The synthetic peptide LyeTxI-b derived from *Lycosa erythrognatha* spider venom is cytotoxic to U-87 MG glioblastoma cells

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Abstract

Antimicrobial peptides present a broad spectrum of therapeutic applications, including their use as anticancer peptides. These peptides have as target microbial, normal, and cancerous cells. The oncological properties of these peptides may occur by membranolytic mechanisms or non-membranolytics. In this work, we demonstrate for the first time the cytotoxic effects of the cationic alpha-helical antimicrobial peptide LyeTx I-b on glioblastoma lineage U87-MG. The anticancer property of this peptide was associated with a membranolytic mechanism. Loss of membrane integrity occurred after incubation with the peptide for 15 min, as shown by trypan blue uptake, reduction of calcein-AM conversion, and LDH release. Morphological studies using scanning electron microscopy demonstrated disruption of the plasma membrane from cells treated with LyeTx I-b, including the formation of holes or pores. Transmission electron microscopy analyses showed swollen nuclei with mild DNA condensation, cell volume increase with an electron-lucent cytoplasm and organelle vacuolization, but without the rupture of nuclear or plasmatic membranes. Morphometric analyses revealed a high percentage of cells in necroptosis stages, followed by necrosis and apoptosis at lower levels. Necrostatin-1, a known inhibitor of necroptosis, partially protected the cells from the toxicity of the peptide in a concentration-dependent manner. Imaging flow cytometry confirmed that 59% of the cells underwent necroptosis after 3-h incubation with the peptide. It is noteworthy that LyeTx I-b showed only mild cytotoxicity against normal fibroblasts of human and monkey cell lines and low hemolytic activity in human erythrocytes. All data together point out the anticancer potential of this peptide.

Keywords Glioblastoma multiform · U-87 MG cells · Anticancer peptide · LyeTx I-b

Abbreviations

Cryo-EM	Cryo-electron microscopy
TEM	Transmission electron microscope
SEM	Scanning electron microscope

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Nec-1	Necrostatin
GBM	Glioblastoma multiform
RIPK1	Receptor interacting protein kinas-1
TNF	Tumor necrosis factor
CAMPs	Cationic antimicrobial peptides
LDH	Lactate dehydrogenase
PBMC	Peripheral blood mononuclear cell

Introduction

In spite of abundant efforts by researchers and clinicians in the neuro-oncology field to treat patients with glioblastoma multiform (GBM), there is still a very poor clinical outcome (Esmaili et al. 2018). Among brain cancers, glioblastoma comprises one of the most aggressive and lethal malignant brain tumors due to apoptosis resistance, intolerable side effects of chemotherapy, and incomplete surgical remove surgical resection (Mellinghoff and Gilbertson 2017; William et al. 2018).

Arthropod venoms are rich in small peptides (> 10 kDa), being many of them toxins that target a variety of different proteins, such as receptors, ion channels, and enzymes, from preys and predators. Most of the lethal toxins found in these venoms are, in fact, peptides able to block or modulate ion channels in a myriad of cell surfaces (Pineda et al. 2014). Since the role of many different ion channels in cancer pathophysiology has become evident, ion channel toxins from spiders and scorpions have been proposed to be used as anticancer peptides (Bubien et al. 2004; Wang and Wang 2016; Nicoletti et al. 2017), as molecular probes to elucidate the functional aspects of these channels in cancer progression and migration (Rooj et al. 2012; Aissaoui et al. 2018) or chemically engineered to be used as tumor imaging agent in both diagnoses and prognoses (Aroui et al. 2015; Moore et al. 2013; Cohen-Inbar and Zaaroor 2016). New drugs aiming at overcoming resistance through the activation of other mechanisms of cell death, like necroptosis, are currently being investigated. Necroptosis, an alternative programmed cell death mechanism, originally described as an accidental cell death, is now known to be a regulated process similar to apoptosis, but it is caspase-independent (Su et al. 2016). Necroptosis is triggered by several types of stimuli, including abnormal cellular metabolism, activation of receptors by ligands such as tumor necrosis factor-TNF, interferon, Toll-like receptor ligands, and anticancer substances. The most well-studied mechanism is triggered by TNF, through recruitment of RIPK1 (receptor interacting protein kinase 1) and inhibition of caspase 8 (Su et al. 2016; Sun et al. 2018). RIPK1 has important kinase-dependent and scaffolding functions that inhibit or trigger necroptosis resulting in expulsion of cellular contents to the extracellular space (Galluzzi et al. 2011). In addition, morphological features

of necroptosis are characterized by nucleus swelling, mild DNA condensation, and increased permeability of lysosomal and plasma membranes (Su et al. 2016). Necroptosis can also be induced after treatment with chemotherapeutic agents (Xu et al. 2017; Galluzzi et al. 2011). Thus, induction of necroptosis could be a promising therapeutic strategy to overcome drug resistance (Su et al. 2016; Pasparakis and Vandenabeele 2015). Cationic anticancer peptides have recently emerged as a new class of promising anticancer reagents owing to their lytic nature and ability to cause rapid cell membrane disruption. Therefore, it is important to test these peptides for their ability to reduce glioblastoma multiform resistance (Liu et al. 2013; Wei et al. 2017).

In this context, we synthesized a cationic peptide, LyeTx I-b, based on a peptide isolated from the venom of *Lycosa erythronatha* spider. This peptide showed strong antimicrobial activity against Gram-positive and negative bacteria including planktonic and multispecies biofilms of periodontal pathogens under various conditions with slight hemolytic activity (Santos et al. 2010; Reis et al. 2018; Cruz Olivo et al. 2017). LyeTxI-b is a synthetic 24-mer peptide (IWL-TALKFLGKNLGKLAQQLAKL-NH₂), which comprises a His16 deletion and N-terminal acetylation, resulting in an improved effect against resistant bacteria. In addition, NMR data revealed that LyeTxI-b adopts a slightly curved helical structure, spanning from the second amino acid residue to its amidated C-terminus, without having, however, an obvious amphipathic secondary structure (Reis et al. 2018). To demonstrate the anticancer potential of this peptide, we focused on its cytotoxic activity against brain tumor cells, investigating its mechanism of action on glioma cells (U-87 MG), compared to non-tumoral human cells, as well as morphological studies using scanning and electronic microscopy, supported necroptosis as the main mechanism triggered by the peptide. Our findings permitted to characterize the cytotoxicity and the anticancer potential of the antimicrobial peptide LyeTx I-b.

