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Design, Synthesis, and Evaluation of Linear and Cyclic Peptide Analogues of Carnosine as Anticancer Agents

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ABSTRACT:
Introduction: Carnosine (β-alanyl-L-histidine) is a naturally occurring dipeptide, widely and abundantly distributed in the muscle and nervous tissues of animal species. Carnosine contains several beneficial biological properties, such as antiglycating and antioxidant activities. It also contains antineoplastic effects in human cell culture as well as in animal experiments; however, the clear molecular basis of this activity has not been known yet. In the present study, in order to further examine structural basis of Carnosine for the anticancer activity, some linear and cyclic Carnosine peptide analogues were synthesized and their cytotoxicity were examined. **Methods:** Linear and cyclic Carnosine peptide analogues were synthesized with appropriate protected amino acids and reagents using solid-phase peptide synthesis strategy, and anti-neoplastic activity of the synthesized compounds was examined on cancer cell lines of HepG2 (Human Liver Cancer Cell Line) and HT-29 (Human Colorectal Adenocarcinoma Cell Line) using MTT assay and flow cytometry analysis. Safety profile of the synthesized Carnosine analogues was also examined using skin fibroblast cells. **Results:** Our results showed that Carnosine analogues were toxic against HepG2 and HT-29 cell lines with a mean IC50 value of 12.7 µg/mL. Flow cytometry analysis showed that such toxic activity could be, at least partly, through apoptosis induction. **Conclusion:** According to our experiments, in overall, compound 3b can be a good candidate for the further development of safe anticancer agents. On the other hand, the considerable toxicity of cyclic peptide analogues of Carnosine, 1c and 2c, on the cancerous cell lines along with their high safety profiles on the normal skin cells, make them attractive for further works on finding anticancer agents with peptide structure, giving better physicochemical properties for oral administration.

Keywords: Carnosine analogues; Anticancer agents; Solid-phase peptide synthesis; MTT assay; Flow cytometry analysis.

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1. Introduction

L-Carnosine, a natural dipeptide consisting of two amino acid residues, β-alanine and L-histidine, was discovered by Gulevich and Amiradzibi in 1900 from Liebig's meat extract [1]. The related compounds, such as anserine¬ (β-alanyl-3-methyl-L-histidine) and homo Carnosine ¬(γ-aminobutyryl-L-histidine) are also naturally occurring dipeptides, existing in skeletal muscles, heart, and brain of mammalian species, at high concentrations [2, 3].

Many proposals have been made regarding the biological actions of Carnosine, including its role as a physiological intracellular buffer [4], a neurotransmitter [5], a metal chelator [6] and an antiglycating agent [7].
In addition, many biochemical studies have demonstrated that Carnosine possesses oxygen-free radical-scavenging and antioxidant properties [8-13]. Furthermore, an interesting observation with regard to the therapeutic value of Carnosine was made by Holliday and McFarland [14].
They demonstrated that Carnosine has an inhibitory effect on cultured neoplastic cells. In their study seven human cell lines and two rodent cell lines were used. Two of the human cell lines were derived from SV40-transformed fibroblasts and the others were from cervical cancer, lung cancer, osteogenic sarcoma, bladder cancer, and prostate cancer [14]. Other studies showed that Carnosine inhibited tumor growth in animals [15, 16]. The molecular basis of the cytotoxic activity of Carnosine has not been known yet [17], however, some mechanisms have been suggested [18]. In the present work, to shed further light on the understanding of the cytotoxic basis of Carnosine structure, we investigated the effect of Carnosine and Carnosine analogues, synthesized by solid phase peptide synthesis (SPPS) method, on some human cancerous cell lines, including HepG2 and HT-29 using MTT assay and flow cytometry analysis. Safety profiles of these peptides were also studied using normal skin fibroblast cells. Carnosine analogues designed and studied in this work were three linear and two cyclic peptides.

