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

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http://dx.doi.org/10.5772/65730

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 antitumor 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).

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 (leczymes) found in Rana catesbeiana (RC-RNase) and Rana japonica (RI-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 leczymes 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 \textit{Aspergillus}; the two well-known T1 ribonuclease members from \textit{Bacillus}, binase (\textit{B. intermedius}), and barnase (\textit{B. amyloliquefaciens}); and RNase Sa (\textit{S. aureofaciens}) \[28, 74\] as well as plant RNases from ginseng, wheat leaf, mung bean, black pine pollen, seeds of bitter gourd, 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 \(\text{Figure 2A–C}\). 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 \(\text{Figure 2D–F}\). Several approaches have been used to fulfill these requirements that will be reviewed in this section and are summarized in \textbf{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 noncytotoxic 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 $5.9 \times 10^3$-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 trials.

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 citosol, 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 equilibriums, and PE5 will progressively accumulate into the nucleus [128]. PE5 kills the cells by apoptosis mediated by the induction of p21WAF/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
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 noncys- tototoxic cross-linked dimers of RNase A, both covalently linked to polysermine 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 (nonsa-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(amoidoamine) 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 (IC\textsubscript{50} 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$_4$S)$_3$, 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$_4$S)$_3$ [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\textsubscript{H}3 or C\textsubscript{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 \textit{in vitro} cytotoxicity against diverse breast cancer cell lines. In addition, both seemed well tolerated at clinically relevant concentrations. However, C\textsubscript{K}-based construct exhibited superior Fc-effector functions \textit{in vitro}, as well as improved pharmacokinetics, stability, and activity \textit{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 \textit{in vitro} and significant \textit{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 \textit{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.

\textbf{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-life 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 overcome 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\textsubscript{EGFR}) has demonstrated feasible. ONC\textsubscript{EGFR} expression by oncolytic viruses is possible with an optimized, replication-dependent gene expression strategy. Very interestingly, virus-encoded ONC\textsubscript{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\textsubscript{EGFR} released from transfected cells. Thus, ONC\textsubscript{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.

Acknowledgements

This work is supported by grants BIO2013-435217 from Ministerio de Economía y Competitividad (MINECO), Spain; SGR2014-70 from Generalitat de Catalunya, Spain; and MPCUdG2016-18 from Universitat de Girona. We are indebted to Dr. Pere Tubert for the design of Figure 2.

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