Binase deletion mutant with enhanced cytotoxic properties

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Abstract. Antitumor peptides are a new class of small molecules characterized by high specificity and therapeutic efficacy. One of the strategies for developing peptide-based therapeutic agents is the modification of the physicochemical properties and structural characteristics of known peptides and cytotoxic proteins. Bacillus pumilus ribonuclease, binase, possesses antitumor activity. Using bioinformatics tools, we have developed a strategy for modifying of amino acid sequence of the antitumor binase peptide to enhance its cytotoxic properties. the binase peptide. A genetic construct enabling the peptide biosynthesis in recombinant strain was engineered. The peptide preparation was purified by metal chelate affinity chromatography. Cytotoxic properties of the purified peptide were assessed according to metabolic activity of tumor cells. Intracellular localization of the peptide was determined by means of fluorescence microscopy. In the study, we have genetically modified the binase peptide in order to change its physicochemical and structural properties. It made it possible to enhance its cytotoxic potential towards human lung adenocarcinoma line A549, but specificity was decreased. Thus, that modification of structural and physicochemical properties of therapeutic peptides is an important step in the development of antitumor drugs.

1 Introduction

Cancer is one of the major problems in the scope of a modern public health. Despite of achievements of contemporary medicine, traditional cancer therapy has a distinct high efficacy only on the initial stages of the disease. In the course of the disease, standard antitumor protocols lose their therapeutic effect due to the low selectivity and high systemic toxicity of chemotherapy agents and acquired drug resistance [1]. Therefore, a search for alternative anticancer therapy methods retaining high therapeutic potential even on the later stages of the disease is a promising approach.

Antitumor peptides are the new class of therapeutic substances and a hopeful alternative to the traditional chemotherapy. These molecules are low-sized and usually positively charged and amphipathic [2]. Peptides, unlike full-sized proteins and monoclonal antibodies utilized in antitumor therapy, have a lower immunogenicity due to their small size. Peptide

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molecules penetrate tumor cells easily with high selectivity and therapeutic efficacy [3]. Peptides are easy to modify and their production costs are low [4].

Binase, a small positively charged ribonuclease (RNase) secreted by *Bacillus pumilus*, is renowned for its antitumor activity. Due to cationicity, stability, catalytic activity towards RNA and resistance to the eukaryotic RNase inhibitor, it is able to bind to the membranes of tumor cells, penetrate them, interact with intracellular proteins and hydrolyze RNA [5]. Binase disrupts the functioning of tumor cell calcium channels, changes the redox potential and increases membrane permeability of mitochondria, transforms the profiles of miRNA, mRNA and protein, impairs the MAPK/ERK signal transduction pathway, which leads to the cell death through apoptosis [6, 7]. At the same time, binase has no significant effect on the viability of normal cells [8]. The selectivity of the action of cationic binase is attributed to the fact that tumor cells carry a more negative total charge on their surface, which mediates its preferential interaction with them. In addition, the binase is able to block signaling pathways that are often hyperactivated in tumor cells [7]. Even binase fragments, namely regions 21 to 50 (peptide B21-50) and 74 to 94 (peptide B74-94) amino acid residues [9], can inhibit cancer cell proliferation. It was shown that binase peptides reduce the antiproliferative potential of tumor cells in HeLa cervical cancer cell line, MCF-7 and BT-20 breast cancer cell lines, but only at high concentrations, and have no effect on human lung adenocarcinoma A549 cells. Meanwhile, the cytotoxicity of the full-length protein remained significantly higher even at lower concentrations. The selectivity of the antitumor action of binase peptides is probably mediated by their penetration ability and interaction with specific oncogenes. Their amino acid composition, secondary structure and physicochemical properties are known to contribute to cytotoxic properties of antitumor peptides [4].

The aim of this study was modification of the physicochemical and structural characteristics of the binase peptide to create RNase deletion mutant with enhanced cytotoxic properties.