2. Materials & Methods

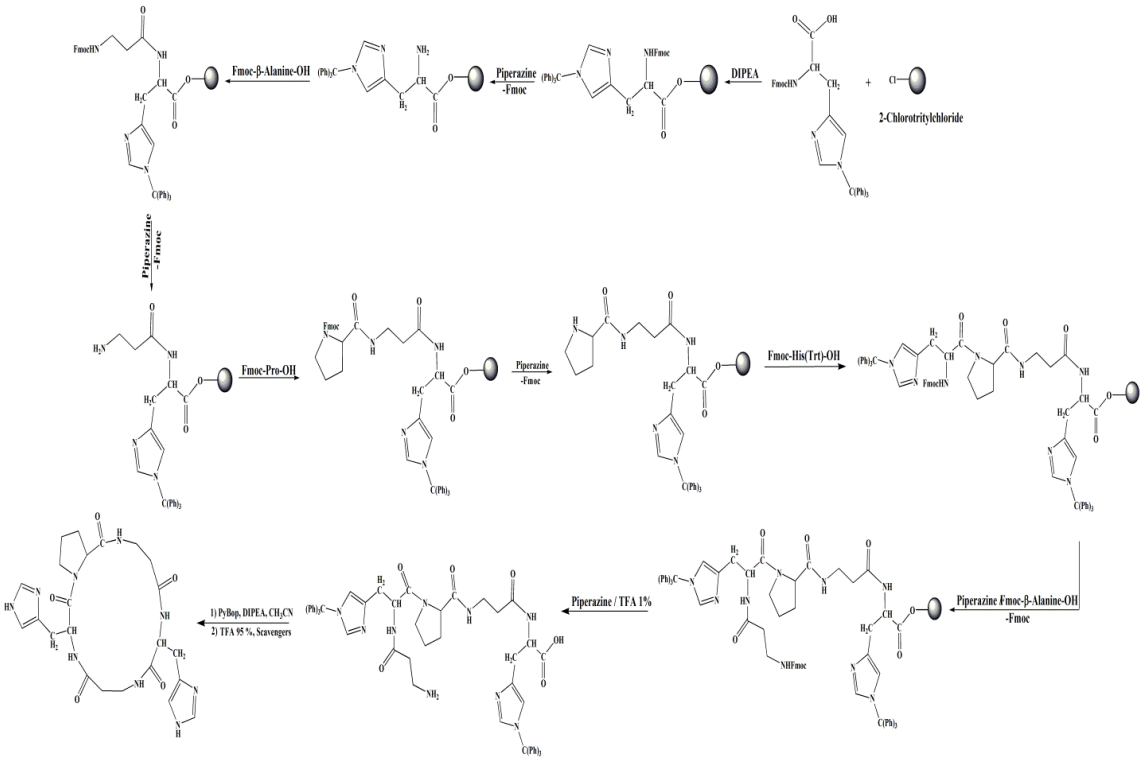
All Fmoc protected amino acids were purchased from Bachem, Switzerland. Reagents for peptide synthesis and solvents were bought from Merck Co, Germany. Resin was supplied from Santa Crus, USA. FT-IR spectra were recorded by a Nicolet Magna 550 Infra-Red spectrometer, USA. Mass spectra were collected employing a 6410 Agilant LCMS triple Quadruple mass spectrometer with electrospray ionization interface, USA. NMR 1H spectra were obtained from a 300MHz Bruker Avance DRX Nuclear Magnetic Spectrometer, Germany. For MTT assay, absorbance was measured by a spectrophotometer plate reader (Infinite® M200, TECAN, Switzerland). DNA fragmentation analysis was performed by a FACSCalibur flow cytometry equipment (Becton Dickinson, CA, USA), supplied with the flowing software 2.5.1.
Fluorescence measurement in lysosomal membrane integrity assay was implemented using a Shimadzu RF5000U fluorescence spectrophotometer, Japan. Cell lines were provided by Iranian Biological Resource Center, Tehran, Iran.

