Ribonucleases and their Applications

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Abstract: Ribonuclease (RNase) is a type of nuclease that catalyzes degradation of RNA into smaller components. RNase can be classified into two broader categories namely endoribonucleases and exoribonucleases on the basis of their site of action. RNases play key roles in the maturation of all RNA molecules; endoribonucleases cleave the RNA molecules from the interior at 5′ end while exoribonucleases degrade RNA molecules in a 3′–5′ direction. With the advent of new frontiers in biotechnology, the applications of ribonucleases besides molecular biology have expended into many other fields like medicinal, clinical, and analytical chemistry. RNase A that belongs to pancreatic ribonucleases super family plays an important key role in structural, biochemical and evolutionary studies. Discovery of eukaryotic orthologues of the bacterial double stranded (ds) RNA-specific ribonuclease III (RNase III) suggests a central role for these enzymes in the regulation of ds-RNA and eukaryotic RNA metabolism. The more recent studies have shown that the mammalian and some fungal RNases are also bestowed with antiproliferative, antiangiogenic and/ or antitumor/ anticancer activities. Some of the members of RNase A superfamily such as RNase 6 and RNase 7 appears to be evolutionary conserved peptides with potent antimicrobial activities for upkeep of sterility in the urinary tract.

Keywords: Microbial ribonucleases, antimicrobial, antiviral, antitumor/ anticancer, apoptosis.

INTRODUCTION

The use of enzyme-based processes has been employed by ancient civilization. The field of industrial enzymes is now experiencing a major research and development initiatives, resulting in both the devolvement of the number of products and processes as well as improvement in the performance of several existing products. As on today, nearly 4000 enzymes are known out of which about 2000 are being used commercially because of their affordability [1]. Use of enzymes in medicine and biochemical industry has greatly expanded their demand. The world's enzymes demand is met by 12 major producers and 400 minor suppliers. Europe alone produces the 60% of the total world supply of industrial enzymes [1]. Bacteria contain many RNases of diverse classes that degrade the virusal RNA. RNases play key roles in the maturation of RNA molecules, both for the messenger RNAs, that carry genetic material for synthesize proteins, as well as for non-coding RNAs that play important role in various cellular processes and their decay. Recently, a novel RNase associated with function of HIV-1 reverse transcriptase inhibition has been purified from the fungus *Ramaria formosa* [2].

Initially, it was a thought that mRNA decay might be a result of a random molecular recycling in which salvaged nucleotides could be reused and thought to provide rapid mRNA turnover that was non-specific and inevitable for all types of transcripts regardless of their structure and length [3, 4]. Since that time, much progress has been made till date with respect to the knowledge of RNA decay, which is now believed to involve a series of specific, controlled events in which specific enzymes target the specific RNA species including mRNA [3, 4]. Consequently, our understanding of RNA decay and the enzymes responsible for the metabolic processes has broadened greatly. Ribonuclease uses different types of RNAs as substrates. Such substrates include single stranded (ss) RNA, DNA/RNA duplex, messenger (m) RNA, ribosomal (r) RNA and transfer (t) RNA and each type of RNase shows specificity for a particular type of substrate.

Sources of RNases

RNases have been isolated and biochemically characterized from many organisms including parasites, bacteria, fungi, plants and a variety of tissues from mammals [5]. RNases possess potent biological therapeutic activities like antitumor, antiproliferative, antiviral, immunosuppressive, antifungal, antiangiogenic and induction of apoptosis. These multitude of diverse activities have drawn the attention of many scientists to exploit RNases as therapeutic tools to deal with malignancies [6-8]. *E. coli* is being used as a model organism for most of RNA decay studies. RNA decay has not been well characterized in Gram-positive bacteria with the exception of some preliminary studies conducted in *Bacillus subtilis* [9, 10], *Streptococcus pyogenes* [11], *S. pneumoniae* [12] and *Staphylococcus aureus* [13,