Materials and methods

Peptide

The peptide LyeTx I-b was synthesized by GenOne (Rio de Janeiro, RJ, Brazil) and purified (98%) using HPLC. The samples were maintained at –80° C and solubilized in PBS immediately before the experiments.

Cell lines

Human glioblastoma U-87 MG and neuroblastoma SHSY5Y cells were kindly donated by Dr. Marcel Leist/University of Konstanz, Germany; Astrocytoma U-373 MG cells were donated by Dr. Rodrigo Resende, Federal University of Minas

Gerais; VERO (African green monkey kidney epithelial cells) and GM-637 (human fibroblast) cells lines were used as a model of normal cells and were kindly provided by Dr. Erna Kroon and Adriana Abalen (Federal University of Minas Gerais). Cells were maintained in culture in DMEM high glucose medium enriched with 2 mM L-glutamine (GIBCO UK, Grand Island, NY), 1% antibiotic solution (100 IU/ml penicillin and 100 µg/ml streptomycin (GIBCO BRL, Grand Island, NY) and supplemented with 10% fetal bovine serum. All lineages were incubated at 37 °C in a humidified atmosphere containing 5% CO₂, and split twice weekly. The cells were regularly examined and used until 20 passages. Human peripheral blood mononuclear cells (PBMC) were obtained from six healthy donors. About 30 ml of blood was collected from each individual in heparinized tubes. This project was approved by the UFMG Ethics in Research Committee (COEP), under protocol number 666.658/2016.

MTT cell viability assay

U-87 MG, U-373 MG, SHSY5Y, GM637, and Vero cells were plated at 1 or 2×10^4 cells/well in a 96-well/plate in 100 µl DMEM complete medium and allowed to adhere for 24 h at 37 °C with 5% CO₂. After treatment of the various lineages for 48 h in the presence or the absence of the peptide, 2.5 mg/ml of MTT (Sigma® Co., USA) (30 µl/well) were added. The plates were incubated for 4 h at 37 °C with 5% CO₂ until formation of formazan crystals. The supernatant was carefully removed using a syringe needle coupled with a suction pump to preserve crystals, and then, 200 µl of 0.04 (4 mM) HCl in isopropanol were added. After stirring gently for 5 min at room temperature, absorbance of solubilized MTT formazan product was measured at 595 nm on an ELISA reader (Spectramax-Molecular Devices®) (Mosmann 1983). The results were expressed as percentage of the viable cells with the y-axis values relative to the control (PBS), considered as having 100% viability. The IC₅₀ values were calculated by nonlinear or linear regression using the GraphPad Prism® Version 5.01 software.

Resazurin cell viability assay

PBMC from 6 healthy individuals were isolated by Ficoll paque gradient separation solution centrifugation lymphocyte separation medium “LSM”, Organon Teknika, Durham, NC (Gomes et al. 2003). The cells were washed with RPMI 1640 medium and cultured in flat-bottom 96-well plates (Nunc). Proliferative responses were evaluated by incubating 2.5×10^5 cells/well in the presence or the absence of the peptide or phytohemagglutinin (2.5 µg/ml) at a final volume of 200 µl of complete RPMI-1640 (10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Incubation was carried out in a humidified 5% CO₂ incubator

at 37 °C for 3 days for PHA-stimulated cultures. Peptide was added using eight 1:2 serial dilutions (100–1.56 µM) for IC₅₀ determination. Cell viability was evaluated by the resazurin assay (O’Brien et al. 2000). The solution containing resazurin was blue, and when added to a cell culture, it is converted to the reduced pink and fluorescent form, resorufin, which can be measured by spectrophotometric reading of the absorbance at 570 and 600 nm in a plate reader (Spectramax-Molecular Devices®). The number of viable cells was correlated with the percentage reduction of resazurin and was expressed as % inhibition.

Hemolytic activity

Briefly, 1 ml of fresh peripheral blood from 5 healthy volunteers was mixed with 400 µl of heparin (5000 IU/ml) and centrifuged at 1200 rpm for 10 min at room temperature. The red blood cells (RBC) were washed three times with sterile PBS and prepared as a 1% (v/v) suspension of erythrocytes in PBS. 190 µl of diluted RBCs were seeded in a 96-well plate with 10 µl of LyeTx I-b at concentrations ranging from 100 to 1.56 µM in the experimental groups, with 100 µl of 2% (v/v) Triton X-100 diluted in PBS in the positive control group, or with 10 µl of PBS in the negative control group. After incubation at 37 °C for 1 h, samples were centrifuged at 1200 rpm for 5 min, and then, 150 µl were transferred from each well to a new 96 wells/plate and the absorbance was measured at 450 nm using (Spectramax-Molecular Devices®) a microplate reader (Yu et al. 2011). The percentage of hemolysis was calculated as:

$$\text{Hemolysis \%} = \frac{[(\text{Sample absorbance} - \text{negative control}) / (\text{positive control} - \text{negative control})] \times 100\%}{100\%}$$

Investigation of the cell death mechanism induced by LyeTx I-b in U-87 MG cells

Quantification of DNA and cell-cycle analysis by flow cytometry

Quantification of the DNA content and cell-cycle analysis was performed by flow cytometry (Nicoletti et al. 1991). 1 or 2×10^5 U-87 MG cells were seeded in 24-well plates and incubated overnight at 37 °C for stabilization. U-87 MG cells were then incubated with 30 µM LyeTx I-b for 24 h. After treatment, the cells were transferred into 1 ml tubes after trypsinization and centrifuged at 10,000 rpm for 5 min in a microcentrifuge (Denver Instrument Company, USA). The supernatant was discarded and 300 µl of a hypotonic fluoro-chromic solution (HFS) containing 50 µg/ml of Propidium Iodide—IP (Sigma, Saint Louis, Missouri USA) and 0.1% of Triton X-100 (Sigma, Saint Louis, Missouri USA) in 0.1% sodium citrate (Sigma, Saint Louis, Missouri

USA) was added. Plates were incubated for 4 h at 4 °C. After incubation, the samples were submitted to flow cytometry analysis. The FL2 voltage was adjusted, so that the G0/G1 and G2/M phases formed peaks of values of 200 and 400, respectively, in FL2-A. The values of FSC-H, SSC-H, FL2-A, and FL2-W were acquired for histograms and statistics analyses in the FlowJo 7.6.4® program.

