Published in final edited form as:

J Control Release. 2018 April 28; 276: 17–29. doi:10.1016/j.jconrel.2018.02.014.

RNA-based micelles: A novel platform for paclitaxel loading and delivery

Yi Shu^b, Hongran Yin^a, Mehdi Rajabi^b, Hui Li^a, Mario Vieweger^a, Sijin Guo^a, Dan Shu^a, and Peixuan Guo^a

^aCenter for RNA Nanobiotechnology and Nanomedicine; College of Pharmacy, Division of Pharmaceutics and Pharmaceutical Chemistry; College of Medicine, Dorothy M. Davis Heart and Lung Research Institute and James Comprehensive Cancer Center; The Ohio State University, Columbus, OH 43210, United States

^bNanobiotechnology Center, Markey Cancer Center and Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY 40536, United States

Abstract

RNA can serve as powerful building blocks for bottom-up fabrication of nanostructures for biotechnological and biomedical applications. In addition to current self-assembly strategies utilizing base pairing, motif piling and tertiary interactions, we reported for the first time the formation of RNA based micellar nanoconstruct with a cholesterol molecule conjugated onto one helical end of a branched pRNA three-way junction (3WJ) motif. The resulting amphiphilic RNA micelles consist of a hydrophilic RNA head and a covalently linked hydrophobic lipid tail that can spontaneously assemble in aqueous solution via hydrophobic interaction. Taking advantage of pRNA 3WJ branched structure, the assembled RNA micelles are capable of escorting multiple functional modules. As a proof of concept for delivery for therapeutics, Paclitaxel was loaded into the RNA micelles with significantly improved water solubility. The successful construction of the drug loaded RNA micelles was confirmed and characterized by agarose gel electrophoresis, atomic force microscopy (AFM), dynamic light scattering (DLS), and fluorescence Nile Red encapsulation assay. The estimate critical micelle formation concentration ranges from 39nM to 78nM. The Paclitaxel loaded RNA micelles can internalize into cancer cells and inhibit their proliferation. Further studies showed that the Paclitaxel loaded RNA micelles induced cancer cell apoptosis in a Caspase-3 dependent manner but RNA micelles alone exhibited low cytotoxicity. Finally, the Paclitaxel loaded RNA micelles targeted to tumor *in vivo* without accumulation in healthy tissues and organs. There is also no or very low induction of pro-inflammatory response. Therefore, multivalence, cancer cell permeability, combined with controllable assembly, low or non toxic nature, and tumor targeting are all promising features that make our pRNA micelles a suitable platform for potential drug delivery.

Graphical Abstract

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

pRNA three-way junction; RNA nanotechnology; RNA micelles; self-assembly; drug loading; systemic delivery

Introduction

Functional nanoparticles fabricated via molecular self-assembly hold great promise for applications in biotechnology and biomedicine [1–4]. Among these, RNAs have been utilized as unique biomaterials to construct a wide variety of nanoparticles *via* self-assembly [3,5–16]. These RNA nanoparticles have been demonstrated to be further functionalized for biomedical applications [2,17–22]. The strategies for RNA nanoparticles self-assembly were previously reported. Exploiting RNA base pairing and tertiary interactions provides versatile RNA assemblies with accurate control over their composition, structure, and function at the nanometer scale [10,23]. In this present study, we explore another novel strategy to fabricate RNA self-assemblies with micellar properties through intermolecular hydrophobic interactions.

We have discovered and manufactured a stable phi29 pRNA three-way junction (3WJ) motif that can be used as a scaffold to construct multivalent RNA nanoparticles with high chemical and thermodynamic stability [5,8]. The resulting branched RNA nanoparticles are uniform in size and shape. They can harbor different functionalities while retaining their tertiary fold and independent functionalities both *in vitro* and *in vivo* [8,12,19,21,24–28]. We have also shown that fluorescent dye molecules [29,30] and specific cell targeting ligands [19–21,27,31] can be covalently attached onto RNA strands either through RNA solid phase synthesis or by post-transcriptional chemical conjugation methods [7,10,32]. The feasibility of modifying RNA molecules with different chemical and functional moieties proved the potential of RNA nanoparticles as a multi-functional drug delivery platform.

Further inspired by DNA micelle construction [33–42], we are able to convert a hydrophilic RNA molecule into an amphiphilic construct by fusing a lipophilic moiety onto one of pRNA-3WJ branched helical ends. This amphiphilic construct behaves similarly as a phospholipid while it spontaneously self-assembles into monodispersed, three-dimensional micellar nanostructures with a lipid inner core and a branched exterior RNA corona as the results of intermolecular hydrophobic interactions in aqueous solution. Unlike current DNA micellar systems, which are limited in application by their mono-functionality [34,37], our pRNA micelles are capable of covalently attaching diverse types of functional moieties to a

single particle, including chemo-drugs for cancer therapeutics, imaging moieties for nanoparticles tracking, and co-delivered RNAi components for combination treatment.

Paclitaxel (PTX), isolated from the bark of Pacific Yew (*Taxus brevifolia*) [43], is one of the most effective chemotherapeutic drugs for a number of cancer types [44–46]. The mechanism of paclitaxel for cancer treatment is to promote and stabilize microtubules and further inhibit G2 or M phases of the cell cycle followed by cell death [47]. However, Paclitaxel has been classified to IV chemical drugs according to the Biopharmaceutical classification system (BCS), because it has both low water solubility (~0.4 µg/mL) and low permeability. The first formulation of paclitaxel to be used was in a 1:1 (v:v) blend of Cremophor EL (polyethoxylated castor oil) and dehydrated ethanol diluted 5–20 fold with 0.9% sodium chloride or 5% dextrose solution for i.v. administration [48]. However, formulation with Cremophor oil has been observed to cause severe side effects [49] as well as unpredictable non-linear plasma pharmacokinetics [50]. Therefore, alternative Paclitaxel formulations have been explored extensively, particularly with nanoparticle-based delivery systems [51]. Taking advantage of nano-scaled size, tumor targeted delivery, and biocompatibility, the encapsulation of Paclitaxel in a nano-delivery system can increase drug circulation half-life, lower its systemic toxicity, reduce side effects, improve pharmacokinetic and pharmacodynamic profiles, and demonstrate better patient compliance. Paclitaxel albumin-bound nanoparticles (Abraxane®) have been approved by the FDA in 2005. Paclitaxel liposomes (Lipusu®), polymeric micelles (Genexol PM®) and polymeric conjugate with polyglutamate (Xyotax®) are currently commercially available paclitaxel formulations. In addition, there are various types of Paclitaxel nanoparticle formulations either under development or in clinical trials, such as polymer-based nanoparticles [52–54], lipid-based nanoparticles [55,56], polymer-drug conjugates [57,58], inorganic nanoparticles [59], carbon nanotubes [60], nanocrystals [61], etc. However, formulating Paclitaxel in a RNA based nano-delivery platform has never been reported.

The present study demonstrates, to the best of our knowledge, the first design and construction of a well-defined RNA-micelles system to load Paclitaxel through conjugation to pRNA-3WJ-lipid. It is also the first time to report RNA-Paclitaxel micelles with significantly enhanced Paclitaxel water solubility and tumor permeability. The resulting RNA micelles showed low critical micelle formation concentration (CMC), excellent cell binding and permeability, and efficient inhibition of cancer cell proliferation by induction of Caspase-3 dependent cell apoptosis *in vitro*. Finally, we demonstrated that these drug-loaded RNA micelles can be systemically delivered into tumor with advantageous tumor targeting, minimizing retention of drug in healthy tissues and crucial organs. There is also no or very low induction of pro-inflammatory response. All of these findings indicate the strong potential of the RNA-micelle system as a suitable drug delivery platform for cancer treatment. We are aimed at achieving tumor specific targeting, reducing the drug effective dose and further diminishing the adverse effects of chemotherapy. Our ultimate goal is to explore the application of the RNA micelles as a robust and safe nano-delivery system to carry anti-cancer drugs for specific tumor targeting and treatment with minimum adverse effects to combat cancer and improve the life quality of patients.

