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Review Article
An Overview on Ribonucleases and their Therapeutic Effects

Gupta Shruti, Singh Sukhdev and Kanwar Shamsher Singh

Department of Biotechnology, Himachal Pradesh University, Summer Hill, 171005 Shimla, India

Abstract

The maturation and degradation of ribonucleic acid (RNA) into smaller nucleotides are carried out by a wide variety of cellular ribonucleases (RNases) which are also responsible for regulating the functional expression of several fundamental genes in living systems. They exist in eukaryotes to prokaryotes as well as in viruses. Structure, functions and catalytic mechanism of RNases have been well understood especially in *Escherichia coli* model. In addition to regulate the RNA metabolisms RNases demonstrate wide range of therapeutic effects also. They exhibit antitumor, antifungal, antiviral and immunosuppressive functions. Bovine pancreatic RNase (RNase A), bovine seminal RNase (BS-RNase), onconase and angiogenin are significant antitumor RNases. Onconase (Rampinase) is a stable amphibian homolog which displays significant cytotoxicity towards tumor cells and is the only ribonuclease which has reached up to the clinical levels. Onconase has proved potential therapeutic applications in the treatment of prostate, cervical, colon, breast and ovarian cancers as well as in lymphocytic leukaemia. The RNases demonstrate antiviral properties also. The Eosinophil Derived Neurotoxin (EDN) and the Eosinophil Cationic Protein (ECP) also known as RNases 2 and 3 are such RNases. The EDN shows broad range of antiviral activity towards several RNA viruses including HIV-1. Recombinant EDN constructs for example EDNsFv and rhEDN express cytotoxic effects against transfer receptor(s) expressing leukemia cells and respiratory syncytial virus. Some other RNases i.e., RNase 5, RNase 7 and RNase 8 have also been known for their antimicrobial effects indicating the role of RNases in immune defence. In the light of above, features and therapeutic applications of the typical RNases have been summerized in this review for the attention of healthcare research.

Key words: RNases, antitumor, onconase, antiviral, EDN, ECP, immune defence

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Corresponding Author: Kanwar Shamsher Singh, Department of Biotechnology, Himachal Pradesh University, Summer Hill, 171005 Shimla, India
Tel/Fax: +91-177-2832153/2832154

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INTRODUCTION

Ribonuclease (RNase) is an omnipotent nuclease which catalyzes the degradation of ribonucleic acid (RNA) into smaller components and it has been widely acknowledged as a therapeutic candidate. RNases play pivotal role in RNA metabolism and regulation of fundamental genes expression in living organisms[1]. They participate in several cellular functions ranging from DNA replication to protein function and defence against foreign microorganisms. RNase-controlled RNA degradation is a determining step in gene regulation, maturation and turnover which is further associated with progression of cancers and infectious diseases. Cytotoxic effects of RNases are the result of catalytic cleavage of available RNAs, byproducts and non-catalytic electrostatic interactions of exogenous enzymes with cell components.

Broadly, RNases are classified as endo-and exo-ribonucleases. Endo-ribonucleases cleave RNA molecule endo-ribonucleolytically (in 5'-3' direction) while exo-ribonucleases degrade RNA molecule in 3'-5' direction[2]. RNases display antitumor, antiviral, antifungal and immunosuppressive properties[3,4]. They produce genetic damage in cancer cells and destroy their RNA[5]. Resulted damaged molecular patterns stimulate immune sensors such as toll-like receptors (TLRs) and activated TLRs provoke immunokines which further induce production of cytokines, growth factors and angiogenic modulators and these determine tumor progression[6]. The anticancer function of immunotoxins which target normal cells also can be improved by introduction of RNases[7]. An intervention based on combination of RNase with other anticancer molecules can be a promising therapeutic preparation for effective tumor killing.

Since, the discovery of first RNases in 1961, several types of RNases have been explored till date[8]. Many of them have been studied in great detail with major emphasis on their applications as therapeutic molecule and RNA sequence determination. Exo-ribonucleases remove nucleotides from the 3′-5′ ends by cleaving the phosphodiester bonds at the ends of the polynucleotide chain. These enzymes are highly specific in their cleavage and produce staggered or blunt ends. Endo-ribonucleases cleave the phosphodiester bonds within the single stranded or double stranded RNA molecules. Features of some well known exo-and endo-ribonucleases are described in the following paragraphs.

