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Original Article

Specific Targeting of Recombinant Human Pancreatic Ribonuclease 1 using Gonadotropin-Releasing Hormone Targeting Peptide toward Gonadotropin-Releasing Hormone Receptor-Positive Cancer Cells

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Abstract

Background: Targeted drug delivery is a novel method to specifically deliver anticancer therapeutics to tumor sites. Gonadotropin-releasing hormone (GnRH) is a decapeptide, and its target binding property has attracted attention as a means of targeted drug delivery. Human pancreatic ribonuclease 1 (hpRNase1) has been shown to exert anticancer properties, when fused to a targeting moiety. The goal of the present study was to add a GnRH targeting peptide to the N-terminus of hpRNase1 to specifically target GnRH receptor (GnRH-R) expressing cells.

Methods: This *in vitro* study was conducted at Shiraz Institute for Cancer Research (Shiraz, Iran) in 2019. The coding sequence of GnRH and hpRNase1 were fused, and the chimeric protein together with non-fused hpRNase1 were produced in *E. coli* (BL21). The recombinant proteins were purified, and their biological activity was evaluated using MTT and apoptosis assays. Non-parametric Kruskal–Wallis tests with Dunn's *post hoc* tests were performed to determine the significant differences between the study groups.

Results: GnRH-hpRNase1 chimeric protein specifically inhibited the proliferation of PC-3 (P=0.021), LNCaP (P=0.034), and AD-Gn (P=0.041) cells, while the growth of negative cells (AD-293) was not significantly affected (P=0.081). GnRH-hpRNase1 decreased the IC50 values more than non-fused hpRNase1, by approximately 26.5-fold (P=0.036) for PC-3 cells, and exerted its growth inhibitory effects through apoptosis induction.

Conclusion: Fusion of GnRH to hpRNase1 structure produced an enzyme, which could specifically target tumor cells. This approach can be used to eliminate tumors that harbor GnRH-R.

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Keywords ● Drug delivery systems ● Ribonucleases ● Pancreatic ● Gonadotropin-releasing hormone

What's Known

- Human pancreatic ribonuclease 1 (hpRNase 1) induces apoptosis in mammalian cells. However, their binding to the cells is inefficient and non-specific.
- Various studies have fused targeting moieties such as antibodies and their derivative to RNases to become more specific to tumor cells.

What's New

- Similar to antibodies, the addition of cell-targeting peptides to the human pancreatic RNase1 structure has made the enzyme specific to tumor cells.
- Gonadotropin-releasing hormone-human pancreatic RNase 1 (GnRH-hpRNase1) fusion protein efficiently enters cells and inhibits cell growth through induction of apoptosis in concentrations lower than that of hpRNase1.

Introduction

Targeted drug delivery has become an important tool in anticancer therapy. The method is based on delivering an active pharmaceutical ingredient to a specific part of the body and, subsequently, reduce its toxic off-target effects. During the last

decade, cell-targeting peptides (CTPs) have gained increased attention as novel targeting moieties in targeted tumor therapy, as well as tumor imaging and diagnosis.[1-4] CTPs are very small, easy to produce, tolerable by patients, can penetrate tumor mass, and have low bone marrow accumulation.[5] Gonadotropin-releasing hormone (GnRH) is considered to be an appropriate CTP to target cancer cells, since its receptor (GnRH-R) is expressed on various tumor types such as ovary, endometrium, prostate, and breast tumor.[6, 7] In addition, GnRH is not immunogenic in humans and is very small (only 10 amino acid long,1200 Dalton), allowing it to easily fuse with any cytotoxic protein.[8, 9] Various studies have successfully used GnRH peptide to produce chimeric anticancer proteins and have shown that these proteins can specifically target and eliminate tumor cells.[3, 10, 11]

Certain members of the RNase A superfamily (ranpirnase [an amphibian RNase], bovine seminal RNase, human eosinophil-derived neurotoxin, and eosinophil cationic protein) have been reported to have cytotoxic effects on various cancer cell lines.[12, 13] Ranpirnase, also called onconase, is the only member of the family that has proceeded to phase III (treatment of unresectable malignant mesothelioma),[14] and phase I (treatment of clinical human papillomavirus infections)[15] human clinical trials. Despite the promising potential of onconase for cancer treatment, human-origin RNases such as human pancreatic ribonuclease 1 (hpRNase1) have gained much attention. This is because they are less likely to be immunogenic, and have a high therapeutic index, favorable tissue distribution, and higher ribonucleolytic activity than amphibian RNases. Note that the ribonucleolytic activity of hpRNase1 is 10^4-10^5 fold greater than that of the amphibian enzyme.[16]

The hpRNase1 is a 128 amino acid protein, which catalyzes the cleavage of RNA on the 3'-side of pyrimidine bases.[17] Although the enzyme by itself is not cytotoxic, it does kill cancer cells once it reaches the cytoplasm. It exerts its cytotoxicity via abrogating protein biosynthesis through RNA degradation leading to cell cycle arrest or apoptosis.[12] Hence, targeted delivery of this enzyme can enhance the specificity and increase the likelihood of the enzyme uptake by target cancer cells.