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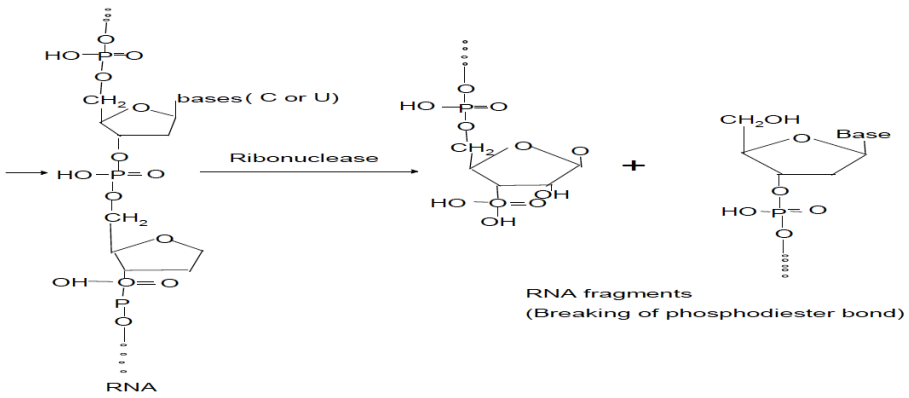

Figure 1: The general mechanism of action of ribonucleases.

14]. Ribonucleases degrade ribonucleotides of both Gram-positive and Gram-negative organisms (Figure **1**) and two broad types of ribonucleases exist that are categorized as endo- and exo-ribonucleases (Table **1**) depending on their function(s). The RNases have molecular sizes varying between 26 to 118 kDa. They also perform diverse functions in the organisms.

Nine endoribonucleases have been known to exist in *E. coli* including RNase G, RNase E, RNase III, RNase I and RNase P [4, 15]. RNase E is the predominant endoribonuclease that degrades mRNA in *E. coli*. Gram-positive organisms lack RNase E. However, *S. pyogenes* possesses endoribonucleases J1 and J2 which, like RNase E in *E. coli*, were found to be essential for bacterial growth [16]. A temperature-sensitive mutant of RNase E, ams-1 (altered mRNA stability), showed a gross mRNA-decay deficiency [17]. RNase E, is autoregulated at the post-transcriptional level mediated by its own 5′ untranslated region [18-20]. RNase E is a protein of 1061-amino-acid residues (118 kDa) with three distinct domains forming a homo-tetramer [21]. This RNase appears to be involved in both r-RNA and t-RNA maturation as well as in mRNA degradation [4]. The first 500 residues at the amino terminus contain both the catalytic and the S1 RNA-binding domains of the endoribonuclease RNase E. The 597–684 residues encode an arginine-rich RNA binding domain while carboxy-terminus of 734–1060 residues serve as a scaffolding region at which various components of a multi-protein complex and the degradosome bind including the PNPase, a DEAD box (core sequence of eight amino acids including D, E, A and D), RNA helicase B (RhlB), RhlE or CsdA of *E. coli* [22], and the glycolytic enzyme enolase [23-25]. There are other minor protein components of the

Table 1: Key Bacterial Ribonucleases, their Sizes and Functions

Ribonuclease	*Mr* (kDa)	Function(s)
RNase E	118	Endoribonuclease processes rRNA and degrades mRNA; acts as a scaffolding protein to which PNPase, enolase and DEAD box RNA helicases associate to form multi-protein complex 'the degradosome' which continuously enhances RNA processing and degradation in the cell.
RNase G	55	Non-essential endoribonuclease that shares N-terminal homology with RNase E has no C-terminal scaffolding region on which the degradosome assembles.
RNase III	26	Endoribonuclease that cleaves ds rRNA during rRNA maturation; involved in degradation of mRNA including the pnp transcript that encodes PNPase.
RNase II	72	Hydrolytic exoribonuclease that degrades mRNA in a 3′–5′ direction but it poorly degrades structured mRNA.
RNase R	95	The second most abundant hydrolytic exoribonuclease in the cell that capable to easily degrade mRNA with extensive secondary structure processes rRNA and is cold-inducible.
RNase PNPase*	80	Primary phosphorolytic exoribonuclease that is cold-inducible and associates with RNase E in the degradosome for cooperative degradation of mRNA. PNPase is required for growth at low temperature (15°C) and like RNase II, it poorly degrades structured RNA
RNase PH	45-50	Second phosphorolytic exoribonucleases in the cell that shares homology with the catalytic domains of PNPase (which contains two RNase PH catalytic domains); it has been shown to physically associate with RNase E and it also processes tRNA.