2 Materials and methods

2.1 Bacterial strains and cultivation conditions

The bacterial strain *Escherichia coli* NEB5α (New England Biolabs, USA) for genetic engineering and *E. coli* BL21(λDE3) (Novagen, Germany) for recombinant protein expression were used. Bacteria were grown on Luria-Bertani (LB) medium for 14-16 h at 37 °C. To cultivate recombinant bacteria the selective antibiotic ampicillin at a concentration of 100 μg/mL was added to the nutrient media..

2.2 Construction of expression strain for binase mutant production

Amplification of the binase gene fragment was performed using DNA polymerase PfuSE (SibEnzyme, Russia) and oligonucleotides Bin-Xho-F (5`-cagaacctcgagaaaagcatcggtggtggagatg-3`) and Bin-Bpu-R (5`-tagattgctcagcttcttatcgaatcgaatacgtgtgtgaaag-3`). Plasmid pML163 carrying a full-length binase gene was used as a DNA template [10]. The PCR amplification program included the following steps: primary denaturation for 1 min at 95 °C, 25 cycles of secondary denaturation at 95 °C for 1 min, annealing at 50 °C for 30 s and elongation at 72 °C for 23 s, followed by a final elongation at 72 °C for 10 min.

For further enzymatic reactions, the amplified gene was purified by GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA). Enzymatic hydrolysis of expression vector pET15b (Novagen, Germany) and the obtained PCR product was carried out by a pair of

restriction endonucleases BlpI and XhoI (Thermo Fisher Scientific, USA) in buffer G for 3 hours at 37 °C. The obtained linear DNA fragments were purified from agarose gel using LumiPure DNA Gel Extraction Kit (Lumiprobe, Russia). The linear DNA fragments (vector and cloned gene) were mixed in a 1:6 vector to gene molar ratio and incubated with T4 DNA ligase (SibEnzyme, Russia) for 16 hours at 4 °C. The ligase mixture was used to transform *E. coli* strain NEB5α by heat shock. The correctness of the obtained pET15b-Bin48N assembly was evaluated by Sanger sequencing.

The expression strain *E. coli* BL21(λDE3) was transformed with the pET15b-Bin48N plasmid. The 16-h bacterial culture was diluted 1:50 with LB medium and grown until the mid-logarithmic phase. Next, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the medium at a concentration of 0.1-1 mM and bacteria were grown for 3-5 h at 16 °C, 25 °C, 30 °C or 37 °C. At the end of induction, cell lysates were obtained and examined by electrophoresis in the presence of 1% sodium dodecyl sulfate (SDS) in 16% polyacrylamide gel (PAAG) using standard Laemmli methodology. The efficiency of biosynthesis was evaluated by the size and color intensity of protein bands on the gel using the GelAnalyzer 19.1 program. In the concentration calibration mode, a calibration graph was constructed using the protein content in the molecular weight marker and the reference preparation of binase, and then the amount of protein with a molecular mass of about 10 kDa in the samples was calculated.

2.3 Isolation and purification of the binase mutant

For isolation and purification of the recombinant protein, bacterial cells were precipitated by centrifugation, the precipitate was resuspended in binding buffer (20 mM sodium phosphate buffer, pH 7.4, 0. 5 M NaCl) supplemented with lysozyme at a concentration of 0.1 mg/mL, 1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 1 h at room temperature with agitation. Next, the lysates were centrifuged for 30 min at 12000 g, the supernatant was mixed with Ni-NTA Sepharose (GE HealthCare, USA) pre-equilibrated with binding buffer in a 4:1 ratio and incubated with stirring for 1 h at 4 °C. The resulting suspension was loaded onto the column. The chromatography conditions were varied to achieve the highest efficiency of protein production: the concentration of imidazole in the binding and washing buffer was varied from 0 to 20 mM, and in the elution buffer from 50 to 500 mM. Throughout the chromatography, fractions were sampled and examined by SDS-PAAG electrophoresis. After elution, the fractions were pooled and desalted on a Bio-Scale™ Mini Bio-Gel® P-6 column (Bio-Rad, USA).

