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Research Paper

Phi29 pRNA Vector for Efficient Escort of Hammerhead Ribozyme Targeting Survivin in Multiple Cancer Cells

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KEY WORDS

pRNA, bacteriophage phi29, ribozyme, gene therapy, nanomotor

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ABSTRACT

Ribozymes are potential therapeutic agents which suppress specific genes in disease-affected cells. Ribozymes have high substrate cleavage efficiency, yet their medical application has been hindered by RNA degradation, aberrant cell trafficking, or misfolding when fused to a carrier. In this study, we constructed a chimeric ribozyme carried by the motor pRNA of bacteriophage phi29 to achieve proper folding and enhanced stability. A pRNA molecule contains an interlocking loop domain and a 5'/3' helical domain, which fold independently of one another. When a ribozyme is connected to the helical domain, the chimeric pRNA/ribozyme reorganize into a circularly permuted form, in which the 5'/3' ends are relocated and buried in the original 71'/75' positions. Effective silencing of an anti-apoptotic gene survivin by an appropriately designed chimeric ribozyme, as demonstrated at mRNA and protein levels, led to programmed cell death in various human cancer cell lines, including breast, prostate, cervical, nasopharyngeal, and lung, without causing significant non-specific cytotoxicity. Through the interlocking interaction of right and left loops, monomer pRNA/ribozyme chimeras can be incorporated into multi-functional dimer, trimer and hexamer complexes for specific gene delivery. Using the phi29 motor pRNA as an escort may revive the ribozyme's strength in medical application again.

INTRODUCTION

The recent development of molecular therapy is one of the most promising applications of modern biological science. Ribozymes,[1-3] anti-sense RNA[4,5] and small interfering RNA[6-10] all show significant potential to be used as new molecular approaches to down-regulate specific gene expression in cancerous or viral-infected cells. At the end of 2002, RNA was selected by Science as the #1 Breakthrough of the Year.[11] Compared with protein and peptides, RNA molecules are non-immunogenic and have fewer side effects when used for therapeutic application. However, recent application of ribozymes in therapy has been hindered due to the finding that ribozyme can not yield high enough efficiency in cells as revealed by several clinical trials, even though they have been proven to be highly effective in vitro.

The lack of an efficient delivery system is believed to be one of the reasons that cause the unsuccessful therapeutic application of ribozyme. Currently, various systemically delivery procedures of therapeutic RNA have been applied in mammals, including intravenous administration using hydrodynamic delivery, intravenous administration of modified RNA of RNA-coding DNA plasmid, viral vector mediated delivery, etc.[12] Approaches to achieve specific delivery by changing viral tropism for are in development.[13] At this time, the development of a safe, efficient, specific and non-pathogenic system for the delivery of therapeutic RNA is highly desirable. The RNA sequence of ribozymes dictate their enzymatic activity and specificity, and therefore must be carefully designed for recognition and cleavage of the correct target RNA molecule. In addition, incorrect folding and degradation by RNase add another important issue for low in vivo efficiency of ribozymes. Alternative approaches have been sought to enhance the stability and efficiency of ribozyme by embedding it to various vector sequences. For example, the stem and anitcodon region of tRNA have been employed to accommodate ribozyme sequences.[14-17] In summary, successful application of ribozymes and other therapeutic RNA awaits improvements in the following aspects: (1) specific recognition of target cells, penetration of the cell membrane; (2) the ability to survive degradation by RNase within serum and cells; (3) intracellular trafficking to appropriate cell compartment; (4) correct folding of

ribozymes in a cellular environment, especially when it is fused to a carrier; and 5) low toxicity to normal, non-targeted cells.

A bacteriophage phi29-encoded small RNA has been shown to play a novel and essential role in the DNA packaging motor of phi29.[18] This 117nt RNA is termed packaging RNA or "pRNA". It has been revealed that six copies of pRNA form a hexameric ring[19,20] to drive the DNA-packaging motor using ATP as an energy source (reviewed in refs. 21 and 22). The pRNA contains two functional domains. The central domain of pRNA located between bases #23 and 97 contains two interlocking left hand and right hand loops that can be engineered to form stable dimers, trimers or hexamers via hand-in-hand interactions. The helical DNA packaging domain is located at the 5'/3' paired ends.[23] Removal of the helical domain does not alter the nature of pRNA's intermolecular interaction, i.e., replacement or insertion of nucleotides before residue #23 or after residue #97 does not interfere with the formation of dimers, trimers, and hexamers.[24-26]

The structure of pRNA has been studied extensively and three-dimensional computer models of pRNA monomers, dimers, trimers and hexamers have been constructed[27] based on a variety of experiments including photo-affinity cross-linking,[28] chemical modification and chemical modification interference,[29-31] base deletion and mutation,[23,32-35] ribonuclease probing,[36,37] oligo targeting,[38,39] competition or inhibition assays,[39-41] UV crosslinking[28] and cryo-AFM.[30,31,42] Our recent work indicates that RNA can serve as building blocks to build patterned superstructure as nanomaterials via bottom-up assembly.[43]

We have taken advantage of the structure and molecular features of phi29 pRNA and constructed a polyvalent delivery vehicle to perform specific delivery of siRNA into cancer cells. Firstly, by replacing or fusing the double stranded helical domain of pRNA with foreign functional sequences, a set of chimeric pRNA subunits have been designed to carry siRNA or cell-recognition units such as folate or cell surface receptor binding RNA aptamer.[44,45] Secondly, pRNA subunits are designed to have complementary interlocking loops so that stable RNA nanoparticles including dimers, trimers or hexamers can be produced when chimeric pRNA with desired gene silencing/cell recognition motifs are mixed together. The size of the fabricated RNA complex is around 30 nanometer in diameter, which can fulfill the size requirement for endocytosis, as nanoscale devices larger than 100 nanometer have difficulties to enter cells and molecules smaller than 20 nanometer would be cleaned out from blood vessels quickly during circulation.[46] Via a simple incubation procedure, the chimeric RNA nanoparticles recognized targeted cells, bound and entered the cell to silence targeted gene expression and induced cell death specifically. Specificity and efficiency in cancer therapy were confirmed in animal trials. The use of RNA as a protein-free delivery vehicle could avoid the immune response and avoid the rejection of protein vectors after repeated long-term drug administration.