PNPase*: Polynucleotide phosphorylase.

Table 2: Source and Properties of Eukaryotic and Prokaryotic RNases

Ribonuclease	Nature	Source	*Mr* (kDa)	Cofactor	Function(s)	Reference(s)
RNase A	Exo	Bovine pancreatic	14.0	Mg^{++}	Hydrolyzes RNA (but not DNA) phosphodiester bonds which covalently link ribonucleotides particularly those linked to pyrimidine bases such as uracil. $(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$	[29]
RNase H	Endo	Bacteria, archaea and eukaryotes	32.2	Mg^{++}	Cleaves the 3'-O-P bond of RNA in a DNA/RNA duplex to produce 3'-hydroxyl and 5'-phosphate terminated products. RNase H mediated viral RNA degradation	[30, 31]
RNase PNase	Exo	Plants and humans	-	-	Nucleotidyltransferase	[32]
RNase PH	Exo	Archaea and eubacteria	25.0	-	Involved in tRNA processing	[33]
RNase II	Exo	*E. coli*		-	3' to 5' degradation of ssRNA	[34]
RNase R	Exo	Bacteria	92.0	-	Close homolog of RNase II, but it can, unlike RNase II degrade RNA with secondary structures without the help of accessory factors.	[35]
RNase D	Exo	*E. coli*	43.0	-	Helps in the 3'-to-5' maturation of many stable RNA molecules.	[36]
RNase T1	Exo	*Aspergillus oryzae*	23.5	-	Sequence specific for ssRNA.	[37]
RNase U2	Exo	*Trichoderma koningi*	-	-	Specific for ssRNA. It cleaves 3'-end of unpaired A residues	[38]
RNase BN	Exo	*E. coli*	60	-	-	[39]
RNase I	-	*Saccharomyces cerevisiae*	27	-	Cleaves 3'-end of ssRNA at all dinucleotide bonds leaving a 5'-hydroxyl, and 3'-phosphate *via* a 2',3'-cyclic monophosphate intermediate.	[40]
RNase III	-	*E. coli, S. pombe and S. cerevisiae*	52.6	-	Cleaves dsRNA	[41]
RNase P	Endo	Bacteria, archeae and eukaryotes	32.0	-	To cleave off an extra, or precursor, sequence on tRNA molecules	-
RNase E	Endo	*E. coli*	116.0	Mg^{++}	RNase E-dependent coupled degradation of target mRNA and ssRNA	[42, 43]
RNase HI	Endo	Humans	17.6	Mg^{++}	Cleavage of RNA via a hydrolytic mechanism	[44]
RNase L	-	Humans	-	-	Interferon-induced ribonuclease which, upon activation, destroys all RNA within the cell (both cellular and viral)	[45]

degradosome that include DnaK, GroEL and polyphosphate kinase [26, 27]. The degradosome is a large multi-protein complex that patrols the cytoplasm targeting RNA molecules destined for their decay or processing. The PNPase-bound RNA is fed to RNase E that attacks the molecule in a 5′ end fashion exposing a free 3′ end. RhlB, known to play a role in rRNA processing that facilitates the process by unwinding structured RNA which often hinders RNA degradation. Degradosome of *Pseudomonas syringae* contains RNase E, exoribonuclease RNase R and the DEAD box helicase RhlE [28]. The presence of PNPase in the *E. coli* degradosome as well as PNPase-like structurally related proteins in the exosome of yeast highlights the importance of PNPase in the multi-protein degradosome and degradosome-

like complexes. RNases have evolved to possess many extracellular functions in organisms. RNase 7, a member of the RNase A superfamily is secreted by human skin and it also serves as a potent antipathogenic agent. A broad range of biological roles for these ribonucleases have been suggested, including scavenging of nucleic acids, degradation of self-RNA, serving as modulating host immune responses and extra- or intra-cellular cytotoxins. The members of RNaseT2 family have been implicated in human pathologies like cancer and parasitic diseases.

