ANTITUMOR RIBONUCLEASES

Marc Ribó*, Antoni Benito*, Maria Vilanova*

Laboratori d'Enginyeria de Proteïnes, Departament de Biologia, Facultat de Ciències, Universitat de Girona, Campus de Montilivi, Maria Aurèlia Capmany, 69, 17071 Girona (Spain) and Institut d'Investigació Biomèdica de Girona Josep Trueta, (IdIBGi), Girona, Spain

*To whom correspondence should be addressed: Tel: +34-972-418173; Fax: +34-972-418150; E-mail: marc.ribo@udg.edu; antoni.benito@udg.edu; maria.vilanova@udg.edu

Abstract

Ribonucleases are small basic proteins that have shown remarkable antitumor activity linked to their ability to destroy RNA. Therefore, they are a second line of cancer chemotherapeutics as they are not genotoxic. This chapter summarizes the main biochemical characteristics of these enzymes and the key factors responsible for their cytotoxic mechanism. Some of them are shared by most cytotoxins but each RNase has particular cancer cell-killing abilities. The effects on the cell cycle and the induced apoptosis mechanism are cell dependent. The knowledge obtained from the cytotoxic mechanism of natural cytotoxic RNases has been used to artificially engineer more potent and selective RNA-degrading enzymes. These approaches are also described. The chapter ends with a brief description of the results of the clinical trials performed with RNases.

1. Introduction

The expression of specific genes can be controlled at both the DNA and RNA levels (Figure 1).The development of drugs with the ability to target nucleic acids (DNA, RNA) for degradation is a powerful way to control gene expression. In recent decades, this approach, among others, has been used to treat diseases and in particular cancer. However, drugs acting at the DNA level may have the disadvantage of being mutagenic (for a review see (Gurova 2009)). On the contrary, destroying RNA may have similar effectiveness leaving DNA undamaged. There is a vast array of

current available technologies for the destruction of RNA with therapeutic potential (for a review see (Tafech et al. 2006)). The use of enzymes with ribonucleolytic activity is one of them.

Until recently the efficient turnover of RNA molecules in higher eukaryotes was thought to occur mainly through exonucleolytic activity. This view has been challenged by recent work on endoribonucleases (for a review see (Li et al. 2010)). It is now possible to say that the endonucleolytic cleavage of RNA, as opposed to exonucleolytic decay, probably plays a larger role in RNA metabolism than has been previously imagined.

Pancreatic ribonucleases (RNases) are endoribonucleases (Cuchillo et al. 1997), so their ability to cleave RNA internally and without high sequence specificity make them exceptional candidates for antitumor drugs provided that they can be selectively and efficiently delivered to the cells. In fact, in a recent volume of the *European Journal of Pharmacology* dedicated to novel anticancer strategies, the introductory review (Los 2009) includes a member of the pancreatic RNase family, Onconase (ONC) (Ardelt et al. 1991), among the new, exciting developments in experimental therapies of the early 21st century. Bovine pancreatic ribonuclease A (RNase A, EC 3.1.27.5), the most well-known member of the RNase pancreatic family, both structurally and functionally (for a review see (Cuchillo et al. 1997; Raines 1998)), was the first RNase to be tested in vitro (Ledoux and Baltus 1954; Ledoux and Revell 1955; Ledoux 1956) and in vivo (Ledoux 1955; Ledoux 1955; Aleksandrowicz 1958; Telford et al. 1959) as an anticancer drug. However, contradictory results were obtained (Roth 1963) and RNases were not considered as potential antitumor agents until the discovery of natural RNases with anticancer activities exhibited at much lower concentrations than RNase A. Cytotoxic RNases are not restricted to the pancreatic RNase family. Prokaryotic and eukaryotic microbial RNases (Irie 1997; Yoshida 2001) as well as plant RNases (Matousek 2010) are also cytotoxic enzymes.

Although a significant number of studies have been carried out to elucidate the cytotoxic mechanism of antitumor RNases, the molecular basis is still not well understood. Nevertheless, there is a general consensus about their mechanism of action (for a review see (Makarov and Ilinskaya 2003; Benito et al. 2005; Makarov et al. 2008)), which, in some parts, is shared by most cytotoxins. Briefly, to act as a cytotoxin, an RNase should follow the following steps (Figure 2). It

has to interact with a specific or a non-specific component of the target cell surface in order to be endocytosed, then to follow a productive intracellular route through the different membranous subcellular organelles. From one of these organelles it has to translocate to reach the cytosol. Once there, it has to be stable enough and keep its ribonucleolytic activity in order to degrade RNA and induce cell death. In the cytosol, there are three different ways for an RNase to preserve its ability to cleave RNA: first, to be insensitive to the ribonuclease inhibitor protein (RI) present in the cytosol of all mammalian cells (Dickson et al. 2005); second, to saturate the RI, meaning that enough protein reaches the cytosol to leave free RNase molecules (Leich et al. 2007); and third, to be captured by other molecules, which impairs the interaction with the RI (Bosch et al. 2004).

A walk through the antitumor members of the pancreatic RNases will unveil what is known about its cytotoxic mechanism.

2. Natural cytotoxic ribonucleases and their mechanism of action

2.1. Amphibian RNases

Onconase (ONC) and Amphinase (Amph) are two homologous RNases first isolated by Alfacell Corporation from *Rana pipiens* (leopard frog). The former was isolated two decades ago (Ardelt et al. 1991) and the latter much more recently (Singh et al. 2007). Their structure and function and the their cytostatic and cytotoxic mechanisms, either alone or together with other cytotoxic RNases, have been the subject of several reviews (Youle and D'Alessio 1997; Rybak and Newton 1999; Leland and Raines 2001; Matousek 2001; Makarov and Ilinskaya 2003; Saxena et al. 2003; Benito et al. 2005; Arnold and Ulbrich-Hofmann 2006; Ramos-Nino 2007; Ardelt et al. 2008; Arnold 2008; Benito et al. 2008; Lee and Raines 2008; Lu et al. 2008; Ardelt et al. 2009, Altomare 2010). Although the main characteristic of these RNases will be briefly summarized, readers should refer to the reviews mentioned above for more detailed information.

ONC has about 30% sequence identity with RNase A (Ardelt et al. 1991) and a similar fold (V or kidney-shaped), conserves three of the four disulfide bonds of RNase A, and is more compact. The N- and C-termini are blocked by pyroglutamic acid and a disulfide bond respectively (Mosimann et al. 1994). Its compact structure makes it a very stable protein and resistant to proteolysis (Leland et al. 2000; Notomista et al. 2000; Notomista et al. 2001; Arnold et al. 2006;

Schulenburg et al. 2010). Folding studies provide further explanation of this high conformational stability (Pradeep et al. 2006; Gahl et al. 2008; Gahl and Scheraga 2009). ONC is 10^2-10^5-fold less active against polymeric substrates and single-stranded RNA than RNase A (Ardelt et al. 1991; Boix et al. 1996) although it has the same key catalytic residues present in RNase A (Lee and Raines 2003). It possesses two additional active-site residues, Lys9 and an N-terminal pyroglutamic residue (Lee and Raines 2003), The cytotoxic activity depends on its ability to degrade RNA (Ardelt et al. 1991; Wu et al. 1993). Its substrate specificity is different from that of RNase A (Singh et al. 2007) and depends on the structure (Lee et al. 2008).

ONC evades the RI (for a review see (Rutkoski and Raines 2008)), a property not shared by the monomeric members of the pancreatic family, but which is critical for cytotoxicity. The dissociation constant of the complex formed between ONC and RI is about 10^7-fold greater than that of RNase A (Boix et al. 1996). This lower affinity has been attributed to a reduction of the length of some exposed loops responsible for the interaction with the RI (Mosimann et al. 1994; Kobe and Deisenhofer 1996), which precludes the complex formation at physiological salt concentration (Turcotte and Raines 2008). This in vivo evasion is supported by the absence of any effect on ONC cytotoxicity due to either increasing (Haigis et al. 2003) or silencing (Monti and D'Alessio 2004; Dickson and Raines 2009) the intracellular levels of RI. Nevertheless, ONC presents a disulfide bond (30-75), which in the reducing conditions of the cytosol could be cleaved, affecting the ONC-RI interaction (Torrent et al. 2008).

