*Anti-Cancer Drugs 2002,* **13,** pp. 149–154

## Preclinical report

# Coupling of the antitumoral enzyme bovine seminal ribonuclease to polyethylene glycol chains increases its systemic efficacy in mice

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**Bovine seminal ribonuclease (BS-RNase) is an antitumoral active enzyme exhibiting specific antitumoral action against a number of different cancer cell lines. However, its systemic use is limited by its pharmacokinetic properties and antigenicity. Therefore, it was conjugated to polyethylene glycol (PEG) chains to overcome these problems. Measurement of aspermatogenic effects of the preparation after s.c. injection and injection into the scrotum was chosen as a model for the distribution of the enzyme in the body mediated by the linkage to PEG chains. Additionally, the antigenicity of BS-RNase coupled to PEG chains (BS-RNase–PEG) was compared to that of free BS-RNase, as antigenicity is known to be one of the main obstacles in the use of protein-based drugs. BS-RNase–PEG caused aspermatogenic effects after systemic administration to mice in very low concentrations at which free BS-RNase is not effective. Moreover, BS-RNase possessed a very low antigenicity as long as it was coupled to the PEG chains. In order to investigate the antitumoral efficacy of BS-RNase–PEG *in vivo*, preliminary experiments on the effect of the conjugate on neuroblastoma growth in mice were performed in a UKF-NB-3 xenotransplantate model, demonstrating a drastically increased antitumoral activity of the conjugate compared to the free enzyme.** 

*Key words:* Aspermatogenicity, BS-RNase, neuroblastoma, polyethylene glycol.

The authors gratefully acknowledge support given by the organization 'Hilfe für krebskranke Kinder, Frankfurt/Main eV', by the foundation 'Frankfurter Stiftung für krebskranke Kinder der Hilfe für krebskranke Kinder Frankfurt eV' and by the Grant Agency of the Czech Republic (grant 523/01/014).

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## Introduction

The enzyme bovine seminal ribonuclease (BS-RNase), a dimeric RNase, was discovered as an antitumorally active substance at the beginning of the 1970s.[1] Different authors further investigated its antitumoral efficacy against different cancer types.[2–5] In addition to its antitumoral activity, it exhibits a number of other biological effects including aspermatogenic effects after injection into the scrotum.[6,7]

The cytotoxicity of BS-RNase is caused by its insensitivity to cytosolic RNase inhibitor.[8] Interestingly, this cytotoxicity is limited to a number of malignant cells, whereas non-malignant cells are not affected.[9,10] The specificity of the cytotoxic action remains not fully understood. It is explained with different intracellular pathways after internalization of the enzyme. Due to these different pathways BS-RNase degrades rRNA in sensitive tumor cells but not in non-malignant cells or in insensitive tumor cells. Its antitumoral action includes multidrug-resistant tumor cells.[11–13]

In contrast to the intratumoral administration that was highly effective in different tumor models, systemic administration was hardly efficient.[4,14,15] Most probably, the *in vivo* use is limited by the antigenicity of BS-RNase and by its insufficient pharmacokinetics, leading to the rapid inactivation and elimination of the enzyme. The present work describes the preparation of conjugates consisting of BS-RNase and polyethylene glycol (PEG) chains.

These conjugates (BS-RNase–PEG) were prepared since conjugation to PEG previously was shown to improve pharmacokinetic properties of proteins in different ways. Binding to PEG decreased antigenicity of proteins, protected proteins from proteolysis and reduced the rate of kidney clearance if the conjugates had a molecular weight of approximately 30 kDa or more. Pegylated forms of the proteins adenosine deaminase and asparaginase were already approved for human use by the FDA.[16,17] In addition, macromolecules are known to accumulate in tumors due to the enhanced permeability and retention (EPR) effect.[18] Therefore, in the present study, BS-RNase–PEG was tested for antitumoral activity against neuroblastoma cells *in vitro*. Aspermatogenic effects were detected after injection of the conjugate into the testes to examine the *in vivo* activity of the conjugate. Moreover, to examine if the conjugation of BS-RNase to PEG chains improves distribution of the enzyme in the organism, the antigenicity of the conjugate was evaluated in mice and aspermatogenic effects were detected after s.c. application and application into the scrotum (i.scr.). To estimate the relevance of the obtained data for *in vivo* antitumoral efficacy of the BS-RNase–PEG, preliminary experiments with the conjugate were performed in a neuroblastoma (UKF-NB-3) xenotransplantate model.

