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Approaches to Endow Ribonucleases with Antitumor Activity: Lessons Learned from the Native Cytotoxic Ribonucleases

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Abstract

Typical antitumor drugs disrupt the flow of biochemical information from DNA to proteins with the aim of precluding uncontrolled cell proliferation and inducing cancer cell apoptosis. However, most of the currently used small antitumor drugs are genotoxic because they act over DNA. Pharmaceutical industry is now searching for a new line of cancer chemotherapeutics without genotoxic effects. Ribonucleases (RNases) are small basic proteins, present in all life forms, which belong to this kind of chemotherapeutics. Some of them present with remarkable selective antitumor activity linked to their ability to destroy RNA, a powerful way to control gene expression, leaving DNA unharmed. In the last two decades, the knowledge gained on the cytotoxic mechanism of these RNases has been used to engineer more powerful and selective variants to kill cancer cells. In this chapter, we describe the advances reached in endowing an RNase with antitumor abilities.

Keywords: ribonucleases, antitumor activity, protein engineering, mechanism of anti-tumor action, delivery

1. Introduction

In their review, Hanahan and Weinberg [1] described ten hallmarks of cancer cells: genome instability and mutation, sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, tumor-promoting inflammation,

inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction. Involved in these metaprocesses, there is a deregulation of gene expression. A significant part of the current chemotherapeutic compounds used to treat cancer patients target different over- or under-expressed genes that take part in the abovementioned processes that drive to malignant cell transformation and/or metastasis.

1.1. Why target RNA to treat cancer diseases?

Control of gene expression can be carried out at different levels in the flow of genetic information from DNA to proteins (**Figure 1**).

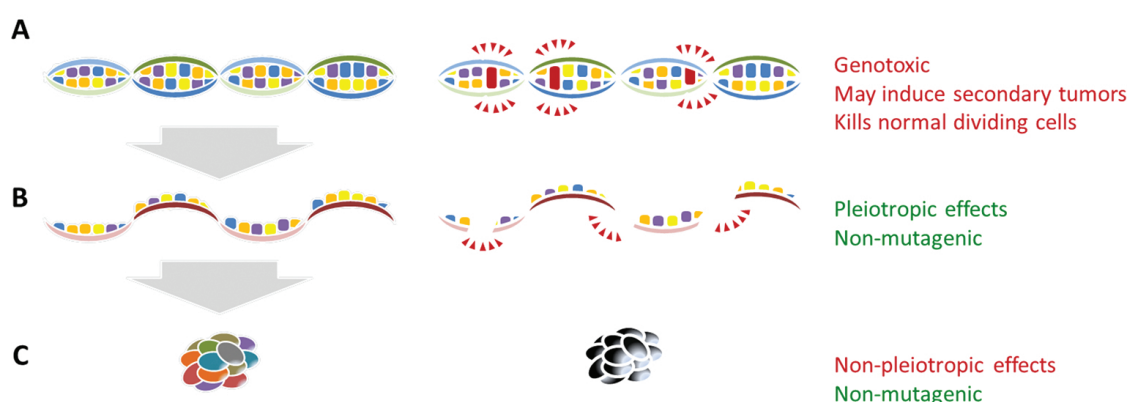


Figure 1. Targets of genotoxic and non-mutagenic antitumoral drugs along the information pathway. Transmission of biological information in tumor cells occurs from DNA to RNA and proteins that exert their biological function. (A) Classical antitumor therapies like radiotherapy and chemotherapy affect DNA inhibiting cell replication but may also kill normal dividing cells, and since they are genotoxic, they may induce secondary tumors. (B) Alternative damaging RNA therapies inhibit gene expression and its regulation. These therapies exert pleiotropic effects because they affect multiple RNA substrates and are not mutagenic. (C) Therapies affecting a single protein or pathway of the cell are highly specific but sometimes cannot cope with the multifactorial nature of cancer although are also non-mutagenic.

Drugs that act over DNA have the drawback of being mutagenic and are responsible for the appearance of new cancers, time after the patients have been cured of or controlled their first cancer disease [2]. Instead, drugs that destroy or inactivate RNA are similarly powerful without the associated risk of genotoxicity. In addition, drugs that specifically target a single protein or pathway have the advantage of being highly specific, but they are often insufficient to cope with the multifactorial complexity of the cancer phenotype. Several approaches are used to target RNAs, the use of antisense oligonucleotides, small interfering RNA (siRNAs), and the use of ribozymes or proteins with ribonucleolytic activity [3]. In the present chapter, we will focus on ribonucleases (RNases) as antitumor agents and how the knowledge gained so far about their mechanism of action has inspired researchers in the design of more powerful and selective RNases that can overcome tumor resistance as well as minimize the toxic effects to normal cells, properties strongly desired for any antitumor drug.

RNases are enzymes present in all life kingdoms that degrade RNA and in cells are responsible for RNA turnover [4]. Their interest as antitumor agents started early in the fifties of the last

century when the bovine pancreatic ribonuclease (RNase A) demonstrated to have antitumor activity both *in vitro* and *in vivo* [5–10] although with contradictory results [11]. This interest vanished until the discovery of non-engineered RNases with natural anticancer activity when used at much lower concentrations than RNase A. Among them we can find prokaryotic and eukaryotic RNases [12], from microbe [13, 14], plants [15], or vertebrates. The latter belong to the known vertebrate-secreted ribonuclease family [16] from which RNase A is the paradigm [17]. Recently, in animal models, even RNases that natively are not cytotoxic, like RNase A, are shown to have antimetastatic properties when ultralow doses are administered everyday. It is suggested that this effect is related to its ability to degrade circulating noncoding RNAs assuming that in blood plasma the enzyme is not inhibited (see below) [18].

1.2. What makes a ribonuclease selectively cytotoxic for cancer cells?

Although some RNases have reached clinical trials for treatment of different types of cancer [19–25], their mechanism of action is not well understood. Nevertheless, RNases share some steps of cell intoxication with most cytotoxins.

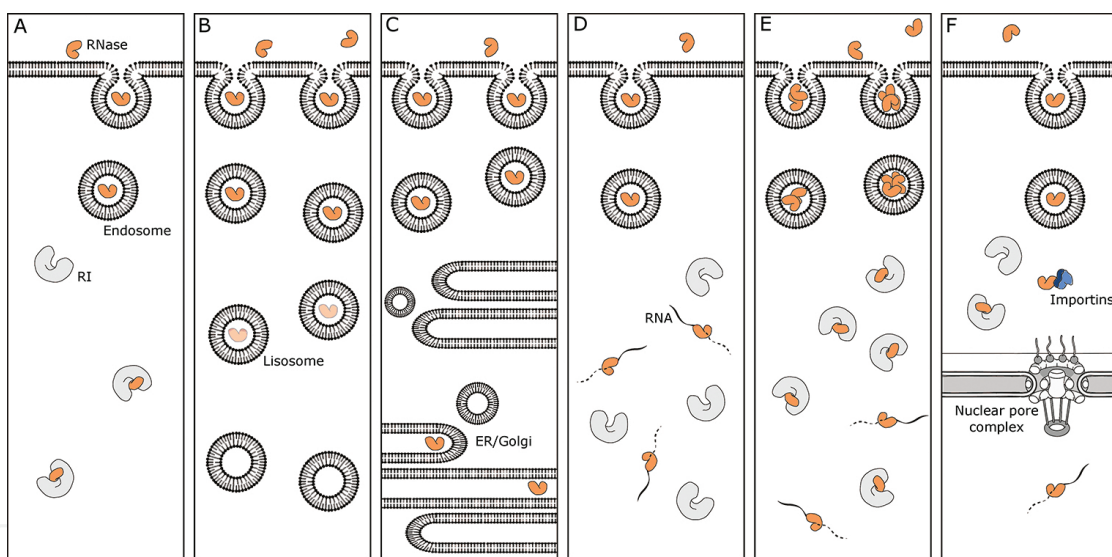


Figure 2. Multifactorial causes of RNase cytotoxicity. Some RNases are able to reach the cytosol but are not cytotoxic because they are unable to evade the action of the RI (A). Other RNases are not cytotoxic because they cannot reach the cytosol, either because they are degraded during its internalization (B) or because they follow an intracellular pathway that does not allow them to reach this compartment (C). Some RNases are cytotoxic because they reach the cytosol and are not inhibited by the RI (D). Other RNases are cytotoxic although they do not evade the RI either because they reach the cytosol with high efficiency allowing to saturate the RI present in the cytosol (E) or because they can reach the nucleus where the RI cannot inhibit them (F).

To be cytotoxic an RNase has to reach the tumor cells. This implies two basic steps: to attain target cells from the administration point (RNases are mainly administered *i.v.*) and to be able to enter these cells. The first step means that the RNase has to be stable enough in blood to reach their target cells and not to be cleared rapidly from circulation through glomerular filtration. The second step [26–28] implies an interaction with a specific or a nonspecific component of the target cell surface in order to be endocytosed. Then, during its journey, at

some point of the endocytic pathway (**Figure 2B**), the RNase has to translocate to the cytosol to avoid lysosomal degradation and, obviously, follow a productive endocytic pathway. Once in the cytosol, it has to be stable and resistant to proteases, and at the same time, it has to evade the ribonuclease inhibitor (RI) to preserve its ribonucleolytic activity and therefore be able to degrade RNAs and induce cell death by apoptosis. The RI is a protein present in the cytosol of mammalian cells that binds to some RNases with high affinity [29]. It is hypothesized that the RI acts as a safeguard for the potential entry of any external RNase [30]. Alternatively to the evasion strategy, an RNase can also have the ability to enter the cell very efficiently to saturate the RI and to leave free RNase molecules able to degrade RNAs. Finally, an RNase can also be driven to any organelle devoid of RI where it can degrade RNAs, for instance, the cell nucleus [31] (**Figure 2F**).

The paradigmatic native cytotoxic RNase that evades the RI is onconase (ONC), a member of the vertebrate-secreted RNase family of amphibian origin (isolated from oocytes and early embryos of *Rana pipiens*). ONC reached phases II/IIIb for treatment of malignant pleural mesothelioma [21] although it presents renal toxicity that is reversed when the treatment is discontinued [32]. It exhibits selective cytostatic and cytotoxic activities against many tumor models both *in vitro* and *in vivo* [22, 33] and presents synergy, proved also *in vivo* and *in vitro*, with a significant number of compounds [34]. ONC induces apoptosis or in some cases autophagy previously to apoptosis [35, 36]. These processes present characteristics different from those of indiscriminate protein synthesis arrest and are due to the degradation of different target RNAs, rRNAs [37], mRNAs [38], tRNAs [39], and miRNAs or their precursors [40–42]. It has been described that ONC up- or downregulates genes that code for proteins involved in cell cycle control or transcription factors that are also responsible for its cytotoxicity [43]. Although from the literature the apoptotic effects seem to be cell-type dependent [34], recently it has been found that the activating transcription factor 3 (ATF3) controls ONC-induced apoptosis in a cell-type independent manner (Vert et al., submitted). Other tumoricidal amphibian RNases are Amphinase (Amph), also isolated from oocytes of *R. pipiens* [44] and the sialic acid-binding lectins (leczyms) found in *Rana catesbeiana* (RC-RNase) and *Rana japonica* (RJ-RNase) oocytes [45, 46]. Unlike ONC and Amph, these latter ones agglutinate cancer cells [47–49] binding to cell membrane glycoproteins with a high content of sialic acids [47, 49]. It is also proposed, like for ONC and Amph, that these leczyms require an internalization process to trigger apoptosis [50]; however compared to them, clinical trials and studies on animal models are needed to unveil their mechanism of antitumor activity and clinical potential.

The critical process of ONC internalization is still an open question. This is not a minor issue because it is strongly related to the RNase cytotoxic selectivity for cancer cells. For ONC it has been described both the existence of a specific receptor [37] and an entry through a non-saturable process [51] as well as an entry through the clathrin/AP-2-mediated endocytic pathway [52] and a non-dynamin-dependent pathway [51]. These discrepancies may be explained by the model cell lines used in the different works. In addition, electrostatics are described as necessary for the cellular uptake of ONC, while for other RNases, an specific interaction with cell surface structures seems to contribute more decisively to their internali-

zation [53]. Essentially, RNases are cationic proteins, and since the surface of most cancer cells is more electronegative [54] than that of normal cells, the electrostatic interactions that they establish may dictate their selectivity. Very recently, both RNase A and its human counterpart, the human pancreatic ribonuclease (HP-RNase), have been described to interact with a neutral hexasaccharide glycosphingolipid, Globo H [55], a component of a glycolipid or a glycoprotein located on the outer membrane of epithelial cells and detected in high levels in the outer membrane of several tumor cells [56]. The authors suggest that this interaction is not only substantial for the internalization of these RNases but for their release from the lumen of endosomes allowing for the access to the cytosol [55], although if they are not engineered to avoid the RI, they are not cytotoxic (see below). In addition, for RNase A, it has been described a multipathway of internalization that involves both clathrin-coated vesicles and macropinosomes [57]. Finally, through an *in silico* study by sliding-window hydrophobicity analysis, it has been hypothesized that some cytotoxic RNases have a hydrophobic segment sterically available for a hydrophobic interaction with both tumor cell and endosomal membranes that would facilitate their internalization [58]. The more it is known about the membrane structures that are recognized by RNases or the productive pathway, by which they enter the cell, the better they can be engineered to increase their selectivity and potency.

