doi:10.4149/neo_2010_04_339

Antitumor activity of apoptotic nuclease TBN1 from *L. esculentum*.

J. MATOUSEK[1], T. PODZIMEK[1,2], P. POUCKOVA[3], J. STEHLIK[1], J. ŠKVOR[3], P. LIPOVOVA[2], J. MATOUSEK[4]

[1]Biology Centre, Academy of Sciences of the Czech Republic v.v.i., Institute of Plant Molecular Biology, 370 05 České Budějovice; [2]Department of Biochemistry and Microbiology, Institute of Chemical Technology Prague, 166 28 Prague; [3]Institute of Biophysics and Informatics, 1st Medical Faculty of the Charles University, 128 00 Prague; [4]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, 277 21 Liběchov, e-mail: matousek@iapg.cas.cz

Received August 3, 2009

Nuclease from tomato (TBN1) was produced by *in planta* biotechnology purified and tested for its anticarcinogenic properties. The nuclease was cytostatic after its intratumoral administration to nude mice bearing human melanoma or prostate carcinoma or after tumor targeting by TBN1 administration intravenously as conjugate with polyethylene glycol (PEG). Inhibitory effects of TBN1 on tumor growth were comparable to effects of bovine seminal RNase (BS-RNase), but the inhibition was reached at about ten times lower protein concentration. Simultaneously, TBN1 exhibited a lower degree of embryotoxicity compared to BS-RNAse and other nucleases. TBN1 showed significant stability *in vivo*, because it was readily detected after its administration intratumorally or intravenously by the fluorescence methods. Intravenous administration of TBN1-PEG caused significant inhibition of tumor proliferation without obvious degenerative changes, while direct administration of TBN1 into melanoma tumors led to rapid tumor tissue degeneration. The fact can be essential for the mode of TBN1 biological action that mature nuclease is a small (36 kDa) thermostable glycoprotein that has ability to destroy human 28S, 18S, 7S and 5.8S RNA, circular RNAs, double-stranded RNA *in vitro* and shows DNase and 3'nucleotidase activities.

Key words: anticarcinogenic and antiproliferative nuclease; dsRNase; embryotoxicity; human solid malignant tumors; Nicotiana benthamina; plant infiltration;

Plants as producers of new engineered pharmaceuticals are becoming a very attractive tool in attempts to improve human health [1, 2]. New plant-produced medicines include various antigenic proteins, vaccines [3] and anticarcinogenic compounds as recently summarized by Pujol et al. [4]. Nucleolytic enzymes like ribonucleases (RNases) belong, as mild toxins, to potential alternatives to non-mutagenic antitumor drugs and chemotherapeutics. In the past, exclusively animal RNases were studied in this respect [5, 6, 7, 8, 9]. Among the enzymes of practical significance, onconase, an RNase from the Northern Leopard frog, is currently undergoing phase III clinical trials for the treatment of malignant mesothelioma [10]. Onconase and its variants have been found to reduce the tumor size in animals and to enhance the cytotoxic efficacy of chemotherapeutics [8]. Together with anticarcinogenic properties, some cytotoxic side effects of RNases of human and animal origin including bovine seminal ribonuclease (BS-RNase) and onconase have been reported [8, 11]. In this respect, it is of interest to analyze nucleolytic enzymes of plant origin for their potential antitumorigenic effects and biological activities.

The antitumorigenic potential has recently been described for several RNases and nucleases of higher plants [12–16] including plant nuclease I (E.C.3.1.30.x) [17]. Antiproliferative and cytotoxic effects of Mung bean sprout nuclease I (PhA) [18] and extracellular nuclease I from black pine pollen (PN) [19] were described in our previous studies.

Sequences of nuclease I class designated as bifunctional nucleases [20] were reported, for instance, for barley [21, 22], *Arabidopsis* [20], zinnia [20, 23] daylily [24], tomato [25] and hop [26]. Bifunctional plant nucleases are closely related, forming a cluster of amino acid sequences with high identity [25]. Some of bifunctional plant nucleases are activated under stress conditions and play an important role in the cell apoptosis [22, 23]. Availability of nuclease I sequences enables a construction of recombinant nuclease variants for their biotechnological production and detailed molecular analysis.

In our previous work, we identified and cloned cDNA of bifunctial nuclease TBN1 from tomato sufferred from viroid disease [25]. It was proposed that disease-induced upregulation of this nuclease leads to apoptosis and collapse of the

plant vascular system [25]. In the present work we analyzed degradative potential of TBN1 nuclease-dsRNAse and its targeting as a cytostatic agent against human malignant tumors grown on nude mice.

Materials and methods

In planta preparation of recombinant nuclease in Nicotiana benthamiana.

N. benthamiana plants, the source of infiltrated leaf tissue, were maintained in clima boxes at a temperature of 25±3°C. The plants were grown under natural light with supplementary illumination [90 μmol m^{-2} s^{-1} PAR] to keep a 16 h-day period. Nuclease TBN1 cDNA (GenBank accession number AM238701) [25] was used for construction of the plant expression vector. This cDNA was re-amplified using primers Xho5'TBN1 (5'gacCTCGAGGGCCCATGTTGAGGTTAAC TTTATTAAGC 3') and Xba3'TBN1 (5'aatTCTAGATTAAG TTGCAACAACTGAATCTTC3') (restriction ends designed for construction of a plant vector suitable for the expression of the recombinant TBN1 nuclease are underlined and added nucleotides are designated by small letters). cDNA fragment was treated with *Xho*I and *Xba*I restriction enzymes and ligated into the intermediary vector pLV-68 [26] to attach the 35S promoter of CaMV. Finally, the *Pac*I-*Asc*I fragment from pLV-68 was cleaved out and re-cloned into the plant vector pLV-07 [26]. This vector was introduced into the *A. tumefacens* strain LBA 4404 by the freeze-thaw method [27] and used for leaf infiltration. *N. benthamiana* leaves were infiltrated by the method described by [28] and lyophilized until used for extraction of recombinant TBN1.

Nuclease Substrates and Assays. To determine nuclease activity we followed the production of ethanol-soluble material from highly polymerized calf thymus ss- of dsDNA (Serva, Heidelberg) or from high weight RNA (Worthington, USA). One unit of nuclease activity is defined as an amount of enzyme that releases 0.001 A_{260} unit of acid-soluble products in 1 min at 37 °C.

In crude leaf homogenates, nuclease was assayed by radial diffusion method. The diffusion was performed in gels containing 30 μg ml^{-1} heat-denatured calf thymus ssDNA (Serva, Heidelberg) [26], the gels were stained with ethidium bromide. The radial diffusion method was used also to assay the activity towards double-stranded RNA [29]. dsRNase activity was assayed using the radial diffusion in 1.5 % agarose gels containing radioactivelly-labelled double-stranded RNA [29]. 25 μl of diluted nuclease samples were applied into holes 0.4 mm in diameter made in agarose and gels were incubated in covered Petri dishes at 37 °C for 15 h. Then the agarose gels were covered with DEAE-paper (Whatmann) and capillary blot of dsRNA was performed. DEAE-paper was then dried and scanned for immobilized dsRNA using *TYPHOON* PhosphoImager.