Previously, we have examined the use of pRNA as a novel ribozyme escort to target mRNA of Hepatitis B virus. It was found that the additional pRNA vector sequence can ensure appropriate folding of the ribozyme and to protect the ribozyme from exonucleases degradation[24] and this chimeric ribozyme cleaved the substrate RNA completely in vitro. Cell culture studies revealed that the chimeric ribozyme significantly inhibited replication of the Hepatitis B virus with an efficiency much higher than ribozyme alone. In this report, we explore the possibility of using pRNA as a chaperone to carry a ribozyme against survivin, which is indispensable for tumor development. The successful construction of chimeric pRNA ribozyme will provide another robust subunit in the pRNA based-polyvalent therapeutic vehicle against cancerous or viral-infected cells.

MATERIALS AND METHODS

Construction and synthesis of RNA. The synthesis of RNA was described previously.[23] 10 mM of magnesium was included in all buffers to maintain the intermolecular interaction and folding of pRNA.[31,42] The nomenclature of pRNA and the resulting chimeric pRNA subunits for the construction of deliverable RNA nanoparticles have been reported.[25,46] pRNA/RZ(Sur) represents a pRNA chimera that harbors a hammerhead ribozyme targeting survivin, following the same strategy for the construction of pRNA/ribozyme (HBV), a chimeric RNA with a pRNA-based vector to carry a hammerhead ribozyme for successful cleavage of the hepatitis B virus (HBV) polyA signal.[24] pRNA/ribozyme(mut1) and pRNA/ribozyme(mut2) chimeras contained mutations in the catalytic core of the ribozyme that disturbed the cleavage ability of the ribozyme, yet possessed the same 14-base stem sequences as the therapeutic ribozyme. pRNA/ribozyme(HBV) is also used in this study, and referred as pRNA/RZ(mut3). The oligo sequences are listed in the table of supplementary data.

Assay for gene silencing efficiency of pRNA/RZ (Sur) by transfection. The methods for the transfection of cells with pRNA chimera have been reported.[46] Human cervical cancer cells Hela T4 were plated in a 24-well culture dish and incubated overnight at 37°C with 5% CO_2. The following morning, the medium was replaced with an antibiotic-free medium and the cells were transfected separately with pRNA constructs at 0.5 μg, 0.1μg or 0.02 μg using Lipofectamine 2000 (Invitrogen) with three duplicates per treatment. After six hours, the transfection solution was replaced with a standard medium supplemented with 10% FBS and antibiotics. Tetrazolium-based MTT assays were performed to determine the cell viability in the next day. Similar assays were performed for the KB, LNCaP and MDA-MB-231 cells.

Western blot assay. T47D human breast cancer cells were seeded in 60 mm dishes and grown to 70% confluency in DMEM supplemented with 10% FBS and penicillin/streptomycin. Prior to transfection, cells were switched to antibiotic free medium and then transfected with pRNA chimera targeting survivin, or mutant chimeric ribozyme, as negative control. Lipofectamine 2000 was used according to the manufacturer's instructions. Cells were rinsed and harvested in lysis buffer at 12, 16, 20 and 24 hours after transfection. Protein concentrations were determined and equal amounts of protein were loaded into a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and membrane was blotted using Amersham ECL kit).

Assay for apoptosis of MCF-7 induced by pRNA/RZ(Sur) using PI/Annexin V double staining in flow cytometry. The methods for apoptosis assay have been reported.[44] Briefly, human breast cancer cells MCF-7 were grown in DMEM medium supplemented with 10% FBS and penicillin/streptomycin, and plated into 24-well plates at a density of 0.5×10^5 cells per well. Transfections were performed with a 0.5 μg ribozyme per well and three duplicates per treatment. 48 hours after transfection, apoptosis in breast cancer cell MCF-7 was assessed with the annexin V-propidium iodide (PI) double staining method.

Real-time PCR assay. MCF-7 cells were seeded into 24-well plates at a density of 0.5×10^5 cells per well. Transfections were performed with a 0.5 μg RNA per well and three duplicates per treatment.

