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Review Article

New Promising Horizons for the Antitumor Activity of Onconase

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Abstract

Cancers represent, together with cardiovascular diseases, the most important cause of death in the industrialized countries. Fortunately, many cancer types have been successfully counteracted by following different strategies, comprising chemio- and/or radio-therapies, new drugs design and immuno-therapy. The success of these therapies, however, often depends on early diagnosis, that is very difficult to get for some tumors, especially the ones affecting internal organs, such as lung, ovary, liver, pancreas. Therefore, the curability of these tumors remains low and, consequently, the related deaths high. In this scenario, although rarely representing the first choice, protein therapy could be a fruitful approach to counteract incurable tumors. RNases, which are able to attack many RNA types, can become tools to block an uncontrolled cell replication and, consequently, cancer development. In particular, the amphibian RNase ranpirnase, commonly called onconase (ONC), showed in the recent past to be active against many tumors either *in vitro* or *in vivo*. Nevertheless, its renal toxicity, although reversible, has limited its use in therapy. However, the most recent results obtained *in vitro* with ONC are presented here, and possible therapeutic strategies based on ONC self- or hetero-oligomerization are as well suggested to overcome renal toxicity.

Keywords: Ribonucleases; Onconase; Antitumor Activity; ImmunoRNases; Oligomeric RNases; 3D Domain Swapping

Abbreviations: AA: Aminoacid; RNase(s): Ribonuclease(s); ONC: Onconase; ANG: Angiogenin; pt-RNases: Pancreatic type-RNases; BS-RNase: Bovine seminal RNase; RI: RNase Inhibitor; EDC: 1-ethyl-3-(3-dimethylaminoisopropyl) carbodiimide

Introduction

Ribonucleases

Ribonucleases (RNases) form a very large bacterial or eukaryotic enzymes group [1] and are known to catalyze the hydrolysis of many RNA substrates [2]. This makes their classification not easy, also because a cell contains about twenty different distinct RNases often characterized by different substrate specificities [3]. However, a possible classification can differentiate intracellular RNases from the ones secreted extracellularly. These are called secretory RNases [1,4] and many of them form a large super-family [5] in which is also included an amphibian RNase, called Onconase, that displays a remarkable antitumor activity [6,7]. Its main features are described in this report.

Pancreatic-type RNases and Onconase: crucial features for cytotoxicity

In the group of the secretory RNases, an increasing number have been characterized since the 60ies, and many of them have been classified as "pancreatic-type" (pt)-RNases [5,8]. This term originates from the most studied enzyme, the 13.7 kDa and 124 aminoacid (AA) residues-long bovine pancreatic, monomeric RNase A (Figure 1A) [9,10]. Incidentally, the members of this super-family refer sometimes to human pancreatic RNase, called HP-RNase, or RNase 1 (Figure 1B) [8,11]: although displaying a high identity sequence with RNase A, it is definitely more basic than it and is not expressed only in the pancreas, but almost in all tissues [12]. Besides RNase A and RNase 1, other variants are included in the mentioned super-family, even if, again, some of them are not secreted by the pancreas. The most important members display remarkable biological activities, as it is for the natively dimeric cytotoxic bovine seminal RNase (BS-RNase), that exists as an equilibrium between two isoforms, as reported in (Figure 1C) [13,14]. and for human RNase 5 [15]. This latter variant is also called angiogenin (ANG, (Figure 1D) because it crucially contributes to the formation of new blood vessels thanks to its ribonucleolytic activity [16,17]. Also

other important RNases, although belonging to non-mammalian species, such as birds or amphibians, are known [18,19]. Some of them are included in the pancreatic-type super-family principally because of their high structural homology with the mammalian pt-RNases [6]. In particular, the 114 AA residues amphinase and, above all, the 104 AA residues frog ranpirnase, or P-30 protein, extracted from Rana *Pipiens oocytes*, deserve to be noted [7,20]. This 11.8 kDA variant is commonly called onconase (ONC, (Figure 1F) because it exerts a remarkable antitumor activity against many cancer types [6,7]. Moreover, ONC is known to display also an antiviral activity against HIV-1 or, more recently, Ebola [21,22]. ONC is considered a pt-RNase because it satisfies the three main features for which a RNase can be associated with the super-family [5]:

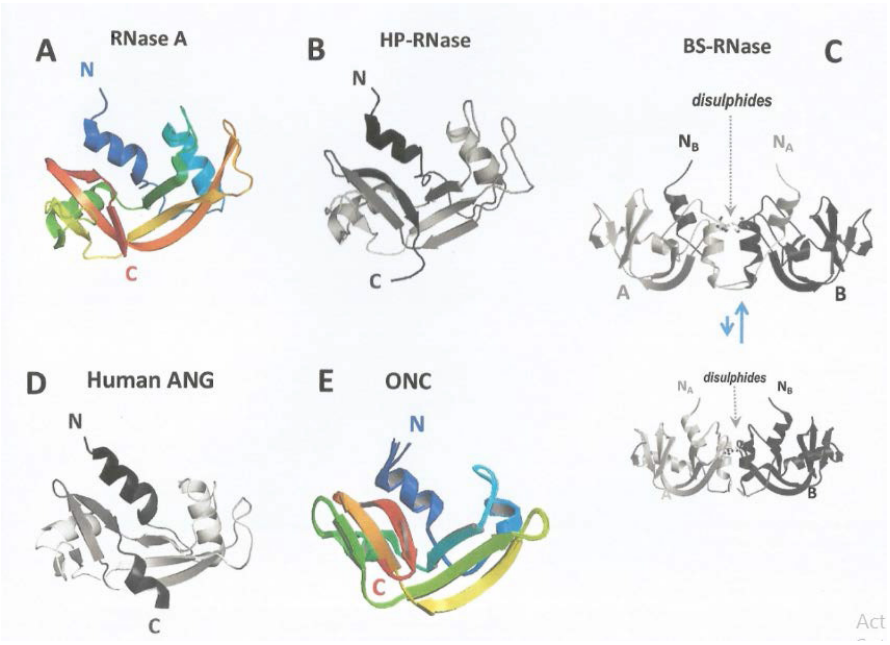

Figure 1: Structures of some important pt-RNases.
RNase A; B) HP-RNase, or RNase 1; C) natively dimeric BS-RNase: two isoforms in equilibrium exist, one natively swapping their N-termini, the other dimeric only through the indicated disulphides; D) human Angiogenin, ANG; E) amphibian Onconase, ONC.

1. a high homology folding represented by a "V-like", or "kidney-like", shape to accommodate the RNA substrate in the relative cavity [23],

2. the catalytic triad, formed by one Lys and two His residues, i.e., H10/K31/H97 for ONC, while for RNase A is H12/K41/H119,

3. the distribution of the basic charged residues, the majority of which must be located in the proximity of the active site [5]. Importantly, at least a minimal ribonucleolytic activity is mandatory for RNases to exert their remarkable biological activities, as it is for ANG [24], but also for ONC [6]. Indeed, ONC cytotoxicity against malignant cells definitely emerges [25-27]. In this context, beyond BS-RNase and, especially, ONC, also the bacterial Barnase and the natively dimeric Binase [28], are definitely cytotoxic [29]. However, ONC is the most considered RNase anticancer tool [30].

Determinants of the Antitumor activity of pt-RNases and ONC

The efficacious action of ONC revealed to be particularly true for incurable solid tumors, as emerged from the use of ONC against human lymphoma [31], glioma [32], pancreatic carcinoma [33], or, more recently, melanoma cell lines [34,35]. In particular, an autophagic cell death effect has been detected in ONC-treated Panc-1 and PaCa-44 tumor cells [33], while ONC affects also NF-κB and TNF-α expression in A375 melanoma cells [34,35]. Strong synergism was reported in early studies with ONC combined *in vitro* with tamoxifen [36], or trifluoroperazine [37], or also lovastatin [38] to counteract pulmonary A549 carcinoma cells. Importantly, an ONC antitumor action had been registered also *in vivo*, in particular against non-resectable mesothelioma and non-small-cell lung cancer [39-41]. However, this application resulted to be not completely successful because nephrotoxicity emerged in many patients after repeated ONC administrations, although this side-effect disappeared after discontinuing the treatment [42].

