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The antitumour effect of ribonucleases and antiangiogenins on human tumours in nude mice

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ABSTRACT

Bovine seminal ribonuclease (BS-RNase) and bovine pancreatic ribonuclease (RNase A) have potent antitumour activity. In this study, the antitumour effect of selected low-toxic antiangiogenins of natural origin and bovine ribonucleases conjugated with polyethylene glycol (PEG) was tested. The antiangiogenins were selected according their toxicity. The antitumour activity of PEGylated BS-RNase and RNase A was enhanced when injected together with antiangiogenins into the athymic nude mice bearing human pancreatic tumours and human melanoma C-32 tumours.

KEYWORDS: angiogenin, antitumour, aspermatogenesis, bovine, embryotoxic, human, injection, pancreatic, substance

1. INTRODUCTION

Biological and antitumour properties of bovine seminal and pancreatic ribonucleases have been studied from the beginning of 1970. Many works have been published by researchers from many countries. Much attention has been focused on their antitumour efficacy and processes associated with this activity [1-5]. In recent years, in addition to studying their direct antitumour effect, attention has also been paid to the substances participating in the initial process of the tumour growth and antiangiogenins, which slow down the entry of the blood into tumour veins. The formation of new blood vessels in tumours is known as angiogenesis. This process is very important for the growth of the tumour and metastases [6-11]. The mechanisms of this activity have not yet been completely understood. Considerable therapeutic efforts have been focused on the treatment of tumours based on angiogenesis inhibition and the related vascular activity in tumours [12].

Angiogenic substances are presumably released from the tumour and/or from its extracellular matrix by the action of the tumour-secreted molecules [12]. These substances diffuse radially until they reach microvessels, activate their endothelial cells, and induce capillary sprouting in microtubules. This microtubule expression is of essential importance in the progression and metastasis production in different types of carcinomas. The cell proliferation increased in the presence of bFGF (basic fibroblast factor) sensible transfectants [12]. These results demonstrate that the endogenous bFGF may not only positively regulate the angiogenin expression but also stimulate the overall proliferation of human tumour cells. The results of the study presented here aim at being helpful in finding a

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potential therapeutic approach for the treatment of human tumour cells. bFGF participates considerably in the angiogenesis and growth of various tumours, but its inhibition alone does not offer a promising strategy for the angiogenesis inhibition [13].

As the cancer chemotherapeutic agents have serious side effects and damage the normal dividing cells, considerable effort has been directed to the use of antiangiogenic substances that could disrupt the growth of blood vessels in tumours. A number of inhibitors targeting the tumour vasculature have already been identified [14]. To reduce the toxicity of the classic chemotherapy and increase the efficacy of the treatment, the administration of low doses of chemotherapeutic drugs combined with specific antiangiogenic agents was proposed as a suitable therapeutic approach.

In the work presented here, we studied the antimour effect of BS-RNase (bovine seminal ribonuclease) and RNase A (bovine pancreatic ribonuclease) combined with antiangiogenins on human tumours in Nu/Nu mice. Thirty two antiangiogenins were purchased from the Sigma-Aldrich and MB Biomedicals. They were tested for their toxicity in ICR mice in terms of spermatogenicity and embryotoxicity [15, 16].

2. MATERIAL AND METHODS

Isolation of BS-RNase: For the isolation of BS-RNase, one volume of the bull seminal vesicle fluid or seminal plasma was diluted with 2.5 volumes of 2% acetic acid and the protein precipitate was removed by centrifugation and the supernatant concentration was adjusted to 3 M with solid ammonium sulphate. It was then dialyzed against water and freeze-dried. The protein material obtained was purified on CM Sephadex C-50 column in 0.05 M sodium phosphate buffer, pH 8.0, by using a linear gradient of 0.1 to 0.5 M NaCl. The major aspermatogenic and antitumour activity peak was obtained at a concentration of 0.36 M NaCl. This fraction was applied to a column of Sephadex G-100 equilibrated with 0.1 M Tris-hydrochloric acid buffer, pH 7.5 [17].

Conjugation of BS-RNase and RNase A to PEG: BS-RNase and RNase A were covalently attached to polyethylene glycol polymer chains (PEGylation) to "mask" BS-RNase from the host's immune system and to reduce the renal clearance [18].

Antiangiogenins and their toxicity: Thirty two antiangiogenins (see Table 1) were purchased from Sigma-Aldrich and MP Biomedicals. The spermatotoxicity and embryotoxicity of antiangiogenins was tested as described in [19-21] and only the antiangiogenins with low toxicity were used in further experiments.

