

The Cytotoxicity of Analogues Carnosine Dipeptide on Human Glioblastoma Multiforme

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Abstract: Background & Aims: The present study evaluated the effect of some synthesized linear and cyclic Carnosine analogues on the cells of human glioblastoma multiforme (GBM). The purpose of this research was to study the exposure of Carnosin analogues on astrocytoma using several experiments. Methods: The mass spectral measurements were performed on a 6410 Agilent LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface. All tests were carried out six times. The concentration used for peptides (10 µg/mL) was selected based on MTT assay. The effect of peptides on the activity of SDH was assayed by MTT test. 100 µL mitochondrial suspension from GBM and normal groups were incubated with applied concentration of peptides (10 µg/mL) at 37°C for 30 min. DCFH (final concentration, 10 µM) was added to cells of both groups and incubated at 37°C for 15 min. The fluorescence intensity of DCFH which is an indicator of ROS concentration was then assayed by a Shimadzu RF-5000U fluorescence spectrophotometer. The cationic fluorescent dye, rhodamine 123 (concentration 10 µM), from mitochondria into the cytosol have been used for the determination of MMP collapse. Mitochondria from astrocytoma and normal astrocyte groups were suspended in corresponding assay buffer and incubated at 37°C with 10 µg/mL peptides. The release was assayed by the Quantikine Cytochrome c. Carnosine peptide analogues on the activation of caspase-3 in the mitochondria isolated from GBM and normal groups using Sigma's caspase-3 colorimetric assay kit. Results: Our study showed that a various range of toxicity on GBM cells was resulted by the linear and cyclic Carnosine analogues using MTT assay. Also, applying peptides on the mitochondria of GBM cells, a raise of mitochondrial reactive oxygen species (ROS) level, mitochondrial swelling, mitochondrial membrane potential ($\Delta\psi_m$) collapse, release of cytochrome c and caspase-3 activation of the affected mitochondria were detected. Conclusion: Based on the overall results, cyclic Carnosine analogues, especially compound 1c [Cyclo-(β -alanine-His-Pro- β -alanine-His)] showed more toxic activity than linear Carnosine analogues, which would be supporting to develop Carnosine cyclic peptide analogues as new anticancer and complementary therapeutic agents for the treatment of glioblastoma.

Keywords: Human Glioblastoma, Cytotoxicity, Mitochondria, Linear and Cyclic Peptides

1. Introduction

Cancerous cells are introduced by uncontrolled growth into nearby tissues. The cell division and growth are caused by genetic mutations that either turn on oncogenes or silent tumor suppressor genes. The continuation of this type of genetic destruction over time can lead to the progressive

transformation of the cells and survival of uncontrolled cells populations that can form tumors. Astrocyte is a type of glial cell in the central nervous system (CNS). They are also known as astrocytic glial cells. Glial cells, within CNS support and protect neurons. when glial cells become malignant, Glial tumors or gliomas increased. Astrocytomas may be categorized by their rate of growth: lowly grade (slow

growth, grade I and II), moderate grade (moderate growth, grade III) and highly grade (rapid growth, grade IV) [1-3]. Lowly and highly grade tumors are more common in children and adults, respectively [4]. Astrocytomas can be eventually introduced as grade IV astrocytoma, the most aggressive malignant brain tumor in humans, also known as glioblastoma multiforme (GBM) [5]. The diagnosis is typically detected with a combination of CT scan, MRI scan, and tissue biopsy [6]. Temozolamide is used to treat certain types of brain tumors. This drug is prescribed for adults who have recently been diagnosed with brain cancer called glioblastoma multiforme. At first, temozolamide is used together with radiation therapy to treat this type of tumor, and then it is used alone. [7, 8]. Anticancer drugs, however, have side effects on normal cells. Possible functions of the dipeptide Carnosine (β -alanyl-L-histidine) include buffer, anti-oxidant, antiglycation, aldehyde and carbonyl scavenger, chelator of metal ion, immuno-stimulant, wound healing and neurotransmitter agent [9-18]. In 1986, it was reported that Carnosine can inhibit growth of tumor cells [19]. In recent years, Carnosine was shown to inhibit growth of cultured glioblastoma cells [20], most probably via effects on glycolysis [21, 22]. Other works investigated and researched that Carnosine could suppress tumor growth in animals [23, 24]. Carnosine analogues designed and studied in this work were seven linear and six cyclic peptides.