Materials and Methods

Synthesis of Paclitaxel conjugated RNA strand via “Click chemistry”

To synthesize PTX-Azide, 1:2:2:1 eq. of Paclitaxel (Alfa Aesar), 6-Azido-hexanoic acid (Azido-HA) (Chem-IMPEX INT'L Inc), N, N'-Dicyclohexyl-carbodiimide (DCC) (Acros Organics), and 4-(Dimethylamino) pyridine (DMAP) (Sigma Aldrich) were weighed into a two neck round bottom flask. The reaction mixture was dissolved in ~20 mL dried Dichloromethane (DCM) and react at room temperature while stirring under nitrogen atmosphere for 24 hr. The reaction solution was filtered and concentrated on a rotary evaporator. The concentrated reaction was further purified by silica gel chromatography under serial solvent wash with n-Hexane:ethyl acetate. Fractions containing pure product were combined and dried. The purified final product PTX-Azide was characterized by Nuclear Magnetic Resonance (NMR) Spectroscopy.

5'-alkyne-RNA was synthesized via standard RNA solid phase chemical synthesis using 5'-Hexynyl phosphoramidite (Glen Research Corp.). The theoretical yield of labeled RNA strand is ~68% ($0.98^{19} = 0.68$) with an average coupling efficiency of 98%.

To 5 μ L of a 2 mM RNA-Alkyne solution in water, 2 μ L of PTX-Azide (50 mM, 5 eq. in 3:1 (v/v) DMSO (Dimethyl sulfoxide, extra dried, Acros Organics)/tBuOH (tert-Butanol, anhydrous, Sigma Aldrich)), 3 μ L of freshly prepared “Click Solution” containing 0.1 M CuBr (Copper(I) bromide, Sigma Aldrich) and 0.1 M TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, Sigma Aldrich) in a 1:2 molar ratio in 3:1 DMSO/tBuOH were added. The reaction mixture was thoroughly mixed and reacted under room temperature for 3 hr. The success of the reaction was determined by 20% 8 M Urea PAGE in TBE (89 mM Tris base-borate, 2 mM EDTA) buffer. The reaction was subsequently diluted with 100 μ L 0.3 M NaOAc (Sodium Acetate) and 1 mL 100% Ethanol for RNA precipitation. The precipitates were dissolved in water for purification using Ion-Pair Reverse Phase HPLC on an Agilent PLRP-S 4.6 X 250 mm 300A column. PTX-labeled RNA was separated from unreacted RNA-Alkyne in an Acetonitrile ramp. The column was equilibrated in 95% solvent A (0.1 M TEAA, HPLC grade H₂O) and 5% solvent B (0.1 M TEAA, 75% Acetonitrile, 25% HPLC grade H₂O) at a flow rate of 1.5 mL/min. The samples were filtered through a 0.2 μ m spinfilter, loaded onto the column and then eluted by ramping from 5% to 100% B over the course of 30 min. Fractions were collected, combined and buffer exchanged. The final RNA-PTX conjugate was characterized by Mass Spectrometry.

Design and construction of 2'-F modified pRNA-3WJ-PTX micelles

The RNA micelles were constructed using a bottom-up approach [6]. The pRNA-3WJ-PTX micelles consisting of three fragments, a_{3WJ}, b_{3WJ} and c_{3WJ}, were functionalized with Paclitaxel (Alfa Aesar) on a_{3WJ} 5'-end (a_{3WJ}-5'PTX), as a therapeutic module; Cholesterol on b_{3WJ} 3'-end (b_{3WJ}-3'chol), as lipophilic module; and Alexa647 (Alexa Fluor® 647, Invitrogen) on c_{3WJ} 3'-end (c_{3WJ}-3'Alexa647), as a near-infra-red (NIR) imaging module. The control RNA nanoparticles are particles without therapeutic module named as pRNA-3WJ micelles, without lipophilic module named as pRNA-3WJ-PTX, and without both lipophilic module and therapeutic module named as pRNA-3WJ.

The following pRNA-3WJ scaffold [6] sequences were used: a_{3WJ} 5'-UUgCCaUgUgUaUgUggg-3'; b_{3WJ} 5'-CCCaCaUaCUUUgUUgaUCC-3'; c_{3WJ} 5'-ggaUCaaUCaUggCaa-3' (upper case indicates 2'-Fluoro (2'-F) modified nucleotide). The RNA fragments were synthesized by standard solid phase chemical synthesis [60] with commercially available phosphoramidite monomers of 2'-TBDMS Adenosine (n-bz) CED, 2'-TBDMS Guanosine (n-ibu) CED, 2'-Fluoro Cytidine (n-ac) CED, and 2'-Fluoro Uridine CED, followed by deprotection according to by manufacturer (Azco Biotech) provided protocol. Paclitaxel (PTX) was conjugated onto a_{3WJ} 5'-end by chemoselective Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction ("Click chemistry") and purified through reverse phase HPLC as mentioned above. Cholesterol was attached to the 3'-end of b_{3WJ} strand by using 3'-Cholesteryl-TEG CPG support (Glen Research Corp.), following manufacturer instructions. The theoretical yield of b_{3WJ}-3'chol is ~68% ($0.98^{19} = 0.68$) with an average coupling efficiency of 98%. Synthesized RNA is purified from terminated strands using an ion-pair reverse phase column which typically yields complete labeling with cholesterol during solid phase synthesis. Alexa647 labeled RNA strand was purchased from TriLink Bio Technologies, LLC.

pRNA-3WJ-PTX micelles were assembled by mixing 3WJ strands (a_{3WJ}, b_{3WJ} and c_{3WJ}) at equal molar concentrations in TMS buffer (50 mM Tris pH = 8.0, 100 mM NaCl, 10 mM MgCl₂) or PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄, pH 7.4) followed by heating to 80 °C for 5 minutes and slowly cooling to 37°C over the course of 40 min with a subsequent 1hr incubation at 37 °C.

Characterization of the assembled pRNA-3WJ micelles

The assembly of the functionalized 3WJ nanoparticles was characterized by 1% (w/v) agarose gel shift assays in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. After electrophoresis, the gel was stained by ethidium bromide and visualized by Typhoon FLA 7000 (GE healthcare).

The apparent hydrodynamic diameters of preassembled pRNA-3WJ-PTX (20 μM in PBS buffer) and pRNA-3WJ-PTX micelles (10 μM in PBS buffer) were measured by Zetasizer nano-ZS (Malvern Instrument, LTD) at 25°C, respectively. The data were obtained from three independent measurements. The Zeta potential of pRNA-3WJ-PTX micelles (1 μM in PBS buffer) was also measured by Zetasizer nano-ZS (Malvern Instrument, LTD) at 25°C.

The size and shape of the RNA micelles were also determined by Atomic Force Microscopy (AFM). For AFM, 10 μM solution of the pRNA-3WJ-PTX micelles in TMS buffer were deposited onto freshly cut mica surface and dried overnight. After two consecutive rinsing steps with HPLC grade water, the mica was mounted onto Bruker Multimode IV AFM and imaged in tapping mode.

Successful formation of the RNA micelles was also determined by a Nile Red encapsulation assay as reported previously [62,63]. A 5 mM stock solution of Nile Red in acetone was used for all experiments. Briefly, 0 μM, 1 μM, 5 μM, and 10 μM of assembled pRNA-3WJ micelles were incubated with 100 μM of Nile Red in TMS buffer, respectively. The mixture was heated to 80 °C for 5 min and slowly cooled to 37°C over 40 min followed by 1hr

incubation at 37°C. The fluorescence intensity of Nile Red versus RNA micelles concentration was measured by Fluorolog spectrofluorometer (Horiba Jobin Yvon) with an excitation wavelength of 535 nm and emission spectra taken from 560 nm to 760 nm. The pRNA-3WJ was used as a control.

Determination of critical micelle formation concentration (CMC)

CMC of pRNA-3WJ micelles was determined by both fluorescent Nile Red encapsulation assay as previously reported [63] and agarose gel electrophoresis. Briefly, 2-fold serial diluted RNA micelle samples (in the range of 5 μ M to 0.005 μ M) were incubated with 100 μ M of Nile Red in a final volume of 50 μ L. The samples were heated to 80 °C for 5 min and slowly cooled to 37°C over 40 min followed by 1hr incubation at 37 °C. The fluorescence intensity of Nile Red versus RNA micelles concentration was measured by Fluorolog spectrofluorometer (Horiba Jobin Yvon) with an excitation wavelength of 535 nm and emission spectra taken from 560 nm to 760 nm. 2-fold serial diluted RNA micelle samples (in the range of 2.5 μ M to 0.0390625 μ M) were directly loaded onto a 1% (w/v) agarose gel for electrophoresis under 120 V in TAE buffer. The gel was stained by ethidium bromide and visualized by Typhoon FLA 7000.

