Some biological actions of PEG-conjugated RNase A oligomers*

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Received May 2, 2005

Previously we have shown that monomeric RNase A has no significant biological activity, whereas its oligomers (dimer to tetramer) prepared by lyophilizing from 50% acetic acid solutions, show remarkable aspermatogenic and antitumor activities. Furthermore, conjugates prepared by chemical binding of native RNase A to polyethylene glycol (PEG) have shown a significant aspermatogenic and antitumor activities. In this work we show that the chemical conjugation of PEG to the RNase A C-dimer, and to the two RNase A trimers (NC-trimer and C-trimer) decreases the aspermatogenic activity of the oligomers while increasing their inhibitory activity on the growth of the human UB900518 amelanotic melanoma transplanted in athymic nude mice. Moreover, the PEG-conjugated RNase A oligomers are devoid, like the free oligomers, of any embryotoxic activity.

Key words: RNase A oligomers, polyethylene glycol conjugates, anti-tumor activity, aspermatogenese, melanoma

About 50 years ago Ledoux reported that bovine pancreatic ribonuclease A (RNase A), administered at high concentration, was able to inhibit the development of various experimental tumors [1], while administration of this enzyme at low concentrations lacks any significant biological activity.

On the contrary, bovine seminal ribonuclease (BS-RNase), a naturally dimeric ribonuclease having 83% identity with its pancreatic counterpart [2], shows several biological activities, including a potent antitumor activity [3], which is lost when BS-RNase is artificially monomerized [4].

It is known that RNase A under various experimental conditions [5, 6] oligomerizes by a 3D domain swapping mechanism [7] forming dimers, trimers, tetramers and higher oligomers, with each oligomeric size being present as at least two conformational isomers, one less basic, one more basic [8]. RNase A in its oligomeric form acquires novel catalytic properties [9], as well as remarkable aspermatogenic and antitumor activities that were studied both *in vitro* and *in vivo* [10]. Therefore, at least for pancreatic type ribonucleases, it appears that dimerization or generally oligomerization is a key factor for the display of novel biological actions. This has been, at least partly, ascribed to the possibility for oligomeric ribonucleases to escape the interaction with the well-known ribonuclease inhibitor (RI) present in the cytosol of eucaryotic cells [11], although recent data do not completely agree with this hypothesis [32]. Support to this interpretation is given by the finding by MATOUSEK et al [12] proving that the covalent binding of polyethylene glycol (PEG) to monomeric, native RNase A makes the enzyme able to exert efficient antitumor and aspermatogenic activities. Indeed, over the last years the efforts of several researchers have been focused on designing mechanisms enabling ribonuclease to escape from interaction with RI. Besides the effects produced by a point mutation in the synthetic gene of ribonuclease A [13], the conjugation of monomeric RNase A with poly[N-(2 hydroxypropyl) methacrylamide] (HPMA) [14] has also been helpful to understand and solve this important problem.

*This work was supported by grant No. 523/04/0755 from the Grant Agency of the Czech Republic, by grant No. NR 8233-3 from the Grant Agency of the Czech Ministry of Health, by grant BQU2003-05257 from the Spanish Ministerio de Ciencia y Tecnologia, and by a grant from the Italian M.I.U.R., Progetti di Rilevante Interesse Nazionale, 2004. We are also indebted to the League Against Cancer, Prague, for its financial support.

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In addition to the inhibited interaction with RI, even other mechanisms could be responsible for the interesting effect due to the covalent binding of PEG to RNase A, like a longer survival of the enzyme protein in the circulation, as well as a decreased interaction with antibodies directed against the enzyme [12]. In other words, the ability to escape the interaction with RI could not be the only reason for the potent antitumor and aspermatogenic action of dimeric or oligomeric ribonucleases of the pancreatic type. It is worth bearing in mind here that other ribonucleases, like onconase or the RNase from *Rana catesbeiana* [15], are potent cytotoxic molecules notwithstanding their monomeric nature, possibly because of their very low affinity to the cellular RI due to their quite different origin.