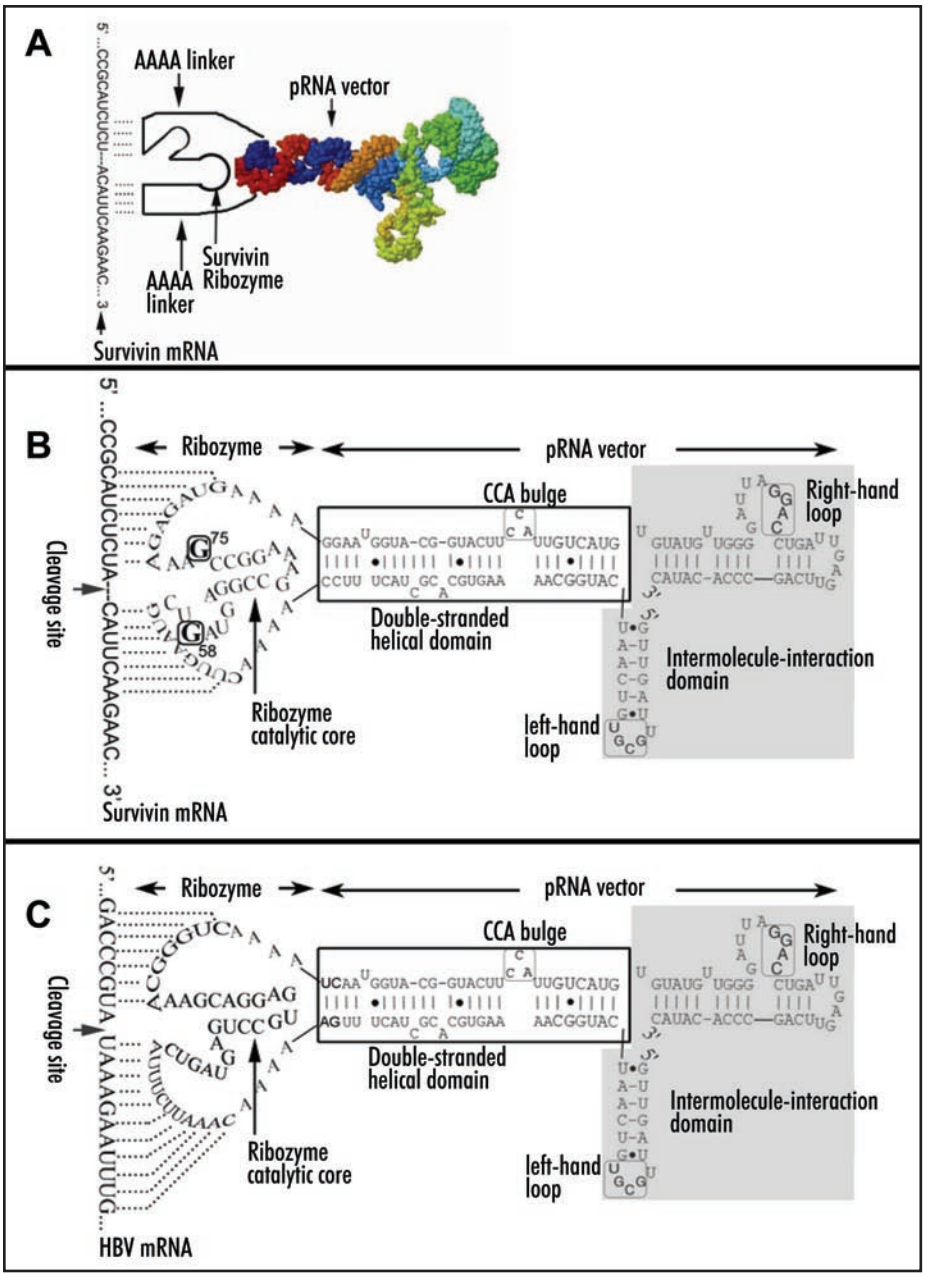

Figure 1. Sketch of sequence and structure of pRNA chimera. (A) Sketch of chimeric ribozyme harbored in pRNA vector. (B) Sequence and secondary structure of pRNA/RZ(Sur) and two cleavage disabling mutants. Both pRNA/RZ(mut1) and pRNA/RZ(mut2) have the identical pRNA vector sequence, while the sequence of catalytic core are altered. The highlighted G75 is deleted in pRNA/RZ(mut1). G75 is deleted and G58 is replaced with an "A" from pRNA/RZ(mut2). (C) Sequence of pRNA/RZ(mut 3) which targets the HBV mRNA.

Cells were harvested 48 hours after transfection and total RNA was extracted with a QIAamp RNA kit (Qiagen). Reverse transcription was carried out on 1 μg of RNA with RevertAidTM First Strand Synthesis Kit (Fermentas).

Equal amounts of cDNA were submitted to PCR, in the presence of SYBR green dye with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN) and the ABI PRISM 6700 Real time PCR detection machine (Fengling Biotechnology Inc.). Primers for survivin were 5'-AAA GAG CCA AGA ACA AAA TTG C-3' and 5'-GAG AGAGAA GCA GCC ACT GTT AC-3', which were published previously.[47] PCR was performed by 40 cycles of 0.5 seconds at 95°C, 10 seconds at 60°C and 10 seconds at 72°C. PCR without template was used as a negative control. The β-actin endogenous housekeeping gene was used as an internal control. Both β-actin and negative control were amplified on the same plate as the experimental gene of interest. Each sample was normalized by using the difference in critical thresholds (CT) between survivin and β-actin. The following equation was used to describe the result:

$$\Delta\Delta CT_{survivin} = \Delta\ CT_{survivin} - \Delta\ CT_{\beta\text{-actin}}$$

where $\Delta\ CT_{survivin}$ was the difference in CT between survivin and negative control, and $\Delta\ CT_{\beta\text{-actin}}$ was the difference between β-actin and negative control. The mRNA levels of each sample were then compared using the expression $2^{\Delta\Delta CTsurvivin}$. The results of each group were averaged. The expression level for non-transfected sample was arbitrarily assigned value 1 and the final results were expressed as fold number compared to non-transfected sample.

In vitro cleavage by hammerhead ribozyme. The survivin mRNA targeting ribozyme pRNA/RZ (Sur) and control ribozyme pRNA/RZ (mut2) cleavage reactions were performed at 37°C for 60 min in the presence of 20 mM Tris pH 7.5, 20 mM $MgCl_2$, and 150 mM NaCl. Ribozyme RNA (5 μg) was used to cleave partial sequence of survivin mRNA (200 ng). The 127nt [^{32}P] labeled RNA substrate is expected to be cleaved into a 77 nt and a 50 nt fragment. The cleavage products were separated by 8% PAGE/8 M urea-denaturing gel.

5' Cy3 Labeling of pRNA. The 5' Cy3 labeling of RNA are achieved by including ADOF550/570 (Adegenix, Inc) in the in vitro transcription reaction. The final concentration for all components will be 40 mM Tris-HCl, pH 8.0, 5 mM DTT, 6 mM $MgCl_2$, 2 mM spermidine, 0.01% Triton X-100, 0.25 mM ATP, 1 mM each of UTP, GTP, and CTP, 2 mM ADO F550/570, 0.05 -0.5 μM dsDNA containing the T7 ∅2.5 promoter, 500 units of T7 RNA polymerase per 100 μL reaction, 10–20 units of RNase inhibitor per 100 μL reaction. The RNA was then purified by PAGE/Urea.