2.4 Evaluation of ribonuclease activity

The catalytic activity of the purified binase mutant was measured by a modified Anfinsen method. A unit of activity is equal to the amount of enzyme that increases the extinction of acid soluble hydrolysis products of high-polymer yeast RNA at 260 nm by one optical unit in 1 h at 37°C.

2.5 Evaluation of cytotoxicity

Cytotoxicity of binase mutant was evaluated on human alveolar lung adenocarcinoma cell lines (A549) and human embryonic lung epithelial fibroblasts (WI-38) obtained from ATCC collection (USA). Cells were grown on Dulbecco medium (DMEM). The medium was supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (penicillin and streptomycin 100 units/mL each). Culturing was performed at 37 °C in a humidified 5% CO2

atmosphere. Cell viability was determined by the activity of mitochondrial NADPH-dependent cellular oxidoreductases, using a standard technique based on the reduction of the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble formazan product being stained purple. Cells were inoculated in a 96-well plate at an initial concentration of 10^4 per well and grown for 24 h, then the culture liquid was removed and fresh medium with binase mutant was added at concentrations of 100 and 300 μg/mL. After 48 h, the medium was changed by the medium with MTT (5 mg/ml) and incubated until formazan crystals appeared, which were then dissolved in dimethyl sulfoxide (DMSO) and analyzed at 570/630 nm (XMark, Bio-Rad, USA). The viability of untreated cells was taken as 100%. The cytotoxicity of the binase mutant was compared with the full-length wild-type protein (Institute of Organic Synthesis, Latvia, Riga).

2.6 Investigation of the intracellular localization of the mutant binase

The localization of the deletion mutant of binase was assessed by immunofluorescence assay on A549 tumor cells. A549 cells (25000 cells/well) were inoculated into 4-well slide chambers and incubated for 24 h on RPMI medium containing 10% fetal bovine serum. The medium was replaced with fresh medium containing binase or its deletion mutant at a final concentration of 100 μg/mL. After 30 min of incubation, the medium was removed, cells were washed three times with ice-cold phosphate buffer (PBS), fixed in 4 % paraformaldehyde solution for 15 min, and permeabilized in PBS supplemented with 0.1% Triton X-100 solution for 10 min. Cells were washed three times in PBS, incubated for 30 min in PBS with 0.1% Tween-20 (PBST) supplemented with 1% bovine serum albumin (BSA) and glycine (22.5 mg/mL). Cells were washed and incubated overnight in the dark at 4°C with primary rabbit anti-binase antibodies (1:50) and mouse anti-6xHis tag antibodies conjugated to Alexa Fluor 647 (1:50) (sc-53073, Santa Cruz Biotechnology, USA). The cells were then washed three times with PBST. Binase-treated cells were incubated at room temperature for 45 min in the dark with secondary anti-rabbit antibodies conjugated with fluoresceinisothiocyanate (FITC) (31584, Thermo Fisher Scientific, USA) at a concentration of 2 μg/mL. Nuclear DNA was stained with 40,6-diamidino-2-phenylindole (DAPI) for 15 min at 37 °C. Preparations were viewed using an LSM 700 confocal laser scanning microscope (Carl Zeiss AG, Germany) with a Plan-Apochromat 63×/1.4 objective (Carl Zeiss AG, Germany) at laser excitation wavelengths of 405 nm for DAPI, 647 nm for Alexa Fluor 647, and 488 nm for FITC.

2.7 Bioinformatic analysis of binase and its derivatives

ExPASy ProtParam tool and Prot pi Protein Tool programs were used to calculate the physicochemical parameters of the peptides, namely length, molecular weight, isoelectric point, total charge, instability index, aliphatic index, and hydrophobicity index - grand average of hydropathicity (GRAVY). To evaluate changes in the secondary and tertiary structure of peptides, they were modeled in the SWISS-MODEL program using the homology method; the tertiary structure of binase in solution was chosen as a template for construction [11]. The constructed models were visualized in the VMD program.

2.8 Statistical analysis

Statistical processing and visualization of quantitative data were performed using GraphPad Prism 8 software (GraphPad Software, USA). Data on histograms were presented as mean (M) ± standard deviation (SD). The Kruskal-Wallis test was used to compare the parameters in groups. Differences were considered statistically significant at $p < 0.05$.