2. Materials and Methods

Trifluoroacetic acid (TFA), Triisopropylsilane (TIS), Fmoc amino acids and coupling reagents O-(7-Azabenzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate (HATU), (benzotriazol-1-yloxy) - tripyrrolidinophosphonium hexafluorophosphate (PyBop), were supplied by Merck. Solvents like acetonitrile (MeCN), Piperazine, *N,N*-diisopropylethylamine (DIPEA), Diethylether, Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), and methanol (MeOH) were purchased from Merck. 2-Chlorotriethylchloride resin (1% DVB, 200-400 mesh, 1mmol/g) was purchased from Aldrich, UK. Flash column chromatography were carried out using silica Gel 60 (particle size 0.04–0.06mm / 230–400 mesh). The mass spectral measurements were performed on a 6410 Agilent LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface.

Experimental

Isolation of mitochondria from human glioblastoma multiforme

Mitochondria were obtained from the tumor tissue of patients with histopathologically confirmed glioblastoma. The freshly removed tumor tissue was suspended in sterile culture tubes and immediately transferred to the laboratory. The tissue was washed with PBS (phosphate buffered saline) to be cleaned from blood and cauterized tissue. The cleaned tissue was minced by a scalpel blade. Differential centrifugation was used for isolation of mitochondria from glioblastoma [20]. All tests were carried out six times. The concentration used for

peptides (10 μ g/mL) was selected based on MTT assay.

Determination of Cytotoxicity

Astrocytes from grade IV human astrocytoma cells (1×10^7 /well) were transferred into 96-well plates and treated with 10 μ g/mL concentration of Carnosine analogues for 6h (cells were maintained in RPMI 1640, supplemented with 10% FBS and antibiotics (50 U/mL of penicillin and 50 μ g/mL streptomycin)). After treatment, MTT (5mg/mL in RPMI 1640) reagent was added to each well. After 4 h, the reaction was stopped by addition of 50 μ L DMSO.

Succinate Dehydrogenase (SDH) activity assay

The effect of peptides on the activity of SDH was assayed by MTT test. Briefly, to carry out this assay, 100 μ L mitochondrial suspension from GBM and normal groups were incubated with applied concentration of peptides (10 μ g/mL) at 37°C for 30 min. Then, 50 μ L of MTT was added to the medium and incubated at 37°C for 30 min. Finally, 50 μ L DMSO was added to the medium to dissolve product of formazan crystals and the absorbance at 570 nm was assayed with an ELISA reader (Tecan, Rainbow Thermo, Austria).

ROS formation assay

Dichloro-dihydro-fluorescein diacetate as DCFH-DA was used for ROS measurement. Astrocyte and astrocytoma cells isolated from tumor tissue of brain were suspended in respiration buffer (0.32mM sucrose, 10 mM Tris, 20mM Mops, 50 μ M EGTA, 0.5Mm $MgCl_2$, 0.1mM KH_2PO_4 , and 5 mM sodium succinate). Then, DCFH (final concentration, 10 μ M) was added to cells of both groups and incubated at 37°C for 15 min. The fluorescence intensity of DCFH which is an indicator of ROS concentration was then assayed by a Shimadzu RF-5000U fluorescence spectrophotometer at $EX\lambda = 488$ nm and $EM\lambda = 527$ nm [25, 26].

Mitochondria membrane potential (MMP) assay

The cationic fluorescent dye, rhodamine 123, from mitochondria into the cytosol have been used for the determination of MMP collapse. Rh 123 (10 μ M) was added to the astrocyte and astrocytoma cells suspensions (1000 μ g mitochondrial protein/mL) in MMP assay buffer (220 mM sucrose, 68 mM, D-mannitol, 10 mM KCl, 5 mM KH_2PO_4 , 2 mM $MgCl_2$, 50 μ M EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μ M rotenone). The cytosolic Rh 123 fluorescence intensity which represents the redistribution of the dye from mitochondria into the cytoplasm was determined using Shimadzu RF-5000U fluorescence spectrophotometer at the $EX\lambda = 490$ nm and $EM\lambda = 535$ nm [26].

Mitochondrial swelling assay

Mitochondria from astrocytoma and normal astocyte groups were suspended in corresponding assay buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, and 1 μ M rotenone) and incubated at 37°C with 10 μ g/mL peptides. The absorbance was measured at 540 nm on 15-min intervals using an ELISA reader (Tecan, Rainbow Thermo). A decrease in the absorbance indicates an increase in mitochondrial swelling [25].

