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Report

Bovine seminal ribonuclease attached to nanoparticles made of polylactic acid kills leukemia and lymphoma cell lines *in vitro*

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Bovine seminal ribonuclease (BS-RNase) is a protein with a number of biological effects. It shows antitumoral, aspermatogenic, antiembryonic, immunosuppressive and antiviral properties. The cytotoxic effects appear to be specific for tumor cells as non-malignant cells seem to be unaffected *in vitro*. Unfortunately, the *in vivo* application of BS-RNase so far was successful only when it was administered intratumorally. Therefore, the objective of the present investigation was to improve the properties of BS-RNase by attachment to nanoparticles made of polylactic acid (PLA-NP) using an adsorption method. This preparation was tested *in vitro* against leukemia (MOLT-4) and lymphoma (H9) cell lines sensitive and resistant to cytarabine. No difference between the nanoparticle preparation and pure BS-RNase was found in these tests. To examine the *in vivo* effects, the preparations were tested for their aspermatogenic and antiembryonal efficacy compared to the pure BS-RNase as a rapid test for antitumoral activity. The aspermatogenic and antiembryonal effects were enhanced by the nanoparticle preparation. Consequently, BS-RNase loaded adsorptively to PLA-NP holds promise for the *in vivo* use as an antitumoral agent. Further research will investigate the efficacy of this preparations in an *in vivo* tumor model. [© 2000 Lippincott Williams & Wilkins.]

Key words: Aspermatogenesis, BS-RNase, leukemia, lymphoma, nanoparticles, polylactic acid.

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Introduction

The enzyme bovine seminal ribonuclease (BS-RNase) was identified as an antitumorally active substance at the beginning of the 1970s.[1] In the following years its antitumoral efficacy against a number of different cancer types was shown by different authors.[2–5] Moreover, it appeared to be a substance exhibiting a number of diverse pharmacological effects. In addition to the antitumoral activity, aspermatogenic,[6] antiembryonic,[7] immunosuppressive[8] and antiviral[9] properties were shown.

The toxicity of BS-RNase seems to be limited to malignant cells, whereas non-malignant cells were not affected.[10] The enzyme becomes internalized after binding to the cell surface and inhibits protein synthesis by degrading the rRNA. Non-malignant cells also bind and internalize BS-RNase but their protein synthesis does not become affected. Up to now the reason for this has remained unclear. In addition, BS-RNase was found to possess a stronger effect against material taken from tumor metastases than from the primary tumor. In *in vivo* experiments BS-RNase reduced the appearance of metastases in the lung of mice inoculated intramuscularly with Lewis lung carcinoma by more than 90%.[11,12] Furthermore, BS-RNase was shown to be effective against multidrug-resistant neuroblastoma cells.[13,14]

At present the major obstacle in the *in vivo* use of BS-RNase is that an antitumoral effect only was obtained after intratumoral (i.t.) administration and i.p. administration of BS-RNase was much less effective.[4,15] Especially in metastasizing tumors i.t.

administration in each metastasis is not possible. Drug carrier systems such as nanoparticles might offer the possibility to modulate the body distribution or even the transport of BS-RNase to the tumor after systemic administration due to the enhanced permeability and retention (EPR) effect. They were shown to prolong the half-life of drugs in the organism, to protect them from degradation, to deliver them to tumors and even to overcome multidrug-resistant tumors.[16–20]

The objective of the present study was to improve the effects of BS-RNase by adsorption to biodegradable, biocompatible nanoparticles made of polylactic acid (PLA-NP). PLA degrades *in vivo* by hydrolysis to lactic acid, which becomes incorporated into the tricarboxylic acid cycle followed by excretion.[21] The BS-RNase-PLA-NP preparation (RNase-NP) and pure BS-RNase were tested against human T cell leukemia MOLT-4 and human T lymphoma H-9 cell lines sensitive and resistant against cytosine arabinoside (Ara-C) which is commonly used for the therapy of these cancers.[22,23] Human foreskin fibroblast (HFF) and human amnion epithelial (HA) cells were used to examine the toxicity of the BS-RNase with and without the nanoparticles on non-malignant tissues. In order to investigate the suitability of these preparations for *in vivo* application of the BS-RNase the aspermatogenic and antiembryonal effects in mice were studied since these effects are considered to be indicative of the biological properties of BS-RNase, including the antitumoral potential.[7]