Formulation Stability Assay

Final 2 μ M of pRNA-3WJ micelles were incubated in acidic (pH=4), neutral (pH=7.4), and basic (pH=12) buffers @ 37°C for 1hr. Final 2 μ M of pRNA-3WJ micelles were also incubated at different temperature (4°C, 37°C, and 65°C) for 1hr. All samples after incubation were loaded onto a 1% (w/v) agarose gel for electrophoresis under 120 V in TAE buffer. The gel was stained by ethidium bromide and visualized by Typhoon FLA 7000.

Cell culture

Human KB cells (American Type Culture Collection, ATCC) were grown and cultured in RPMI-1640 (Thermo Scientific) containing 10% FBS in a 37 °C incubator under 5% CO₂ and a humidified atmosphere. Mouse macrophage-like RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium(DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in humidified air containing 5% CO₂.

***In vitro* binding assay using Flow Cytometry**

250 nM, 500 nM, and 1 μ M Alexa647 labeled pRNA-3WJ-PTX micelles and the control pRNA-3WJ-PTX nanoparticles without lipophilic module were each incubated with 2×10^5 KB cells at 37°C for 1 hr. After washing with PBS twice, the cells were resuspended in PBS. Flow Cytometry was performed by the University of Kentucky Flow Cytometry & Cell Sorting core facility to observe the cell binding efficacy of the Alexa647 labeled pRNA-3WJ-PTX micelles. The data was analyzed by FlowJo 7.6.1 software.

***In vitro* binding and internalization assay using Confocal Microscopy**

KB cells were grown on glass slides overnight. 1 μ M Alexa647 labeled pRNA-3WJ-PTX micelles and the control pRNA-3WJ-PTX nanoparticles without lipophilic module were

each incubated with the cells at 37°C for 1 hr. After washing with PBS, the cells were fixed by 4% paraformaldehyde (PFA) and washed 3 times by PBS. The cytoskeleton of the fixed cells was treated with 0.1% Triton-X100 in PBS for 5 min to improve cell membrane permeability and then stained by Alexa Fluor 488 Phalloidin (Life Technologies) for 30 min at room temperature and then rinsed with PBS for 3×10 min. The cells were mounted with Prolong® Gold antifade reagent with DAPI (Life Technologies) and DAPI was used for staining the nucleus. The cells were then assayed for binding and cell entry by FluoView FV1000-Filter Confocal Microscope System (Olympus Corp.).

***In vitro* drug releasing assay for pRNA-3WJ-PTX**

10 μ M of The pRNA-3WJ-PTX conjugate was incubated with 50% FBS at 37 °C and samples were collected at different time point (1hr, 4hr, 8hr, 12hr, 15hr, 22hr, 24hr, 28hr, and 36hr) and froze immediately at -80°C . After completing sample collection through entire time course, all samples were run on 15% native PAGE in TBM buffer (89 mM Tris base-borate, 5 mM MgCl_2). The gel bands were quantified by Image J and the percentage of intact particle was calculated by $\text{intact particle \%} = \frac{(\text{intensity of upper band})}{(\text{intensity of upper band} + \text{lower band})} \times 100\%$. Upper band indicates intact RNA-drug conjugate while lower band indicates RNA oligo after drug releasing.

MTT assay

In order to assay the cytotoxicity effects after pRNA-3WJ-PTX micelles treatment, CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) was used to assay cell viability changes following manufacturer instructions. Briefly, 1×10^4 KB cells were seeded into 96-well plates a day prior to the assay. On the second day, 1 μ M, 800 nM, 600 nM, 400 nM, and 200 nM of pRNA-3WJ-PTX micelles were added to the wells in triplets. Paclitaxel alone, pRNA-3WJ micelles, pRNA-3WJ-PTX, and pRNA-3WJ were used as controls in the same testing concentrations. The plate was incubated at 37°C for 48 hr in a humidified, 5% CO_2 atmosphere. After incubation, 15 μ L of the Dye Solution was added to each well and the plate was incubated at 37°C for up to 4 hr in a humidified, 5% CO_2 atmosphere. Next, 100 μ L of the Solubilization Solution/Stop Mix was added to each well and incubated for 1hr. Finally, the contents of the wells were mixed to get a uniformly colored solution and their absorbance at 570nm was recorded using Synergy 4 microplate reader (Bio-Tek).

Apoptosis study in *in vitro* cell model

FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) and Caspase-3 Assay Kit (BD Pharmingen) were used as previously reported [64] to study cell apoptosis induced by pRNA-3WJ-PTX micelles treatment. For FITC Annexin V staining assay, KB cells were seeded on 12-well plates overnight. Cells (~30% confluence) were then treated with 1 μ M of pRNA-3WJ-PTX micelles. The controls include Paclitaxel, pRNA-3WJ micelles, and pRNA-3WJ. According to manufacturer's instruction, 48 hr after incubation with the RNA micelles, KB cells were trypsinized to single cell suspension. After two times PBS wash, the cells were re-suspended in 100 μ L 1x Annexin V-FITC binding buffer. Then 5 μ L AnnexinV-FITC and 5 μ L propidium iodide (PI) were added into each sample and incubated at room temperature for 25 min. The samples were finally added to a flow tube which contained 200 μ L 1x binding buffer for FACS analysis within 1 hr.

For Caspase-3 assay, KB cells were seeded on 24-well plates overnight. Cells (~30% confluence) were then treated with 1 μ M of pRNA-3WJ-PTX micelles. The controls include Paclitaxel, pRNA-3WJ micelles, and pRNA-3WJ. The cellular Caspase-3 activity was measured and compared by Caspase-3 Assay Kit (BD Pharmingen) according to manufacturer instructions. Briefly, Cell lysates (1–10 $\times 10^6$ cells/mL) at different time points (4 hr, 8 hr, 12 hr, and 24 hr) after induction of apoptosis were prepared using cold Cell Lysis Buffer provided by the kit, and incubated for 30 min on ice. For each sample, 25 μ L of cell lysate was added with 2 μ L reconstituted Ac-DEVD-AMC in 80 μ L of 1x HEPES buffer and incubated at 37°C for 1 hr. The amount of AMC liberated from Ac-DEVD-AMC was measured at an excitation wavelength of 380 nm over an emission wavelength range of 400–500 nm on a Fluorolog spectrofluorometer (Horiba Jobin Yvon).

Animal models

All protocols involving animals are performed under the supervision of the University of Kentucky Institutional Animal Care and Use Committee (IACUC). To generate xenograft model, female athymic nu/nu mice, 4–8 weeks old, were purchased from Taconic. Subcutaneous tumor xenografts were established by injecting 2×10^6 cells/site KB cells resuspended in sterile PBS into the left shoulder of nude mice. When the tumor nodules had reached a volume of 50 mm³, approximately 5 days post-injection, the mice were used for tumor targeting studies.

NIR fluorescence imaging to detect the targeting of RNA micelles to cancer xenografts *in vivo*

To investigate the delivery of pRNA-3WJ-PTX micelles *in vivo*, a fluorescence imaging study was performed after tail vein injection of 100 μ L 20 μ M Alexa 647 labeled pRNA-3WJ-PTX micelles and pRNA-3WJ-PTX (estimated final concentration in blood is about 1 μ M) into mice bearing KB tumor, respectively. PBS injected mice were used as fluorescence negative controls. The whole body images were taken at 30min, 1hr, 2 hr, 4 hr, 8 hr, 24 hr. Mice were sacrificed at 24 hr post injection by inhalation of CO₂ followed by cervical dislocation, and major internal organs including heart, lungs, liver, spleen, kidneys together with tumor from the sacrificed mice were collected and subjected to fluorescence imaging for assessment of biodistribution profiles using IVIS Spectrum station (Caliper Life Sciences) with excitation at 640 nm and emission at 680 nm.

Evaluation of pro-inflammatory induction of pRNA-3WJ micells

For *in vitro* evaluation of pro-inflammatory cytokines induction, 2.5×10^5 RAW 264.7 cells per well were seeded in 24-well plate and cultured overnight. pRNA-3WJ micelles (1 μ M or 200 nM) as well as Lipopolysaccharide (LPS, 3.6 μ g/mL, equal amount as 200 nM pRNA-3WJ micelles) were diluted in DMEM (Life Technologies Corporation) and added to cells for incubation in triplet. After 16 hours incubation, cell culture supernatants were collected and stored at –80°C immediately for further assay. TNF- α and IL6 in collected supernatants were examined by using Mouse ELISA MAX Deluxe sets (BioLegend, San Diego, CA, USA) while IFN- α was examined using Mouse IFN Alpha ELISA Kit (PBL Assay Science, Piscataway, NJ, USA), following the manufacturers' instruction respectively.