Assay of nuclease activity towards melanoma rRNA, 7SL RNA and viroid RNA in vitro by Northern Blot Analyses. RNA from melanoma tumors was isolated according to the manufacturer's protocol supplied for the RNeasy Plus Mini Kit, (Qiagen, Hilden). For cleavage of ribosomal and 7SL RNA, the reaction mixtures contained 15 μg of total RNA isolated from melanoma tumors and 1 μl of purified diluted TBN1 from stock sample (0.6 μg x $μl^{-1}$). Cleavage was performed in 0.2 M acetate puffer pH 6.0 at the room temperature. Reactions were terminated by phenolization, samples were then precipitated by ethanol precipitation, and separated by electrophoresis in a denaturating 1.5% agarose gel containing formaldehyde. After capillary blotting the samples onto Biodyne A transfer membrane (Pall, Hampshire, England), prehybridization and hybridization were carried out in 50% formamide-based (pre)hybridization buffer [30] at 32 °C overnight. The washing procedure included an incubation of membranes in 2x SSC for 10 min at 0 °C followed by incubation in 2x SSC buffer containing 0.1% SDS for 30 min at 32 °C. RNA samples were hybridized to 23S, 18S, 5.8S and 7SL cDNA probes labelled with [α-^{32}P]dCTP using the Redivue™ [α-^{32}P]dCTP 3000 Ci/mmolRediprime™ II random prime labeling system (Amersham Pharmacia Biotech, Freiburg, Germany).

In some experiments we used partly purified potato spindle tuber viroid (PSTVd) RNA as substrate and assayed viroid cleavage after separation of RNA in polyacrylamide denaturing gels, electroblot and hybridization as described by Matoušek et al. [26].

The autoradiograms were scanned using the TYPHOON PhosphoImager (Amersham Biosciences, Sunnyvale, California, USA) and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, California, USA). Human 7SLRNA and rRNA probes were amplified from RNA extracts using Titan, one step RTPCR system (Roche) and cloned to PCRScript (Stratagene). The following primer combinations were used to amplify human 7SL cDNA probe 299bp: 5'HSRN7SL1 (5'GCCGGGCGCGGTGGC3') and 3'HSRN7SL1 (5'AGAGACGGGGTCTCGCTATGTTGC3'); 28S cDNA probe 360bp: 5'H28S (5'CGCGACCTCAGATCAGAC3') and 3'H28S (5'CGGTACTTGTTGACTATC3'); 18S cDNA probe 360 bp: 5'H18S (5'TACCTGGTTGATCCTGCC3') and 3'H18S (5'TAGGGCAGACGTTCGAATG3') and 5.8S cDNA probe 156bp: 5'H5.8S (5'GACTCTTAGCGGTGGATC3') and 3'H5.8S (5'AAGCGACGCTCAGACAGG3').

Nuclease TBN1 purification and PEGylation, other nucleolytic enzymes for comparative analyses. The lyophilized *N. benthamiana* leaves containing recombinant TBN1 were homogenized in 50 mM Tris buffer pH 7.5 containing 1 mM PMSF (serine protease inhibitor) and 10 % sucrose (stabilizer). The process was performed in a frozen mortar with sand and pestle. The debris was separated by centrifugation (12 000 g, 15 min, 4 °C) and the extract was further treated. The proteins were precipitated by ammonium sulfate in an ice-cold water bath. The precipitate obtained between 35 and 80% of saturation was centrifuged (15 000 g, 10 min. 4 °C) and the pellet was dissolved in 50 mM Tris buffer pH 7.5. Ammonium sulfate was removed by gel filtration on PD 10 column (Amersham

Biosciences, USA) equilibrated with 50 mM Tris buffer pH 7.5. The sample was further purified by two chromatography techniques – ionex and affinity chromatography. The sample was diluted four-times with distilled water to decreasing ionic strength and applied to HiTrap Q FF column 3x5 ml (Amersham Biosciences, USA) containing strong anex and eluted with gradient of sodium chloride. The starting buffer was 50 mM Tris buffer pH 7.5 and the elution buffer was 50 mM Tris buffer pH 7.5 + 1 M NaCl. The fractions showing ssDNase activity were pooled and applied to HiTrap Heparin HP 2x5 ml column (Amersham Biosciences, USA) and eluted with gradient of sodium chloride. The same buffers were used as by ionex chromatography. The fractions with ssDNase activity were collected, pooled, diluted four-times with distilled water and concentrated on HiTrap Heparin HP 2x5 ml column. Finally, the pure sample of nuclease was desalted on PD 10 column equilibrated with 50 mM Tris buffer pH 7.5 and stored at -20 °C.

In order to improve the stability of TBN1 against hydrolytic cleavage after intravenous administration, purified nuclease was chemically modified by PEGylation as described earlier [15]. Conjugated TBN1 was purified by size exclusion chromatography on Sephacryl S-300 in above mentioned buffer.

In addition to TBN1, Mung bean (*Phaseolus aurens*) nuclease (PhA) was purchased from MP Biomedicals, Irvine California 92618, USA. Black pine *(Pinus nigra)* pollen nuclease was prepared and purified as described previously [19], Bovine seminal ribonuclease (BS-RNase) was prepared from the bull seminal vesicle fluid as described by Matoušek [29].

Preparation of anti-TBN1 antibody, nuclease deglycosylation and N-terminal sequencing. Polyacrylamide gel electrophoresis and gel treatments for nuclease renaturation were performed as described previously [25] except for the nuclease reaction buffer: in this study we used 0.1 M acetate buffer pH 6.0 as a final reaction buffer. For induction of anti-TBN1 antibody, TBN1 purificate (3 mg) was further separated by electrophoresis on preparative SDS gel. The gel was briefly stained for proteins using Coomassie R-250 for 10 minutes and contrasted for 15 min to recognize major 36 kDa TBN1 bands. After electrophoresis, the gel was incubated twice for 10 min in 0.01 M Tris-HCl buffer pH 7.0 (wash buffer) containing 25% isopropanol and afterwards twice for 5 min in wash buffer containing 2 μM $ZnSO_4.x7H_2O$. Finally, the gels were washed twice with 1xPBS for 5 min and the protein bands were precisely excised. The pieces of gels were homogenized in 1xPBS using a mortar and pestle and used as antigen for rabbit immunization. The immunization scheme included three times subcutaneous injections on the back weekly with TBN1 antigen emulsified with an equal volume of the Freund's complete adjuvant. A booster injection was given 3 weeks after the third priming immunization with TBN1 antigen without the Freund's adjuvant and the rabbit was exsanguinated two weeks after the boost. The IgG fraction was isolated from the serum using DEAE-TRISACRYL [31].

For Western blot analysis semi-dry electroblots with Panther device (Thermo Scientific Owl Separation Systems, Portsmouth, USA) were performed in 0.05 M Tris-0.04 M Glycine buffer, pH 9.2 containing 20% methanol and 0.04% SDS. Proteins were transferred to 0.45 μM Immobilon–NC Transfer membrane (Millipore). After standard blocking and washing, the membrane was incubated with anti-TBN1 IgG as primary antibody and subsequently, with secondary goat anti-rabbit (Sigma) antibody conjugated to alkaline phosphatase. Bands were developed after incubation with NBT/BCIP substrate at the room temperature for 6-12 h in the dark.