Cytochrome c release

The release was assayed by the Quantikine Cytochrome c Immunoassay kit provided by R & D Systems, Inc.

(Minneapolis, MN, USA).

Caspase-3 activity assay

Carnosine peptide analogues on the activation of caspase-3 in the mitochondria isolated from GBM and normal groups using Sigma's caspase-3 colorimetric assay kit (Sigma-Aldrich, Taufkirchen, Germany).

3. Results

3.1. The Protected and Deprotected Carnosine Analogues

Synthesis of β -alanine-His (1)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (1a) 468.22, Found m/z = 469.5(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (1b) 226.11, Found m/z = 227.2(M+1).

Synthesis of β -alanine-His- β -alanine-His (2)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (2a) 918.42, Found m/z = 919.6 (M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (2b) 434.2, Found m/z = 435.5(M+1).

Synthesis of β -alanine-His-Pro- β -alanine-His (3)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (3a) 1015.48, Found m/z = 1016.6(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (3b) 531.26, Found m/z = 532.3(M+1).

Synthesis of Pro- β -alanine-His- β -alanine-His (4)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (4a) 1015.48, Found m/z = 1016.6(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (4b) 531.26, Found m/z = 532.3(M+1).

Synthesis of His- β -alanine- β -alanine-His (5)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (5a) 918.42, Found m/z = 919.6(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (5b) 434.2, Found m/z = 435.5(M+1).

Synthesis of His- β -alanine-Pro- β -alanine-His (6)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (6a) 1015.48, Found m/z = 1016.6(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (6b) 531.26, Found m/z = 532.3(M+1).

Synthesis of Pro-His- β -alanine- β -alanine-His (7)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (7a) 1015.48, Found m/z = 1016.6(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (7b) 531.26, Found m/z = 532.3(M+1).

Synthesis of Pro- β -alanine-His-His- β -alanine (8)

a) Synthesis of protected peptide

LC-MS (ESI) m/z Calcd for (8a) 1015.48, Found m/z = 1016.6(M+1).

b) Synthesis of deprotected peptide

LC-MS (ESI) m/z Calcd for (8b) 531.26, Found m/z = 532.3(M+1).

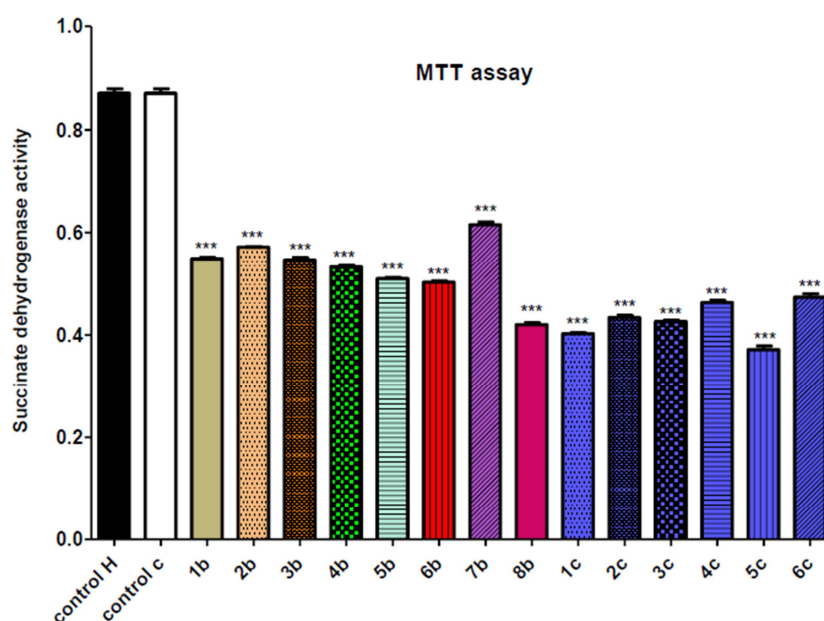


Figure 1. Cytotoxicity assay. Cytotoxic effects of linear and cyclic Carnosine analogues on astrocyte from normal group and comparative effects of linear and cyclic Carnosine analogues at 10 μ g/mL on the cytotoxic of human glioblastoma. The cells were treated with peptides for 6 h, and cytotoxic effects were determined by MTT assay. Control H represents normal (astrocyte) cells and control c shows glioblastoma (astrocytoma cells). Data represent the mean \pm SD of three replicates in three independent experiments ($n=3$). The stars show that values were significantly different from the corresponding control (***) $p < 0.001$.