3 Results

The binase-derived peptide B74-94, characterized by its small size (2.5 kDa) and negative charge (pI 4.4), has the highest cytotoxic potential among binase peptides. According to NMR analysis, the B74-94 peptide represents a region of the five-strand antiparallel β-sheet of the binase molecule (Fig. 1) [11]. To increase the cationicity of the peptide and to include amino acid residues involved in catalysis, the length of the molecule was extended and comprised the entire five-strand antiparallel β-sheet of the binase molecule (region from 48 to 109 amino acid residues). Thus, the modified binase peptide was a mutant form of RNase with a deletion of 47 amino acid residues from the N-terminus. The constructed binase mutant was designated B-His-48-109. A polyhistidine tag was inserted into its sequence to facilitate the purification process. The spatial structure and physicochemical properties of the deletion mutant B-His-48-109 were evaluated using bioinformatics tools (Fig. 1, Table 1).

Table 1. Physico-chemical properties of binase and its derivatives. a.a. – amino acids; MW – molecular weight in Da; q – net charge at pH 7; II – instability index; AI – aliphatic index; GRAVY – grand average of hydropathy.

Protein	Length, a.a.	MW	pI	q	II	AI	GRAVY
Binase	109	12212	9.5	+3.9	27	79	-0.42
B74-94	21	2491	4.4	-1.2	19	88	-0.37
B-His-48-109	84	9581	9.7	+3.6	30	60	-0.81

It was shown that the B-His-48-109 mutant, like the full-length protein, retains cationic properties. The instability index, despite the increase, remained within the acceptable limit (less than 40). The aliphatic index and hydrophobicity index indicate a slight decrease in the thermostability of the binase mutant and an increase in its solubility.

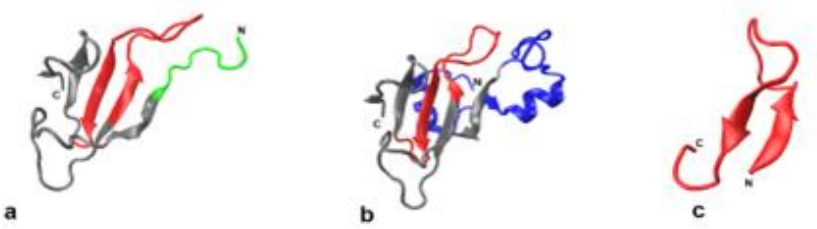

Fig. 1. Model of binase mutant B-His-48-109 spatial structure (*a*) in comparison with full-size binase (*b*) and its peptide B74-94 (*c*). Fragment of β-sheet, corresponding to peptide B74-94, is highlighted in red, N-terminal polyhistidine affinity tag is highlighted in green, binase fragment missing in B-His-48-109 is highlighted in blue. N and C stand for terminal amino and carboxyl group of proteins, correspondingly.

To produce a deletion mutant of binase, we created a pET15b-Bin48N expression plasmid (Figure 2a, 2b) bearing the binase gene fragment under the control of the IPTG-inducible T7 promoter. This construct enables the production of recombinant B-His-48-109 protein with a N-terminal polyhistidine tag in *E. coli* BL21(λDE3) cells. Synthesis of the mutant binase was induced by addition of IPTG to the medium. The IPTG concentration (0.1-1 mM), temperature (16 ℃, 25 ℃, 30 °C and 37 °C) and incubation time (3 and 5 h) were varied to

optimize the induction conditions. The maximum level of recombinant protein production was observed when bacteria were grown at 37 °C for 3 h in the presence of 0.1 mM IPTG (Table 2).

Table 2. Optimization of peptide B-His-48-109 expression in *E. coli* BL21(λDE3) recombinant strain. * Biosynthesis efficiency was evaluated according to the amount of protein of interest in induced culture lysates analyzed by SDS-PAGE and expressed in "+", where one "+" corresponds to minimum level of recombinant protein production and "++++" corresponds to maximum level. IPTG – isopropyl-β-D-1-thiogalactopyranoside.