Analysis of DNA fragmentation by agarose gel electrophoresis

Profiles of DNA fragmentation were verified by 1.5% agarose gel electrophoresis to observe the typical apoptosis profile with the formation of bands containing multiples of 180–200 bp “ladder standard” (Wlodkowic et al. 2011). U-87 MG cells were grown in 6 well plates at 300,000 cells/well for 24 h. 30 µM LyeTx1-b was added and the plate was incubated again for 24 h in a CO₂ incubator at 37 °C. DNA extraction was performed after this treatment period as described (Grimberg et al. 1989), and DNA quantity was determined by reading absorbance at 260 nm in NanoDrop apparatus (Thermo Scientific, USA). Electrophoresis was conducted in TAE-1× buffer at 100 V and 500 mA, and subsequently, the gel was stained with ethidium bromide solution (0.5 µg/ml) and photographed using a trans-illuminator.

Quantification of autophagic cells

Detection of autophagic vesicles in U-87 MG cells was labeled with acridine orange dye, which is commonly used to study autophagy. The U-87 MG cells were seeded at the density of 200,000 cells/well in 24-well plates and incubated at 37 °C overnight in a CO₂ incubator. After this period, treatment with substances or negative control (PBS) was performed for 24 h. Cells were washed with ice-cold PBS, and then labeled with 10 µg/ml acridine orange solution (Sigma Aldrich) prepared in Milli-Q water. After 10 min of incubation at room temperature, the cells were collected (as above) and read using a flow cytometer (Kanematsu et al. 2010).

Cell death analysis with imaging flow cytometry

U-87 MG cells (3×10^5 cells) were seeded into each well of a 6 well/plate and cultured until reaching 80% confluence. Cell death was induced by adding LyeTx I-b for 3, 6, and 12 h. Cells were trypsinized, carefully suspended in the medium, transferred to microfuge tubes, centrifuged at $1200 \times g$ for 5 min at 4 °C and the supernatant then aspirated. Cells were washed with 1 ml PBS and resuspended in 100 µl staining buffer (BD Annexin V FITC Assay) by mixing 2.5 µl of Annexin V and 2.5 µl propidium iodide (PI) in the

incubation buffer according to manufacturer's instructions. The cells were incubated for 15 min at room temperature and protected from light. Samples were immediately analyzed by Amnis® Imaging Flow Cytometry according to Analysis of Apoptosis and Necroptosis by Fluorescence-Activated Cell Sorting (Wallberg et al. 2016; Pietkiewicz et al. 2015).

Lactate dehydrogenase enzyme activity

U-87 MG cells were plated and the effect of the LyeTx I-b on the release of LDH enzyme was evaluated at 15, 30, and 60 min after treatment. Lactate production was determined by assaying the formation of NADH and pyruvate from lactate in the presence of excess NAD⁺ (Meira et al. 2005). The reaction is catalyzed by lactate dehydrogenase, the lactate concentration being calculated from the extinction coefficient of NADH at 340 nm. 50 mM sodium phosphate buffer (pH 7.4) was prepared with 0.6 mM sodium pyruvate. 6.3 mM NADH solution was prepared at the time of use and protected from light and added immediately prior to reading. The LDH reaction was monitored after 30 min of reaction with reading absorbance at 340 nm in a Varioskan TM Flash spectrophotometer (Thermo Scientific). As a positive control, Triton X-100 (0.5%) was used, and ultrapure water was as the negative control. For the calculation of the percentage of lysis, cells treated with Triton X-100 were considered as 100% lysis (Chan et al. 2013).

Quantification of the effect of Necrostatin-1 inhibitor on the viability of U-87 MG treated with the peptide

Briefly, U-87 MG cells (5×10^3 /well) were seeded into 96-well plates and pre-incubated with (100 µM) Necrostatin-1 for 1 h. The peptide was added in different concentrations and Triton X-100 (0.1, 0.05, and 0.025% v/v), and was used as a positive control (that induces necrosis). After 24 h of treatment, cell viability was evaluated by resazurin metabolism assay (Gartlon et al. 2006).

Evaluation of U-87 MG cell membrane integrity after treatment with the peptide using trypan blue exclusion

Briefly, after 15, 30, 60, 120, and 180 min of incubation of 1×10^4 U-87 MG cells per well (96 well plate) with 30 µM LyeTx I-b, 20 µl of 0.2% trypan blue in PBS were added and incubated for 5 min at room temperature, and then, cells were examined under a microscope. Only dead cells take up the dye and appear as darkly stained cells (Simon and Langdon 2003).

Morphological alteration analysis using time-lapse microscopy

The same field of U-87 MG cells was imaged every 10 min during a 180 min treatment period with 30 μ M LyeTx I-b using a 20 $\times$ objective on an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany) equipped with an AxioCam MRm camera and an incubation system for a constant temperature and CO₂ level.

Time course using high-content automated imaging microscopy (Cellomics) to evaluate early events

U-87 MG cells were treated with 30 μ M of peptide or Triton 0.1% (positive control of necrosis) for 15 min, labeled with Calcein/AM and Hoechst 3342, and then analyzed using a Cellomics system according to the manufactures protocols.

Electron microscopic ultrastructural analysis of cryofixed, freeze-substituted cells

To U-87 MG detached after peptide treatment, the control and treated cells were trypsinized, centrifuged at 960 $\times g$ at room temperature for 5 min, resuspended in DMEM medium supplemented with 0.1% FBS in microfuge tubes, and then allowed to recover in the incubator for 2 h. The cells were centrifuged and resuspended in DMEM medium without serum, and the cell count determined using a hemocytometer. The suspension was divided into 4 groups: one control, untreated group and three groups treated with LyeTx I-b for 30 min, 1 h and 3 h, respectively, in a water bath at 37 °C. After each incubation period, cells were centrifuged at 960 $\times g$ at room temperature for 5 min, the supernatant was discarded, and 5 μ l (~9000 cells) from the pellet of each group were immediately mixed with sterile PBS or cryoprotectant (20% dextran) and cryofixed by high-pressure freezing (HPF) in a Leica EM HPM100 (Leica Microsystems, Wetzlar, Germany). Ultrarapid cryofixation methods were used to preserve the plasma membrane in as close to the native state as possible. Samples were also evaluated using standard EM fixatives and similar results were obtained (data not shown). Cryofixed samples were stored in liquid nitrogen (196 °C) overnight and then transferred to 2.0 ml cryotubes containing liquid nitrogen above a 1.0 ml frozen mixture of 1% osmium tetroxide in anhydrous acetone. Freeze substitution was carried out in a Leica EM AFS2, starting at –90 °C and slowly warming up to 20 °C over a 4 day period. During freeze substitution, frozen water was removed from the samples by anhydrous acetone at a temperature low enough to prevent the cellular water from recrystallizing (Thompson et al. 2016; Zabeo et al. 2017). Samples were brought

to room temperature and then processed for light, transmission, and scanning electron microscopy using standard techniques.