Another RNase that naturally shows antitumor activity by RI evasion is bovine seminal ribonuclease (BS-RNase), present in the bull seminal plasma. In this case the quaternary structure attained by this enzyme is responsible for its low RI affinity due to steric hindrance, while the monomeric form is strongly inhibited by the RI [59]. BS-RNase exists as a mixture of two dimeric forms, M=M and MxM, each monomer being a structural homolog of RNase A [60]. The MxM dimer exchanges the N-terminal α -helices forming a 3D-swapped structure and is the form that even in the reducing conditions of the cell cytosol is cytotoxic [61] (for a comparative review on the RNase structures, see [62, 63]). BS-RNase binds to the extracellular matrix, and this interaction seems to be important for its cytotoxic effect [64, 65] even though it does not bind to cell membranes, suggesting an adsorption cell entry mechanism [66]. BS-RNase has been localized in endosomes and its cytotoxicity is blocked by inhibition of this energy-dependent entry mechanism [65]. It has also been localized in the trans-Golgi network of treated malignant cells, which may be indicative that this organelle is an effective site for translocation providing an explanation for its selectivity [65, 67]. Although, it has been described that BS-RNase can destabilize artificial membranes [68, 69], it is not exactly known how BS-RNase permeates the trans-Golgi membranes. Like for ONC, rRNA is a target of BS-RNase and its cleavage induces apoptosis [64], but the enzyme has also been found in the nucleolus of cancer cells [65], and although it is not known how it reaches the cell nucleus, a correlation between cytotoxicity and a decrease of telomerase activity and its associated RNA has been found [70]. Recently, it has been described that BS-RNase triggers Beclin1-mediated autophagy in treated cancer cells being ineffective in normal cells, suggesting that autophagy more than apoptosis can be the mechanism of cancer cell death induced by BS-RNase [71]. Comparable to ONC, this selective autophagy for cancer cells seems to be related to the basic charge distribution in the surface of these RNases [36, 71].

Apart from RNases of animal origin, it is worth mentioning that there are a vast array of RNases from fungal, bacterial, and plant origin that natively present remarkable cytotoxic properties. Among them we can mention mushroom RNases [72, 73]; microbial RNases such as α -sarcin from *Aspergillus*; the two well-known T1 ribonuclease members from *Bacillus*, binase (*B. intermedius*), and barnase (*B. amyloliquefaciens*); and RNase Sa (*S. aureofaciens*) [28, 74] as well as plant RNases from ginseng, wheat leaf, mung bean, black pine pollen, seeds of bitter melon, tomato, and hop [12, 15, 75–79]. Although adverse effects due to immunogenicity and nonspecific binding [12] have been described for some of them, others are described to have a lower immunogenicity than ONC [28]. In addition, they have a remarkable resistance to RI, and in some cases, the cytotoxic effect is comparable to that of ONC. However, in terms of knowledge of their cytotoxic mechanism and clinical applications, they are still lagging behind when compared to the animal counterparts. In the last years, especially for binase, a significant advance has been attained in the understanding of its mechanism of cell intoxication. The cytotoxic effect of binase is effected via induction of both intrinsic and extrinsic apoptotic pathways [80], and evidence is provided that targets KIT and AML1-ETO oncogenes in human leukemia Kasumi-1 cells [81]. It has also shown a positive effect on the liver of tumor-bearing mice, articulated as a tumor reduction in the volume of destructive changes in the live parenchyma as well as of being effective in tumor growth suppression [82].

2. How to endow a ribonuclease with selective antitumor activity

In the last two decades, the knowledge gained on the cytotoxic mechanism of natively tumoricidal RNases, described in the previous section, as well as in the references therein, has been used to engineer more powerful and selective RNase variants able to kill cancer cells. From this knowledge, it is clear that RNases will be cytotoxic if they are able to reach the cytosol avoiding lysosomal degradation or nonproductive intracellular pathways and if, once in the cytosol, they can evade the action of RI (**Figure 2A–C**). Consequently, an RNase will be cytotoxic either if it can avoid the RI inhibition or it can efficiently reach the cytosol saturating all the RI present in this compartment (**Figure 2D–F**). Several approaches have been used to fulfill these requirements that will be reviewed in this section and are summarized in **Figure 3**.

2.1. Engineered RNases that evade the RI

In the literature two main approaches used to engineer noncytotoxic RNases to render them resistant to the RI and endow them with cytotoxicity are described: The most evident one consists of precluding RNase-RI complex formation through steric hindrance or coulombic repulsion. The variant's design is based on the known 3D structure of the RI-RNase A complex described by Kobe and Deisenhofer [83]. However, another approach is to hide RNases from the inhibitor. This is accomplished by targeting monomeric RNases to an organelle free of RI making needless neither the RI evasion nor the RI saturation.

2.1.1. RNases in a monomeric form

The first reported approaches to endow an RNase with the ability to evade the RI were carried out by Raines and coworkers, who introduced single or few amino acid changes in wild-type nontoxic RNase A that created steric hindrance to decrease RI binding [84]. Replacement of Gly 88 of RNase A by bulky charged residues, like Arg or Asp, resulted in a variant with 10^4 -fold less affinity for the RI and which was only about 20-fold less cytotoxic than ONC. Similar approaches were used on HP-RNase [85, 86] or monomeric BS-RNase [87, 88]. This first approach was concomitant or followed by the introduction of other changes that disturbed the electrostatic interaction between the RNase and the RI [89–92] creating new variants each time more cytotoxic. For instance, the RNase A variant Asp38Arg/Arg39Asp/Asn67Arg/Gly88Arg had 5.9×10^9 -fold lower affinity for the RI keeping the activity and stability of the parental enzyme with a cytotoxicity equivalent to that of ONC [91]. Although this approach has attained success, in some cases the use of the same rationale has not worked to get variants with the expected properties [89, 93]. This is due to the fact that the replacement of some residues to disrupt the RNase-RI interaction at the same time alters other factors important for the enzyme cytotoxicity, such as the catalytic activity or the stability of the enzyme that counterbalances the obtained gain on RI evasion. Nevertheless, one of the engineered RNases to evade the RI has reached clinical trials. The QBI-139 RNase variant (Evade™ ribonucleases from Quintessence Biosciences Inc. (<http://www.quintbio.com/>)) is now in Phase I of clinical

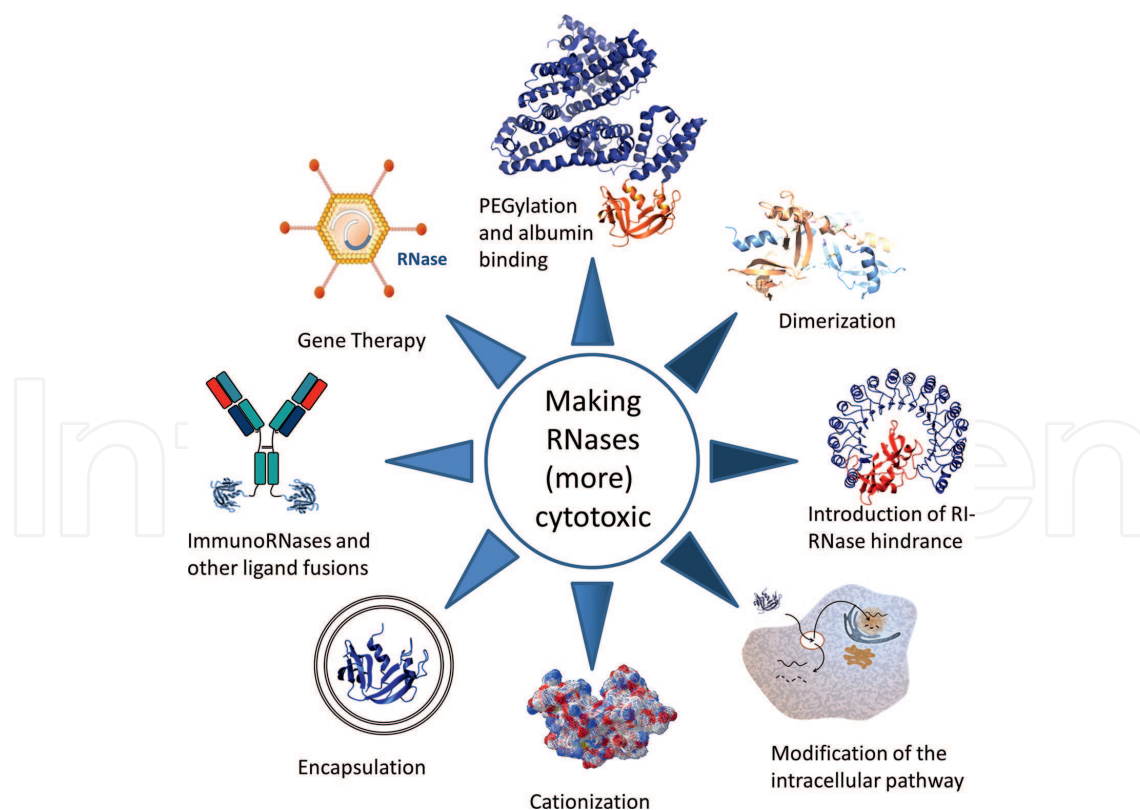


Figure 3. Strategies to create cytotoxic RNases or to improve its antitumor activity. Two groups of strategies are considered: those allowing the RNase to avoid the inhibition by the RI (dark blue arrows) and those that improve the delivery of the RNase into the cell (light blue arrows). Some of the indicated strategies can be included in both groups.

trials for the treatment of solid tumors [25]. On the other hand, different strategies have been carried out to avoid some of the non-desired side effects. The Gly88Arg RI-evading RNase A, described above, was engineered to introduce nonnative disulfide bonds to increase its conformational stability [94], resulting in a more cytotoxic variant. Also, an increase in stability has also been attained by the glycosylation of the protein. For instance, the production of ONC in *Pichia pastoris* yields a glycosylated protein more stable and 50-fold more cytotoxic [95].

2.1.2. RNase dimerization or oligomerization

The formation of oligomeric structures, such as the BS-RNase dimers, has inspired the design and production of new RNase variants with the aim of precluding their binding to the RI by steric hindrance mimicking the way of action of BS-RNase [96, 97]. The pursuit of dimeric or oligomeric variants is very attractive because they are more cationic proteins and can potentially strongly interact with the negative surfaces of cancer cells gaining selectivity and, at the same time, reducing kidney clearance due to the increase of molecular mass. As stated in Section 1.2, the current model for BS-RNase cytotoxicity is that in the reducing conditions of the cell cytosol, the unswapped isomer from (M=M) dissociates into monomers, which are strongly inhibited by the RI, whereas the swapped isomer (M×M) remains as a non-covalent dimer able to evade the RI [64, 98]. In addition, analysis of the structure of the non-covalent dimer of BS-RNase [61] and different mutated forms [99] suggested that it adopts a compact quaternary structure that is critical for the RI interaction, explaining its trapping. One of the first approaches to get cytotoxic dimeric RNase variants was to reproduce the structural determinants of BS-RNase swapping [62] in different members of the vertebrate-secreted RNase family. Thus, different combinations of those residues identified as responsible of dimer formation (Cys31, Cys32, Leu28, Gly16, Ser80) of BS-RNase were introduced in the sequence of either HP-RNase or RNase A. Alternatively, the full N-terminal hinge sequence (the peptide that links the N-terminal α -helix of V-shaped RNase structure with the rest of the protein body) of RNase A was replaced by that of BS-RNase [63] in order to endow RNase A with dimerization abilities. These changes resulted in the formation of different ratios of swapped and unswapped forms, which was critical for their cytotoxicity [63]. Among these constructs it is remarkable that of a dimeric form of HP-RNase containing the mutations Glu111Gly, Gln28Leu, Arg31Cys, Arg32Cys, and Asn34Lys that was more cytotoxic and selective than BS-RNase for cancer cells [100]. As another approach, covalent linkers to stabilize the dimeric structures have also been used. In this sense, first cytotoxic RNase A dimers [101, 102] and more recently higher oligomers cross-linked with dimethyl suberimidate [103] were obtained. Although these constructs were cytotoxic, they presented heterogeneity, a drawback for their use as antitumor agents. The use of more specific cross-linkers like the introduction of thioether bonds between different Cys residues of BS-RNase [104] and RNase A [105], in some cases, allowed the production of variants with an increased cytotoxicity. Finally, an evaluation of cross-linkers and selection of positions to introduce different Cys was carried out in the work of Rutkoski et al. [106]. In this case, some of the constructs were as cytotoxic as the RI-evading RNases. However, as far as we know, none of the described constructs has reached clinical trials yet. An interesting and different way to get dimeric RNases consists in the fusion of two RNase genes using a linker to get a tandem RNase [107]. This construct although inhibited by

the RI showed a cytotoxicity of the same order of that shown by BS-RNase. Modeling studies of this tandem RNase bound to the RI revealed that the engineered enzyme binds the RI with a 1:1 stoichiometry, and the authors suggested that the cytotoxic effect was due to an improved endocytosis efficiency [108] likely due to a higher cationization (see below).