Temperature, °C	IPTG, mM	Time, h	Biosynthesis efficiency*
16	0.1	3	+
25	0.1	3	+++
30	0.1	3	+++
37	0.1	3	++++
16	0.5	3	+
25	0.5	3	++
30	0.5	3	+++
37	0.5	3	+++
16	1.0	3	+
25	1.0	3	++
30	1.0	3	+++
37	1.0	3	+++
16	0.1	5	+
25	0.1	5	+++
30	0.1	5	+++
37	0.1	5	++++
16	0.5	5	+
25	0.5	5	++
30	0.5	5	+++
37	0.5	5	+++
16	1.0	5	+
25	1.0	5	++
30	1.0	5	+++
37	1.0	5	+++

The recombinant protein was isolated using metal affinity chromatography. Minor modifications were made to the standard purification protocol. The protein was sorbed onto Ni-NTA Sepharose and the first washing step was carried out without imidazole because the deletion mutant B-His-48-109 did not bind efficiently to the sorbent. The second wash was performed using buffer with reduced concentration of imidazole (10 mM). Protein elution was carried out stepwise, varying the imidazole concentration from 50 to 500 mM. The maximum protein yield was detected from 150 mM to 300 mM imidazole. To facilitate the

subsequent step of purification from low molecular weight impurities, elution of mutant B-His-48-109 was performed at 150 mM imidazole. The protein was desalted, and the homogeneity of the resulting preparation was assessed by 16% SDS-PAAG. As a result, a highly purified binase mutant B-His-48-109 with a molecular weight of about 10 kDa was obtained (Fig. 2c). The protein yield was 28 mg per liter of bacterial culture. The obtained binase mutant showed no catalytic activity.

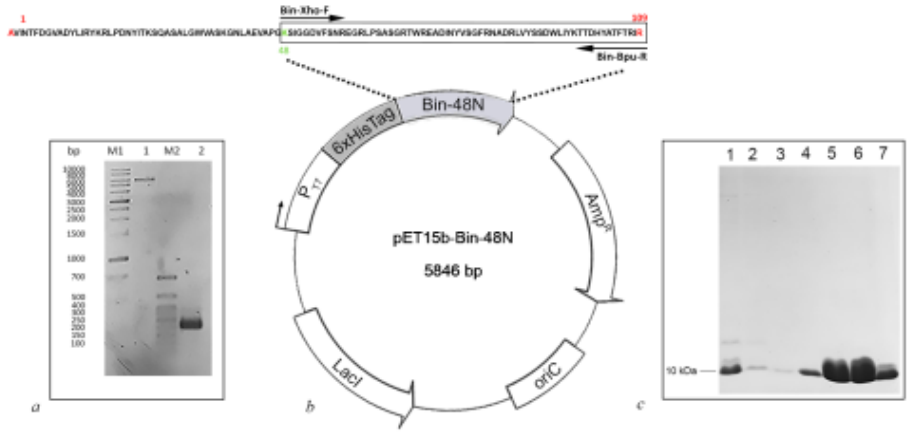

Fig. 2. Genetic construct pET15b-Bin48N. (*a*) Electrophoresis of vector pET15b (1) and PCR product of binase gene fragment Bin-48N (2), M1 and M2 – molecular weight ladders. (*b*) Plasmid map of pET15b-Bin48N constructed for mutant 48-109 production. LacI – lac-repressor, oriC – origin of replication, Amp^R – selective marker of resistance to ampicillin, P_{T7} – T7 phage promoter, 6xHisTag – polyhistidine affinity tag, Bin-48N – binase fragment from 48 to 109 amino acid residue, Bin-Xho-F and Bin-Bpu-R – oligonucleotides for binase gene fragment PCR amplification. (*c*) SDS-PAGE analysis of protein fractions acquired during the purification of binase mutant B-His-48-109 from the cells of recombinant strain *E. coli* BL21(λDE3) pET15b-Bin48N. 1 – intracellular protein fraction, 2 – "flow-through" fraction (proteins that did not bind with sorbent), 3 – sorbent washing fraction, 4-7 – fractions of mutant elution from the sorbent with 150 mM imidazole.