EXO-RIBONUCLEASES

RNase PH: RNase PH is a 25 kDa *E. coli* RNase encoded by the *rph* gene which maps at 81.7 min at the genome. It was first identified due to its phosphorolytic activity against tRNAs[9,10]. It requires a divalent cation and uses phosphate as a co-substrate to degrade RNA thereby producing 5′ diphosphates. This RNase displays sequence and functional similarity with other *E. coli* RNases and poly nucleotide phosphorylase (PNPase) also. The RNase PH plays pivotal role in degradation of structural RNAs and provides a potential explanation for the growth defects caused by the absence of the phosphorolytic RNases[11].

Polynucleotide phosphorylase: PNPase is encoded by the *pnp* gene and maps at 69 min at the *E. coli*[12]. It is associated with the RNase E and degradosome in the cytoplasm[13]. Like RNase PH, PNPase also uses phosphate as co-substrate to carry out the phosphorolytic cleavage of RNA and nucleotide diphosphate[14]. PNPase catalyzes the typical 3′-5′ phosphorolysis of RNA and generates nucleoside diphosphate products[15,16]. The *E. coli* PNPase activity is blocked by RNA secondary structure while, the *Bacillus subtilis* PNPase is hindered by RNA hairpin structures. The *E. coli* PNPase also repairs the 3′ terminal CCA sequence of tRNA which is also executed by tRNAs[17]. Crystallographic structure analysis revealed that PNPase is a homotrimeric circular-shaped complex[18]. Amino acid sequences of bacterial PNPases share high degree of similarity with the PNPases of nuclear genome of plants and mammals[19].

RNase II: The RNase II family exo-ribonucleases are present in all domains and degrade RNA from the 3'-end releasing 5'-nucleotide monophosphates[20] They participate in the processing, degradation and quality control of all types of RNAs. The *E. coli* RNase II (72 kDa) is a prototype member of RNase II family exoribonucleases. It is encoded by the *rnb* gene which is mapped at 29 min on chromosome[21]. The RNase II has several orthologs and *Saccharomyces cerevisiae* RNase Rrp44p is one of them. Structural analyses revealed that RNase II contains two N-terminal Cold Shock Domains (CSDs), one C-terminal S1 domain and a central catalytic RNB domain[21]. The RNase II is a Mg^{2+} dependent enzyme and its activity is also inhibited by the RNA secondary structure[22]. The RNase II expression is regulated at transcriptional and post-transcriptional levels[23]. The RNase II is essential for growth as mutations in RNase II genes have been demonstrated with abnormal chloroplast biogenesis, mitotic control and cancer[24,25].

RNase R: It is a 92 kDa RNase encoded by the *rnr* gene which maps at 95 min in the *E. coli* genome[26,27]. The RNase R degrades linear and Y-structure RNAs and doesn't act on the

loop portion of a lariat RNAs. RNase R exhibits 60% sequence homology with RNase II[28]. It has two N-terminal Cold Shock Domains (CSDs), a central nuclease domain and an S1 domain near the C-terminus[29] nuclease domain executes nucleotide degradation wheras CSD and S1 domains give stability to the catalysis. The RNase R expression is essential for the virulence of *Shigella* sp. and *E. coli* strains[30].

RNase D: RNase D (49 kDa) is encoded by the *rnd* gene mapped at 40 min on the *E. coli* chromosome[31]. It belongs to the DEDD superfamily RNases and performs both DNA and RNA degrading functions[32]. The RNase D is a divalent metal ion (e.g., Mg^{2+}, Mn^{2+} and Co^{2+}) dependent RNase and generate ribonucleoside 5′-monophosphate products. The RNase D plays significant roles in tRNA and 5S rRNAs processing also[33]. The RNase D contains a catalytic domain and two helical domains which come together and form a ring shaped structure[33].

RNase T: RNase T is a 23.5 kDa enzyme encoded by the *rnt* gene (maps at 36 min) in *E. coli*[34]. It displays tremendous ribonucleolytic activity among the discovered exo-ribonucleases. It also is a member of DEDD superfamily which is a large family of 3′-5′ exo-nucleases[32]. It is made up of opposing dimers and functions on tRNA to yield mature 3′end of 5S and 23S rRNA[35].

RNase BN and oligo RNase: The RNase BN (encoded by *rbn* gene) is a 60 kDa ribonuclease which performs 3′ end maturation of tRNAs[36]. Oligoribonuclease acts on small oligonucleotides and is encoded by the *orn* gene which maps at 94 min on the *E. coli* chromosome[37]. It is a K2 dimer and essential to complete the degradation process of mRNA[38].