ONC internalization is not completely understood (for a review see (Benito et al. 2008)). Early studies (Wu et al. 1993; Wu et al. 1995) and more recent ones (Haigis and Raines 2003) indicate that ONC enters the cell by energy-dependent endocytosis. Initial uptake takes place through the well-characterized clathrin/AP-2 mediated endocytic pathway (Rodriguez et al. 2007) although a previous study indicated that the entry was not dynamin-dependent (Haigis and Raines 2003). This apparent contradiction might be related to the use of transiently or stably transfected cell lines expressing a dynamin-K44A dominant-negative mutant. The first evidence of the presence of a cell receptor was proposed by Wu et al. (Wu et al. 1993). However, dose-dependent internalization of Oregon-green-labeled D16C-ONC in Hela cells showed a non-saturable process

(Haigis and Raines 2003). It has also been reported that, due to its cationic nature, ONC and, in general, RNases bind to cell surfaces electrostatically, indicating that endocytosis is not mediated by a receptor (Notomista et al. 2006; Johnson et al. 2007). Since the surface of most cancer cells is more electronegative compared to normal cells (Ran et al. 2002), this electrostatic interaction could explain their selectivity. It has recently been shown (Chao et al. 2010) that ONC interacts with membrane glycoproteins that do not seem to mediate a productive internalization. No correlation was apparent in the cell-surface binding, internalization, and cytotoxicity of ONC. Nevertheless, the distribution of cationic residues on the surface of ONC might be responsible for an efficient translocation process, once endocytosed (Turcotte et al. 2009). One major question is from which endocytic vesicle this translocation is produced. The intracellular routing of ONC has been studied using drugs that disrupt intracellular trafficking (Wu et al. 1993; Wu et al. 1995; Newton et al. 1998; Haigis and Raines 2003) and by co-localization studies of fluorescent-labeled ONC with markers of different organelles (Rodriguez et al. 2007). The results indicate that the normal route for ONC to reach the cytosol is through an endosomal compartment, not from the Golgi or endoplasmic reticulum (ER). ONC is localized in the recycling endosomes from where it translocates, again suggesting the existence of a cellular receptor (Rodriguez et al. 2007).

Once in the cytosol, ONC degrades RNA and precludes protein synthesis, inducing cell cycle arrest (initially described at the G1/S checkpoint later found to be cell dependent) and apoptosis (Darzynkiewicz et al. 1988; Deptala et al. 1998; Juan et al. 1998; Smith et al. 1999; Halicka et al. 2000; Iordanov et al. 2000; Grabarek et al. 2002; Tsai et al. 2004). Early in vitro studies identified the rRNAs (28 S and 18 S) as the ONC targets (Wu et al. 1993), but now it is known that the tRNAs are the primary in vivo target of this enzyme (Lin et al. 1994; Iordanov et al. 2000; Suhasini and Sirdeshmukh 2006; Suhasini and Sirdeshmukh 2007). However, ONC-induced apoptosis presents features different from those of an indiscriminate translation inhibition (Iordanov et al. 2000). Indeed, the up- or down-regulation of genes that code for proteins involved cell cycle control, or transcription factors, are observed after ONC cell treatment (Juan et al. 1998; Tsai et al. 2004). Accordingly, two other targets for ONC have been proposed: the mRNA coding for the ubiquitous NF-kB transcription factor either directly or by degrading other RNAs involved in its

turnover (Deptala et al. 1998; Juan et al. 1998; Tsai et al. 2004) and the non-coding RNAs (microRNAs and/or siRNAs) involved in gene expression control (Ardelt et al. 2003; Zhao et al. 2008). In support of these hypotheses, Saxena et al. (Saxena et al. 2009) have reported the degradation of double stranded RNA by ONC.

ONC-induced apoptotic effects are very dependent on the cell type. Thus, some apparently contradictory results are found in the literature. The activation of the stress-activated protein kinase c-Jun NH2 terminal kinase was described as an early event in the induction of apoptosis by ONC (Iordanov et al. 2000). This process seems to promote the activation of pro-caspase-9, -3 and -7, but in the study the involvement of the mitochondrial pathway was not clear (Iordanov et al. 2000). Furthermore, in neuroblastoma cells ONC induces autophagy by lysosomal activation, which leads to caspase-independent apoptosis (Michaelis et al. 2007). However, the typical mechanisms of the intrinsic apoptosis pathway, in addition to an activation of Ser-proteases, were observed in other cell lines (Grabarek et al. 2002; Ardelt et al. 2007). Recently, it has been demonstrated that ONC-induced apoptosis is dependent on Apaf-1 and that ONC enhances apoptosis by reversing the inhibitory effect of the tRNAs on cytochrome c (Mei et al. 2010). These results could explain the synergistic effects with drugs that elicit the intrinsic apoptosis pathway. In addition, since tRNA expression is enhanced in tumor cells, its degradation could provide an explanation for ONC selectivity (Mei et al. 2010).

The Amph variants present most of the characteristics of ONC, however their catalytic efficiency, their substrate specificity, the N-terminal end, and the glycosylation state are different (Singh et al. 2007; Ardelt et al. 2008). All the variants present cytostatic and cytotoxic activity patterns that, to a certain extent, resemble those of ONC (Ardelt et al. 2007). A recombinant form of an Amph that is as active as the natural enzyme suggests that the glycans are not involved in the cytotoxicity (Singh et al. 2007).

Tumoricidal ribonucleases have been also found in *Rana catesbeiana* (RC-RNase) and *Rana japonica* (RJ-RNase) oocytes. They present about 50% sequence identity with ONC, high stability, the same number of disulfide bonds, and both are pyrimidine base-specific RNases and do not present detectable carbohydrate moieties (for a review see ((Youle and D'Alessio 1997; Irie

et al. 1998)). The structure is only known for RC-RNase and resembles that of ONC (Chang et al. 1998). Both are sialic acid-binding lectins that specifically agglutinate cancer cells (Sakakibara et al. 1979; Okabe et al. 1991; Nitta et al. 1994) binding to cell membrane glycoproteins with a high content of sialic acids (Sakakibara et al. 1979; Nitta et al. 1994). By contrast, ONC has been reported not to cause tumor cell agglutination (Ardelt et al. 1991) and shows minimal interaction with cell-surface GAG and sialic acid-containing proteins (Chao et al. 2010).

Preclinical studies not directly related to the elucidation of cytotoxic mechanisms have been carried out with amphibian RNases. ONC presents synergy, proved in vitro and/or in vivo, with a significant number of compounds including: tamoxifen (Mikulski et al. 1990; Mikulski et al. 1992), lovastatin (Mikulski et al. 1992; Mikulski et al. 1992), cis-platin (Mikulski et al. 1992; Lee et al. 2007), AEBS/H_{IC}-binding drugs (Mikulski et al. 1993), vincristine, which was independent of P-gp expression (Rybak et al. 1996), proteasome inhibitors (Mikulski et al. 1998), interferons (Deptala et al. 1998; Vasandani et al. 1999), doxorubicin (Mikulski et al. 1999), small molecule inhibitors of PI3-K (Ramos-Nino et al. 2005), cepharanthine (Ita et al. 2008), and rosiglitazone (Ramos-Nino and Littenberg 2008). Interestingly, ONC-induced apoptosis is independent of the p53 tumor-suppressor protein status (Iordanov et al. 2000). In addition, ONC decreases the content of reactive oxygen intermediates (ROI), which may be an important element of its cytotoxicity towards cancer cells (Ardelt et al. 2007). In vivo studies, using animal models, proved the efficacy of ONC in tumor suppression and prolonged survival (Mikulski et al. 1990; Lee et al. 2000; Lee and Shogen 2008). In vitro, ONC induces cellular radiation sensitivity by inhibiting cellular oxygen consumption (Lee et al. 2000). In vivo, it changes tumor physiological parameters, significantly increasing tumor oxygenation (Lee et al. 2003). Because of that, ONC acts as a radiation sensitizer (Kim et al. 2007; Lee et al. 2007). Apart from the positive effects, the main drawback of ONC is longer retention in the kidneys, which promotes nephrotoxicity, although it is reversed after drug withdrawal (Vasandani et al. 1996; Vasandani et al. 1999). In addition, in vitro and in vivo assays have shown that ONC has stronger aspermatogenic, embryotoxic and immunotoxic activity than other pancreatic RNases (Matousek et al. 2003) and that it also displays significant neurotoxicity when injected intracranially (Slager et al. 2009).