The cytotoxic properties of the obtained binase mutant were analyzed by MTT test on the tumor cell line A549, previously insensitive to the action of binase peptides, and normal cells WI-38. Cells were treated with B-His-48-109 and native binase at concentrations of 100 and 300 μg/mL for 48 h. Binase at the concentration of 100 μg/ml reduced the viability of tumor cells by 15% (Fig. 3); the obtained mutant at the same concentration had no cytotoxicity. Meanwhile, both cell lines showed high sensitivity to the B-His-48-109 mutant at a concentration of 300 μg/mL. Cell viability decreased approximately by 85% (Fig. 3). The native binase at the maximum concentration showed significantly less pronounced cytotoxic effects (Fig. 3). Therefore, binase mutant had enhanced cytotoxicity and decreased specificity as compared to full-length binase. The loss of selectivity in the obtained mutant indicates the probability of accumulation of its positively charged molecules on the cell membrane, which leads to the initiation of apoptosis and cell death, according to the mechanism of action of cationic peptides.

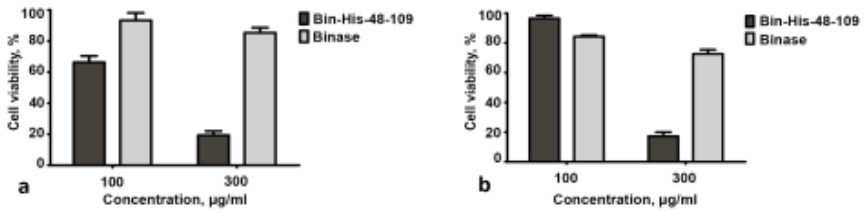

Fig. 3. Evaluation of viability of WI-38 normal cells (a) and A549 tumor cells (b) under the effect of mutant B-His-48-109 in comparison with binase. Viability of untreated cells was taken as 100 %.

To assess the localization of the obtained deletion mutant in A549 tumor cells, immunofluorescence analysis was performed using antibodies against binase and a polyhistidine tag. It was shown that the mutant B-His-48-109, like the native protein, penetrates into tumor cells, localizing in the perinuclear zone, in contrast to binase, which is diffusely distributed in the cytoplasm (Fig. 4).

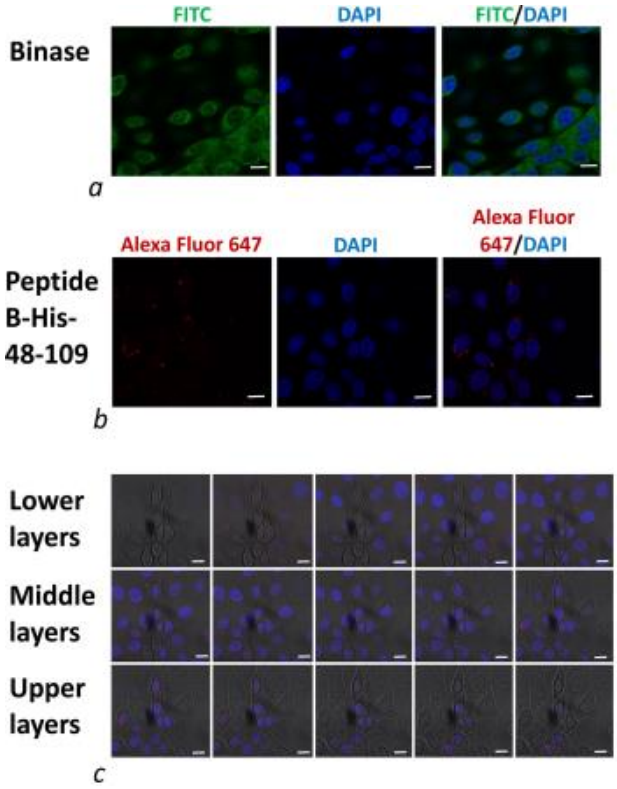

Fig. 4. Fluorescence analysis of mutant B-His-48-109 localization (a,c) in comparison with binase (b) in adenocarcinoma cells A549. Sections (a) and (b) represent the general image of cells, while the section (c) displays layered tissue slices in increments of 1 μm. Binase was detected with primary rabbit antibodies to binase and visualized with secondary antibodies conjugated with FITC (green). Mutant B-His-48-109 was visualized with primary mouse antibodies to polyhistidine tag conjugated with Alexa Fluor 647 (red). Cell nuclei were colored with DAPI. Scale bar – 10 μm.