RESULTS

Construction of Circularly Permuted pRNAs and pRNA Chimera Harboring Ribozyme. To evaluate the effectiveness of therapeutic RNA molecules including ribozymes in treating cancer, it is necessary to suppress genes involved in tumor development and progression. Survivin was chosen as a target since it inhibits apoptosis and is detected only in cancer cells but not in normal adult cells. It has been reported that the suppression of survivin induces the apoptosis of cancer cells.[48,49]

To connect the sequence of ribozyme with pRNA vector, the strategy of circular permutation was employed to construct a chimeric pRNA/ribozyme (Fig. 1A). A ribozyme targeting survivin was connected to the original 5/3 ends of pRNA by two poly-A linker sequences, resulting in the reorganization of pRNA into a circularly permuted form (Fig. 1B). pRNA molecule adopts a unique secondary structure in which the 5' and 3' ends are in close proximity. Relocation of the 5' and 3' ends does not affect the overall secondary structure as well as the function of the two independent domains: the intermolecular interacting domain and the helical domain. We have been able to generate a series of circular permutated pRNA (cp-pRNA) with 5'/3' opening moved to various locations including 71'/75' position used in the construction of chimeric pRNA/ribozyme. The reorganized cp-pRNAs retain the same biological function as wild type pRNA as demonstrated by their full effectiveness in a phi29 viral assembly assay.[50] Thus our previous results suggest that a larger foreign moiety such as a ribozyme sequence can be placed to the nascent 5'/3' ends of pRNA with such a strategy. The approach of circular permutation is to ensure the independent and correct folding of both the ribozyme and the pRNA vector, and to relocate the nascent 5'/3' end of the RNA chimera into a tightly folded region, and protect the pRNA from exonuclease digestion. Three mutative chimeric ribozymes were used in this study (Fig. 1).

pRNA/RZ (Sur) chimera induced apoptosis and cell death specifically in all tested cancer cells. The effects of chimeric pRNA/RZ (Sur) in human breast cancer cells. The pRNA/RZ (Sur) was tested in four breast cancer cell lines: MCF-7, T47D, MDA-MD-231 and MDA-MB-453. After transfection, the majority of cells shrank and detached from the cell culture plate, while the control pRNA/RZ (mut3) did not cause any change in cell morphology (Fig. 2A, and Table 1). When the effects of various chimeric ribozymes were measured by an MTT assay in MDA-MD-231 and MDA-MB-453 cells, pRNA/RZ (Sur) showed strong and dose-dependent inhibition of cell viability, while pRNA/RZ (mut3) showed no effects (Figs. 2C and D). These results indicate that pRNA/RZ (Sur) could induce cell death specifically in a dose-dependent manner when introduced into breast cancer cells by transient transfection.

The effects of chimeric pRNA/RZ (Sur) in human cervix cancer cells. Various amounts of pRNA/RZ (Sur) and the mutant was transfected into the human cervical cancer Hela T4 cells to evaluate its function of inducing apoptosis in human cervix cancer cells. Cell viability was measured at various time points after transfection. As shown in Figure 3A, treatment with pRNA/RZ (Sur) resulted in a dose-dependent decrease of cell viability compared to no RNA control. For pRNA/RZ (mut3) treated cells, no significant difference in survival rates was observed when increasing amounts of RNA were