Here, in order to verify the biological effects of the conjugation of PEG to ribonuclease molecules, we have tested the more basic one of the two RNase A dimers, the so-called C-dimer [6, 11], and the two RNase A trimers (NC-trimer and C-trimer [10], as free molecules or after conjugation to PEG, comparing them with native RNase A and BS-RNase for their aspermatogenic and antitumor activities. The results obtained indicate that PEG, on one side, decreases the aspermatogenic effects of the RNase A oligomers, and on the other side, enhances their ability to inhibit the development of a human melanoma transplanted in nude mice. The results confirm, moreover, the lack of embryotoxic effects in the action of both free [10, 16] or, as observed in this work, PEG-conjugated RNase A oligomers.

Material and methods

Ribonuclease preparations. Ribonuclease A (Type XII-A) was purchased from Sigma Chem. Co. Bovine seminal RNase was prepared and purified as described elsewhere [17]. RNase A oligomers the C-dimer, and the two trimers (NC- and C-trimer) were prepared as described elsewhere [8]. Polyethylene glycol (PEG) derivative was purchased from Shearwater Polymers, Inc., USA. All other chemicals were of the highest purity available.

Conjugation of PEG to the various RNase A oligomers. A derivative of PEG, the highly reactive ester N-hydroxyl succinimidyl propionate PEG, was used to transfer and link PEG chains to the RNase A oligomers. The stable conjugate is resistant to hydrolytic cleavage [18]. PEG was coupled under nitrogen atmosphere in 0.1 M sodium phosphate buffer, pH 7.4, at 4 °C, for 35 min. Immediately after ending the reaction, the excess reagent was removed by ultrafiltration through Amicon PM-10 membrane, and the products were purified by size exclusion chromatography on Sephacryl S-300 equilibrated with the buffer mentioned above. The eluted peaks were spectrophotometrically evaluated, and protein concentration was determined by using the procedure of BRADFORD [19]. As the reagent reacts with amine groups, in theory a maximum of eleven PEG molecules could be attached per RNase A monomer which contains ten Lys and one N-terminal amine. In practice, complete PEG substitution is not achieved due a) to the burial or participation in electrostatic interactions of some Lys, such as Lys-7, Lys-41 and Lys-66, and b) to the fact that the addition of each PEG group could act to sterically inhibit the addition of subsequent PEG groups.

Detection of free amino groups. The number of free amino groups remaining in the PEG conjugated RNase A oligomers was estimated spectrophotometrically at 340 nm after 30 min. reaction in the dark with 2,4,6-trinitrobenzensulfonic acid (TNBS), according to the method of Satake et al modified by WANG et al [20].

Mass spectrometry analyses. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were acquired using an Applied Biosystems Voyager System 6214 apparatus. Two thousand laser shots were applied per spectrum. During mass spectroscopy, RNase A oligomers dissociate into monomers.

Aspermatogenic assays. The aspermatogenic actions of the free or PEG-conjugated monomeric or oligomeric RNase A were assayed by a previously described method [21]. Into the left testis of adult ICR mice 100 μg of free or PEG-conjugated material were injected. It should be borne in mind that PEG conjugation to RNase A oligomers is heterogeneous and incomplete (see later). The injected and non-injected testes were excised from mice sacrificed 10 days after starting the treatment, and histologically examined. Destructive effects were detected by measuring the decrease of the weight index of testes, the width of their spermatogenic layers, and the diameter of the seminiferous tubules. The results were compared with controls obtained using PBS. Moreover, in order to investigate the potential of the free and the PEG-conjugated C-dimer (in comparison with native RNase A, BS-RNase and PEG-conjugated BS-RNase) to be distributed within the body and to reach their site of action, all mentioned samples were administered intraperitoneally (100 μg each) to the animals once a week for five weeks. The degenerative effects on the testes were determined by means of histological criteria.