2.1. Chemistry

Detailed procedures for the synthesis of Carnosine and its peptide analogues were given in reference [19]. Briefly, 2- chlorotrityl chloride resin was employed to load fmoc amino acids, one by one, on the solid support using Fmoc strategy in peptide synthesis. Diisopropylethylamine (DIPEA) was used as a reagent for the first amino acid loading. CH3OH was then included in the reagents to cap remaining free tritylchloride groups of the resin. For all other amino acid attachments, O-(7-Azabenzotriazol-1-yl)-¬N, N, N′, N′- tetramethyluronium hexafluorophosphate (HATU), as a coupling reagent, and DIPEA were applied. After each amino acid attachment, piperazine was used to deprotect the loaded amino acid/ peptide on the resin. For linear peptide synthesis, peptides were cleaved from the resin by concentrated trifluoroacetic acid (TFA) plus scavengers, precipitated in diethyl ether, and then collected. In the cyclic peptide synthesis, after loading peptides on the resin, partial cleavage was applied by TFA 1% to obtain protected peptides. Cyclization was carried out in a large amount of acetonitrile using DIPEA and PyBOP (Benzotriazol-1-yloxy) tri-pyrrolidino-phosphonium hexafluorophosphate, as a coupling reagent. After cyclization, deprotected cyclic peptides were achieved by concentrated TFA containing scavengers. The peptides were then collected after precipitation in diethyl ether. Scheme 1 shows the whole procedure of the cyclopeptide synthesis of compound 1c.

2.2. In vitro cytotoxic activity

MTT assay and flow cytometry analysis were performed for all the synthesized peptides using HepG2 (Human Liver Cancer) and HT-29 (Human Colorectal Adenocarcinoma) cell lines and skin fibroblast cells, as a control normal cell line, with the same procedures as published in previous work [19].



Scheme 1. The solid-phase synthesis of linear and cyclic Carnosine analogue 1c, *cyclo(β-Ala-His-Pro-β-Ala-His) using* 2-chlorotrityl chloride resin.

2.3. Determination of lysosomal membrane integrity of HepG2 cells

Lysosomal damage using acridine orange (AO), as a probe, was assayed following incubation of HepG2 cells with the peptides samples. Aliquots of the cell suspension (0.5 mL), previously incubated with the peptides samples (10 µg/mL), were stained with AO (5 µM) and precipitated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then suspended in 2 mL fresh incubation medium. This washing process was carried out twice to remove the fluorescent dye from the media. The AO redistribution in the cell suspension was then measured fluorimetrically using fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths. Lysosomal membrane damage was determined as difference in redistribution of acridine orange from lysosomes into cytosol between the treated cells and the control cells at the time of preparation [20].

2.4. Statistical analysis

Graph Pad Prism 5 (Graph Pad Software, La Jolla, CA, USA) was used to analyze data by one way ANOVA, completed by Tukey's post hoc test. All data were shown as arithmetic mean ± S.E.M of at least triplicate measurements. Data significance was assumed as $P<0.05$.

3. Results and discussion

The protected linear Carnosine analogues
β-Ala-His(Trt)-β-Ala-His(Trt) **(1a)**

Yield: 78%; Yellow oily liquid; IR (KBr): ν (cm^{-1}) 3434.17 (NH), 2166.47 and 2555.28 (C=N in amino acid Histidine), 1664.92 (C=O amide), 1546.34 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 2.89-3.13 (m , CH_2), 4.09-5.03 (m, 2H, CH), 5.38-5.57 (m, CH_2), 7.31-7.43 (m, 12H, CH-Ar trityle of Histidine), 7.81-7.89 (t of t, 2H,

CH-Ar trityle of Histidine), 8.36-8.40 (d, 2H, NHCO); LC-MS (ESI) *m/z* Calcd for (1a) 918.434, Found *m/z* = 917.90000[M-H].