3.2. The Deprotected Cyclic Carnosine Analogues

Synthesis of Cyclo-(β-alanine-His-Pro-β-alanine-His) (1c)

LC-MS (ESI) m/z Calcd for (1c) 513.26, Found m/z = 258.2 ($\frac{M+2}{2}$).

Synthesis of Cyclo-(Pro-β-alanine-His-β-alanine-His) (2c)

LC-MS (ESI) m/z Calcd for (2c) 513.26, Found m/z = 258.2 ($\frac{M+2}{2}$).

Synthesis of Cyclo-(His-β-alanine-Pro-β-alanine-His) (3c)

LC-MS (ESI) m/z Calcd for (3c) 513.26, Found m/z = 258.2 ($\frac{M+2}{2}$).

Synthesis of Cyclo-(Pro-His-β-alanine-β-alanine-His) (4c)

LC-MS (ESI) m/z Calcd for (4c) 513.26, Found m/z = 258.2 ($\frac{M+2}{2}$).

Synthesis of Cyclo-(β-alanine-His-Pro-His-β-alanine) (5c)

LC-MS (ESI) m/z Calcd for (5c) 513.26, Found m/z = 258.2 ($\frac{M+2}{2}$).

Synthesis of Cyclo-(Pro-β-alanine-His-His-β-alanine) (6c)

LC-MS (ESI) m/z Calcd for (6c) 513.26, Found m/z = 258.2 ($\frac{M+2}{2}$).

3.3. Effect of Carnosine Analogues in Cytotoxicity

The MTT test after 6h incubation of cells with different concentrations of analogues. The IC_{50} value obtained with the mean of the three independent experiments for Carnosine analogues was 10 μ g/mL. Figure 1 shows the human glioblastoma viability decreased by Carnosine analogues after 6 hr treatment.

3.4. Effect of Carnosine Analogues in Producing ROS

Linear Carnosine analogues 2b, 6b and at 30 min and 1-6b and 8b at 60 min showed significant difference in ROS production compared with the GBM control group (Figure 3). ROS generation for all the cyclic Carnosine analogues (1-6c) was significant in comparison with the GBM group at two different times.

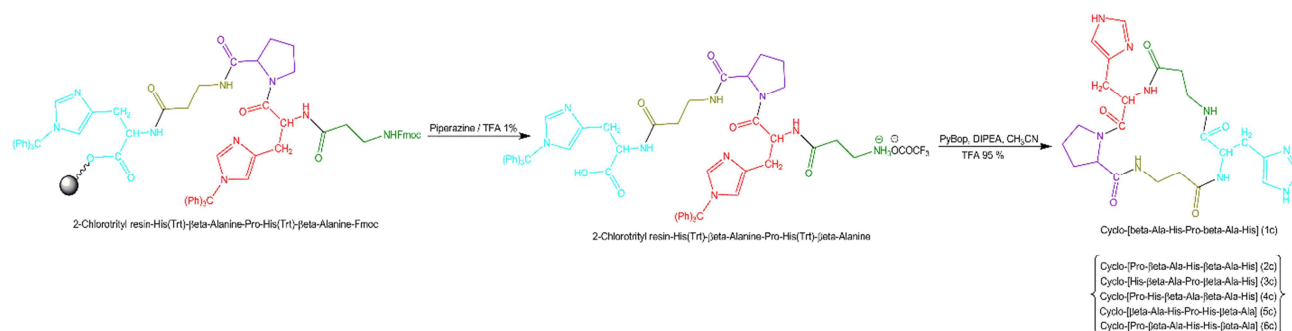


Figure 2. The synthesis of cyclic Carnosine analogues.