TBN1 deglycosylation was performed using PNGase F (Roche). N-terminal amino acid sequences were determined on the Procise - Protein Sequencing System (PE Applied Biosystems, 491 Protein Sequencer) by the Edman degradation. The resulting PTH-amino acids were analyzed sequentially (by RP HPLC) to determine the amino acid sequence of the protein or peptide.

Treatment of melanoma and prostatic carcinoma tumors with TBN1. The antitumoral activity of TBN1 and TBN1+PEG nucleases as well as BS-RNase was tested on athymic (nu/nu) female mice CD-1 weighing 19 - 21 g. The mice were housed under aseptic conditions in cages with bedding (SAWI Research bedding sterilized by irradiation) and fed with sterile diet as referred previously [32].

Analysis of melanoma tumors with real-time PCR. For molecular analysis of melanoma tumors, two human genes (PLAB and L1CAM), were analyzed using Real-time PCR with primer pairs PLAB-F (5'AATGGCTCTCAGATGCTC CTG3') plus PLAB-R (5'GGTCGGTGTTCGAATCTTCC3') and L1CAM-F (5'ACTTCAGGTTCCATATCTTGTTCA3') plus L1CAM-R (5'AGTGGATCTCGTAGTCAGTGTCAG3'), respectively [33]. Four micrograms of total RNA were reverse transcribed using oligo dT^{18} primer and SuperscriptII reverse transcriptase (Invitrogen) at 42°C/60 min. A total of 5 **μl** of 50x diluted cDNA was used for a 20**μl** PCR reaction: 0.6 units of Hot Start Ex Taq polymerase (TaKaRa, Shiga, Japan), Taq buffer 1X, dNTPs 200 μM each, Syber green 1:20000 (Molecular Probes, Leiden, The Netherlands) and primers 375 nM each. All amplifications were carried out on a Bio-Rad IQ5 cycler for 40 cycles (94°C/20sec, 58°C/30sec, 72°C/30sec) following an initial denaturation/Taq activation step (94°C/5min). Product size was confirmed by melting analysis and 2% agarose gel electrophoresis. Data were analyzed and quantified with the Bio-Rad IQ5 Optical System version 2.0 software. The abundance of a reference transcript, hydroxymethylbilane synthase (HMBS) [33] was estimated in parallel in each sample. HMBS was amplified using primers HMBS-forward (5'TCTTGGATCTGGTGGGTGTG3') and HMBS-reverse (5'GTAGCCTGCATGGTCTCT TGTAT3') and normalized to the sample with highest expression (calibrator, set to 100%).

Histology and immunofluorescence cryomicroscopy. All animals injected with TBN1 and comparative substances were subjected to excision of testes and tumor enucleation. The small pieces of these tissues were consequently fixed in

Bouin solution for histology examination. The fixed samples of tissues were embedded in paraffin blocks. Tissues slides (5 μm) were cut and stained with hematoxylin-eosin.

Cryosections from control and TBN1-injected melanoma tumors were fixed in pre-cooled (-20°C) acetone for 10 min washed three times with PBS for 5 min and blocked for 60 min with 10% inactivated goat serum (IGS). Subsequently, sections were incubated with Rabbit anti-TBN1 IgG (1:200) in 10% IGS at 4 °C overnight and washed three times for 5 min. The sections after incubation for 1h with the secondary antibody [Goat anti Rabbit CY3-conjugate (1:1000 in IGS)] were washed 3 times for 5 min with PBS. Finally, cell nuclei were counterstained with 0.01% 4',6-diamidino-2-phenylindole (DAPI) (Sigma) in distilled water for 1 min, washed three times with PBS, rinsed for 10 second with distilled water before mounting to Mowiol and then viewed with a fluorescent microscope. Images from fluorescent microscope were preprocessed with gradient morpho RGB intensity option of the program LUCIA v.5.0 software (Laboratory Imaging, Prague, Czech Republic) and integral value of brightness was measured.

Assays of nuclease embryotoxicity, statistical analyses and ethics. To assay embryotoxicity, effects of TBN1, PN and PhA nucleases and BS-RNase on the development of mouse embryos *in vitro* were analyzed [34]. The data were analyzed statistically using the Fisher´s t-test. All the mentioned experiments adhered to ethical standards and were approved by the institutional committee (approval no. 3/04) and all researchers handling experimental animals possess certificates from the Central Committee for Animal Welfare.

Results

In planta preparation and some essential properties of bifunctional apoptotic nuclease from tomato. According to our previous work [18, 19], nuclease I purified from natural plant sources exhibited antiproliferative activities. Because this group of enzyme is known to undergo posttranslational modifications, we selected the *in planta* concept for preparation of recombinant TBN1 nuclease. In order to overexpress TBN1 by leaf disc infiltration method, we constructed plant expression vector pLV-07 [26] containing nuclease cDNA driven from strong 35S CaMV promoter. Young leaves of *N. benthamiana* were infiltrated with a mixture of activated agrobacterium bearing the TBN1 expression vector and suppressor of post-translational gene silencing (PTGS) p19 to achieve a higher yield of the overexpressed TBN1 protein. For the nuclease purification, ammonium sulfate fractionation, ion exchange chromatography on a HiTrap Q FF column and affinity chromatography on HiTrap Heparin HP were used (see Material and Methods). The nuclease yield was about 30 % and purified fold was 29. The purification process led to clean TBN1 samples showing one predominant band of nuclease having apparent molecular mass of 36 kDa and usually a very minor band of nuclease showing 34 kDa (Fig. 1A). In Westernblots, the two bands exerted a specific cross reaction with the anti-TBN1 polyspecific serum (Fig. 1B). The nuclease purity was also proven on silver-stained gels (not shown). Typically about 5-10 mg of pure TBN1 nuclease was prepared from 100 g of infiltrated *N. benthamiana* leaves. This amount of nuclease hydrolyzes 0.6-1.2 g of highly polymerized ssRNA to ethanol-soluble products within one minute at 37 °C. N-terminal sequencing of the purified nuclease protein indicated its cleavage at amino acid position 25 and removal of signal peptide corresponding to the sequence MLRLTLLSSIFFLCVAFINQHGVEA. Mature nuclease has pI 5.42 and molecular mass of 31.6 kDa predicted according to the amino acid sequence. The difference between predicted and observed molecular masses suggests that the mature recombinant nuclease is a glycoprotein containing about 12 % of sugar residue. The glycosylation of TBN1 was proven by a deglycosylation reaction with PNGase F. In these experiments, deglycosylated nuclease band of 32 kDa was detected on activity gels containing ssDNA (not shown).