Embryotoxic effects determination. All free or PEG-conjugated RNase A oligomers were assayed for their possible effects on the development of mouse embryos *in vitro*. Two-cell embryos from superovulated C57/BL6 mice were flushed from oviducts about 36 hrs after mating. Embryos were cultured in CZB medium [22], supplemented with bovine serum albumin (3 mg/ml) and oligomers for 72–96 hrs at 37.5 °C in a humidified atmosphere containing CO_2 (5% v/v). Controls were prepared as above, but without the addition of the RNase A oligomers. The stage of development of the embryos was monitored with a stereomicroscope [22]. The amount of free oligomers tested was 100 μg/ml.

Assay for antitumor activity 'in vivo'. The tumor line used was a human amelanotic melanoma (UB900518) originally obtained from surgical material. Pieces (two by two mm in size) of the tumor were implanted subcutaneously, together

with 0.1 ml of Matrigel, into the right flank of each of the nude mice used. These were 28 outbred female CD-1 strain nude mice of 18–20 g in body weight, supplied by AnLab Ltd., Prague, Czech Rep. The mice were kept in an air laminar flow box for small laboratory animals, KAT-F-SZ/1. They were provided with radiation-sterilized SAWI Research bedding and fed with ST-radiation sterilized diet (AnLab Ltd.). They also received autoclaved water *ad libitum*. The transplanted tumor grew in the subcutaneous layer of all 28 mice. The mice were divided in 7 groups with four animals each. When the growing tumors reached the volume of 0.103–0.235 cm^3 the treatment with the RNase A oligomers was started. Free and PEG-conjugated oligomers were administered intravenously at a dose of 100 μg/20 g (5 mg/kg) contained in a volume of 0.1 ml. The oligomers were administered five times, once every two days. During the course of the experiment, on each third or fourth day the mice were weighed, and the tumors measured with a caliper for their longest (a), and shortest (b) dimensions, as well as for their height (c). The volume of the tumor was calculated according to the formula V = a x b x c x π/6. The experiment was ended 30 days after starting the treatment. Evaluation of results was performed by comparing the growth curves of each experimental group with that of the control animals. During the course of the experiment, and at the end of it, the per cent of tumor growth inhibition (TGI) was calculated for the mean tumor volume %TGI = (1 – (mean tumor volume in the treated group)/(mean tumor volume in controls) x 100. Photo-documentation of the mice to show the efficacy of treatment in comparison with control was also performed.

Results and discussion

Coupling of PEG to RNase A oligomers. Three different approaches, namely, gel filtration, TNBS assay and Mass Spectroscopy, were used to measure the extent of PEG coupling to the RNase A oligomers. Gel filtration results indicated that 8–10 PEG chains were attached to each monomer. The results of gel filtration could be affected by the possibly remarkable modification of the globular shape of the protein species. The TNBS assay measure the number of free -NH_2 groups (and consequently the number of Lys residues modified in the conjugation reaction [12]). The TNBS analysis revealed that only five or six out of eleven amine groups were modified by the conjugation reaction in case of RNase dimers (data not shown). Moreover, the Mass Spectrometry analyses, carried out with the PEG-NC-trimer, clearly showed the heterogenic nature of the sample. The mass spectra revealed 10% molecules free of PEG chains, 22% molecules bearing one PEG chain per monomer, 31% with two PEG chains per monomer, 27% with three PEG Chains per monomer, and 10% with four PEG chains per monomer. In summary, all three methods revealed extensive but incomplete and heterogeneous coupling of PEG to RNase A oligomers in the course of mildly basic reaction conditions, which show downward trend of monomeric PEGylation index in relation to ascending degree of RNase A oligomerization. Therefore, we are presently unable to correlate precisely the loss of Lys residues with the extent of the action of the RNase A oligomers linked to polyethylene glycol chains.