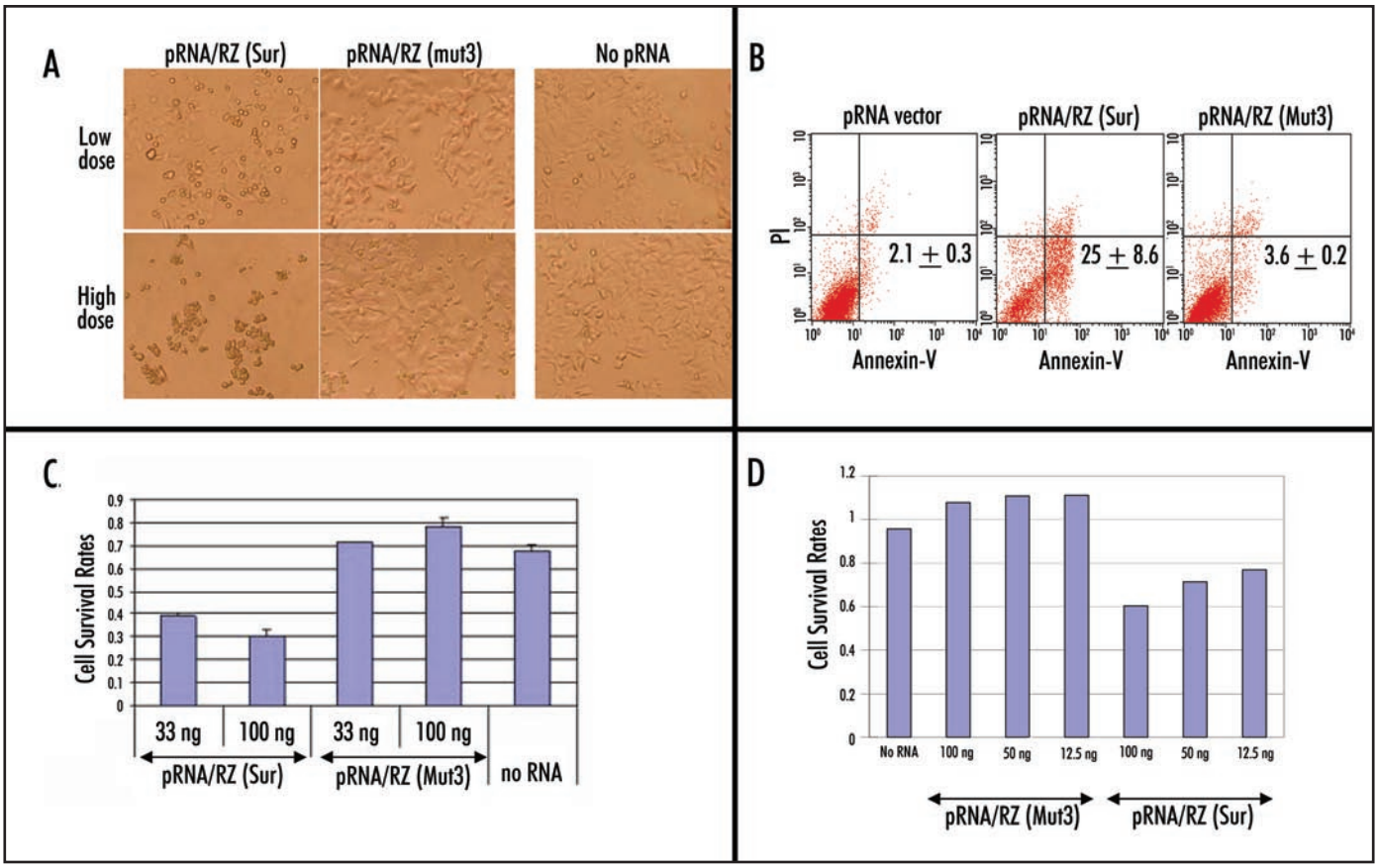

Figure 2. The effects of chimeric survivin ribozyme on breast cancer cells. (A) Cell morphology of MCF-7 after treatment of pRNA/RZ(Sur) chimera. MCF-7 were transfected with pRNA/RZ(Sur), pRNA/RZ(mut3) at high and low doses. One day after transfection, images were taken using an inverse microscope. (B). PI/annexin V double-staining to differentiate apoptosis from necrosis. Breast cancer MCF-7 cells were transfected with pRNA/RZ(sur) and apoptosis was monitored using PI/annexin V double-labeling followed by flow cytometry. Three parallel experiments were performed and the percentage of apoptotic cells was shown with standard deviation. The effects of chimeric survivin ribozyme on other breast cancer cell lines. (C). MDA-MB-231 and (D). MDA-MB-453 cells were transfected with indicated amount of RNA and cell viability was measured in the next day.

Table 1 **Functional assay of chimeric pRNA/ribozyme targeting survivin**

Cell lines	MCF-7	T47D	PC-3	A-549
pRNA/RZ (Mut3)	+++	++++	++++	++++
pRNA/RZ (Sur)	<+	<+	<+	<+

Several cancer cells were transfected with 500 ng of RNA as indicated in 24-well plates. Cells in the well were assigned a survival viability score from 0–20% to 80–100%, labeled as + to ++++, relative to the viability of untreated cells. Results were collected from two independent experiments.

used, indicating that pRNA/RZ (Sur) acts on Hela T4 cells without causing non-specific toxicity.

The effects of chimeric pRNA/RZ (Sur) in nasopharyngeal cancer cells. Human nasopharyngeal cancer KB cells were treated with pRNA/RZ (Sur), as well as three mutants. As shown in Figure 3B, KB cells responded strongly to pRNA/RZ (Sur) after transfection. Mutant 1 and 2 also led to reduced cell viability, with the latter showing inhibitory effects to a lesser extent (see discussion). Mutant 3 that target HBV sequences did not affect cell viability, even with increased dose.

The effects of chimeric pRNA/RZ (Sur) in prostate cancer and lung cancer cells. Human prostate cancer cell lines LNCaP were transfected with different doses of pRNA/RZ (Sur) or pRNA/RZ (mut3), with the latter serving as negative control. LNCaP cells reacted strongly only to the treatment of pRNA/RZ (Sur), while the control RNA did not affect cell survival rate significantly (Fig. 3C). Similar phenomenon was observed in human lung cancer line A-549 cells as shown in Table 1. These results suggest that the reduction of cell viability depended on the sequence corresponding to the survivin ribozyme, instead of being caused by the non-specific RNA toxicity.

Specific inhibition of survivin expression by pRNA/RZ (Sur) in mRNA and protein level. To test its predicted function in suppressing the expression of survivin, pRNA/RZ (Sur) was introduced into MCF-7 and T47D human breast cancer cells in which survivin was abundantly expressed. Real-time PCR and immuno-blotting analysis revealed that the mRNA and protein expression of survivin were significantly reduced and almost eliminated 24 hours after transient transfection (Fig. 4). In contrast, neither cells transfected with non-specific mutant control nor untreated cells showed significantly decreased expression of survivin, further demonstrating the specificity with which the pRNA/RZ (Sur) acted. The cleavage of survivin mRNA by pRNA/RZ (Sur) was shown in Figure 5B. The specificity was demonstrated since the mutant pRNA/RZ (mut2), which contains a two-base mutation in the catalytic core, did not produce RNA cleavage product.

The chimera causes apoptosis instead of necrosis. To determine whether pRNA/RZ (Sur) induces apoptosis by inhibiting the anti-apoptosis factor survivin, or promotes the necrosis non-specifically, annexin V-propidium iodide (PI) double-staining was performed on MCF-7 cells for flow cytometry analysis after transfection with pRNA/RZ (Sur) or its mutant. As shown in Figure 2B, 25% ± 8.6 of cells underwent apoptosis after pRNA/RZ (Sur) treatment, as shown in the cell population in the lower right quadrant representing apoptotic cells. On the contrary, cells treated with mutant 3 show only a slight increase (3.6% ± 0.2) in the population of apoptotic cells, compared to (2.1% ± 0.3) of cells treated with a pRNA vector alone. The necrotic cells, as indicated in the upper right quadrant, did not show marked increase after pRNA/RZ (Sur) treatment, which indicates the chimeric ribozyme caused apoptosis instead of necrosis in cancerous cells.