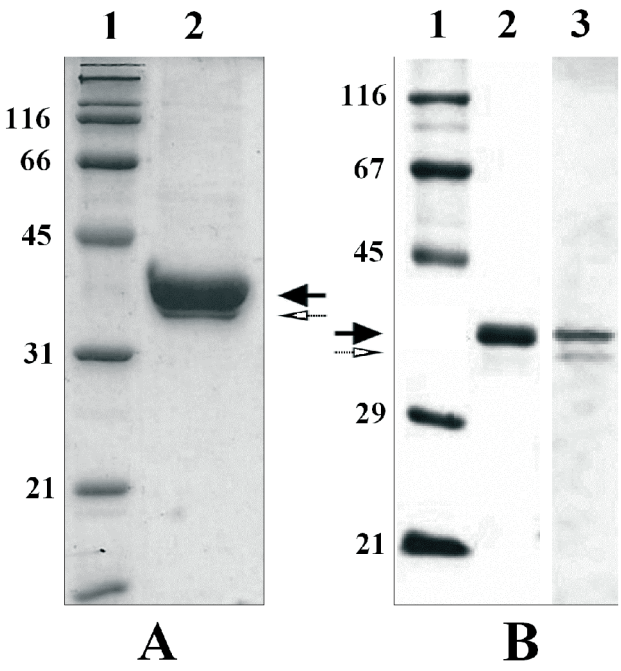

Fig. 1 PAGE (A) and Westernblot analyses (B) of purified nuclease samples. Recombinant nuclease was extracted from *N. benthamiana* leaves, purified by subsequent purification steps including ammonium sulfate fractionation, ion exchange chromatography on HiTrap Q FF column and affinity chromatography on HiTrap Heparin HP and applied on denaturing SDS PAGE as described in Material and Methods. Panel A: 1, SDS-PAGE Standards - Broad Range (BioRad); 2, application of 10 μg of nuclease purificate. Panel B: 1, unstained SDS PAGE Protein Marker from Serva (Heidelberg); 2, application of 1.2 μg of nuclease purificate; 3, Westernblot of nuclease (0.12 μg) after incubation with polyspecific TBN1 serum. Gels were stained for proteins with Coomassie blue R250. Major nuclease bands of approx. 36kDa are indicated by filled arrows, minor bands of 34 kDa are indicated by hollow arrows.

Some essential biochemical properties of purified recombinant nuclease were analyzed. Nuclease has an optimum pH of 5.9 for dsDNA and 6.3 for both, ssRNA and ssDNA. Ac-

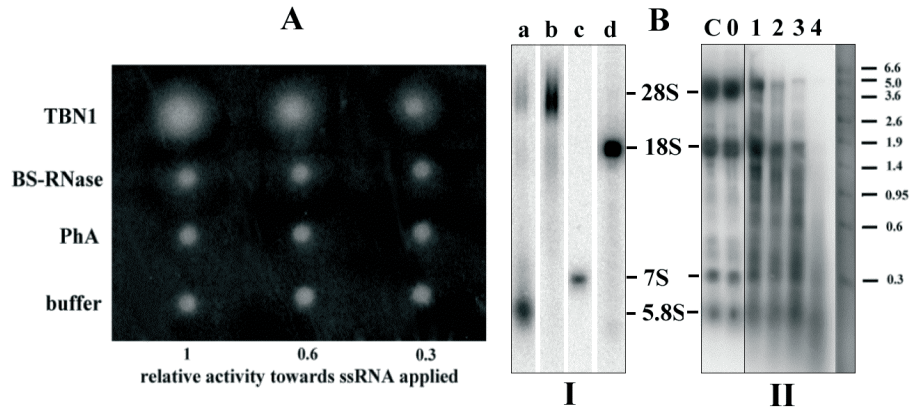

Fig. 2 Detection of dsRNase activity of nuclease TBN1 (A) and its capability of hydrolyzing human ribozomal and 7SL RNAs *in vitro* in crude RNA extracts (B). dsRNase activity was assayed using the radial diffusion in 1.5 % agarose, see Material and Methods. Samples in panel A are designated according to comparative enzymes applied; 0.1M reaction acetate buffer pH 6.0 was added as a control sample. An equal amount of ssRNase activity containing 1830, 1100 and 550 units was applied per sample corresponding to 1, 0.6 and 0.3 relative concentrations, respectively. Sizes and intensities of the white spots represent dsRNase activity. Panel B I: Positions of individual RNA species after hybridization of total RNA from melanoma to specific probes. 1, 5.8S probe; 2, 18S; 3, 28S and 4, 7SL RNA probe. II: Cleavage of RNA with TBN1 as described in Material and Methods, C-control without cleavage; 1, 50x diluted TBN1, 5 min cleavage; 2, dilution 50x, 10 min; dilution 50x, 25 min; dilution 10x, 10 min. RNA was hybridized to mixed 5.8S,18S, 28S and 7SL probe. Positions of RNA Molecular Weight Marker III from Boehringer Mannheim (kb) are given on the right side.

cording to the kinetics, it cleaves substrates ssRNA : dsDNA : ssDNA at the ratio 1 : 1.4 : 1.6, respectively, showing no general preference for single-stranded nucleic acids. The cleavage of RNA and DNA substrates has an optimum temperature of about 60 °C, suggesting high thermal and structural stability of nuclease. Our experiments demonstrated that the TBN1 activity is capable of cleaving dsRNA (Fig. 2A), while BS-RNase did not show any significant activity towards this substrate under the same conditions, as well as single strand-specific Mung bean nuclease (Fig. 2A). The dsRNase activity of TBN1 could be an important factor determining its ability to destroy highly cooperative structures like 7SL RNAs, or other thermodynamically stable RNA elements. According to our experiments, TBN1 is able to completely destroy viroid RNAs (not shown), confirming the endonucleolytic mode of its action and its ability to cleave partly double-stranded circular RNAs. Nuclease effects on human ribosomal and 7SL RNAs were analyzed by Northern blotting using total RNA extracted from human melanoma tumors (Fig. 2B). These results indicate that TBN1 readily cleaved 28S, 18S, 7S and 5.8S RNA *in vitro,* because only smear ranging from 1kb to low molecular mass cleavage products was detectable in the sample after a more pronounced reaction (Fig. 2B, sample No 4). In addition to phosphodiesterase activities, the recombinant purified TBN1 was found to exhibit 3'nucleotidase (phospohomonoesterase) activity reaching 0.54±0.02 μmol Pi/min/μg of protein as assayed in reaction with 3'AMP at pH 6.0. Simultaneously, this enzyme was found to exert no 5'nucleotidase activity.

Antitumorigenic activity and embryotoxicity of TBN1 in comparison with other selected nucleases and BS-RNase. In further experiments, antiproliferative activities of purified recombinant tomato nuclease, as well as TBN1 stabilized by conjugation with polyethylene glycol (TBN1+PEG) were examined. It was found that TBN1 caused strong inhibition of the tumor proliferation *in vivo.* These effects were similar to BS-RNase, however, they were achieved at about ten times lower doses (Table 1). TBN1 after its one intratumoral administration (12-15 μg per injection) into melanoma tumors (Table 1) and TBN1+PEG after their intravenous administration (seven injections) to athymic mice bearing melanoma and/or prostate tumors exerted reductions in the tumor growth by more than 60% and 90%, respectively. The degradation of these tumors was accompanied with certain body weight losses of the injected mice (Table 1, Fig. 3). These results showing strong suppression of *in vivo* tumorigenesis with TBN1 nuclease suggest a high stability of TBN1 as a potential cytostatic. Immunofluorescence microscopic analyses of melanoma tumors 6 hours post injection of TBN1 showed nuclease in tumor cells (Fig. 4, compare panels 1-3). In these image analyses, strong immunofluorescence reaching the integral brightness of 1.05×10^6 was counted per cell, whereas about 19 % of integral brightness level was detected as an unspecific signal (Fig. 4, panel 3). Light microscope analyses of tumors subjected to direct nuclease action revealed degeneration and apoptotic bodies [35] at the midstage (Fig. 5, panel 2) and a broad fibrocytic degeneration (Fig. 5, panel 3), suggesting tissue and cell degenerative processes caused by direct intratumoral injection of TBN1. The analysis using fluorescence-labelled PEG clearly localised TBN1+PEG complex signal (Fig. 4, compare panels 4 and 5) and confirmed TBN1 delivery to melanoma tumors after its intravenous application. Despite dramatic inhibition of tumorigenesis by TBN1+PEG (Fig. 3), microscopy analysis did not reveal histological differences between control and af-