Materials and methods

Isolation of BS-RNase from bull seminal vesicle fluid

Bull seminal vesicle fluid was obtained from healthy sexually mature bulls at the slaughterhouse or from seminal plasma of bulls bred in Insemination Stations. One volume of the seminal fluid was diluted by 2.5 volume of 2% acetic acid. The protein precipitate was removed by centrifugation and the supernatant was adjusted with solid ammonium sulfate to 3 M. The new supernatant was than dialysed against water and freeze-dried. The raw material was further purified by size-exclusion chromatography. For this purpose, the material was dissolved in demineralized water at a concentration of 20 mg/ml. This solution was filtered through 0.22 μm membrane filters (Millex-GV filter units; Millipore, Bedford, MA). Aliquots (1.0 ml) of the BS-RNase solution were purified using a size-exclusion chromatographic system consisting of a HPLC Pump 64 (Knauer, Berlin, Germany) and a size exclusion column (Superformance Säule 600-16 Fractogel EMD BioSEC (S); Merck, Darmstadt, Germany) attached to a spectrophotometer (Lambda-Max model 481 LC spectrophotometer; Waters, Eschborn, Germany). The wavelength was adjusted to 260 nm. Fractions were concentrated in a Model 402 microconcentrator unit equipped with a DiafloYC05 membrane (Amicon, Witten, Germany), purified by washing with demineralized water and then freeze-dried (Lyovac GT 2; Leibold Heraeus, Huerth, Germany).

Preparation of nanoparticles

A pre-emulsion was prepared by emulsifiing a 0.5% (w/v) aqueous polyvinyl alcohol (PVA)/5% (w/v) with a PLA solution (ResomerL104; Boehringer Ingelheim, Ingelheim, Germany) in dichloromethane at a ratio of 5:1 with an Ultra Turrax (Janke Kunkel, IKA Labortechnik, Staufen, Germany) at 8000 r.p.m. for 2 min. This pre-emulsion was then high-pressure homogenized at 400 bar, five cycles, using a Rannie high pressure laboratory homogenizer, model MINI-LAB, type 8.30H (APV Homogenizers, Albertslund, Denmark). The dichloromethane was evaporated for 24 h at room temperature. Then the nanoparticles were washed by 5-fold centrifugation at 46 500 *g* (Ultrazentrifuge Optima L-80; Beckman, Munich, Germany) and redispersed in demineralized water. The polymer contents of the resulting suspensions were determined by gravimetry. The resulting nanoparticles were autoclaved (121°C, 2 bar, 20 min), and characterized with regard to particle size and surface charge.

Particle size measurement

The particle size of the obtained nanoparticles was measured by photon correlation spectroscopy (PCS) (24) using a BI-200SM Goniometer version 2.0 (Brookhaven Instruments, Holtsville, NY) equipped with a 30 mW He–Ne laser and connected to a BI-2030AT digital correlator. The count rate was adjusted to between 15 and 30 kHz by diluting the samples with water filtered through 0.22 μm filter units (Millex-GS; Millipore, Molsheim, France).

Surface charge measurement of the nanoparticles

The surface charge of the nanoparticles was determined by microelectrophoresis (25) with a laser Zee Meter Model 501 (Penkam, Bedford Hills, NY) equipped with a CCD video camera head LDH 0460/

xx and a video monitor LDH 2132/10 (Philips, Eindhoven, Netherlands). An electric field of 150 V was applied to observe the electrophoretic mobility of the particles. Aliquots (100 μl) of the nanoparticle suspensions were diluted to 100 ml with demineralized water. The surface charge was expressed as the ξ potential at 20°C.