Testing of the safety of the pRNA chimera. The safety of the pRNA chimera was tested by an incubation assay with high dose of various pRNA chimeras. Incubation of cells with various concentration of pRNA chimera did not cause noticeable toxicity in cells (please refer to Fig. 8 in ref. 44). Incubation of cancer cells with pRNA/siRNA (survivin) or pRNA/RZ (Sur) did not cause cell death for up to 72 hours in the absence of transfection reagent. However, when introduced into cells by transfection, the pRNA chimera caused death in cells derived from breast cancer, prostate cancer and lung cancer. Only chimeric pRNA containing the survivin ribozyme sequence inhibited cell viability. As shown in Figures 3 and 4), the control pRNA/RZ (mut3), which contains vector sequences identical to pRNA/RZ (Sur) except the ribozyme sequence, did not show marked inhibitory effect on cell viability even in high RNA concentration.

Competence of pRNA/RZ(Sur) in the assembly of dimer and trimers nanoparticles. Specific cell recognition and gene silencing are required for an effective RNA-based targeted therapy. However, direct fusion or conjugation of a ribozyme to a targeting moiety such as RNA aptamer may lead to misfolding and loss of function. Construction of pRNA dimmer, instead, may offer a better solution to acquire both functions. Recently, a multivalent RNA complex has been constructed using phi29 pRNA chimera. Dimers or trimers were assembled by interlocking loop/loop interaction of the engineered chimeric pRNA harboring both receptor-binding RNA aptamers and siRNA.[44,46] Here we demonstrated that pRNA/RZ (Sur) also retains capability to form RNA dimer and trimer, as documented by native gel electrophoresis (Fig. 5A) and other physical approaches such as ultracentrifugation and single molecule counting (data not shown). RNA dimers were generated by mixing pRNA/RZ(Sur)(A-b') with pRNA(B-a'). Formation of trimers was achieved by mixing pRNA/RZ(Sur)(A-b') with pRNA(B-e') and (E-a').[19,20,25,46] Therefore, pRNA/RZ(Sur) can be used to assemble the dimeric/trimeric RNA nanoparticles and will be an additional member of the polyvalent RNA delivery system.

DISCUSSION

RNA therapeutics has been thought to be one of the most promising approaches in modern medicine. As in other therapeutics, toxicity and specificity are two major issues in the development. We have put our effort into the quest for low toxicity therapeutic RNA complex. Previously, we found that phi29 pRNA can be a vector to escort the ribozyme for inhibition of hepatitis virus B replication.[24] In this study, we found that pNA/RZ (Sur) is highly efficient in inducing cell death compared with controls with mutated ribozyme sequences. Safety issue may exist since the response to RNA transfection depends on the type of cells, and is a case-by-case issue. For example, breast cancer cell lines MCF-7 are far more sensitive to RNA transfection due to an unknown mechanism, compared to other breast cancer cell lines (personal communication). Therefore, we have tested a variety of cancer cell lines, including breast cancer, prostate cancer, cervix cancer, nasopharyngeal cancer, and lung cancer. Although the transfection efficiency may vary among different cell lines, the four constructs of chimeric ribozyme with similar molecular weight and secondary structure are expected to have very similar transfection efficiency within a specific cell line. Thus the conclusion of effectiveness of chimeric pRNA/ribozyme drawn from the comparison among these constructs in each cell line is valid.

We cannot say this RNA chimera is entirely safe for cancer therapy by itself, since the specificity rendered by survivin ribozyme