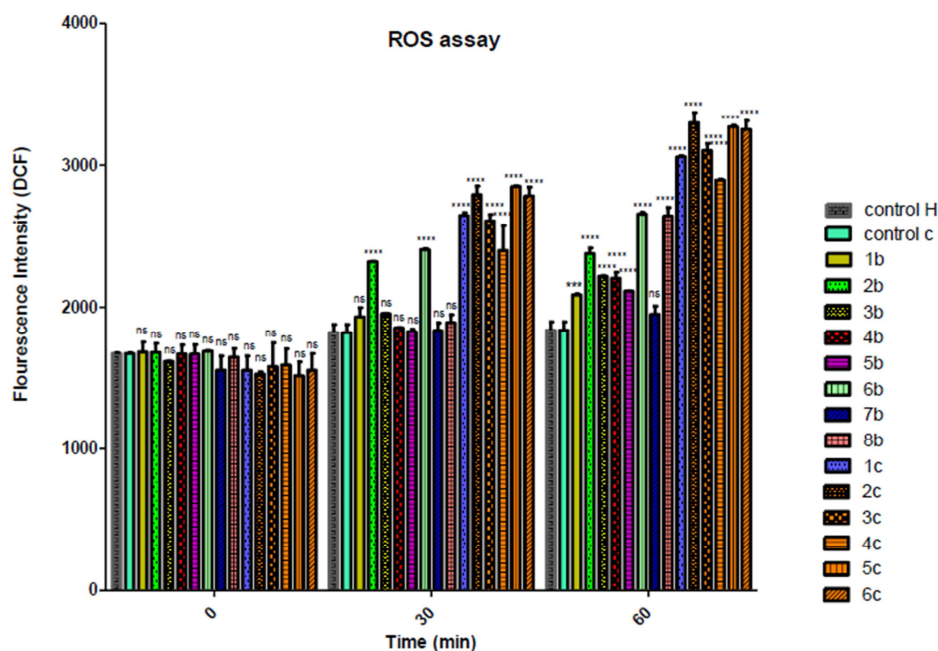


Figure 3. ROS formation assay. The effect of linear and cyclic Carnosine analogues at 10 μ g/mL on ROS generation at different times (0, 30, and 60 min) in astrocyte and human glioblastoma cells isolated from tumor tissue of brain. Data represent the mean \pm SD of three replicates in three independent experiments ($n=3$). The two-way ANOVA test was performed. *** and **** significantly different from the corresponding control ($p<0.001$ and $p<0.0001$, respectively).

3.5. Effects of Carnosine Analogues in Collapsing MMP

Results in Figure 4 show that, all Carnosine analogues have significant effect on increasing fluorescein intensity (i.e., MMP reduction) at 30 and 60 min in comparison with the GBM group.

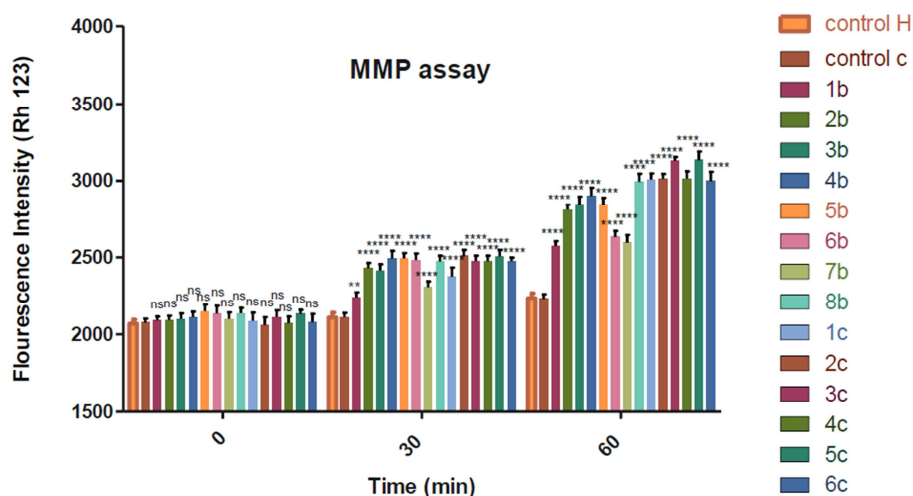


Figure 4 Mitochondrial membrane potential (MMP) assay. The effect of 10 $\mu\text{g/mL}$ of linear and cyclic Carnosine analogues on collapse MMP at different times (0, 30 and 60 min) in astrocyte and human glioblastoma cells isolated from tumor tissue of brain. Data represent the mean \pm SD of three replicates in three independent experiments ($n=3$). The two-way ANOVA test was performed. ** and **** significantly different from the corresponding control ($p<0.01$ and $p<0.0001$, respectively).