A previous study reported that receptors of GnRH are overexpressed in prostate, breast, and ovary cancer, while their expression is not detectable in normal human organs.[6] We hypothesized that the addition of GnRH peptide to the structure of hpRNase1 would enhance the tumor inhibitory effect of the enzyme through specific targeting of GnRH-R overexpressing cells and induce apoptosis by disabling the cell protein machinery via RNase1 activity. Therefore, the present study aimed to design and produce a recombinant fusion protein capable of targeting tumor cells that overexpress GnRH-R.

Materials and Methods

Vector Design and Construction

This *in vitro* study was conducted at Shiraz Institute for Cancer Research (Shiraz, Iran) in 2019. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.S930).

Three variants of human pancreatic ribonuclease1 were produced, namely hpRNase1, human immunodeficiency virus-1 transactivator of transcription (Tat-hpRNase1), and GnRH-hpRNase1. One common reverse primer (5′-AAAACTCGAGGGTGCTGTCCTCAACGC-3′) was used for the amplification of all three variants. The coding sequence of hpRNase1, Tat-hpRNase1, and GnRH-hpRNase1 were all PCR amplified from a synthetic pBluescript II cloning vector using h-f primer (AAAACCATG GGTAAGGAGAGCTGCGCGAAGAAGTTTC), t-f primer (AAAACCATGGGTTATGGCCGCAAA A A A C G C C G C C A G C G C C G C C G C G GCAAGGAGAGCTGCGCGAAGAAGT), and g-f primer (5′-AAAACCATGGGTCAA CACTGGAGCTACG-3′), respectively. Specific forward and common reverse primers contained *NcoI* and *XhoI* restriction sites, respectively, for further cloning. All three genes were separately sub-cloned into pET28a (+) expression vector (Novagen, Madison, WI, USA) at their corresponding restriction sites with a C-terminal hexa-histidine tag (His-tag). Chemically competent *E. coli* DH5α cells (Novagen, USA) were then transformed with recombinant constructs. The plasmids were extracted from transformants, using GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, USA), and the accuracy of cloning was confirmed by nucleic acid sequencing (Eurofins Genomics, Germany).

Protein Expression and Purification

E. coli BL21 (DE3) (Novagen, USA) hosts were transformed using the verified recombinant constructs for protein expression. Bacteria were grown in Luria Bertani broth (Sigma-Aldrich, USA), supplemented with 50 µg/mL kanamycin (Sigma-Aldrich, USA) at 37 ˚C and were agitated (160 rpm) until the absorbance at 600 nm reached 0.5.[18] Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside

(IPTG, Sigma-Aldrich, USA) to the final concentration of 0.25 mM, and incubated at 28 ˚C for eight hours. Cells were then harvested by centrifugation at 6000 ×g at 4 ˚C for 20 minutes. Bacterial pellets were resuspended and homogenized in lysis buffer (Urea 8M, NaH_2Po_4 50 mM, β-mercaptoethanol 10 mM; pH 7.2) (Merck, Germany) and were sonicated on ice (six times at 55% amplitude) for 30 seconds to disrupt the bacterial cell wall and membrane. The bacterial lysates were centrifuged at 12000 ×g, at 4 ˚C for 30 minutes. The solubilized proteins were purified using Ni-NTA column (Invitrogen, NY, USA). The denatured proteins were refolded *in vitro* by sequential dialysis with gradual reduction of the urea concentration. Green fluorescent protein (GFP) was produced using the same vector, host, as well as the same expression and purification procedures. They served as a control in all experiments. In addition, a fusion variant of hpRNase1, named Tat-hpRNase1 was produced. This protein contained HIV1 transactivator of transcription-protein transduction domain (TAT-PTD) peptide and was used as a control for the internalization of GnRH-hpRNase1 fusion protein.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting of Purified Proteins

The purified proteins were analyzed using SDS-PAGE to demonstrate the presence of recombinant proteins and verified with Western blot analysis. For SDS-PAGE analysis, all fractions (supernatant, flow-through, wash, and elution fractions) of the recombinant proteins were electrophoresed on 12.5% polyacrylamide gel and the corresponding bands were visualized using coomassie Brilliant Blue (BioRad, UK). For western blot analysis, 50 ng of each purified protein was separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel, and transferred onto a 0.2 µm polyvinylidene difluoride (PVDF) membrane (GE Healthcare, USA) using Trans-Blot® Turbo™ blotting system (Bio-Rad, CA, USA). Membranes were blocked in PBS-T (phosphate-buffered saline 1× supplemented with 0.15% Tween 20), and 5% nonfat dried milk (Sigma-Aldrich, USA), at 4 °C, for 24 hours. The membranes were incubated with mouse horseradish peroxidase-conjugated anti-His-tag antibody (1:3200, BioLegend, USA) for 40 minutes at room temperature. The membranes were treated with enhanced chemiluminescence substrate (Bio-Rad, CA, USA) to visualize the corresponding bands using ChemiDoc™ MP system (Bio-Rad, CA, USA).