ENDO-RIBONUCLEASES

RNase I and III: The *rna* gene at 4.3 min on the chromosome of *E. coli* encodes a 27 kDa RNase I[39] which cleaves within unstructured regions of RNA and forms 2′-3′ cyclic phosphodiester RNA termini. RNase I plays crucial role in the turnover of RNAs and does not dependent on the divalent cations for the hydrolysis function[40]. The RNase III is an essential enzyme for RNA processing and post-transcriptional gene regulation[41]. It is a Mg^{2+}-dependent nuclease and is encoded by the *rnc* gene which maps at 55 min on the chromosome in *E. coli*. This RNase cleaves phosphodiester bonds of double stranded (ds) RNA and generates 3′ hydroxyl and 5′ phosphate termini. It is a 52 kDa homodimer RNase and contains an N-terminal nuclease domain and a C-terminal dsRNA binding domain (dsRBD). Several orthologs of RNase III have been discovered in prokaryotes and eukaryotes[42]. The eukaryotic ortholog dicer process the dsRNAs in short interfering (si) RNAs which target RNAs having complementary sequences.

RNase E, P and HI: RNase E is another important RNase for the processing of mRNA, rRNA and tRNA[43]. It is a 180 kDa Mg^{2+}-dependent phosphodiesterase encoded by *rne* gene, which maps at 24 min on the chromosome and cleaves adenine and uracil-rich sequences, generating 5′ phosphate and 3′ hydroxyl termini[44]. Catalytic site of RNase E is present in N-terminal half and C-terminal half contains the RNA binding site[45]. The RNase P is a divalent cation-dependent ribonucleoprotein RNase and is made up of one RNA subunit and one or more protein subunits[46]. This ribozyme is encoded by two genes; the protein subunit is coded by *rnpA* gene (maps at 83 min) and the RNA subunit is coded by *rnpB* gene which maps at 70 min[47].

RNase HI is also a Mg^{2+}-dependent phosphodiesterase that cleaves the RNA strand of RNA-DNA hybrids and is encoded by the *rnhA* gene (maps at 5 min) in *E. coli* chromosome[48,49]. The RNase HI plays pivotal role in ColE1 plasmid replication. The *rnhB* gene maps at 4.5 min and encodes another ribonuclease namely RNase HII[50].

THERAPEUTIC APPLICATIONS OF RNases

Anticancer effects: Anticancer effects of RNases have been extensively studied with animal ribonucleolytic enzymes, viz., bovine pancreatic RNase A, bovine seminal RNase (BS-RNase), onconase and angiogenin[51]. Among these, BS-RNase and onconase demonstrated significant anticancer potential with bovine pancreatic RNase A, a modest angiogenin activity was observed to work in opposite direction and initiate vascularization of tumor and subsequent tumor growth[52]. Bovine seminal plasma RNase, onconase and binase are well known anticancer RNases which have been described in following paragraphs. The major types of RNase with their therapeutic applications have been illustrated in Table 1. RNases have the capacity to degrade mRNAs efficiently and thus prevent their translation into biologically active proteins (Fig. 1).

Bovine seminal ribonuclease: The BS-RNase (EC 3.1.27.5) is expressed in the seminal vesicles and testes of *Bos taurus*[53]. It is a secretory ribonuclease[54]. Its native form exists as a dimer in which both subunits are held together by two disulfide linkages between Cys-31 and Cys-32[55]. The disulfide linkage

Table 1: Major types of RNases, their characteristics and applications

RNase type	Source organism	MW (kDa)	Important characteristics	Applications	References
BS-RNase	*Bos taurus*	27	Secretory, homodimeric enzyme. Binds to and destabilizes the membrane bilayer and thus reaches the cytosol and degrades the cellular RNA	Antitumor activity, treatment of thyroid cancer	Spalletti-Cernia *et al.*[61]
Onconase/Rampinase/ P 30 protein	*Rana pipiens*	12	Exhibits thermal and guanidine stability. Degrades tRNA, inhibits protein synthesis which leads to apoptosis of cells. Only RNase in clinical trials	Antitumor and anticancer activity. Treatment of malignant mesothelioma, human lung carcinoma, human pancreatic aden carcinoma	Zwolinska and Smolewski[76]
Binase	*Bacillus intermedius*	12.2	Extracellular cationic RNase. Cleaves the RNA at purine residues, does not require any cofactor	Antitumor activities against K562, A 549 and ovary cancer cells, Kasumi-1 cells. Antiviral effect aginst rabies virus, plant virus, influenza virus strains	Garipov *et al.*[77] and Mahmud and Ilinskaya[78]
RNase 7	*Homo sapiens*	14.5	Cationic residues in RNase 7 bind to negatively charged components on bacterial surface and facilitate the insertion of RNase	Antimicrobial activity against *Enterococcus faecium, Pseudomonas aeruginosa* and *Pichia pastoris*	Spencer *et al.*[79]
Angiogenin	*Homo sapiens, Mus musculus, Rattus norvegicus*	14	Enters the cell via its receptor binding site and then into the nucleus via nuclear localization signal and degrades the tRNA through its catalytic activity	Antibacterial activity	Li and Hu[80]
RNase 2/EDN	*Homo sapiens*	18.6	Elicits immune response via leukocyte activation, maturation and chemotaxis	Antiviral activities against respiratory syncytial virus, HIV-1 and some RNA viruses	Liu *et al.*[81] and Yang *et al.*[82]

