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The Combined Action of Binase and Bleomycin on Human Lung Adenocarcinoma Cells

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Abstract—Some microbial ribonucleases (RNases) demonstrate selective cytotoxic effect against a wide range of tumor cells. In this context combined use of cytotoxic RNases in complex therapy with other chemotherapeutic agents appears to be especially promising. In this study we have investigated the apoptosisinduced effect of *Bacillus pumilus* RNase (binase) in combination with known antitumor antibiotic bleomycin on human lung adenocarcinoma A549 cells. The combined effect of high concentrations of these agents did not have any mutual increase in their apoptosisinduced action, while a combination of nonapoptotic concentrations resulted in the increase of proportion of apoptotic cells up to 22% as compared with individual effect of bleomycin (6%) and binase (12%) used separately. These results indicate that binase and bleomycin are effective in combination of their low concentrations and ineffective in combination of their high concentrations.

Keywords: cytotoxic ribonucleases, *Bacillus pumilus*, binase, bleomycin, antitumor activity, lung adenocarcinoma

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INTRODUCTION

Some ribonucleases (RNase) of different origin exhibit antitumor activity. In this context the therapeutic potential of RNases causing a pronounced selective cytotoxic effect on tumor cells is especially interesting. Identification of selected RNase cytotoxicity to cells expressing certain oncogenes, provides a basis for the development of RNase preparations as agents for targeted therapy. In the case of RNase from *Bacillus pumilus* (formerly known as a *B. intermedius strain* [1]) selective cytotoxicity has been recognized for cells expressing oncogenes *ras* [2], *kit* [3], *AML1ETO* [4], and *TNF* [5]. The most wellknown RNase with antitumor activity, onconase (leopard frog *Rana pipiens* RNase), demonstrated an inhibitory effect on the expression of 34% genes of lung malignant mesothelioma cells; it caused a 3-fold decrease in expression of genes influencing apoptosis (*IL-24, TNFAIP3*), transcription (*ATF3*, *DDIT3*, *MAFF*, *HDAC9*, *SNAPC1*) and the immune response (*IL-6*, *COX-2*) [6]. Onconase monotherapy of lung malignant mesothelioma did not produce expected results and so clinical trials of its combination with an antitumor antibiotic doxorubicin started; they are currently undergoing phase III [7]. Among known antitumor agents onconase cytotoxicity increased in combination with tamoxifen, cisplatin, and vincristine, but the most effective was onconase combination with doxorubicin [7, 8]. Interaction of bacterial RNases with known anticancer agents has not been studied so far. Taking into consideration a high apoptogenic activity of binase towards human lung carcinoma cells [9], partially determined by expression of the mutant *k-RAS*, we aimed to investigate the cytotoxic potential of this RNase in combination with bleomycin, a glycopeptide antibiotic synthesized by bacteria *Streptomyces verticillus* and demonstrating both DNA damaging and RNA fragmentation effects [10].

MATERIALS AND METHODS

Enzyme

Binase, guanyl specific RNAse from wild type *B. pumilus* strain 319 (EC 3.1.27.3, molecular mass of 12.3 kDa, 109 residues, pI = 9.5), was used in experiments. The enzyme was isolated as a homogeneous protein from the culture medium of the recombinant strain *Escherichia coli* BL21, carrying plasmid pGEMGX1/ent/Bi [11]. Catalytic activity of binase was described previously using synthetic substrates and highpolymer yeast RNA [12, 13].

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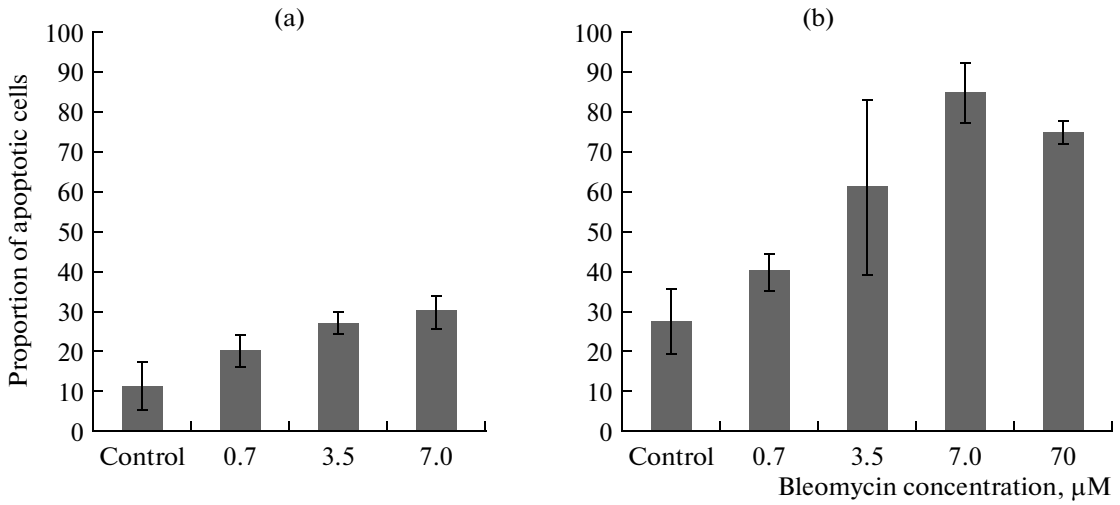

Fig. 1. The apoptosis inducing effect of bleomycin after its incubation with human lung adenocarcinoma cells A549 for 24 h (a) and 72 h (b).