Cellular internalization

The main obstacle encountered by extracellular RNases to exert their action is represented by cell internalization. This occurs through endocytosis [43], but is possible only through a fruitful interaction with the cell membrane occurs. However, the possibility for ONC to enter the cell thanks to the mediation of a receptor has been reported as well [44,45]. Then, Sundlass et al. [46] revealed

that both electrostatic forces and specific interactions are crucial for a RNase to determine the time spent near the cell surface, a determinant for its consequent internalization [46]. In addition, Notomista & coll. reported that either native or artificial dimeric cytotoxic RNases strongly affect membrane aggregation, fluidity and fusion [47]. Importantly, if we consider that a RNase should be selectively cytotoxic against malignant cells, that are characterized by a more negatively charged membrane than the normal ones, the basicity of each RNase is important to win this challenge. Then, also the specific RNase orientation is important for a successful approach to the membrane, as it has been demonstrated for ONC or also BS-RNase [46-49]. Hence, the orientation of the basic charges might affect also the cytotoxic potential of other RNases. In addition, ONC seems to approach the cell membrane differently from other pt-RNases [46]. Moreover, the cellular internalization event can be evaluated as to be residue-specific because wt-ONC is less efficiently internalized than the so called "R-mutant", in which all Lys residues except the catalytically active one are replaced by arginines [50]. However, if the RNase net basic charge is randomly increased, the relative advantage can be counteracted by the increase of the enzyme affinity toward the negatively charged cellular RI [51].

Some discordant data have been reported about the mechanism of ONC cell internalization: Haigis & Raines wrote that ONC is internalized in early endosomes of HeLa and K562 cells by a clathrin- and caveolae-independent mechanism [45], while Rodriguez & coll. reported that Jurkat cells can endocytate ONC through a dynamin-dependent route, presumably through a pathway mediated by clathrin/AP-2 [52]. These data, although apparently controversial, suggest that ONC may follow different routes to cross the membrane of different cell lines.

Evasion from the RNase inhibitor

Another huge obstacle for the biological activity of a RNase is represented by its interaction with the cellular RNase Inhibitor (RI). RI is a 50 kDa negatively charged, horseshoe-shaped, and cysteine-plus leucine-rich macromolecule ubiquitously expressed in almost all cells [53,54]. For many years, RI has been considered present only in the cytosol, but its presence has been detected also in cell mitochondria and nuclei [55]. RI can form very tight complexes with many pt-RNases, such as RNase A [56], RNase 1 [57], ANG [58], and also with RNase 2, that is the eosinophil derived neurotoxin (EDN) [59]. The RNase-RI complexes are accompanied with Kd values comprised between the pico- and the femto-molar range [60]. Their structures explain why RI inactivates the RNase moiety that remains caged inside the RI cavity [61]. RI is highly conserved in mammals, but is present also in non-mammalian species [62]. Instead, in contrast with almost all secretory pt-RNases, ONC can evade RI because it is devoid of the flexible regions, or loops, in which reside the key-residues allowing RNases to fruitfully interact with RI [44,61]. For this reason, ONC can actually display a remarkable cytotoxicity by exerting its ribonucleolytic activity toward t-RNAs [63] and arresting the G1 cell cycle phase [7,20]. Besides, it is important to note that also the mammalian BS-RNase is cytotoxic because, being natively dimeric, can sterically evade RI [64,65]. On the contrary, its monomeric derivative, although being enzymatically active, is not cytotoxic because is sequestered by RI [66,67]. However, the sensitivity toward RI is not the unique determinant hindering cell cytotoxicity: indeed, non-cytotoxic RNases were unable to reduce HeLa cells viability also after silencing the RI expression [68]; instead, non-covalent dimers of RNase A, although being partially RI-sensitive [69] and definitely inactive against pancreatic cancer cell lines [70], showed to be cytotoxic against leukemia cells and also against melanoma in mice [71,72].