3.6. Effect of Carnosine Analogues on Mitochondrial Swelling

As Figure 5 shows, compounds 2b and 6b at 30 min have mitochondrial swelling effects with significant in comparison

with GMB group. At 60 min, all linear analogues with the exception of 7b and all cyclic analogues show significant difference in swelling effect, compared with the GBM group.

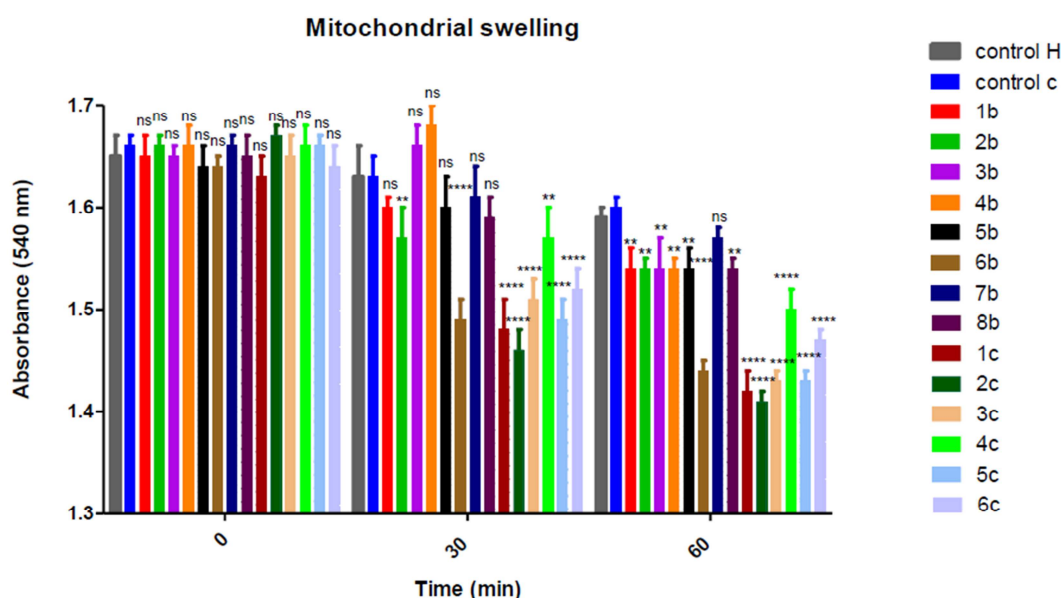


Figure 5. Mitochondrial swelling assay. The effect of 10 $\mu\text{g/mL}$ of linear and cyclic Carnosine analogues on mitochondria swelling at different times (0, 30 and 60 min) in astrocyte and human glioblastoma cells isolated from tumor tissue of brain. Data represent the mean \pm SD of three replicates in three independent experiments ($n=3$). The two-way ANOVA test was performed. ** and **** show a significant difference in comparison with the corresponding control ($P<0.01$ and $P<0.0001$, respectively).

3.7. Effect of Carnosine Analogues on Cytochrome c Release

As shown in Figure 6, Carnosine analogues, with the exception of 6b and 7b, induced a significant release of Cytochrome c (with various P values) from glioblastoma mitochondria in comparison with that from mitochondria of the GBM group.