Bleomycin

Bleomycin sulfate from *Streptomyces verticillus* was purchased from Sigma (USA).

Cell Culture

Human adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (Rockville, USA). Cells were cultured in RPMI 1640 medium (Sigma), containing 10% fetal calf serum, 2 mM glutamine, and 100 U/mL penicillin and streptomycin, at 37°C in a humidified atmosphere of 5% CO_2.

Cells passaged into 6-well culture plates (GBO, Germany) were grown until a confluent monolayer of 50% and the medium was replaced by a similar one, containing binase, bleomycin, their combinations and/or $FeSO_4$ as a source of Fe(II). Incubation carried out for 24 and 72 h.

Flow Cytometry

Apoptotic cell changes were recorded by means of a flow cytometer BD FACSCanto II (BD, USA) using merocyanine 540 dye (Sigma) [14].

Statistical treatment of the results obtained from triplicates of each experiment were performed by standard methods in Microsoft Excel 2010.

RESULTS AND DISCUSSION

Bleomycin is widely used in cancer chemotherapy, especially in squamouscell cancer of mucosa of the mouth mucosa, nose, throat, esophagus and lung [15]. In our experiments bleomycin used in the range of concentrations of 0.7 to 7 μM exhibited a dosedependent apoptosis inducing action on the human lung carcinoma cell line A549. After incubation with bleomycin (7 μM) for 24 h the proportion of apoptotic cells in the population of A549 cells increased to 30% (Fig. 1a); after incubation for 72 h this proportion increased to 85% (Fig. 1b). The increase in the bleomycin level in the medium to 70 μM did not increase the proportion of apoptotic cells in the population (Fig. 1b).

According to Carter et al. [16] divalent iron acts as a bleomycin cofactor; however, addition of equimolar amounts of Fe(II) to bleomycin did not increase its apoptogenic activity (data not shown). It appears that trace amounts of Fe(II) in the initial medium are sufficient for manifestation of apoptosis inducing activity of bleomycin.

As expected, binase exhibited the cytotoxic effect on malignant cells; this effect was comparable to the effect of bleomycin: the proportion of apoptotic cells A549 after incubation with 4.15 and 25 μM binase for 24 h was 11 and 40%, respectively, while in control (untreated) cells this parameter did not exceed 9% (Fig. 2a).

We evaluated cytotoxicity of combinations of bleomycin with binase using a certain concentration of one compound with increasing concentrations of another one. After incubation with 0.1 and 7 μM bleomycin for 24 h the number of apoptotic cells was 6 and 24%, respectively. In the case of simultaneous exposure to 0.1 μM bleomycin/4.15 μM binase the proportion of apoptotic cells was 22%. At higher concentration of binase (0.1 μM bleomycin/25 μM binase), the proportion of apoptotic cells reached 31% (Fig. 2a). However, even in this case the level of apoptotic cells was lower than that induced only binase: after the treatment of cells with 25 μM binase the proportion of apoptotic cells was 40% (Fig. 2a). Thus, the combination

