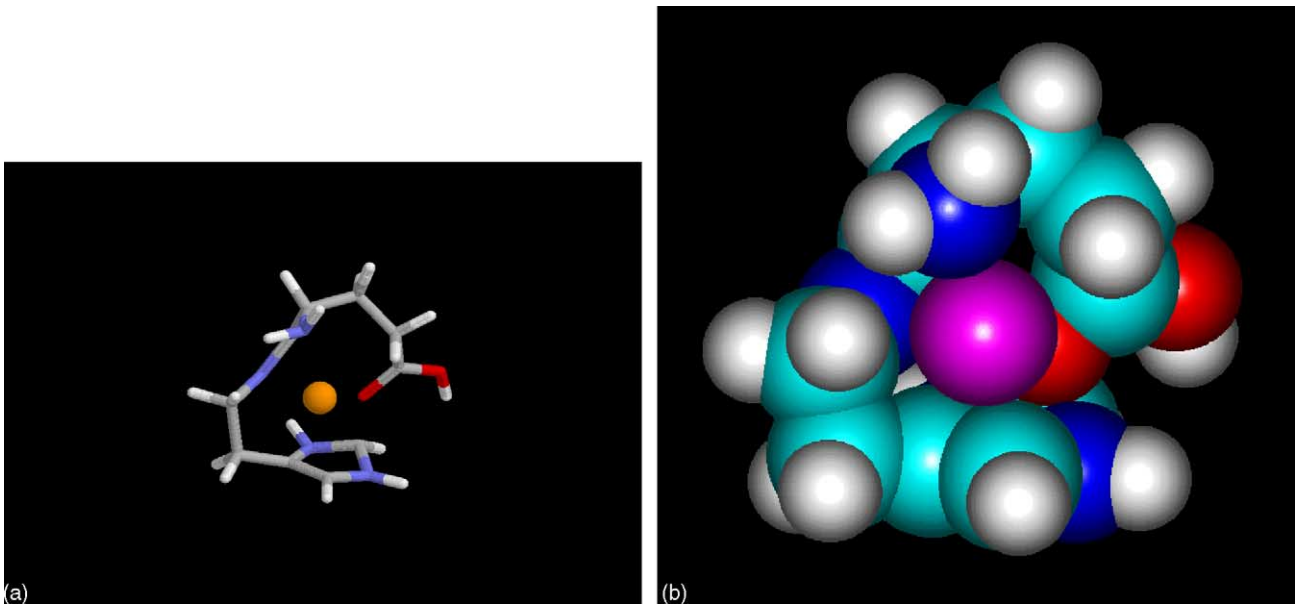

Fig. 2. Ferrous iron chelating structures of L-glutamyl-histamine, shown as the 3D lowest energy conformation: (a) stick model; (b) space filling model.

3.3. Action of L-Glu-Hist on superoxide anion radical ($O_2^{\bullet -}$)

A mixture of xanthine and xanthine oxidase at pH 7.4 generates $O_2^{\bullet -}$, which can be detected by its ability to reduce ferricytochrome c^{3+}. Any added compound that is itself able to react with $O_2^{\bullet -}$ should decrease the rate of reduction of this substance. The tested compound L-Glu-Hist itself does not reduce cytochrome *c*. Addition of the free metal ions chelator 0.1 mM EDTA to incubation medium containing reactive compounds significantly diminished the rate of cytochrome *c* reduction in xanthine–xanthine oxidase system. L-Glu-Hist had appreciable both inhibitory and stimulating effects on the rate of reduction of cytochrome *c* by $O_2^{\bullet -}$ (Table 1). It had essentially inhibitory effect at high concentrations (10–20 mM) of peptidomimetic. The effects of L-Glu-Hist on reduction of cytochrome *c* were not connected with inhibition of the xanthine oxidase enzyme itself by L-Glu-Hist because the used concentrations do not effect conversion of xanthine into hypoxanthine (as judged by the decrease in xanthine), recorded as the change in optical density at 250 nm. EDTA (0.1 mM) obviously does not abolish the inhibitory action of L-Glu-Hist but acts as an inhibitor itself to the reduction of cytochrome *c* in much the same way as L-Glu-Hist because the reduction rate upon addition of EDTA changes more (from 5.6 to 3.9) than