Drug loading

For the drug loading experiments, BS-RNase concentrations between 0.1 and 4.0 mg/mg PLA nanoparticles in water were incubated by stirring over 24 h. After incubation the suspension was centrifuged at 20 800 *g* in an Eppendorf centrifuge 5417 (Gerätebau Eppendorf, Engelsdorf, Germany) for 45 min. Aliquots (450 μl) of the supernatant were transferred to Microcon100 microconcentrators (Amicon, Witten, Germany) and centrifuged at 10 600 *g* for 45 min. The content of BS-RNase in the ultrafiltrate was detected at 278 nm (U3000 spectrophotometer; Hitachi, Reading, UK) and the amount of unbound drug was calculated relative to a control treated in the same manner without addition of the nanoparticles. The bound drug was calculated as the difference between total BS-RNase and unbound BS-RNase. Absolute loading (w/w) was calculated as the amount of drug bound to 1 mg of the carrier system.

Cells

Human T cell leukemia MOLT-4 and human T lymphoma H-9 cell lines were obtained from ATCC (Rockville, MD). $H9^{r}Ara\text{-}C^{600}$ and $MOLT\text{-}4^{r}ArAa\text{-}C^{250}$ cells were established by exposure of parental H9 and MOLT-4 cells to increasing concentration of Ara-C as described previously.[26] $H9^{r}Ara\text{-}C^{600}$ and $MOLT\text{-}4^{r}Ara\text{-}C^{250}$ grown for more than 1 year in medium with 600 and 250 μM Ara-C, respectively, were used in the experiments. Normal HFF and HA[27] were taken between one and three subcultures after their isolation. All cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS.

Assessment of cytoxic effects

Cytotoxic effects of RNase-NP and BS-RNase were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Cells were plated in 96-well microtiter plates at a density 2×10^4 cells/well. The cells were incubated in a culture medium at concentrations between 0.75 and 50 μg/ml of BS-RNase, RNase-NP at the same BS-RNase concentrations and PLA-NP at concentrations of 3 μg/ml to 20 mg/ml. After 5 days of treatment the MTT substrate (1 mg/ml) was added and plates were incubated at 37°C for 4 h. After incubation, cells were lysed in a buffer containing 20% (w/v) SDS and 50% (v/v) *N*,*N*-dimethylformamide with the pH adjusted to 4.5. Absorbance at 570 nm was determined for each well using a 96-well multiscanner. After correcting for the background, the results were expressed as percentage viability relative to a control culture which received no drug.

Measurement of induction of apoptosis

Induction of apoptosis was measured in $H9^{r}Ara\text{-}C^{600}$ and HFF cells. Cytoplasmic histone-associated DNA fragments were quantified (mono- and oligonucleosomes) to determine if the tested preparations induced apoptosis. Measurements were performed using photometric enzyme immunoassays with specific monoclonal antibodies directed against DNA and histones using Cell Death Detection ELISAplus (Roche, Mannheim, Germany) according to the manufacturer's instructions. Quantification of apoptosis by ELISA was performed in cultures treated for 3 days with the drugs at concentrations ranging from 12.5 to 50 μg/ml pure BS-RNase and RNase-NP at the same BS-RNase concentrations.

Aspermatogenic effects

The effect of RNase-NP, BS-RNase and PLA-NP was determined as previously described.[6] Adult male ICR mice were injected with the preparations containing 100 μg BS-RNase in only one of the testes and sacrificed after 10 days. Both testes with epididymises were excised, weighed and histologically studied. Aspermatogenic effect was expressed by index weight, i.e. testis weight in mg $\times$ 10/body weight in g. Destructive effect on the testes was evaluated by the loss of spermatogenic cells, the decrease in the width of spermatogenic layers and the diameter of seminiferous tubules. The degree of testicular damage was asserted on a scale from 0 (normal histological appearance) to 4 (disappearance of all cells of the spermatogenic layers and spermatogonia of the convoluted tubules). Between 50 and 60 tubules from the central part of the testes were measured microscopically.