Broader Applications of Ribonucleases

Microbial ribonucleases have a variety of potential applications in the fields of molecular biology as well as in recombinant DNA technology (Figure **2**). Recently, many effective roles of ribonucleases have been explored such as antimicrobial activity of the human ribonuclease A, is a super family member of RNase 8 against potential pathogenic microorganism that is effective at the micro- to nano-molar concentration. Thus RNAse was identified as the novel antimicrobial protein that contributes to the host defense system mechanism [46].

It is widely acknowledged that stability of RNA molecules play important role in bacterial adaptation and survival in adverse environments like those encountered when bacterium infects a host. Bacterial ribonuclease activity along with regulatory RNase or RNA binding protein is the mediator of the regulatory outcome of RNase stability. Ribonuclease of *Bacillus subtilis*, a Gram positive model microorganism is imposing a major health concern worldwide [47]. The combined expression of RNase7 along with RNaseA, HBD-3 and Boriasinin in the fetal skin provides a developmental mechanism which exerts a broad spectrum of antimicrobial activities to maintain sterility in the amniotic cavity [48]. In the host innate immunity, the members of the RNase A super family such as mRNase3 and RNase7 participate in response against pathogen infections. RNase 3 was identified as an eosinophil product secreted by activated eosinophils during inflammation and its levels in biological fluid is important for diagnosis and monitoring of allergy & eosinophilia disorders. RNase3 displays specific *E. coli* cell agglutination activity, which precedes the bacterial cell death and lysis, while the RNase7 prompts the release of bacterial cell content without inducing any cell aggregation [49].

RNase E is a ribonuclease of plant origin, which modulates SOS responses in bacteria, for a response to the stress of DNA damage by activation of the SOS mechanism by the RecA/LexA-dependent signal transduction pathway that transcriptionally depresses a multiplicity of genes leading to transit arrest of cell division as well as initiation of DNA repair. *E. coli* endoribonuclease RNase E precludes normal initiation of SOS after deletion or inactivation of temperature-sensitive RNase E protein. Down regulation of RNase E followed by DNA damage by mitomycin C resulted in SOS termination and restoration of RNase E function leads to restoration of a previously aborted response [50]. RNase H is a prospective drug target as it is one of the two viral enzymes considered essential for virus replication. An active recombinant Hepatitis B virus RNase H is suitable for antiviral drug screening [51].

With ever extending applications of biotechnology, the spectrum of uses of ribonucleases has also extended into many other fields, such as clinical, medicinal and analytical chemistry. There are several

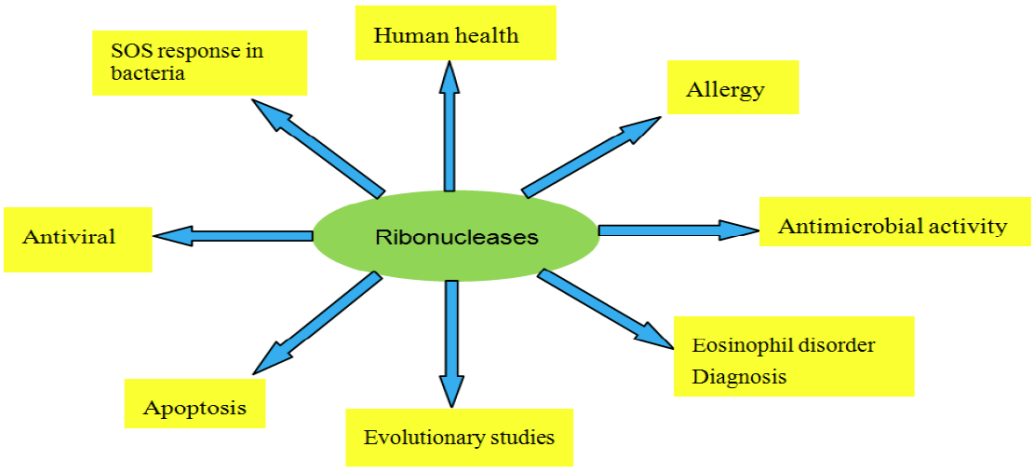

Figure 2: Broader applications of ribonucleases.

