BioNanoSci.
DOI 10.1007/s12668-016-0269-y

Obvious and Hidden Reasons of Breast Cancer Cell Sensitivity to Antitumor RNase

Pavel Zelenikhin[1] · **Victoria Pukhovskaya**[1] · **Azat Garipov**[1] · **Anna Makeeva**[1] · **Evgenia Sokolova**[1] · **Olga Ilinskaya**[1]

Abstract Breast cancer is the second most common cancer in women, but chemotherapy and targeting antibodies possess certain side effects. Today's efforts are aimed to search of new effective methods of breast cancer treatment, especially triple-negative breast cancer, which cannot be affected by conventional therapeutics. Binase, the RNase secreted by *Bacillus pumilus*, possesses unusual biological activities as selective anticancer agent inducing apoptosis in malignant cells. Many aspects of this activity have been elucidated, but the details of its specific mechanisms remain unknown. Here, we found for the first time that the sensitivity of breast cancer cells to binase was not connected with the level of cellular RNA catalytic degradation. Gene expression analysis in different breast cancer cell lines and breast cancer cells from patient samples revealed that the PI3K/AKT pathway activity could be considered as a potential marker of binase effectiveness.

Keywords Breast cancer · BT-20 · HBL-100 · ZR-75-1 · MCF-7 · *Bacillus pumilus* RNase · Binase · RNase A · RNA content · Apoptosis · Oncogene expression

1 Introduction

Breast cancer is the most common invasive cancer in females worldwide; 18.2 % of all cancer deaths worldwide, including both males and females, are caused by breast cancer. Now, chemotherapy and hormone therapy referring to estrogen or progesterone receptor-positive cancers are supplemented by targeted drugs. Further, there are monoclonal antibodies, which target and destroy HER2 (epidermal growth factor receptor 2)-positive cancer cells. Nevertheless, drugs of lower toxicity and higher effectiveness, especially against triple-negative breast cancer, would be desirable.

Cytotoxic ribonucleases (RNases) of various organisms are currently studied as potential antitumor agents. The commercial preparation of RNase from the frog *Rana pipiens* oocytes (onconase) is now at the third stage of clinical trials as a drug against lung mesothelioma [1]. Antitumor activity was found to be characteristic of RNase from bovine testes (BS-RNase), RNases of human eosinophilic granules, and of a number of RNases of lower organisms, including fungi, actinomycetes, and bacteria [2]. RNases possess therapeutic opportunities for cancer treatment, as RNA damage caused by RNases could be an important alternative to high toxic standard DNA-damaging chemotherapeutics. In most cases, the ribonucleolytic activity of exogenously applied RNases is essential for their cytotoxicity [2, 3].

Binase is a highly cationic guanyl-preferring RNase secreted by *Bacillus pumilus* (former *B. intermedius* [4], genome sequence is known [5]) that catalyzes RNA cleavage without the need for metal ions and cofactors. Cloning and sequencing of the binase gene have been reported [6, 7], and a three-dimensional structure at 1.65 A° resolution was determined [8]. Earlier, we have shown that *Bacillus pumilus* RNase (binase) shuts down processes that are vital for cancer cells to survive, but does not affect or poorly affects healthy cells [9]. For human embryonic kidney cells (HEK) and myeloid progenitor cells (FDC-P1), we have demonstrated that binase action leads to decrease in the total amount of RNA, but the significant decrease of RNA in kidney HEKhSK4 cells artificially expressing the human small conductance Ca^{2+}-activated K^+ channel type hSK4 (up to 44 % compared to binase-untreated cells) did not induce cell death and apoptosis, while decrease of 20 % RNA in FDC-P1iR1171 cells (transgenic

✉ Pavel Zelenikhin
pasha_mic@mail.ru

[1] Kazan (Volga-region) Federal University, Kazan, Russia

Published online: 17 September 2016

myeloid progenitor cells expressing the activated *kit* oncogene) is accompanied by apoptosis. Interesting, the small decrease of cellular RNA (about 5 %) in myeloid FDC-P1 cells did not lead to apoptosis [10]. Thus, in order to establish the correlation between RNA alteration and apoptotic activity of binase, the cell lines of identical origin have to be analyzed. Based on this assumption, we investigated how binase affects the total amount of intracellular RNA in four different breast cancer cell lines and whether the alteration of RNA content is connected with sensitivity of cells towards binase cytotoxic action.