Table 1. Growth of human melanoma (M) and prostatic carcinoma (PC) in athymic mice after intratumorally (i.t.)or intravenously (i.v.) injections of tomato nuclease (TBN1) in comparison with *Phaseolus aureus nuclease (PhA), Pinus nigra pollen nuclease (PN) and bovine seminal ribonuclease (BS-RNase)*

Substances injected (μg i.t. or i.v.) on melanoma (M) and prostatic carcinom (PC)	Number of		Tumor volume after injections (cm^3)	Percent loss of tumor volume compared with control (%)	Degree of tumor degeneration (D)	Loss (-) and addition (+) of body mass (in g) after the injections
	Mice	injections				
PBS (control) (- i.t.) M	5	1	2.41 ± 0.4	0	0	+ 2
BS-RNase (100 – i.t.) M	5	1	0.52 ± 0.07	79	4	- 3
PhA nuclease (10 – i.t.) M	5	1	0.83 ± 0.04	67	4	0
PN nuclease (10 – i.t.) M	5	1	1.16 ± 0.29	54	3	0
TBN1 nuclease (12 – i.t.) M	4	1	0.96 ± 0.14	60	3	- 3
TBN1+PEG nuclease (15 – i.v.) M	5	7	0.74 ± 0.05	70	4	- 2
TBN1+PEG nuclease (15 – i.v.) PC	4	7	0.24 ± 0.09	90	4	- 1

Degree of tumor degeneration (D) = 0% without degeneration, 3 – from 40% to 70%, 4 – from 70% to 100%

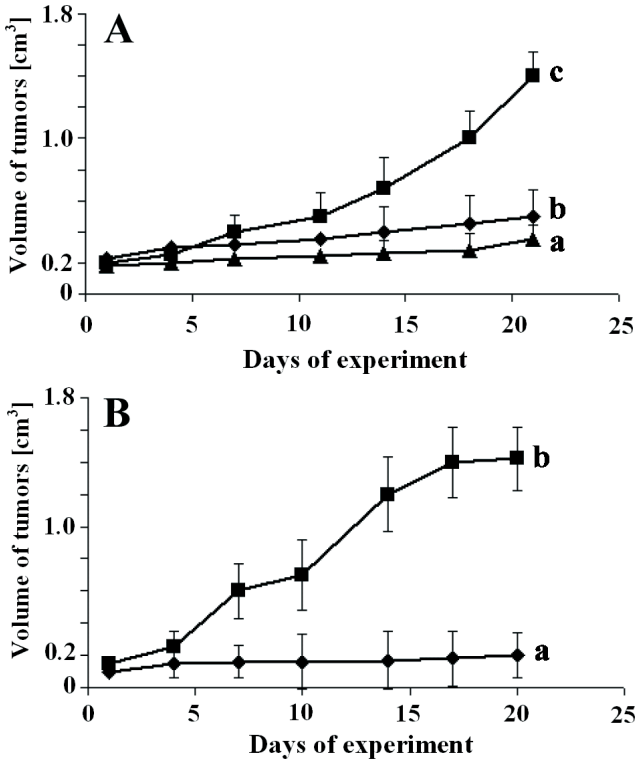

Fig 3 A: Human melanoma tumor growth in athymic nu/nu mice after intravenous administration of TBN1 (a) and BS-RNase (b) conjugated with PEG. Athymic nude mice were injected 7 times over a period of 21 days at the doses of 15 μg of TBN1+PEG. Tumor volume was determined twice a week. PBS buffer was administrated in controls (c) B: Human prostate tumor growth in athymic nu/nu mice after intravenous administration of TBN1 conjugated with PEG (a) or PBS buffer (b). Athymic nu/nu mice were injected 7 times over a period of 20 days at the doses 15 μg of nuclease.

fected (significantly reduced in size) tumors, i.e. no pronounced degenerative processes in these tumors were observed like after direct TBN1 aplication by intratumoral injections (compare Fig 4, panel 6 A and B with Fig. 5). Results of RT real-time PCR analysis of melanoma markers PLAB and L1CAM normalized against expression of HMBS internal reference transcript did not reveal any significant decrease of their mRNA levels in melanoma affected by intravenous application of TBN1+PEG when compared to control sample (not shown). This suggests that the real-time PCR analysis was in accordance with histological analysis.

In order to estimate a potential cytotoxicity of TBN1, its embryotoxicity in comparison with other nucleases were examined. The embryotoxic effect of TBN1 nuclease was tested after addition of nucleases to medium used for early mouse embryos culture at doses comparable to that administered to inhibit tumorigenesis. Final parameters such as the number of degenerated embryos and embryo splitting were estimated (Table 2). As can be judged from these results, the TBN1 embryotoxicity was lower compared to those observed with all the other enzymes examined (Table 2). In all the events, the embryotoxic activity of TBN1 nuclease was also not as strong as that of onconase that we described earlier [11].

The results presented make it possible to conclude that recombinant TBN1 shows high cytotoxicity for tumors, but lower obryototoxic effects in comparison with BS-RNase and other nucleases analyzed.

Discussion

1. Apoptotic nuclease TBN1 as a perspective anticarcinogenic agent. Apoptotic tomato nuclease, which was analyzed in the

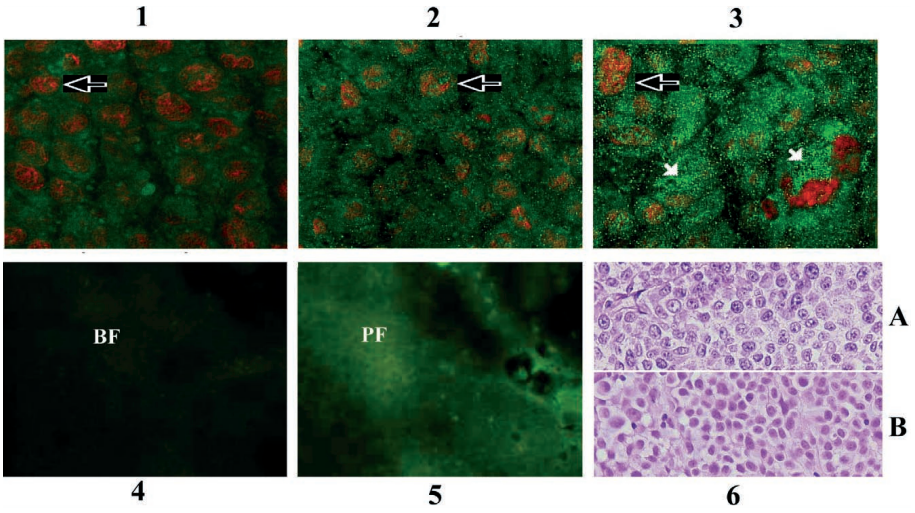

Fig. 4 A: Image analysis of immunofluorescence signal of TBN1 nuclease six hours post injection in developing human melanoma tumors (1-3); immunofluorescence (4-5) and histological analysis (6) of human melanoma after intravenous TBN1+PEG administration. Cryomicroscopical analysis and immunodetection were performed as described in Material and Methods, red-labelled nuclei are indicated by black arrows, specific TBN1 immunocomplexes in panel 3 are seen as bright green signals indicated by white arrows. 1, the control sample with secondary antibody but without anti-TBN1 IgG; 2, the control sample with anti-TBN1 IgG, 3, the tissue sample after nuclease injection and cross-reaction with anti-TBN1 IgG, 4, background fluorescence signal (BF); 5, fluorescence from TBN1-PEG complexres (PF); 6, histology analysis of control (A) and TBN1+PEG-affected (B) melanoma tissues stained with hematoxylin-eosin.