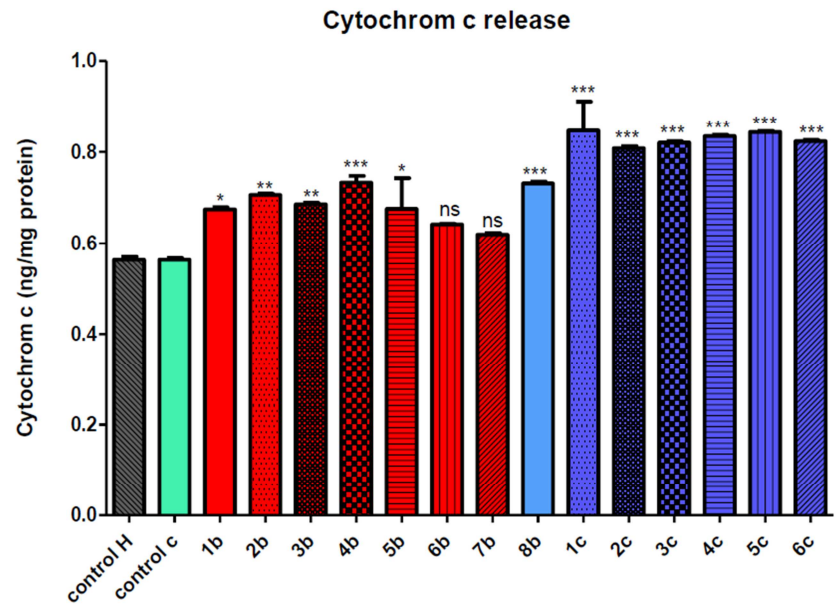


Figure 6. Cytochrome *c* release assay. The amount of expelled cytochrome *c* from astrocyte and glioblastoma fractions into the suspension buffer was determined using cytochrome *c* ELISA kit. Data represent the mean \pm SD of three replicates in three independent experiments ($n=3$). The one-way ANOVA test was performed. *, ** and *** show significant difference in comparison with the corresponding control ($p<0.05$, $p<0.01$ and $p<0.001$, respectively).

3.8. Effect of Carnosine analogues on Caspase-3 Activation

Peptides at concentration of 10 $\mu\text{g/mL}$ induced a significant ($P<0.001$) increase in activity of caspase-3 only in the Mitochondria isolated from the GBM group (see Figure 7).

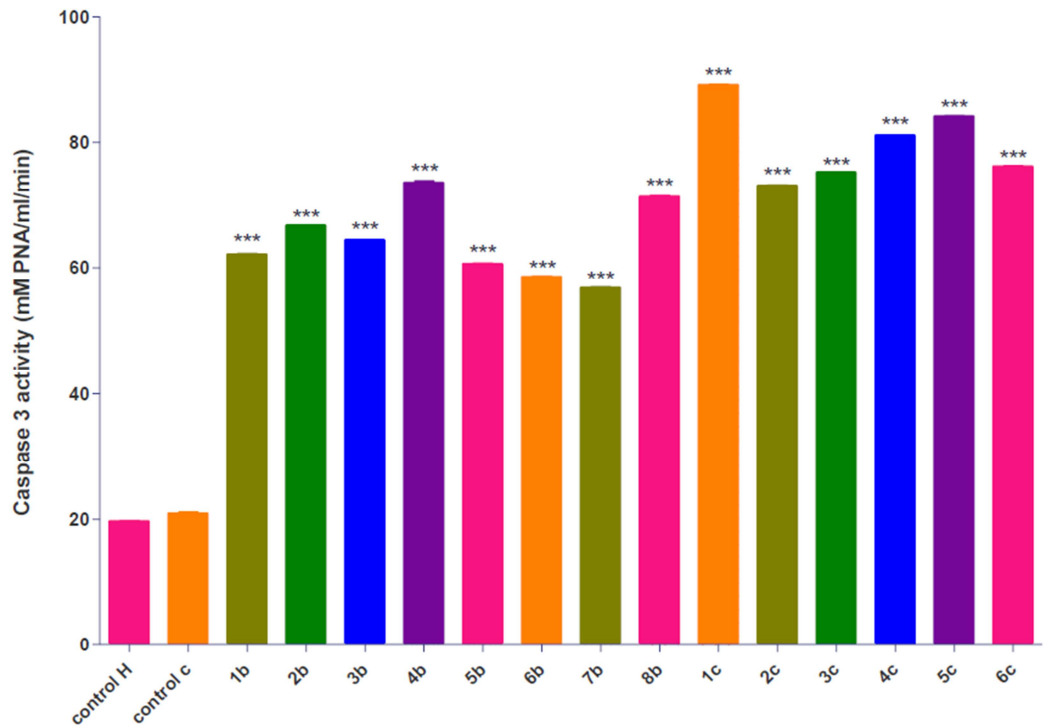


Figure 7. Evaluation of caspase 3 activity. The caspase-3 activity was measured by using Sigma-Aldrich kit. Caspase-3 activation in the both GBM and untreated control following the exposure to peptides (10 $\mu\text{g/mL}$). Data represent the mean \pm SD of three replicates in three independent experiments ($n=3$). The one-way ANOVA test was performed *** Significant difference in comparison with corresponding control HCC group ($p < 0.001$).