Finally, related with the formation of oligomeric structures, it is worth mentioning that RNase A can form 3D domain-swapped multimers, ranging from trimers to hexamers [109, 110] and up to decatetramers [111]. These oligomers are enzymatically and biologically active [110, 112] and what is more interesting they exhibit cytotoxicity [99, 113]. The study of these oligomeric structures could reveal new scaffolds for the design of potential antitumor RNase variants [63].

2.1.3. Targeting organelle RI-free

The tumor cell nucleus is the final destination of multiple conventional antitumor drugs [114, 115] as well as a critical compartment for suicide gene therapy [116]. In addition, drugs that do not have a native tendency to accumulate in the cell nucleus have been conjugated/engineered/encapsulated by different means to reach this compartment. Literature is full of examples, for instance, drugs that have been modified by the introduction of a nuclear localization signal (NLS) as a modular component of a construct [117–119] and that have been encapsulated in nanoparticles directed to the cell nucleus [120] or the viral-based vectors, which are an elegant choice as vehicles to deliver DNA that encodes therapeutic proteins or RNAs to this organelle [121–123]. Based on this, an alternative strategy to bypass the RI action was to guide the RNases to the cell nucleus, which is described as free of RI [124] or at least the nucleolus [125]. Initially, an HP-RNase variant was produced, namely PE5, that carries a noncontiguous extended bipartite NLS [31, 126]. Although this variant is inhibited by the RI, at the same time it is recognized by α -importin [126] and cleaves nuclear but not cytoplasmic RNA *in vivo* [127]. At present, the mechanism by which the engineered HP-RNase reaches the cell nucleus is different from the one described above for BS-RNase (Section 1.2). It is postulated that the concentrations of RI and α -importin are similar in the cytosol. Thus, the affinity of PE5 for each protein will determine to which it will mainly bind. However, those RNase molecules captured by the α -importin will be released into the nucleus and, therefore, removed from the two competing equilibria, and PE5 will progressively accumulate into the nucleus [128]. PE5 kills the cells by apoptosis mediated by the induction of p21^{WAF/CIP1} and inactivation of JNK and increases the number of cells in the S-G₂/M-phases of cell cycle [129]. Moreover, the cytotoxic mechanism of PE5 is not prevented by a mutated p53 or a multidrug-resistant (MDR) phenotype [129], and it is synergic with doxorubicin [130] on doxorubicin-resistant NCI/ADR-RES cell line [130]. Very recently, using microarray-derived transcriptional profiling, it has been shown that PE5 remarkably downregulates multiple genes that code for enzymes involved in the deregulated metabolic pathways in cancer cells [131], one of the hallmarks of cancer. In addition, new cytotoxic RNase variants directed to the cell nucleus, collectively named ND-RNases, have been engineered either by reverting some of PE5 changes to render the variant more similar to wild-type enzyme or by the addition of an extra NLS to its N-terminus. In the latter case, a tenfold more cytotoxic enzyme than PE5 [132] has been obtained. Due to their

cytotoxic mechanism, which differs from that of RNases that exert its action on the cell's cytosol, ND-RNases are very interesting antitumor agents that can cope with the complexity of cancer cell phenotype, and their multiple effects allow anticipating synergism with many currently clinically used antitumor agents. In *in vivo* studies with animal models, the ND-RNases have shown very low toxicity (it has not been possible to calculate the maximum tolerated doses (MTD) but the maximum feasible dose (MFD) which is of 80 mg/kg) (Castro et al., results not published).

2.2. Engineered RNases that might saturate the intracellular RI and/or gain selectivity

The efficiency of cell internalization is another important determinant of the cytotoxicity of the RNases because an RI-sensitive RNase is still a potential danger provided that enough enzyme molecules reach the cell cytosol. The most basic strategy to increase the internalization of RNases is their cationization by chemical or genetic modification, that is, to make the RNases even more basic to increase their interaction with the anionic membranes of tumor cells. As stated above, this fact may also increase their selectivity for cancer cells [133]. Several examples of this approach can be found in the literature. The chemical modification of the carbonyl groups of RC-RNase with a water-soluble carbodiimide in the presence of nucleophiles or the amidation with ethylenediamine, 2-aminoethanol, taurine, or ethylenediamine of HP-RNase and RNase A increases their cytotoxicity [134–136]. The preparation of RNase A and noncytotoxic cross-linked dimers of RNase A, both covalently linked to polyspermine to increase their basicity, slightly increased their cytotoxicity [137]. In general, the higher cationic variants were more efficiently internalized into the cells. However, in some cases, the chemical modifications seriously compromised the ribonucleolytic activity of the modified enzymes [134, 135] and generated heterogeneous products difficult to use as antitumor drugs. RC-RNase and RNase Sa variants were engineered substituting acidic residues by Asn, Gln, or Arg [138] or by positively charged residues [139, 140], respectively, showing antitumor activity and enhanced internalization. Gly38Lys-BS-RNase that bears an enforced cluster of positive charges at the N-terminal surface also presented an increased cytotoxicity relative to its parental RNase and a higher membrane interaction capability [141]. Fuchs et al. [142] replaced two residues of a cytotoxic variant of RNase A to create a patch of Arg residues on its surface that rendered a threefold increase in cytotoxicity and added a protein translocation domain (nona-arginine) to a previously cytotoxic RNase variant that increased their cytotoxicity [142, 143]. However, the same group has proposed that the internalization of pancreatic RNases by cationization can be counterbalanced by an increased affinity for the anionic RI in the cytosol [92]. Like for RI evasion, one has to be very cautious in the design of these variants in order to not counterbalance the increased internalization by the loss of other important characteristics responsible for the RNase cytotoxicity. In the same line but with a different approach, co-treating cells with a cationic 2 poly(amidoamine) dendrimer [144] increase the cytotoxicity of the RNase probably by increasing its translocation from the endosomes without affecting its ribonucleolytic activity or conformational stability observed upon cationization of some RNases.

In addition to merely increasing the positive charge of an RNase, other approaches that can enhance its delivery to the cytosol or to a specific organelle are related to the construction of targeted RNases either by chemical conjugation or fusion with a specific component that directs them to cancer cells. These procedures have been used with other drugs combining a targeting molecule, mainly antibodies, with an effector moiety getting what has been called immunotoxins (see below) [145, 146]. Small molecule drugs are still the modality of choice for addressing intracellular targets due to the barriers to cell entry that proteins have to face. Nevertheless, despite the considerable research efforts and advances attained, there remain many protein-protein interactions that small molecules cannot modulate effectively [147], and proteins have a lower propensity for off-targets. Thus, the strategies described below include both small molecules and proteins as drivers of payload RNases, including that nonsensitive to the RI.

2.2.1. Intracellular pathway

The engineering of RNases to amend the intracellular pathway, apart from the ND-RNases described in Section 2.1.3 that have a different goal, is the less explored approach. This is likely because the productive intracellular route followed by cytotoxic RNases is the part of the intoxication process that looks like more as a black box. Nevertheless, there are some examples to deliver RNases to cellular compartments other than the cytosol. The KDEL consensus sequence, which drives proteins to the endoplasmic reticulum (ER), was added to the C-terminal end of BS-RNase resulting in a loss of cytotoxicity compared to the parental enzyme [65] indicating that this compartment is not an essential intracellular station for the arrival of BS-RNase to the cell cytosol. In addition, it has been tried to decrease the lysosomal degradation of one of the cytotoxic Evade™ RNases, the Gly88Arg variant, by introducing the change Lys7Arg in the Lys-Phe-Glu-Arg-Gln sequence that targets proteins to the lysosomes. In this case, Lys7Arg/Gly88Arg RNase A is nearly tenfold more cytotoxic than Gly88Arg RNase A variant but has more than tenfold less affinity for RI [90]. Once again, the changes introduced to enhance one of the aspects of RNase cytotoxicity affect other important features of this process precluding a clear conclusion.

2.2.2. Chemical conjugation

Targeting RNase molecules to tumor cells was early carried out by chemical conjugation of both RNase A to transferrin (Tf) or mAb against Tf receptor (TfR) [148] as well as to the T-cell antigen CD5 [149] and ONC to anti-Tf receptor mAb 5E9 [150]. These studies showed that the antibody conjugates were more efficient than Tf conjugates and that ONC and RNase A conjugated to antibodies by a reducible disulfide bond were equally potent (IC₅₀ values in the nM range). However, ONC conjugates showed increased efficiency likely due to the fact that ONC is not inhibited by the RI, while RNase A might not be able to saturate it even when conjugated to a particular cell driver [151]. In these primary studies also, ONC was conjugated to CD22-specific mAb LL2 and RFB4, which resulted in a several thousand-fold increase in cytotoxicity comparable to that of anti-CD22 immunotoxin conjugated to plant or bacterial toxin cargoes [152]. These results confirmed that RNases are as potent as these toxins when

properly targeted. Nevertheless, although these chemical conjugates reduced the tumor size in animal models while not showing appreciable toxicity, their lot-to-lot heterogeneity was a serious drawback for further development [153]. ONC was also conjugated to P-glycoprotein (P-gp) neutralizing mAb MRK16. This construct increased ONC cytotoxicity and at the same time sensitized the multidrug-resistant cancer cells that overexpress MDR1 gene to vincristine [154]. These results may be explained by both the mAb binding to P-gp that diminishes its drug-expelling ability and its ability to internalize ONC.

More recently, the amino groups of Lys side chains of RNase A and variant Lys41Arg (Lys41 is an amino acid critical for the ribonucleolytic activity of the enzyme [155]) were randomly conjugated to folate since folate receptors are overexpressed on the surface of many types of cancer [156, 157]. However, the results showed an abolition of the catalytic activity of RNase A, while the variant Lys41Arg only retained 54 % of its catalytic activity. In the same work, a folate analogue was designed, produced, and used to specifically S-alkylate Cys residues introduced by site-directed mutagenesis at positions 19 or 88. Only those proteins modified at positions that endow them with the ability to evade the RI were able to diminish cell proliferation. Thus, even in this case, enhanced internalization had to be accompanied by an RI evasion [158].

2.2.3. Fusion RNases and ImmunoRNases

The progress attained in the technology of construction and production of recombinant fusion proteins particularly using antibodies [145, 146] has been applied to RNases. Several RNases have been used either as scaffold onto which a targeting domain is engineered or fused, including antibodies. In the latter case, several antibody constructs such as scFv, diabodies, scFv-Fc, and F(ab)₂ antibody fragments were used as a fusion partner [159]. Generally speaking, small antibody constructs are best suited to penetrate and distribute into solid tumors. However, the smaller the construct, the faster it disappears from blood circulation. Thus, a compromise in the molecular mass has been agreed, between 60 and 120 kDa for a therapeutic protein in order to ensure a good pharmacokinetics [160]. On the other hand, when choosing a particular RNase to be fused to an antibody or any targeting domain, it has to be taken into account the connecting linker and the orientation of the RNase relative to the carrier molecule in order not to alter some of the previously described RNase properties important for their cytotoxicity.

Primarily, HP-RNase as well as other members of the same family [161] such as angiogenin (ANG) and other RNases such as eosinophil-derived neurotoxin (EDN) and some engineered variants of them were tested in experimental sets of RNase-antibody fusion targeting Tf. They showed about 10³ times more potency than the respective chemically conjugated RNase-antibody (Section 2.2.2) [149, 162]. The main concern with these constructs was the host selected for production indicating that the final yield was dependent on the expression system used [154]. To overcome production concerns and to get more specific clinical targets, different immunoRNases have been produced directed to antigens expressed on certain types of leukemia but not in hematopoietic stem cells, such as CD22 and CD30 or the ErbB2 that showed significant advantages over the equivalent chemical conjugates [153]. As alternatives, ANG,

HP-RNase, or RapLR1 (*R. pipiens* liver RNase 1), a close relative of ONC, were fused to two CD22-specific scFV antibody fragments generated either by reengineering the variable domain core structure of mAb LL2 or grafting the complementary-determining regions (CDR) of the clinically established mAb RF4B into consistent human scFv scaffolds [163–165]. Some of them were successfully produced and exhibited potent cytotoxicity (IC_{50} in the nM range) [164] which drove to a second generation of anti-CD22 immunoRNases in diabody format fusing LL2 or humRFB4 to ANG or RapLR1. Bivalent anti-CD22 immunoRNases showed a superior cytotoxicity toward CD22⁺ tumor cells when compared to their monovalent counterparts due to antigen binding by avidity and enhanced internalization [163, 164]. Different CD30-targeting constructs have also been produced fusing HP-RNase or ANG to CD30-specific murine or human scFvs that inhibited tumor growth [166], but the entirely human bivalent scFv-Fc-HP-RNase showed better properties and inhibited the growth of CD30⁺ Hodgkin lymphoma cells [167]. Even better results were obtained for immunoRNases resulting from the fusion of CD30-specific scFv Ki4 to ANG [168]. Recently, a humanized anti-epidermal growth factor receptor (EGFR) scFV was used to target ONC to EGFR-expressing tumor cell lines [169]. Fusion was accomplished by a flexible linker (G_4S)₃, but the construct resulted in very poor cytotoxicity, likely due to endosomal accumulation and lysosomal degradation. To avoid this drawback, the authors substituted the linker by a peptide from dengue virus that has been reported to be involved in the endosomal escape of the virus. The modified immunoRNase exhibited exceptionally high cytotoxicity toward EGFR-expressing head and neck cancer cell lines without affecting specificity. More recently, the same research group constructed a derived diabody fragment with the specificity of the clinically established mAb Cetuximab to deliver ONC to EGFR-expressing tumor cells. The dimeric immunoRNase was several orders of magnitude more cytotoxic toward EGFR-expressing tumor cell lines than its monomeric counterpart and exhibited significant antitumor activity in a murine A431 xenograft model, but in this case, the linker was (G_4S)₃ [170]. Thus, not only the linker between the ONC and the antibody fragment is important but the structure of the antibody moiety.