Immunosuppressive activity of antiangiogenins: Immunosuppressive effect of antiangiogenins on human lymphocytes stimulated in a mixed lymphocyte culture was assessed [22].

Anti-tumour effects *in vivo*: CD-1 athymic female mice (Anlab-Charles River, Czech Republic) weighing 18-20 g were used for all experiments. The mice (5-41 individuals) were kept and fed under aseptic condition. Human tumours were transplanted subcutaneously. The treatment of mice was initiated when the area of the tumours transplanted reached about 5 x 5 mm. Bovine seminal ribonucleases and antiangiogenins, alone or in combination, were injected 3 times a week. The dose and method of the drug administration are indicated in the legend to the figures. The tumour dimensions were measured twice a week with a slide calliper (length x width). The average body weight of mice in experimental groups was simultaneously determined to evaluate the toxic effects of the injected drug(s) during the treatment. The data presented are mean values obtained for each group of the animals injected [23-27].

Histology: All the animals injected with the substances studied were subjected to the tissue excision and examination by histology. Small pieces of these tissues were consequently fixed in the Bouin solution before the examination. Fixed samples of tissues were embedded in paraffin blocks or frozen for freeze microtome. Tissue slides (5 μm) were cut and stained with hematoxylin-eosin.

Statistical analysis: The results are presented as the mean ± standard error of the mean (S.E.M.). The data were statistically analyzed by using the Fisher's t-test.

Ethics: All the experiments adhered to ethical standards and were approved by the Ethical Committee of the institute.

Table 1. Aspermatogenic activity and embryotoxicity of antiangiogenins.

Substances injected in quantities 20 µg into left testes	No. of mice	Aspermatogenesis		Diameter of tubules in µg ± SEM	Embryotoxicity % of embryos in blastocyst stage
		Index mass of injected testes in µg ± SEM	Width of spermatogenic layers in µg ± SEM		
PBS (control)	5	41 ± 7	63 ± 7	151 ± 6	86
Apigenin crystalline	6	35 ± 5	41 ± 4^{++}	129 ± 4	76
Betulinic acid	6	28 ± 11^{++}	16 ± 13^{++}	-	0
Colagen	6	42 ± 6	55 ± 8	142 ± 13	75
Curcumin	6	31 ± 5^{++}	50 ± 6^{++}	138 ± 16	58
Cyclophosphamide monohydrate	5	26 ± 9^{++}	38 ± 7^{++}	132 ± 18^{++}	83
D-glucuronic acid	5	36 ± 4	51 ± 9	133 ± 8^{++}	5
Docetaxel	5	26 ± 9^{++}	56 ± 6	143 ± 22	0
Dopamine hydrochloride	5	33 ± 8	43 ± 9^{++}	117 ± 45^{++}	0
Epigallocatechin galate	7	39 ± 9	49 ± 6^{++}	146 ± 18	65
Fisetin	4	25 ± 7^{++}	42 ± 13^{++}	128 ± 9^{++}	0
Genistein	5	35 ± 3	40 ± 9^{++}	143 ± 9	0
Ginkgo biloba	5	39 ± 10	56 ± 1	131 ± 10^{++}	65
Heparan sulphate	4	44 ± 6	45 ± 9^{++}	152 ± 4	73
Heparin sodium salt	5	38 ± 4	38 ± 5^{++}	145 ± 11	78
Hyaluronidase bovine	5	40 ± 7	56 ± 8	147 ± 12	92
Kanamycin disulphate	5	43 ± 8	55 ± 8	134 ± 10^{++}	15
Matrine	6	42 ± 5	53 ± 11	151 ± 7	0
Melatonin	5	39 ± 4	52 ± 11	144 ± 8	82
Paclitaxel	5	37 ± 3	50 ± 6	146 ± 12	0
Resveratrol	5	41 ± 7	27 ± 5^{++}	118 ± 11^{++}	0
Retinoic acid	5	36 ± 10	43 ± 8^{++}	116 ± 13^{++}	0
Rutin	5	43 ± 7	53 ± 8	144 ± 6	73
Sulphorane	6	29 ± 6^{++}	44 ± 6^{++}	140 ± 8	0
Sylimarin	4	33 ± 6	33 ± 5^{++}	127 ± 6^{++}	0
Taurolidine	4	35 ± 8	37 ± 16^{++}	111 ± 14^{++}	0
Thiabendazole	6	44 ± 7	57 ± 8	132 ± 22^{++}	71
Thymoquinone	5	33 ± 9	56 ± 9	148 ± 13	0
Ursolic acid	5	34 ± 5^{++}	36 ± 13^{++}	124 ± 11^{++}	0
Vitamin D2	5	46 ± 11	54 ± 6	144 ± 16	60
Xylitol	6	32 ± 6	49 ± 5^{++}	133 ± 22^{++}	0

Significantly lower values are labeled with $^{++}$($p < 0.01$).
When substances with enhanced toxicity were tested a high aspermatogenic activity and lower blastocyst rate was detected.