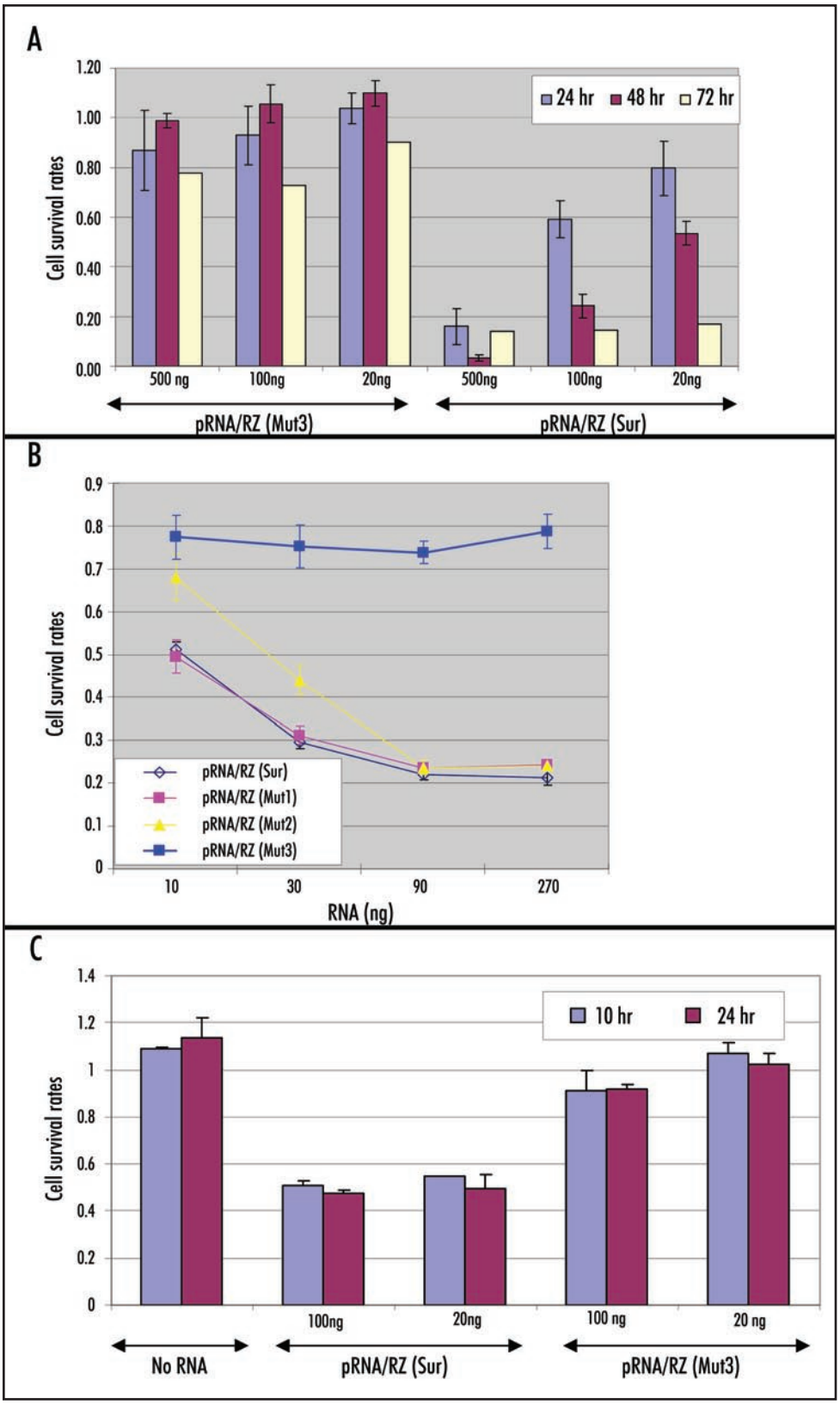

Figure 3. The effects of chimeric survivin ribozyme on (A) cervix cancer cells, (B) nasopharyngeal cancer cells and (C) prostate cancer cells. Human cervix, nasopharyngeal or prostate cancer cells were transfected with pRNA/RZ(sur) with indicated amount. Cell viability was measured by MTT assay in the next day or as indicated.

sequence was not 100% for all cell types. We tested the RNA chimeras on the most fragile and sensitive MCF7 cell by real time PCR to evaluate the silencing efficiency on the transcript of the survivin gene, and found that the expression of survivin mRNA is also slightly reduced by mutant 3 (Fig. 4A). Obviously, such reduction is caused by a non-specific RNA effect. In addition, cell viability reduction was also found when mutant 1 and 2 were used (Fig. 3B). Comparing the sequence of pRNA/RZ (Sur) and mutant 1 and 2, the only difference between them is the sequence of the catalytic core of the ribozyme. The 14 nt stem sequences, which are complementary to the survivin mRNA, are identical. Therefore, it is highly possible that the stem sequence in the mutant ribozyme can still recognize the survivin mRNA, and inhibit the expression of survivin mRNA by an antisense effect, thus inducing apoptosis. A very similar phenomenon has been reported by Zhang et al.[51] It was found in their study that a mutant ribozyme had no cleavage activity in vitro, but exhibited an antisense effect in vivo.

Some degree of non-specific effects raises the safety issue of the pRNA/RZ (Sur) monomer. However, since the goal in cancer therapy is to eliminate cancer cells, the non-specific inhibition of cancer cells might, in some cases, be desirable as long as the pRNA can enter the cancer cell specifically and as long as the un-entered pRNA is not toxic to other cells. From our previous report, we found that incubation of cells with high concentration of pRNA chimera did not cause noticeable cell death.[44] Thus, a pRNA chimera is promising in that it can enter the cell specifically by being engineered into multimers that contains a cell targeting function. Our effort will focus on the specificity of cell entry of such multi-functional RNA complexes. The advantage of using a phi29 pRNA chimera is to develop a powerful system that combines the functions of specific delivery of RNA chimera to target cells and effective tumor cell killing.

As we know, phi29 pRNA has a tendency to form dimers (a linking of 2 pRNA), trimers (3 pRNA), and hexamers (6 pRNA) as a result of the interaction of interlocking loops. Thus, two to six pRNA chimeras can be incorporated into the RNA nanocomplex, with multiple positions available to carry RNA molecules for targeting, therapy, or detection. For

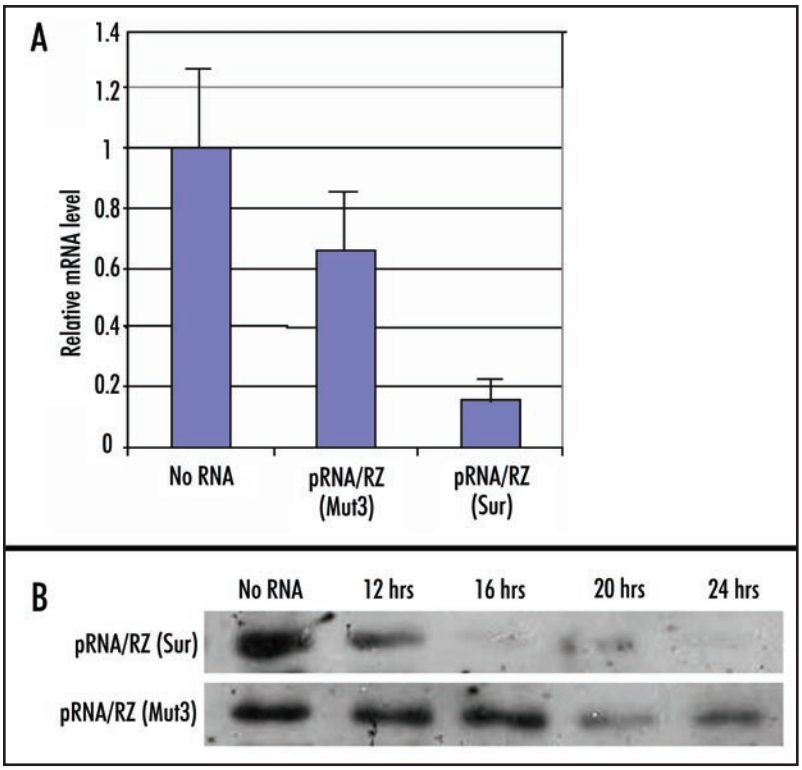

Figure 4. The effect of chimeric pRNA/RZ on mRNA and protein level. (A) Comparison of mRNA levels of different pRNA chimera treated samples revealed by real-time PCR. Gene expression level was compared to the level of gene expression found in non-transfected sample, arbitrarily assigned the value 1. Bars represent the fold number in gene expression over the expression level in the non-transfected samples. The mRNA level of pRNA/RZ(sur) treated cells decreased to 16% of that of the non-transfected cells, or 24% of pRNA/RZ(mut3) treated cells. (B). Comparison of survivin protein level by Western blot after cells were transfected with chimeric pRNA/RZ(sur) or mutant chimeric ribozyme.