Here, we show for the first time that the sensitivity of breast cancer cells to binase does not depend on the level of cellular RNA catalytic destruction. Gene expression analysis in different breast cancer cell lines and breast cancer cells from patient samples revealed the possible targets of binase apoptotic action. We assume that interaction of binase with oncogenic RNA as well as with oncoproteins could be a reason of binase selective cytotoxic activity.

2 Experimental

2.1 Materials and Methods

Bacterial RNase The guanyl-specific RNAse from *B. pumilus*, binase (12.2 kDa, 109 amino acid residues, p*I* 9.5), was homogenously isolated from the culture fluid of *Escherichia coli* BL21 carrying the pGEMGX1/ent/Bi plasmid, according to Schulga et al. [11]. The molecular weight of binase, as well as its catalytic activity against synthetic substrates and high-polymeric yeast RNA, was reported earlier [12]. The commercial preparation of pancreatic RNase A (Vector, Novosibirsk, Russia) was used for comparison with binase. Both enzymes have similar catalytic activity about 14×10^6 unit/mg.

Cell Cultures Breast cancer cell lines BT-20, HBL-100, ZR-75-1, and MCF-7 were obtained from All-Russian cell culture collection (Saint-Petersburg). Cells were cultivated in RPMI supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), 2 mM glutamine (Sigma-Aldrich, USA), and 10 % fetal bovine serum (HyClone, USA) in 12-well plates (SPL Lifesciences, Korea) at 37 °C and 5 % CO_2; 5×10^4 cells/ml were seeded into each well and grown to 60 % monolayer formation, then cultural fluid was changed onto fresh RNase-containing medium, and cells were grown 24 and 48 h before cytometric analysis.

Cytometry Apoptosis was measured using BD FACSCanto II flow cytometer (BD, USA) and merocyanine 540 dye (MC540, Sigma-Aldrich, USA). The dye was added to 1-ml cell suspension containing 2×10^5 cells/ml in complete medium and incubated for 20 min in the dark before analysis. The four cell lines that were treated or left untreated with different concentrations of RNase (100 and 300 μg/ml) were tested. As standard apoptosis inductor, 10 μM camptothecin (Sigma-Aldrich, USA) was used.

RNA Analysis RNA analysis was accomplished using the metachromatic dye acridine orange (AO, Sigma-Aldrich, USA). Cells were fixed with 70 % ethanol and permeabilized, and then cell suspension (2×10^5 cells/ml) was dyed by AO in final concentration of 4 μg/ml. Total RNA amount was evaluated using BD FACSCanto II flow cytometer immediately after staining.

Database Gene expression data sets from patient samples were obtained from Gene Expression Omnibus (GEO) database and GSE15852 data set, which includes gene expression patterns of 43 breast tumors and their paired normal control (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15852).

Statistics The results were processed using the Statistica 6.0 software. The nonparametric Mann–Whitney test was applied in order to determine the significance of differences. The significance level was $p \leq 0.05$.

2.2 RNase-Induced Apoptosis of Breast Cancer Lines

Using flow cytometry, we estimated capacity of binase and pancreatic RNase A to induce apoptosis of breast cancer cells HBL-100, BT-20, MCF-7, and ZR-75-1. It was shown that ZR-75-1 line possesses maximal sensitivity to binase. The enzyme at concentration 100 μg/ml induced apoptosis of 13 % cells during 24 h of treatment that was twice more that of non-treated cells (Fig. 1a). After 48 h, the number of apoptotic cells was 18 % of whole population, and at binase concentration 300 μg/ml, this value reached 29 % (Fig. 1b). The BT-20 cell line has middle sensitivity to binase (Fig. 1c, d); the MCF-7 line demonstrated significant susceptibility to binase at 300 μg/ml after 24 h (Fig. 1e), but this effect was eliminated after 48 h (Fig. 1f). Binase at all concentrations used did not induce apoptosis of HBL-100 cells (Fig. 1g, h). Pancreatic RNase A never induced apoptosis in all the cell lines (Fig. 1a–h).