4 Discussion

The search for new antitumor peptides and their modification are one of the promising approaches in the development of modern drugs. The net charge, hydrophobicity and helicity are known to influence the biological effects of peptides [4]. Modification of the amino acid composition, secondary and tertiary structures, and shape of peptides leads to changes in their biophysical parameters and, as a consequence, to an increase in the activity and stability of their molecules, and, in some cases, to a change in their mechanism of action. For example, modification of the amino acid composition of the antitumor peptide temporin-1CEa increased its cytotoxic properties by changing the cationicity and hydrophobicity of its molecule, while the amphipathicity and secondary structure of the molecule remained unchanged [12]. Currently, increasing cationicity is one of the approaches in modifying peptides to enhance their antitumor potential.

Cytotoxic binase-based peptides have shown their ability to inhibit proliferation of tumor cell lines HeLa, MCF-7 and BT-20 [9]. However, their antiproliferative potential was much weaker than that of the full-length protein. Moreover, A549 cells were absolutely insensitive to the action of peptides. In the present work, the most active binase peptide B74-94 was modified to enhance cytotoxic properties. Since one of the mechanisms responsible for the selective action of binase against tumor cells is the high level of cationicity and its ability to hydrolyze intracellular RNA, the length of the amino acid sequence of the peptide was extended to increase the charge of the molecule and to include amino acid residues involved in RNA hydrolysis and a polyhistidine tag to facilitate purification (Table 1, Fig. 1, 2). Despite the expected results, the obtained deletion mutant B-His-48-109 had no catalytic activity. The inability of the mutant to hydrolyze RNA is probably due to a change in the conformation of the beta-sheet, which comprises the amino acid residues of the active center, induced by the removal of the first two alpha helices that stabilize the structure of the full-length protein.

Modification of the binase peptide, in particular, the increased cationicity led to enhancement of the cytotoxic properties of the new mutant. Thus, mutant B-His-48-109 became much more cytotoxic in comparison with the full-size protein, however, it lost the selectivity towards the cancer cells. Probably, the reduction of the extended region from the N-terminus of the binase resulted in the disruption of the stability of the protein molecule and, as a consequence, its aggregation.

Depending on the biological effects, antitumor peptides can be divided into three groups: peptides that exhibit inhibitory activity against tumor cells, and peptides that cause their necrosis or apoptosis. Peptides of the first group act on integrin receptors on the surface of cancer cells and inhibit their migration and metastasis [13]. The target of necrosis-inducing peptides is the cell membrane [14]. Due to electrostatic interactions, peptides bind to the negatively charged membrane of cancer cells, disrupt its integrity, which leads to necrotic changes in the cell. Peptides of the third group are internalized into tumor cells and act on various intracellular targets, triggering apoptosis in them [15]. According to the data obtained in this work, it is not possible to accurately determine the mechanism of the antitumor effect of the binase mutant. However, taking into account its ability to penetrate into tumor cells and localize in the nuclear region (Fig. 4), we assume that it could belong to the group of apoptosis-inducing peptides.

5 Conclusions

Thus, in this work we have created an expression system that allows to obtain a homogeneous preparation of a deletion mutant of binase with increased antitumor activity in preparative quantities. The obtained mutant of binase did not show selectivity towards normal cells,

which is probably due to its ability to aggregate and requires additional modifications of its amino acid sequence. The obtained data show that modification of structural and physicochemical properties of antitumor peptides is an important step in the development of drugs based on them.

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