β-Ala-His(Trt)-Pro-β-Ala-His(Trt) **(2a)**

Yield: 75%; Yellow oily liquid; IR (KBr): ν (cm^{-1}) 3428.45 (NH), 2167.10 and 2559.85 (C=N in amino acid Histidine), 1666.66 (C=O amide), 1546.62 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 3.38-3.42 (m, 15H, CH_2), 5.40-5.71 (s, br, 2H, NH_2), 7.29-7.32 (s, br, CH=C Histidine), 7.39-7.42 (s, br, CH=N Histidine), 7.50-7.61 (m, 24H, CH-Ar trityle of Histidine), 7.95-8.05 (t of t, 2H, CH-Ar trityle of Histidine), 8.69-8.73 (d, 3H, NHCO); LC-MS (ESI) *m/z* Calcd for (2a) 1015.49, Found *m/z* = 1014.00000[M-H].

Pro-β-Ala-His(Trt)-β-Ala-His(Trt) **(3a)**

Yield: 75%; Yellow oily liquid; IR (KBr): ν (cm^{-1}) 3451.30 (NH), 2164.07 and 2551.33 (C=N in amino acid Histidine), 1664.07 (C=O amide), 1545.98 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 3.24-3.29 (m, 15H, CH_2), 7.50-7.55 (m, 2H, CH=C Histidine), 7.55-7.60 (m, 2H, CH=N Histidine), 7.94-8.04 (t of t, 2H, CH-Ar trityle of Histidine), 8.28-8.40 (s, br, 24H CH-Ar trityle of Histidine), 8.56-8.69 (s, br, 4H, NHCO); LC-MS (ESI) *m/z* Calcd for (3a) 1015.49, Found *m/z* = 1014.00000[M-H].

β-Ala-His(Trt) **(4a)**

Yield: 80%; Yellow oily liquid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2165.88 and 2556.05 (C=N in amino acid Histidine), 1664.42 (C=O amide), 1546.45 (C=C in amino acid Histidine), 600-800 (out-o-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 7.50-7.55 (m, 1H, CH=C Histidine), 7.55-7.60 (m, 1H, CH=N Histidine), 7.94-8.04 (t of t, 1H, CH-Ar trityle of Histidine), 8.28-8.40 (s, br, CH-Ar trityle of Histidine), 8.56-8.69 (s, br, 1H, NHCO); LC-MS (ESI) *m/z* Calcd for (4a) 468.228, Found *m/z* = 467.30000[M-H].

The deprotected linear Carnosine analogues

β-Ala-His-β-Ala-His **(1b)**

Yield: 78%; White solid; IR (KBr): ν (cm^{-1}) 3440.64 (NH), 2163.68 and 2565.33 (C=N in amino acid Histidine), 1666.61 (C=O amide), 1546.72 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 2.54-2.60 (s, br, 4H, CH_2-CO β-alanine), 2.66-2.71 (s, br, 4H, CH_2 Histidine and CH_2-NH_2 β-alanine), 3.11-3.22 (m, 2H, CH_2 Histidine), 3.42-3.47 (s, br, CH_2-NH β-alanine), 6.33-6.56 (m, 1H, CH Histidine), 6.36-6.48 (t, 2H, NH_2), 6.72-6.87 (m, 1H, CH Histidine), 7.65-7.79 (t, 2H, CH=C Histidine), 8.14-8.29 (t, 1H, NHCO), 8.51-8.71 (d, 2H, CH=N Histidine), 16.49-16.84 (s, 1H, NH imidazole cyclic Histidine); LC-MS (ESI) *m/z* Calcd for (1b) 434.215, Found *m/z* = 432.90000[M-H].