Morphometric analyses of cryofixed samples

High-resolution images of 300-nm-thick sections of cryofixed cells were captured at 1000 $\times$ using a Zeiss Axio Imager Z2 with the Zen 2 Blue Edition V1.0 software (Carl Zeiss Microscopy GmbH, Jena, Germany). Images from different experiments ($n=4$) were randomly collected to distinguish the different cell death processes and then subjected to morphometric analyses. For each group, a total of 200 cells were counted. The identification of glioblastoma cells was based on the presence of typical cell features such as normal round euchromatic nucleus as well as intact plasma and nuclear membranes. Cell death processes were characterized as: (a) apoptosis: clearly identified by its high toluidine blue affinity and morphological changes such as cell and nuclear shrinkage, chromatin condensation, membrane budding, and apoptotic bodies formation; (b) necroptosis: low toluidine blue affinity, swollen nucleus, increased cell volume, with intact or disarranged plasma and nuclear membranes; and (c) necrosis: loss of plasma membrane integrity and consequent cytoplasmic content leakage.

Scanning electron microscope (SEM)

Scanning electron micrographs of U-87 MG cells ranging in magnification from 10,000 $\times$ to 20,000 $\times$ and showing most of the cell surface were randomly taken from cryofixed, freeze-substituted samples, and membrane structure alterations like slits, pores, or hole formations were analyzed.

Transmission electron microscopy (TEM)

To study the cytoplasmic ultrastructural features of U-87 MG glioma cells and of the different cell death processes, a total of 80 U-87 MG electron micrographs ranging from 4200 $\times$ to 26,500 $\times$ and showing the cell profile and nucleus were randomly taken from the cryofixed cells. The same characteristics as visualized in the high-resolution light microscopy images were analyzed at the ultrastructural level, including the organelles organization within glioma cells.

Statistical analysis

For all parameters, data from different groups were compared by the Mann–Whitney *U* test or one-way ANOVA followed by Kruskal–Wallis test, using the GraphPad Prism® 6.01 software (San Diego, CA, USA).

Results

Cytotoxicity of LyeTx I-b peptide against tumor and non-tumoral cell lines

The cytotoxicity of LyeTx I-b peptide was evaluated against three cell lines representative of brain tumors: U-87 MG (glioblastoma), U373-MG (astrocytoma), and SHSY5Y (neuroblastoma). The IC₅₀ values of the peptide for each cell line are shown in Table 1. LyeTx I-b demonstrated a different potency against each cell line: U-87 MG and U373-MG were more susceptible with IC₅₀ values < 30 µM as compared to SHSY5Y (93 µM). General toxicity of the peptide was also evaluated against human PBMC, erythrocytes, fibroblast (GM637), and kidney cells (Vero). While non-tumor cells such as GM637 and Vero cells showed higher values of IC₅₀ (> 100 µM), human PBMC displayed lower IC₅₀ values (Table 1). LyeTx I-b induced mild hemolytic activity, displaying 30–35% hemolysis of human erythrocytes even at the highest concentration of the peptide (100 µM), as shown in Table 1.

The increase of subdiploid DNA content after exposure to LyeTx I-b peptide is not associated with apoptosis

To understand the mechanism of cytotoxicity induced by LyeTx I-b against brain tumor cells in vitro, we investigated the effect of the peptide on U-87 MG cells. Cell death mechanisms involved in the cytotoxicity of many anticancer drugs include apoptosis, autophagy, necrosis, and necroptosis among others. To evaluate if apoptosis was involved, the subdiploid DNA content of U-87 MG cells treated with

their IC₂₅ and IC₅₀ concentrations of peptide (10 and 30 µM, respectively) was measured by flow cytometry after a 24 h treatment. Cells undergoing apoptotic cell death display an increase of subdiploid DNA content, which can be associated with the DNA fragmentation induced during late apoptosis or necrosis. The histograms in Fig. 1A show a significant, concentration-dependent, increase in the percentage of subdiploid (Sub-G1) content in U-87 MG cells after treatment with IC₅₀ concentrations of LyeTx I-b as compared with control (PBS). To confirm if the increase of the DNA content correlated with DNA fragmentation, agarose gel electrophoresis was performed to evaluate genomic DNA. Results in Fig. 1B display the profile of genomic DNA of U-87 MG cells after treatment with the IC₂₅ concentration (30 µM) of LyeTx I-b, and no DNA fragmentation ladder pattern was observed for treated or untreated control cells (i.e., DNA ladders of 180–200-bp oligomers are usually displayed in apoptotic cells). This observation indicates that the DNA fragmentation observed in U-87 MG cells after treatment with LyeTx I-b was not associated with apoptosis.

Evaluation of autophagy induction by LyeTx I-b in U-87 MG cells

To identify if autophagy was involved in the toxicity of the peptide, the autophagic acidic cellular compartments were evaluated by FACS using acridine orange-staining dye. The U-87 MG cells were treated with the same concentrations used to quantify the DNA content 10 and 30 µM LyeTx I-b. Flow cytometric analysis of untreated U-87 MG cells after acridine orange staining showed that most of the cells were labeled with green fluorescence and a minimal red fluorescence (4.37%). Similarly, after treatment with the peptide, the cells still exhibited a green fluorescence comparable to the control, and showed a discreet increase in red fluorescence (14.4%), indicating the presence of acidic vesicle organelles (AVO), which characterizes autophagy. These data suggest that this cell death is not the principal mechanism that contributes to the cytotoxicity of LyeTx I-b on U-87 MG cells (Fig. 2).