2.3 Total Cellular RNA Alteration Under Binase Action

Analysis of cellular RNA content after 24 h of treatment by binase-sensitive ZR-75-1 cell line and non-sensitive HBL-100 cells showed the equal RNA decease in both cell lines at 16 h of treatment. This decrease was no more than 20 % of total RNA in non-treated cells. No difference between sensitive and resistant cell lines was observed (Fig. 2).

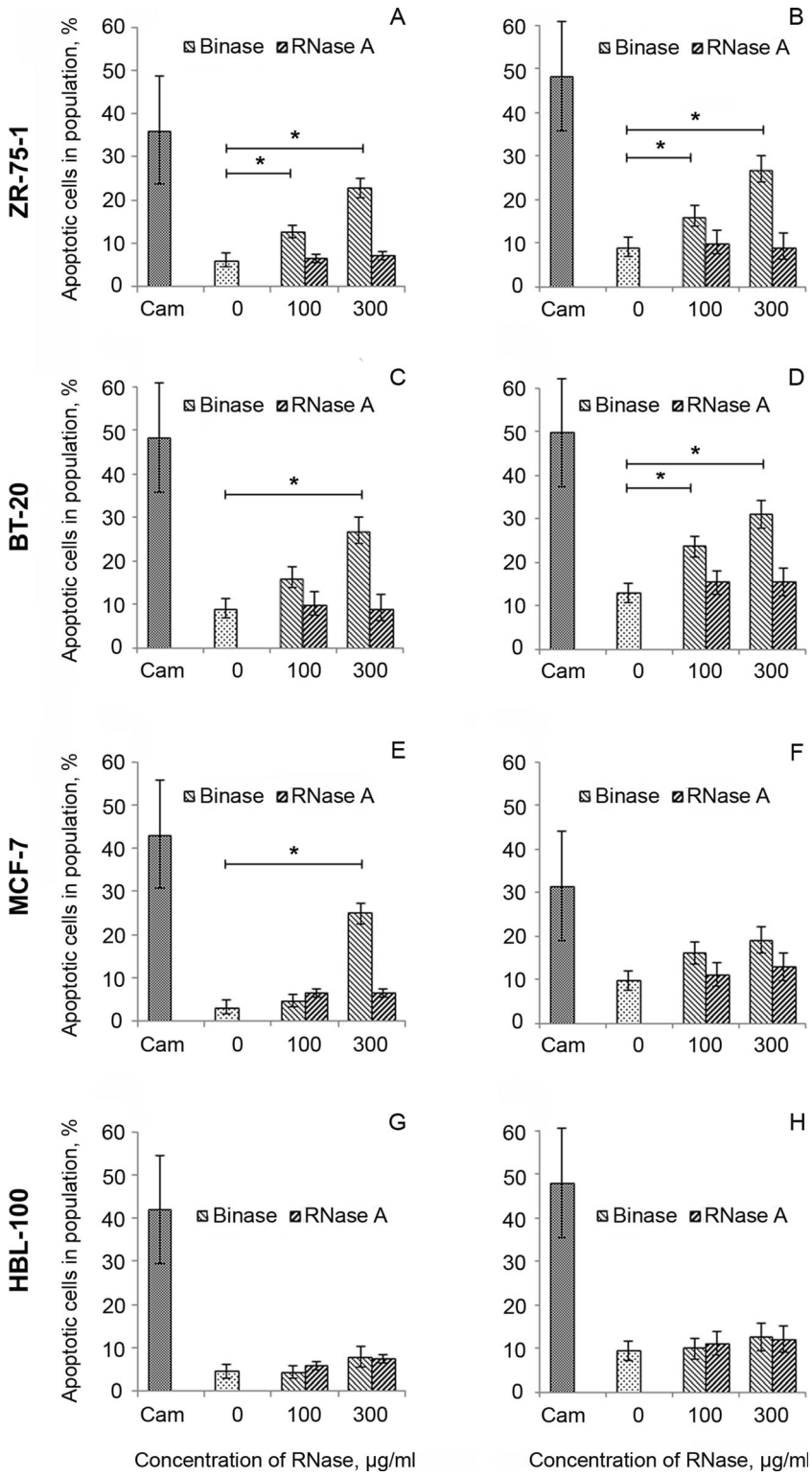

Fig. 1 Apoptosis induction in ZR-75-1 (**a**, **b**), BT-20 (**c**, **d**), MCF-7 (**e**, **f**), and HBL-100 (**g**, **h**) cells by binase and RNAse A after 24 h (left column) and 48 h (right column) of incubation. *Cam*—camptothecin (10 μM). The data represent mean ± SD of the mean from three independent experiments performed in triplicates, $^{*}p < 0.05$ vs. control

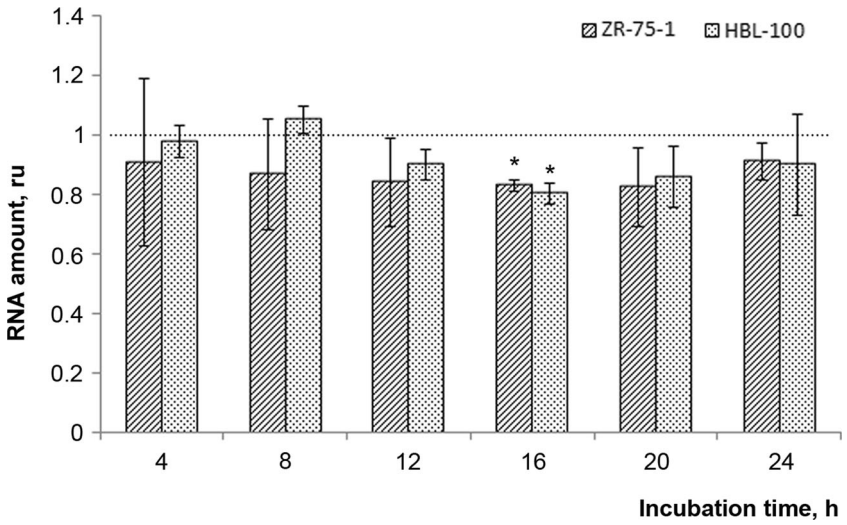

Fig. 2 Relative amount of RNA in binase-treated (100 μg/ml) ZR-75-1 and HBL-100 cells in dependence on the incubation time. The data are shown in relative units (ru) in comparison with binase-untreated cells. Values represent mean ± SD of the mean from three independent experiments performed in triplicates, $*p < 0.05$ vs. control

2.4 Oncogene Expression in Breast Cancer Cell Lines and Patient Samples with Diagnosed Breast Cancer

Based on the available data, one of the crucial pathways that regulates the state and aggressiveness of breast cancer is PI3K/AKT pathway (Table 1).

We propose that PI3K/AKT pathway activity could be connected with cancer cell susceptibility to binase cytotoxic action. All sensitive to binase cell lines carry activation mutations or possess amplificated proteins of PI3K/AKT pathway. Interestingly, receptor tyrosine-protein kinase (human epidermal growth factor receptor 2 (HER2)) and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) are pre-membrane proteins. Probably, binase can interact with these proteins directly during its internalization that leads to inhibition of their functions. All tested cell lines that appeared to be sensitive to binase (ZR-75-1, MCF-7, and BT-20) harbor mutations that lead to PI3K/AKT pathway activation. On the opposite, the HBL-100 cell line, which was resistant to binase, did not have activated components of that pathway.

Based on analysis of GSE15852 dataset in GEO database, we revealed increased expression AKT1 and decreased expression of PTEN, which is a negative regulator of PI3K/AKT pathway (Fig. 3), in breast cancer samples compared to corresponding normal tissue.