For *in vivo* evaluation of pro-inflammatory cytokines and chemokines induction, pRNA-3WJ micelles (1 μ M), LPS (10 μ g per mouse), and DPBS control were injected into 4~6 weeks male C57BL/6 mice *via* tail vein. 3 hr post-injection, blood samples were harvested from mice by cardiac puncture and centrifuged at $12,800 \times g$ for 10 min to collect serum. Concentrations of TNF- α , IL6 and IFN- α in serum were examined by ELISA as described above follow manufactures' instruction. Chemokine induction was conducted by using Mouse Chemokine Array Kit (R&D Systems, Minneapolis, MN, USA) following manufacturer's instruction. LiCor was used for blotting spot quantification.

Statistical Analysis

Each experiment was repeated 3 times in duplicates for each sample tested. The results are presented as mean $\pm$ SD unless otherwise indicated. Statistical differences were evaluated using Student's t-test, and $p < 0.05$ was considered significant.

Results and Discussion

Construction and characterization of pRNA-3WJ micelles

The pRNA-3WJ-PTX micelles utilize a modular design composed of three short RNA fragments from pRNA 3WJ motif [6] (Fig. 1A). The lipophilic module, cholesterol, was conjugated to the 3'-end of b_{3WJ} by considering the global folding structure of the pRNA 3WJ motif. The crystal structure of the pRNA 3WJ motif reveals that the angles among three helices (H1, H2, and H3) of the pRNA 3WJ are $\sim 60^\circ$ (H1-H2), $\sim 120^\circ$ (H2-H3), and $\sim 180^\circ$ (H1-H3) [65] (Fig. 1B). In order to avoid interference of micelle formation by steric hindrance due to the branched 3WJ structure, the cholesterol molecule was placed onto H3 where it is furthest from the other two helices, H1 and H2. Our current design functioned H1 with therapeutic module (Paclitaxel) and imaging module (Alexa 647 dye) without interfering in micelle formation (Fig. 1C, Fig. 1D). The function of conjugated chemotherapeutic drug and detection dye was also well retained as shown in the following study. The unoccupied helix H2 can be further activated with RNAi modules, such as siRNA or microRNA, for combined treatment to generate enhanced or synergetic therapeutic effects.

Upon mixing the individual strands in equal molar ratio in PBS or TMS buffer, the complex assembled with high efficiency, as shown in the 1% Agarose gel shift assay (Fig. 1E). Alexa 647 labeled RNA micelles also showed fluorescent bands corresponding to the band indicating successful micelle formation (Fig. 1E). The assembly of the RNA micelles was further demonstrated by AFM which showing homogeneous sphere shaped architectures (Fig. 2A). DLS assays showed that the average hydrodynamic diameter of pRNA-3WJ-PTX micelles was 118.7 ± 14.50 nm compared to 7.466 ± 1.215 nm for pRNA-3WJ core scaffold (Fig. 2B). The constructed pRNA-3WJ-PTX micelles were also negatively charged. The Zeta potential was -26.1 ± 10.4 mV as shown in Fig. 2C. Finally, the RNA micelles formation was also assayed by a Nile Red encapsulation assay. Nile Red is a hydrophobic dye and nearly non-emissive in bulk aqueous solution, but its inclusion in a nonpolar microenvironment such as the lipid core of the micellar structure results in an intense fluorescence enhancement [66]. Therefore, the increase in fluorescence intensity associated

with the incubation of RNA micelles at varying concentrations with fix amounts of Nile Red dye indicate the formation of the RNA micelles in the buffer solution (Fig. 2D). In contrast, no significant increase of fluorescent intensity was observed in the control pRNA-3WJ without a lipid core (Fig. 2D).

To make the pRNA-3WJ micelles chemically stable *in vivo*, we used 2'-F modified U and C nucleotides [67,68] during RNA strand synthesis. The 2'-F modified RNA nanoparticles were proved to be chemically stable and exhibit longer half-life in circulation compared to its unmodified RNA counterparts [6,67]. The presence of 2'-F nucleotides not only makes the RNA nanoparticles resistant to RNase degradation, but also enhances the melting temperature of pRNA-3WJ [69], without compromising the authentic folding and functionalities of the core and incorporated modules [6,70].

The pRNA-3WJ formulation stability was further assayed versus pH (acidic pH 4, neutral pH 7.4, and basic pH 12) and temperature (4°C, 37°C, and 65°C). The results indicated that pRNA-3WJ micelles were stable across a wide range of temperature and in acidic and neutral condition. Although pRNA-micelles showed dissociation problem at basic condition as reported in Supplementary Fig. 1, pRNA-3WJ micelles formulation was chemically and thermodynamically stable in physiological condition (pH 7.4, 37°C).

Synthesis of Paclitaxel conjugated RNA strand *via* “Click chemistry”

In order to load pRNA micelles with therapeutic module, PTX was firstly functionalized with -Azide ($-N_3$) in order to further react with alkyne modified RNA. Azide group on 2'-OH group was introduced to PTX using 6-azidohexanoic acid linker *via* esterification, in the presence of N,N'-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) in dry dichloromethane to afford azide-functionalized PTX (PTX- N_3) as the predominant product (Supplementary Fig. 2A). Although both hydroxyl groups at the 7' and 2' positions of PTX are chemically reactive, the 2'-OH group typically showed higher reactivity than the 7'-OH group, because acetylation of 7'-OH group is very unstable in aqueous media, losing the C-7 substituent rapidly [71]. PTX- N_3 with functionality at the 2' position was obtained in 73% yield after purification. The 1H NMR spectroscopy is employed to confirm the chemical structure of the PTX- N_3 in $CDCl_3$. In the 1H NMR spectrum of PTX- N_3 , the 7'-CH resonates at 4.4 ppm and no significant chemical shift change was observed for the 7'-CH-OH signal (at 4.40 ppm) before and after esterification (Supplementary Fig. 2B) while the resonance of 2'-CH proton was shifted from 4.78 ppm (before esterification) to 5.46 ppm (after esterification) (Supplementary Fig. 2C).

PTX- N_3 reacted with -Alkyne modified RNA (a_{3WJ}) at above 90% efficiency via Copper I mediated Click reaction as shown in Fig. 3A. The successful conjugation was confirmed by 20% 8M Urea PAGE (Fig. 3B) and the Mass Spectrometry. The experimental mass of a_{3WJ} -PTX determined by Mass Spectrometry is 6939.2 (m/z) which is close to the theoretic masses (6936.82 (m/z)) calculated based on the chemical formula (Fig. 3C). The ester bond formed between Paclitaxel and RNA can be then hydrolyzed in presence of either esterase or in aqueous solution, which allows slow releasing of the loaded drug in a controllable manner as indicated by *in vitro* drug releasing assay (Fig. 3A, D). This is the first report to “click” a RNA molecule with a chemotherapeutic drug, Paclitaxel. PTX conjugation did not interfere

with the stepwise assembly of pRNA-3WJ (Supplementary Fig. 3A). It is also the first time demonstrating that the water insolubility of Paclitaxel can be altered by fusing with a RNA oligo. 1 mM of RNA-Paclitaxel conjugates dissolved in DEPC H₂O showed clear solution compared to cloudy solution prepared using equal concentration of Paclitaxel in DEPC H₂O (Supplementary Fig. 3B). The significantly improved water solubility of Paclitaxel after conjugating with RNA oligo and assembling into micellar nanostructures allows the use of normal saline solution for *in vivo* administration instead of Cremophor EL formulation. In addition to Click chemistry, functionalizing RNA oligos and candidate drugs with other chemical coupling reactive groups, such as –NH₂/–NHS –NH₂/–COOH, or –SH/–maleimide, are also feasible RNA-drug conjugation approaches which can be applied for drug candidates that are not suitable for Click chemistry.