LyeTx I-b peptide induces rapid loss of cell membrane integrity of U-87 MG cells associated with morphological alterations

Since the results up to this point demonstrated that LyeTx I-b peptide does not induce apoptosis or autophagy on U-87 MG cells, we investigated the involvement of necrosis, in cell membrane integrity and cell morphology. For that, time kinetics experiments were performed using MTT with IC₅₀ (30 µM). The results in Fig. 3A indicate that U-87 MG cells lose cell viability very quickly in the presence of 30 µM LyeTx I-b (i.e., only after 15 min of treatment), as observed

Table 1 IC₅₀ value of LyeTxI_b cytotoxicity against cancer and normal cell lines

Cell line	LyeTxI_b IC ₅₀ (µM)
U87-MG	29.20 ± 7.96
U373-MG	20.94 ± 5.18
SHSY5Y	93.80 ± 2.17
GM637	≥ 100
Vero	≥ 100
PBMC	4.16 ± 1.94
hRBC	155 ± 13.04

Cytotoxicity of LyeTx I-b peptide against neuronal cancer cells and non-tumoral cells. The IC₅₀ values were calculated from the linear regression of the dose-log response curves after 48 h exposure to peptide, determined by the MTT and resazurin assays. Values are mean ± SD; U-87 MG: Glioblastoma, U-373 MG: Astrocytoma, SHSY5Y: Neuroblastoma, GM637: human lung fibroblasts, Vero: African green monkey kidney, PBMC: Peripheral Blood Mononuclear Cells and hRBC: human blood red cells. The peptide was tested in erythrocytes from the peripheral blood of five different donors

Fig. 1 The increase of subdiploid DNA content in U-87 MG cells treated with LyeTx I-b is not associated with DNA fragmentation. Illustrative histograms showing cell cycle changes 24 h after exposure to LyeTx I-b at IC_{25} and IC_{50} concentrations (10 and 30 μM , respectively). Statistical analysis of independent triplicate experiments showed significant differences between the negative control and the

treated cells in terms of the subG1 (sub-diploid) population. **B** Analysis of U-87 MG cells genomic DNA treated with 30 μM LyeTx I-b. Lane 1: DNA marker; Lane 2: U-87 MG DNA. Note, no DNA fragmentation ladder laddering formation was obviously viewed on ethidium bromide-stained gels (2%) and photographed by UV illumination. Data shown are representative of three independent experiments

by LDH release. The increase in extracellular LDH levels indicate cell membrane damage, and a reduction in cell viability was observed after 15 min, upon labeling with calcein-AM and Hoechst, as shown in Fig. 3B. In control cultures, viable cells were labeled with both green calcein-AM and blue Hoechst after treatment with the peptide, the number of cells displaying green fluorescence decreased, while the number of blue nuclei became evident upon staining with Hoechst. Therefore, LyeTx I-b peptide induces damage in cell membrane integrity and concomitantly reduces the viability of U-87 MG cells in a time-dependent manner.

The morphological alterations were identified by light microscopy after stain with trypan blue (Fig. 4A). Phase contrast microscopy confirmed the time-dependent morphological alterations observed with Calcein-AM and Hoechst labeling. Representative images of time-lapse analysis (Fig. 4B) are corroborate with our previous findings, and show morphological alterations, including changes in cell

shape, and reduction in cell size, probably due to depletion of intracellular constituents after plasma membrane damage (Fig. 4B, arrows). The loss of membrane integrity was confirmed by scanning microscopy studies (Fig. 5). Electron micrographs of control, untreated U-87 MG cells exhibited plasma membranes with a continuous, smooth, morphology, while cells exposed to 30 μM LyeTx I-b for 30, 60, and 180 min exhibited significant alterations in the cell membrane (Fig. 5), including numerous cell membrane pores, holes, and slits. These observations demonstrate that LyeTx I-b peptide is able to disrupt the plasma membrane, and suggests that necrotic cell death is the mechanism involved in the toxicity of peptide against U-87 MG cells.

Fig. 2 Representative images showing flow cytometric measurement of U-87 MG cells after exposed to LyeTx-I_b peptide for 24 h and labeled with acridine orange. FL1-H indicates green color intensity,

while FL3-H shows red color intensity (autophagy cells, AVO). Three independent experiments performed in duplicate (color figure online)

Electron microscopy studies displayed necrosis and regulated necrosis (necroptosis) as possible cell death processes involved with the LyeTx I-b peptide toxicity against U-87 MG cells

To confirm that necrosis is induced by LyeTx I-b, a detailed ultrastructure study of cryofixed U-87 MG cells was carried out using transmission electron microscopy. Morphological structure alterations, indicating various cell death processes, were evaluated after 30, 60, and 180 min of 30 μM peptide treatment. Transmission electron micrographs consistently displayed ultrastructure features that indicating a regulated necrosis process such as necroptosis as the principal findings in most of the observed fields. These characteristics include nuclei with mild DNA condensation, an increased cell volume with an electron-lucent nucleus and cytoplasm, and organelle vacuolization, but

without rupture of nuclear or cell membranes (Fig. 6C). In addition, a progression of the necroptotic process was clearly observed after 30 min of treatment, no plasmatic membrane alterations could be observed in the early stage (Fig. 6C), and progressive enlargement of membrane pores could be observed from the intermediate late stages (Fig. 6D, E). These findings suggest that the aggressive action of LyeTx I-b at 30 μM concentration precludes these cells from undergoing a regulated apoptotic cell death process. Moreover, ultrastructural studies showed no morphological alterations indicating autophagy such as presence of autophagosomes with intracellular organelles and disappearance of some cytoplasmic organelles, corroborating with the flow cytometry analysis (Fig. 1).

Considering these data, the percentages of cells undergoing necroptosis, necrosis, and apoptosis after treatment with LyeTx I-b were determined using high-resolution light

Fig. 3 Effect of LyeTx I-b on LDH release and on the morphology of U-87 MG cells. **A** U-87 MG cells were treated with peptide (30 μ M) for 15, 30 and 60 min and LDH levels were evaluated ($n=3$). Triton X-100 (0.1%), a nonionic surfactant, was used as a positive control of cell membrane disruption. Relative LDH concentration was calculated as a % of the positive control. **B** U-87 MG cells were treated

with peptide (30 μ M) for 15 min and then labeled with Calcein-AM and Hoechst 3342. Double positive cells (green and blue) were counted as viable. The number of viable cells per field was detected using a high-content screening microscope (Scale bar, 50 μ m). At least 3 independent experiments were performed (color figure online)

Fig. 4 Partial disintegration of U-87 MG cell membranes after exposure to 30 μ M LyeTx I-b. Control or treated cells were treated with the peptide for 0, 15, 30, 60, and 180 min, stained with trypan blue (**A**) and during time-lapse analysis (**B**). Red and black arrows repre-

sent alterations in both cells. Scale bars: (**A**)=50 μ m; (**B**)=100 μ m. Three independent experiments were done in duplicate for trypan blue, and time-lapse two experiments performed in triplicates (color figure online)

microscopy (HRLM) morphometric analysis. The results confirmed necroptosis as the main cell death process, while necrosis was measured as a minor proportion. The percentage of cells under apoptosis was similar to the control cells, as shown in Table 2. Apoptosis was identified by the chromatin condensation levels followed by nuclear and

organelles shrinkage and the formation of apoptotic bodies (supplementary Fig. 1A). Necrosis was best characterized by the huge plasma membrane rupture with extravasation of intracellular contents and organelles to the extracellular medium (supplementary Fig. 1B).