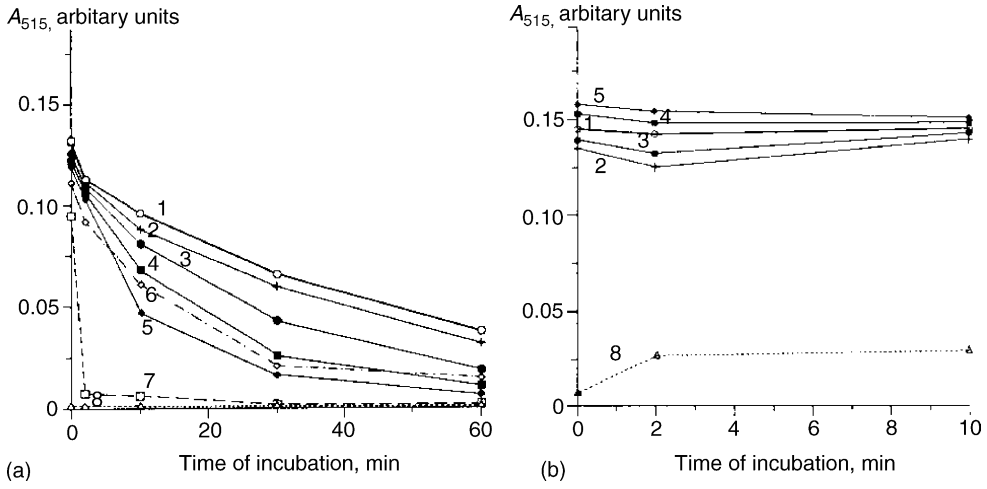

Fig. 3. Effect of L-glutamyl-histamine on the decrease of ferrous iron determined by l,10-*o*-phenanthroline assay in the presence of 12.5 μM ferrous sulfate (a) or in the presence of 12.5 μM ferrous sulfate + 5 mM ascorbic acid (b). The data points are the means of two independent determinations and are representative of three independent experiments. The standard error of the mean value for each point is ≤3% of the mean value. Details of incubations are presented in Section 2: (1) Fe^{2+}, control incubation; (2–5) Fe^{2+} + L-Glu-Hist (3, 5, 10, and 20 mM, respectively); (6–8) Fe^{2+} + EDTA (3, 33, and 330 μM, respectively). Samples taken at zero time and at the time intervals indicated in (a) and (b) and were used immediately for measurements.

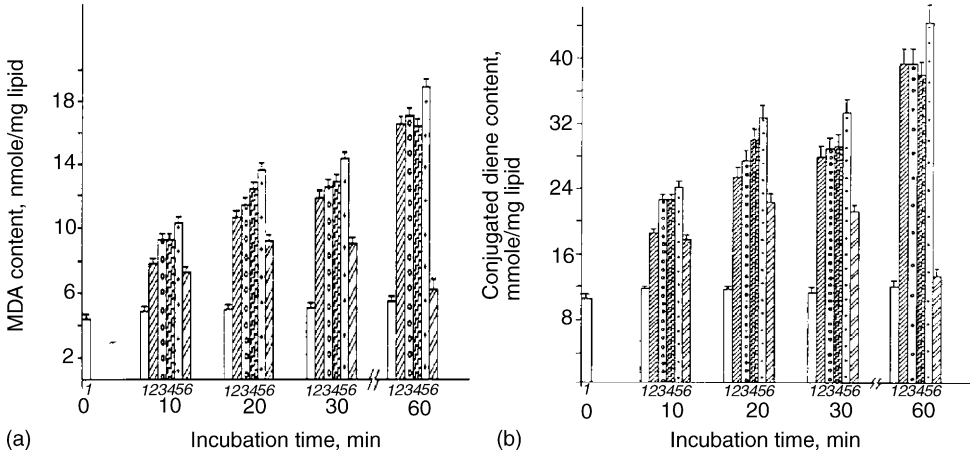

Fig. 4. Accumulation of lipid peroxidation products (TBARS), measured as MDA (a) and conjugated diene, (b) in liposomes (1) (l mg/ml) incubated alone for 60 min and with the addition of the peroxidation-inducing system Fe^{2+} + ascorbate (2). Different concentrations of L-Glu-Hist were added prior to the incubation to the system containing the peroxidation inducers: (3) 210 nM; (4) 42 μM; (5) 1.33 mM; (6) 10 mM. Samples were taken at zero time and at the indicated time intervals (10, 20, 30, and 60 min) and were used immediately for measurement of TBARS (see Section2). A similar amount of sample was partitioned through chloroform and used for detection of conjugated diene dissolved in 2–3 ml of methanol–heptane mixture (5:1, v/v). Data represent mean ± S.D. (n = 3–5).

the largest effect seen with L-Glu-Hist (5.6–4.6). However, 0.05 mM L-Glu-Hist concentration showed effectiveness in stimulation of $O_2^{\bullet-}$ formation in the absence or presence of EDTA (Table 1). Due to the ferroxidase-like activity of small (0.05 mM) L-Glu-Hist concentrations this peptidomimetic can facilitate superoxide free radical release affecting the concentration of catalytically active ferrous ions in reaction:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$$