Biological Assays

Cell Culture

PC-3, LNCaP (cells of prostate cancer), and AD-293 cells (human embryonic kidney cells; National Cell Bank of Iran, Tehran, Iran) were grown in Roswell Park Memorial Institute, 1640 medium, supplemented with 10% (V/V) heat-inactivated fetal bovine serum, 100 µg/ml streptomycin (both from Gibco, Life Technologies, NY, USA), and 100 U/mL penicillin (Sigma-Aldrich, USA). All cell lines were cultured at 37 °C in 5% CO_2 in a humidified atmosphere.

Stable Transfection of AD-293 Cells

The coding sequence of GnRH-R was optimized, synthesized, and cloned into the mammalian expression vector, pcDNA3.3 (Invitrogen, NY, USA). The resultant construct was linearized using PvuI restriction enzyme (Thermo Scientific, USA) and then gel purified to remove the transfection inhibitors. AD-293 cells were transfected with linearized pcDNA-GnRHR recombinant plasmid by electroporation (capacitance: 950 µF, voltage: 300 V, resistance: ∞). Cells were transferred to a T-25 culture flask (Jet Biofil, China) and incubated at 37 °C in 5% CO_2 in a humidified atmosphere for 48 hours. To obtain a stable cell line capable of overexpressing GnRH-R, the cells were then grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium (Thermo Scientific, USA), supplemented with 10% fetal bovine serum and 500 µg/ml Geniticin® (both from Thermo Scientific, USA) for three weeks. At the end of the selection, survived cells were analyzed for GnRH-R expression by western blotting analysis.

Cell Viability Assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay was used to evaluate the viability of the cells. PC-3 cells were detached using 0.25% trypsin/EDTA (Thermo Scientific, USA) and seeded in 96-well microtiter plates at a density of 12.5×10^3 per well. After 24 hours, the cells were subjected to increasing concentrations of each protein (0-50 µM). Control groups were treated with 50 µM GFP as well. In addition, three wells remained as untreated controls. The plates were then incubated at 37 °C for 48 hours. Finally, 0.5 mg/ml MTT solution (Sigma-Aldrich, USA) was added to the wells followed by an additional four hours of incubation. The optical density was measured using a microplate reader (Anthos microplate reader, UK) at a wavelength of 570 nm, and the half-maximal inhibitory concentration (IC50) values were calculated. The entire experiment was performed in triplicate with three repeats.

Apoptosis Assay using Annexin V-Phycoerythrin (PE)/7-Amino-Actinomycin (7-AAD) Staining

4×10^5 of PC-3, AD-Gn (GnRH-R positive), and AD-293 (GnRH-R negative) cells were seeded into 6-well plates and were separately subjected to 1 µM of recombinant hpRNase1 and GnRH-hpRNase1 proteins. After 18 hours of incubation at 37°C, cells were harvested, washed and stained using an Annexin V-PE/7-AAD kit (BD Biosciences, USA) according to the manufacturer's instruction. Apoptosis was then examined using FACSCalibur™ flow cytometer (BD Biosciences, USA). Cells treated with GFP and unstained cells were used as controls. All experiments were performed in triplicate.

Statistical Analysis

GraphPad Prism version 6.0 (GraphPad Software, CA, USA) was used to analyze the numerical data and plotting the graphs. The data were presented as median and interquartile range. Non-parametric Kruskal–Wallis tests with Dunn's *post hoc* tests were performed to determine significant differences between the study groups. Data of flow cytometric analyses were analyzed using FlowJo software version 7.6.2 (FlowJo, LLC, CA). The sum of early and late apoptotic cell percentages was calculated as total apoptosis and represented as mean±SEM (standard error of the mean). P values<0.05 were considered statistically significant.

Results

Construction, Expression, and Purification of Recombinant Proteins

The coding sequence of recombinant hpRNase1 (414bp), Tat-hpRNase1 (450 bp), and GnRH-hpRNase1 (447 bp) were sub-cloned into pET28a (+) vectors at *NcoI* and *XhoI* restriction sites. The nucleic acid sequencing confirmed the accuracy of the sequence of all three inserts. Protein purification was performed using nickel-immobilized metal affinity chromatography column with the yield of 1.7 mg/mLfor Tat-hpRNase1, 1.4 mg/mL for hpRNase1, and 0.9 mg/mL for GnRH-hpRNase1 after dialysis. The purity and molecular weight (MW) of the proteins (hpRNase1: 16 kDa, Tat-hpRNase1: 17.6 kDa, and GnRH-hpRNase1: 17.2 kDa) were demonstrated using SDS-PAGE analysis (figures 1A-1C). Moreover, western blot analysis confirmed authenticity of all three proteins (figure 1D).