## 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 the Insemination Stations. One volume of the seminal fluid was diluted by 2.5 volumes of 2% acetic acid. The protein precipitate was removed by centrifugation and the supernatant was adjusted with solid ammonium sulfate to 3 M concentration. The new supernatant was then dialyzed against water and freeze-dried. The raw material was further purified by size-exclusion chromatography as described before.[13] For this purpose, the material was dissolved in demineralized water at a concentration of 20 mg/ml. This solution was filtered through 0.22 $\mu$m 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; Waters, Eschborn, Germany). The wavelength was adjusted to 260 nm. Fractions were concentrated in a Modell 402 microconcentrator unit equipped with a Diaflo YC05 membrane (Amicon, Witten, Germany), washed with demineralized water and then freeze-dried (Lyovac GT 2; Leibold Heraeus, Huerth, Germany).

### Conjugation of BS-RNase to PEG chains

The BS-RNase–PEG conjugates were custom-synthesized by Seva Imuno Praha (Prague, Czech Republic). In principle, amino-reactive *N*-methoxy-PEG-succinimidyl propionate (molecular weight 22 000) was coupled to the lysine residues of BS-RNase producing a stable amide linkage. The ratio of BS-RNase to PEG in the resulting conjugate was 30:70 (w/w).

### Cells

The NB cell line UKF-NB-3 was established from metastasis harvested in relapse in one of our patients with Evans stage 4 NB.[19,20] The cells were propagated in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% $CO_2$ incubator.

### Assessment of cytotoxic effects

Cytotoxic effects of BS-RNase–PEG and BS-RNase were determined by a MTT dye reduction assay. Cells were plated in 96-well microtiter plates at a density $2 \times 10^4$ cells/well. The cells were incubated in culture medium at concentrations between 0.75 and 100 $\mu$g/ml of BS-RNase applied as a BS-RNase–PEG conjugate or as free BS-RNase. 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) sodium dodecylsulfate 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.

### Aspermatogenic effects

The aspermatogenic effects of BS-RNase–PEG and BS-RNase were determined as described previously.[6,13] Adult male ICR mice were injected with 100 $\mu$g of either free BS-RNase or BS-RNase–PEG conjugate (containing 30 $\mu$g of BS-RNase) dissolved in phosphate-buffered saline into the testes and sacrificed after 10 days. The injected testes with epididymises were excised and histologically studied. Destructive effects on the testes were detected by the decrease in the width of spermatogenic layers and the diameter of seminiferous tubules. Between 50 and 60 tubules from the central part of the testes were measured microscopically. Results were compared to aspermatogenic effects of RNase A (a non-toxic monomeric RNase) and PEG.

Additionally, in order to investigate the potential of the preparation to distribute within the body and to reach its site of action, BS-RNase and BS-RNase–PEG were administered i.scr., and s.c. BS-RNase or BS-RNase–PEG were administered once a week during 5 or 10 weeks. Degenerative effects of the testes were detected by measurement of testes weight and width of spermatogenic layers.

The mice were weighed before and after the end of experiments.

### Antigenicity testing

The antigenicity of BS-RNase and BS-RNase–PEG was determined as described before.[21] A non-competitive ELISA test was performed. The preparations were injected i.scr. or s.c. to mice Microtiter plate wells were coated with 25 $\mu$g of BS-RNase. After washing, antisera from mice treated with BS-RNase or BS-RNase–PEG and control sera from mice injected with citric acid were serially diluted in wells and the plate was incubated for 1 h at room temperature. The SwAMPx (USOL, Prague, Czech Republic) conjugate diluted 1:1000 was added and after a further 20-min incubation with the substrate solution the reaction was stopped by addition of 4 N $H_2SO_4$. The solutions were measured photometrically at 450 nm (Titertek Uniskan; Flow Laboratories, Irvine, UK). Serum tests were defined as positive if the optical density of the tested serum was at least 2 SEM higher than that of mice injected by sodium citric solution. The titer of antibodies against BS-RNase and the titer of antibodies against BS-RNase–PEG was detected.

### Neuroblastoma xenotransplantate model

Preliminary antitumoral experiments in a neuroblastoma xenotransplantate mouse model were performed as described before.[22] Female, 4–6 weeks old, outbred CD-1 strain athymic nude mice (*nu/nu*) (AnLab, Charles River, Prague, Czech Republic) weighing 18–20 g were kept under sterile conditions, and provided with sterile nutrition and water. To establish xenotransplantate NB tumors $5 \times 10^6$ UKF-NB-3 tumor cells were injected s.c. into the right flank of mice. Cells were administered together with Matrigel in a total volume of 0.2 ml. Tumor size was measured using a caliper and calculated by the formula volume = (length $\times$ width$^2$)/2.