β -Ala-His-Pro-β-Ala-His **(2b)**

Yield: 75%; White solid; IR (KBr): ν (cm^{-1}) 3439.64 (NH), 2164.39 and 2556.76 (C=N in amino acid Histidine), 1665.21 (C=O amide), 1546.14 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 0.88-0.96 (t, 2H, CH_2 proline), 3.18-3.27 (d of d, 1H, CH_2 Histidine), 3.49-3.53 (m, 1H, CH_2 Histidine or s, 1H, NH Proline), 6.48-6.59 (d of d, 2H, CH=C Histidine), 6.82-6.90 (t, 1H, CH-N Proline), 7.67-7.85 (t, 2H, CH Histidine), 8.18-8.32 (t, 1H, NHCO), 8.57-8.79 (d, 2H, CH=N Histidine), 16.67-16.77 (s, 1H, NH imidazole cyclic Histidine), ^{13}C NMR ($CDCl_3$-d_6,75 MHz,) δ = 115.25, 119.20 (C=C Histidine), 126.98, 129.26 (C-Histidine), 141.65, 145.44 (C=N), 156.97, 161.29, 161.77, 162.24 (C=O), 170.64 (COOH); LC-MS (ESI) *m/z* Calcd for (2b) 531.268, Found *m/z* = 529.90000[M-H].

Pro-β-Ala-His-β-Ala-His **(3b)**

Yield: 75%; White solid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2165.77 and 2569.91 (C=N in amino acid Histidine), 1666.21 (C=O amide), 1546.57 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 1.07-1.13 (t, 2H, CH_2 proline), 1.15-1.19 (m, 2H, CH_2 proline), 1.87-2.03 (s, br, 1H, NH proline), 2.72-2.81 (m, 1H, CH_2 Histidine), 2.82-2.92 (m, 1H, CH_2 Histidine), 3.34-3.44 (d of d simillar q, *J*=7.02 Hz, CH-NH proline), 3.63-3.74 (m, 1H, CH_2 of each Histidine), 6.65-6.75 (m, 1H, CH Histidine), 7-7.08 (m, 1H, CH Histidine), 7.71-7.92 (t, 2H, CH=C Histidine), 8.22-8.40 (t, 1H, NHCO), 8.69-8.89 (d, 2H, CH=N Histidine), 17.37-17.55 (s, 1H, NH imidazole cyclic Histidine); LC-MS (ESI) *m/z* Calcd for (3b) 531.268, Found *m/z* = 529.90000[M-H].

β-Ala-His (Carnosine) **(4b)**
Yield: 80%; White solid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2164.07 and 2561.28 (C=N in amino acid Histidine), 1666.62 (C=O amide), 1547.02 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 7.80-7.89 (t, 1H, CH=C Histidine), 8.28-8.37 (t, 1H, NH β-alanine), 8.77-8.85 (d, 1H, CH=N Histidine), 17.51-17.64 (s, 1H, NH imidazole cyclic Histidine), ^{13}C NMR ($CDCl_3$-d_6,75 MHz,) δ = 115.41 (C=C Histidine), 129.38 (C-Histidine), 144.52 (C=N), 162.48 (C=O), 174.39 (COOH); LC-MS (ESI) *m/z* Calcd for (4b) 226.119, Found *m/z* = 224.90000[M-H].

The deprotected cyclic Carnosine analogues
Cyclo(β-Ala-His-Pro-β-Ala-His) **(1c)**
Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3438.45 (NH), 1673.24 (C=O amide), 1537.69 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 4.01-4.22 (m, 1H, CH-N proline), 4.35-4.48 (m, 1H, CH-NH Histidine), 4.79-4.84 (m, 1H, CH-NH Histidine), 6.81-6.88 (d, 2H, CH=C Histidine), 7.13-7.21 (t, 1H, NH β-alanine), 8.26-8.33 (d, 1H, NH Histidine), 8.42-8.51 (t, 1H, NH β-alanine), 8.89-9.16 (m, 2H, CH=N Histidine); LC-MS (ESI) *m/z* Calcd for (1c) 513.268, Found *m/z* = 514.3$[M+H]^+$.
Cyclo(Pro-β-Ala-His-β-Ala-His) **(2c)**