Antiembryonic effects

The antiembryonic effects were detected as described before.[6] Cryoconserved mouse embryos (8–64 blastomers) were washed free of the cryoprotecting substance, and incubated with BS-RNase, RNase-NP and PLA-NP in 1 ml of MEMD medium supplemented with 20% (v/v) fetal calf serum (FCS) at 37°C. Cultivation Falcon plates (Nunc, Roskilde, Denmark) covered with paraffin oil were kept in the atmosphere of N_2 (90% v/v) and CO_2 (5% v/v) at 37°C. The mortality of embryos was assessed 24, 48 and 72 h after addition of the preparations. Mortality was detected as a stopping of cell division and subsequent degranulation.

Results

Particle size and surface charge

The particle size of the PLA-NP ranged between 200 and 250 nm with a surface charge (ξ potential) of about -22 mV in demineralized water.

Drug loading

Drug loading studies were performed in triplicate. Up to a concentration of 0.34 ± 0.01 mg BS-RNase/mg PLA-NP, BS-RNase was bound quantitatively to the particles. Above this concentration, no further binding occured. In the following experiments 0.25 mg BS-RNase/mg nanoparticles were used.

Cytotoxic effects of BS-RNase and RNase-NP

Cytotoxic effects of the substances were tested in cultures (cell types listed under Materials and methods) continuously treated for 5 days with concentrations ranging from 0.75 to 50 μg BS-RNase/ml by the MTT assay. As shown in Figure 1, both BS-RNase and RNase-NP showed comparable toxicities for tumor cell lines but were non-toxic for HFF and HA. Cytotoxic effects of the drugs for $H9^{r}Ara\text{-}C^{600}$ and $MOLT\text{-}4^{r}Ara\text{-}C^{250}$ cells resistant against cytarabine were similar to those for parental cells (H9 and MOLT-4). PLA-NP showed no toxicity up to a concentration of 20 mg/ml in culture medium (data not shown).

Effect of BS-RNase and RNase-NP on apoptosis

BS-RNase is able to induce apoptosis in neuroblastoma cell lines.[13] The potential of RNase-NP to induce apoptosis was tested in lymphoma H9 cells. Quantification of apoptosis by ELISA performed in cultures treated for 3 days with RNase-NP or BS-RNase indicated a similar formation of DNA fragments in a dose-dependent manner in H9 cells for both BS-RNase preparations as shown in Figure 2. Treatment of HFF was not associated with the induction of programmed cell death.

Aspermatogenic effects

The data of the aspermatogenic effects are given in Table 1. The RNase-NP are more aspermatogenic compared to BS-RNase as shown by the reduction of spermatogenic layers of testes. The reference preparations, PBS or the pure PLA-NP, showed no or only a slight influence of spermatogenesis, respectively.

A remarkable effect could be seen in one mouse of the group treated with the RNase-NP. In this mouse the aspermatogenic effect in the second testis was stronger than in the injected one, indicating the RNase-NP possess a tendency to distribute within the organism.

Table 1. Aspermatogenic effects of male mice injected with BS-RNase and BS-RNase-NP

Injected substances	No. of mice	Index weight of testes		Width of spermatogenic layers of testes (μm)		Diameter of seminiferous tubules of testes (μm)		Testicular damage score of injected testes
		Injected testes	Non-injected testes	Injected testes	Non-injected testes	Injected testes	Non-injected testes	
BS-RNase	10	20 ± 6	32 ± 3	31 ± 8	61 ± 7	135 ± 23	162 ± 10	2–3
BS-Nase-NP	7	19 ± 8	31 ± 6	15 ± 10	51 ± 19	126 ± 47	151 ± 26	2–4
PLA-NP	6	28 ± 5	29 ± 5	54 ± 3	58 ± 6	151 ± 19	151 ± 11	0–1
PBS	5	28 ± 4	29 ± 5	62 ± 8	63 ± 6	160 ± 14	159 ± 15	0