Table 2. Development of mice embryos after 72 hours incubation of tomato bifunctional nuclease (TBN1) in comparison with bovine seminal ribonuclease, mung bean nuclease (PhA) and black pine pollen nuclease (PN).

Enzymes species added to medium used for embryos culture (μg/ml)	No. of mice embryos	Number of embryos in cell stages				Embryous splitting	
		Expanded blastocyst	Blastocyst	4-8 cell embryos	No. of degenerated embryos	No.	%
Control	16	6	3	1	6	10	62
BS-RNase (100)	12	0	0	0	12	0	0^{++}
PhA (10)	19	0	3	6	10	9	47^{+}
PN (10)	17	0	1	4	12	5	30^{++}
TBN1 (10)	16	1	4	4	7	7	56

$^{+}P < 0.05$, $^{++}P < 0.01$

present study, belongs to a group of widely spread major plant endonucleases [36]. These enzymes show a tissue- or organ-specific expression and existence of multiple isoforms, which are strongly regulated during different developmental processes [17]. In addition to our previous studies, where we described anticarcinogenic properties of Mung bean nuclease [18] and pine pollen extracellular nuclease [19], both from natural plant

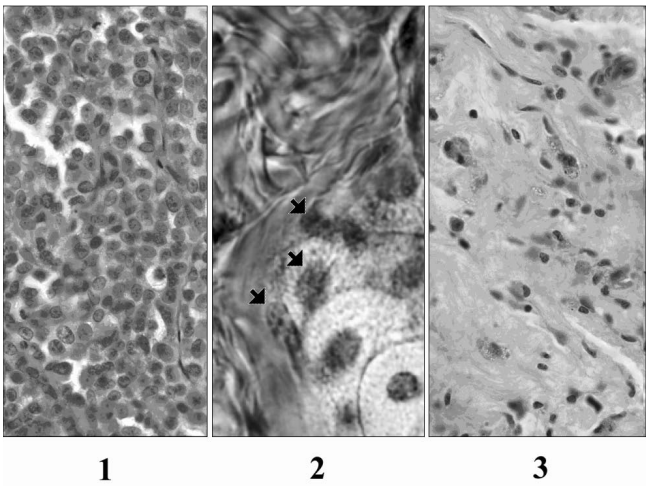

Fig. 5 Light microscope observation of tumor degeneration after intratumoral application of tomato apoptotic nuclease. Control melanoma tumor (1), tumor tissues in midstage of degeneration after application of TBN1 nuclease (apoptotic bodies are indicated by arrows) and complete degeneration of tumor tissue (3). Tissue was collected by the end of 20-day experiment, fixated and stained with hematoxylin-eosin. Control tumors are without substantial degeneration, while a broad fibrocytic degeneration is seen in tissue after nuclease injection. Magnification 400x.

sources, in this study we employed for the first time *in planta* biotechnology for the production of recombinant tomato nuclease. TBN1 was produced in a closely related species belonging to the same family *Solanaceae*, *N. benthamiana*. According to our results, recombinant TBN1 appeared to be modified post-translationally by N-glycosylation and showed electrophoretic pattern similar to that described for natural TBN1 expression in tomato [25]. In addition, recombinant nuclease was found to be correctly processed by the cleavage of signal peptides at amino acid position as predicted earlier from sequence comparisons [25]. It is obvious that *in planta* biotechnology described here enables preparation of stably expressed sequence-specified antitumorigenic nuclease under standard conditions. Standardized conditions belong to requirements necessary for the production of plant-derived pharmaceuticals [3].

In our recent experiments we compared tomato bifunctional nuclease with non-recombinant black pine and commercial Mung been nucleases, for which we previously described some anticarcinogenic activity [18, 19]. Antitumorigenic effects of TBN1 *in vivo* on human melanoma tumors growing on athymic mice were very significant and comparable to PhA and PN nucleases. TBN1, PhA and PN nucleases intratumorally injected into melanoma tumors caused almost the same antitumor degenerative effects as observed previously after the administration of BS-RNase and wheat leaf ribonuclease (WLN-RNase) [15]. However, in the case of BS-RNase, ten-fold amount of protein was needed to achieve the same effects. Similarly to the results with WLN-RNase, the antitumor activity of TBN1 was also proved by intravenous injection of this enzyme conjugated with PEG to mice with prostate carcinoma. Immunofluorescence experiments clearly suggest affinity of TBN1+PEG for cancer cells, where specific signal was localized similarly as detected after intratumoral injections. It is not known, whether or not the TBN1 antitumorigenic activity could involve a broad spectrum of malignant tumors. According to our unpublished results, in addition to the anticarcinogenic effect detected in melanoma and prostate carcinoma, the proliferation of neuroblastoma was also efficiently blocked by the application of TBN1+PEG.

Spermatogenic and embryonic cells are very effective markers for studying the toxic and side effects of various substances administered to animals. In our experiments, a lower toxicity of TBN1 nuclease to embryonic cells was observed in comparison with BS-RNase and onconase [11]. These results are promising for further studies of this and other plant nucleases given their possible lower side effects.

*2. Essential biochemical properties of in planta produced recombinant nuclease in relation to its antitumorigenic and biological effects***.** In this study, a purified recombinant TBN1 nuclease was found as a potential antitumorigenic agent with significant molecular stability including thermostability to cause specific biological effects. Similarly to biologically active BS-RNase [37], the structure of plant nuclease I is stabilized by polypeptide chain cross-linking with two disulfide bonds. This structural feature is probably one of the factors contributing to the biochemical and biological stability and activity of the nuclease I. This feature is also essential for the conformation of the active centre and for a strong single-strand specificity of fungal S1/P1 nucleases catalytically related to nuclease I [38]. Another important feature of TBN1 that could contribute to its structural stability and biological activity is its obvious postranslational N-glycosylation. From calculations based on the predicted primary sequence [25] we assume that mature TBN1 should contain about 12% of sugar oligomers. It is important to note in this respect that N-glycosylation of onconase significantly increased its conformational stability and cytotoxic properties [39].

All cytotoxic animal RNases investigated, showed a catalytic activity, whereas catalytically non-active variants essentially did not show any biological and antitumorigenic effects [7]. For instance, direct connection between the structural flexibility of the catalytic centre, catalytic activity and cytotoxicity were recently described for onconase by Lee [40]. The antitumorigenic activity and catalytic activity *per situ* depend on many factors including the presence of specific inhibitors [41, 42]. Approximately 10-times higher concentration of BS-RNase was required to achieve the same anticarcinogenic effect like TBN1 and other plant nucleases [18, 19]. This difference could be explained by insensitivity of plant nuclease I to endogenous levels of RNase and nuclease inhibitors specific for animal and human systems [43, 44]. According to our unpublished results, TBN1 is insensitive to RNase inhibitor (RI) [44] representing a widely spread type of ribonuclease inhibitor in animals. Hundred times lower sensitivity of P1 nuclease to animal RI was reported by Saxena et al. [45].