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3415.11 (NH), 1673.93 (C=O amide), 1544.00 (C=C in amino acid Histidine), 600-800 (out-of-plane bending vibration C-H in amino acid Histidine); ^{1}H NMR ($CDCl_3$-d_6, 300 MHz,): δ = 2.39-2.56 (m, 1H, CH_2 Histidine or CH_2 proline), 2.60-2.74 (m, 1H, CH_2 Histidine or CH_2 proline), 2.95-3.21 (m, 1H, CH_2 Histidine), 3.38-3.43 (m, 2H, CH_2-N proline), 3.46-3.70 (m, 1H, CH_2 Histidine), 3.97-4.11 (m, 1H, CH-NH Histidine), 4.27-4.43 (m, 1H, CH-NH Histidine), 4.68-4.72 (m, 1H, CH proline), 7.43-7.54 (d of d, 2H, CH=C Histidine), 8.03-8.14 (t, 1H, NH β-alanine), 8.68-8.77 (d, 2H, CH=N Histidine), 8.91-9 (d, 1H, NH Histidine); LC-MS (ESI) *m/z* Calcd for (2c) 513.268, Found *m/z* = 514.3$[M+H]^+$.

MTT assay results
Table 1 shows the IC_{50} results of HepG2 and HT-29 cancer cell lines, affected by the linear and cyclic peptide analogues of Carnosine (values ranging from 9.18 to 16.23 µg/mL). IC_{50} results for 5-flurouracil, as a standard toxic drug, are given for comparison. Table 2 contains viability percentage data for the three cell lines; skin fibrobast, HepG2, and HT-29 cancer cells.

Table 1. IC_{50} values (µg/mL) for toxicity of the peptides on HepG2 and HT-29 cells in MTT assay. Values are presented from the corresponding control (*P <0.05, *** P <0.001).

Entry	IC_{50} Cell line HepG2	IC_{50} Cell line HT-29
1b	9.5 ± 0.1583 ***	10.73 ± 0.0014 ***
2b	9.33 ± 0.0919 *	10.11 ± 0.0028 ***
3b	10.21 ± 0	14.23 ± 0.0035 ***
4b (Carnosine)	10.01 ± 0.0063 ***	16.23 ± 0.0021 ***
1c	9.18 ± 0.17 ***	9.64 ± 0.11 ***
2c	9.21 ± 0.42 ***	10.53 ± 0.11 ***
5-Fluorouracil (Standard drug)	3.16	6.08

Table 2. Validity percentage of the cell lines of skin fibroblast, HepG2 and HT-29 exposed to the linear and cyclic Carnosine analogues, in MTT assay

Entry	Viability percentage (Fibroblast)	Viability percentage (HepG2)	Viability percentage (HT-29)
1b	70.93	83.8	13.81
2b	95.25	99.04	27.63
3b	70.72	3.28	13.97
4b	94.98	8.01	27.79
1c	75.39	9.2	11.76
2c	78.87	19.31	12.44
5-FU	8.64	3.22	9.28
control	100	100	100

Table 3. Apoptosis percentage of the cell lines HepG2 and HT-29 exposed to the linear Carnosine analogues, obtained in flow cytometry analysis.

Entry	Apoptosis percentage (HepG2)	Apoptosis percentage (HT-29)	Viability percentage (HepG2)	Viability percentage (HT-29)
1b	84.56	89.63	12	0.67
2b	89.18	90.21	0.43	0.33
3b	99.68	67.52	0.33	32.43
4b	86.69	84.65	7.86	0.42
1c	99.46	99.82	0.55	0.18
2c	99.55	99.74	0.45	0.26