2.2. Bovine seminal ribonuclease

Bovine seminal ribonuclease (BS-RNase) is the only natural dimeric member of the pancreatic-type RNases. The enzyme was first discovered in 1963 in bull seminal plasma (for a review see (D'Alessio et al. 1991; D'Alessio et al. 1997)). Each subunit presents 83% amino acid sequence identity to RNase A and a similar fold. They are linked by two disulfide bonds (Mazzarella et al. 1993). BS-RNase exists as a mixture of two distinct forms, M=M and MxM. The latter swaps its N-terminal α-helices (for a comparative review on the structures see (Benito et al. 2008)) and at equilibrium exists in slight molar excess (Piccoli et al. 1992).

BS-RNase conserves the catalytic residues of RNase A, although its dimeric structure endows it with allosteric properties (Piccoli et al. 1988), the ability to efficiently degrade dsRNA (Libonati and Floridi 1969), and the RNA moiety of RNA:DNA hybrids (Taniguchi and Libonati 1974; Libonati et al. 1975). The quaternary structure of BS-RNase is also responsible for its antitumor activity (for a review see (Youle and D'Alessio 1997)). Enzymatic activity and the quaternary structure are necessary for its cytotoxicity (Vescia et al. 1980; Kim et al. 1995; Antignani et al. 2001). The structure of the complex between RNase A and RI (Kobe and Deisenhofer 1996) shows that a BS-RNase-RI complex is not possible, due to steric hindrance, while a single chain subunit is strongly inhibited (Murthy and Sirdeshmukh 1992). It is not clear whether the dimeric structure is maintained in the reducing conditions of the cytosol. Cells in which RI has been silenced are more sensitive to BS-RNase action (Monti and D'Alessio 2004). Nevertheless, it has been shown that the presence of RNA as a substrate stabilizes the MxM reduced form of BS-RNase (Murthy et al. 1996), and may be counterbalance the conversion of the MxM form to the M=M form due to monomer sequestering by RI .

Unlike frog RNases, results denying the existence of a cellular receptor for BS-RNase are more convincing. BS-RNase binds to the extracellular matrix (ECM) of different cell lines and this interaction seems to be important for the cytotoxic effect (Mastronicola et al. 1995; Bracale et al. 2002) but it does not bind to cell membranes, suggesting that it enters the cell by adsorption mechanisms (Kim et al. 1995). Alternatively, it was proposed that BS-RNase binds to the cell membrane through sulfhydryl-disulfide interchange reactions between cell surface sulfhydryls and

the disulfides that link both subunits (Bracale et al. 2003). However, this hypothesis is not sustained by results that demonstrate that a semisynthetic enzyme remains dimeric and conserves the cytotoxic properties of BS-RNase (Kim et al. 1995).

BS-RNase has been localized in endosomes and its cytotoxicity is blocked when this energy-dependent mechanism is inhibited (Bracale et al. 2002). The intracellular routing of BS-RNase, studied using drugs that disrupt this traffic and immunofluorescence methods, show that it is localized in the trans-Golgi network of treated malignant cells, suggesting that this organelle, but not the ER, is an effective site for translocation and provides an explanation for its selectivity (Wu et al. 1995; Bracale et al. 2002). BS-RNase can destabilize artificial membranes (Mancheno et al. 1994; Notomista et al. 2006) and it is tempting to speculate that this mechanism is used to permeate the trans-Golgi network membranes, allowing BS-RNase to reach the cytosol. The main targets described for BS-RNase are the rRNAs (Mastronicola et al. 1995) resulting in protein synthesis inhibition and subsequent apoptosis. Interestingly, BS-RNase has also been found in the nucleolus of malignant cells (Bracale et al. 2002; Viola et al. 2005). It is not known how the enzyme reaches the nucleus or the significance of this localization. However, a correlation has been shown between cytotoxicity and a decrease of telomerase activity, as well as levels of RNA associated with the enzyme in proliferating cells treated with BS-RNase (Viola et al. 2005). Therefore, telomeric RNA might be another target of BS-RNase.

BS-RNase induces apoptosis in a dose-dependent manner in a wide range of cells and reduces tumor growth in vivo (Marinov and Soucek 2000; Sinatra et al. 2000). It was shown to be selectively toxic to human neuroblastoma cell lines with or without the MDR phenotype (Cinalt et al. 1999) and to thyroid cancer cells. In vivo it induces significant tumor regression and shows no cytotoxicity for healthy mice (Kotchetkov et al. 2001; Spalletti-Cernia et al. 2003; Spalletti-Cernia et al. 2004). The apoptotic mechanism has been studied in thyroid carcinoma cells and is associated with the activation of caspase-8, -9 and -3 accompanied by a reduced phosphorylation of Akt/protein kinase B (Spalletti-Cernia et al. 2003).

2.3. Eosinophil cationic protein (ECP)

Activated eosinophils release toxic proteins among which ECP and eosinophil-derived neurotoxin (EDN) are members of pancreatic-type family (for a review see (Venge and Bystrom 1998; Boix 2001). ECP is a potent cytotoxic protein able to kill normal and malignant mammalian cells (Motojima et al. 1989; Maeda et al. 2002; Carreras et al. 2005), non-mammalian cells such as parasites and bacteria, as well as viruses (for a review see (Boix et al. 2008)). The antipathogen capacities have classified ECP as a human host defense RNase involved in inflammatory processes mediated by eosinophils (see Chapter XX). Although not described as an antitumor agent, its RNase nature and potent cytotoxicity merits a brief description.

ECP is a highly stable protein (Maeda et al. 2002; Nikolovski et al. 2006). Its structure has been solved by x-ray crystallography (Boix et al. 1999; Mallorqui-Fernandez et al. 2000) and in solution (Laurents et al. 2009), and corresponds to the "RNase A fold", although it has low ribonucleolytic activity (Barker et al. 1989). The relationship between the cytotoxic effect of ECP and its RNase activity is controversial. While its ability to kill bacteria and parasites (Rosenberg 1995) and some mammalian cells (Navarro et al. 2008; Chang et al. 2010) does not depend on the ribonucleolytic activity, the neurotoxic and antiviral activities do (Durack et al. 1979; Domachowske et al. 1998).

ECP induces apoptosis in mammalian cells (Navarro et al. 2008; Chang et al. 2010; Navarro et al. 2010) in a cell-dependent manner (Benito et al. 2008). In the human bronchial epithelial cell line, Beas-2B, ECP is internalized following a clathrin-and caveolin-independent but lipid raft-dependent macropinocytosis, being the cell surface-bound heparin sulfate its major receptor (Fan et al. 2007). Recently, the ECP heparin binding affinity (Fan et al. 2008) has been shown to depend on the catalytic sites (Torrent et al. 2010). In Beas-2B, ECP causes apoptosis mainly through the TNF-α –mediated caspase-8 specific pathway in a mitochondria independent manner (Chang et al. 2010). In contrast, apoptosis does not require internalization in human acute promyelocytic leukemia HL-60, cervix adenocarcinoma HeLa cell lines, and primary cultures of cerebellar granule cells and astrocytes. ECP binds and aggregates on the cell surface, altering membrane permeability, which results in a modification of the ionic intracellular equilibrium (Navarro et al. 2008; Navarro et al. 2010). The membrane destabilization promoted by ECP, using

synthetic lipid vesicles as membrane models, is mediated by a carpet-like mechanism (Torrent et al. 2007).

2.4. Ribonucleases of different origins

Over the years, not only the pancreatic-type RNases have drawn increasing attention due to their remarkable antitumor properties but RNases purified from multiple origins. Representative RNases of fungal, bacterial and plant origin have also shown their medicinal potential in the treatment of tumors. Among them, the most promising RNases are mushroom RNases (Ng 2004; Wong et al. 2010), α-sarcin from *Aspergillus* (Olmo et al. 2001), binase and barnase, two well-known T1 ribonuclease members from *Bacillus* (Makarov et al. 2008), RNase Sa3 from *Streptomyces* (Sevcik et al. 2002), and ginseng RNases (Fang and Ng 2010). Besides, RNases from wheat leaf, mung bean, black pine pollen, tomato and hop have been reported to exhibit antitumor activities (Skvor et al. 2006; Soucek et al. 2006; Lipovova et al. 2008; Matousek 2010; Matousek et al. 2010). However, future studies on these RNases with prominent medicinal activities that would open a new perspective for them as potential antineoplastic drugs and their translation from the bench to the clinic are still needed. Fast developing protein engineering of these RNases, which display more potent cytotoxic activity and greater selectivity for malignant cells, is now the aim of researchers.