According to our results, recombinant TBN1 is a degradative endonuclease with capability of hydrolyzing thermodynamically stable RNA structures, circular, as well as double-stranded RNAs. It readily cleaved *in vitro* all RNA components of human ribosomes, as well as RNA component of signal recognition particles that it could target. Ribosomal 28S RNA was described as a target of BS-RNase [46]. According to our unpublished results, tRNA, a putative primary target of onconase [40], is also readily degraded by TBN1. In addition, significant dsRNase activity of TBN1 could interfere with intracellular processes of PTGS or affect regulation by noncoding RNAs [47] as micro RNA-mediated regulation in the stroma. The disruption of regulatory processes connected to stroma and to tumor "nutrition" could be the major mechanism mediating strong inhibition of tumor proliferation after intravenous TBN1+PEG application. As the sugar non-specific nucleases TBN1 and anticarcinogenic plant nucleases described previously [18, 19] could, in principle, target genomic or mitochondrial DNA, providing nuclear or intramitochondrial transportation of these enzymes and DNA accessibility. In addition to RNase and DNase activities of TBN1 and related nucleases, 3'nucleotidase activity could play some role in the dephosphorylation of some nucleoproteins or receptors. Despite this broad degrading potential, possible primary target(s) of TBN1 leading to its anticarcinogenic action and to specific degenerative processes remain to be determined.

Acknowledgements. The authors would like to thank Ing. Lidmila Orctová, Ing. Olga Horáková and Helena Matoušková from Biological Centre AS CR v.v.i., Institute of Plant Molecular Biology for excellent technical assistance. Authors would like to thank Dr. Zdeněk Voburka from Institute of Organic Chemistry and Biochemistry, AS CR v.v.i. Prague for N-terminal protein determination. We thank Dr. Ján Strnadel and Dr. Vratislav Horák from Institute of Animal Physiology and Genetics, AS CR v.v.i., Liběchov for their help in antibody preparation and cryomicroscopy. The same thanks belong to Václav Pech and Libuše Koberová from Liběchov Institute for histology preparations and administrative help. The authors wish to thank also to Dr. Marie Zadinová and Daniela Hloušková from the 1st Medical Faculty of Charles University Prague for their help with antitumor testing. The work was supported predominantly by grant of GAČR 521/06/1149 and 521/09/1214, by AV0Z50510513, I RP I APG no. 50450515 projects, by scientific purpose MSM 0021620808 and MSM6046137305, by the grant of Ministry of Health of the Czech Republic No 00023736 and by the grant of League against Cancer, Prague

References

[1] RASKIN I, RIBNICKY DM, KOMARNYTSKY S, ILIC N, POULEV A et al. Plants and human health in the twenty-first century. Trends Biotechnol 2002; 20: 522–531. doi:10.1016/S0167-7799(02)02080-2

[2] BALL P Natural strategies for the molecular engineer. Nanotechnol 2002; 13: 15-R28. doi:10.1088/0957-4484/13/5/201

[3] STREATFIELD SJ, HOWARD JA Plant-based vaccines. Int J Parasitol 2003; 33: 479–493. doi:10.1016/S0020-7519(03)00052-3

[4] PUJOL M, GAVILONDO J, AYALA M, RODRÍGUEZ M, GONZÁLEZ EM et al. Fighting cancer with plant-expressed pharmaceuticals. Trends Biotechnol 2007; 25: 455–459. doi:10.1016/j.tibtech.2007.09.001

[5] MATOUŠEK J Ribonucleases and their antitumor activity. Comp Biochem Physiol C 2001; 129: 175–191.

[6] MAKAROV AA, ILINSKAJA ON Cytotoxic ribonucleases: molecular weapons and their targets. FEBS Lett 2003; 540: 15–20. doi:10.1016/S0014-5793(03)00225-4

[7] BENITO A, RIBÓ M, VILANOVA M On the track of antitumor ribonucleases. Molecular BioSystems 2005; 1: 294–302. doi:10.1039/b502847g

[8] ARNOLD U, ULBRICH-HOFMAN R Natural and engineered ribonucleases as potential cancer therapeutics. Biotechnol Lett 2006; 28: 1615–1622. doi:10.1007/s10529-006-9145-0

[9] RAMOS-NINO ME Cytotoxic ribonuclease-based cancer therapies. Drugs Fut 2007; 32: 517–526. doi:10.1358/dof.2007.032.06.1110573

[10] FAVARETTO A Overview on ongoing or planned clinical trials in Europe. Lung Cancer 2005; 49: S117–S121. doi:10.1016/j.lungcan.2005.03.022

[11] MATOUŠEK J, SOUČEK J, SLAVÍK T, TOMÁNEK M, LEE EJ et al. Comprehensive comparison of the cytotoxic activities of onconase and bovine seminal ribonuclease. Comp Biochem Physiol C 2003; 136: 343–356.

[12] LAM SK, NG TB Isolation of a novel thermolabile heterodimeric ribonuclease with antifungal and antiproliferative activities from roots of the Sanchi ginseng Panax notoginseng. Biochem Biophys Res Commun 2001; 285: 419–423. doi:10.1006/bbrc.2001.5193

[13] XIA HC, FENG LI, ZHEN LI, ZHANG ZC Purification and Characterization of Moschatin, a novel type I ribosome-inactivating protein from the mature seeds of pumpkin (Cucurbita moschata) and preparation of its immunotoxin against human melanoma cells. Cell Res 2003; 13: 369–374. doi:10.1038/sj.cr.7290182

[14] XU H, HE WJ, LIU WY A novel ribotoxin with ribonuclease activity that specifically cleaves a single phosphodiester bond in rat 28S ribosomal RNA and inactivates. Arch Biochem Biophys 2004; 427: 30–40. doi:10.1016/j.abb.2004.04.018

[15] ŠKVOR J, LIPOVOVÁ P, POUČKOVÁ P, SOUČEK J, SLAVÍK T et al. Effect of wheat leaf ribonuclease on tumor cells and tissues. Anti-Cancer Drugs 2006; 17: 815–823. doi:10.1097/01.cad.0000217430.75078.cc

[16] NAWROT R, WOLUN-CHOLEWA M, GOZDZICKA-JOZEFIAK A Nucleases isolated from Cheliodonium majus L. milky sap can induce apoptosis in human cervical carcinoma HeLa cells but not in chinese hamster ovary CHO cells. Folia Histol Cytobiol 2008; 46: 79–83. doi:10.2478/v10042-008-0011-x

[17] WILSON CM Plant nucleases: biochemistry and development of multiple molecular forms. In: Rattazi MC, Scandalios JG, Whitt GS, eds. Isozymes: Current Topics in Biological and Medical Research, Vol. 6; New York: Alan R. Liss, 1982: 33–54.

[18] SOUČEK J, ŠKVOR J, POUČKOVÁ P, MATOUŠEK J, SLAVÍK T et al. Mung bean (Phaseolus aureus) sprout nuclease and its biological and antitumor effects. Neoplasma 2006; 53: 402–409.