Treatment was started after tumors grew to a size greater than 100 mm$^3$. The mice were divided into two groups. Group A (five animals) was treated with 0.1 ml of BS-RNase–PEG (0.75 mg BS-RNase/kg body weight) i.v. every third day 7 times during 22 days. Group B (for animals) was treated with 0.1 ml PBS solution at the same times. Tumor sizes were measured and body weights controlled every third day.

## Results

### Cytotoxic effects of BS-RNase and BS-RNase–PEG *in vitro*

The $IC_{50}$ of BS-RNase was $2.18 \pm 0.18$ $\mu$g/ml in UKF-NB-3 cells. BS-RNase–PEG showed no cytotoxicity against UKF-NB-3 cells at the tested concentrations up to 100 $\mu$g BS-RNase/ml.

### Aspermatogenic effects

The aspermatogenic effects of the different preparations after one-time intratesticular application of free BS-RNase (100 $\mu$g) and BS-RNase–PEG (containing 30 $\mu$g BS-RNase) can be seen from Figure 1. Untreated controls were defined as 100% for the diameter of the seminiferous tubules and for the width of spermatogenic layers. Non-toxic RNase A and PEG neither influenced the diameter of seminiferous tubules nor the width of spermatogenic layers compared to control. BS-RNase (100 $\mu$g) and BS-RNase–PEG (30 $\mu$g BS-RNase) strongly decreased the diameter of seminiferous tubules in a comparable manner.

Systemic application (s.c.) of BS-RNase, PEG or RNase A did not cause aspermatogenic effects compared to control (data not shown). In contrast,

administration of BS-RNase–PEG (90 $\mu$g BS-RNase per injection, 10 times, once a week) provoked a significant decrease in the width of spermatogenic layers ($43 \pm 3\,\mu$m) compared to control ($62 \pm 8\,\mu$m, $p < 0.01$). Comparable results were obtained by injection of BS-RNase–PEG i.scr. of mice, but not in the testes. Ten times administration of BS-RNase–PEG containing 90 $\mu$g of BS-RNase led to a width of spermatogenic layers of $44 \pm 4\,\mu$m; 5 times administration of BS-RNase–PEG containing 180 $\mu$g led to a width of $43 \pm 4\,\mu$m ($p < 0.01$).

Neither administration of BS-RNase nor administration of BS-RNase–PEG influenced body weights of mice significantly compared to control animals treated with PBS (data not shown).

### Antigenicity testing

The results are shown in Table 1. BS-RNase (90 $\mu$g) as BS-RNase–PEG was administered s.c. or i.scr. and compared to the free BS-RNase. Both s.c. and i.scr. application of free BS-RNase and BS-RNase–PEG caused comparable antibody titers against free BS-RNase. However, very low levels of antibodies directed against the BS-RNase–PEG conjugate were found, indicating that BS-RNase is hardly antigenic as long as it is conjugated to the PEG chains. Most probably, generation of antibodies against BS-RNase by the PEG conjugates happens after release of BS-RNase from the conjugate.

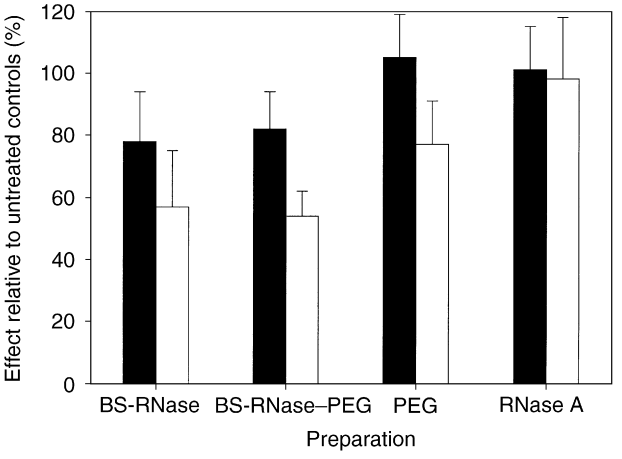


**Figure 1.** Diameter of seminiferous tubules (black bars) and width of spermatogenic layers (white bars) after one time injection of 100 $\mu$g of free BS-RNase, BS-RNase–PEG (BS-RNase covalently bound to PEG chains containing 30 $\mu$g BS-RNase), PEG and 100 $\mu$g RNase A relative to untreated controls.