Native or artificial oligomeric RNases and their possible application in therapy

If a RNase can be induced to oligomerize, this would make it bulkier than its native monomer, and the charge density of its moiety augment likewise. This event would help the enzyme internalization in tumor cells and would allow its evasion from the cellular RI as well [67]. Then, the augment of ONC derivatives dimensions could represent a successful strategy if we consider that ONC-based therapy had been limited by adverse effects at the expense of the kidneys: ONC antitumor activity would be conjugated, in fact, with a simultaneous low renal uptake. Protein oligomerization may occur spontaneously, or could be induced also by the cell environment or, again, by an *in vivo* context [65,73,74]. Within mammalian RNases, only BS-RNase is nowadays known to be natively homo-dimeric [14]. This is principally ascribable to two antiparallel disulphides involving the two Cys31 and 32 residues that are present in both subunits [13,75], while are absent in ONC and also in other monomeric RNases.

Covalent or non-covalent ONC oligomerization

RNase oligomerization can be provoked also *in vitro* to obtain different products. Incidentally, RNases or ONC oligomerization can be induced to form covalently linked derivatives upon conjugation with bifunctional or multifunctional cross-linkers, like diimidoesters or maleimides. In this way, stable hetero- or homo-oligomers can be produced, upon modifying one or more AA residue(s). Therefore, any chemical modification can somehow affect the properties of native RNases and, thus, negatively affect its biological activities. Nevertheless, this approach has been often exploited by RNase A permitting to obtain products displaying promising, but not conclusive, results in terms of high cytotoxic potential. This was, very probably, because of the excessive involvement and modification of the basic Lys residues [76-78]. Again, maleimides have been instead used with a properly designed ONC mutant to obtain bulky dimer(s) or trimer(s) (Figure 2A)

that, however, were not more cytotoxic, *in vitro*, than monomeric ONC [79]. Nevertheless, and importantly, these derivatives were characterized also by dimensions that overpassed the calibre of the glomeruli [79], and their use could be reconsidered in the next future.

Immuno-RNase protein fusion strategy for ONC

To obtain larger RNase moieties without affecting their activity properties, the protein-fusion technique has been widely used especially with RNase 1 [80,81], but sometimes also with ONC [82]. Thanks to protein engineering, ONC derivatives have been designed with different peptide linkers and expressed in conjugation with many adducts [82], such as antibody fragments, human serum albumin, dengue virus-derived peptide, and also the transferrin N-terminal domain ((Figure 2B), upper panel) [83-85]. All derivatives displayed remarkable cytotoxicity against many cancer cell types, and in some cases also in an *in vivo* context, in mice [83,86]. Furthermore, this augmented antitumor activity could have been paralleled, in a possible *in vivo* application, by a low undesired renal filtration [79]. Then, differently from microbial or plant immunotoxins, human Immuno-RNases lack immunogenicity or nonspecific binding and toxicity that could damage also normal cells [87]. Indeed, clinical trials performed with non-mammalian toxins drove sometimes toward even fatal events [88]. Instead, the Immuno-RNase 1 fusion derivatives were benign outside cells, and not immunogenic as well [80]. Furthermore, the immuno-protein fusion approach has been applied also to form ONC "diabodies" (Figure 2B), lower panel), i.e., covalent dimers of single chain antibody fragments (scFv) connected with the RNase moiety, or in other words dimers of the Immuno-ONC derivatives [86,89].