Fig. 5 Ultrastructural changes on the U-87 MG cell surface exposed to 30 μ M LyeTx I-b. **A, B** Control cells at 0 min and after 180 min of incubation, respectively, displaying a smooth and homogeneous surface, with few membrane disruptions. **C, D, Di** and **E** After 30, 60, and 180 min of treatment with LyeTx I-b slits (**C**), pores (arrows) (**Di**) and hole (arrowheads) were detected in the plasma membrane. **Di** is a higher magnification view of the white-boxed area in **D**. U-87

MG cells were cultured, fixed using high-pressure freezing (HPF), freeze-substituted and then processed for scanning electron microscopy (SEM). A total of 165 electron micrographs was randomly taken and analyzed at magnifications ranging from 10,000 to $\times 20,000$. Bars: **A–E**=5 μ m; **Di**=2 μ m. Three independent experiments were performed, $N=3$

Pre-treatment with necrostatin-1 reduces loss of viability induced by the peptide on U-87 MG cells

To further confirm that necroptosis was involved in the cytotoxicity of the peptide against U-87 MG cells, assays with necrostatin-1, a specific inhibitor of necroptosis, were performed. Triton X 100 was used as a positive control. Pre-treatment of U87 cells with necrostatin-1 partially protected the cells from the toxicity of the peptide at 12.5, 25, and 50 μ M, as observed by the higher values of resorufin fluorescence intensity, compared to the same treatment without necrostatin-1 (Fig. 7).

LyeTx I-b peptide induction of necroptosis confirmed by imaging stream flow cytometry

Experiments using flow cytometric analyses were performed to differentiate between apoptosis, late apoptosis, and necroptosis after treatment of U-87 MG with the

peptide. Microscopy was combined with flow cytometry in one measurement to analyze morphological and biochemical features of cell death in a quantitative way, using classical Annexin V/PI iodine staining to identify cells with phospholipids exposure and loss of membrane integrity. This strategy allowed classification of cells only labeled with Annexin V in early apoptosis, double positive cells (Annexin V and PI) in late apoptosis or necroptosis, and cells labeled only with PI in necrotic cells. To evaluate necroptotic cells, an image-based analysis was used to measure nuclear morphology, distinguishing cells with secondary necrosis due to late apoptosis and necroptosis, simultaneously (Pietkiewicz et al. 2015). The results demonstrated that LyeTx I-b induced necroptotic death in U-87 MG cells after a 3 h treatment, observed by double positive staining with Annexin V and PI, differently than observed in control cells. Control cells maintained a round shape and staining for either Annexin V or PI could not be detected (Fig. 8A). Moreover, the number of double positive cells increased in correlation with increasing

Fig. 6 Intracellular ultrastructure of U-87 MG cells treated with 30 μ M LyeTx I-b. **A, B** Control cells showing ultrastructural characteristics indicative of cellular activation such as the presence of a well round and central euchromatic nucleus (N) with fine distinction from the nuclear envelope (arrows). A higher magnification in (Bi) shows a prominent Golgi apparatus (G) and mitochondria (asterisk). **C–E** Cells after 30 min of treatment, in different stages (early, intermediate, and late) of the necroptosis process, identified by the increase in the cell volume, swelling of the nucleus and cytoplasmic organelles (arrowheads), and electron-lucent nucleus and cytoplasm.

Differences in plasma membrane alterations ranging from membrane integrity maintenance (early stage) to the progressive membrane ruptures (middle-to-late stages) were detected. Human glioblastoma cells were cultivated and processed for transmission electron microscopy using high-pressure freezing and freeze substitution. A total of 80 electron micrographs showing the entire cell profile and nucleus was randomly taken and analyzed at magnifications ranging from $\times 4200$ to $\times 26,500$. Bars: **A–E** = 2 μ m; **Bi** = 500 nm. The micrographs shown here are representative of three experiments performed in triplicate.

peptide concentration and time of incubation, as observed in Fig. 8B. After 3 h of treatment with 30 μ M LyeTx I-b, the percentage of double positive cells was 59.09%, while after 6 h, it was 80.9% (Fig. 8C).

Table 2 Morphometric analysis of transmission electron micrograph (TEM) of U-87 MG cell line after exposure to 30 μ M LyeTx I-b for 30, 60, and 180 min

%	Normal	Apoptosis	Necroptosis	Necrosis
Control	89.7	2.6	3.9	3.9
30 min	24.4	6.5	53.6	15.5
1 h	5.7	1.9	64.2	28.3
3 h	4.7	1.7	63.3	30.3

The total count was at least 300 cells for each group

Discussion

Cationic antimicrobial peptides (AMPs) are antimicrobial peptides that are able to stimulate innate immunity to fight against infections and have dual activities as anticancer and antimicrobial agent (Felício et al. 2017; Pane et al. 2017). The presence of unsaturated lipid chains in cancer cell membranes confers negative charges that allow them bind with the positive charges present in cationic peptides, increasing the selectivity of these peptides for cancer cells (Chen et al. 2014; Deslouches and Di 2017). Furthermore, anticancer cationic peptides have many features such as mild toxicity without accumulation in different tissues and eventually selectivity and specificity for cancer targets and receptors (Perez-Pitarch et al. 2017). In this work, we focused on brain cancer cells, specifically in one representative lineage

Fig. 7 Effect of LyeTx I-b in cell membrane integrity and viability in the presence of necrostatin-1, a specific inhibitor of necroptosis. U-87 MG cells were pre-treated or not with 100 μ M necrostatin-1 (nec-1) for 1 h, followed by incubation with various concentrations of the peptide or Triton $\times 100$. Cell viability was measured by resazurin assay. The data shown here are representative of two experiments performed in triplicate. *** $P < 0.01$, one-way ANOVA followed by Tukey multiple comparison test

of glioblastoma multiform, U-87 MG cells as a model to understand the cytotoxic mechanism of LyeTx I-b peptide and evaluate its anticancer potential. This peptide displayed excellent antimicrobial activities *in vitro* and *in vivo* (Reis et al. 2018). The aggressive nature of this class of tumor and the poor prognosis of patients with GBM underscore the clear requirement for more precise and powerful treatments (Marqus et al. 2017).