Determine the critical micelle formation concentration

In order to determine the Critical Micelle Formation Concentration (CMC) of pRNA-3WJ-PTX micelles, the Nile Red assay was used as previously reported [63]. Nile red is a hydrophobic dye which has low fluorescence in water and other polar solvents but emits strong fluorescence in nonpolar environments such as the lipid core of the micellar structure. Therefore, Nile Red fluorescence emission intensity is utilized as an indicator for micellar structure formation. To determine CMC, the fluorescence intensity of Nile Red was plot as a function of the sample concentration. As shown in Supplementary Fig. 4A, Nile Red exhibits low fluorescence intensity at the concentration below 0.078 μ M indicating that the Nile Red was in water and few micelles were present. With increasing concentration, the fluorescence intensity increased dramatically which demonstrating that Nile Red was encapsulated in the lipid core of RNA micelles. The CMC can be estimated as the range from 39nM to 78nM since the Nile Red fluorescence intensity exhibits obvious increase starting at 39nM to 78nM (Supplementary Fig. 4B). 1% TAE agarose gel further confirmed the CMC is ~150nM due to sensitivity limit (Supplementary Fig. 4C). All the pRNA micelles concentration *in vitro* and *in vivo* experiments we performed is above CMC to ensure all the micelles are formed.

Binding and internalization of pRNA-3WJ-PTX micelles into KB cells

In order to assay the tumor cell targeting *in vitro*, the pRNA-3WJ-PTX micelles and control pRNA-3WJ-PTX without lipophilic module were incubated with KB cells, trypsinized, washed and then analyzed by Fluorescence-Activated Cell Sorting (FACS) assay. Strong binding (almost 100%) was observed for pRNA-3WJ-PTX micelles compared to pRNA-3WJ-PTX scaffold control (0% binding) (Fig. 4A). Confocal microscopy images further confirmed the efficient binding and internalization of pRNA-3WJ-PTX micelles into cancer cells, as demonstrated by excellent overlap of fluorescent RNA nanoparticles (red color in Fig. 4B) and cytoplasm (green color in Fig. 4B). Very low signal was observed for control pRNA-3WJ-PTX without lipophilic modules. These results indicate that the RNA micelles have a high affinity for cancer cell binding. Although the current RNA micelles design did not include a tumor specific targeting module, the highly charged RNA micelles (as RNA is negatively charged, also shown in Zeta potential study) were able to disintegrate themselves and insert into the cell membrane when interacting with the cells similar as DNA

micelles as reported previously [35]. We suspect the internalization of the RNA micelles was mediated possibly by subsequent endocytosis after inserting into the cell membrane.

Effects of pRNA-3WJ-PTX micelles on growth and apoptosis of cancer cells *in vitro*

In order to determine the cellular effects of RNA micelles treatment, MTT assay was performed to assay the cell viability after treatment. The RNA micelles harboring PTX can successfully inhibit tumor cell growth at or above 250 nM, compared to RNA micelles only and 3WJ controls without Paclitaxel conjugation (Fig. 5A). We did not test the concentrations below 125 nM in order to keep experiment condition higher than CMC. Cell growth inhibition caused by pRNA-3WJ-PTX micelles indicates release of the Paclitaxel from RNA strands due to the linker ester hydrolysis in aqueous solution. There is very little possibility of free Paclitaxel contamination to the testing constructs because of all the RNA-PTX conjugates went through thorough HPLC purification. In addition, pRNA-3WJ micelles without Paclitaxel loading showed no effects on cell viability and proliferation which indicating the low cytotoxicity of RNA micelles scaffolds and their potential as a safe drug delivery platform.

FITC labeled Annexin V staining confirmed that the majority of the cancer cell death is due to cell apoptosis as shown in Fig. 5B. Loss of plasma membrane is one of the earliest features in apoptotic cells. The externalization of membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane allows FITC labeled Annexin V to bind PS for detecting cells that are undergoing apoptosis. Staining with FITC Annexin V is used in conjunction with PI to identify early apoptotic cells (Q3: PI negative, FITC Annexin V positive) and cells that are in late apoptosis or already dead (Q2: FITC Annexin V and PI positive). In our study, over 30% of the cells were undergoing apoptosis after 48 hr treatment with pRNA-3WJ-PTX micelles compared to the control micelles without PTX (4.52%) or pRNA-3WJ without PTX (3.7%), indicating that the cancer viability change is due to the induction of cell apoptosis.

Caspase-3 is an early cellular apoptotic marker. The increase of the Caspase-3 activity is closely related to cell apoptosis. The elevation of Caspase-3 activity, as reflected by increased fluorescence intensity, appeared 12 hr after treatment with pRNA-3WJ-PTX micelles (Supplementary Fig. 5). We found that pRNA-3WJ-PTX micelles treated cell lysates showed the highest fluorescence emission similar to Paclitaxel alone compared to the control nanoparticles (pRNA-3WJ micelles and pRNA-3WJ), which indicate the induction of cell apoptosis in a Caspase-3 dependent manner (Fig. 5C).

Specific targeting of tumors in xenografted animal models *via* imaging using NIR fluorescent pRNA-3WJ-PTX micelles

Tumor targeting efficiency by pRNA-3WJ-PTX micelles was investigated by collecting *in situ* fluorescence images of tumor xenografts in nude mice at different post injection time points. Images of the tumor area became readily defined after 4 hr post injection (Fig. 6A, Supplementary Fig. 6). *Ex vivo* images of normal tissues, organs, and tumors harvested from the RNA micelles-injected mice showed that the tumors harvested at 24 hr post injection exhibited the strongest signal (Fig. 6B). In terms of tumor accumulation kinetics, RNA

nanoparticles reached their highest accumulation 4 hr post injection and remained longer in the tumor compared to healthy organs and tissues, which indicates a high tumor targeting efficiency and tumor retention capability of the constructed RNA micelles. Such distinct tumor retention behavior of RNA micelles suggests this delivery system makes the advantage of the EPR (enhanced permeability and retention) effect due to its nano-scale size and particle shape. In addition, the RNA micelles constructs showed prolonged tumor retention possible due to its larger particle size. The specificity of *in vivo* tumor targeting of RNA micelle nanoparticle can be further secured by including a tumor targeting modules, such as folate [6,27,65,72,73] and RNA aptamers [19,21,74], to the empty helical branch on the RNA micelles.

No or low induction of pro-inflammatory response by pRNA-3WJ micelles

Pro-inflammatory response could be potentially induced by both RNA component [75] and cholesterol component [76] in pRNA-3WJ micelles formulation. In order to address this concern, we evaluated the production of pro-inflammatory cytokines and chemokines upon pRNA-3WJ micelles treatment both *in vitro* and *in vivo*. Tumor necrosis factor- α (TNF- α) is cytokine involved in systemic inflammation and one of the cytokines that make up the acute phase reaction [77]. Interleukin 6 (IL6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. IL6 is secreted to stimulate immune response during infection [78,79]. IFN- α , which belongs to type I interferon, is also cytokines involved in pro-inflammatory reaction released in response to the presence of viral pathogens. The results in Fig. 7A showed that pRNA-3WJ micelles at higher dose (1 μ M) and lower dose (200 nM) did not induce neither IL6 nor IFN- α production compared to LPS positive control while incubating with mouse macrophage-like RAW 264.7 cells *in vitro*. TNF- α induction is not detectable with lower dose of pRNA-3WJ-micelles treatment but was slightly raised up upon higher dose of RNA micelles treatment. All three cytokines were not induced after *in vivo* injection of pRNA-3WJ-micelles into immune competent C57BL/6 mice compared to LPS control as shown in Fig. 7B.

Chemokines are the main proinflammatory mediators [80,81]. We profiled 25 chemokine production upon pRNA-micelles treatment *in vivo*. From the result as shown in Fig. 7C, we can conclude that pRNA-3WJ micelles did not induce new chemokines compared to PBS group. There were three chemokines, macrophage inflammatory protein-1 gamma (MIP-1 γ), Chemokine10 (C10), and Monocyte chemoattractant protein 2 (MCP2) showed elevated induction compared to PBS control as highlighted by red square in the Supplementary Fig. 7. In summary, pRNA-3WJ micellar formulation induced no or very low pro-inflammatory response.