Aspermatogenic activity of free or PEG-conjugated RNase A oligomers. Spermatogenesis can be particularly sensitive to cytotoxic ribonucleases. The intratesticular injection of BS-RNase in mice, rats, and rabbits, as well as its subcutaneous, intraperitoneal or intrascrotum injection evokes aspermatogenesis that can revert to normality [23]. The level of RNA in testes decreases, and returns to normal values about 50 days after the subcutaneous injection of BS-RNase, whereas no RNA decrease is detectable in liver and kidney tissues of mice. After administration of BS-RNase to rats or mice, Leydig cells were not damaged and the levels of testosterone remained unchanged [24].

Table 1 shows the results obtained after injection of the C-dimer or the NC- and C-trimers of RNase A into the left testis of mice. The destruction of tubular structures in all animals treated is clearly observed. The width of spermatogenic layers of the testes was reduced by 36% by the action of the C-dimer, and by 49% and 42%, respectively, by the action of the NC-trimer and the C-trimer. The PEG-conjugated forms of these oligomers reduced clearly the damage to the spermatogenic layers. Thus the PEG-C-dimer reduced the width of the spermatogenic layers by 28%, and the reduction caused by the two PEG-trimers was only 23% (PEG-NC-trimer) and 24% (C-trimer). However, in no cases Leydig cells were destroyed in the testicular parts of the injected testes (Fig. 1). Native RNase A was totally inactive.

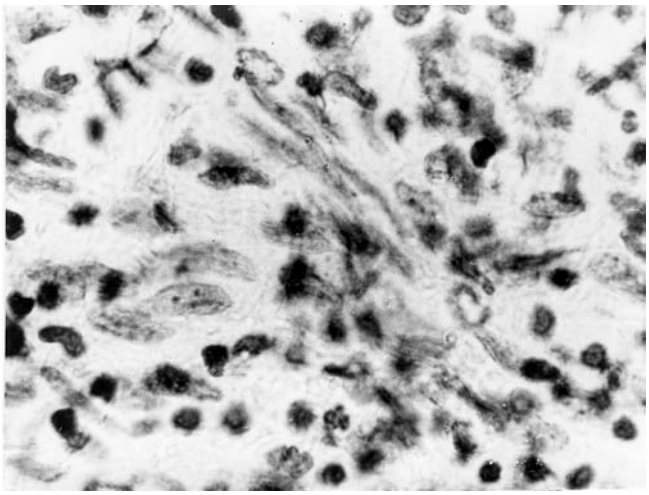

Figure 1. Effect of RNase A C-dimer injections into the left testis of a mouse. The image was obtained 10 days after injection of 100 μg of C-dimer. Interstitial tissue of the injected testicle with Leydig cells and fibroblasts. x 1200.

Aspermatogenesis by intraperitoneal injection of the free or PEG-conjugated RNase A C-dimer. The action of both free or PEG-conjugated C-dimer of RNase A injected intraperitoneally in mice, side-by-side with RNase A and BS-RNase, is shown in Table 2. Regarding the width of the spermatogenic layers of both testes, it is quite clear that while native RNase A did not affect the width of the spermatogenic layers of the testes, BS-RNase or its PEG-conjugated form expressed high aspermatogenic activity. Interestingly, the free C-dimer of RNase A caused serious damage to the spermatogenic layers, of the same order of magnitude as that produced by BS-RNase, whereas the PEG-conjugated C-dimer was practically harmless. In the case of the diameter of the seminiferous tubules of both testes, the PEG-conjugated BS-RNase showed a slight effect, while the RNase A C-dimer and its PEG-conjugated counterpart were harmless, as was native RNase A. The body weight of all animals subjected to the experiments described was not significantly affected. In conclusion, it is remarkable that while the conjugation with PEG could not reduce the aspermatogenesis induced by the naturally dimeric BS-RNase, it was successful in the case of the artificial C-dimer of bovine pancreatic RNase A.