processes in the medicinal and clinical areas that involve the applications of ribonuclease, like RNase A, a representative of pancreatic ribonucleases superfamily is considered important in biochemical, structural and evolutionary studies. RNase 2, 3, 5, and 7, have been associated with antipathogen activities. Many scientists suggested that the RNase-superfamily started off a host-defense mechanism in vertebrates. Consistent with this hypothesis, all members of the superfamily exhibit high rates of amino acid substitution as is commonly seen in immunity genes [52]. Mammalian RNases have been associated with diverse physiological functions including cytotoxicity, digestion, angiogenesis, male reproduction and host defense [53]. Viruses also exploit cellular RNases or their own RNases in maintaining their reproductive cycles. Studies on bacterial ribonucleases and in particular those from *E. coli* are providing greater insight into RNase structure, mechanism and regulation [54].

The crystal structure of a newly reported *Thermotogamaritima* sp. RNase P holoenzyme in complex with $tRNA^{Phe}$ is a 154 kDa complex consists of a large catalytic RNA (P RNA), a small protein cofactor and a mature t-RNA. The structure shows that RNA–RNA recognition occurs through shape complementarity, specific intermolecular contacts and base-pairing interactions [55]. RNase III enzymes occur ubiquitously in animals and also have diverse applications including conversion of RNA precursors into functional RNAs that actively participate in RNA interference, translation and a range of cellular activities. Members of the RNase III enzyme family, including *E*. *coli* RNase III, Rnt1, Dicer and Drosha have the ability to recognize and cleave dsRNA at specific positions or sequences. The biochemical and structural analysis of RNase have shed new light on how RNase III class of enzymes catalyze dsRNA hydrolysis and maintain substrate specificity [56]. Bacterial cells not only respond to the stress of DNA damage by the SOS response activation but RecA/LexA-dependent signal transduction pathway transcriptionally depresses a multiplicity of genes leading to transient arrest of cell division that forces to initiate the DNA repair. Reports explain the vital role of *E. coli* endoribonuclease RNase E in regulation of the SOS response(s). RNase E deletion or inactivation of temperature-sensitive RNase E protein precludes normal initiation of SOS. The ability of RNase E to regulate SOS is dynamic. The down regulation of RNase E following DNA damage by mitomycin C resulted in SOS termination and restoration of RNase E function as well as resumption of a previously aborted response [50]. RNase P is responsible for processing the 5′ end of pre t-RNAs as well as other RNA molecules. RNase P is formed by a RNA molecule responsible for catalysis and one or more proteins. Structural studies of the proteins from different organisms, the bacterial RNA component and a bacterial RNase P holoenzyme/tRNA complex provide insights into the mechanism of this universal ribozyme [57].

The discovery of eukaryotic orthologues of the bacterial dsRNA-specific RNase III suggested a pivotal role for these enzymes in the regulation of dsRNA and eukaryotic RNA metabolism. Yeast RNase III involved in the maturation of a majority of snRNAs, snoRNAs and rRNA. In addition, perturbation of the expression level of yeast RNase III alters meiosis and causes sterility [58]. RNase P is Mg^{2+} dependent endoribonuclease which is responsible for the 5'-maturation of transfer RNAs. It is a ribonucleoprotein complex containing an essential RNA and a number of protein subunits depending on the source; its number may be one, four and nine in bacteria, archaea and eukarya, respectively. Since bacterial RNase P is required for viability and differs in structure/subunit composition from its eukaryal counterpart, it is a potent antibacterial agent [59]. RNase III has important cellular functions in bacteria and generally, its gene is not essential, with the remarkable exception of that of *B. subtilis*. Essential role of RNase III in this bacterium is to protect it from the expression of toxic genes coded by two prophages, Skin and SPβ through antisense RNA [60]. Ribonucleases H are enzymes that cleave the RNA of RNA/DNA hybrids that are formed during replication and repair and which could lead to DNA instability if they were not processed. There are two main types of RNases H and at least one of them is present in most of the organisms. Eukaryotic RNases H are larger and more complex than those of prokaryotes. Although prokaryotes and some single-cell eukaryotes do not require RNases H for viability yet in higher eukaryotes RNases H is essential. *Rnaseh1* null mice arrest development around E8.5 because RNase H1 is necessary during embryogenesis for mitochondrial DNA replication. Mutations in any of the three subunits of human RNase H2 cause Aicardi-Goutiéres Syndrome, a human neurological disorder [61]. RNase T is one of eight distinct 3′→5′ exo-ribonucleases found in *E. coli*. This enzyme plays an important role in normal RNA metabolism including t-RNA end turnover and 3′ maturation of most stable RNAs. However, it is RNase that can efficiently remove residues near a double-stranded stem. RNase T also