An ONC variant with a modified putative N-glycosylation site (Asn69Gln) has also fused to humanized antibody hRS7 raised against Trop-2, a cell surface glycoprotein expressed in a variety of epithelial cancers [171]. The construct contained two ONC molecules fused to each of the N-terminus of light chains of the antibody and was produced in stably transfected myeloma cells. The purified immunoRNase inhibited the proliferation of Trop-2-expressing cell lines with an IC_{50} in the nM range and was able to suppress tumor growth in a prophylactic model of nude mice bearing Calu-3 human non-small cell lung cancer xenografts with an increase of the median survival time from 55 to 96 days [172]. More recently, a second generation of these immunoRNases has been produced by the dock-and-lock (DNL) method. This methodology consists in the use of a pair of distinct protein domains that are involved in the natural association between protein kinase A (PKA; cyclic AMP-dependent protein kinase) and A-kinase-anchoring proteins. The dimerization and docking domain (DDD) found in the regulatory subunit of PKA and the anchoring domain (AD) of an interactive-A-kinase anchoring protein are each attached to a biological entity through specific linkers and the resulting derivatives, when combined, readily form a stably tethered complex of defined composition that fully retains the functions of individual constituents. That is, the docked

complex can be made irreversible using a pair of linker modules that introduce Cys residues into both the DDD and the AD domains at strategic positions that facilitate the formation of disulfide bridges [173]. The integration of genetic engineering and conjugation chemistry of the DNL method has been used to get two constructs containing four ONC molecules linked to either the C_{H3} or C_K C-termini of hRS7 that have been evaluated as potential therapeutics for triple-negative breast cancer (TNBC). Both constructs showed specific cell-binding and rapid internalization in MDA-MB-486, a Trop-2-expressing TNBC, and displayed potent *in vitro* cytotoxicity against diverse breast cancer cell lines. In addition, both seemed well tolerated at clinically relevant concentrations. However, C_K-based construct exhibited superior Fc-effector functions *in vitro*, as well as improved pharmacokinetics, stability, and activity *in vivo*. Further studies are needed regarding their immunogenicity although they are potentially a new class of immunoRNases that warrant future research [174].

Not only animal RNases have been used to construct immunoRNases. For instance, the construct formed by two barnase molecules fused serially to scFv of humanized 4D5 antibody directed to the extracellular domain of epidermal growth factor receptor 2 (HER2 or ErbB2) was produced [175]. This scFv 4D5-dibarnase showed cytotoxicity *in vitro* and significant *in vivo* inhibition of human breast cancer xenografts in nude mice without severe side effects [176].

The first entirely human immunoRNase was produced fusing HP-RNase to an ErbB2-specific scFv named Erbicin [177, 178]. The construct recognizes an epitope distinct to that of trastuzumab and pertuzumab [179], the two humanized antibodies currently used to treat HER2⁺ metastatic mammary carcinomas [180, 181] and do not induce cardiac dysfunction as the other two do [182–184]. Although this immunoRNase was inhibited by the RI to an extent comparable to that of HP-RNase, the quantity that entered the cell cytosol saturated the RI, and it exhibited a clear RNA degradation ability [185]. Due to this limitation, a second generation of immunoRNases was obtained by fusing an RI-insensitive HP-RNase variant (Arg39Asp/Asn67Asp/Asn88Ala/Gly89Asp/Arg91Asp) [186] to ErbB2-specific scFv showing resistance to RI inhibition and the ability to kill mammary ErbB2⁺ tumor cells more efficiently [187]. This variant does not show cardiotoxic effects *in vitro* and does not impair cardiac function in mouse models [188]. In addition, since bivalent immunoRNases are more powerful than monovalent ones, a dimeric variant of HP-RNase was fused to two Erbicin molecules, one per subunit [189]. The new construct was able to bind to ErbB2-positive cancer cell lines with an increased avidity with respect to the monovalent variant and was a more cytotoxic, likely due to an increased RI evasion.

2.2.4. Delivery strategies

Although RNases have reached clinical trials, one important aspect that researchers have still to cope with is to improve their tissue delivery. This means, to enhance the RNases circulating half-live in the blood and to avoid a high glomerular filtration rate. These factors contribute to an optimal pharmacokinetics and biodistribution. Related to these issues, some formulations have been carried out. One of the ways to increase persistence in circulation of small proteins such as RNases is PEGylation. Early, RNase A-PEG conjugates were randomly made [190–194]. However, although they presented increased persistence in circulation, they showed a

significant reduced catalytic efficiency due to modification of the critical catalytic residue Lys 41 that abolished their cytotoxic properties. More recently, previously acylated RNase A [195] or HP-RNase [196] has been PEGylated at specific positions (Gly88Cys in RNase A and Gly89Cys in HP-RNase). Although the conjugates show a significantly reduced cytotoxicity *in vitro*, they are effective in inhibiting tumors in xenograft mouse models, likely because the diminished renal clearance *in vivo* compensates the potential loss of cytotoxicity due to the PEG moiety. Due to the efficacy of this approach, mono-PEGylation of RNase A has been studied using two chemicals, N-hydroxysuccinimide ester of S-acetylthioacetic acid (SATA) and 2-iminothiolane (IT). Both react with primary amino groups to introduce thiol groups, a process followed by PEGylation using maleimide chemistry. Interestingly, by thiolation, the original positive charges of RNase A can be conserved, an important feature in order not to lose the cationic residues. In addition, in both cases the enzymatic activity of the RNase A was essentially maintained [197].

Another method to increase the half-life of a protein in the blood is its conjugation with bovine serum albumin (BSA) [198]. However, depending on the way used to get the conjugate, albumin can decrease the enzymatic activity of RNase A. Thus, different strategies have been described to prepare RNase A-BSA conjugates to keep the bioactivity of the enzyme, although the pharmacokinetic and pharmacodynamic properties still need to be determined [199].

In an attempt to get an ONC that can circumvent renal clearance, improve tumor cell targeting, and gain endosomal escape, a modular construct has recently been described. ONC was fused to human serum albumin (HSA) through its C-terminus, and this former construct was also C-terminus appended to scFv 4D5MOCB, which targets epithelial cell adhesion molecule (EpCAM), a validated target for anticancer therapy [200] (construct Onc-HSA-4D5MOCB). In addition, in the same work, the link between ONC and the rest of the construct was also carried out through a cleavable disulfide linker (construct Onc-SS-HSA-4D5MOCB) that potentially enables the release of ONC from its carrier after endocytosis and avoids HSA inactivation of ONC catalytic activity. Although both constructs overcame most of the *in vitro* barriers, *in vivo* toxicity studies with animal models showed that they increased liver toxicity while ONC is described to produce renal toxicity [32]. Unfortunately, only the construct Onc-SS-HSA-4D5MOCB showed a reduction of tumor growth, but it was similar to that of ONC alone, and the tumor started to regrowth when treatment was discontinued [201]. Nevertheless, this all-in-one drug delivery system may inspire other constructs that can accomplish the pursued goal.

The genetic delivery of ONC using oncolytic adenovirus has just been tested. A combination of viral oncolysis with intratumoral genetic delivery of an EGFR-binding scFv antibody fragment fused to ONC (ONC_{EGFR}) has demonstrated feasible. ONC_{EGFR} expression by oncolytic viruses is possible with an optimized, replication-dependent gene expression strategy. Very interestingly, virus-encoded ONC_{EGFR} induced a potent and EGFR-dependent bystander killing of tumor cells. That is, some of the non-transformed cells die by the entry of ONC_{EGFR} released from transfected cells. Thus, ONC_{EGFR}-encoding oncolytic adenovirus showed dramatically increased cytotoxicity specifically to EGFR-positive tumor cells *in vitro* and significantly enhanced therapeutic activity in a mouse xenograft tumor model. The authors

claim that this virus-antibody therapy platform can be further developed for personalized therapy by exploiting antibody diversity to target further established or emerging tumor markers or combination of thereof [202].

Finally, to avoid cerebellar neuronal toxicity while affecting glioma cells, ONC has been encapsulated in biodegradable poly(ricinoleic-co-sebacic acid) for local controlled delivery in the parietal lobe of the brain [203]. In this way ONC was released in a controlled manner and was cytotoxic against 9L glioma cells xenograft into the brain while evading neurotoxicity in the cerebellum.

3. Conclusion

The efforts to construct, produce, and characterize RNase variants to get more potent and selective non-genotoxic antitumor drugs have been successful because both natural and engineered RNases have reached clinical trials for the treatment of different types of cancer. RNases do not cleave a specific RNA molecule. Instead, their effects on gene expression are pleiotropic. This ensures a broad spectrum of synergistic interactions with other chemotherapeutics and, as stand-alone compounds, makes difficult the appearance of resistance to the drug by treated cancer cells. Thus, RNases are considered a new class of modern antitumor drugs very interesting for the pharmaceutical industry with fewer side effects than conventional chemotherapeutic treatments.

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References

- [1] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674. DOI: 10.1016/j.cell.2011.02.013
- [2] Gurova K. New hopes from old drugs: revisiting DNA-binding small molecules as anticancer agents. *Future Oncology*. 2009;5:1685–1704. DOI: 10.2217/fon.09.127
- [3] Tafech A, Bassett T, Sparanese D, Lee CH. Destroying RNA as a therapeutic approach. *Current Medicinal Chemistry*. 2006;13:863–881. DOI: 10.2174/092986706776361021
- [4] Li WM, Barnes T, Lee CH. Endoribonucleases—enzymes gaining spotlight in mRNA metabolism. *FEBS Journal*. 2010;277:627–641. DOI: 10.1111/j.1742-4658.2009.07488.x
- [5] Ledoux L, Baltus E. The effects of ribonuclease on cells of Ehrlich carcinoma. *Experientia* 1954;10:500–501. DOI: 10.1007/BF02166182
- [6] Ledoux L. Action of ribonuclease on two solid tumours *in vivo*. *Nature*. 1955;176:36–37. DOI: 10.1038/175258b0
- [7] Ledoux L, Revell SH. Action of ribonuclease on neoplastic growth. I. Chemical aspects of normal tumour growth: the Landschütz ascites tumour. *Biochimica et Biophysica Acta*. 1955;18:416–426
- [8] Ledoux L. Action of ribonuclease on neoplastic growth. II. Action on Landschütz ascites cells *in vitro*. *Biochimica et Biophysica Acta*. 1956;20:369–377
- [9] Aleksandrowicz J. Intracutaneous Ribonuclease in chronic myelocytic leukaemia. *Lancet*. 1958;272:420. DOI: 10.1016/S0140-6736(58)90139-9
- [10] Telford IR, Kemp JF, Taylor EF, Yeaman MW. Effect of ribonuclease on survival of ascites tumor bearing mice. *Proceedings of the Society for Experimental Biology and Medicine*. 1959;100:829–831.
- [11] Roth JS. Ribonuclease activity and cancer: a review. *Cancer Research*. 1963;23:657–666.
- [12] Fang EF, Ng TB. Ribonucleases of different origins with a wide spectrum of medicinal applications. *Biochimica et Biophysica Acta—Reviews on Cancer*. 2011;1815:65–74. DOI: 10.1016/j.bbcan.2010.09.001
- [13] Irie M. RNase T1/RNase T2 Family RNases. In: D’Alessio G, Riordan JF, editors. *Ribonucleases: Structures and Functions*. New York: Academic Press; 1997. p. 101–130. DOI: 10.1016/B978-012588945-2/50004-2
- [14] Yoshida H. The ribonuclease T1 family. *Methods in Enzymology*. 2001;341:28–41. DOI: 10.1016/S0076-6879(01)41143-8
- [15] Matousek J, Podzimek T, Pouckova P, Stehlik J, Skvor J, Lipovova P, et al. Antitumor activity of apoptotic nuclease TBN1 from *L. esculentum*. *Neoplasma*. 2010;57:339–348. DOI: 10.4149/neo_2010_04_339