Embryotoxicity of free or PEG-conjugated oligomers. All free or PEG-conjugated RNase A oligomers were assayed for their effect on the development of mouse embryos *in vitro*. As shown in Table 3, no embryotoxic activity was displayed by both free or PEG-conjugated RNase A oligomers, while assays performed in parallel with BS-RNase revealed a clear embryotoxic effect. This result confirmed previous data concerning the strong embryotoxicity of BS-RNase and the lack of embryotoxicity in the biological actions of the RNase A oligomers [10, 16]. It is worth mentioning that the PEG-conjugated oligomers partly precipitated, which might

Table 1. Aspermatogenic effect in mice after injection into testicle of oligomers of RNase A or oligomers of PEG- conjugated RNase A

Substances	No of. mice	Index weight of testicles		Width of spermatogenic layers of testicles		Diameter of seminiferous tubules of testicles	
		injected	non-injected	injected	non-injected	injected	non-injected
PBS	5	44 ± 5	45 ± 7	63 ± 5	62 ± 9	163 ± 8	165 ± 11
RNase A	5	43 ± 6	42 ± 3	64 ± 6	66 ± 7	161 ± 7	161 ± 7
C-dimer	5	41 ± 3	42 ± 4	39 ± 7^{+}	61 ± 6	158 ± 14	160 ± 13
PEG-C-dimer	5	37 ± 23$^{\#}$	43 ± 3	41 ± 9^{+}	57 ± 7	157 ± 6	158 ± 5
NC-trimer	5	35 ± 24*	33 ± 21^{*2}	30 ± 6^{+}	59 ± 5	148 ± 18	162 ± 13
PEG-NC-trimer	4	32 ± 22*	31 ± 22^{*3}	47 ± 5^{+V}	61 ± 7	155 ± 6	159 ± 8
C-trimer	5	40 ± 5	39 ± 5	32 ± 6+	55 ± 3	154 ± 13	160 ± 10
PEG-C-trimer	5	42 ± 6	44 ± 3	44 ± 6^{+V}	58 ± 6	159 ± 9	156 ± 13

The values are mean ±SD.
$^{+}$ and * – p<0.01. $^{+V}$ – p<0.01 higher for PEG-NC-trimer and PEG-C-trimer compared with free NC-trimer and C-trimer, $^{\#}$ – the weight of one testicle from 5 mice injected with PEG-C-dimer was lower, ^{*2} – both testicles from 5 mice injected with NC-trimer were degenerated, ^{*3} – both testicles from 4 mice injected with PEG-NC-trimer were degenerated.

Table 2. Aspermatogenic effect in mice after intraperitoneal injection of RNase C-dimer or PEG-conjugated RNase C-dimer, compared with the effects due to RNase A or BS-RNase

Substances injected	Mice (*n*)	Index weight of both testicles	Width (in μm) of spermatogeni c layers of both testicles	Diameter of seminiferous tubules of both testicles	Body weight (in g) before and after the experiments	
PBS	5	92 ± 9	64 ± 11	166 ± 9	22 ± 1	25 ± 1
RNase A	5	96 ± 5	65 ± 7	162 ± 11	21 ± 1	23 ± 1
BS-RNase	5	83 ± 7	45 ± 6	161 ± 9	21 ± 1	22 ± 1
PEG-BS-RNase	5	79 ± 8	46 ± 5^{+}	156 ± 6	20 ± 2	23 ± 1
C-dimer	5	102 ± 8	42 ± 12^{+}	163 ± 7	21 ± 1	24 ± 2
PEG-C-dimer	5	101 ± 9	61 ± 10	161 ± 8	21 ± 1	24 ± 1

The values are mean ±SD.
$^{+}$ p<0.01

Table 3. Development of mice embryos after 72 hours culture in CZB medium supplemented with bovine serum albumin (3 mg/ml), RNase A, BS-RNase, RNase A oligomers or PEG-RNase A oligomers

Enzyme species used for embryo culture	Number of embryos	Number of embryos in cell stages			
		Blastocysts	Expanded blastocysts	Total blastocysts	Blastocysts %
Control (medium)	12	7	1	8	66
RNase A	10	4	2	6	60
BS-RNase	11	1	0	1	9^{+}
C-dimer	13	6	4	10	69
PEG-C-dimer	15	9	2	11	73
NC-trimer	14	6	5	11	78
PEG-NC-trimer	12	4	3	7	58
C-trimer	10	4	3	7	70
PEG-C-trimer	13	4	4	8	61

$^{+}$ p<0.01

explain a certain degree of retardation in embryos' development.