The superoxide radicals generated in the xanthine oxidase/xanthine system can stimulate the reduction back to the ferrous ions to be catalytic:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

Table 1
Reduction of cytochrome *c* in xanthine–xanthine oxidase (X–XO) system in the presence of L-Glu-Hist

Conditions	Velocity of reduction of cytochrome *c* (10^{-5} M^{-1} s^{-1})
X (0.025 mM) + XO (0.02 U/ml) + cytochrome *c* (50 μM), pH 7.4	
Control	5.6 ± 0.1
Addition of	
L-Glu-Hist (0.05 mM)	6.3 ± 0.2*
L-Glu-Hist (10 mM)	5.1 ± 0.1**
L-Glu-Hist (20 mM)	4.6 ± 0.1***
X (0.025 mM) + XO (0.02 U/ml) + EDTA (0.1 mM) + cytochrome *c* (50 μM), pH 7.4	
Control	3.9 ± 0.1
Addition of	
L-Glu-Hist (0.05 mM)	6.3 ± 0.2***
L-Glu-Hist (1mM)	3.9 ± 0.1
L-Glu-Hist (5 mM)	4.2 ± 0.2
L-Glu-Hist (10 mM)	3.0 ± 0.5
L-Glu-Hist (20 mM)	3.3 ± 0.3

Results are the mean ± S.E.M. of three to five experiments.
* $P < 0.05$ (significant difference from control).
** $P < 0.01$ (significant difference from control).
*** $P < 0.001$ (significant difference from control).

3.4. Effects of L-Glu-Hist on lipid peroxidation

L-Glu-Hist did not produce any significant effect on peroxidation of PC liposomes in the absence of Fe^{2+} ascorbate catalytic oxidation system. Fig. 4a and b presents the kinetic data of MDA and diene conjugates accumulation during peroxidation of PC liposomes, catalyzed by the $O_2^{\bullet-}$-dependent Fe^{2+}–ascorbate oxidation system in the presence and absence of L-Glu-Hist. The results demonstrate that L-Glu-Hist both exhibited significant stimulatory peroxidative effect for PC liposomes at 210 nM, 42 μM (10 μg/ml) and 1.33 mM concentrations of peptidomimetic and significantly inhibited LPO at 10 mM L-Glu-Hist concentration in the Fe^{2+}–ascorbate-catalyzed reaction (Fig. 4). These results together with above-mentioned data indicate that L-Glu-Hist has modest oxidizing activity at low concentrations (<1 mM), and works as an antioxidant at high concentrations. The prooxidant effect of L-Glu-Hist impurities gives maximal excess of 2.9 nmol MDA per mg of lipids accumulation over the control incubation in the presence of Fe^{2+}–ascorbate by the interval of 20 min which takes place most likely due to the exhibited during the first minutes of incubation ferroxidase-like activity of L-Glu-Hist more powerful as LPO promoter than free ferrous ions. The inhibition of TBA-reactive substances (TBARS) accumulation at higher L-Glu-Hist concentration is due to Fe^{2+} chelation by the peptidomimetic and prevention of radical generation. Fig. 4 shows that maximum TBARS and lipid hydroperoxides assessed from the absorbance of diene conjugates reached in the presence of 10 mM L-Glu-Hist at 20 min of incubation decreases at later time points which must be due to a loss of existing TBARS or hydroperoxide

Table 2
Effect of imidazole-containing compounds on the iron-ascorbate cata1yzed lipid peroxidation

Testing compound (concentration)	Percentage of control
None	100
Carcinine (10 mM) (β-alanylhistamine)	58
Histamine (10 mM)	163
Histamine (1 μM)	145
L-Carnosine (10 mM) (β-alanyl-L-histidine)	47
Histidine (10 mM)	148
Histidine (10 mM) + EDTA (50 μM)	51
L-Glu-Hist (1.33 mM)	119
L-Glu-Hist (10 mM)	76

Peroxidation was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbate in 0.1 M Tris/HC1 buffer, pH 7.4 to the reaction mixture. Lipid peroxidation products were measured by reaction with TBA (see Section 2). Data represent mean of three to five experiments. Oxidation substrate: PC liposomes (1 mg/ml); catalyst Fe^{2+} + ascorbate; time of incubation at 37 °C 60 min. Data represent mean (%) (n = 3–5).