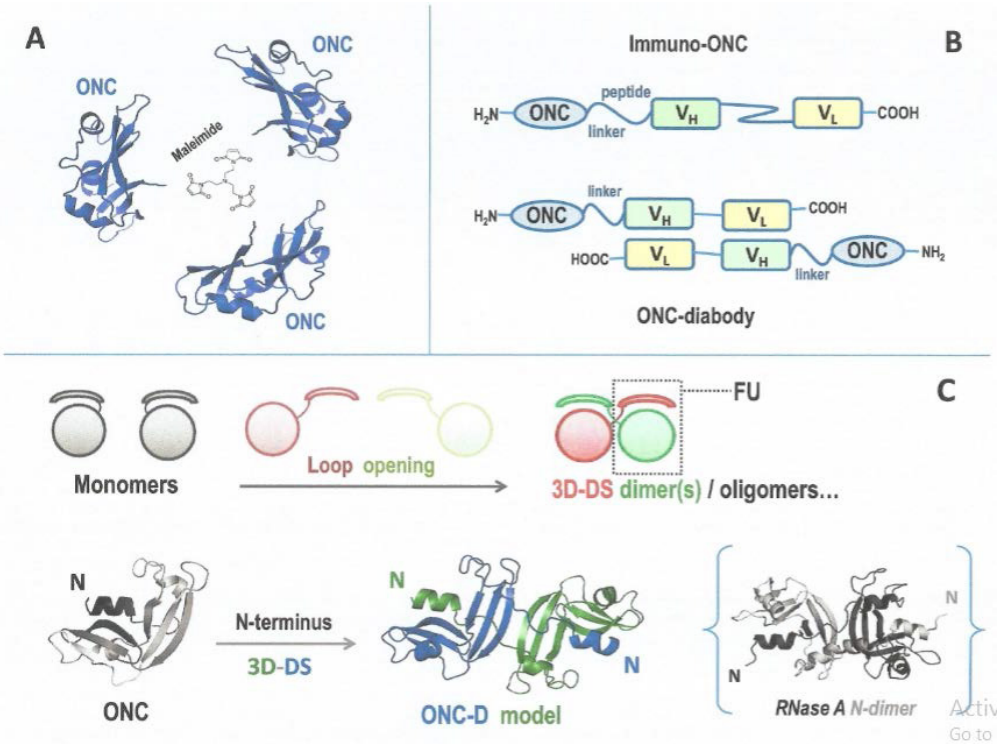

Figure 2: Strategy for ONC oligomerization.
A) Covalent oligomerization (trimerization) of ONC; adapted from [79]; B) Immuno - RNase (ONC) fusion protein [84,85] and ONC diabody schematic structures [86,89]; C) 3D-DS mechanism [91,95-97] and N-swapped ONC dimer model [105] compared with the crystal structure of the RNase A N-dimer [107]. FU represents the functional unit reconstituted in the dimer after 3D-DS [96].

Oligomerization through the three dimensional domain-swapping (3D-DS) mechanism

RNases can form supramolecular structures also through a non-covalent self-association mechanism called three dimensional domain-swapping (3D-DS). Firstly described and analyzed by Eisenberg, but then also by other groups [90-95], 3D-DS involves many proteins [90,96,97] and partially violates the "Anfinsen dogma" which states that a protein AA sequence dictates a unique folding [98]. Indeed, the flexible loop(s) of a protein can adopt variable conformations corresponding to more than one energy minimum [91]. This possibility allows the domains linked to the flexible protein segments to adopt different orientations and undergo a reciprocal exchange (Figure 2C), upper panel). This drives to form a non-covalent dimer, or dimers, or even larger oligomers, as it is for RNase A and BS-RNase [65,99-101] if more than a single flexible loop is present [96,97]. The domain detached from the native monomer can reconstitute the original contacts

in each composite, functional unit (FU) of the oligomer [96]. The FU overlaps the native monomer (Figure 2C), upper panel), while the folding of the dimer/oligomer parallels the monomer one, with the exception of additional, so called open interface(s) [91]. The domains involved in 3D-DS are often the protein N- or C-termini, or both, as it is, again for RNase A and BS-RNase [65,96,99,101]. Oligomerization is often accompanied with increased RNases enzymatic and biological activities, being the latter ones sometimes absent in the native monomer [72].