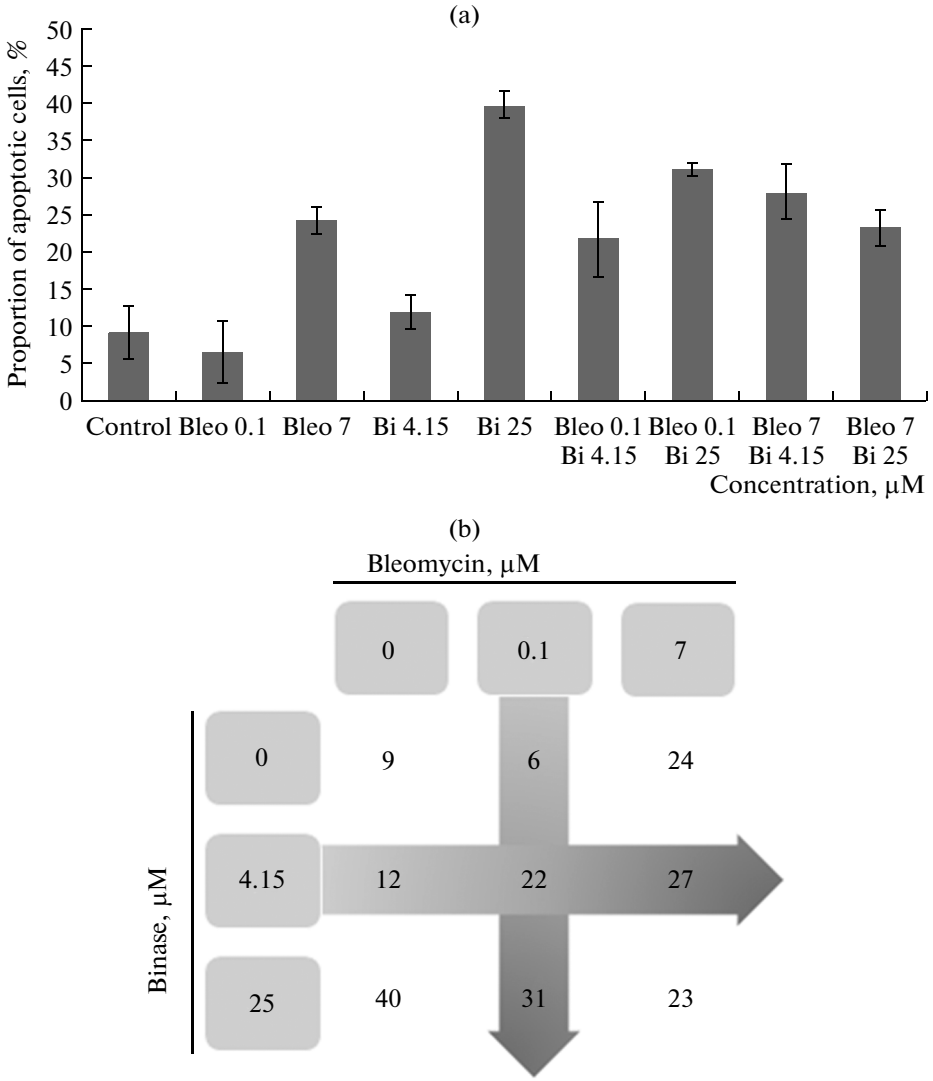

Fig. 2. Combined apoptosis-inducing effect of bleomycin (Bleo) with binase (Bi) on human lung adenocarcinoma cells A549 after incubation for 24 h.

of a low (weakly toxic) concentration of bleomycin with high concentration of binase was ineffective.

Combination of a low binase concentration (4.15 μM) with increasing concentrations of bleomycin (0.1 and 7 μM) increased the proportion of apoptotic cells to 22 and 27%, respectively. In this context it should be noted that the combined effect of the maximal concentrations of both compounds used in this study ineffective (23% of apoptotic cells), whereas the combination of their nonapoptogenic concentrations increased the proportion of apoptotic cells to 22% as compared to the individual effects of bleomycin (6%) and binase (12%) (Fig. 2b). These data suggest that combinations of low concentrations binase and bleomycin are effective while combinations of their high concentrations are ineffective.

Molecular mechanisms of action of both compounds share some common features. For example, bleomycin action of pneumocytes MLE-12, and primary alveolar type II cells induces apoptosis via the mitochondrial pathway associated with activation of stress kinases JNK and proapoptotic proteins Bak and Bax [17]. On the other hand, there is evidence that bleomycin activates caspase-8 and induces apoptosis of primary bronchial epithelial cells and pulmonary artery endothelial cells via the extrinsic pathway [18]. Apoptosis induced by binase also combines extrinsic and intrinsic pathways [5].

Besides degradation of DNA bleomycin also causes tRNA fragmentation, and the second reaction is more specific [10]. The mechanism of bleomycin action at the level of RNA shares some similarity with the cytotoxic effect of onconase. Onconase exhibits its antitumor properties by inhibiting protein synthesis due to degradation of one or more tRNAs; it is suggested that both anticancer drugs specifically cleave $tRNA^{Lys}$ and

$tRNA^{Phe}$ [19, 20]. For binase its catalytic activity is also an important factor for manifestation of the antitumor effect [21–23]. It is possible that the combination of high concentrations of binase and bleomycin results in competition between these ribonucleolytic agents for binding to available cellular RNA and corresponding decrease in the apoptotic activity that does not occur when combination of low concentrations of both antitumor agents is used.

The antitumor antibiotic doxorubicin tested, in combination with onconase, interacts with DNA and thus disrupts repair of topoisomerase IImediated DNA damage. In addition, doxorubicin damages DNA due to the generation of free radicals, leading to oxidative stress and apoptosis [24]. In vitro combination of onconase and doxorubicin was more toxic to diffuse large cell lymphoma than either drug alone: the cytotoxic index of onconase, doxorubicin, and their combination was 25, 15 and 35%, respectively [25]. According to our analysis, the proportion of apoptotic cells of lung adenocarcinoma, after treatment with binase, bleomycin, and their combination was 12, 6, and 22%, respectively. This suggests that subsequent studies of the combined effect of binase and bleomycin are rather promising.

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