4. Discussion

At the present time, cancer is one of the main causes of

diseases in the world leading to human death, therefore, there is a highly demand for discovering a new and effective treatment to overcome this aggressive disease. Current techniques for cancer treatment include chemotherapy,

surgery and radiation therapy [27]. Recently, other studies on structural differences between normal and cancerous cells (for instance, various changes in the size, shape, and number of mitochondria) have been used to design new anticancer drugs [25, 28]. The aim of this research was to study the exposure of Carnosin analogues on astrocytoma using several experiments. First, primarily results in MTT assay indicated that the percentage of cell viability (enzyme succinate dehydrogenase activity) was more decreased by the synthesized cyclic peptides compared with the linear ones using Glioblastoma Multiforme (GBM) group as control cells (Figure 1). The results showed that all the synthesized linear and cyclic peptides increased ROS in various levels in comparison with unaffected mitochondria isolated from the GBM group. A significant increase of ROS was resulted by all the cyclic and some linear Carnosine analogues (compounds 2b and 6b) at 30 min (Figure 3). ROS generation at 60 min was more pronounced for the linear as well as cyclic peptide analogues. Also, it was shown MMP (Mitochondrial Membrane Potential) is disrupted via the formation of transition pores due to the effect of various agents [29]. Here, Rh 123 fluorescence staining (see Figure 4) indicated that the integrity of the mitochondria was damaged by the all peptides. In this regard, Compounds 3c and 5c significantly increased the collapse of MMP among the Carnosine analogues in comparison with mitochondria isolated from the GBM group. Thus, it could be deduced that the synthesized peptides caused apoptosis by the mechanism of MMP collapsing. All the cyclic peptides (compared with the linear ones) significantly increased mitochondrial swelling (reduction of absorbance in the experiment) in comparison with untreated mitochondria isolated from the GBM group (see Figure 5). Among the cyclic peptides, the effect of compound 2c was more pronounced, whereas the effect of compound 4c was the lowest. Among the linear peptides, compound 6b showed exceptionally the highest effect on mitochondria swelling. The release of cytochrome c from mitochondria to media buffer as a subsequent event after mitochondrial swelling and collapse of MMP was also determined. The most important result was that all the linear and cyclic peptides significantly increased the release of cytochrome c in comparison with unaffected mitochondria isolated from the GBM group. Here also, cyclic peptides showed more release of cytochrome c compared with the linear ones (see Figure 6). In this regard, compound 1c gave relatively higher effect than the other cyclic peptides. Caspase-3 plays a terminable role of the apoptosis signaling as an important executioner. When activated, it cleaves a series of substrates, orchestrating apoptosis [30]. To investigate the caspases cascade in apoptosis pathway, we studied the activity of caspase-3 in Carnosine analogues--treated human glioblastoma. Our results showed that the synthesized peptides could activate caspase-3. Moreover, the cyclic peptides, compared with the linear ones, caused relatively higher caspase-3 activation (see Figure 7). Also, among the cyclic peptides, compound 1c was the best in caspase-3 activation. Among the linear peptides, compounds

4b and 8b showed exceptionally high caspase-3 activation. In overall, the results showed that the cyclic Carnosine peptides showed more pronounced effect on GBM mitochondria, compared with the linear ones, these phenomena can be interpreted that these cyclic peptides generally have better permeability properties as a result of giving smaller size than linear peptides and also cyclic peptides have better stability in biological media than linear congeners. Since, compound 1c showed more effect than the other cyclic peptides, it may be due to having dimer building blocks of Carnosine in its structure. This finding can confirm the results of the previous works [31] on demonstrating the activity of Carnosine on brain cancer. Moreover, Carnosine dimer along with a proline amino acid within the two monomers, constructed in a cyclic structure, gives better compound to show anticancer activity in brain.

5. Conclusion

The present study showed the apoptosis effects of synthesized linear and cyclic Carnosine analogues on GBM of human. Based on the increase in mitochondrial reactive oxygen species (ROS) level, swelling in mitochondria, mitochondrial membrane potential ($\Delta\psi_m$) collapse and release of cytochrome c after exposure of mitochondria of the brain carcinoma with the synthesized peptides, cyclic Carnosine analogues rather than linear Carnosine analogues would be encouraging to develop new anticancer agents and they may be considered as a promising complementary therapeutic agents for the treatment of GBM.

Compliance with Ethical Standards

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

Ethical approval this work did not involve any studies on human or animal experiment undertaken by any of these authors.

Informed consent Informed consent was obtained from all individual participants included in this study.

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