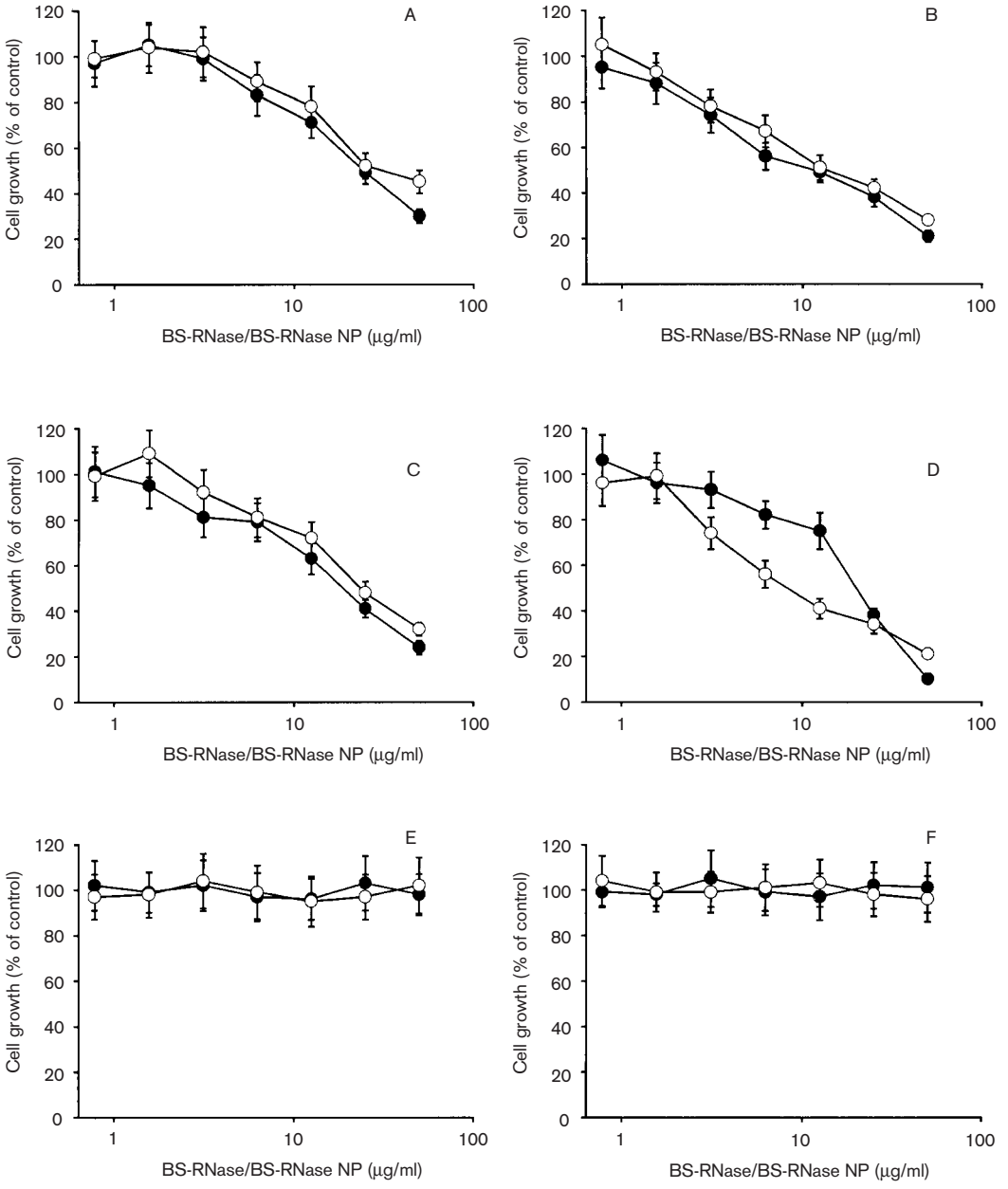

Figure 1. Cytotoxicity (MTT assay) of pure BS-RNase (○) and RNase-NP (●) in (A) MOLT4 cells, (B) H9 cells, (C) MOLT-4^{r}Ara-C^{250} cells, (D) H9^{r}Ara-C^{600} cells, (E) HFF cells and (F) HA cells (mean ± SD, *n*=3).

Antiembryonal effects

Antiembryonal effects of the tested preparations are shown in Table 2. In the untreated control group, 14% of the embryos died after 24 h and 65% survived for 72 h. After this time the experiments were truncated. In contrast, 72% of the embryos treated with BS-RNase died within 24 h and no embryo survived for more than 48 h. In the RNase-NP group, all embryos died in the first 24 h, indicating a much stronger effect than with BS-RNase alone. Only slight differences could be seen between the PLA-NP and the control.