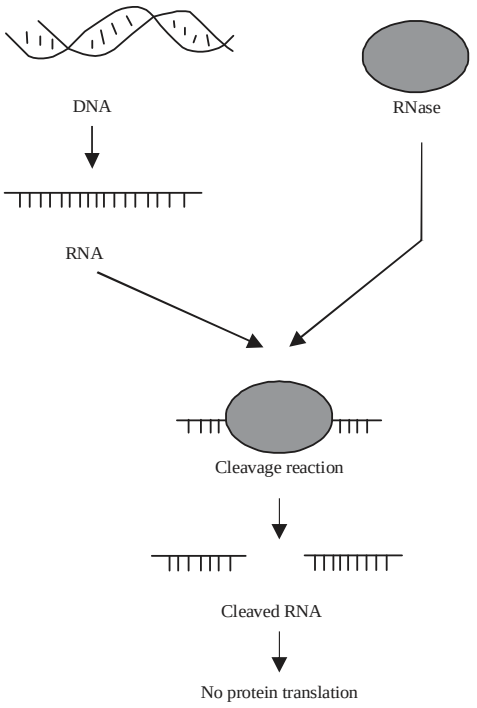

Fig. 1: Action of RNase on the RNA in the target cell

undergoes cleavage under reducing conditions of cytosol and the dimer is converted into two monomers. The monomeric form is neutralized by the ribonuclease inhibitor[56]. Amino acid sequence and crystallographic analysis revealed that BS-RNase belongs to the pancreatic RNase A superfamily[57]. Each subunit of BS-RNase shares 82% sequence identity with RNase A[58]. The BS-RNase exerts antiproliferative and apoptotic effects on cancer cells via Beclin-1-mediated autophagy[54]. It has cytotoxic, aspermagenic and immunosuppressive properties for protecting sperm cells from the female immune system. The BS-RNase suppresses the activation of proliferating lymphocytes by reducing the expression of interleukin (IL-2)[59]. The BS-RNase treated lymphocytes undergo apoptosis via DNA fragmentation, chromatin migration, disorganised mitochondria and cell shrinking[60]. The BS-RNase induces cell death in thyroid carcinoma cells also[61].

Onconase: Onconase is a 104 amino acid member of RNase A superfamily. It is a promising candidate for the treatment of malignant mesothelioma[62]. It was isolated from oocytes and early embryos of *Rana pipiens.* It shares ~30% homology with RNase A and resembles in 3D structure also. Crystallographic and homology studies revealed that onconase has three disulfide linkages at positions 19-68, 30-75 and 48-90 and an imidazol ring of His97 residue rotated at 180° angle[63,51]. Catalytic site of onconase composed of His10, Lys31 and His97 residues[64]. Onconase tolerates thermal and guanidine induced