In conclusion, we have designed and constructed well-defined pRNA based micelles composed of a hydrophobic lipid core and a hydrophilic pRNA-3WJ corona. Chemotherapeutic drug Paclitaxel has been loaded into RNA micelles with significantly improved water solubility. We also demonstrated that the Paclitaxel loaded pRNA micelles exhibit excellent tumor cell binding and internalization as well as effective induction of cytotoxicity effects on tumor cells as PTX *in vitro*. There is also no or very low induction of pro-inflammatory responses upon pRNA-micelles injection. Tumor targeting was achieved

upon systemic injection of pRNA micelles into xenografted mouse models without accumulation into normal organs and tissues. The branched pRNA-3WJ corona gives incomparable versatility that can be designed and constructed in any desired combination. For example, the multifunctional pRNA-3WJ micelles can be achieved by combing targeting, imaging and therapeutic modules all in one nanoparticle. pRNA-3WJ micelles can also simultaneously deliver siRNAs or microRNAs against multiple genes or different location of one gene to generate synergetic effects. In addition, different types of anti-cancer drugs can be loaded onto one pRNA-3WJ micelles to enhance the therapeutic effect or overcome the drug resistance by combination therapy. Therefore, this innovative RNA based micellar nano-delivery platform holds great potential for clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The research in P.G.'s lab was supported by the National Institutes of Health [R01EB019036, U01CA151648, U01CA207946] to Peixuan Guo as well as [P50CA168505, R21CA209045] and DOD Award [W81XWH-15-1-0052] to Dan Shu. The authors would like to thank Daniel Jasinski for the helpful discussion and assistance in RNA solid phase synthesis. We also would like to thank Dr. Haining Zhu at University of Kentucky for the help of performing Mass Spectrometry. P.G.'s Sylvan G. Frank Endowed Chair position in Pharmaceutics and Drug Delivery is funded by the CM Chen Foundation. PG is the consultant of Oxford Nanopore Technologies and Nanobio Delivery Pharmaceutical Co. Ltd, as well as the cofounder of Shenzhen P&Z Biomedical Co. Ltd and its subsidiary US P&Z Biological Technology LLC, and cofounder of ExonanoRNA LLC.

References

1. Paulmurugan R, Bhethanabotla R, Mishra K, Devulapally R, Foygel K, Sekar TV, Ananta JS, Massoud TF, Joy A. Folate Receptor-Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple-Negative Breast Cancer. *Mol Cancer Ther*. 2016; 15:221–231. [PubMed: 26553061]
2. Lee JB, Hong J, Bonner DK, Poon Z, Hammond PT. Self-assembled RNA interference microsponges for efficient siRNA delivery. *Nat Mater*. 2012; 11:316–322. [PubMed: 22367004]
3. Li H, Zhang K, Pi F, Guo S, Shlyakhtenko L, Chiu W, Shu D, Guo P. Controllable Self-Assembly of RNA Tetrahedrons with Precise Shape and Size for Cancer Targeting. *Adv Mater*. 2016; 28:7501–7507. [PubMed: 27322097]
4. Douglas SM, Dietz H, Liedl T, Hogberg B, Graf F, Shih WM. Self-assembly of DNA into nanoscale three-dimensional shapes. *Nature*. 2009; 459:414–418. [PubMed: 19458720]
5. Guo P. The emerging field of RNA nanotechnology. *Nature Nanotechnology*. 2010; 5:833–842.
6. Shu D, Shu Y, Haque F, Abdelmawla S, Guo P. Thermodynamically stable RNA three-way junctions for constructing multifunctional nanoparticles for delivery of therapeutics. *Nature Nanotechnology*. 2011; 6:658–667.
7. Shu Y, Cinier M, Shu D, Guo P. Assembly of multifunctional phi29 pRNA nanoparticles for specific delivery of siRNA and other therapeutics to targeted cells. *Methods*. 2011; 54:204–214. [PubMed: 21320601]
8. Haque F, Shu D, Shu Y, Shlyakhtenko L, Rychahou P, Evers M, Guo P. Ultraprecise synergistic tetravalent RNA nanoparticles for targeting to cancers. *Nano Today*. 2012; 7:245–257. [PubMed: 23024702]
9. Afonin KA, Kireeva M, Grabow WW, Kashlev M, Jaeger L, Shapiro BA. Co-transcriptional assembly of chemically modified RNA nanoparticles functionalized with siRNAs. *Nano Lett*. 2012; 12:5192–5195. [PubMed: 23016824]

10. Shu Y, Shu D, Haque F, Guo P. Fabrication of pRNA nanoparticles to deliver therapeutic RNAs and bioactive compounds into tumor cells. *Nat Protoc.* 2013; 8:1635–1659. [PubMed: 23928498]
11. Khisamutdinov E, Li H, Jasinski D, Chen J, Fu J, Guo P. Enhancing immunomodulation on innate immunity by shape transition among RNA triangle, square, and pentagon nanovehicles. *Nucleic Acids Res.* 2014; 42:9996–10004. [PubMed: 25092921]
12. Jasinski D, Khisamutdinov EF, Lyubchenko YL, Guo P. Physicochemically Tunable Poly-Functionalized RNA Square Architecture with Fluorogenic and Ribozymatic Properties. *ACS Nano.* 2014; 8:7620–7629. [PubMed: 24971772]
13. Khisamutdinov EF, Jasinski DL, Li H, Zhang K, Chiu W, Guo P. Fabrication of RNA 3D Nanoprism for Loading and Protection of Small RNAs and Model Drugs. *Advanced Materials.* 2016; 28:100079–100087.
14. Afonin KA, Viard M, Koyfman AY, Martins AN, Kasprzak WK, Panigaj M, Desai R, Santhanam A, Grabow WW, Jaeger L, Heldman E, Reiser J, Chiu W, Freed EO, Shapiro BA. Multifunctional RNA nanoparticles. *Nano Lett.* 2014; 14:5662–5671. [PubMed: 25267559]
15. Dibrov SM, McLean J, Parsons J, Hermann T. Self-assembling RNA square. *Proc Natl Acad Sci USA.* 2011; 108:6405–6408. [PubMed: 21464284]
16. Afonin KA, Bindewald E, Yaghoobian AJ, Voss N, Jacovetty E, Shapiro BA, Jaeger L. In vitro assembly of cubic RNA-based scaffolds designed in silico. *Nat Nanotechnol.* 2010; 5:676–682. [PubMed: 20802494]
17. Lee TJ, Haque F, Shu D, Yoo JY, Li H, Yokel RA, Horbinski C, Kim TH, Kim SH, Nakano I, Kaur B, Croce CM, Guo P. RNA nanoparticles as a vector for targeted siRNA delivery into glioblastoma mouse model. *Oncotarget.* 2015; 6:14766–14776. [PubMed: 25885522]
18. Cui D, Zhang C, Liu B, Shu Y, Du T, Shu D, Wang K, Dai F, Liu Y, Li C, Pan F, Yang Y, Ni J, Li H, Brand-Saberi B, Guo P. Regression of gastric cancer by systemic injection of RNA nanoparticles carrying both ligand and siRNA. *Scientific reports.* 2015; 5:10726. [PubMed: 26137913]
19. Shu D, Li H, Shu Y, Xiong G, Carson WE, Haque F, Xu R, Guo P. Systemic delivery of anti-miRNA for suppression of triple negative breast cancer utilizing RNA nanotechnology. *ACS Nano.* 2015; 9:9731–9740. [PubMed: 26387848]
20. Rychahou P, Haque F, Shu Y, Zaytseva Y, Weiss HL, Lee EY, Mustain W, Valentino J, Guo P, Evers BM. Delivery of RNA nanoparticles into colorectal cancer metastases following systemic administration. *ACS Nano.* 2015; 9:1108–1116. [PubMed: 25652125]
21. Binzel D, Shu Y, Li H, Sun M, Zhang Q, Shu D, Guo B, Guo P. Specific Delivery of MiRNA for High Efficient Inhibition of Prostate Cancer by RNA Nanotechnology. *Molecular Therapy.* 2016; 24:1267–1277. [PubMed: 27125502]
22. Afonin KA, Grabow WW, Walker FM, Bindewald E, Dobrovolskaia MA, Shapiro BA, Jaeger L. Design and self-assembly of siRNA-functionalized RNA nanoparticles for use in automated nanomedicine. *Nat Protoc.* 2011; 6:2022–2034. [PubMed: 22134126]
23. Khisamutdinov EF, Bui MN, Jasinski D, Zhao Z, Cui Z, Guo P. Simple Method for Constructing RNA Triangle, Square, Pentagon by Tuning Interior RNA 3WJ Angle from 60 degrees to 90 degrees or 108 degrees. *Methods Mol Biol.* 2015; 1316:181–193. [PubMed: 25967062]
24. Shu Y, Haque F, Shu D, Li W, Zhu Z, Kotb M, Lyubchenko Y, Guo P. Fabrication of 14 Different RNA Nanoparticles for Specific Tumor Targeting without Accumulation in Normal Organs. *RNA.* 2013; 19:766–777.
25. Khisamutdinov EF, Jasinski DL, Guo P. RNA as a boiling-resistant anionic polymer material to build robust structures with defined shape and stoichiometry. *ACS Nano.* 2014; 8:4771–4781. [PubMed: 24694194]
26. Li H, Rychahou PG, Cui Z, Pi F, Evers BM, Shu D, Guo P, Luo W. RNA Nanoparticles Derived from Three-Way Junction of Phi29 Motor pRNA Are Resistant to I-125 and Cs-131 Radiation. *Nucleic Acid Ther.* 2015
27. Lee TJ, Yoo JY, Shu D, Li H, Zhang J, Yu JG, Jaime-Ramirez AC, Acunzo M, Romano G, Cui R, Sun HL, Luo Z, Old M, Kaur B, Guo P, Croce CM. RNA Nanoparticle-Based Targeted Therapy for Glioblastoma through Inhibition of Oncogenic miR-21. *Mol Ther.* 2017