precursors of MDA and not due to a decreased formation of peroxide compounds. The reduction of lipid hydroperoxides may result from the cleavage of a lipid hydroperoxide with a transition metal complex and supplement with electrons for the reductive reaction LOOH → LOH. Different constituents and imidazole-containing compounds were compared for their ability to inhibit the iron–ascorbate-catalyzed LPO (Table 2). The imidazole-containing compounds histamine and histidine exposed peroxidative effects for PC liposomes while carcinine (β-alanylhistamine) and L-carnosine (β-alanyl-L-histidine) showed the ability to inhibit the iron–ascorbate-dependent oxidation of PC liposomes. The addition of 50 μM EDTA significantly inhibited the pro-oxidant activity of 10 mM L-histidine and histamine (Table 2), indicating the role of free iron catalysts. The other tested compounds, i.e. imidazole, β-alanine were inactive to LPO (see Ref. [10]), suggesting that the redox potential of the whole peptidomimetic molecule is essential for exhibition of its pro- or anti-oxidant activities.

3.5. Stimulation of lymphocyte proliferation

The estimation of [^{3}H]-thymidine incorporation into DNA is used as a tool for the determination of the proliferation of mouse spleen lymphocytes and immunoregulatory mononuclear cells from human blood in vitro. The pooled data concerning the dose-related effect of L-Glu-Hist and related peptides L-Glu-Trp and L-Pro-Glu-Trp on the incorporation of [^{3}H]-thymidine into DNA of mouse spleen lymphocytes are presented in Table 3. The reference compound mitogen Con A produced significant stimulating effect on [^{3}H]-thymidine incorporation at doses of 2.5–25 μg/ml with maximum stimulating activity at 10 μg/ml (stimulation index 61.6). As can be seen from the data (Table 3), L-Glu-Hist can specifically regulate proliferation of mouse spleen lymphocytes within the doses range of 2.5–25 μg/ml of L-Glu-Hist peptidomimetic. Within tested low doses of L-Glu-Hist, this compound appeared necessary for cell viability as judged by trypan blue staining. Cell cultures exposed to L-Glu-Hist in the indicated dose range revealed no cell damage or an obvious 1oss of cellular density. The dose of 10 μg/ml L-Glu-Hist significantly stimulated the [^{3}H]-thymidine incorporation

Table 3
The effect of L-Glutamyl-histamine, related peptides L-Glu-Trp, Pro-Glu-Trp and concanavalin A on [^{3}H]-thymidine incorporation into DNA of mouse spleen lymphocytes in vitro

Compounds	n	c.p.m./5 × 10^5 cells ± S.E.M.	Stimulation index	P
Control (free of mitogen or testing compound)	7	796 ± 276		
Concanavalin A (μg/ml)				
2.5	5	6620 ± 720	8.3	<0.001*
5.0	5	26607 ± 6689	33.4	<0.01*
10.0	4	49002 ± 13003	61.6	<0.01*
25.0	5	5055 ± 1167	6.4	<0.01*
L-Glu-Hist (μg/ml)				
2.5	4	6222 ± 2608	7.8	<0.1
5.0	5	34042 ± 17765	42.8	<0.1
10.0	6	33589 ± 10336	42.2	<0.05*
25.0	4	4528 ± 3293	5.7	n.s.
L-Glu-Trp (μg/ml)				
2.5	5	3184 ± 346	4.0	<0.001*
5.0	4	29634 ± 8350	37.2	<0.01*
10.0	5	13636 ± 3501	17.1	<0.01*
25.0	5	1000 ± 225	1.3	n.s.
L-Pro-Glu-Trp (μg/ml)				
2.5	6	2070 ± 852	2.6	n.s.
5.0	5	2318 ± 1075	2.9	n.s.
10.0	4	4935 ± 3076	6.2	n.s.
25.0	5	3741 ± 2405	4.7	n.s.

n: number of determinations; n.s.: non significant difference with control. Results are the mean ± S.E.M. of three to five experiments. The data do not indicate any specific effect of L-Glu-Hist vs. L-Glu-Trp, but indicate on the input and important role of L-Glu residue in the structure of peptidomimetic in the signaling activity.

* Significant differences with control.

Table 4
The effect of L-glutamyl-histamine, carcinine 2HCl (β-alanylhistamine) and concanavalin A on the proliferation of mononuclear cells (lymphocytes) obtained from blood of the normal human donor, 48 years (in vitro studies)