Table 1 Breast cancer cell lines and associated oncogenes

Cell line	Genes	
	Mutated	Amplified
ZR-75-1	PTEN [13], ER [14], PR [14], HER2 [14, 15]	
MCF-7	ER [14, 16], PIK3CA [13], PR [14]	N-ras [17]
BT-20	p53 [18], PIK3CA [13], HER1 [14]	
HBL-100		myc [16]

3 Discussion

We have shown that the four breast cancer cell lines demonstrate different susceptibility to binase cytotoxic action. According to increased level of apoptosis, the cell lines could be aligned from MCF-7 and BT-20 to ZR-75-1. Binase did not affect the HBL-100 line representing triple-negative breast cancer (TNBC), which is defined by the absence of estrogen and progesterone receptors and the absence of HER2 overexpression. Interestingly, the other TNBC cell line BT-20 carrying PI3K/AKT activation mutation of PIK3CA was sensitive to binase apoptogenic action. This fact supports the concept of microbial RNase perspective use as an agent against TNBC, which usually does not respond to conventional therapeutics.

Applying a new method for integrating several genome-wide datasets in order to characterize the molecular aberration landscape of human cancer, Schlicker et al. [19] revealed that breast cancer shows strong similarity between cell lines and tumors. Our analysis of gene expression in patient samples also revealed the similarity of PI3K/AKT pathway activation in tumor samples and cell lines.

Our results testify that the reduction in the total RNA level in breast cancer cells is not connected with the induction of the apoptosis process by binase. Earlier, we found the high susceptibility of transgenic myeloid progenitor cells expressing the activated kit oncogene (FDC-P1iR1171 cells) to binase, which decreased about 20 % of RNA content in these cells. In the presence of interleukin, viability of binase-treated cells retained, despite the fact that the RNA content in these cells is 23 % lower than without the binase [20]. Another cytotoxic

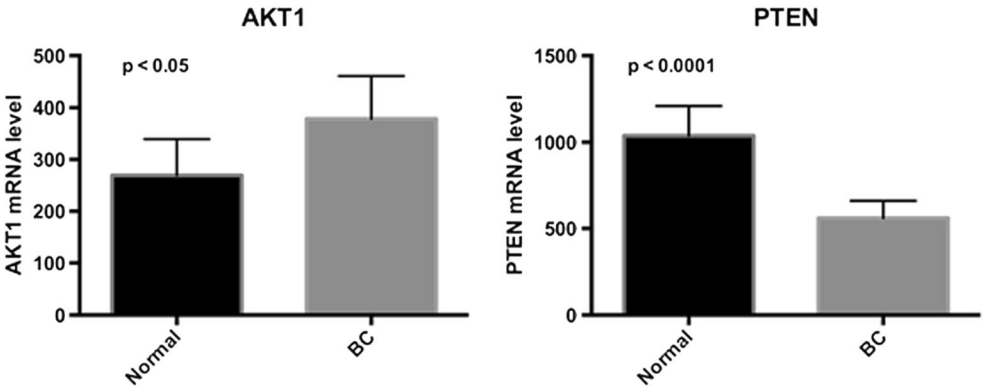

Fig. 3 Relative mRNA expression of AKT1 and PTEN in human breast cancer (BC) and normal breast tissues

RNase from bull frog *Rana pipiens* (onconase) induces apoptosis in mitogen-stimulated lymphocytes, but does not affect the total RNA content [21]. Thus, we cannot consider apoptogenicity as well as selective cytotoxicity of binase as a direct consequence of its catalytic activity.

4 Conclusion

Here, we have shown that total RNA decrease itself could not be an obvious reason for cancer cell death. On the other side, we cannot exclude that breakdown of certain mRNA of oncogenes is partially involved in binase-induced apoptosis. Comparison of gene expression profiles in patients with diagnosed breast cancer and their paired normal tissue, as well as analysis of cancer cells viability in response to binase treatment, allowed us to conclude that PI3K/AKT pathway activity could be considered as a potential marker of binase effectiveness, thus making it one of the hidden reasons of different sensibility of breast cancer cells toward binase.

Acknowledgments The study was performed within the Russian Government Program of Competitive Growth of Kazan Federal University and was supported by the Russian Science Foundation (project no. 14-14-00522).

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