3. Approaches to endow a ribonuclease with cytotoxic properties

The discovery of the natural RNases described above has stimulated the construction over the past 15 years of other cytotoxic RNases with enhanced properties. The main objectives of these efforts have been to increase tumor cell selectivity and to construct more powerful antitumor drugs that could bypass the main drawbacks of ONC, which are its renal toxicity (Vasandani et al. 1996; Vasandani et al. 1999) and its non-human origin. In addition, RNases are low molecular mass proteins that are rapidly cleared from the organism (Maack et al. 1979).

3.1. Monomeric ribonucleases

The cytotoxic pathway of RNases, described in the introduction section, has inspired different strategies to endow an RNase with cytotoxic activity. Researchers have tried to make a

particular RNase more efficient to carry out some steps of this model in order to obtain more potent cytotoxins.

The first artificial monomeric RNase to be described was constructed by Raines and coworkers. In this seminal work changes were rationally introduced in the RNase A sequence to decrease its affinity for the RI (Leland et al. 1998). They replaced residue Gly88, which was positioned in a hydrophobic pocket of the RI in the three-dimensional structure of the complex (Kobe and Deisenhofer 1996), by different bulky charged residues (Arg or Asp). The replacement by Arg produced an RNase with a 10^4-fold less affinity for the RI, which was approximately only 20-fold less cytotoxic than ONC. The success of this approach was confirmed using other monomeric RNases like HP-RNase (Gaur et al. 2001; Leland et al. 2001) or monomeric BS-RNase (Antignani et al. 2001; Lee and Raines 2005). Further mutations were introduced in RNase A and HP-RNase to disturb their electrostatic attraction for the RI (Bretscher et al. 2000; Haigis et al. 2002; Rutkoski et al. 2005; Johnson et al. 2007) obtaining new variants with an increased cytotoxicity. The substitutions D38R/R39D/N67R/G88R in RNase A yielded a variant that maintained ribonucleolytic activity and conformational stability but had 5.9×10^9-fold lower affinity for RI. This variant showed a cytotoxicity that was nearly equal to that of ONC (Rutkoski et al. 2005). However, when creating a new RNase variant it is difficult to discern how a specific mutation may affect other characteristics important for the cytotoxicity. An illustrative example refers to variants evading the RI. In some cases, the introduced mutations produced variants that were more stable or that had less affinity to the RI but also were less catalytically active and therefore, the increase in cytotoxicity was minor (Bretscher et al. 2000; Dickson et al. 2003).

An alternative strategy to bypass the RI action has been to redirect the RNase to the nucleus, which is believed to be devoid of this inhibitor (Roth and Juster 1972). An HP-RNase variant, named PE5, which carries a non-contiguous extended bipartite nuclear localization signal (NLS), has been reported (Rodriguez et al. 2006). This NLS is constituted by at least three different regions of the protein comprising residues 1, 31–33 and 89–91. This variant recognizes α-importin (Rodriguez et al. 2006) and cleaves nuclear but not cytoplasmic RNA in vivo. In addition, the modification of residues important for this NLS significantly decreases the variant's cytotoxicity

(Tubert et al. 2010). All these results show that this NLS endows this HP-RNase variant with cytotoxic activity (Bosch et al. 2004; Tubert et al. 2010). The variant is internalized and reaches the cytosol where it can interact with the RI and with the α-importin. It is likely that the concentrations of RI and α-importin are similar in the cytosol (RI concentration is 4 μM (Haigis et al. 2003) and α-importin concentration is 3μM in Xenopus oocytes (Gorlich et al. 1994)), thus the affinity of the RNase 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 (Figure 2).

The Gly88R RI-evading RNase A variant described above was modified by the addition of cystine bonds in order to increase its conformational stability (Klink and Raines 2000) resulting in a more cytotoxic variant. A different strategy to increase the stability and therefore the toxicity is glycosylation of the protein; the expression of ONC in *Piscia factoris* produces a glycosylated protein at Asn69 which is more stable, more resistant to protein degradation and also is 50-fold more cytotoxic (Kim et al. 2004).

The efficiency of internalization is another important determinant of cytotoxicity (Wu et al. 1995; Leich et al. 2007). One strategy to increase the internalization efficiency of the RNases is their cationization by chemical or genetic modification. This strategy is based on the rationale that a more efficient internalization could be achieved by using electrostatic interactions to efficiently adsorb highly cationic proteins into the negatively charged surface. It has been reported that the chemical modification of the carboxyl 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 (Futami et al. 2001; Iwama et al. 2001; Futami et al. 2002). The enhancing effect was dependent on the increase in positive net charge and the higher cationic variants were more efficiently internalized into the cells. In addition, the preparation of RNase A and non-cytotoxic cross-linked dimers of RNase A, both covalently linked to polyspermine to increase their basicity, has also been described (Pouckova et al. 2007). In this case, only the dimeric structures, which were much more basic, slightly increased the cytotoxicity exerted by the free polyspermine (two-fold increase). In some cases, the chemical

modifications seriously compromised the ribonucleolytic activity of the modified enzyme (Futami et al. 2001; Futami et al. 2002) and were not specific, generating heterogeneous products that would be difficult to use as antitumor drugs. In any case, the effect of cationization has been confirmed by site-directed mutagenesis. In RC-RNase variants where Asp or Glu residues were replaced by Asn, Gln or Arg (Ogawa et al. 2002), antitumor activity and internalization was enhanced. In addition, the replacement of acidic residues by positively charges residues increased the cytotoxicity of *Streptomyces aureofaciens* RNase Sa (Ilinskaya et al. 2002; Ilinskaya et al. 2004). However, it has been also shown that promoting the internalization of pancreatic RNases by introducing positive charges into the molecule can be counterbalanced by an increased affinity to the anionic RI in the cytosol of the resulting variants (Johnson et al. 2007). To improve the internalization Raines and colleagues (Fuchs et al. 2007) also replaced two residues of a cytotoxic variant of RNase A to create a patch of Arg residues on its surface. The cytotoxicity of the resulting variant was slightly improved (three-fold increase). On the other hand, the addition of a protein translocation domain (nona-arginine) to improve the translocation of the RNase to the cytosol has been shown to increase the cytotoxicity of previously cytotoxic RNase A variants (Fuchs and Raines 2005; Fuchs et al. 2007). Recently, it has been reported that an alternative strategy to enhance the cellular internalization of the RNases consists in co-treating cells with a cationic 2 poly(amidoamine) dendrimer (Ellis et al. 2010). This treatment increases the cytotoxicity of the RNase probably by increasing endosomal escape. In addition, it avoids the deleterious consequence of decreasing ribonucleolytic activity or conformational stability observed upon cationization of the RNases.

The engineering of RNases to improve their intracellular pathway has not been extensively explored, and the studies conducted until now have not given positive results. It has been shown that the introduction of a Lys-Asp-Glu-Leu consensus sequence to direct BS-RNase to the endoplasmic reticulum significantly reduces its cytotoxicity (Bracale et al. 2002)). The alteration of intracellular routing has been also assayed with the Gly88R RI-evading RNase A variant to diminish its routing to the lysosomes and therefore favor the translocation of more protein. This variant carries a Lys-Phe-Glu-Arg-Gln sequence (residues 7-11) that targets it for lysosomal

degradation. However, the replacement of Lys7 with Ala had no effect on the cytotoxicity (Haigis et al. 2002).

Finally, formulations for improving tissue delivery have also been described. ONC has been encapsulated in biodegradable poly(ricinoleic-co-sebacic acid) for local controlled delivery in the parietal lobe of the brain in an attempt to overcome cerebellar neuronal toxicity while affecting glioma cells (Slager et al. 2009). ONC was released in a controlled manner and was cytotoxic against 9L glioma cells xenografted into the brain, while evading neurotoxicity in the cerebellum.