3. RESULTS

Toxicity of the substances studied: The eventual toxicity of antiangiogenic substances used in our experiments was examined. The impact of these substances on mouse spermatogenesis *in vivo* and mouse embryo development *in vitro* was evaluated. Only the non-toxic or low-toxic substances were used in further experiments (Table 1).

Aspermatogenesis: The production of spermatozoids in the animal testicle tissue is well suited for a quick assessment of the cell production process (spermatogenesis). Every toxic substance affects the production of spermatogenic cells with stepwise reduction in their numbers, and aspermatogenesis is encountered over time. The aspermatogenesis can be studied histologically and the toxic effects can be established from the decrease in the number of spermatogenic cells. The spermatogonia do not produce primary and secondary spermatocysts, and the width of spermatogenic layers and the diameter of spermatogenic tubules are reduced. The results of evaluation of the toxic effect on spermatogenesis are shown in Table 1.

Toxicity to embryos: The mouse embryo assay (MEA) is also a valuable tool for the evaluation of

Table 2. Antitumour effect of BS – RNase, RNase A and HP RNase (human pancreatic RNase) combined with antiangiogenins.

Type of tumours	Injected substances (µg/ml)	No. of injected athymic mice	No. of antitumour injections	Body mass (in g)		Tumour loss in % at the end of treatment
				Before injections	After injections	
Melanoma C-32	RNase A 100 µg	8	9	19.8	21.5	39
Melanoma C-32	RNase A + PEG 200 µg	8		20.1	20.4	51
Melanoma C-32	HP RNase (human) 100 µg	8	9	19.5	20.2	22
Melanoma C-32	HP RNase + PEG 200 µg	8	9	18.8	19.6	50
Melanoma C-32	RNase A + PEG 200 µg Curcumin 20 µg Docetaxel 50 µg	5	11	21.4	22.3	59
Melanoma C-32	BS-RNase + PEG 200 µg Neamine 10 µg Curcumin 20 µg D-glucuronic acid 50 µg	5	11	25.0	25.2	75
Melanoma C-32	RNase A + PEG 200 µg Docetaxel 50 µg Heparin 50 µg Melatonin 500 µg	5	11	26.4	26.5	74
Melanoma C-32	RNase A 100 µg Hyaluronidase + PEG 500 µg Epigallocatechin gallate 50 µg	5	9	26.5	26.9	71

potential toxicity of different substances. *In vitro* cultured mouse preimplantation embryos are highly sensitive to the toxicity of culture media. During the first cleavages of an early embryo, the embryonic nuclei become progressively transcriptionally active. The transcriptional activity increases during the transition of the early embryo development from the zygote to the blastocyst stage. The events in early embryos like the embryonic genome activation and gene transcription require precise conditions of the *in vitro* culture. Some antiangiogenins evaluated here do not exhibit aspermatogenic effect in living animals, but their low toxicity perturbs the development of early embryos to the blastocyst stage in MEA testing. According to the results of the MEA the antiangiogenins were shown to be strongly toxic (less than 10% of blastocysts), moderately toxic (10%-70% of blastocysts) and non-toxic (more than 70% embryos developed to the blastocyst stage) (see Table 1).

Antitumour effect of bovine RNases and antiangiogenins: Suppression of the human Melanoma C-32 growing in nude mice (Table 2, Figure 1) and injected with selected single antiangiogenins was studied. The antitumour effect of free RNase A injected into nude mice bearing growing human pancreatic tumour Capan 2, was very low (Figure 1); however, PEGylation of RNase A enhanced its antitumour activity. Free BS-RNase and PEGylated BS-RNase exhibited high antitumour effect (Figure 1). When the effect of PEGylated BS-RNase was tested on three human brain tumours growing in athymic mice, a high antitumour effect on meduloblastoma and neuroblastoma was observed; however, the growth of glioblastoma tumour was not affected (Figure 2). However, when PEG was conjugated to hyaluronidase the loss of melanoma tumours was much distinct (Figure 3). The combination of both bovine ribonucleases with various antiangiogenins was very destructive on human pancreatic tumours (Figure 3). When antiangiogenins alone were applied only a small antitumour activity was observed (Figure 4).