Compounds	*n*	c.p.m./5 × 10^5 cells ± S.E.M.	Stimulation index	*P*
Control (addition of medium RPMI 1640)	6	1201 ± 120		
Concanavalin A (μg/ml)				
2.5	4	5785 ± 320	4.8	<0.001*
5.0	3	35544 ± 5400	29.6	<0.001*
10.0	4	25369 ± 2480	21.1	<0.001*
25.0	4	872 ± 120	0.7	n.s.
L-Glu-Hist (μg/ml)				
2.5	4	9884 ± 986	8.2	<0.001*
5.0	4	47816 ± 1580	39.8	<0.001*
10.0	3	28329 ± 1520	23.5	<0.001*
25.0	5	3039 ± 1240	2.5	n.s.
Carcinine 2HCl·β-alanylhistamine (μg/ml)				
2.5	4	5765 ± 1980	4.8	<0.1
5.0	5	18856 ± 6800	15.7	<0.05*
10.0	4	20297 ± 6820	16.9	<0.05*
25.0	4	22339 ± 4470	18.6	<0.001*
50.0	3	15372 ± 3888	12.8	0.1
100.0	4	1922 ± 420	1.6	n.s.
Carcinine 2HCl·β-alanylhistamine (10 μg/ml) + concanavalin A (10 μg/ml)				
	3	30145 ± 5500	25.1	<0.001*

n: number of determinations; n.s.: non significant difference with control. Results are the mean ± S.E.M. of three to five experiments.
* Significant differences with control.

into DNA of mouse spleen lymphocytes (stimulation index 42.2). The related L-Glu-containing dipeptide L-Glu-Trp showed significant stimulation of [^{3}H]-thymidine incorporation into DNA of mouse spleen lymphocytes in vitro at doses of 2.5–10.0 μg/ml, whereas L-Pro-Glu-Trp tripeptide exhibited no stimulatory activity on thymidine incorporation into the mouse spleen lymphocytes in the range of doses studied in the same test. Despite the fact that the structure of L-Glu-Hist is related to histamine, it appears doubtful that the stimulating effects on the proliferation of lymphocytes/monocytes observed with L-Glu-Hist are related with pure histamine residue, but rather with introduction of L-Glu residue into the peptidomimetic molecule since the dipeptide L-Glu-Trp has virtually the similar effect (stimulation index 37.2) as L-Glu-Hist (stimulation index 42.8; standard deviation: about 50% of the mean value). In the human blood mononuclear cells L-Glu-Hist and mitogen Con A applied at the similar dose range did not negatively change cell viability and showed significant stimulating activity on the proliferation of lymphocytes in the range of 2.5–10 μg/ml doses with maximum of stimulating effect on the [^{3}H]-thymidine uptake at 5 μg/ml (see Table 4). A histamine derivative of L-Glu-Hist, carcinine, 2HCl·β-alanylhistamine showed similar good biocompatibility and exposed induction of proliferation of mononuclear cells at doses of 5.0–25.0 μg/ml when it was used alone and in admixture with Con A (Table 4). In both types of cells, L-Glu-Hist stimulated the [^{3}H]-thymidine incorporation into DNA and lymphocyte proliferation in our assay system at a low concentration (ranged about 42 μM) of peptidomimetic coinciding with the range that this compound also appears to have a pro-oxidant activity to lipid membranes in the Fe^{2+}–ascorbate system interlaced with release of low concentrations of oxygen free radicals due to ferroxidase-like activity of L-Glu-Hist. This finding provides a possible mechanism by which L-Glu-Hist can act as a very fast specific and sensitive trigger for lymphocyte proliferation and immunoregulation. A number of different mechanisms may contribute to the mitogen-like activity of the matched tested compounds: Con A, L-Glu-Trp, L-Pro-Glu-Trp and carcinine (β-alanylhistamine).

4. Discussion

A histamine-containing peptidomimetic (L-Glu-Hist) was synthesized and characterized in this study and its role to act as immune cells cytokine mimetic leading to cellular lymphocytes proliferation responses was revealed. The L-Glu-Hist peptidomimetic showed the stabilization in the formation of ferrous ions chelating complexes and accelerated the decrease of ferrous iron ions concentration in the aqueous medium demonstrating the ferroxidase like activity at lower less or equal to 3 mM concentrations of L-Glu-Hist peptidomimetic. L-Glu-Hist demonstrated pure ferrous iron chelating properties at higher >5–20 mM L-Glu-Hist concentrations responsible for its antioxidant activity in lipid membranes in this concentrations range. L-Glu-Hist both exhibited a stimulatory effect for PC liposomes peroxidation catalyzed by superoxide anion radical generating system at 210 nM, 42 μM (10 μg/ml) and 1.33 mM concentrations of peptidomimetic.

L-Glu-Hist stimulated DNA synthesis and the proliferation of mouse splenocytes and human peripheral blood lymphocytes. Structural analogues of L-Glu-Hist (L-Glu-Trp, carcinine (β-alanyl-histamine)) were also active in stimulat-

ing thymidine incorporation as compared to that mediated by Con A. The provided data suggest that L-Glu-Hist can act rapidly as a specific trigger for lymphocyte proliferation and immune regulation. The study analyzes the mechanisms of biological activity of L-Glu-Hist including its modulating effects on free radical-induced oxidation and function of specific oxygen radicals and radical-derived species as well as its role as a chemical "messenger" to lymphocyte reactivity and spleen cells functions when added directly to the cell culture medium.