- [16] D'Alessio G. The Superfamily of Vertebrate-Secreted Ribonucleases. In: Nicholson AW, editor. *Nucleic Acids Mol. Biol.* 26. Springer Berlin Heidelberg; 2011. p. 1–34. DOI: 10.1007/978-3-642-21078-5_1
- [17] Cuchillo CM, Vilanova M, Nogués MV. Pancreatic Ribonucleases. In: D'Alessio G, Riordan JF, editors. *Ribonucleases: Structures and Functions*. New York: Academic Press; 1997. p. 271–304.
- [18] Patutina O, Mironova N, Ryabchikova E, Popova N, Nikolin V, Kaledin V, et al. Inhibition of metastasis development by daily administration of ultralow doses of RNase A and DNase I. *Biochimie*. 2011;93:689–696. DOI: 10.1016/j.biochi.2010.12.011
- [19] Costanzi J, Sidransky D, Navon A, Goldsweig H. Ribonucleases as a novel proapoptotic anticancer strategy: review of the preclinical and clinical data for ranpirnase. *Cancer Investigation*. 2005;23:643–650. DOI: 10.1080/07357900500283143
- [20] Favaretto A. Overview on ongoing or planned clinical trials in Europe. *Lung Cancer*. 2005;49 Suppl 1:S117–S121. DOI: 10.1016/j.lungcan.2005.03.022
- [21] Pavlakis N, Vogelzang NJ. Ranpirnase-an antitumour ribonuclease: its potential role in malignant mesothelioma. *Expert Opinion on Biological Therapy*. 2006;6:391–399. DOI: 10.1517/14712598.6.4.391
- [22] Beck AK, Pass HI, Carbone M, Yang H. Ranpirnase as a potential antitumor ribonuclease treatment for mesothelioma and other malignancies. *Future Oncology*. 2008;4:341–349. DOI: 10.2217/14796694.4.3.341
- [23] Lee JE, Raines RT. Ribonucleases as novel chemotherapeutics: the ranpirnase example. *BioDrugs*. 2008;22:53–58. DOI: 10.2165/00063030-200822010-00006
- [24] Porta C, Paglino C, Mutti L. Ranpirnase and its potential for the treatment of unresectable malignant mesothelioma. *Biologics: Targets & Therapy*. 2008;2:601–609. DOI: <https://dx.doi.org/10.2147/BTT.S2383>
- [25] Strong LE, Kink JA, Baigen M, Shahan MN, Raines RT. First-in-human phase I clinical trial of QBI-139, a human ribonuclease variant, in solid tumors. In: ASCO Annual Meeting, editor. *J. Clin. Oncol.* Chicago, Illinois: ASCO University; 2012. p. suppl; abstr TPS3113.
- [26] Benito A, Ribó M, Vilanova M. On the track of antitumour ribonucleases. *Molecular BioSystems*. 2005;1:294–302. DOI: 10.1039/b502847g
- [27] Benito A, Vilanova M, Ribó M. Intracellular routing of cytotoxic pancreatic-type ribonucleases. *Current Pharmaceutical Biotechnology*. 2008;9:169–179. DOI: 10.2174/138920108784567281
- [28] Makarov AA, Kolchinsky A, Ilinskaya ON. Binase and other microbial RNases as potential anticancer agents. *Bioessays*. 2008;30:781–790. DOI: 10.1002/bies.20789

- [29] Rutkoski TJ, Raines RT. Evasion of ribonuclease inhibitor as a determinant of ribonuclease cytotoxicity. *Current Pharmaceutical Biotechnology*. 2008;9:185–189. DOI: 10.2174/138920108784567344
- [30] Haigis MC, Kurten EL, Raines RT. Ribonuclease inhibitor as an intracellular sentry. *Nucleic Acids Research*. 2003;31:1024–1032. DOI: 10.1093/nar/gkg163
- [31] Bosch M, Benito A, Ribó M, Puig T, Beaumelle B, Vilanova M. A nuclear localization sequence endows human pancreatic ribonuclease with cytotoxic activity. *Biochemistry*. 2004;43:2167–2177. DOI: 10.1021/bi035729+
- [32] Vasandani VM, Burris JA, Sung C. Reversible nephrotoxicity of onconase and effect of lysine pH on renal onconase uptake. *Cancer Chemotherapy and Pharmacology* 1999;44:164–169. DOI: 10.1007/s002800050962
- [33] Ardelt W, Shogen K, Darzynkiewicz Z. Onconase and amphinase, the antitumor ribonucleases from *Rana pipiens* oocytes. *Current Pharmaceutical Biotechnology*. 2008;9:215–225. DOI: 10.2174/138920108784567245
- [34] Ribó M, Benito A, Vilanova M. Antitumor Ribonucleases. In: Nicholson AW, editor. *Nucleic Acids and Molecular Biology* 26. Heidelberg: Springer Berlin Heidelberg; 2011. p. 55–88. DOI: 10.1007/978-3-642-21078-5_3
- [35] Michaelis M, Cinatl J, Anand P, Rothweiler F, Kotchetkov R, Deimling A Von, et al. Onconase induces caspase-independent cell death in chemoresistant neuroblastoma cells. *Cancer Letters*. 2007;250:107–116. DOI: 10.1016/j.canlet.2006.09.018
- [36] Fiorini C, Cordani M, Gotte G, Picone D, Donadelli M. Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner. *Biochimica et biophysica acta*. 2015;1853:549–560. DOI: 10.1016/j.bbamcr.2014.12.016
- [37] Wu Y, Mikulski SM, Ardelt W, Rybak SM, Youle RJ. A cytotoxic ribonuclease. Study of the mechanism of onconase cytotoxicity. *The Journal of Biological Chemistry*. 1993;268:10686–10693.
- [38] Goparaju CM, Blasberg JD, Volinia S, Palatini J, Ivanov S, Donington JS, et al. Onconase mediated NF κ B downregulation in malignant pleural mesothelioma. *Oncogene*. 2011;30:2767–2777. DOI: 10.1038/onc.2010.643
- [39] Saxena SK, Sirdeshmukh R, Ardelt W, Mikulski SM, Shogen K, Youle RJ. Entry into cells and selective degradation of tRNAs by a cytotoxic member of the RNase A family. *The Journal of Biological Chemistry* 2002;277:15142–15146. DOI: 10.1074/jbc.M108115200
- [40] Ardelt B, Ardelt W, Darzynkiewicz Z. Cytotoxic ribonucleases and RNA interference (RNAi). *Cell Cycle*. 2003;2:22–24. DOI: 10.4161/cc.2.1.232

- [41] Zhao H, Ardelts B, Ardelts W, Shogen K, Darzynkiewicz Z. The cytotoxic ribonuclease onconase targets RNA interference (siRNA). *Cell Cycle*. 2008;7:3258–3261. DOI: 10.4161/cc.7.20.6855
- [42] Qiao M, Zu L-D, He X-H, Shen R-L, Wang Q-C, Liu M-F. Onconase downregulates microRNA expression through targeting microRNA precursors. *Cell Research* 2012;22:1199–1202. DOI: 10.1038/cr.2012.67
- [43] Altomare DA, Rybak SM, Pei J, Maizel J V, Cheung M, Testa JR, et al. Onconase responsive genes in human mesothelioma cells: implications for an RNA damaging therapeutic agent. *BMC Cancer*. 2010;10:34. DOI: 10.1186/1471-2407-10-34
- [44] Singh UP, Ardelts W, Saxena SK, Holloway DE, Vidunas E, Lee HS, et al. Enzymatic and structural characterisation of amphinase, a novel cytotoxic ribonuclease from *Rana pipiens* oocytes. *Journal of Molecular Biology* 2007;371:93–111. DOI: 10.1016/j.jmb.2007.04.071
- [45] Youle RJ, D'Alessio G. Antitumor RNases. In: D'Alessio G, Riordan JF, editors. *Ribonucleases: Structures and Functions*. New York: Elsevier; 1997. p. 491–514. DOI: 10.1016/B978-012588945-2/50016-9
- [46] Irie M, Nitta K, Nonaka T. Biochemistry of frog ribonucleases. *Cellular and Molecular Life Sciences* 1998;54:775–784. DOI: 10.1007/s000180050206
- [47] Sakakibara F, Kawauchi H, Takayanagi G, Ise H. Egg lectin of *Rana japonica* and its receptor glycoprotein of Ehrlich tumor cells. *Cancer Research*. 1979;39:1347–1352.
- [48] Okabe Y, Katayama N, Iwama M, Watanabe H, Ohgi K, Irie M, et al. Comparative base specificity, stability, and lectin activity of two lectins from eggs of *Rana catesbeiana* and *R. japonica* and liver ribonuclease from *R. catesbeiana*. *Journal of Biochemistry*. 1991;109:786–790.
- [49] Nitta K, Ozaki K, Ishikawa M, Furusawa S, Hosono M, Kawauchi H, et al. Inhibition of cell proliferation by *Rana catesbeiana* and *Rana japonica* lectins belonging to the ribonuclease superfamily. *Cancer Research*. 1994;54:920–927.
- [50] Tatsuta T, Sugawara S, Takahashi K, Ogawa Y, Hosono M, Nitta K. Leczyme: a new candidate drug for cancer therapy. *BioMed Research International*. 2014;2014:421415. DOI: 10.1155/2014/421415
- [51] Haigis MC, Raines RT. Secretory ribonucleases are internalized by a dynamin-independent endocytic pathway. *Journal of Cell Science*. 2003;116:313–324. DOI: 10.1242/jcs.00214
- [52] Rodríguez M, Torrent G, Bosch M, Rayne F, Dubremetz J-F, Ribó M, et al. Intracellular pathway of Onconase that enables its delivery to the cytosol. *Journal of Cell Science*. 2007;120:1405–1411. DOI: 10.1242/jcs.03427

- [53] Sundlass NK, Eller CH, Cui Q, Raines RT. Contribution of electrostatics to the binding of pancreatic-type ribonucleases to membranes. *Biochemistry*. 2013;52:6304–6312. DOI 10.1021/bi400619m
- [54] Ran S, Downes A, Thorpe PE. Increased exposure of anionic phospholipids on the surface of tumor blood vessels. *Cancer Research*. American Association for Cancer Research; 2002;62:6132–6140. DOI: 10.3109/09687689409160430
- [55] Eller CH, Chao T-Y, Singarapu KK, Ouerfelli O, Yang G, Markley JL, et al. Human cancer antigen Globo H is a cell-surface ligand for human ribonuclease 1. *ACS Central Science* 2015;1:181–190. DOI: 10.1021/acscentsci.5b00164
- [56] Zhang S, Cordon-Cardo C, Zhang HS, Reuter VE, Adluri S, Hamilton WB, et al. Selection of tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. *International Journal of Cancer*. 1997;73:42–49. DOI: 10.1002/(SICI)1097-0215(19970926)73:1<42::AID-IJC8>3.0.CO;2-1
- [57] Chao T-Y, Raines RT. Mechanism of ribonuclease A endocytosis: analogies to cell-penetrating peptides. *Biochemistry*. 2011;50:8374–8382. DOI: 10.1021/bi2009079
- [58] Shirshikov F V., Cherepnev G V., Ilinskaya ON, Kalacheva N V. A hydrophobic segment of some cytotoxic ribonucleases. *Medical Hypotheses*. 2013;81:328–334. DOI: 10.1016/j.mehy.2013.04.006
- [59] Murthy BS, Sirdeshmukh R. Sensitivity of monomeric and dimeric forms of bovine seminal ribonuclease to human placental ribonuclease inhibitor. *Biochemical Journal*. 1992;281:343–348. DOI: 10.1042/bj2810343
- [60] D'Alessio G, Di Donato A, Mazzarella L, Piccoli R. Seminal ribonuclease: the importance of diversity. *ribonucleases: structures and functions*. New York: Academic Press; 1997. p. 383–423.
- [61] Sica F, Di Fiore A, Merlino A, Mazzarella L. Structure and stability of the non-covalent swapped dimer of bovine seminal ribonuclease: an enzyme tailored to evade ribonuclease protein inhibitor. *The Journal of Biological Chemistry*. 2004;279:36753–36760. DOI: 10.1074/jbc.M405655200
- [62] Benito A, Laurents D V, Ribó M, Vilanova M. The structural determinants that lead to the formation of particular oligomeric structures in the pancreatic-type ribonuclease family. *Current Protein and Peptide Science* 2008;9:370–393. DOI: 10.2174/138920308785132695
- [63] Gotte G, Laurents D V., Merlino A, Picone D, Spadaccini R. Structural and functional relationships of natural and artificial dimeric bovine ribonucleases: new scaffolds for potential antitumor drugs. *FEBS Letters*. 2013;587:3601–3608. DOI: 10.1016/j.febslet.2013.09.038