example, one subunit of the complex could be altered to carry an RNA aptamer that binds the cell surface receptor, or a ligand such as folate,[45] thereby helping to carry the RNA complex for cellular entry. The remaining subunits could be modified to carry specific therapeutic siRNAs, ribozymes, antisense RNAs, chemotherapy drugs, fluorescent dyes, heavy metals, quantum dots, or radioisotopes for cancer cell elimination or detection. Endosome-disrupting chemicals may also be incorporated into the RNA complex to promote the release of RNA from the endosome. In addition, the use of these RNA nanoparticles (with sizes of 30–40 nanometers) avoids the problem of a short half-life encountered in vivo by smaller molecules due to short retention times and also avoids the problem of poor delivery efficiency encountered by larger molecules (greater than 100 nanometers). It has been well accepted that immunogenicity of RNA is very low, except when complexed with protein.[52] The construction of protein/peptide-free nanoparticles can avoid the immune response, which could allow long-term administration.

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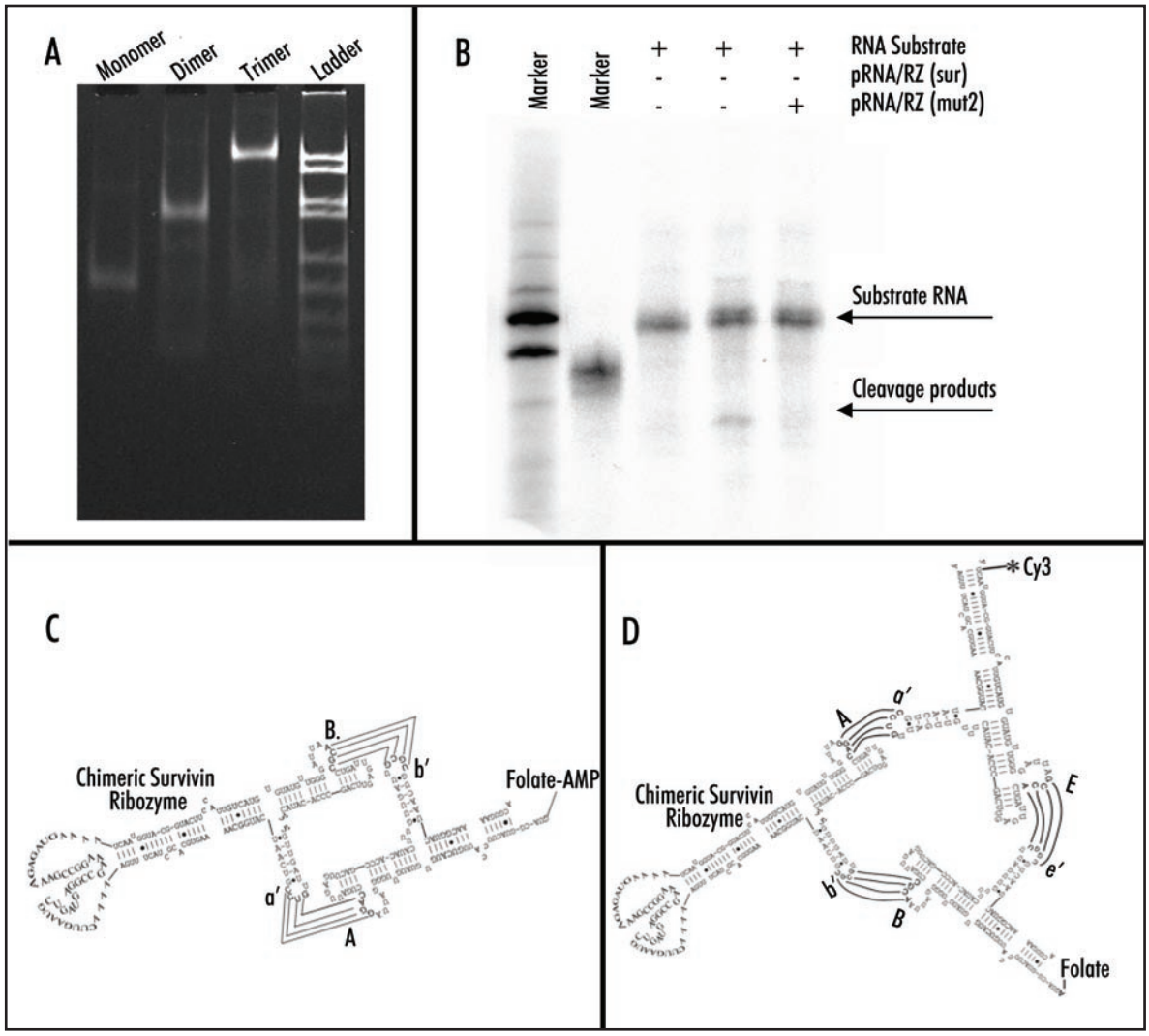

Figure 5. (A) Dimer and trimer formation of pRNA/RZ(sur) as shown in 8% native PAGE. (B) The cleavage of partial sequence of survivin mRNA by pRNA/RZ(sur). The substrate RNA is partial sequence of mRNA of human survivin labeled with [^{32}P]. (C) Deliverable dimer and trimeric complex. Each subunit of the dimer or trimer complex will be used to carry different functional motifs such as ribozyme and cell-surface recognizing aptamer or folate. In the trimeric complex, folate is used to target the folate receptor on cancer cell surface and trigger receptor mediated endocytosis. pRNA subunit with Cy3 labeling can be used to locate the complex.

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