3.2. Oligomeric and tandem ribonucleases

BS-RNase has inspired the generation of other cytotoxic RNases through the formation of oligomeric structures that hinder the binding of the RI by steric hindrance (for a review see (Libonati 2004; Libonati et al. 2008)). The cytotoxicity of BS-RNase seems to be restricted to the swapped form (Cafaro et al. 1995; Mastronicola et al. 1995), which likely remains dimeric in the reductive conditions of the cytosol. However, the reasons for the cytotoxicity are not completely understood and the activity of different variants seems to contradict this model. For example, a dimeric HP-RNase variant in which the enzyme had been engineered to reproduce the sequence of BS-RNase helix-II and to eliminate the Glu111 charge on the surface was highly cytotoxic (Merlino et al. 2009) but this activity was also associated with the unswapped form of the protein. Moreover, RNase A dimeric variants covalently linked by two Cys at positions 31 and 32 with swapping propensities ranging between 14 and 60% are poorly cytotoxic (Ercole et al. 2009). This shows that other features must be needed for a dimeric RNase to be cytotoxic. Nevertheless, the design of RNase oligomers is attractive since they present more positive charges and therefore must interact more tightly with the anionic surface of cancer cells and, in most cases, they cleave dsRNA. Furthermore, the increase in molecular mass could help to avoid the clearance of the drug from the organism (Bartholeyns and Moore 1974).

Cytotoxic HP-RNase (Piccoli et al. 1999; Di Gaetano et al. 2001) and RNase A (Di Donato et al. 1994) variants have been created by introducing some of the residues of BS-RNase that are believed to be important for the swapping process. In all these cases, two disulfide interchain bonds were established between the Cys introduced at positions 31 and 32. The deletion of five

residues of the loop that connects the N-terminal α-helix of HP-RNase with the rest of the protein produced a dimeric enzyme (Russo et al. 2000) but until now no cytotoxicity data have been reported.

Other covalent linkers have been proposed to stabilize the RNase dimeric structure. Dimers of RNase A cross-linked with dimethyl suberimidate have been shown to display an antitumor action both in vitro and in vivo (Bartholeyns and Baudhuin 1976; Tarnowski et al. 1976). Higher oligomers cross-linked with the same strategy displayed higher cytotoxicity in vitro (Gotte et al. 1997). These variants were heterogeneous and more specific cross-linking strategies were subsequently evaluated. They were based on the introduction of additional Cys resudues to provide reaction sites for specific chemical reagents. Raines and colleagues (Kim et al. 1995) introduced a thioether bond between both Cys31 residues of BS-RNase to increase the dimer stability but they did not obtain an increase in cytotoxicity. On the other hand, the formation of covalent dimers by the introduction of a thioether bond between the cysteines of the RNase A Cys89 variant or the EDN Cys87 variant produced cytotoxic enzymes (Suzuki et al. 1999). Finally, an exhaustive evaluation of cross-linkers and of sites introducing the additional cysteine has recently been reported (Rutkoski et al. 2010). Some of these constructs were as effective as monomeric RNase A variants that highly evade RI to inhibit tumor growth.

The construction of recombinant proteins with duplicated RNase genes is an alternative strategy to covalently link two RNase units. Arnold and colleagues (Leich et al. 2006) constructed tandem dimers of RNase A in which it was expected that one unit of the RNase tandem enzyme should remain unbound due to steric hindrance, but that the other could be trapped by the RI. Surprisingly, the tandem construction, although fully inhibited by the RI, was cytotoxic. As stated above, interpretations other than RI inhibition can explain the acquisition of cytotoxic properties of oligomeric or multimodular RNases.

3.3. Targeted ribonucleases

Most of the natural RNases are devoid of innate anticancer activity and have not evolved mechanisms for efficiently entering and killing cells. However, nontoxic RNases can be linked to targeting molecules and acquire or perform cell type-specific cytotoxic activity. RNase-based

targeted therapeutics, which have been developed in parallel with the so-called smart drugs or targeted drugs together with monoclonal antibodies (mAb) and recombinant antibody technology, will be the aim of this section. Figure 3 shows schematically the strategies to target RNases by conjugation or fusion either to antibodies or ligands described below.

3.3.1. Chemical RNase conjugates

One approach to improve the therapeutic efficacy of a drug is to combine a targeting molecule (hormone, interleukins, antibody, etc.) with the effector moiety (radioisotope, toxin, drug-activating enzyme, etc.) in the same molecule. In particular, the combination of the antigen-specific targeting abilities of antibodies with the toxicity of a payload toxin from plants such as ricin from *Ricinus communis,* or bacterial enzymes such as diphtheria toxin from *Corynebacterium diphtheria* or *Pseudomonas* exotoxin A, are referred to as 'immunotoxins' (Pastan et al. 1986) (for review see (Wu and Senter 2005; Dübel 2007)).

The most promising diphtheria toxin conjugates are the human transferrin receptor (TfR) specific CRM107 conjugate (Johnson et al. 1989) and the CD3 specific A-dmDT390-bisFv(UCHT1) (Woo et al. 2008), which has entered phase I/II clinical trials. Also, ricin, a ribosome inactivating protein from plants, has been thoroughly used as an immunotoxin payload but it did not progress further than phase I trials due to product heterogeneity and individual differences in patients' responses (Messmann et al. 2000). These immunotoxins demonstrated a potent in vitro tumor cell killing activity, but when applied in human patients they caused toxic side effects and immunological antidrug responses that limited their therapeutic potential.

In addition to the non-specific binding and toxicity of immunotoxins using microbial or plant toxins that have resulted in several fatalities in clinical trials, the strong immunogenicity of the toxic heterologous compounds disallows repeated or long-term clinical applications and even short-term treatment requires prophylactic protocols. Although some successful clinical results may arise after continued refinement of the engineered plant and bacterial toxins, an alternative to all these problematic approaches is offered by the use of pancreatic-type RNases.

The tumor targeting of RNases was first demonstrated by the chemical conjugation of RNase A to transferrin (Tf) or mAb against the TfR (Rybak et al. 1991) and against the T cell

antigen CD5 (Newton et al. 1992). Soon after, ONC was compared to RNase A as a conjugated cargo of anti-TfR mAb 5E9 (Rybak et al. 1993) (for review see (Rybak 2008)). The anti-TfR-RNase A conjugates were comparable in vivo to a ricin A chain conjugate, although in vitro results had shown that the RNase conjugate was much less efficient. These studies also showed that the antibody conjugates were more efficient than the Tf conjugates, due likely to an enhanced binding to the cell and consequent internalization. Interestingly, the antibody conjugates linked by a reducible disulfide bond to ONC and RNase A were equally potent in the nanomolar range, even though ONC, which is cytotoxic in the micromolar range (0.1-0.8 μM for the majority of tested cell lines (Haigis et al. 2003)), is several orders of magnitude more cytotoxic than RNase A when assayed as non-conjugated drugs. In addition, while ONC did not change its internalization pathway, it is still not known whether RNAse A evades the cytosolic RI, or saturates it. ONC has also been conjugated to the CD22 specific mAb LL2 and RFB4, which resulted in a many thousand-fold increase in cytotoxicity comparable to the specificity and potency of anti-CD22 immunotoxin conjugates with a plant or bacterial toxin payload (Newton et al. 2001), confirming that RNases are as potent as these toxins when properly targeted.

ONC has also been conjugated to P-glycoprotein (P-gp) neutralizing mAb MRK16. When investigated against MDR1 overexpressing human carcinoma cells in vitro and in vivo it was observed that the ONC conjugate increased its cytotoxicity and sensitized the multidrug-resistant cancer cells to vincristine in vivo (Newton et al. 1996). Although P-gp is not a rapidly internalizing transmembrane protein, the increase in cytotoxicity and the vincristine sensitizing effect of the MRK16-ONC conjugates could be explained by the P-gp cell-binding enhancement of the mAb, which at the same time diminishes the drug-expelling activity of the P-gp, and the internalizing capabilities of ONC by itself.

3.3.2. RNase fusion proteins and immunoRNases

The progress of recombinant antibody engineering and fusion protein technology has led to the rapid expansion of drug-targeting approaches including the development of an antibody-RNase fusion protein.