- [64] Mastronicola MR, Piccoli R, D'Alessio G. Key extracellular and intracellular steps in the antitumor action of seminal ribonuclease. *European Journal of Biochemistry*. 1995;230:242–249. DOI: 10.1111/j.1432-1033.1995.0242i.x
- [65] Bracale A, Spalletti-Cernia D, Mastronicola M, Castaldi F, Mannucci R, Nitsch L, et al. Essential stations in the intracellular pathway of cytotoxic bovine seminal ribonuclease. *Biochemical Journal*. 2002;362:553–560. DOI: 10.1042/bj3620553
- [66] Kim JS, Soucek J, Matousek J, Raines RT. Mechanism of ribonuclease cytotoxicity. *The Journal of Biological Chemistry*. 1995;270:31097–31102. DOI: 10.1074/jbc.270.52.31097
- [67] Wu Y, Saxena SK, Ardelt W, Gadina M, Mikulski SM, De Lorenzo C, et al. A study of the intracellular routing of cytotoxic ribonucleases. *The Journal of Biological Chemistry*. 1995;270:17476–17481. DOI: 10.1074/jbc.270.29.17476
- [68] Mancheno JM, Gasset M, Onaderra M, Gavilanes JG, Dalessio G. Bovine Seminal Ribonuclease Destabilizes Negatively Charged Membranes. *Biochemical Biophysical Research Communications*. 1994;199:119–124. DOI: 10.1006/bbrc.1994.1202
- [69] Notomista E, Mancheño JM, Crescenzi O, Di Donato A, Gavilanes J, D'Alessio G. The role of electrostatic interactions in the antitumor activity of dimeric RNases. *FEBS Journal*. 2006;273:3687–3697. DOI: 10.1111/j.1742-4658.2006.05373.x
- [70] Viola M, Libra M, Callari D, Sinatra F, Spada D, Noto D, et al. Bovine seminal ribonuclease is cytotoxic for both malignant and normal telomerase-positive cells. *International Journal of Oncology*. 2005;27:1071–1077. DOI: 10.3892/ijo.27.4.1071
- [71] Fiorini C, Gotte G, Donnarumma F, Picone D, Donadelli M. Bovine seminal ribonuclease triggers Beclin1-mediated autophagic cell death in pancreatic cancer cells. *Biochimica et Biophysica Acta—Molecular Cell Research* 2014;1843:976–984. DOI: 10.1016/j.bbamcr.2014.01.025
- [72] Ng T. Peptides and proteins from fungi. *Peptides*. 2004;25:1055–1073. DOI: 10.1016/j.peptides.2004.03.013
- [73] Wong JH, Ng TB, Cheung RCF, Ye XJ, Wang HX, Lam SK, et al. Proteins with antifungal properties and other medicinal applications from plants and mushrooms. *Applied Microbiology and Biotechnology*. 2010;87:1221–1235. DOI: 10.1007/s00253-010-2690-4
- [74] Hameş EE, Demir T. Microbial ribonucleases (RNases): production and application potential. *World Journal of Microbiology and Biotechnology*. 2015;31:1853–1862. DOI: 10.1007/s11274-015-1945-8
- [75] Skvor J, Lipovová P, Poucková P, Soucek J, Slavík T, Matousek J. Effect of wheat leaf ribonuclease on tumor cells and tissues. *Anticancer Drugs*. 2006;17:815–823. DOI: 10.1097/01.cad.0000217430.75078.cc

- [76] Soucek J, Skvor J, Poucková P, Matousek J, Slavík T, Matousek J. Mung bean sprout (*Phaseolus aureus*) nuclease and its biological and antitumor effects. *Neoplasma*. 2006;53:402–409.
- [77] Lipovova P, Podzimek T, Orctova L, Matousek J, Pouckova P, Soucek J, et al. Antitumor and biological effects of black pine (*Pinus nigra*) pollen nuclease. *Neoplasma*. 2008;55:158–164.
- [78] Matousek J, Matousek J. Plant ribonucleases and nucleases as antiproliferative agents targeting human tumors growing in mice. *Recent Patents on DNA and Gene Sequences*. 2010;4:29–39. DOI:10.2174/187221510790410813
- [79] Fang EF, Zhang CZY, Fong WP, Ng TB. RNase MC2: a new *Momordica charantia* ribonuclease that induces apoptosis in breast cancer cells associated with activation of MAPKs and induction of caspase pathways. *Apoptosis*. 2012;17:377–387. DOI:10.1007/s10495-011-0684-z
- [80] Mironova NL, Petrushanko IY, Patutina OA, Sen'kova A V, Simonenko O V, Mitkevich VA, et al. Ribonuclease binase inhibits primary tumor growth and metastases via apoptosis induction in tumor cells. *Cell Cycle*. 2013;12:2120–2131. DOI: 10.4161/cc.25164
- [81] Mitkevich VA, Petrushanko IY, Spirin P V, Fedorova T V, Kretova O V, Tchurikov NA, et al. Sensitivity of acute myeloid leukemia Kasumi-1 cells to binase toxic action depends on the expression of KIT and AML1-ETO oncogenes. *Cell Cycle*. 2011;10:4090–4097. DOI: 10.4161/cc.10.23.18210
- [82] Sen'kova AV, Mironova NL, Patutina OA, Mitkevich VA, Markov OV, Petrushanko IY, et al. Ribonuclease binase decreases destructive changes of the liver and restores its regeneration potential in mouse lung carcinoma model. *Biochimie*. 2014;101:256-259. DOI: 10.1016/j.biochi.2014.02.006
- [83] Kobe B, Deisenhofer J. Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A. *Journal of Molecular Biology*. 1996;264:1028–1043. DOI: 10.1006/jmbi.1996.0694
- [84] Leland PA, Schultz LW, Kim B-M, Raines RT. Ribonuclease A variants with potent cytotoxic activity. *Proceedings of the National Academy of Sciences*. 1998;95:10407–10412. DOI: 10.1073/pnas.95.18.10407
- [85] Leland PA, Staniszewski KE, Kim BM, Raines RT. Endowing human pancreatic ribonuclease with toxicity for cancer cells. *The Journal of Biological Chemistry*. 2001;276:43095–43102. DOI:10.1074/jbc.M106636200
- [86] Gaur D, Swaminathan S, Batra JK. Interaction of human pancreatic ribonuclease with human ribonuclease inhibitor. Generation of inhibitor-resistant cytotoxic variants. *The Journal of Biological Chemistry*. 2001;276:24978–24984. DOI: 10.1074/jbc.M102440200

- [87] Antignani A, Naddeo M, Cubellis M V, Russo A, D'Alessio G. Antitumor action of seminal ribonuclease, its dimeric structure, and its resistance to the cytosolic ribonuclease inhibitor. *Biochemistry*. 2001;40:3492–3496. DOI: 10.1021/bi002781m
- [88] Lee JE, Raines RT. Cytotoxicity of bovine seminal ribonuclease: monomer versus dimer. *Biochemistry*. 2005;44:15760–15767. DOI: 10.1021/bi051668z
- [89] Bretscher LE, Abel RL, Raines RT. A ribonuclease A variant with low catalytic activity but high cytotoxicity. *The Journal of Biological Chemistry*. 2000;275:9893–9896. DOI: 10.1074/jbc.275.14.9893
- [90] Haigis MC, Kurten EL, Abel RL, Raines RT. KFERQ sequence in ribonuclease A-mediated cytotoxicity. *The Journal of Biological Chemistry*. 2002;277:11576–11581. DOI: 10.1074/jbc.M112227200
- [91] Rutkoski TJ, Kurten EL, Mitchell JC, Raines RT. Disruption of shape-complementarity markers to create cytotoxic variants of ribonuclease A. *Journal of Molecular Biology*. 2005;354:41–54. DOI: 10.1016/j.jmb.2005.08.007
- [92] Johnson RJ, Chao TY, Lavis LD, Raines RT. Cytotoxic ribonucleases: the dichotomy of Coulombic forces. *Biochemistry*. 2007;46:10308–10316. DOI: 10.1021/bi700857u
- [93] Dickson KA, Dahlberg CL, Raines RT. Compensating effects on the cytotoxicity of ribonuclease A variants. *Archives of Biochemistry and Biophysics*. 2003;415:172–177. DOI: 10.1016/S0003-9861(03)00214-5
- [94] Klink TA, Raines RT. Conformational stability is a determinant of ribonuclease A cytotoxicity. *The Journal of Biological Chemistry*. 2000;275:17463–17467. DOI: 10.1074/jbc.M001132200
- [95] Kim B-M, Kim H, Raines RT, Lee Y. Glycosylation of onconase increases its conformational stability and toxicity for cancer cells. *Biochemical and Biophysical Research Communications*. 2004;315:976–983. DOI: 10.1016/j.bbrc.2004.01.153
- [96] Libonati M. Biological actions of the oligomers of ribonuclease A. *Cellular and Molecular Life Sciences*. 2004;61:2431–2436. DOI: 10.1007/s00018-004-4302-x
- [97] Libonati M, Gotte G, Vottariello F. A novel biological actions acquired by ribonuclease through oligomerization. *Current Pharmaceutical Biotechnology*. 2008;9:200–209. DOI: 10.2174/138920108784567308
- [98] Cafaro V, De Lorenzo C, Piccoli R, Bracale A, Mastronicola MR, Di Donato A, et al. The antitumor action of seminal ribonuclease and its quaternary conformations. *FEBS Letters*. 1995;359:31–34. DOI: 10.1016/0014-5793(94)01450-F
- [99] Merlino A, Ercole C, Picone D, Pizzo E, Mazzarella L, Sica F. The buried diversity of bovine seminal ribonuclease: shape and cytotoxicity of the swapped non-covalent form of the enzyme. *Journal of Molecular Biology* 2008;376:427–437. DOI: 10.1016/j.jmb.2007.11.008

- [100] Di Gaetano S, D'alessio G, Piccoli R. Second generation antitumour human RNase: significance of its structural and functional features for the mechanism of antitumour action. *Biochemical Journal*. 2001;358:241–247. DOI: 10.1042/bj3580241
- [101] Bartholeyns J, Baudhuin P. Inhibition of tumor cell proliferation by dimerized ribonuclease. *Proceedings of the National Academy of Sciences of the United States of America*. 1976;73:573–576.
- [102] Tarnowski GS, Kassel RL, Mountain IM, Blackburn P, Wilson G, Wang D. Comparison of antitumor activities of pancreatic ribonuclease and its cross-linked dimer. *Cancer Research*. 1976;36:4074–4078.
- [103] Gotte G, Testolin L, Costanzo C, Sorrentino S, Armato U, Libonati M. Cross-linked trimers of bovine ribonuclease A: activity on double-stranded RNA and antitumor action. *FEBS Letters*. 1997;415:308–312. DOI: 10.1016/S0014-5793(97)01147-2
- [104] Kim JS, Soucek J, Matousek J, Raines RT. Catalytic activity of bovine seminal ribonuclease is essential for its immunosuppressive and other biological activities. *Biochemical Journal*. 1995;308:547–550. DOI: 10.1042/bj3080547
- [105] Suzuki M, Saxena SK, Boix E, Prill RJ, Vasandani VM, Ladner JE, et al. Engineering receptor-mediated cytotoxicity into human ribonucleases by steric blockade of inhibitor interaction. *Nature Biotechnology*. 1999;17:265–270. DOI: 10.1038/7010
- [106] Rutkoski TJ, Kink J.A., Strong LE, Schilling CI, Raines RT. Antitumor activity of ribonuclease multimers created by site-specific covalent tethering. *Bioconjugate Chemistry*. 2010;21:1691–1702. DOI: 10.1021/bc100292x
- [107] Leich F, Köditz J, Ulbrich-Hofman R, Arnold U. Tandemization endows bovine pancreatic ribonuclease with cytotoxic activity. *Journal of Molecular Biology*. 2006;358:1305–1313. DOI: 10.1016/j.jmb.2006.03.007
- [108] Arnold U, Leich F, Neumann P, Lilie H, Ulbrich-Hofmann R. Crystal structure of RNase A tandem enzymes and their interaction with the cytosolic ribonuclease inhibitor. *FEBS Journal*. 2011;278:331–340. DOI: 10.1111/j.1742-4658.2010.07957.x
- [109] Gotte G, Bertoldi M, Libonati M. Structural versatility of bovine ribonuclease A. Distinct conformers of trimeric and tetrameric aggregates of the enzyme. *European Journal of Biochemistry* 1999;265:680–687. DOI: 10.1046/j.1432-1327.1999.00761.x
- [110] Gotte G, Laurents D V., Libonati M. Three-dimensional domain-swapped oligomers of ribonuclease A: identification of a fifth tetramer, pentamers and hexamers, and detection of trace heptameric, octameric and nonameric species. *Biochimica et Biophysica Acta—Proteins Proteomics*. 2006;1764:44–54. DOI: 10.1016/j.bbapap.2005.10.011
- [111] López-Alonso JP, Gotte G, Laurents DV. Kinetic analysis provides insight into the mechanism of Ribonuclease A oligomer formation. *Archives of Biochemistry and Biophysics*. 2009;489:41–47. DOI: 10.1016/j.abb.2009.07.013