### Preliminary anti-NB *in vivo* experiments

Treatment of UKF-NB-3 tumors was performed using BS-RNase–PEG, containing 15 $\mu$g BS-RNase per i.v. injection (750 $\mu$g BS-RNase/kg body weight). Tumor size at day 1 was defined as 100%. At day 22 animals of the control group had developed tumors with a volume of $650 \pm 174\%$ relative to day 1, whereas animals treated with BS-RNase–PEG developed tumors to a size of $280 \pm 41\%$. Student's *t*-test indicated this difference to be statistically significant ($p = 0.002$).

The body weights of mice treated with BS-RNase–PEG ($26.7 \pm 4.5$ g) were not influenced compared to control animals ($27.3 \pm 4.0$ g).

## Discussion

In the present study BS-RNase was covalently bound to PEG chains. Linkage to PEG chains led to the loss of antitumoral efficacy *in vitro*. However, the conjugate showed considerable aspermatogenic effects *in vivo*. The mechanism of the cleavage of the amide bond between BS-RNase and PEG under *in vivo* conditions remained uninvestigated and unclear. The aspermatogenic effects after s.c. and i.scr. injection proved BS-RNase–PEG to be distributed through the body, allowing it to reach the site of action, whereas free BS-RNase in the same quantities as in BS-RNase–PEG was inefficient.

**Table 1.** Antigenicity of BS-RNase and BS-RNase linked to PEG chains (BS-RNase–PEG) after 5-fold application s.c. or i.scr. (mean $\pm$ SEM)

| | Titer of antibodies | |
|---|---|---|
| | Against BS-RNase | Against BS-RNase–PEG |
| s.c. application | | |
| BS-RNase | 6400–12800 | 80–160 |
| BS-RNase–PEG | 6400–25600 | 32–240 |
| i.scr. application | | |
| BS-RNase | 6400–12800 | 80–160 |
| BS-RNase–PEG | 12800–51200 | 64–120 |

In addition to the fast clearance, antigenicity is a further main obstacle in the use of BS-RNase. Antigenicity of BS-RNase and BS-RNase–PEG was determined by detection of antibodies against free BS-RNase and BS-RNase–PEG after treatment with free BS-RNase or with the conjugate. Both preparations caused high antibody titers against BS-RNase. However, the conjugate itself was hardly antigenic. This indicates that recognition of the BS-RNase from the conjugate by the immune system might happen after the release of the bound BS-RNase.

Consequently, the BS-RNase–PEG conjugate represents a promising candidate for antitumoral *in vivo* use. The antigenicity of BS-RNase as BS-RNase–PEG conjugate was drastically reduced. This might cause a prolonged circulation time of the conjugate compared to the free BS-RNase and therefore enable BS-RNase to reach its site of action. Additionally, the molecular weight of the conjugate is greater than 40 kDa, which represents the cut-off size for molecules for direct drainage by the kidneys. Moreover, the conjugate might accumulate in tumors due to the EPR effect.[18]

These results and the hypothesis that these results indicate a greater antitumoral efficacy of BS-RNase–PEG *in vivo* compared to free BS-RNase were confirmed by our preliminary investigations in a mouse neuroblastoma xenotransplantate model. BS-RNase–PEG inhibited neuroblastoma tumor growth significantly at a very low concentration of 0.75 mg BS-RNase/kg body weight. In previous studies BS-RNase (12.5 mg/kg body weight) was ineffective after i.v. or i.p. administration in the inhibition of *in vivo* growth of human prostate carcinoma cells,[4,5] although animals were treated more frequently (5 times a week for 4 weeks). The same treatment regimen was needed to inhibit neuroblastoma tumor growth in former experiments by i.p. administration of free BS-RNase (12.5 mg/kg body weight, 5 times a week for 4 weeks[15]). Therefore, the antitumoral efficacy of BS-RNase could be increased by at least about 16- to 17-fold by the use of BS-RNase–PEG, although treatment was less intensive (7 times within 3 weeks). Consequently, coupling of BS-RNase to PEG chains led to a superior preparation that is a suitable and promising candidate for further evaluation as an antitumoral agent.

## Conclusion

Linkage of the antitumorally active enzyme BS-RNase to PEG enhanced its systemic activity, as demonstrated by aspermatogenic effects after systemic administration in mice. The antigenicity of the conjugate was also decreased by the conjugate. Preliminary antitumoral experiments showed these results to be relevant for antitumoral use *in vivo* in a neuroblastoma xenotransplantate model. From this data BS-RNase–PEG represents a promising antitumoral agent that is worth being further evaluated.

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*(Received 22 October 2001; accepted 6 November 2001)*