Generation of Stable AD-Gn Cell Line

The western blotting analysis confirmed the expression of GnRH-R in AD-293 cells (figure 2A). These cells (designated as AD-Gn) along with PC-3 and LNCaP cells were used as GnRH-R-positive cell lines to evaluate the effects of GnRH-hpRNase1 in all experiments.

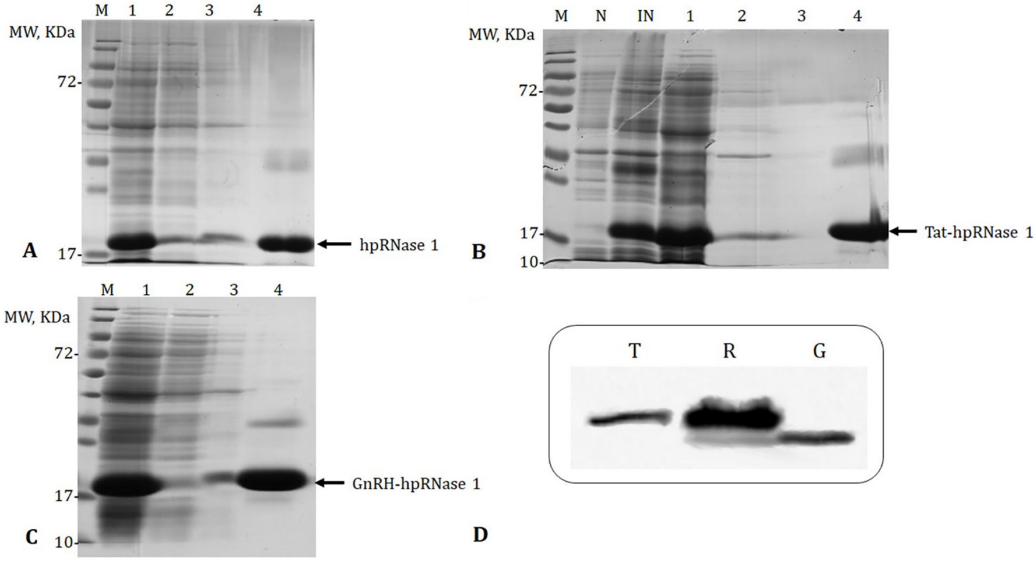

Figure 1: Recombinant variants of hpRNase1 analyzed using SDS-PAGE and western blot analysis. Purified hpRNas1 (A), Tat-hpRNase1 (B), and GnRH-hpRNase1 (C) were electrophoresed on 12.5% polyacrylamide gel, and were stained with Coomassie Brilliant Blue. Figures 1A-1C: Lane 1: Supernatant of bacterial lysates following solubilization of inclusion bodies, Lane 2: Flow-through fraction following binding of recombinant proteins to the resin, Lane 3: Wash fraction, Lane 4: Elution fraction, M: Fermentas PageRuler™ Protein Ladder. Figure 1D: Western blot analysis of reduced recombinant variants of hpRNase1 performed following purification. The polyvinylidene difluoride membrane was blotted with 50 ng of purified hpRNase1 (R), GnRH-hpRNase1 (G), and Tat-hpRNase1 (T). The bands were detected with anti-His-tag antibody. The molecular size of hpRNase1, GnRH-hpRNase1, and Tat-hpRNase1 were 16 kDa, 17.2 kDa, and 17.6 kDa, respectively. hpRNase1: Human pancreatic ribonuclease1 protein; Tat-hpRNase1: HIV-1 transactivator of transcription-hpRNase1 fusion protein; GnRH-hpRNase1: Gonadotropin-releasing hormone-hpRNase1 fusion protein