- [112] Matousek J, Gotte G, Pouckova P, Soucek J, Slavik T, Vottariello F, et al. Antitumor activity and other biological actions of oligomers of ribonuclease A. *The Journal of Biological Chemistry*. 2003;278:23817–23822. DOI: 10.1074/jbc.M302711200
- [113] Cafaro V, Bracale A, Di Maro A, Sorrentino S, D'Alessio G, Di Donato A. New muteins of RNase A with enhanced antitumor action. *FEBS Letters*. 1998;437:149–152. DOI: 10.1016/S0014-5793(98)01221-6
- [114] Balderas-Renteria I, Gonzalez-Barranco P, Garcia A, Banik BK, Rivera G. Anticancer drug design using scaffolds of β -lactams, sulfonamides, quinoline, quinoxaline and natural products. *Drugs advances in clinical trials. Current Medicinal Chemistry*. 2012;19:4377–4398. DOI: 10.2174/092986712803251593
- [115] Schapira M. Pharmacogenomics opportunities in nuclear receptor targeted cancer therapy. *Current Cancer Drug Targets*. 2002;2:243–256.
- [116] Vassaux G, Martin-Duque P. Use of suicide genes for cancer gene therapy: study of the different approaches. *Expert Opinion on Biological Therapy*. 2004; 4:519–530. DOI: 10.1517/14712598.4.4.519
- [117] Kuusisto H V, Wagstaff KM, Alvisi G, Roth DM, Jans DA. Global enhancement of nuclear localization-dependent nuclear transport in transformed cells. *FASEB Journal*. 2012;26:1181–1193. DOI: 10.1096/fj.11-191585
- [118] Costantini DL, Chan C, Cai Z, Vallis KA, Reilly RM. ¹¹¹In-Labeled Trastuzumab (Herceptin) Modified with Nuclear Localization Sequences (NLS): an Auger Electron-Emitting Radiotherapeutic Agent for HER2/neu-Amplified Breast Cancer. *Journal Nuclear Medicine, Society of Nuclear Medicine*. 2007;48:1357–1368. DOI: 10.2967/jnumed.106.037937
- [119] Costantini DL, McLarty K, Lee H, Done SJ, Vallis KA, Reilly RM. Antitumor effects and normal-tissue toxicity of ¹¹¹In-nuclear localization sequence-trastuzumab in athymic mice bearing HER-positive human breast cancer xenografts. *Journal of Nuclear Medicine*. 2010;51:1084–1091. DOI: 10.2967/jnumed.109.072389
- [120] Misra R, Sahoo SK. Intracellular trafficking of nuclear localization signal conjugated nanoparticles for cancer therapy. *European Journal of Pharmaceutical Sciences*. 2010;39:152–163. DOI: 10.1016/j.ejps.2009.11.010
- [121] Arís A, Villaverde A. Engineering nuclear localization signals in modular protein vehicles for gene therapy. *Biochemistry and Biophysical Research Communications*. 2003;304:625–631. DOI: 10.1016/S0006-291X(03)00644-2
- [122] Akhlynina T V, Jans DA, Statsyuk N V, Balashova IY, Toth G, Pavo I, et al. Adenoviruses synergize with nuclear localization signals to enhance nuclear delivery and photodynamic action of internalizable conjugates containing chlorin e6. *International Journal of Cancer*. 1999;81:734–740. DOI: 10.1002/(SICI)1097-0215(19990531)81:5<734::AID-IJC12>3.0.CO;2-J

- [123] Akhlynnina T V, Jans DA, Rosenkranz AA, Statsyuk N V, Balashova IY, Toth G, et al. Nuclear targeting of chlorin e6 enhances its photosensitizing activity. *The Journal of Biological Chemistry*. 1997;272:20328–20331. DOI: 10.1074/jbc.272.33.20328
- [124] Roth JS, Juster H. On the absence of ribonuclease inhibitor in rat liver nuclei. *Biochimica et Biophysica Acta—Nucleic Acids Protein Synthesis*. 1972;287:474–476. DOI: 10.1016/0005-2787(72)90291-2
- [125] Furia A, Moscato M, Cali G, Pizzo E, Confalone E, Amoroso MR, et al. The ribonuclease/angiogenin inhibitor is also present in mitochondria and nuclei. *FEBS Letters*. 2011;585:613–617. DOI: 10.1016/j.febslet.2011.01.034
- [126] Rodríguez M, Benito A., Tubert P, Castro J, Ribó M, Beaumelle B, et al. A cytotoxic ribonuclease variant with a discontinuous nuclear localization signal constituted by basic residues scattered over three areas of the molecule. *Journal of Molecular Biology* 2006;360:548–557. DOI: 10.1016/j.jmb.2006.05.048
- [127] Tubert P, Rodríguez M, Ribó M, Benito A, Vilanova M. The nuclear transport capacity of a human-pancreatic ribonuclease variant is critical for its cytotoxicity. *Investigational New Drugs*. 2011;29:811–817. DOI: 10.1007/s10637-010-9426-2
- [128] Castro J, Ribó M, Benito A, Vilanova M. Mini-review: nucleus-targeted ribonucleases as antitumor drugs. *Current Medicinal Chemistry*. 2013;20:1225–1231. DOI: 10.2174/0929867311320100003
- [129] Castro J, Ribó M, Navarro S, Nogués MV, Vilanova M, Benito A. A human ribonuclease induces apoptosis associated with p21WAF1/CIP1 induction and JNK inactivation. *BMC Cancer*. 2011;11:9. DOI: 10.1186/1471-2407-11-9
- [130] Castro J, Ribó M, Puig T, Colomer R, Vilanova M, Benito A. A cytotoxic ribonuclease reduces the expression level of P-glycoprotein in multidrug-resistant cell lines. *Investigational New Drugs*. 2012;30:880–888. DOI: 10.1007/s10637-011-9636-2
- [131] Vert A, Castro J, Ribó M, Benito A, Vilanova M. A nuclear-directed human pancreatic ribonuclease (PE5) targets the metabolic phenotype of cancer cells. *Oncotarget*. 2016;7:18309–18324. DOI: 10.18632/oncotarget.7579
- [132] Vert A, Castro J, Ruiz-Martínez S, Tubert P, Escibano D, Ribó M, et al. Generation of new cytotoxic human ribonuclease variants directed to the nucleus. *Molecular Pharmaceutics* 2012;9:2894–2902. DOI: 10.1021/mp300217b
- [133] Chao T-Y, Lavis LD, Raines RT. Cellular uptake of ribonuclease A relies on anionic glycans. *Biochemistry*. 2010;49:10666–10673. DOI: 10.1021/bi1013485
- [134] Futami J, Maeda T, Kitazoe M, Nukui E, Tada H, Seno M, et al. Preparation of potent cytotoxic ribonucleases by cationization: enhanced cellular uptake and decreased interaction with ribonuclease inhibitor by chemical modification of carboxyl groups. *Biochemistry*. 2001;40:7518–7524. DOI: 10.1021/bi010248g

- [135] Futami J, Nukui E, Maeda T, Kosaka M, Tada H, Seno M, et al. Optimum modification for the highest cytotoxicity of cationized ribonuclease. *Journal of Biochemistry*. 2002;132:223–228.
- [136] Iwama M, Ogawa Y, Sasaki N, Nitta K, Takayanagi Y, Ohgi K, et al. Effect of modification of the carboxyl groups of the sialic acid binding lectin from bullfrog (*Rana catesbeiana*) oocyte on anti-tumor activity. *Biological and Pharmaceutical Bulletin*. 2001;24:978–981. DOI: <http://doi.org/10.1248/bpb.24.978>
- [137] Poučková P, Morbio M, Vottariello F, Laurents D V., Matoušek J, Souček J, et al. Cytotoxicity of polyspermine-ribonuclease A and polyspermine-dimeric ribonuclease A. *Bioconjugate Chemistry*. 2007;18:1946–1955. DOI: 10.1021/bc700253c
- [138] Ogawa Y, Iwama M, Ohgi K, Tsuji T, Irie M, Itagaki T, et al. Effect of replacing the aspartic acid/glutamic acid residues of bullfrog sialic acid binding lectin with asparagine/glutamine and arginine on the inhibition of cell proliferation in murine leukemia P388 cells. *Biological and Pharmaceutical Bulletin*. 2002;25:722–727. DOI: <http://doi.org/10.1248/bpb.25.722>
- [139] Ilinskaya ON, Dreyer F, Mitkevich VA, Shaw KL, Pace CN, Makarov AA. Changing the net charge from negative to positive makes ribonuclease Sa cytotoxic. *Protein Science* 2002;11:2522–2525. DOI: 10.1110/ps.0216702.toward
- [140] Ilinskaya ON, Koschinski A, Mitkevich VA, Repp H, Dreyer F, Pace CN, et al. Cytotoxicity of RNases is increased by cationization and counteracted by KCa channels. *Biochemical and Biophysical Research Communications*. 2004;314:550–554. DOI: 10.1016/j.bbrc.2003.12.110
- [141] D'Errico G, Ercole C, Lista M, Pizzo E, Falanga A, Galdiero S, et al. Enforcing the positive charge of N-termini enhances membrane interaction and antitumor activity of bovine seminal ribonuclease. *Biochimica et Biophysica Acta—Biomembranes* 2011;1808:3007–3015. DOI: 10.1016/j.bbamem.2011.08.009
- [142] Fuchs SM, Rutkoski TJ, Kung VM, Groeschl RT, Raines RT. Increasing the potency of a cytotoxin with an arginine graft. *Protein Engineering, Design and Selection*. 2007;20:505–509. DOI: 10.1093/protein/gzm051
- [143] Fuchs SM, Raines RT. Polyarginine as a multifunctional fusion tag. *Protein Science*. 2005;14:1538–1544. DOI: 10.1110/ps.051393805
- [144] Ellis GA, Hornung ML, Raines RT. Potentiation of ribonuclease cytotoxicity by a poly(amidoamine) dendrimer. *Bioorganic & Medicinal Chemistry Letters*. 2011;21:2756–2758 DOI: 10.1016/j.bmcl.2010.11.028
- [145] Dübel S. *Handbook of Therapeutic Antibodies*. Dübel S, Reichert JM, editors. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2014. 2188 p. DOI: 10.1002/9783527682423

- [146] Guillard S, Minter RR, Jackson RH. Engineering therapeutic proteins for cell entry: the natural approach. *Trends in Biotechnology*. 2015;33:163–171. DOI: 10.1016/j.tibtech.2014.12.004
- [147] Arkin MR, Tang Y, Wells JA. Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. *Chemistry and Biology*. 2014;21:1102–1114. DOI: 10.1016/j.chembiol.2014.09.001
- [148] Rybak SM, Saxena SK, Ackerman EJ, Youle RJ. Cytotoxic potential of ribonuclease and ribonuclease hybrid proteins. *The Journal of Biological Chemistry*. 1991;266:21202–21207.
- [149] Newton DL, Ilercil O, Laske DW, Oldfield E, Rybak SM, Youle RJ. Cytotoxic ribonuclease chimeras. Targeted tumoricidal activity *in vitro* and *in vivo*. *The Journal of Biological Chemistry*. 1992;267:19572–19578.
- [150] Rybak SM, Newton DL, Mikulski SM, Viera A, Youle RJ. Cytotoxic onconase and ribonuclease a chimeras: comparison and *in vitro* characterization. *Drug Delivery*. 1993;1:3–10. DOI: 10.3109/10717549309031335
- [151] Rybak SM. Antibody-onconase conjugates: cytotoxicity and intracellular routing. *Current Pharmaceutical Biotechnology*. 2008;9:226–230. DOI: 10.2174/138920108784567272
- [152] Newton DL, Hansen HJ, Mikulski SM, Goldenberg DM, Rybak SM. Potent and specific antitumor effects of an anti-CD22-targeted cytotoxic ribonuclease: Potential for the treatment of non-Hodgkin lymphoma. *Blood*. 2001;97:528–535. DOI: 10.1182/blood.V97.2.528
- [153] Schirrmann T, Krauss J, Arndt MAE, Rybak SM, Dübel S. Targeted therapeutic RNases (ImmunoRNases). *Expert Opinion on Biological Therapy*. 2009;9:79–95. DOI: 10.1517/14712590802631862
- [154] Newton DL, Pollock D, DiTullio P, Echelard Y, Harvey M, Wilburn B, et al. Antitransferrin receptor antibody-RNase fusion protein expressed in the mammary gland of transgenic mice. *Journal of Immunological Methods*. 1999;231:159–167. DOI: 10.1016/S0022-1759(99)00154-4
- [155] Cuchillo CM, Nogués MV, Raines RT. Bovine pancreatic ribonuclease: fifty years of the first enzymatic reaction mechanism. *Biochemistry*. 2011;50:7835–7841. DOI: 10.1021/bi201075b
- [156] Garin-Chesa P, Campbell I, Saigo PE, Lewis JL, Old LJ, Rettig WJ. Trophoblast and ovarian cancer antigen LK26. Sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *The American journal of pathology*. 1993;142:557–567.
- [157] Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues *in vivo* and in established cell lines. *Physiologic and*