4. DISCUSSION

Ribonucleases as enzymes degrading ribonucleic acid might also be important in the restriction of the tumour growth in addition to their function in RNA turnover. The anti-tumour activity of various RNases has already been reported [28-30]. All these published data suggest that RNase A and BS-RNase did not adversely affect the survival of injected experimental animals (mice, rats, hamsters,

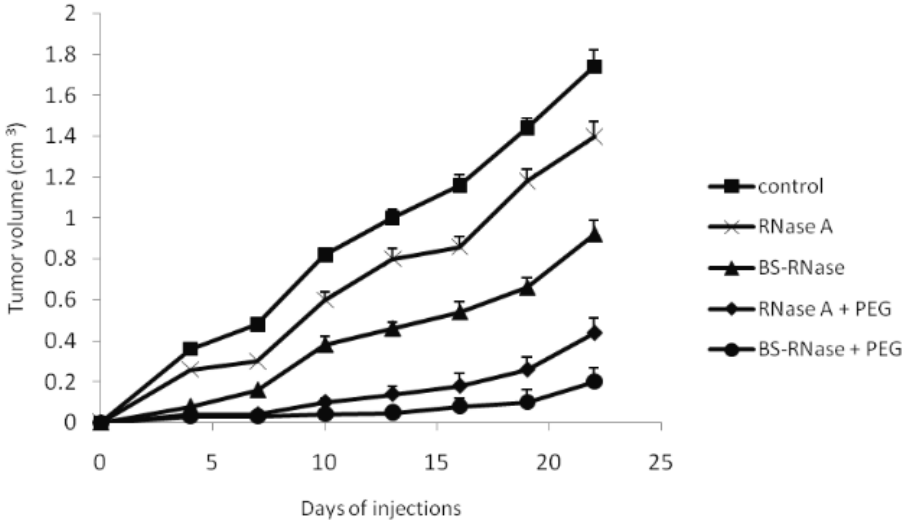

Figure 1. The antitumour effect of free RNase A injected into nude mice bearing growing human pancreatic tumour Capan 2 was very weak. Combination of RNase A with PEG enhanced its antitumour activity. BS-RNase + PEG or BS-RNase alone exhibit high antitumour effect. The concentration of all RNases alone or with PEG was 100 µg/ml.

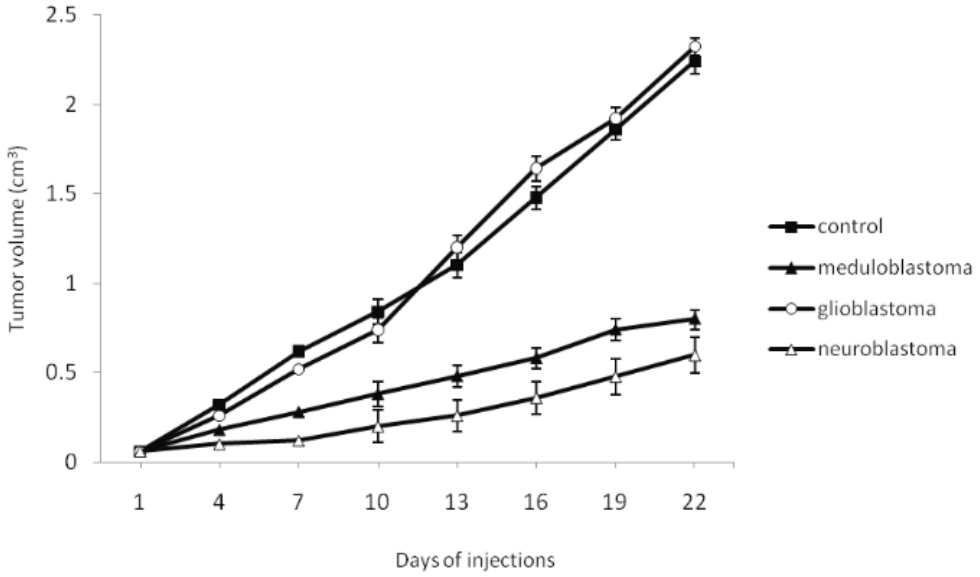

Figure 2. Effect of BS-RNase + PEG on three brain tumours growing in nude mice. Meanwhile BS-RNase + PEG has relatively high antitumour effect on meduloblastoma and neuroblastoma, BS-RNase + PEG (100 µg/ml) did not supress the growth of glioblastoma.