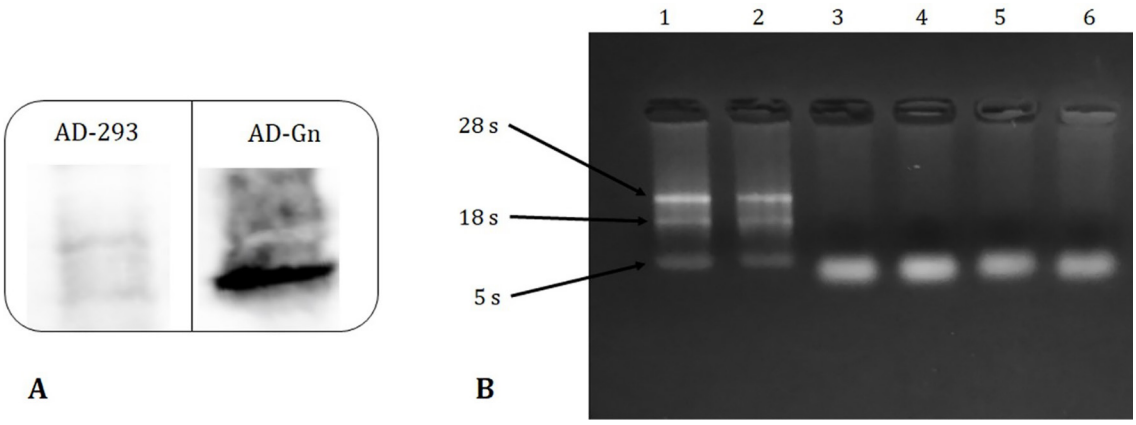

Figure 2: (A) AD-293 and AD-Gn cells analyzed for the expression of GnRH-R using western blot analysis. The polyvinylidene difluoride membrane was blotted with 50 ng of AD-293 and AD-Gn cell lysates. The bands were then detected by anti-GnRHR antibody. The molecular size of GnRH-R was 37.73 kDa. (B) Ribonucleolytic activity of purified hpRNAse1 variants examined using the qualitative gel-based assay. Untreated eukaryotic total RNA (1), as well as eukaryotic total RNA treated with GFP (as negative control) (2); RNase A (as positive control) (3); hpRNase1 (4); Tat-hpRNase1 (5); and GnRH-hpRNase1 (6) were electrophoresed on 1% w/v agarose gel. The presence of 28 s, 18 s, and 5 s rRNA bands indicated that the extracted RNA had a good quality. RNA samples subjected to RNase A, as well as all hpRNAse1 variants were degraded, while GFP protein did not affect the RNA samples. hpRNase1: Human pancreatic ribonuclease1 protein; Tat-hpRNase1: HIV-1 transactivator of transcription-hpRNase1 fusion protein; GnRH-hpRNase1: Gonadotropin-releasing hormone-hpRNase1 fusion protein

Bioactivity Assessment of Purified Recombinant Variants of hpRNase1

The ability of all three variants of the recombinant protein to degrade eukaryotic RNA molecules was evaluated using the qualitative gel-based assay. Like the positive control (commercial RNase A), all three enzymes degraded the RNA molecule, while RNAs were not affected upon exposure to the purified GFP (negative control) (figure 2B).

Cytotoxic Effect of Recombinant Variants of hpRNase1

48 hours incubation of PC-3 cells with purified recombinant hpRNase1 variants revealed that all three recombinant proteins inhibited the proliferation of PC-3 cells in a dose-dependent manner with IC50 values of 8.49±0.94 μM (P=0.027), 0.55±0.07 μM (P=0.011), and 0.32±0.06 μM (P=0.036) for hpRNase1, Tat-hpRNase1, and GnRH-hpRNase1, respectively (figure 3A). Moreover, in another experiment, AD-293, which does not express GnRH-R, as well as PC-3, LNCaP, and AD-Gn, which all express GnRH-R on their surface were treated with 0.55 μM of each enzyme variant and GFP protein for 48 hours. The data showed that, at this concentration, Tat-hpRNase1 inhibited the proliferation of all cell lines (P=0.013 for PC-3, P=0.028 for LNCaP, P=0.020 for AD-293, and P=0.035 for AD-Gn cells). However, this effect was not significant compared to that of hpRNase1 (figure 3B). Additionally, GnRH-hpRNase1 significantly inhibited the proliferation of PC-3 (P=0.021), LNCaP (P=0.034), and AD-Gn (P=0.041), while it did not significantly affect the growth of