- clinical implications. *Cancer*. 1994;73:2432–2443. DOI: 10.1002/1097-0142(19940501)73:9<2432::AID-CNCR2820730929>3.0.CO;2-S
- [158] Smith BD, Higgin JJ, Raines RT. Site-specific folate conjugation to a cytotoxic protein. *Bioorganic & Medicinal Chemistry Letters*. 2011;21:5029-5032. DOI: 10.1016/j.bmcl.2011.04.081
- [159] Rybak SM, Newton DL. Immunotoxins and beyond: targeted RNases. In: Dübel S, editor. *Handbook of Therapeutic Antibodies*. Weinheim, Germany: Wiley-VCH Verlag GmbH; 2007. p. 379–410. DOI: 10.1002/9783527619740.ch16
- [160] Hudson PJ, Souriau C. Engineered antibodies. *Nature Medicine*. 2003;9:129–134. DOI: 10.1038/nm0103-129
- [161] Sorrentino S. The eight human “canonical” ribonucleases: Molecular diversity, catalytic properties, and special biological actions of the enzyme proteins. *FEBS Letters*. 2010;584:2194–2200. DOI: 10.1016/j.febslet.2010.04.018
- [162] Newton DL, Nicholls PJ, Rybak SM, Youle RJ. Expression and characterization of recombinant human eosinophil-derived neurotoxin and eosinophil-derived neurotoxin-anti-transferrin receptor sFv. *The Journal of Biological Chemistry*. 1994;269:26739–26745.
- [163] Arndt MAE, Krauss J, Vu BK, Newton DL, Rybak SM. A dimeric angiogenin immunofusion protein mediates selective toxicity toward CD22+ tumor cells. *Journal of Immunotherapy*. 2005;28:245–251.
- [164] Krauss J, Arndt MAE, Vu BK, Newton DL, Seeber S, Rybak SM. Efficient killing of CD22+ tumor cells by a humanized diabody–RNase fusion protein. *Biochemical and Biophysical Research Communications*. 2005;331:595–602. DOI: 10.1016/j.bbrc.2005.03.215
- [165] Krauss J, Arndt MAE, Vu BK, Newton DL, Rybak SM. Targeting malignant B-cell lymphoma with a humanized anti-CD22 scFv-angiogenin immunoenzyme. *British Journal of Haematology*. 2005;128:602–609. DOI: 10.1111/j.1365-2141.2005.05356.x
- [166] Braschoss S, Hirsch B, Dübel S, Stein H, MD HD. New anti-CD30 human pancreatic ribonuclease-based immunotoxin reveals strong and specific cytotoxicity *in vivo*. *Leukemia & Lymphoma*. 2009;48:1179-1186. DOI: 10.1080/10428190701272264
- [167] Menzel C, Schirrmann T, Konthur Z, Jostock T, Dübel S. Human antibody RNase fusion protein targeting CD30+ lymphomas. *Blood*. 2008;111:3830–3837. DOI: 10.1182/blood-2007-04-082768
- [168] Stöcker M, Tur MK, Sasse S, Krüßmann A, Barth S, Engert A. Secretion of functional anti-CD30-angiogenin immunotoxins into the supernatant of transfected 293T-cells. *Protein Expression and Purification*. 2003;28:211–219. DOI: 10.1016/S1046-5928(02)00709-X

- [169] Kiesgen S, Liebers N, Cremer M, Arnold U, Weber T, Keller A, et al. A fusogenic dengue virus-derived peptide enhances antitumor efficacy of an antibody-ribonuclease fusion protein targeting the EGF receptor. *Protein Engineering, Design and Selection*. 2014;27:331–337. DOI: 10.1093/protein/gzu040
- [170] Kiesgen S, Arndt MAE, Körber C, Arnold U, Weber T, Halama N, et al. An EGF receptor targeting Ranpirnase-diabody fusion protein mediates potent antitumour activity *in vitro* and *in vivo*. *Cancer Letters*. 2015;357:364–373. DOI: 10.1016/j.canlet.2014.11.054
- [171] Rapani E, Sacchetti A, Corda D, Alberti S. Human TROP-2 is a tumor-associated calcium signal transducer. *International Journal of Cancer*. 1998;76:671–676. DOI: 10.1002/(SICI)1097-0215(19980529)76:5<671::AI
- [172] Chang C-H, Gupta P, Michel R, Loo M, Wang Y, Cardillo TM, et al. Ranpirnase (frog RNase) targeted with a humanized, internalizing, anti-Trop-2 antibody has potent cytotoxicity against diverse epithelial cancer cells. *Molecular Cancer Therapeutics*; 2010;9:2276–2286. DOI: 10.1158/1535-7163.MCT-10-0338
- [173] Chang C-H, Rossi EA, Goldenberg DM. The dock and lock method: a novel platform technology for building multivalent, multifunctional structures of defined composition with retained bioactivity. *Clinical Cancer Research*; 2007;13:5586s–5591s. DOI: 10.1158/1078-0432.CCR-07-1217
- [174] Liu D, Cardillo TM, Wang Y, Rossi EA, Goldenberg DM, Chang C-H, et al. Trop-2-targeting tetrakis-ranpirnase has potent antitumor activity against triple-negative breast cancer. *Molecular Cancer*. 2014;13:53. DOI: 10.1186/1476-4598-13-53
- [175] Glinka EM, Edelweiss EF, Sapozhnikov AM, Deyev SM. A new vector for controllable expression of an anti-HER2/neu mini-antibody-barnase fusion protein in HEK 293T cells. *Gene*. 2006;366:97–103. DOI: 10.1016/j.gene.2005.06.042
- [176] Balandin TG, Edelweiss E, Andronova N V., Treshalina EM, Sapozhnikov AM, Deyev SM. Antitumor activity and toxicity of anti-HER2 immunoRNase scFv 4D5-dibarnase in mice bearing human breast cancer xenografts. *Investigational New Drugs*. 2011;29:22–32. DOI: 10.1007/s10637-009-9329-2
- [177] De Lorenzo C, Palmer DB, Piccoli R, Ritter MA, D'Alessio G. A new human antitumor immunoreagent specific for ErbB2. *Clinical Cancer Research*; 2002;8:1710–1719. DOI: 10.1038/319230a0
- [178] De Lorenzo C, Arciello A, Cozzolino R, Palmer DB, Laccetti P, Piccoli R, et al. A fully human antitumor immunoRNase selective for ErbB-2-positive carcinomas. *Cancer Research*. 2004;64:4870–4874. DOI: 10.1158/0008-5472.CAN-03-3717
- [179] Troise F, Monti M, Merlino A, Cozzolino F, Fedele C, Russo Krauss I, et al. A novel ErbB2 epitope targeted by human antitumor immunoagents. *FEBS Journal*. 2011;278:1156–1166. DOI: 10.1111/j.1742-4658.2011.08041.x

- [180] Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CEJ, Davidson NE, et al. Trastuzumab plus Adjuvant Chemotherapy for Operable HER2-Positive Breast Cancer. *The New England journal of medicine*. 2005;353: 1673-1684 DOI: 10.1056/NEJMoa052122
- [181] Chung C, Lam MSH. Pertuzumab for the treatment of human epidermal growth factor receptor type 2-positive metastatic breast cancer. *American Journal of Health-System Pharmacy*. 2013;70:1579–1587. DOI: 10.2146/ajhp120735
- [182] Gelardi T, Damiano V, Rosa R, Bianco R, Cozzolino R, Tortora G, et al. Two novel human anti-ErbB2 immunoagents are active on trastuzumab-resistant tumours. *British Journal of Cancer*. 2010;102:513–519. DOI: 10.1038/sj.bjc.6605499
- [183] Perez EA, Suman VJ, Davidson NE, Martino S, Kaufman PA, Lingle WL, et al. HER2 testing by local, central, and reference laboratories in specimens from the north central cancer treatment group n9831 intergroup adjuvant trial. *Journal of Clinical Oncology*. 2006;24:3032–3038. DOI: 10.1200/JCO.2005.03.4744
- [184] Lenihan D, Suter T, Brammer M, Neate C, Ross G, Baselga J. Pooled analysis of cardiac safety in patients with cancer treated with pertuzumab. *Annals of Oncology*. 2012;23:791–800. DOI: 10.1093/annonc/mdr294
- [185] De Lorenzo C, Di Malta C Di, Call` G, Troise F, Nitsch L, D'Alessio G. Intracellular route and mechanism of action of ERB-hRNase, a human anti-ErbB2 anticancer immunoagent. *FEBS Letters*. 2007;581:296–300. DOI: 10.1016/j.febslet.2006.12.034
- [186] Johnson RJ, McCoy JG, Bingman CA, Phillips GN, Raines RT. Inhibition of human pancreatic ribonuclease by the human ribonuclease inhibitor protein. *Journal of Molecular Biology* 2007;368:434–449. DOI: 10.1016/j.jmb.2007.02.005
- [187] Riccio G, D'avino C, Raines RT, De Lorenzo C. A novel fully human antitumor ImmunoRNase resistant to the RNase inhibitor. *Protein Engineering, Design and Selection*. 2013;26:243–248. DOI: 10.1093/protein/gzs101
- [188] D'Avino C, Paciello R, Riccio G, Coppola C, Coppola M, Laccetti P, et al. Effects of a second-generation human anti-ErbB2 ImmunoRNase on trastuzumab-resistant tumors and cardiac cells. *Protein Engineering, Design and Selection*. 2014;27:83–88. DOI: 10.1093/protein/gzt065
- [189] Riccio G, Borriello M, D'Alessio G, De Lorenzo C. A novel human antitumor dimeric immunoRNase. *Journal of Immunotherapy*. 2008;31:440–445. DOI: 10.1097/CJI.0b013e31816bc769
- [190] Veronese FM, Largajolli R, Boccù E, Benassi CA, Schiavon O. Surface modification of proteins. Activation of monomethoxy-polyethylene glycols by phenylchloroformates and modification of ribonuclease and superoxide dismutase. *Applied Biochemistry and Biotechnology*. 1985;11:141–152. DOI: 10.1007/BF02798546

- [191] Caliceti P, Schiavon O, Veronese FM, Chaiken IM. Effects of monomethoxypoly(ethylene glycol) modification of ribonuclease on antibody recognition, substrate accessibility and conformational stability. *Journal of Molecular Recognition*. 1990;3:89–93. DOI: 10.1002/jmr.300030206
- [192] Schiavon O, Caliceti P, Sartore L, Veronese FM. Surface modification of enzymes for therapeutic use: monomethoxypoly (ethylene glycol) derivatization of ribonuclease. *Il Farmaco*. 1991;46:967–978.
- [193] Matoušek J, Poučková P, Souček J, Škvor J. PEG chains increase aspermatogenic and antitumor activity of RNase A and BS-RNase enzymes. *Journal of Controlled Release*. 2002;82:29–37. DOI: 10.1016/S0168-3659(02)00082-2
- [194] Matoušek J, Poučková P, Hloušková D, Zadinová M, Souček J, Škvor J. Effect of hyaluronidase and PEG chain conjugation on the biologic and antitumor activity of RNase A. *Journal of Controlled Release*. 2004;94:401–410. DOI: 10.1016/j.jconrel.2003.10.025
- [195] Rutkoski TJ, Kink JA, Strong LE, Raines RT. Site-specific PEGylation endows a mammalian ribonuclease with antitumor activity. *Cancer Biology and Therapy*. 2011;12:208–214. DOI: 10.4161/cbt.12.3.15959
- [196] Rutkoski TJ, Kink JA, Strong LE, Raines RT. Human ribonuclease with a pendant poly(ethylene glycol) inhibits tumor growth in mice. *Translational Oncology*. 2013;6:392–397. DOI: 10.1593/tlo.13253
- [197] Liu S, Sun L, Wang J, Ma G, Su Z, Hu T. Mono-PEGylation of ribonuclease A: high PEGylation efficiency by thiolation with small molecular weight reagent. *Process Biochemistry*. 2012;47:1364–1370. DOI: 10.1016/j.procbio.2012.05.003
- [198] Kratz F. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *Journal of Controlled Release*. 2008;132:171–183. DOI: 10.1016/j.jconrel.2008.05.010
- [199] Li C, Lin Q, Wang J, Shen L, Ma G, Su Z, et al. Preparation, structural analysis and bioactivity of ribonuclease A-albumin conjugate: Tetra-conjugation or PEG as the linker. *Journal of Biotechnology*. 2012;162:283–288. DOI: 10.1016/j.jbiotec.2012.09.008
- [200] van der Gun BTF, Melchers LJ, Ruiters MHJ, de Leij LFMH, McLaughlin PMJ, Rots MG. EpCAM in carcinogenesis: the good, the bad or the ugly. *Carcinogenesis*. 2010;31:1913–1921. DOI: 10.1093/carcin/bgq187
- [201] Zhao HL, Xue C, Du JL, Ren M, Xia S, Cheng YG, et al. Sustained and cancer cell targeted cytosolic delivery of Onconase results in potent antitumor effects. *Journal of Controlled Release*. 2012;159:346–352. DOI: 10.1016/j.jconrel.2012.02.019
- [202] Fernández-Ulibarri I, Hammer K, Arndt MAE, Kaufmann JK, Dorer D, Engelhardt S, et al. Genetic delivery of an immunoRNase by an oncolytic adenovirus enhances

anticancer activity. *International Journal of Cancer*. 2015;136:2228–2240. DOI: 10.1002/ijc.29258

- [203] Slager J, Tyler B, Shikanov A, Domb AJ, Shogen K, Sidransky D, et al. Local controlled delivery of anti-neoplastic RNase to the brain. *Pharmaceutical Research*. 2009;26:1838–1846. DOI: 10.1007/s11095-009-9893-3

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