*"In vivo" anti-tumor action of free or PEG-conjugated RNase A oligomers***.** The C-dimer, and the NC-trimer of RNase A were injected side-by-side with their PEG-conjugated counterparts into nude mice, that had been transplanted subcutaneously with the human non-pigmented UB900518 melanoma. Figure 2 shows the effects of the C-dimer and PEG-conjugated C-dimer. Both substances clearly inhibit the growth of the tumor, and within 30 days of treatment the PEG-conjugated dimer was slightly more efficient than the free C-dimer. The action of the less basic NC-trimer and of its PEG-conjugated counterpart are shown in Figure 3. The inhibitory effect of NC-trimer is of the same order of magnitude as that of the C-dimer. However, the PEG-conjugated sample was clearly more active than the free NC-trimer along the entire treatment. As for the inhibition of tumor growth exerted by the C-trimer, it was on the same order of magnitude as that of the NC-trimer but its PEG-conjugated derivative did not increase the antitumor effect (data not shown). Within 30 days of treatment the capacity of inhibitory effect was in the order PEG-NC-trimer > PEG-C-dimer > NC-trimer > C-dimer. In conclusion, we could say that in cases of C-dimer and NC-trimer of RNase A the PEG-conjugated oligomers were more efficient than their free counterparts.

While the anti-tumor and other biological activities of monomeric bovine RNase A are only minor or nil, the efficiency of BS-RNase in these activities is very significant. Previously we have demonstrated that conjugation with various polymers may influence markedly the behavior of the both mentioned ribonucleases. Conjugates of BS-RNase with the HPMA polymer exerted significant anti tumor activity after intraperitoneal and intravenous administration in nude mice bearing various human tumors, while the same application with native BS-RNase caused only negligible effects, and significant anti-tumor action was obtained only after its intratumoral administration [25, 26]. As mentioned above, the activity of RNase A was also changed very much after its conjugation with HPMA or PEG. In both cases, its conjugates administered intravenously in mice strongly reduced the growth of transplanted tumors, whereas native RNase A was inactive [12, 14]. Previously, we have proved that RNase A oligomers inhibit the growth of a human melanoma transplanted in nude mice, and that their action increases as a function of the size of the oligomers [10]. This effect was in agreement with the antiproliferative action exerted by the RNase A oligomers in experiments performed *in vitro* with ML-2 and HL-60 cell lines [10]. Therefore, we actually did not expect any striking increase of antitumor activity in PEG-conjugated oligomers. Anyhow, the results obtained in this work show that two RNase A oligomers, the C-dimer and the NC-trimer, conjugated with PEG were more efficient than their free counterparts. *In vitro* experiments performed with PEG-conjugated oligomers (data not shown)

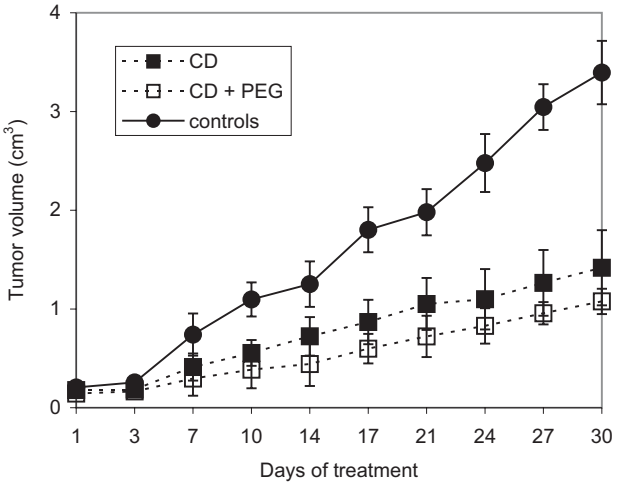

Figure 2. ***In vivo*** **antitumor activity of free or PEG-conjugated RNase A C-dimer. Growth of the human amelanotic melanoma, UB900518, transplanted in the right flank of athymic nu/nu mice. The animals were divided in seven groups of four mice each. When the tumor reached the volume of 0.103–0.235 cm^3, free or PEG-RNase A C-dimers were administered intravenously at a dose of 100 µg/20 g animal (5 mg/kg). For experimental details see the Materials and methods section. C_D – C-dimer; CD+PEG – PEG-conjugated C-dimer.**