transitions (up to 90°C and 4.4 M) and does not interact with mammalian RNase inhibitor hence evade the attack of inhibitor[65]. Onconase demonstrated significant cytotoxic effects against cancer cell lines i.e., HL-60, HT-29, 9L rat glioma, K-562, Colo-320, JCA-1, U937, A549 and ASPC-1[66,67]. Onconase and its derived products exhibited potent antitumor effects against cervical, breast, colon, pancreatic, ovarian and prostate cancers with LD_{50} (median lethal dose) value ~10^{-7} M^3. It catalyzes the formation of interfering RNAs (RNAi), degrades tRNAs and inhibits protein synthesis, which results in apoptosis of the cell[68]. Onconase modulates cytokine-receptor interactions, MAPK, Jak-STAT, Bcl-2, Bax and various other signaling pathways in cancer models[69]. It activates jun-N-terminal kinase (JNK) and caspase-9, -3 and -7 proteins in HeLa cells, serine proteases in HL-60 cells[64] and IL-6, IL-24 and ATF-3 in MM cell line[69]. In lymphocytic leukemia onconase mediates its apoptotic effects by reducing NF-κB expression level[70]. Onconase also pull down the level of reactive oxygen species in cancer cells[71]. The cytotoxic potential of onconase increased when positively charged residues were added to the enzyme by site-directed mutagenesis and chemical modification[72,73]. Onconase modulates tumor cell apoptosis at microRNA expression level and reduces the oncogenic microRNAs in malignant mesothelioma models[74,65]. In clinical trials onconase demonstrated some adverse effects, viz., lymphocyte proliferation suppression, renal failure, bone marrow toxicity, mascular stiffness and tremor[75].

Binase: Bacteria also provide anticancer RNases[3,83]. Several bacterial species producing RNase with cytotoxicity towards cancer models have been discovered and *Bacillus intermedius*[84], *Bacillus amyloliquefaciens*[85,86] and *Streptomyces aureofaciens*[87] are exemplary among them. Binase (EC 3.1.27.3) is a 12.2 kDa extracellular cationic RNase from *Bacillus intermedius*[77]. It cleaves RNA at purine residues and does not require any cofactor to accomplish this hydrolysis[86,87]. Binase was the first anticancer bacterial RNase which demonstrated comparable cytotoxic potential against malignant cells[62]. Binase exhibited significant antiproliferative and apoptotic activities on K562, A549 and ovary cancer cells[77]. It elicited cell death in transformed fibroblasts and myeloid progenitor cells[88] and demonstrated cytotoxic effects on Kasumi-1 cells with half-maximal concentration of 0.56 µM. Binase has suppressive effects on several oncogenes also i.e., KIT, AML1-ETO and FLT3-ITD[89]. Moreover, Binase has low immunogenicity[90] and doesn't affect cell viability of leukocytes and myeloid progenitor cells[91]. Binase also demonstrated antiviral properties against rabies viruses, plant viruses and the influenza strains[78].

Some other RNases have also been reported with anticancer activities. RNase L showed antiproliferative effects against H9 leukemia cells[92]. RNase Sa3 from *Streptomyces aureofaciens*[93] showed cytotoxicity against K562 cells with IC_{50} of 5 µM. RNase Sa3 is not inhibited by the cytosolic RNase inhibitor[94]. Some of the mushroom species also have been known for their anticancer properties. *Hypsizigus marmoreus* RNase (18 kDa) reduced the L1210 proliferation (IC_{50} 60 µM). Another RNase (14.5 kDa) from fresh fruiting bodies of the edible mushroom *Lyophyllum shimeji* exhibited cytostatic potential on liver cancer HepG2 cells (IC_{50} 10 µM) and on breast cancer MCF7 cells (IC_{50} 6.2 µM)[95]. A 28 kDa RNase from ascocarps of *Tuber indicum* showed antiproliferative effects on HepG2 and MCF7 cells with IC_{50} values 12.6 and 16.6 µM, respectively[96].

RNases IN HOST DEFENCE

Eosinophil RNases: Cytoplasmic granules of human eosinophilic leukocytes secrete two major ribonuclease proteins[97], the Eosinophil Cationic Protein (ECP) and the Eosinophil Derived Neurotoxin (EDN). The ECP and EDN belong to the pancreatic type RNase family and share 70 and 90% similarity in their amino acid and nucleotide sequences[98]. The EDN is also known as RNase II or eosinophilic protein-X which demonstrated better RNase activity than ECP[99,100]. The EDN is located on 'q' arm of chromosome 14[101] and shares similarity with human liver and urinary RNase (RNase U)[102]. The EDN is an 18.6 kDa single chain polypeptide with four characteristic disulfide bonds and His15-Lys38-His129 catalytic triad[103,104]. The RNases can elicit immune response via leukocyte activation, maturation and chemotaxis. The EDN showed antiviral activities against respiratory syncytial virus, HIV-1 and some RNA viruses[81,82]. The recombinant EDN (EDNsFv) created by fusing human EDN gene and antibody fragment of human transferring receptor exhibited significant cytotoxic effects against transferring receptor expressing leukemia cells[105]. Another recombinant EDN (rhEDN) reduced the infectivity of respiratory syncytial virus which causes asthma aggravations[106,107]. The EDN levels correlate with neuroinflammation characteristic in Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disorder. Thus EDN is used as a biomarker for ALS disease[81]. The EDN engineered with hepatitis B virus core protein (HBVc), suppressed the hepatitis B infected cells without affecting normal cells RNA[105]. Some other ribonucleases i.e., mEar 11 and mEar 2 (the mouse eosinophil associated RNases) are also reported for antiviral and immunogenic effects. Alveolar macrophages produce mEar 11 upon administration of IL-4 or 13. The mEar 11 is a