Table 2. Toxical effects of BS-RNase, BS-RNase-NP and PLA-NP on mice embryos (100 μg/ml)

Embryo development in culture medium after	Medium (Control)			PLA-NP			BS-RNase			BS-RNase-NP		
	No. of developing embryos	Mortality no.	%	No. of developing embryos	Mortality no.	%	No. of developing embryos	Mortality no.	%	No. of developing embryos	Mortality no.	%
0 h	43	0	0	29	0	0	41	0	0	26	0	0
24 h	37	6	14	23	6	21	12	29	72	0	26	100
48 h	34	9	21	21	8	28	0	41	100	0	26	100
72 h	28	15	35	18	11	38	0	41	100	0	26	100

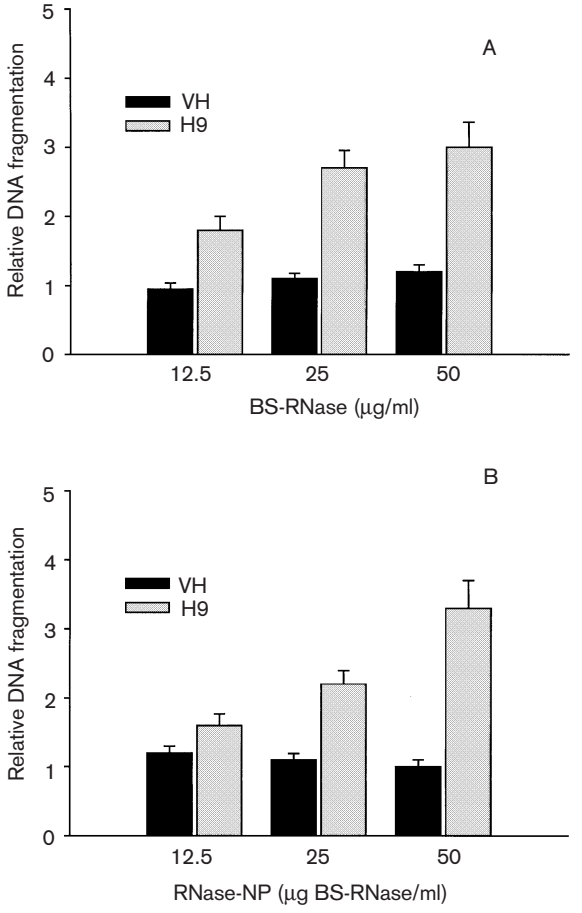

Figure 2. Relative DNA fragmentation (apoptosis assay) in HFF and H9 cells after incubation with (A) pure BS-RNase and (B) RNase-NP (mean ± SD, *n*=3).