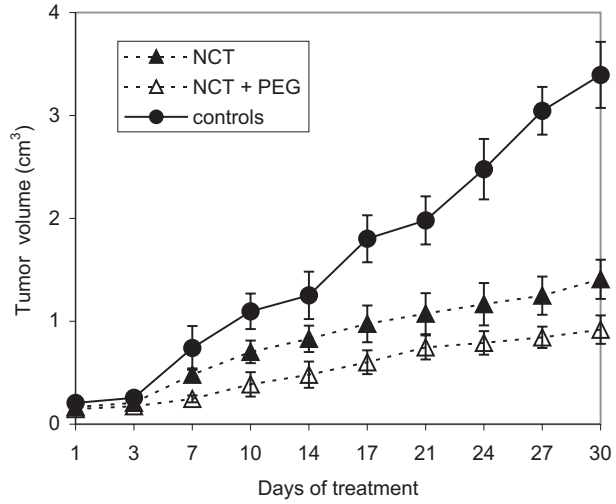

Figure 3. ***In vivo*** **antitumor activity of free or PEG-conjugated RNase A NC-trimer. Experiments were performed as described in the Legend to Figure 2. NC_T – NC-trimer; NC_T+PEG – PEG-conjugated NC-trimer.**

confirmed our results previously obtained with BS-RNase, the potent inhibitor of several tumor cell lines [26] that loses its inhibitory action after conjugation with poly-HPMA. This loss of cytotoxic activity by BS-RNase, and the only slight increase of activity of the RNase A oligomers suggest that the ability of bulky polymeric RNase molecules to enter malignant cells might be significantly reduced in an *in vitro* sys-

tem. Moreover, in trying to explain the rather modest changes in the biological activity of the RNase A PEG-conjugated oligomers if compared with the activities of the free oligomers, we must also consider that the reaction to couple polyethylene glycol to the protein moiety involves Lys residues, subtracting basic charges to the protein molecule. We know from previous results [27, 28, 29] that the positive charges present on the surface of ribonuclease molecules are very important for a number of reasons. They improve (a) the interaction of the protein with the cell membrane [28, 30, 31], and therefore (b) possibly its entrance into the cell. They (c) are essential for the catalytic activity of the oligomers against secondary structures of RNA [27, 29], and (d) increase the antitumor activity of the RNase A oligomers [10, 16] that is indeed, like the catalytic activity, a function of the number and/or the density of basic charges on the RNase molecule [16, 27, 29]. Therefore, also from this point of view, it is quite reasonable that the PEG-conjugated oligomers show a lower activity than expected. This becomes particularly true for the activity of the RNase A oligomers on double-stranded [poly(A)-poly(U)]. While enzyme degradation of single-stranded RNA (Kunitz assay) is not affected, the PEG-conjugated RNase A oligomers show a significantly reduced activity on the double-stranded substrate (data not shown). The importance of the positive charges to the biological actions of RNase A oligomers suggests that chemical coupling to the negatively charged carboxylate groups could lead to an important increase of *in vitro* and/or *in vivo* anti-tumor activity.

When conjugated to PEG, two of three RNase A oligomers show an increased anti-tumor activity, and all three show a reduction of their aspermatogenic activity. Moreover, the PEG-conjugated RNase A oligomers are devoid of any embryotoxic activity. These encouraging results indicate that chemical coupling can improve the pharmacological properties of oligomeric RNase A.

We are very grateful to V. PECH for histology examination and to Miss G. LINDNEROVA for skilful technical assistance.

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