chemo-attractant for $CD11c^+$ dendritic cells and $F4/80^+CD11c^-$ macrophages[108]. These mEars exhibited significant antiviral effects against influenza strains and pneumonia virus of mice[109].

RNase 7 and 8: RNase 7 (~14.5 kDa) is a member of RNase A superfamily and the gene encoding RNase 7 is located on chromosome[110] 14q11.2. It is expressed in skin, liver, kidney, skeletal muscles and heart[99]. It exhibited potential antibacterial activities against *Enterococcus faecium, Pseudomonas aeruginosa* and *Pichia pastoris*[79]. Cationic residues in RNase 7 bind to negatively charged components on bacterial surface and facilitate the insertion of RNase. In *P. aeruginosa*, RNase 7 enters the cell by making complex with the outer membrane protein Opr I[111]. The RNase 7 was reported to be express significantly in response to the external stimuli[112]. Wanke and colleagues[113] studied the host defence aspects of RNase 7 that *Staphylococcus epidermis* induces RNase 7 expression in keratocytes via TLR2, EGFR and NF-κB pathways. Studies with protozoan's exposure also demonstrated that RNase 7 contributes to a responsible role in host defence[114]. RNase 8 is another ribonuclease in the RNase A superfamily. It shares 78% amino acid similarity with RNase 7[115]. The RNase 8 play responsible role in placental host defence by defending the foetus from pathogen from the maternal circulation[116]. RNase 8 exhibited significant antimicrobial activities against *Klebsiella pneumonia, Enterococcus faecium, E. faecalis, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*[117].

Angiogenin: Angiogenin is a 14 kDa ribonuclease which was isolated from HT-29 conditioned media[116,118]. It has the ability to induce the formation of new blood vessels[119,120]. Angiogenin has a receptor binding site (which facilitates the angiogenin entry in the cell), a nuclear localization sequence (by which angiogenin enters the nucleus) and a catalytic site which catalyze the tRNA cleavage[121]. Angiogenin is reported for its host defence features. In mouse, six kinds of angiogenin (1-6) are reported. Mouse angiogenin 4 is reported to be significantly expressed in paneth cells upon bacterial LPS (lipopolysaccharide) challenge. It exhibited significant antibacterial activities against intestinal microbes[80,99].

Besides these applications, RNases have been reported to exhibit antiviral functions also. RNase from *Rana* catesbeiana suppressed the multiplication of Japanese encephalitis virus and accelerated apoptosis of virus-infected cells[122]. Yadav and Batra[123] recognized specific targets of restrictocin, an RNase from *Aspergillus restrictus* in HIV-1 genome. Some mushroom RNases displayed inhibitory effects against Reverse Transcriptase (RT) of HIV-1[3]. The RNases from mushroom species *Thelephora ganbajun* (~30 kDa)[124], *Lyophyllum shimeh*i[96], *Hygrophorus russula* (~28 kDa)[125], *Hohenbuehelia serotina* (~27 kDa)[4] and *Ramaria formosa* (~29 kDa)[126] inhibited HIV-1-RT with IC_{50} concentrations 0.3, 7.2, 4.64, 50 and 3 μM, respectively.

CONCLUSION

Ribonucleases are potential therapeutic candidates and must be converted into druggable forms with sufficient bioavailability. They have promising anticancer and antiviral applications. Till date, very low number of bacterial RNases have been discovered. Also, none of the discovered RNase has crossed the clinical barriers for therapeutic use. Genetic pathways of their synthesis and mechanisms of actions in cancer cells are still a topic of research which needs to be understood for their development into a therapeutic product. So, there is an essential need to discover potential and elaborate the ribonucleases of therapeutic value. RNases mentioned in this review must be further researched for clinical trials and druggability evaluation especially BS-RNase and onconase for anticancer value and RNase 7, 8 and eosinophil RNases for anti-HIV properties. In final words, this review provides general information about RNases and can help the healthcare pharmacy for the treatment of serious metabolic syndromes including cancers and AIDS.

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