Recombinant antibody technology (Dübel 2007) has been used to improve novel antibody formats and drug-targeting devices with superior antigen binding, pharmacokinetic, and effector and production properties. The intended therapeutic purpose strongly influences the choice of a particular antibody format. Features such as molecular size, valence, additional domains or chemical modifications must be carefully considered to achieve the most selective targeting, pharmacokinetic, and therapeutic efficacy.

Generally speaking, small antibody or antibody fusions penetrate and distribute best into solid tissues and tumors. However, these constructions disappear from the blood circulation faster and, thus, have a reduced serum half-life. It has been proposed that engineering therapeutic molecules with a molecular size between 60-120 kDa provides the best equilibrium between tumor penetration and longest half-life (Hudson and Souriau 2003). As a proof of the concept, bivalent antibodies have shown remarkable enhanced tumor retention in comparison to monovalent counterparts (for a review of antibody architectures used in drug targeting see (Rybak and Newton 2007).

Several members of the RNase A superfamily have been used either as a scaffold onto which a targeting domain is engineered or fused to a targeting antibody. When choosing a particular vertebrate RNase, one has to consider that human RNases are believed to have the least immunogenic payload (De Lorenzo et al. 2004);(Menzel et al. 2008) and also that RNases may display functions other than ribonucleolytic activity. Additionally, the connecting linker and the orientation of the RNase and antibody fragment have to be taken into account. For instance, ANG could promote remarkably enhanced selective tumor cell killing provided that the endothelial cell binding and angiogenic activity is maintained, which to a certain extent depends on the C-terminal residues of the molecule, while for amphibian RNases the pyroglutamyl N-terminal residue has been proved essential.

Regarding all the available clinical data for ONC (see sections 1 and 4 in this chapter), this amphibian RNase seems to be the most promising effector to use as an antibody payload, but constructs using HP-RNase or ANG or engineered variants have also shown potential for tumor therapy. Antibody RNase fusions using HP-RNase, ANG or EDN have been tested in experimental

sets targeting the TfR and have shown approximately 10^3 times more potency than the respective chemical antibody RNase conjugates (Rybak et al. 1992; Newton et al. 1994). However the $(Fab)_2$-like CH2-ANG immunoRNase fusion construct exhibited serious production difficulties in myeloma cells with a yield of only 5 ng/mL. Yet, the same fusion protein could be produced in mammary glands of transgenic mice with a final yield of 0.8 mg/mL milk (Newton et al. 1999), indicating that yield is also dependent on the expression system used.

Several studies have investigated different immunoRNase designs to overcome production concerns and to extend this approach to more promising clinical targets, using lineage antigens expressed on certain types of leukemia but not present on hematopoietic stem cells such as CD22 and CD30, or in particular internalizing tumor antigens such as ErbB-2 (Schirrmann et al. 2009).

Chemical conjugates combining mice anti-CD22 mAbs and RNases have shown great potential as novel antitumor drugs. In animal models, these immunoRNases reduced tumors size with high efficacy while not showing appreciable toxicity. However, their heterogeneity affected their binding capacity, caused lot-to-lot variability, and prevented further development. As an alternative, ANG, HP-RNase or RapLRI (Rana pipiens liver RNase I), 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 by grafting the complementarity-determining regions of the clinically established mAb RFB4 into consistent human scFv scaffolds (Arndt et al. 2005; Krauss et al. 2005; Krauss et al. 2005). Among them, CD22 specific ANG-scFv(RFB4) immunoRNase was successfully produced in a mammalian myeloma cell line without degradation, and exhibited potent cytotoxicity with an IC_{50} in the nanomolar range (Krauss et al. 2005). Since bivalency improves retention to the tumor location (Hudson and Souriau 2003), dimeric second generation derivatives were generated from monovalent first generation anti-CD22 immunoRNases to improve the cytotoxicity and pharmacokinetics. Hence, scFv fragments LL2 and humRFB4 were engineered into a diabody format, fused either to ANG or RapLRI, and successfully produced in *E.coli* cells. Bivalent anti-CD22 immunoRNases showed a markedly superior cytotoxicity towards CD22+ tumor cells when compared with monovalent counterparts due to improved antigen binding by avidity effect and enhanced internalization (Arndt et al. 2005; Krauss et al. 2005).

Also, different CD30 targeting approaches fusing HP-RNase or ANG to CD30-specific murine or human scFv have been developed. While scFv(Ber-H2)-HP-RNase fusion protein produced in insect cells inhibited tumor growth in vivo and in vitro (Braschoss et al. 2007), the entirely human bivalent immunoRNase ScFv-Fc-HP-RNase, consisting of human scFv, human IgG1 Fc part, and HP-RNase, showed better properties and inhibited growth of CD30+ Hodgkin lymphoma cells with an IC50 of 3 nM (Menzel et al. 2008). Even better results were observed for immunoRNases produced in HEK293T cells resulting from the fusion of CD30-specific scFv Ki4 to ANG which inhibited the growth of CD30+ lymphoma cells in vitro with an IC50 of 0.5 nM (Stocker et al. 2003).

The first entirely human antibody RNase fusion protein consisting of an ErbB-2 specific human scFv antibody fragment fused to HPR was described by D'Alessio and co-workers (De Lorenzo et al. 2004). The human anti-ErbB2 single-chain variable fragment, Erbicin, specifically distinguished ErbB2-positive cells with high affinity and was internalized upon specific antigen recognition by ErbB2-expressing cells. Erbicin strongly inhibited receptor autophosphorylation and displayed strong inhibitory activity on the growth of ErBB2-positive cell lines. The combination of these properties with the ribonucleolytic activity of HP-RNase, resulted in a remarkable reduction (86%) of ErbB2-positive tumors in mice. Although the immunoRNase was inhibited by the cytosolic RNase inhibitor to an extent comparable to the free HP-RNase (De Lorenzo et al. 2007), the amounts of immunoRNase entering the cytosol saturated the endogenous RNase inhibitor present in this cellular compartment. Based on bivalent immunoRNases being more powerful than monovalent ones, a dimeric variant of HP-RNase was fused to two Erbicin molecules, one per subunit (Riccio et al. 2008). The new immunoRNase, Er-HHP2-RNase, was found to selectively bind ErbB2-positive cancer cells with an increased avidity with respect to monovalent anti-ErbB2 scFv- HP-RNase, and exerted a more powerful cytotoxic activity, likely due to an increased RNase inhibitor evasion. Recently the human anti-tumor immunoconjugate engineered by the fusion of Erbicin with human RNase has been assayed on trastuzumab-resistant cells, and it has proven to be selectively cytotoxic for ErbB2-positive cancer cells both in vitro and vivo; targets an ErbB2

epitope different from that recognized by trastuzumab; and does not show cardiotoxic effects (Gelardi et al. 2010).

Barnase manifests potent antitumor activity, but toxicity to the host cells limits its potential clinical application. Deyev and co-workers established a new plasmid for eukaryotic expression of a scFv 4D5-dibarnase, which consists of two barnase molecules fused serially to the single-chain variable fragment (scFv) of humanized 4D5 antibody. The 4D5 antibody is directed against the extracellular domain of human epidermal growth factor receptor 2 (HER2) and could assist the delivery of barnase to HER2/neu-positive cells and facilitate its penetration into the target cells (Glinka et al. 2006). They further evaluated its antitumor activity and toxicity in mice bearing HER2-overexpressing human breast cancer xenografts. This immunotoxin scFv 4D5-dibarnase manifested a specific apoptosis-associated cytotoxic effect on HER2-overexpressing SKBR-3 and BT-474 human breast carcinoma cells in vitro, and a significant inhibition of human breast cancer xenografts in nude mice without severe side effects (Balandin et al. 2009).

Apart from being used as antibody payload, recombinant RNases have been either fused or chemically conjugated to various internalizing cell-binding ligands such as luteinizing hormone-releasing hormone (LHRH) (Gho and Chae 1999), basic fibroblast growth factor (bFGF) (Suzuki et al. 1999), epidermal growth factor (EGF) (Jinno et al. 1996; Jinno et al. 1996; Psarras et al. 1998; Yoon et al. 1999; Jinno et al. 2002), or interleukin 2 (IL2) (Psarras et al. 2000). Although most of them overcame the concern of RNase inhibition when translocated into the cytosol and were cytotoxic to different tumor cell lines, the different constructs did not selectively target tumor cells.