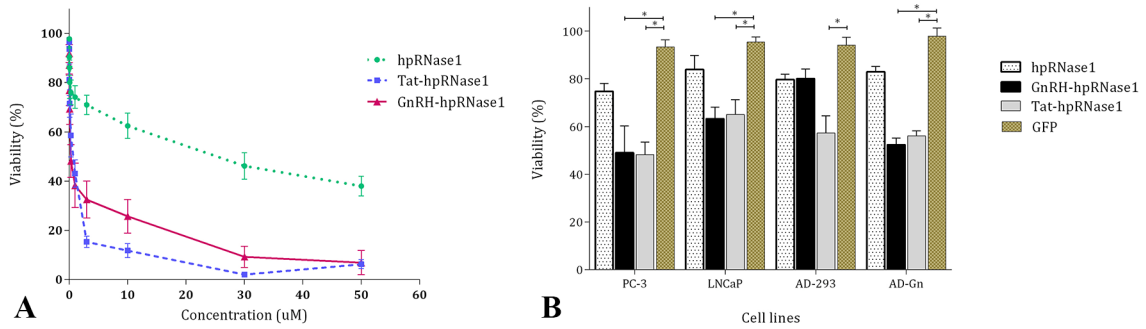

Figure 3: (A) IC50 values of recombinant variants of hpRNase1 on PC-3 cell line determined using MTT assay. The IC50 values were calculated as 8.49±0.94 μM (P=0.027), 0.55±0.07 μM (P=0.011), and 0.32±0.06 μM (P=0.036) for hpRNase1, Tat-pRNase1, and GnRH-hpRNase1, respectively. Dots represent an average value (median of viability) of ten replicates. Data were analyzed using Kruskal-Wallis test, and the results are reported as median (interquartile range). (B) Growth inhibitory effect of recombinant variants of hpRNase1 on different human cancer cell lines evaluated using MTT assay. Cell lines were subjected to 0.55 μM of GFP (as a control) and each hpRNase1 variant. Data were analyzed using Kruskal-Wallis test and Dunn's multiple comparison post-hoc test. The results are presented as median (interquartile range). *P<0.05 considered statistically significant; hpRNase1: Human pancreatic ribonuclease1 protein; Tat-hpRNase1: HIV-1 transactivator of transcription-hpRNase1 fusion protein; GnRH-hpRNase1: Gonadotropin-releasing hormone-hpRNase1 fusion protein

AD-293 cells (P=0.081) (figure 3B). Similar to Tat-hpRNase1, the difference between the cytotoxic effect of GnRH-hpRNase1 and hpRNase1 was not statistically significant (P=0.091 for PC-3, P=0.086 for LNCaP, and P=0.102 for AD-Gn). The viability of each cell line after treatment with the enzyme variants is summarized in table 1. GFP protein did not significantly affect the viability of cells under the same conditions (P=0.074 for PC-3, P=0.068 for LNCaP, P=0.083 for AD-293, and P=0.093 for AD-Gn).

The Effect of hpRNase1 Variants on Apoptosis of Cell Lines

Flow cytometry was used to explore whether recombinant GnRH-hpRNase1 could induce apoptosis in cell lines. The percentage of necrotic PC-3, AD-293, and AD-Gn cells as well as the profile of cell distribution is illustrated in figure 4. The results showed that hpRNase1 and its fusion variant, GnRH-hpRNase1, induced apoptosis in all three cell lines. However, the induction of apoptosis by GnRH-hpRNase1 was

Table 1: The cytotoxic effect of human pancreatic ribonuclease 1 variants on different cancer cell lines

Enzyme variants (0.55 µM)	Viability (%)			
	PC-3	LNCaP	AD-293	AD-Gn
hpRNase1	74.73 (65.31-78.05)	83.87 (77.24-89.84)	79.75 (71.10-81.87)	82.94 (73.94-85.14)
Tat-hpRNase1	48.28 (43.10-53.45)	65.05 (62.34-71.23)	57.33 (53.25-64.47)	56.07 (51.60-58.18)
GnRH-hpRNase1	49.05 (46.81-60.23)	63.25 (56.29-68.12)	80.07 (78.66-84.11)	52.39 (48.35-55.21))

Cells were treated with 0.55 µM of each enzyme variants, and the viability was assessed using MTT assay. The results are presented as median (interquartile range) of three independent experiments. hpRNase1: Human pancreatic ribonuclease1 protein; Tat-hpRNase1: HIV-1 transactivator of transcription-hpRNase1 fusion protein; GnRH-hpRNase1: Gonadotropin-releasing hormone-hpRNase1 fusion protein