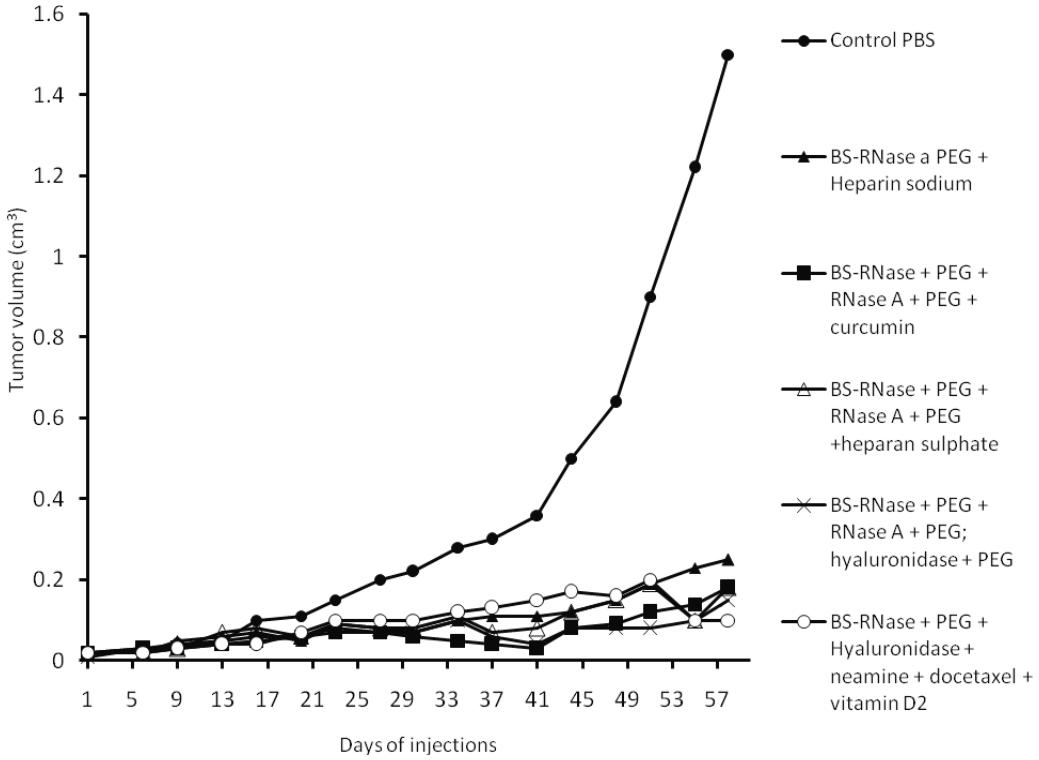

Figure 3. Antitumour effect of BS-RNase + PEG, RNase A + PEG and antiangiogenins on human pancreatic tumour Capan 2. All combined treatments exhibited high antitumour effect. In comparison with the low antitumour activity of antiangiogenins in Figure 4, here it is possible to see the strong antitumour activity of BS-RNase + PEG.