We present an evidence that L-Glu-Hist can both stimulate and inhibit LPO induced with the aid of an $O_2^{\bullet -}$-generating system Fe^{2+} + ascorbate [3]. In comparison with "free" ferrous ion, addition of L-Glu-Hist at high concentrations results in (1) decreased peroxidation of lipids, (2) decreased cytochrome *c* reduction by superoxide anion radicals, (3) decreased lymphocyte proliferation. The magnitude of these effects, however, is modest. These results are attributed to antioxidant properties of the iron–Glu-Hist complex. On the other hand, our major findings were that at low concentrations addition of L-Glu-Hist resulted in (1) modest increase of LPO, (2) modest increase in the rate of cytochrome *c* reduction and (3) striking significant increase of lymphocyte proliferation. These results are attributed to ferroxidase-like activity of the complex at low concentrations.

Although a number of mechanisms may contribute to the underlying processes that give rise to the observed results including release of cytokines by activated lymphocytes capable of stimulating several distinct processes, the expression of immunomodulatory proteins and the synthesis and release of hydrophilic glycosaminoglycans a direct effect of oxygen free radicals released by L-Glu-Hist upon the lymphocyte proliferation is one possible explanation. It is now clear that superoxide and H_2O_2 can stimulate growth responses in a variety of mammalian cell types when added exogenously to the culture medium. Besides hamster and mouse fibroblasts, these include mouse epidermal cells [16], Balb/3T3 cells [49] and human primary fibroblasts [40]. Our results show that the proliferative response to L-Glu-Hist occurs as to immune cells cytokine in a dose-dependent manner (10–50 μM) although maximal stimulation of LPO in the $O_2^{\bullet -}$-generating system (Fe^{2+} + ascorbate) occurred at different (higher: ∼1 mM) L-Glu-Hist concentrations studied. Free radical release may occur in these instances at a rate insufficient to damage cells, but sufficient to stimulate their proliferation. In the presence of the transitional metal iron, ascorbic acid induces LPO with the formation of reactive aldehydes and similarly with our data this step may be necessary for the stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts [17]. Interestingly, tumor necrosis factor-α (TNFα), IL-1 and interleukin 1β (IL-1β), have each been shown to induce the expression of MnSOD, an enzyme charged with the disposal of superoxide radicals through dismutation [55,41]. Since the previous [40,17,47,48] and present experiments used fetal calf serum in the incubations, these cells would accumulate a sizeable amount of iron [45], which upon the addition of L-Glu-Hist could induce or inhibit free radical release and LPO.

The modeling experiments illustrate the high Fe^{2+}-ion binding properties and possible ferroxidase-like activity of L-Glu-Hist. It is not surprising that the metal will bind to the two groups in the peptidomimetic molecule with good ligand properties. The stoichiometry of ligand to metal may change its structure and perhaps reactivity. The lowest energy conformation of the ligand is probably not very important as many other conformations are kinetically accessible for this relatively flexible molecule. As a result, there is a possibility that mononuclear versus binuclear complexes can explain the concentration behavior of the experiments shown.

L-Glu-Hist has maximum of mitogenic activity on lymphocytes at about 10 μg/ml (corresponds to about 40 μM). This level of L-Glu-Hist was shown to have modest oxidizing activity and increase in mitogenic activity is higher than expected from the modest oxidizing activity of L-Glu-Hist. The specificity of mitogenic activity of L-Glu-Hist on lymphocytes can also be related with the L-Glu and Hist moieties of peptidomimetic. This effect can be not solely related to the oxidative nature of the molecule. The structural derivatives of L-Glu-Hist-like L-Glu-Trp and carcinine (β-alanylhistamine), but not L-Pro-Glu-Trp were shown in this study able to induce lymphocyte proliferation in a dose-dependent manner similar to L-Glu-Hist peptidomimetic. A combination of carcinine and Con A did not exhibit additive effect. This experiment means that the immune cellular signaling does not necessarily indicate on combination of more specified cellular factors.