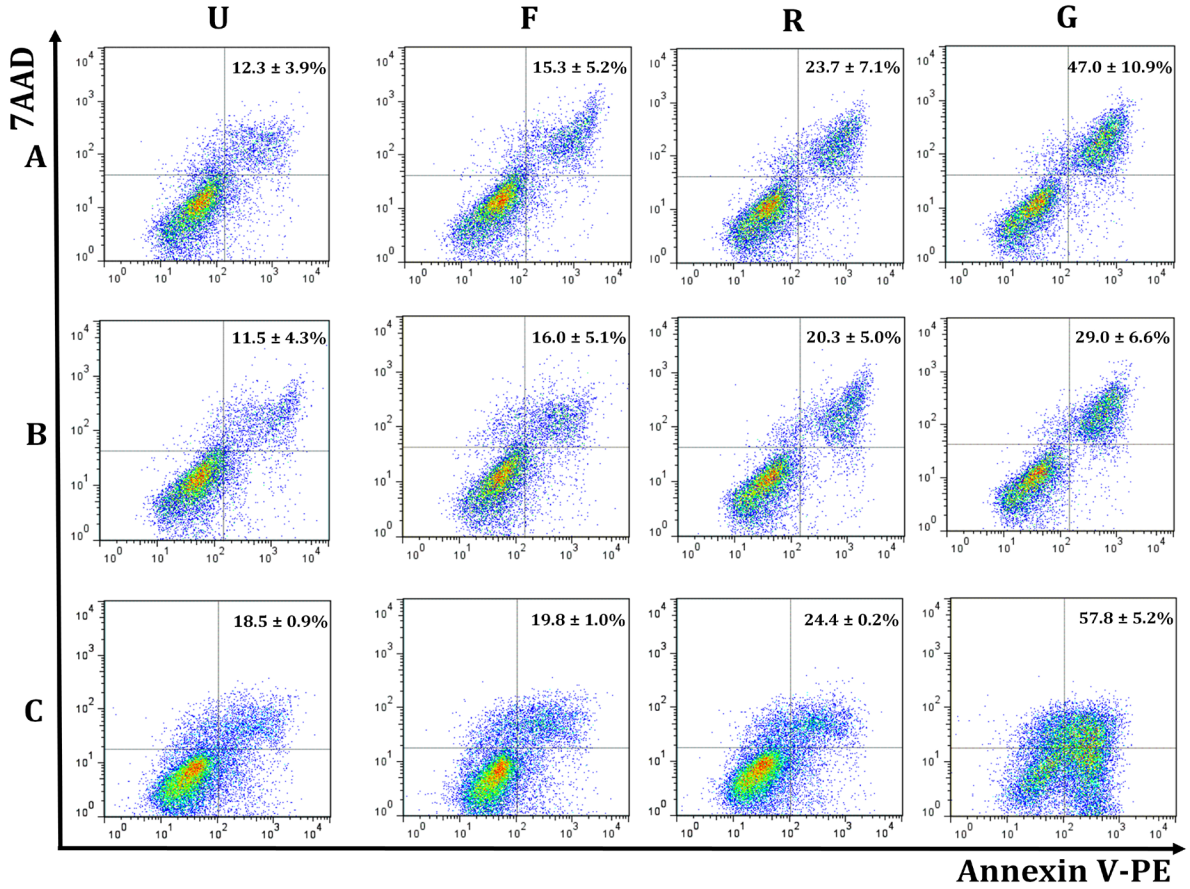

Figure 4: Induction of apoptosis examined in different cell lines after 18 hours treatment with 1 µM of each recombinant hpRNase1 variants using flow cytometry. PC-3 (A), AD-293 (B), and AD-Gn (C) cells were separately treated with 1 µM of green fluorescent protein (F), hpRNase1 (R), and GnRH-hpRNase1 (G) for 18 hours. Control cells (U) remained untreated. Apoptosis was evaluated through annexin V-PE/7AAD staining using FACSCalibur™ flow cytometer. Values on the quadrants represent the sum of early and late apoptotic cells as means±SEM (standard error of the mean) (n=3). hpRNase1: Human pancreatic ribonuclease1 protein; GnRH-hpRNase1: Gonadotropin-releasing hormone-hpRNase1 fusion protein; GFP: green fluorescent protein

higher in PC-3 (47±10.9%, P=0.09) and AD-Gn (57.8±5.2%, P=0.15) cells than in AD-293 cells (29.0±6.6%, P=0.18). Moreover, GnRH-hpRNase1 was more potent than hpRNase1 in inducing apoptosis in PC-3 (P=0.097) and AD-Gn (P=0.088) than in AD-293.

Discussion

The results showed that the recombinant GnRH-hpRNase1 fusion protein could specifically target cancer cells that overexpress GnRH-R, while it did not significantly affect those cells that do not express GnRH-R. Based on the flow cytometry analysis, it was also shown that the recombinant GnRH-hpRNase1 eliminated the target cells through induction of apoptosis.

Inherently, mammalian RNases have promising antineoplastic potential. However, two main barriers have rendered them ineffective. First, they are inhibited by RNase inhibitor (RI), which is ubiquitously present in the cytosol of all mammalian cell types.[19] Researchers have applied different strategies to eliminate the sensitivity of mammalian RNases to RI, including dimerization,[20] chemical modification,[16] fusion to targeting moieties,[21] and the use of site-directed mutagenesis[12] to sterically hinder the binding site or to attenuate the enzyme's affinity for RI. In the current study, we used an engineered version of hpRNase1 harboring six amino acid substitutions (R4C/L86E/N88R/G89D/R91D/V118C). The amino acid sequence of this variant is 95% identical to that of native hpRNase1, nevertheless, it was shown to be RI-evasive, while retaining high conformational stability.[22] However, in this study, the recombinant version of this enzyme was successfully produced in a prokaryotic system, and the qualitative gel-based assay demonstrated that it was active against mammalian RNAs. Although the results of the viability assessment indicated that hpRNase1 could inhibit the proliferation of cell lines, this inhibitory effect was not pronounced and appeared to be non-specific, as it inhibited the proliferation of all cell types.