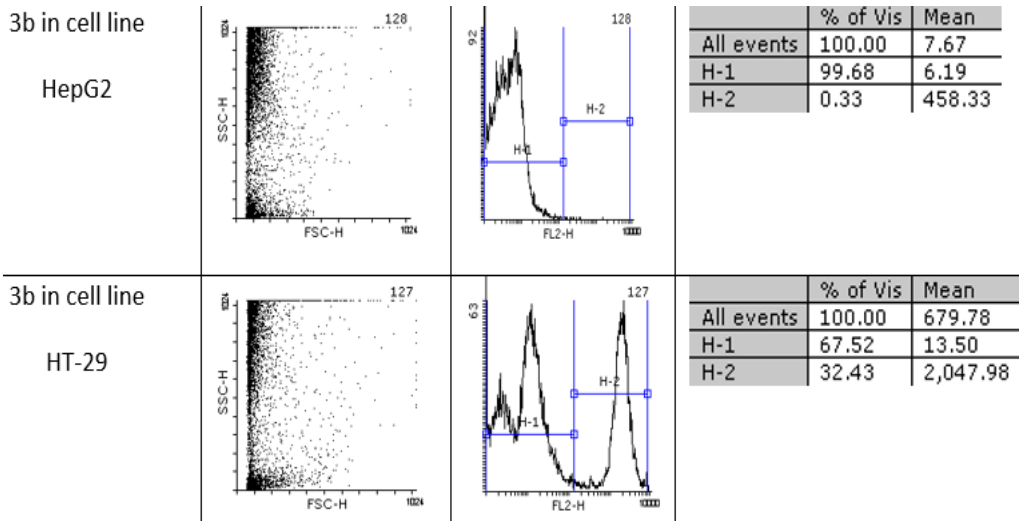


Figure1. Flow cytometry results obtained from the peptide-treated HepG2 and HT-29 cells. The left: Dot plots show the relative size of the cells, in the horizontal axis by the forward scatter (FSC) parameter and the vertical axis demonstrates the granularity of the cells by side scatter (SSC) parameter. The right: Histograms show the fluorescence intensity of the peptide-treated cells in the FL-2 channel scaled on the PI emission wavelength [22].

Flow cytometry analysis results

Table 3 represents flow cytometry results for Carnosine and its linear and cyclic peptides analogues. All the histograms achieved are included in the supplementary file. Fig.1 shows only the histogram of 3b as an example [21].

Lysosomal membrane integrity results

Lysosomal damage was assayed using acridine orange, as a probe, through the peptide-treated HepG2 cells. Values are presented as mean ± SD of three independent experiments, performed for determination of lysosomal membrane integrity of HepG2 cells (Fig 2). As shown in Fig 2, linear peptide 1b caused the highest absorption of acridine orange dye in the cells.

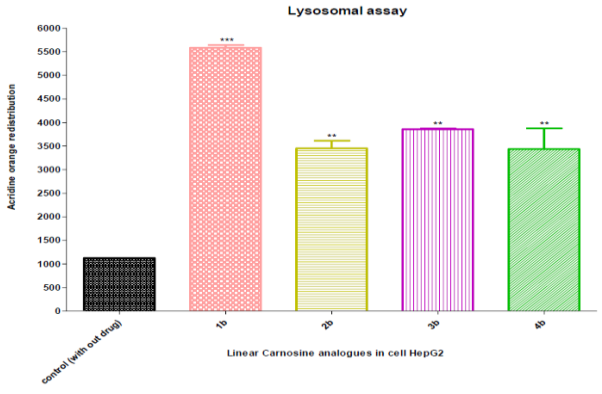


Figure 2. Determination of lysosomal membrane integrity assay on HepG2 cells. The values show acridine orange absorbance of the damaged lysosomes of the peptide-treated HepG2 cells and are presented as mean ± SD of three independent experiments (n= 3). The stars show the values, which are significantly different from the corresponding control (**P <0.01, *** P <0.001).