28. Shu D, Khisamutdinov E, Zhang L, Guo P. Programmable folding of fusion RNA complex driven by the 3WJ motif of phi29 motor pRNA. *Nucleic Acids Res.* 2013; 42
29. Shu D, Zhang H, Jin J, Guo P. Counting of six pRNAs of phi29 DNA-packaging motor with customized single molecule dual-view system. *EMBO J.* 2007; 26:527–537. [PubMed: 17245435]
30. Jasinski DL, Yin H, Li Z, Guo P. The Hydrophobic Effect from Conjugated Chemicals or Drugs on in Vivo Biodistribution of RNA Nanoparticles. *Hum Gene Ther.* 2017
31. Pi F, Binzel DW, Lee TJ, Li Z, Sun M, Rychahou P, Li H, Haque F, Wang S, Croce CM, Guo B, Evers BM, Guo P. Nanoparticle orientation to control RNA surface display on extracellular vesicles for efficient cell targeting, siRNA delivery and cancer regression. *Nature Nanotechnology.* 2017 In Press.
32. Raouane M, Desmaele D, Urbinati G, Massaad-Massade L, Couvreur P. Lipid conjugated oligonucleotides: a useful strategy for delivery. *Bioconjug Chem.* 2012; 23:1091–1104. [PubMed: 22372953]
33. Gosse C, Boutorine A, Aujard I, Chami M, Kononov A, Cogne-Laage E, Allemand JF, Li J, Jullien L. Micelles of lipid-oligonucleotide conjugates: implications for membrane anchoring and base pairing. *J Phys Chem B.* 2004; 108:6485–6497. [PubMed: 18950138]
34. Wu Y, Sefah K, Liu H, Wang R, Tan W. DNA aptamer-micelle as an efficient detection/delivery vehicle toward cancer cells. *Proc Natl Acad Sci US A.* 2010; 107:5–10.
35. Liu H, Zhu Z, Kang H, Wu Y, Sefan K, Tan W. DNA-based micelles: synthesis, micellar properties and size-dependent cell permeability. *Chemistry.* 2010; 16:3791–3797. [PubMed: 20162643]
36. Chen T, Wu CS, Jimenez E, Zhu Z, Dajac JG, You M, Han D, Zhang X, Tan W. DNA micelle flares for intracellular mRNA imaging and gene therapy. *Angew Chem Int Ed Engl.* 2013; 52:2012–2016. [PubMed: 23319350]
37. Jeong JH, Park TG. Novel polymer-DNA hybrid polymeric micelles composed of hydrophobic poly(D,L-lactic-co-glycolic acid) and hydrophilic oligonucleotides. *Bioconjug Chem.* 2001; 12:917–923. [PubMed: 11716682]
38. Dentinger PM, Simmons BA, Cruz E, Sprague M. DNA-mediated delivery of lipophilic molecules via hybridization to DNA-based vesicular aggregates. *Langmuir.* 2006; 22:2935–2937. [PubMed: 16548535]
39. Alemdaroglu FE, Ding K, Berger R, Herrmann A. DNA-templated synthesis in three dimensions: Introducing a micellar scaffold for organic reactions. *Angew Chem Int Ed Engl.* 2006; 45:4206–4210. [PubMed: 16705747]
40. Trinh T, Chidchob P, Bazzi HS, Sleiman HF. DNA micelles as nanoreactors: efficient DNA functionalization with hydrophobic organic molecules. *Chem Commun(Camb).* 2016; 52:10914–10917. [PubMed: 27533528]
41. Li Z, Zhang Y, Fullhart P, Mirkin CA. Reversible and chemically programmable micelle assembly with DNA block-copolymer amphiphiles. *Nano Letters.* 2004; 4:1055–1058.
42. Alemdaroglu FE, Alemdaroglu NC, Langguth P, Herrmann A. DNA block copolymer micelles- A combinatorial tool for cancer nanotechnology. *Adv Mater.* 2008; 20:899–902.
43. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc.* 1971; 93:2325–2327. [PubMed: 5553076]
44. Spencer CM, Faulds D. Paclitaxel. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in the treatment of cancer. *Drugs.* 1994; 48:794–847. [PubMed: 7530632]
45. Rowinsky EK, Donehower RC. Paclitaxel (taxol). *N Engl J Med.* 1995; 332:1004–1014. [PubMed: 7885406]
46. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer.* 2004; 4:253–265. [PubMed: 15057285]
47. Horwitz SB. Mechanism of action of taxol. *Trends Pharmacol Sci.* 1992; 13:134–136. [PubMed: 1350385]
48. Singla AK, Garg A, Aggarwal D. Paclitaxel and its formulations. *Int J Pharm.* 2002; 235:179–192. [PubMed: 11879753]

49. Gelderblom H, Verweij J, Nooter K, Sparreboom A. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *Eur J Cancer*. 2001; 37:1590–1598. [PubMed: 11527683]
50. Sparreboom A, van TO, Nooijen WJ, Beijnen JH. Nonlinear pharmacokinetics of paclitaxel in mice results from the pharmaceutical vehicle Cremophor EL. *Cancer Res*. 1996; 56:2112–2115. [PubMed: 8616858]
51. Kim SC, Kim DW, Shim YH, Bang JS, Oh HS, Wan KS, Seo MH. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J Control Release*. 2001; 72:191–202. [PubMed: 11389998]
52. Hamaguchi T, Kato K, Yasui H, Morizane C, Ikeda M, Ueno H, Muro K, Yamada Y, Okusaka T, Shirao K, Shimada Y, Nakahama H, Matsumura Y. A phase I and pharmacokinetic study of NK105, a paclitaxel-incorporating micellar nanoparticle formulation. *Br J Cancer*. 2007; 97:170–176. [PubMed: 17595665]
53. Dong Y, Feng SS. Methoxy poly(ethylene glycol)-poly(lactide) (MPEG-PLA) nanoparticles for controlled delivery of anticancer drugs. *Biomaterials*. 2004; 25:2843–2849. [PubMed: 14962562]
54. Kim K, Kim JH, Park H, Kim YS, Park K, Nam H, Lee S, Park JH, Park RW, Kim IS, Choi K, Kim SY, Park K, Kwon IC. Tumor-homing multifunctional nanoparticles for cancer theragnosis: Simultaneous diagnosis, drug delivery, and therapeutic monitoring. *J Control Release*. 2010; 146:219–227. [PubMed: 20403397]
55. Yoshizawa Y, Kono Y, Ogawara K, Kimura T, Higaki K. PEG liposomalization of paclitaxel improved its in vivo disposition and anti-tumor efficacy. *Int J Pharm*. 2011; 412:132–141. [PubMed: 21507344]
56. Yuan H, Miao J, Du YZ, You J, Hu FQ, Zeng S. Cellular uptake of solid lipid nanoparticles and cytotoxicity of encapsulated paclitaxel in A549 cancer cells. *Int J Pharm*. 2008; 348:137–145. [PubMed: 17714896]
57. Khandare JJ, Jayant S, Singh A, Chandna P, Wang Y, Vorsa N, Minko T. Dendrimer versus linear conjugate: Influence of polymeric architecture on the delivery and anticancer effect of paclitaxel. *Bioconjug Chem*. 2006; 17:1464–1472. [PubMed: 17105225]
58. Bedikian AY, Plager C, Papadopoulos N, Eton O, Ellerhorst J, Smith T. Phase II evaluation of paclitaxel by short intravenous infusion in metastatic melanoma. *Melanoma Res*. 2004; 14:63–66. [PubMed: 15091196]
59. Hwu JR, Lin YS, Josephrajan T, Hsu MH, Cheng FY, Yeh CS, Su WC, Shieh DB. Targeted Paclitaxel by conjugation to iron oxide and gold nanoparticles. *J Am Chem Soc*. 2009; 131:66–68. [PubMed: 19072111]
60. Lay CL, Liu HQ, Tan HR, Liu Y. Delivery of paclitaxel by physically loading onto poly(ethylene glycol) (PEG)-graft-carbon nanotubes for potent cancer therapeutics. *nanotechnology*. 2010; 21:065101. [PubMed: 20057024]
61. Deng J, Huang L, Liu F. Understanding the structure and stability of paclitaxel nanocrystals. *Int J Pharm*. 2010; 390:242–249. [PubMed: 20167270]
62. Edwardson TGW, Carneiro KMM, McLaughlin CK, Serpell CJ, Sleiman HF. Site-specific positioning of dendritic alkyl chains on DNA cages enables their geometry-dependent self-assembly. *Nature Chemistry*. 2013; 5:868–875.
63. Zhang A, Zhang Z, Shi F, Ding J, Xiao C, Zhuang X, He C, Chen L, Chen X. Disulfide crosslinked PEGylated starch micelles as efficient intracellular drug delivery platforms. *Soft Matter*. 2013; 9:2224–2233.
64. Guo S, Tschammer N, Mohammed S, Guo P. Specific delivery of therapeutic RNAs to cancer cells via the dimerization mechanism of phi29 motor pRNA. *Hum Gene Ther*. 2005; 16:1097–1109. [PubMed: 16149908]
65. Zhang H, Endrizzi JA, Shu Y, Haque F, Sauter C, Shlyakhtenko LS, Lyubchenko Y, Guo P, Chi YI. Crystal Structure of 3WJ Core Revealing Divalent Ion-promoted Thermostability and Assembly of the Phi29 Hexameric Motor pRNA. *RNA*. 2013; 19:1226–1237. [PubMed: 23884902]
66. Greenspan P, Mayer EP, Fowler SD. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol*. 1985; 100:965–973. [PubMed: 3972906]