It is well known that Fe(II) autoxidizes in neutral aqueous solutions and that it reduces O_2 to $O_2^{\bullet -}$. It is further known that anything that ligates Fe(III) more strongly than Fe(II) might favor that autooxidation. This applies to the peptidomimetic L-Glu-Hist used in this study. In the phenanthroline assay the system is equalized and the relative affinities of phenanthroline and the peptidomimetic for ferrous ion are compatible. Since phenanthroline is added in excess over Fe^{2+} a high binding constant in comparison with L-Glu-Hist would mask the true chelating ability of the peptidomimetic. Analysis of the binding kinetics in the presence of ascorbate could help distinguish between chelation and oxidation of ferrous to ferric ion as the main factor of reduced availability of free ferrous ion. The kinetic results give more support for the statement that reduced availability of ferrous ion is due to chelation at high concentrations of L-Glu-Hist but to ferroxidase activity at low concentrations of peptidomimetic.

Chemically the antioxidant activity of L-Glu-Hist shown at high concentrations of peptidomimetic is not solely due to the imidazole moieties of the molecule, since imidazole itself' did not show the antioxidant activity, and histamine exhibited a pro-oxidant action dependent on the concentration of catalytically active free iron ions (this study; Ref. [10]). The antioxidant activity of L-Glu-Hist may involve reduction of oxidative potential or stabilization of the imidazole radical, probably due to the peptide bond. The lack of car-

boxyl group of histamine comparatively to histidine is essential for demonstration of more pro-oxidant than antioxidant activity of the molecule. Since L-Glu-Hist has been shown to be effective chelating agent for ferrous ions, the ligand acts as antioxidant at high concentrations, but at low concentrations the peptidomimetic acts as a generator of the reactive oxygen species that it presumably reduces at high concentrations. With regards to the possible ferroxidase activity, it is well documented in the inorganic chemical literature that iron chelates can modulate the redox behavior of reactive iron species [10]. The current experiments on the action of L-Glu-Hist on LPO show how such modulation would actually operate in this system. Similarly, the pro-oxidant effect of histamine is most likely due to the formation of the chelate Fe(II)–histamine, with redox activity more powerful as LPO promoter than of free ferrous ions.

Reactive oxygen species include the superoxide molecule ($O_2^{\bullet -}$), the hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). The current results do not distinguish whether the reactive oxygen species generated are superoxide, hydrogen peroxide or hydroxyl radical. In aqueous solution two molecules of superoxide can disproportionate into O_2 and hydrogen peroxide, which in turn can generate hydroxyl radical by Fenton type chemistry with ferrous ion. The exact mechanism and contribution of $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$ and LPO products to lymphocyte stimulation and proliferation is undetermined. Preliminary investigations suggest that stimulation of cyclo-oxygenase by peroxides in the prostaglandin cascade may be important [35]. As an alternative, the signaling of proliferation responses in the immune cells involving released superoxide, or hydrogen peroxide, may be mediated through the oxidative inactivation of serum protease inhibitors allowing serum proteases to remodel the cell surface, or glycocalyx, and thereby facilitate, or modulate, the action of L-Glu-Hist and thus retain the necessary all important growth specificity [53]. $O_2^{\bullet -}$ is being formed either spontaneously during normal cellular respiration or produced in activated leukocytes (neutrophils and macrophages) which are involved in the cell:cell interaction with the lymphocyte subpopulations included in immunomodulatory activity. Using the cytochrome *c* reduction test, we have shown that superoxide radicals are at least abundant to stimulate lymphocyte proliferation in vitro, being formed spontaneously due to ferroxidase-like activity of L-Glu-Hist applied at less or about 50 μM concentration.

The majority of the effects of free radicals demonstrated by previous investigators have been toxic [25,7,1,39] and it has been hypothesized that the toxicity arises from the production of reactive oxygen species at a rate exceeding the protective capacity of the cellular detoxification systems [23,57,8,5]. Reactive oxygen species and LPO products are capable of oxidative damage to a number of important cellular components, including cell membranes through LPO, cellular proteins-resulting in loss of function or enzymatic activity, and DNA [28]. The presence of lipid hydroperoxides within cell membranes will perturb membrane organization due to their polarity. However, recently it has been found that LPO has a dual effect on lipid order [13]. A more ordered or disordered state may result depending on the degree of oxidation and the state of lipid order prior to oxidation. Although an adequate explanation is not yet available for the endogenous LPO encountered in cultured mammalian cells, it is nonetheless clear that the level can vary considerably. Lipid peroxides can yield oxidative breakdown products such as the hydroxyalkenals through non-enzymic pathways. The aldehyde products of LPO, in particular 4-hydroxynonenal (HNE), can react with thiol and amino groups of proteins affecting several cellular activities [20,18]. Such effects occur at HNE (aldehyde) concentrations greater than 10 μM. In concentrations of less than 3 mM L-Glu-Hist showed lower "non-toxic" stimulating activity for TBARS accumulation in the $O_2^{\bullet -}$-generating system Fe^{2+} + ascorbate. It is hypothesized that L-Glu-Hist may play a central role in the "down-regulation" of cell proliferation via the production of LPO products at a "steady-state" level which can be rapidly catabolized by normal cells [21].