4. Discussion

The synthesized linear and cyclic Carnosine analogues demonstrated cytotoxic activity on the cells of HepG2 (Human Liver Cancer Cell Line) and HT-29 (Human Colorectal Adenocarcinoma Cell Line) with IC_{50} values in the range of 9.18 to 16.23 µg/mL. 5-Fluorouracil (5-FU) was employed as a standard cytotoxic drug in MTT assay. Among the peptides, compounds 1b and 2b showed a high viability percentage (low toxicity) on the liver cancerous cell line of HepG2, whereas these and other synthesized compounds presented a low viability percentage (high toxicity) on the colon cancerous cell line of HT-29 (see Table 2). In addition, compounds 3b, 1c, and 2c showed high toxicity effects (low viability percentages) on the both cell lines of HepG2 and HT-29, comparable with Carnosine (compound 4b). In this regard, the peptides 3b and 1c were the most effective cytotoxic agents. Moreover, compound 3b showed toxicity on HepG-2 cells as high as 5-Fu did. Table 3 and Fig.1 show the results, obtained from flow cytometry analysis of the peptides-treated cancerous cell lines. The signals, obtained in H-1 area, mean that propidium iodide (PI) was able to enter the cells, bind to DNA, and lead to apoptosis, whereas the signals, appeared in H-2 area, indicate that the cancerous cells, treated with peptides, remained intact (20). According to Table 3, all the synthesized peptides could induce apoptosis on the cancerous cell lines. The peptides 3b and 1c showed pronounced toxic effects on the cancerous cell line of HepG2 compared with the other peptides, which can be attributed to better cell permeability of the synthesized peptides on HepG2 cells. In this view, HT-29 cells were more resilient towards these peptides. As our results showed, it was also true for 5-FU. In overall, among the synthesized peptides, compound 3b gave the highest cytotoxic activity on HepG2, similar to 5-FU. Moreover, compound 3b showed a much higher safety profile on skin normal cells compared to 5-FU. Our results also indicated that Carnosine dimers with a proline amino acid, connected with the two monomers, give compounds with a profound variation in their anticancer activities on cancerous cells. This result is in accordance with our previous study on the synthesized inverse Carnosine peptide analogues [19].

In this work, MTT results for compounds 1b and 2b were not consistent with apoptosis of HepG-2 cells, found using flow cytometry analysis, whereas for the other peptide analogues, the MTT results were in agreement with the data of flow cytometry assay. In order to find cytotoxic activity mechanism of the linear peptides 1b and 2b, lysosomal membrane integrity experiment was employed. As shown in Fig. 2, HepG-2 cells absorbed more acridine orange dye after exposure to the peptide 1b than the other peptides compared with the untreated cells (the control group). This means that lysosomes of HepG-2 cells were more damaged, and thus resulted in more release of cathepsin, which in turn, by binding to acridine orange gave higher absorption intensity, detected by the instrument. The more release of cathepsin means that more apoptosis has occurred [23]. Therefore, the results of lysosomal membrane integrity experiment suggest that the mechanism of apoptotic action of the linear peptide 1b is mediated through the lysosomal damage more than the mitochondrial pathway. For compound 2b, the mechanism of cytotoxicity remained to be determined.

5. Conclusion

Cytotoxic effect was observed for the linear and cyclic Carnosine peptide analogues on HepG2 and Ht-29 cell lines with varying amounts. In MTT assay, compounds 3b and 1c caused low viability of the both cell lines. Moreover, compound 3b showed the lowest cell viability result (i.e., the highest cytotoxic activity) for the cell line HepG2 and this activity was similar to that of 5-Fu on this cell line. In addition, compound 3b showed a much higher safety profile on skin normal cells compared to 5-FU did. Our results also indicated that Carnosine dimers with a proline amino acid, connected with the two monomers, could give compounds with a profound variation in their anticancer activities on cancerous cells.

According to our experiments, in overall, compound 3b can be a good candidate for the further development of safe anticancer agents. On the other hand, the considerable toxicity of cyclic peptide analogues of Carnosine, 1c and 2c, on the cancerous cell lines along with their high safety profiles on the normal skin cells, make them attractive for further works on finding anticancer agents with peptide structure, giving better physicochemical properties for oral administration.

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Conflict of interest

The authors declare that there is no conflict of interest on this research work.

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