In the early phase of drug discovery, the predictive assays based on cells are essential to characterize the activity of the new compound in a specific model of disease. Most of the authors in the literature claim that the potential antitumor activity of AMPs is due to a selective recognition of cancer cells via electrostatic interactions (Chen et al. 2014; Deslouches and Di 2017). To characterize the cytotoxicity of LyeTx I-b peptide and its anticancer potential, we used as predictive models tumor cells derived from non-tumoral cells (human PBMC, human fibroblast, monkey kidney cells) and to measure the general toxicity hazard.

We found that LyeTx I-b peptide effectively inhibits the growth of some cells derived of brain cancers in a dose-dependent manner (Table 1), whereas neuroblastoma cells (SHSY5Y, $IC_{50} = 93 \mu$ M) were less susceptible, comparing with glioma cells ($IC_{50} = 29$ and 20μ M, respectively, to U-87 MG and U373-MG). When evaluated against representative models of non-tumoral cells, the peptide showed more cytotoxicity against human PBMC, a representative model of immunotoxicity ($IC_{50} = 4 \mu$ M), comparing with human fibroblast GM-637 cells and monkey kidney cells Vero ($IC_{50} > 100 \mu$ M). Most of the chemotherapeutic agents in clinics also induce adverse effects such as myelosuppression, cardiotoxicity, ototoxicity, neurotoxicity, and malignancies (Swartz et al. 2015). Treatment with anticancer drugs such as cisplatin, etoposide, and paclitaxel is frequently accompanied by toxic side effects including immunosuppression (Kim et al. 2016; Galluzzi et al. 2015;

Lee and Jeon 2001). To Galluzzi et al. such “side” effects are desirable to suppress immunosuppressive circuitries induced by tumors. Hemolytic effect is a common finding observed to AMPs. We observed that peptide-induced hemolysis on human erythrocytes (30–35%), indicating disruption of RBC membrane, similar to the hemolytic effects of others α -helix peptides as described by Huang et al. (2011). These effects are corroborated by the findings of Bacalum and Radu (2015) evaluating the therapeutic index of 11 MPAs (α -helix, β , or random coil). They used hemolysis and PBMC viability as parameters as more realistic to identify side effects. Some AMPs like Melittin displayed cytotoxicity at lower micromolar range ($< 10 \mu$ M) for both PBMC and hemolytic activity. Another peptides like Dermaseptin 1 and Indolicidin showed cytotoxicity against hRBC and PBMC in similar concentration (ranging from 10 to 50μ M). Hydrophobicity is a key factor of AMPs cytotoxic efficacy against cancer cells and less hemolytic activity because of the presence of alanine residue in α -helical peptides that might help to penetrate and accumulate in cancer cell membrane, leading to disrupt cell membrane rapidly than red blood cells. In addition, V13K, K4R2-Na2-S1, and D-K6L9 are cationic peptides, which have been demonstrated high specificity to kill various cancer cells with hemolytic effect, similar to our results (Chu et al. 2015; Huang et al. 2011).

To elucidate the mechanism of cell death involved in the cytotoxic activity of LyeTx I-b against U-87 MG, classical cell death mechanisms such as apoptosis, necrosis, and autophagy were investigated using flow cytometry and morphological studies. To evaluate apoptosis, cell-cycle analyses demonstrated an increase in the content of subdiploid DNA in U-87 MG cells after treatment with 30μ M peptide (Fig. 1). Although this result indicates DNA fragmentation, agarose gel analyses clearly showed that the treatment of U-87 MG cells with the peptide did not induce apoptotic pattern of apoptosis, since no ladder was observed (Fig. 1).

Fig. 8 Imaging stream flow cytometry of U-87 MG cells treated or not with LyeTx I-b. U-87 MG cells were treated with 30 μ M peptide for 3 and 6 h and labeled with Annexin V (An) and propidium iodide (PI) and classified as live (double negative), early apoptotic (An positive), late apoptotic (double positive) or necroptotic (double positive). Dots were constructed displaying intensity of Annexin, MC-Ch02; aspect ratio of PI stained nucleus, intensity of MC-Ch-05 and con-

trast morphology of PI, contrast MC_Ch04. **A** Representative images of U-87 MG, treated or not, acquired simultaneously with double negative and positive labeling (An+/PI+), as well as in bright field. **B** Percentage of necroptotic cells treated with 30 μ M LyeTx I-b for 3 and 6 h in two independent experiments. Graphs represent a different quantitative parameter that distinguishes between necroptosis and apoptosis

The cells undergoing apoptotic cell death exhibit different morphological and biochemical alterations such as DNA fragmentation due to endonucleases enzymes like caspase-3 that cleave the chromatin into fragments with 180 bp that

appear after running the DNA in agarose gel, similar to a “ladder” (Matassov et al. 2004). These results were also supported by FACS analyses of one early apoptotic phenomena such as phosphatidylserine exposure since less than 15%

of apoptotic cells, and were detected in different times of incubation of U-87 MG cells with the peptide (Table 2 and Fig. 6, respectively).

Furthermore, autophagy, another cell death correlated with chemoresistance, recurrence, and metastasis does not seem to be involved (Ojha et al. 2015). Under ultrastructural studies, this type of cell death usually demonstrates sequestration of cytoplasmic components within a double-membrane structure called the autophagosome, followed by delivery to lysosomes for degradation. These lysosomes can be detected with acridine orange that can be quantified by flow cytometry (Gewirtz 2014). There was no indication of cells in autophagic process, since the presence of AVO was lower than 15%, comparing with the control. Even considering that other markers of autophagy have not been investigated here, such as microtubule-associated protein LC3, which plays a key role for the formation of autophagosomes (Eum and Lee 2011; Brauchle et al. 2014). Morphological analysis using MET (Fig. 6) clearly demonstrated no signs of autophagy alterations such as the presence of double-membrane structure with undigested cytoplasmic organelles including mitochondria or endoplasmic reticulum as described by Mizushima et al. (2010). These results demonstrated that neither apoptosis nor autophagy were the main mechanisms triggered by the peptide on U-87 MG cells.