has single-strand-specific DNase activity. Purified RNase T degrades both ss RNA and ss DNA in a non-processive manner. However, in contrast to its action on RNA, RNase T binds ssDNA much more efficiently and shows low sequence specificity. As with RNA, DNA secondary structure strongly affects its degradation by RNase T. Thus, RNase T action on a dsDNA with a single-stranded 3′-extension efficiently generates blunt-ended DNA molecules. This property of RNase T suggested it to be a useful enzyme for blunt-ended DNA cloning [62].

Pondering at the previous literature on ribonucleases, it seems that RNases are among the most important enzymes employed in the molecular biology. Their applications may be further extended as potent therapeutic antiviral, antitumor/ anticancer, antiproliferative, antiangiogenic and immunity boosting agents in medicine (Table **3**).

In higher plants, embryogenesis starts from a single fertilized egg cell (zygote). In these plants, RNase J is required for chloroplast and embryo development especially in the Arabidopsis [73].

RNases as New Class of Antitumor & and Anticancer Therapeutic Tools

It has been observed that the surface of cancer cells is more anionic than the noncancerous cells because of increase in glycosaminoglycan profile, phospholipids and glycosphingolipid content [74]. The cancer cells also undergo constitutive endocytosis much faster than the matched noncancerous cells, and thus because of rapid endocytosis and enhanced negative charge they perform enhanced uptake of RNases. Reducing the negative charge on a cell surface by diminishing the biosynthesis of heparin sulfate and chondroitin sulfate decreases net charge internalization, as does decreasing the positive charge of an RNase [75]. Thus there is somehow preferential uptake of RNase in these malignant cells and cytotoxicity, thereof. Globo H is a hexasaccharide considered as tumor associated antigen [8] which shows strong affinity for human RNase (RNase 1) and a homologue from cow (RNase A).

On other hand progression of cancers/ tumors requires induction of new vascular network to support rapid proliferation of malignant cells as well as extensive metastatic spread of cancer cells which eventually depends upon adequate supply of oxygen, nutrients and efficient removal of waste products. These activities of malignant cells are supported by angiogenic factors for laying fresh blood vessels [76]. Human RNASET2 i.e. hRNASET2 is a glycoprotein encoded by RNASET2 a tumor suppressor gene located on human chromosome 6 (6q27) and is a acidic hydrolase that shares 35% identity and 52% similarity with ACTIBIND, a fungal RNAse T2 [73]. The hRNASET2 exerts antiangiogenic and antitumorigenic effects via its binding to actin and consequent inhibition of cell motility [72]. The motility of cells requires the