3D-DS dimerization of ONC: ONC can penetrate cancer cells either for its high basicity or to a favorable interaction with the sialic acid moieties present on the membrane of malignant cells [102]. Afterwards, ONC evades RI as mentioned before [60] and can attack tRNAs or other substrates, such as miRNAs, to exert its cytotoxic action [21,103,104]. Hence, it would seem not necessary to produce ONC oligomers to design anticancer therapies [30]: indeed, many positive results have been reached with monomeric ONC both *in vitro* and *in vivo* against several incurable tumors [82]. Furthermore, ONC was recently found to enhance the activity of new generation drugs that are active against the BRAF-mutated A375 melanoma cell line [34], and also to restore cytotoxicity vs the same A375 cells that became resistant to dabrafenib [35]. However, the although reversible renal toxicity lowered ONC therapeutic applications [42], and the possibility to enlarge the dimensions of ONC moiety/ies remains a promising strategy to allow a more efficacious block at the glomerular barrier. This would increase the half-life of circulating ONC derivatives at the same time, and the aforementioned fusion immune-ONC derivatives are in line with this strategy [83]. Then, notwithstanding its remarkable stability (TM ~90°C), ONC has been discovered to form a N-swapped dimer (ONC-D, (Figure 2C), lower panel) [105], upon being lyophilized from 40% acetic acid solutions, like RNase A [106]. The ONC-D structure has been modeled [105] as to be similar to the N-swapped dimer of RNase A (Figure 2C), lower panel) [107]. Notably, low concentrations of ONC-D displayed to be more active against pancreatic cancer cells than native monomer [105]. Unfortunately, ONC can swap only its N-terminus, because its C-terminus is locked by a disulphide bond involving Cys87 and Cys104, i.e., the last AA residue. The impossibility to swap more than one domain definitely reduces the self-association propensity of a protein [97]. Moreover, some ONC variants lacking the disulphide blocking the C-terminus are known to be less stable than the native enzyme [108,109]. Consequently, the only way nowadays feasible to obtain large ONC homo-oligomers is the use of multifunctional maleimides producing covalent, stable derivatives (Figure 2A) [79]. However, a recent study has combined the features of the ONC C-terminal loop with the ones of RNase A to build a chimera that increased the tendency of the mammalian enzyme to oligomerize through 3D-DS. Indeed, the RNase A native cis configuration of the Pro114 residue residing inside the flexible 112-115 residues loop makes its C-terminus difficult to be swapped [110], therefore requiring high energy to succeed [111,112]. Instead, the loop present in ONC, shorter and devoid of this proline residue, makes the RNase A mutant more prone to oligomerize [113]. The antitumor activity of the resulting oligomers was not tested because mutations did not affect the determinants crucial for cytotoxicity. Nevertheless, this result may suggest to deepen the analysis and combine the most advantageous features of RNase A and ONC. This could allow to build chimeras by combining the cytotoxic properties of ONC with the determinants making pt-RNases prone to oligomerize, and contemporarily reduce undesired side-effects. Once oligomers larger than dimer(s) being obtained, these species could be covalently stabilized by using condensing agents, as it had been performed with the RNase A C-dimer [114] with 1-ethyl 3-(3-dimethylaminoisopropyl) (EDC) carbodiimide [115].

Conclusion

The new data registered in the last decade by measuring the *in vitro* ability of ONC to counteract incurable tumors are certainly promising [30-32,34,35,70]. However, to obtain more satisfactory results, the following strategies could be experimented:

I. artificial protein cross-linking (Figure 2 A)

II. immuno-fusion protein derivatives (Figure 2B),

III. protein engineering devoted to enlarge the tendency of ONC 3D-DS self-association (Figure 2C). This could allow to design and produce active derivatives that may become efficacious tools able to counteract incurable cancers , but with negligible side effects.

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Conflict of Interest

The author declares no conflict of interest.

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