Two different observations are made in the experiments with superoxide and liposomes depending on the concentration of the peptidomimetic. The results at high concentrations (decreased cytochrome *c* reduction, decreased liposome oxidation) are qualitatively essentially the same as those seen when EDTA is added but quantitatively much less pronounced (Fig. 3, Table 1). It is proposed that L-Glu-Hist acts in the xanthine oxidase experiments as a one-electron acceptor. However, the fact that EDTA also reduces the rate of cytochrome *c* reduction (and even to a larger extent) suggests that perhaps the ligand properties of both compounds are responsible. Presumably ferrous ion is also present in these reactions judging from the obtained results. One of the mechanisms by which antioxidants can protect their biological targets from oxidative stress is the chelation of transition metals such as copper and iron thus preventing them from participating with peroxides in the deleterious Fenton reaction. At concentrations of 5–20 mM L-Glu-Hist was shown to be efficient ferrous iron chelating agent, which can possess low SOD-like activity in vitro. The complex of iron:L-Glu-Hist was sufficient in reducing the superoxide radicals, only when EDTA was omitted in the incubation medium. The iron chelating complexes of L-Glu-Hist may serve as scavengers of the superoxide radicals rather than as catalysts. Another antioxidant protection possessed by L-Glu-Hist is that at 10 mM concentration this compound removes lipid-derived hydroperoxide intermediates typically catalysed by glutathione-requiring enzymes (Se-dependent GSH peroxidases and certain Se-independent enzymes, such as GSH-S-transferase B) [54]. The reduction of lipid peroxides by L-Glu-Hist at high concentrations was experimentally monitored by diene conjugates measuring test and tested by decrease of TBARS during LPO. Recently, the reduction of phospholipid-derived and fatty acid hydroperoxides to their alcohols was demonstrated for the related compounds L-carnosine

(β-alanyl-L-histidine) and carcinine (β-alanylhistamine) both in aqueous medium and in a phospholipid system [10].

The stimulating activity of L-Glu-Hist at low concentrations is more interesting. Our study describes the most promising results as the peptidomimetic is shown to have very similar activity as concanavalin A. In the phenanthroline strong chelating assay, the excess in concentration of 12.5 μM $FeSO_4$ has been added to the reaction mixture and at the concentrations of peptidomimetic that cell stimulation is seen no information on binding and/or presumed ferroxidase activity is presented (Fig. 3). However, the indicated effects related with anti- or pro-oxidant properties of L-Glu-Hist are seen in the liposome peroxidation experiments (Fig. 4). The discrepancies between ≤1 and 10 mM concentrations of L-Glu-Hist in their action to the iron-catalyzed LPO are consistent with metabolic effects of this peptidomimetic on the cellular activities and their roles in metabolism of lipid-derived peroxide intermediates. Despite the possible role of L-Glu-Hist in millimolar concentrations as an antioxidant preventing cellular LPO, our experiments with lymphocyte cells showed the metabolic actions of this compound on cellular proliferation at quite modest range of concentrations apparently related with little significant effect on stimulation of oxygen free radicals release and endogenous levels of LPO.

In summary, the obtained findings suggest a theoretical avenue through which oxidative damage, acting either directly or as an end-product of cytokine release, may contribute to lymphocyte stimulation. Free radicals perform many critical functions in our bodies in controlling the flow of blood through our arteries, to fighting infection, to keeping our brains alert and in focus. Similar to antioxidants, some free radicals at low levels are signaling molecules, i.e. they are responsible for turning on and off genes. The phenomena outlined in this paper introduce a novel synthetic peptidomimetic L-Glu-Hist as a potent immunotherapeutic signaling agent and a tool with an oxygen radicals modulating activity and a specific role of physiological agonist leading to various above-discussed cellular responses, such as lymphocyte proliferation by modulating DNA synthesis.

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This work was supported by Innovative Vision Products Inc. The biological applications of L-glutamyl-histamine peptidomimetic are protected with the worldwide patents owned by Dr. Mark A. Babizhayev/Exsymol SAM and issued with the PCT International publication numbers WO 95/12581 (pseudodipeptide product having an imidazole grouping and applications) and WO 94/19325 (coupling product obtained from histamine and an amino acid). L-Glutamyl-histamine peptidomimetic product is marketed internationally by Exsymol SAM for the cosmetic use and skin care under the trade name of IMMUDILIN® (INCI: glutamylamidoethyl imidazole, aqueous solution) with the in vivo demonstrated activities of immunostimulation that combats immuno-deficiency due to UV irradiation.

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