67. Behlke MA. Chemical modification of siRNAs for in vivo use. *Oligonucleotides*. 2008; 18:305–319. [PubMed: 19025401]
68. Vestweber H, Pommerehne J, Sander R, Mahrt RF, Greiner A, Heitz W, Bassler H. Majority Carrier Injection from Ito Anodes Into Organic Light-Emitting-Diodes Based Upon Polymer Blends. *Synthetic Metals*. 1995; 68:263–268.
69. Binzel DW, Khisamutdinov EF, Guo P. Entropy-driven one-step formation of Phi29 pRNA 3WJ from three RNA fragments. *Biochemistry*. 2014; 53:2221–2231. [PubMed: 24694349]
70. Liu J, Guo S, Cinier M, Shlyakhtenko LS, Shu Y, Chen C, Shen G, Guo P. Fabrication of stable and RNase-resistant RNA nanoparticles active in gearing the nanomotors for viral DNA packaging. *ACS Nano*. 2011; 5:237–246. [PubMed: 21155596]
71. Skwarczynski M, Hayashi Y, Kiso Y. Paclitaxel prodrugs: toward smarter delivery of anticancer agents. *J Med Chem*. 2006; 49:7253–7269. [PubMed: 17149855]
72. Rychahou P, Shu Y, Haque F, Hu J, Guo P, Evers BM. Methods and assays for specific targeting and delivery of RNA nanoparticles to cancer metastases. *Methods Mol Biol*. 2015; 1297:121–135. [PubMed: 25896000]
73. Guo S, Huang F, Guo P. Construction of folate-conjugated pRNA of bacteriophage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. *Gene Ther*. 2006; 13:814–820. [PubMed: 16482206]
74. Pi F, Zhang H, Li H, Thiviyanathan V, Gorenstein DG, Sood AK, Guo P. RNA nanoparticles harboring annexin A2 aptamer can target ovarian cancer for tumor-specific doxorubicin delivery. *Nanomedicine*. 2016; 13:1183–1193. [PubMed: 27890659]
75. Guo S, Li H, Ma M, Fu J, Dong Y, Guo P. Size, Shape and Sequence-dependent Immunogenicity of RNA Nanoparticles. *Molecular Therapy-Nucleic Acids*. 2017 In Press.
76. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. *Nat Rev Immunol*. 2015; 15:104–116. [PubMed: 25614320]
77. Jaffer U, Wade RG, Gourlay T. Cytokines in the systemic inflammatory response syndrome: a review. *HSR Proc Intensive Care Cardiovasc Anesth*. 2010; 2:161–175. [PubMed: 23441054]
78. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol*. 2014; 6:a016295. [PubMed: 25190079]
79. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta*. 2011; 1813:878–888. [PubMed: 21296109]
80. Wang ZM, Liu C, Dziarski R. Chemokines are the main proinflammatory mediators in human monocytes activated by *Staphylococcus aureus*, peptidoglycan, and endotoxin. *J Biol Chem*. 2000; 275:20260–20267. [PubMed: 10751418]
81. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta*. 2014; 1843:2563–2582. [PubMed: 24892271]

Figure 1. Structure base and assembly principle of pRNA-3WJ-PTX micelles

A. pRNA-3WJ motif from bacteriophage phi29 packaging RNA. **B.** The angled branch structure of pRNA-3WJ. **C.** Conjugating pRNA-3WJ with a lipophilic module (cholesterol, blue), a therapeutic module (PTX, green), and a reporter module (Alexa dye, red). **D.** Illustration of the formation of pRNA-3WJ micelles *via* hydrophobic interaction of conjugated lipophilic module in aqueous solutions. **E.** Assay the assembly of pRNA-3WJ micelles by 1% TAE agarose gel electrophoresis. Upper gel: EtBr channel; lower gel: Alexa647 channel (M: 1kb plus DNA ladder).

Figure 2. Characterization of pRNA-3WJ micelles

A. AFM image. Scale bar: 200 nm. **B.** The apparent hydrodynamic diameters measurement by DLS. **C.** The Zeta potential measurement by DLS. **D.** Validate the assembly of pRNA-3WJ micelles *via* Nile Red binding assay.

Figure 3. RNA-PTX conjugates

A. The rational of design RNA-PTX conjugates. PTX- N_3 can react with end Alkyne labeled RNA *via* Click chemistry and PTX can be later released out from RNA strand by hydrolysis. **B.** Assay of successful RNA-PTX conjugation by 20% 8M Urea PAGE in TBE buffer. **C.** The experimental mass prediction of a_{3wj} -PTX conjugates by Mass Spectrometry. **D.** *In vitro* PTX releasing profile along time.

Figure 4. Assay tumor cell binding and internalization of pRNA-3WJ-PTX micelles *in vitro* by **A.** Flow Cytometry and **B.** Confocal microscopy. Blue: nuclei staining; Green: cytoskeleton staining; Red: pRNA-3WJ-PTX micelles binding.

Figure 5. Cytotoxicity and apoptotic effects of PTX loaded pRNA-3WJ micelles *in vitro*.
A. Assay cytotoxicity effects of pRNA-3WJ-PTX micelles by MTT assay. **B.** Assay apoptotic effects of pRNA-3WJ-PTX micelles by PI/Annexin V-FITC dual staining and FACS analysis. **C.** Caspase-3 assay.

Figure 6. *In vivo* tumor targeting of pRNA-3WJ-PTX micelles in mouse xenografts
A. whole body image obtained 4 hr post injection. **B.** Organ image obtained 24 hr post injection.

Figure 7. Assay inductions of pro-inflammatory cytokines and chemokines by pRNA-3WJ micelle formulation

A. In vitro evaluation of the TNF- α , IL6, and IFN- α production after incubating pRNA-3WJ micelles with mouse macrophage-like RAW 264.7 cells by ELISA assay. **B.** In vivo evaluation of the TNF- α , IL6, and IFN- α production after injecting pRNA-3WJ micelles into C57BL/6 mice by ELISA assay. **C.** In vivo chemokines induction profiling for pRNA-3WJ micelle formulation.