Most of the data presented here indicated that LyeTx I-b induced necrosis or a programmed necrosis through rapid cell membrane damage. Trypan blue uptake in U-87 MG cells occurred after 15 min incubation with 30 μ M LyeTx I-b (Fig. 3A), as well as, time-lapse studies (Fig. 4B) showed cell membrane disruption. This effect was in a concentration dependent, confirmed by measurement of LDH release and calcein-AM. Comparable data were observed by Burns et al. who described the effect of pHLIP-KLAKLAK c antimicrobial peptide on the integrity of MDA-MB-231 cell membrane (Burns et al. 2016). They also showed trypan blue uptake after treating with the toxic peptide, disruption, and permeabilization of the cell membrane as a result of pore formation (Tran et al. 2011).

Disruption of U-87 MG membrane after treatment with the peptide was confirmed herein by scanning microscopy studies. Our data suggest that LyeTx I-b was able to cause damage to the cell membrane after 30 min of treatment, forming “holes” after 180 min, leading to membrane destruction, as shown in Fig. 5. These findings suggested that LyeTx I-b can induce pores formation in the membrane of U-87 MG cells, in a time- and dose-dependent way, leading to plasma membrane permeabilization and consequent cell degeneration. Scanning microscopy analyses of U-87 MG cells after treatment with the cationic anticancer peptide bacteriocin LS10 demonstrated cell membrane disintegration, similar to our findings (Baindara et al. 2017). LyeTx I-b almost perfect helical structure, as observed from NMR data,

also suggests that this peptide is prone to act as a membranolytic peptide (Reis et al. 2018). Currently, in vitro studies of LyeTx I-b using large unilamellar vesicles (LUVs) measured by biophysical techniques such as isothermal titration calorimetry (ITC), aiming to better explain the nature of such interactions, are being carried out by our group (to be published elsewhere). Lytic activities of peptides isolated from other venoms have been described. ABH3 peptide is a BCL-2 homologue artificial lytic peptide, which exhibits the ability to rapidly disrupt the cell membrane in different cancer cell lines HCT116, HeLa, U937, MCF-7, and MDA-MB-231 (Liu et al. 2016). A similar study, antimicrobial peptide temporin-1CEa, extracted from skin secretions of the Chinese brown frog demonstrated that temporin-1CEa able to disrupt cancer cell membranes, causing loss of membrane integrity due to the formation of transmembrane pores that allow an uptake of the peptide into the cytoplasmic compartment of the cancer cell (Wang et al. 2013). However, we did not evaluate how the pores were formed and their size in U-87 MG cells after treatment with the peptide in this approach.

Interesting finding observed after transmission electronic microscopy (TEM) and ultrastructural analysis suggested that the peptide at 30 μ M induced a regulated type of necrotic cell death in U-87 MG. Necrotic cells at the light of TEM studies have unique morphological features, such as swollen organelles, nuclear membrane dilatation, little chromatin condensation and breakdown of the plasma membrane due to oncosis, increasing the volume of cell membranes (Vandenabeele et al. 2010). Necroptosis or regulated necrosis were frequently identified in most of the fields, characterized by swollen nuclei, vacuolization, increase of cell volume, and integrity of cell membrane (Fig. 6), in agreement with previously morphological alterations described this cell death process, like swollen nucleus, increased cell volume and mild chromatin condensation (Su et al. 2016). We not only found cells with a necroptotic profile but also few cells in necrosis and apoptosis (nuclei with condensed chromatin and reduction of cell volume), at lower frequencies. Morphometric analyses (Table 2) demonstrated that after 30 min, more than 50% of the cells were necroptotic, less than 20% were necrotic and less than 3% were apoptotic. These findings were time dependent. TEM analysis characterizes that necroptotic cell death has been described in the literature (Sun et al. 2016). Our data demonstrated morphological necroptosis signs in U-87 MG cells after treating with 30 μ M LyeTx I-b, characterized by the swelling of nucleus and mitochondria, increase in cytoplasm volume, membrane disruption, cytoplasm vacuoles, and release of cellular content. Other studies using TEM showed necroptosis signs after treatment with natural alkaloid compounds (martine) in cholangiocarcinoma cells (CCA) characterized by cytoplasmic vacuolation and extensive swelling of

organelle and loss of plasma membrane integrity (Xu et al. 2017). As a consequence of cellular swelling and rupture of the plasma membrane, they also observed an extravasation of cellular components and increase in LDH, as observed in the present study (Zhao et al. 2016).

To better characterize the necroptotic death triggered by LyeTx I-b on U-87 MG, cells were pre-treated the cells with necrostatin-1 (Nec-1), which is a specific inhibitor of necroptosis in human glioma cells (Melo-Lima et al. 2014; Cho et al. 2011). As observed in Fig. 7, pre-treatment of U-87 MG cells with Nec-1 protected U-87 MG cells against the cytotoxicity induced by the peptide, observed by an increase in the number of viable cells, comparing with control. Flow cytometric studies (Fig. 8) corroborate with these data, showing an increase in the number of necroptotic cells after 3 h treatment, in a time- and dose-dependent manner, 59.9% of cells were necroptotic, and after 6 h, this number increasing to 80.9%. These data were in agreement with those observed after the morphometric analysis by TEM (Table 2). Although other direct molecular markers of necroptosis (TNF- α receptor activation; MLKL phosphorylation, RIP3 involvement) have not been evaluated in this study, the use of the specific necroptosis inhibitor necrostatin, flow cytometry assays, and ultrastructural studies clearly demonstrate that necroptosis is the main type of cell death induced by the peptide.

Conclusion

In the present study, we reported that the cationic antimicrobial peptide LyeTx I-b presents cytotoxicity against brain cancers cells. This effect involves induction of necroptosis in glioblastoma cells as the main cell death process. In addition, the peptide showed undesirable cytotoxicity such as hemolytic effect and immunotoxicity, but this was similar to other anticancer drugs used in clinic. Therefore, this deleterious effect cannot be considered as a restrictive reason to invalidate the antitumor potential of this peptide. We suggested that the novel synthetic LyeTx I-b peptide is an interesting prototype to be used as a model to develop new chemotherapeutics.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest regarding the publication of this paper such as personal or professional relationships, affiliations, knowledge, or beliefs in the subject matter or materials discussed in this manuscript.

Ethical statement The collection of human blood samples was a part of the project approved by the UFGM Research Ethics Committee (COEP), under Protocol Number 666.658/2016, and all healthy donors provided written consent.

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