Discussion

In the present study we attached the protein BS-RNase obtained from bull seminal vesicle fluid to nanoparticles made of polylactic acid (RNase-NP), and compared the effects of this preparation to free BS-RNase *in vitro* and *in vivo*.

For *in vitro* studies, HFF and HA were used, and tumor cells, lymphoma cells (H9), leukemia cells (MOLT-4) as well as Ara-C-resistant $H9^{r}Ara\text{-}C^{600}$ and $MOLT\text{-}4^{r}Ara\text{-}C^{250}$ cells, which were established as described before, were used.[13] Ara-C is used primarily in combination with doxorubicin or daunorubicin in lymphomas[23] and as a secondary therapy for childhood acute lymphocytic leukemia.[22] Moreover, treatment of tumor cells with Ara-C may be associated with development of drug resistance due to multiple mechanisms.[28] The present *in vitro* results indicated no difference between the free BS-RNase and the RNase-NP. Both were cytotoxic against the Ara-C-resistant cell lines and induced apoptosis in these cells in a comparable manner. BS-RNase and the RNase-NP were non-toxic and caused no apoptosis to the parental cell lines. These results are compatible with our previous findings showing that BS-RNase is able to overcome the resistance of tumor cell lines towards common medication.[13,14]

Differences between BS-RNase in solution and bound to PLA-NP were found when investigating the aspermatogenic and antiembryonal effects of the BS-RNase. These effects induced by BS-RNase are associated with the biological efficacy of the substance and these assays are predictive for the antitumoral efficacy *in vivo*. As mentioned, the main obstacle in the use of BS-RNase as an antitumoral agent is the lack of efficacy when it is administered systemically and not i.t.[4,15]

To overcome this problem, nanoparticles as drug carrier systems might be useful. They were shown to be able to alter the body distribution of drugs and to protect them from degradation.[17,18] Therefore we bound BS-RNase to PLA-NP as a biodegradable and biocompatible material. Binding happened by adsorption. This preparation was tested for its aspermatogenic and antiembryonic effects in mice. Aspermatogenic and antiembryonic effects of the RNase-NP were stronger than of BS-RNase alone. In one mouse treated with the nanoparticle preparation the untreated testis was more strongly affected by the

BS-RNase than that which had the injection. This indicates that the nanoparticles were able to distribute within the body, whereas the BS-RNase alone seems not to be able to distribute within the body. This distribution might be due to a hematogenic route as there is no other connection between the two testes.

In summary, our data shows that BS-RNase attached to PLA-NP is as effective as unbound BS-RNase *in vitro* and is equally able to overcome tumor cell resistance to the common treatment. The toxicity of BS-RNase to non-malignant cells also remained unchanged after binding to nanoparticles. In contrast, the aspermatogenic and antiembryonal effects were increased by the nanoparticle preparation. In addition, some tendency of the RNase-NP to distribute in the body could be observed. Therefore, RNase-NP represents a promising preparation for tumor therapy.

Further studies are under way to determine the antitumoral activity of the proposed carrier system in an *in vivo* tumor model.

Conclusion

In this study BS-RNase was shown to kill leukemia and lymphoma cells resistant and sensitive to the common medication. Tests of induction of apoptosis were performed in lymphoma cells and BS-RNase was detected to induce apoptosis in these cells but not in HFF cells used to examine effects on normal cells. In order to overcome the poor bioavailability of BS-RNase *in vivo*, it was attached to nanoparticles made of PLA (RNase-NP) by adsorption. RNase-NP showed the same *in vitro* effects as observed with pure BS-RNase. In contrast to this, RNase-NP showed stronger aspermatogenic and antiembryonic effects in mice than pure BS-RNase. As the strength of these effects are predictive of the *in vivo* antitumoral behavior of BS-RNase preparations, this indicates the preparation to be promising for *in vivo* use. The antitumoral efficacy of the preparation after systemic administration in the organism has to be evaluated in further experiments.

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