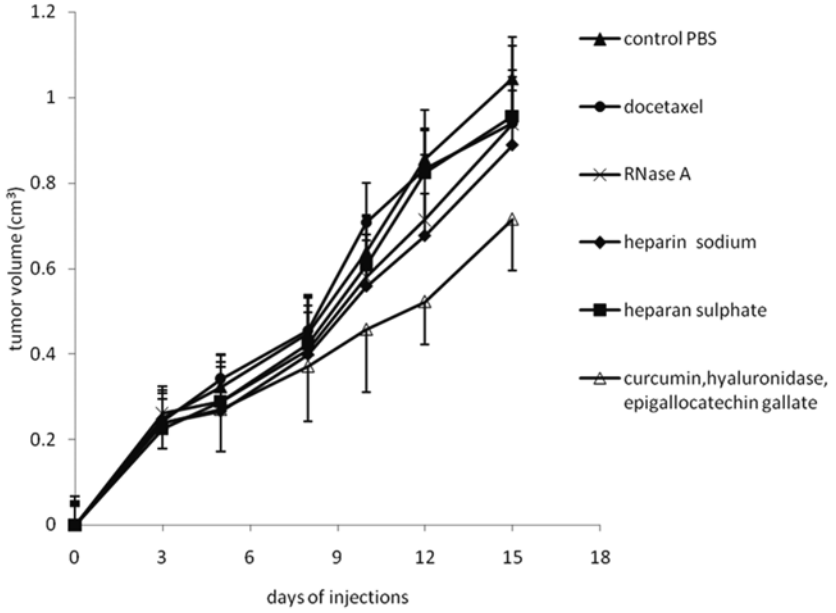

Figure 4. Activity of free antiangiogenins against the human pancreatic tumour Capan 2 growing in nude mice was low. The combination of curcumin, hyaluronidase and epigallocatechin gallate antiangiogenins in one group was partly stronger.

rabbits, mini-pigs). Furthermore, BS-RNase enzyme inhibited more effectively the growth of xenografted tumours derived from metastases, produced for example, in mouse lung through inoculation by cells of Lewis lung carcinoma, compared to cells isolated from primary tumours [31]. The results shown in Figure 3 clearly demonstrate that human pancreatic tumour Capan 2 growing in nude mice, has been very sensitive to injections of BS-RNase combined with heparin sodium, RNase + PEG, curcumin, heparan sulphate, hyaluronidase, neamine, docetaxel and vitamin D2. When the mice bearing growing pancreatic tumour Capan 2 were injected with RNase A alone or RNase A + PEG, the inhibition of pancreatic tumours was low. In contrast, much higher antitumour activity was proved by the injection of mice with BS-RNase + PEG.

Angiogenin (ANG) is a homologue of bovine pancreatic ribonuclease A that induces the neovascularization. ANG is the only angiogenic factor exerting the ribonucleolytic activity in humans and even in animals [32]. This enzyme alone has higher antitumour activity in comparison with RNase A. For the blood vessel growth stimulation, ANG must be transported to the nucleus and must maintain its catalytic activity [33, 34].

The production of angiostatin by the interrelationship among angiogenin, plasminogen, plasminogen-activator, plasmin and elastase also becomes a potent inhibitor of angiogenesis and metastases. All these substances circulate in the plasma at high concentrations. However, the angiogenins without substantial antitumour effect are supposedly released either from the tumour and/or from its extracellular matrix by the action of the tumour-secreted molecules [35]. Our results suggest that their antitumour activity is not strong. These substances diffuse radially until they reach microvessels, activate their endothelial cells, and induce capillary sprouting in microtubules. This can be followed by a selective adhesion or chemotaxis and the process of anastomosis [35]. These angiogenins include members of the basic fibroblast growth factor (bFGF) family, which stimulate endothelial cells to produce more new capillary growth. They also induce the mitogenesis of osteoblasts and morphogenesis of fibroblasts. On the other hand, they inhibit the proliferation of epithelial and endothelial cells.

Bovine seminal ribonuclease and bovine pancreatic ribonuclease with PEG and used together with antiangiogenins against tumours growing in nude

mice were considerably promising in our work. The effect of BS-RNase and RNase A was reviewed in many papers. These bovine ribonucleases are also the main anti-tumour substances we were studying since 1965, and since 2005 we analysed the effect of antiangiogenins too [36]. Most of these antiangiogenins are natural substances. In spite of their natural origin, the majority of antiangiogenins are toxic (Table 1) and their use in larger amounts and in the absence of BS-RNase was not sufficiently effective against human carcinoma (Figure 4). However, when athymic mice bearing growing human pancreatic tumour Capan 2 were injected with PEGylated BS-RNase or PEGylated RNase A combined with selected antiangiogenins, the results were much more favourable (Figure 3). This also applies to a study on athymic mice bearing human melanoma tumours therapeutically injected in the same way. PEGylated BS-RNase exerts good antitumour activity against human meduloblastoma and neuroblastoma brain tumours but not against glioblastoma brain tumour, which is very tenacious (Figure 3).

However, it is necessary to mention that only a limited number of antiangiogenins were studied and BS-RNase or even RNase A need not be suitable antitumour subjects for acting with other unstudied antiangiogenins or binding them firmly to the tumour. It seems that antiangiogenins exhibit only a low antitumour activity in the absence of a strong antitumour substance (Figure 4). The highest antitumour effect was reached when the mice bearing human pancreatic tumours were injected with PEGylated BS-RNase in combination with selected antiangiogenic substances (Figure 3).

The results presented here suggest that PEGylated bovine RNases combined with antiangiogenins of low toxicity are a prospective tool in anticancer research.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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