4. Clinical development

As mentioned above, ONC is the first RNase that reached clinical trials. Although most of them are completed, the full results have not yet been published (http://www.cancer.gov/). Table 1 summarizes the clinical trials performed with RNases and some of the main published results. The reader is addressed to the following reviews for more detailed information (Costanzi et al. 2005; Favaretto 2005; Pavlakis and Vogelzang 2006; Ramos-Nino 2007; Beck et al. 2008; Lee 2008; Porta et al. 2008).

The major advances of ONC have been in the treatment of unresectable malignant mesothelioma (UMM) (confirmatory Phase IIIb). However, in the annual report of the Alfacell Corporation (2009) (at present Tamir Biotechnology Inc.) (http://www.alfacell.com/annualreport2009.pdf), the company indicates that it decided not to pursue further clinical trials for the treatment of UMM based on recommendations of the FDA. The ongoing trials are now focused on the treatment of patients with non-small cell lung cancer.

It is remarkable that an engineered HP-RNase that evades the RI (Evade™ Ribonuclease family from Quintessence Biosciences Inc; http://www.quintbio.com/whoIs.asp) is now in Phase I of clinical trials. This RNase of human origin presents a new challenge for research into the use of these enzymes as chemotherapeutics for cancer treatment.

5. Conclusions

Modern pharmacological anticancer drugs are no longer focused on “small molecules” and the pharmaceutical industry today is searching for a second line of cancer chemotherapeutics without genotoxic effects. In addition, it is exploring different options like cellular targets or new drug-delivery methods, even to a specific cellular compartment. RNases fall within this second line of anticancer drugs. Since they do not cleave a specific RNA molecule, their effects on gene expression are pleiotropic, ensuring a broad spectrum of synergistic interactions with other chemotherapeutics and, if used alone, making the appearance of resistance to the drug by cancer cells difficult. The efforts described in this chapter to understand their cytotoxic mechanism have led to engineering more potent and selective RNases with fewer side effects than conventional chemotherapeutic drugs.

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Figure legends

Figure 1. Flow chart for transmission of biological information.

The therapeutic approach of RNases is to destroy RNA, therefore they are non-genotoxic antitumor drugs like conventional small anticancer drugs or radiotherapy.

Figure 2. Productive and non-productive pathways of cytotoxic RNases.

RNases interact with the cell surface non-specifically or through a receptor either naturally or targeted by binding the RNase to a ligand (1) and they are endocytosed (2). From one of the endocytic vesicles, the RNase translocates to the cytosol (3) evading the lysosomal degradation. This process can be enhanced by RNase modification. Once in the cytosol, the RNase must retain its stability, and one of two conditions is necessary for it to be cytotoxic. To meet the first condition it has to be, either naturally or artificially, resistant to the RI or, provided that enough molecules reach the cytosol, it has to saturate it. In this way, the RNase activity degrades cellular RNA and induces apoptosis (4). Otherwise, if the RNase is captured by the RI, it loses its ribonucleolytic activity leaving the RNA undamaged (5). To meet the second condition, it has to be sequestered by other molecules that hamper its interaction with the RI, driving it to an RNA-containing organelle free of RI like the cell nucleus (6). In this latter case, α-importin will sequester and release the RNase into the nucleus removing it from the RI-RNase and α-importin-RNase competing equilibriums and ultimately inducing cell death.

Figure 3. Schematic drawing of targeted RNase constructs

Different strategies have been developed to target RNases by conjugation or fusion either to antibodies or ligands. RNases used in the different approaches are: (A) HP-RNase, EDN, ANG; (B) HP-RNase, EDN, ANG, BS-RNase, rapLRI and ONC; (C) rapLRI, ANG and HP-RNase (D)HP-RNase; (E) ANG; (F) RNase A, HP-RNase, ECP, ANG; (G) HP-RNase. Cell surface target used in the different constructions are: (A) TfR, CD30; (B) TfR, ErbB2, P-gp, hPLAP, ErbB2, P-gp, CD22, CD30; (C) CD22, CD30; (D) CD30, (E) TfR; (F) Tf, LHRH, EGF; (G) EGF, bFGF, IL-2. N and C indicate the amino and carboxiterminus.

Table 1. Clinical trials performed with cytotoxic ribonucleases

MAIN OBSERVATIONS	
PROTEIN: Onconase	**References**
Two Phase I clinical trials with patients with solid tumors demonstrated that ONC was well tolerated. Dose limiting toxicity was renal but with reversal effects. MTD 960μg/m^2/week and recommended doses for Phase II 480 μg/m^2/week.	(Mikulski et al. 1993)
In a Phase II trial ONC increases the median survival time (7.7 months) of patients with advanced non-small cell lung cancer (NSCLC) compared with patients treated with a variety of chemotherapeutic regimes; Ongoing Phase II clinical trial with the addition of ONC to pemetrexed plus carboplatin in patients with NSCLC (**http://www.alfacell.com/)**.	(Mikulski et al. 1995)
A Phase I/II trial involving patients with advanced pancreatic carcinoma treated with ONC and tamoxifen suggested potential activity of the combination according to pre-clinical results. This trial was discontinued in 1998 because tolerated levels of ONC did not offer a significant advantage over gemcitabine (Gemzar®)	(Chun et al. 1995)
In a Phase II trial patients with prostate cancer recurrence were treated with a combination of ONC and tamoxifen with extremely low clinical benefit.	Not published although completed
A Phase II study of ONC in patients with advanced breast cancer resulted in limited clinical benefit.	(Puccio et al. 1996)
In a Phase II trial of ONC in patients with metastatic kidney cancer it had minimal activity.	(Vogelzang et al. 2001)
In a multicenter Phase II clinical trial carried out in patients with UMM, ONC demonstrated activity, including for those pre-treated with one or more chemotherapeutic regimens, and tolerable toxicity. Overall median survival was 6 months for the intent-to-treat group and 8.3 months for the treatment target group. ONC acted as a cytostatic agent. Obtained results were the basis for the initiation of the randomized Phase III trial.	(Mikulski et al. 2002)
Initial Phase III clinical trial for patients with MM treated either with ONC or doxorubicin showed no significant differences. However, the group treated with ONC revealed an excess of poor prognosis patients. Retrospective analysis clearly favored ONC (median survival 11.6 months for ONC-treated group *vs*. 9.6 months for the doxorubicin group) setting the basis for another Phase III analysis.	Not published although completed
A Phase IIIb study performed on a global scale in patients with UMM, to test the effects of combined use of ONC and doxorubicin *vs.* doxorubicin alone seemed to favor the drug combination.	Not published although completed
PROTEIN: HP-RNase variant QBT-139	
Ongoing Phase I clinical trial to evaluate the toxicity and tolerability (MTD) of QBT-139 in patients with advanced, refractory solid tumors.	http://www.cancer.gov/