[19] LIPOVOVÁ P, PODZIMEK T, ORCTOVÁ L, MATOUŠEK J, POUČKOVÁ P et al. Antitumor and biological effects of black pine (Pinus nigra) pollen nuclease. Neoplasma 2008; 55: 158–164.

[20] PEREZ-AMADOR MA, ABLER ML, DE ROCHER J, THOMPSON DM, VAN HOOF A et al. Identification of BFN1, a bifunctional nuclease induced during leaf and stem senescence in Arabidopsis. Plant Physiol 2000; 122: 169–180. doi:10.1104/pp.122.1.169

[21] MURAMOTO Y, WATANABE A, NAKAMURA T, TAKABE T Enhanced expression of a nuclease gene in leaves of barley plants under salt stress. Gene 1999; 234: 315–321. doi:10.1016/S0378-1119(99)00193-6

[22] ZAINA G, MORASSUTTI C, DE AMICIS F, FOGHER C, MARCHETTI S Endonuclease genes up-regulated in tissues undergoing programmed cell death are expressed during male gametogenesis in barley. Gene 2003; 315: 43–50. doi:10.1016/S0378-1119(03)00820-5

[23] AOYAGI S, SUGIYAMA M, FUKUDA H BEN1 and ZEN1 cDNAs encoding S1-type DNase that are associated with programmed cell death in plants. FEBS Lett 1998; 429: 134–138. doi:10.1016/S0014-5793(98)00563-8

[24] PANAVAS T, PIKULA A, REID PD, RUBINSTEIN B, WALKER EL Identification of senescence-associated genes from daylily petals. Plant Mol Biol 1999; 40: 237–248. doi:10.1023/A:1006146230602

[25] MATOUŠEK J, KOZLOVÁ P, ORCTOVÁ L, SCHMITZ A, PEŠINA K et al. Accumulation of viroid-specific small RNAs and increase of nucleolytic activities linked to viroid-caused pathogenesis. Biol Chem 2007; 388: 1–13. doi:10.1515/BC.2007.001

[26] MATOUŠEK J, ORCTOVÁ L, ŠKOPEK J, PEŠINA K, STEGER G Elimination of hop latent viroid upon developmental activation of pollen nucleases. Biol Chem 2008; 389: 905–918. doi:10.1515/BC.2008.096

[27] HOLSTERS M, DE WAELE D, DEPICKER A, MESSENS E, VAN MONTAGU M et al. Transfection and transformation of Agrobacterium tumefaciens. Mol Gen Genet 1978; 163: 181–187. doi:10.1007/BF00267408

[28] VOINNET O, RIVAS S, MESTRE P, BAULCOMBE D An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J 2003; 33: 949–956. doi:10.1046/j.1365-313X.2003.01676.x

[29] MATOUŠEK, J Aspermatogenic effect of the bull seminal ribonuclease (BS-RNase) in the presence of anti BS-RNase antibodies in mice. Anim Genet 1994; 25: 45–50.

[30] MATOUŠEK J, TRNĚNÁ L, SVOBODA P, ORINIAKOVA P, LICHTENSTEIN CP The gradual reduction of viroid levels in hop mericlones following heat therapy: A possible role for a nuclease degrading dsRNA. Biol Chem Hope Seyler 1995; 376: 715–721.

[31] MATOUŠEK J, TURKOVÁ V. Some immunochemical properties of pollen extracellular nuclease. Biol Plant 1987; 29: 401–408. doi:10.1007/BF02882205

[32] POUČKOVÁ P, ZADINOVÁ M, HLOUŠKOVÁ D, STROHALM J, PLOCOVÁ D et al. Polymer-conjugated bovine pancreatic and seminal ribonucleases inhibit growth of human tumors in nude mice. J Control Release 2004; 95: 83–92. doi:10.1016/j.jconrel.2003.11.008

[33] TALANTOV D, MAZUMDER A, YU JX, BRIGGS T, JIANG Y et al. Novel genes associated with malignant melanoma but not benign elanocytic lesions. Clin Cancer Res 2005; 11: 7234–7242. doi:10.1158/1078-0432.CCR-05-0683

[34] MATOUŠEK J, GOTTE G, POUČKOVÁ P, SOUČEK J, SLAVÍK T et al. Antitumor activity and other biologic actions of oligomers of ribonuclease A. J Biol Chem 2003; 278: 23817–23822. doi:10.1074/jbc.M302711200

[35] CINATL JJR, CINATL J, KOTCHETKOV R, VOGEL JU, WOODCOCK BG et al. Bovine seminal ribonuclease selectivley kills human multidrug-resistant neuroblastoma cells via induction of apoptosis. Internat J Oncol 1999; 15: 1001–1009.

[36] WILSON CM Plant nucleases. Annu Rev Plant Physiol Plant Mol Biol 1975; 26: 187–208.

[37] D'ALESSIO G, RIORDAN JF Ribonucleases: Structures and Functions. San Diego: Academic Press, 1997.

[38] ROMIER C, DOMINGUEZ R, LAHM A, DAHL O, SUCK D Recognition of single-stranded DNA by nuclease P1: high resolution crystal structures of complexes with substrate analogs. Proteins 1998; 32: 414–424. doi:10.1002/(SICI)1097-0134(19980901)32:4<414::AID-PROT2>3.0.CO;2-G

[39] KIM B-M, KIM H, RAINES RT, LEE Y Glycosylation of onconase increases its conformational stability. Biochem Biophys Res Commun 2004; 315: 976–983. doi:10.1016/j.bbrc.2004.01.153

[40] LEE JE, BAE E, BINGMAN CA, PHILLIPS JR GN, RAINES RT Structural basis for catalysis by onconase. J Mol Biol 2008; 375: 165–177. doi:10.1016/j.jmb.2007.09.089

[41] ILINSKAYA ON, MAKAROV AA Why ribonucleases induce tumor cell death. Mol Biol 2005; 39: 3–13.

[42] RODRÍGUEZ M, TORRENT G, BOSCH M, RAYNE F, DUMBREMETZ JF et al. Intracellular pathway of onconase that enables its delivery to the cytosol. J Cell Sci 2007; 120: 1405–1411. doi:10.1242/jcs.03427

[43] SHAPOT VS, CHUDINOVA IA, KRECHETOVA GD, PUSHKINA I Nuclease inhibitor from the nuclear sap of liver and hepatoma cells. FEBS Lett 1971; 13: 13–16. doi:10.1016/0014-5793(71)80653-1

[44] BLACKBURN P, WILSON G, MOORE S Ribonuclease inhibitor from human placenta: purification and properties. J Biol Chem 1977; 252: 5904–5910.

[45] SAXENA SK, RYBAK SM, WINKLER G, MEADE HM, McGRAY P et al. Comparison of RNases and toxins upon injection into Xenopus oocytes. J Biol Chem 1991; 266: 21208–21214.

[46] MASTRONICOLA MR, PICCOLI R, D'ALESSIO G Key extracellular and intracellular steps in the antitumor action of seminal ribonuclease. Eur J Biochem 1995; 230: 242–249. doi:10.1111/j.1432-1033.1995.tb20557.x

[47] ZARATIEGUI M, IRVINE DV, MARTIENSSEN RA Noncoding RNAs and Gene Silencing. Cell 2007; 128: 763–776. doi:10.1016/j.cell.2007.02.016