Table 3: Ribonucleases and their Therapeutic Applications

Ribonuclease	Therapeutic role(s)	Reference
hPNPaseold3	Prevents tumor cell division, drives tumor cells to apoptosis and senescence.	[63]
Onoconase	In clinical trials as an anti-tumor therapy and promotes apoptosis of tumor cells.	-
RNAse 8	A novel antimicrobial protein that may contribute to host defense.	-
RNase H	Suitable for antiviral drug screening.	[47]
RNase 7	Selectively inhibits *Enterococcus faecium* on human keratinocytes/ skin.	[64]
Dicer like enzymes/RNase III	Antiviral properties in fungal, plant and protozoan models.	[65-67]
RNase from *Hohenbuehelia serotina* (a mushroom)	Antiproliferative activity towards leukemia and lymphoma cells, and inhibits HIV-1 reverse transcriptase.	[7, 68]
RNase 6 & 7	Antimicrobial function in human and murine urinary tract.	[69]
RNase A	Efficient catalyst of RNA cleavage and hence anticancer/ cytotoxic.	[70]
RNase 1	Toxicity for cancer cells; binds Globo H a tumor cell-associated antigen and anticancer treatment undergoing clinical trials.	[8]
RNase P	Cleaves specifically chimeric molecules created by chromosomal abnormalities in human cancer; anticancer treatment.	[71]
hRNASET2	Potent antiangiogenic and antitumorigenic agent independent of its ribonuclease capacity.	[72]

formation of actin-rich cell protrusions termed phyllopodia and lamellipodia; and these actin-rich pseudopods are a prerequisite for cancer-cell function. Moreover, endothelial cell proliferation, migration and actin reorganization are utmost vital components of angiogenic response that supports formation and development of malignant cells.

To have an effective treatment of cancer is to distinguish between normal and cancer cells. The molecular characterization of tumor-specific chromosomal abnormalities has revealed that fusion proteins are involved in most types of cancers [77]. The catalytic subunit of RNase P specifically destroyed tumor-specific fusion genes created as a result of chromosome abnormalities that is definitely a step towards a novel approach for cancer treatment. A 27 kDa RNase purified from dried fruiting bodies of a mushroom *Hohenbuehelia serotina* showing strong homology to fungal RNase inhibited HIV-1 reverse transcriptase with an IC_{50} of 50 M and also it was found to reduce uptake of radiolabelled thymidine in leukemia and lymphoma cells [6].

The urinary tract is often a common target of bacterial infections in humans and the urinary tract infections (UTI) are quite problematic in females of all ages. The UTI is complemented by the increasing incidence of infections caused by strains of *E. coli* that is increasingly becoming resistant to most front line antibiotics thus prompting the discovery of new class of potent antimicrobial agents. RNase 7, a member of RNase A superfamily is a potent epithelial-derived protein that maintains human urinary tract sterility, however, RNase 7 expression is restricted to primates thus limiting the experimental evaluation of its antimicrobial activity. A RNase 6 as another member of RNase A superfamily is present in mice has close amino acids sequence homology to RNase 7. It has been suggested that RNase 6 serves as an evolution-ary conserved antimicrobial peptide that participates in the maintenance of urinary tract sterility [69].

CONCLUSION AND FUTURE PERSPECTIVES

Virus infection remains a health challenge and thus a global a persisting problem with over 350 million chronically infected people causing an increased dissemination and risk of viral diseases. RNases play crucial roles at different steps of the cellular metabolism in bacteria and viruses. On the level of post-transcriptional gene regulation the permanent but coordinated and fine tuned degradation of mRNAs provides a direct impact on the availability of mRNA for the translation machinery. RNases are potent antiviral drugs that may be used for selectively treating viral infections. The antiviral drug field has greatly developed through the interaction of several disciplines such as virology, biochemistry, chemistry, structural biology and have acquired enormous achievements. The HIV-1 reverse transcriptase-associated RNase H activity is an attractive non-traditional target for drug development which has been scarcely explored. It is possible to foresee that the HIV-1 RNase H will be the next target in the antiviral drug discovery. The more recent studies have shown that the mammalian and some fungal RNases are also bestowed with the potential of antiproliferative, antiangiogenic and/ or antitumor/ anticancer activities. Some of the members of RNase A superfamily such as RNase 6 and RNase 7 appears to be evolutionary conserved peptides with potent antimicrobial activities for the upkeep of sterility in the urinary tract. The diverse biological activities of the RNases have made them a distinct distinguished class of potent biomolecules bearing antimicrobial, antiviral, antiproliferative and antitumor/ anticancer activities.

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