The second feature of this enzyme is the non-specific binding, as well as low uptake of hpRNase1 by tumor cells, making it ineffective in eliminating cancer cells. To overcome this shortcoming, we generated two recombinant fusion forms of hpRNase1 protein, named Tat-hpRNase1 and GnRH-hpRNase1 to make a cytotoxic RNase. The former contained HIV1 TAT-PTD peptide, and the latter contained GnRH targeting peptide at their N-termini. HIV-1 transactivator of transcription-protein transduction domain peptide (TAT-PTD) is one of the most studied cell-penetrating peptides (CPPs), applied for delivery of a variety of therapeutic molecules including liposomes,[23] proteins, and antibodies.[24, 25] Thanks to the positive net charge of CPPs, the CPP-drug complex can reach negatively charged cell membranes, and the CPPs can traverse the cellular membrane. Inside the cell, the complex can escape the endosome and reach the cytoplasm, where the drug can exert its biological activities.[26] Therefore, we added TAT-PTD peptide to hpRNase1 to examine whether an increase in penetration can enhance the cytotoxicity of the recombinant hpRNAse1. It was revealed that, compared with hpRNAse1, the cell growth inhibition was significantly increased when Tat peptide was introduced into the hpRNase1 structure. Based on our observations, it was inferred that the IC50 value was reduced by approximately 15-fold, when the PC-3 cells were subjected to Tat-hpRNase1. However, further investigation indicated that Tat-hpRNase1 inhibits the proliferation of all examined cells in the same manner without any specificity.

Although cell-targeting peptides have been used to target chemotherapeutics as well as toxic proteins, to the best of our knowledge, GnRH peptide so far has not been employed to generate a targeted hpRNase1. The fusion of GnRH targeting peptide to the N-terminus of hpRNase1 did not adversely affect the ribonucleolytic activity of the enzyme, probably because its N-terminal amino acids are not involved in the catalytic activity of the molecule. Moreover, the growth inhibitory effect of GnRH-hpRNase1 was approximately 26.5-fold higher than that of hpRNase1 on GnRH-R positive PC-3 cells, probably due to the presence of overexpressed GnRH receptors on these cells, which leads to the accumulation of GnRH-hpRNase1.[3] Given that GFP treatment did not affect the viability of the cells, and the growth inhibitory effect of GnRH-hpRNase1 was greater in GnRH-R positive cells (PC-3, LNCaP, and AD-Gn) than in GnRH-R negative counterpart (AD-293), it can be argued that the GnRH-hpRNase1 fusion protein specifically targeted the GnRH-R overexpressing cells. Moreover, the growth inhibitory pattern of GnRH-hpRNase1 in receptor-positive cells was similar to that observed for Tat-hpRNase1 in all cells. Therefore, it is suggested that GnRH-hpRNase1 fusion protein exerted its inhibitory effect following internalization in the target cells. However, the antiproliferative effect of Tat-hpRNase1 and GnRH-hpRNase1 was not statistically significant, when their effects were compared to that of hpRNase1 alone. The inefficient and small quantity uptake of

hpRNase1 by the cells,[27] which led to the growth inhibition of approximately 16-28% of cell lines, may explain why these differences were not statistically significant. A limitation of the present study was the fact that it was difficult to assess RNase biological activity due to its high stability and abundance in the environment.

Incorporation of targeting moieties to the structure of hpRNAse1 is an efficient strategy employed by several studies to target hpRNase1 to specific cells and tumors. De Lorenzo and colleagues generated an immunoRNase consisting of hpRNase1 and anti-ErbB2 single-chain fragment variable (scFv) and demonstrated that the chimeric enzyme was selectively toxic to ErbB2-positive SK-BR-3 and MDA-MB453 cells, whereas free hpRNase1 had no considerable effect on negative control cells.[28] Similarly, Psarras and colleagues fused human interleukin 2 (hIL-2) to hpRNase1 and showed that the fusion protein could selectively arrest protein translation in activated lymphocytes hyper producing high-affinity IL-2 receptors.[29] It was also reported that a fusion hpRNase1 consisting of human epidermal growth factor could specifically inhibit the proliferation of cells overexpressing the human epidermal growth factor receptor.[30] In addition, targeting of hpRNase1 to the transferrin receptor,[31] fibroblast growth factor receptor,[32] CD30+ lymphomas,[33] and recently, nucleolin (NCL) for Triple-Negative Breast Cancer[34] have been shown to increase the specificity and activity of hpRNase1 in eliminating tumor cells.

Conclusion

Ribonucleases, in particular human pancreatic RNase1, have shown promising features for the development of a new class of therapeutics. However, they suffer from two main shortcomings, namely being RI sensitive and not exclusively acting against cancer cells. We designed and produced a novel fusion hpRNase1 harboring GnRH targeting peptide at its N-terminus. This fusion protein retained its ribonucleolytic activity and could specifically target GnRH-R expressing cells and inhibit their proliferation through apoptosis induction. Considering its promising anti-tumor activity, the fusion enzyme should be further examined on GnRH-R-expressing tumor xenografts to evaluate its anti-tumor effects *in vivo*.

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