Figure 1

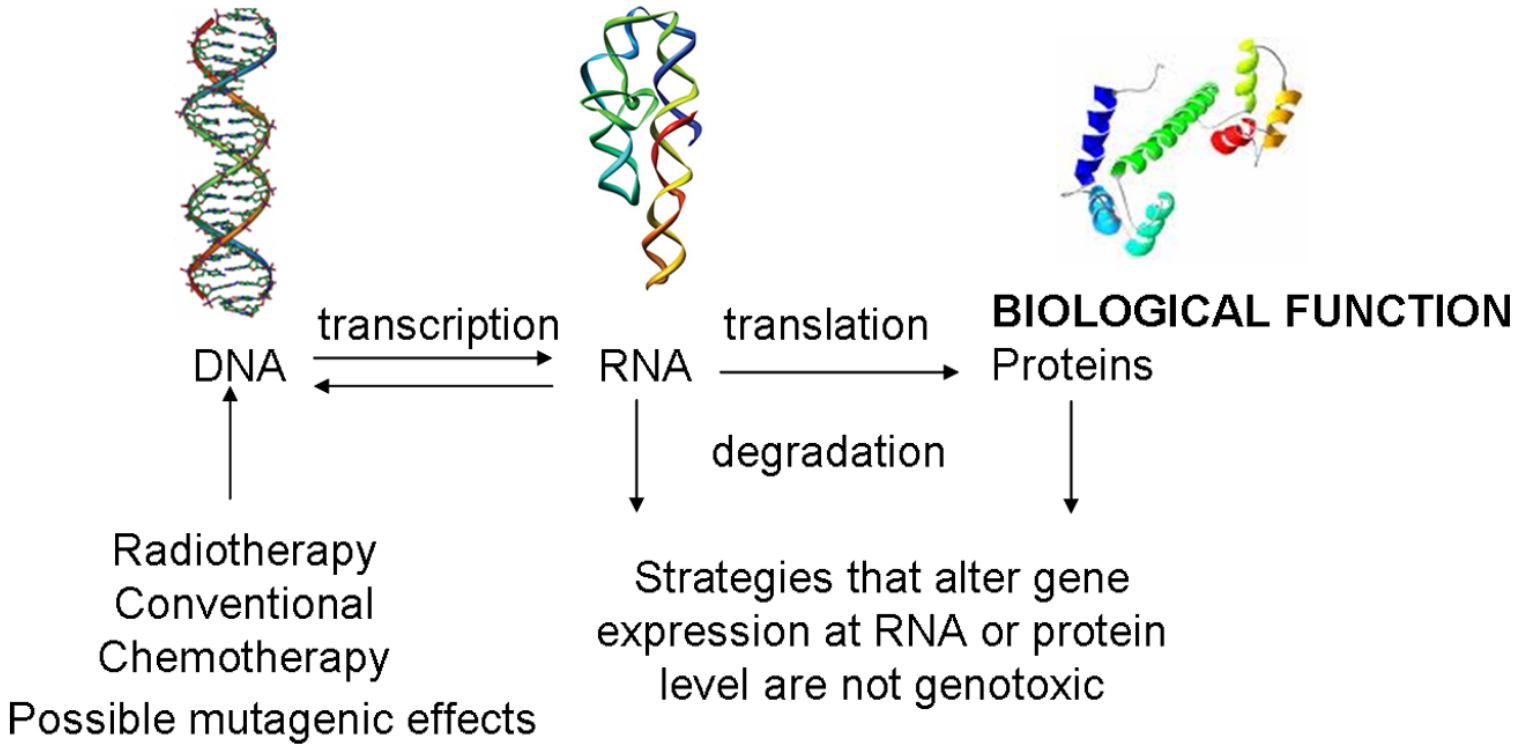
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Figure 2

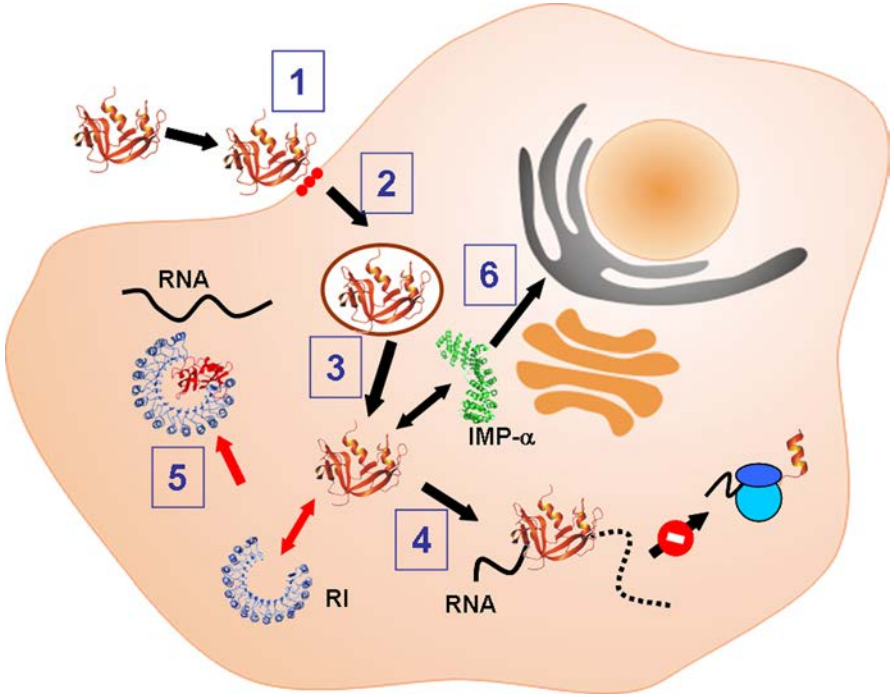
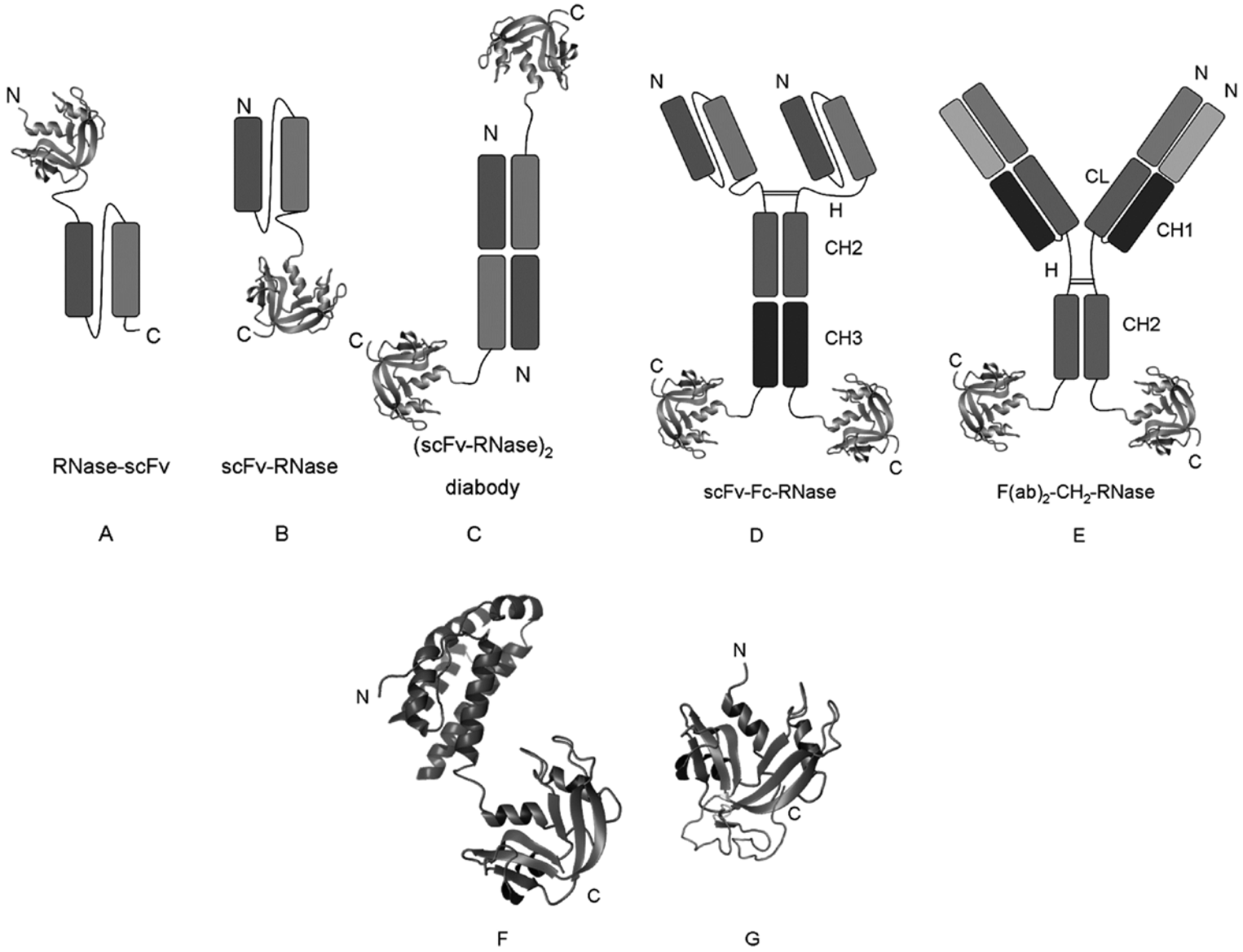


Figure 3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