Cosilencing of PKM-2 and MDR-1 Sensitizes Multidrug-Resistant Ovarian Cancer Cells to Paclitaxel in a Murine Model of Ovarian Cancer

Meghna Talekar1, Qijun Ouyang1, Michael S. Goldberg2, and Mansoor M. Amiji1

Abstract

Tumor multidrug resistance (MDR) is a serious clinical challenge that significantly limits the effectiveness of cytotoxic chemotherapy. As such, complementary therapeutic strategies are being explored to prevent relapse. The altered metabolic state of cancer cells, which perform aerobic glycolysis, represents an interesting target that can enable discrimination between healthy cells and cancer cells. We hypothesized that cosilencing of genes responsible for aerobic glycolysis and for MDR would have synergistic antitumor effect. In this study, siRNA duplexes against pyruvate kinase M2 and multidrug resistance gene-1 were encapsulated in hyaluronic acid–based self-assembling nanoparticles. The particles were characterized for morphology, size, charge, encapsulation efficiency, and transfection efficiency. In vivo studies included biodistribution assessment, gene knockdown confirmation, therapeutic efficacy, and safety analysis. The benefit of active targeting of cancer cells was confirmed by modifying the particles’ surface with a peptide targeted to epidermal growth factor receptor, which is overexpressed on the membranes of the SKOV-3 cancer cells. To augment the studies involving transplantation of a paclitaxel-resistant cell line, an in vivo paclitaxel resistance model was developed by injecting repeated doses of paclitaxel following tumor inoculation. The nanoparticles accumulated significantly in the tumors, hindering tumor volume doubling time (P < 0.05) upon combination therapy in both the wild-type (2-fold) and resistant (8-fold) xenograft models. Although previous studies indicated that silencing of MDR-1 alone sensitized MDR ovarian cancer to paclitaxel only modestly, these data suggest that concurrent silencing of PKM-2 improves the efficacy of paclitaxel against MDR ovarian cancer. Mol Cancer Ther; 14(7): 1521–31. ©2015 AACR.

Introduction

Ovarian cancer is one of the most common female gynecological malignancies and has an average 5-year survival rate of 44% (1). Because the majority of patients present with advanced stages of the disease, monotherapies typically do not confer meaningful efficacy. As a result, innovative strategies to prolong disease remission are being explored. Paclitaxel is a standard treatment for ovarian cancer, though its effectiveness often diminishes over the course of treatment owing to the emergence of multidrug resistance (MDR). MDR develops as a consequence of several factors, including poor systemic drug delivery due to vascular abnormalities, insufficient intracellular availability, and microenvironmental selection pressures (2). Among these, one of the most studied resistance mechanisms is the reduction in intracellular drug concentration by efflux transporter proteins that pump drugs out of cells. Many of these transporters are members of the ATP-binding cassette transmembrane protein super-family, including P-glycoprotein (P-gp) and MDR protein-1 (MRP-1). Although increasing the dosing of anticancer agents can transiently increase intracellular drug concentrations, dose-limiting toxicities can necessitate drug-free recovery periods that lead to reversion to MDR.

Along with drug resistance, cancer cells also undergo adaptations in cellular respiration to enhance their tumorigenic and metastatic potential (3). Cellular respiration is a series of catabolic reactions that yield ATP. In healthy cells, this occurs by the energy-efficient oxidative phosphorylation (OXPHOS) system (yielding between 30 and 36 ATPs for every glucose molecule), whereas cancer cells show a propensity to rely on a less energy-efficient process termed aerobic glycolysis (which yields two ATPs for every glucose molecule) for energy. Although this dependence of cancer cells on aerobic glycolysis would seem detrimental energetically, cancer cells are not limited for ATP and prioritize conversion of glucose to biomass for dividing cells. From a therapeutic perspective, these metabolic differences between cancer cells and healthy cells can be used to discriminate these cell types, thereby avoiding the harsh side effects of cytotoxic therapies.

Pyruvate kinase (PK) is a critical enzyme in the glycolysis pathway that has been avidly explored as a potential target for cancer therapy. It catalyzes the final irreversible rate-limiting step of glycolysis by dephosphorylating phosphoenolpyruvate (PEP) into pyruvate and produces one molecule of ATP in the process. PK has 4 isoforms (L, R, M1, and M2) with a specific
tissue distribution pattern (4). PKM-1 and PKM-2 are different splicing products of the same mRNA transcribed from the PKM gene. The PKM-1 isofrom is expressed in energy-intensive organs such as muscles, brain, heart, and kidney, whereas PKM-2 is expressed in lungs, fat, pancreatic islets, and in cells with a high rate of nucleic acid synthesis, such as embryonic cells, adult stem cells, and tumor cells. Because of a high expression in tumor cells, novel inhibitors (5, 6) and nucleic acid therapies (7–9) have been designed to target PKM-2 to achieve anticancer activity.

RNA interference (RNAi) is a biological process in which RNA molecules (miRNA or siRNA) inhibit gene expression, typically by causing the destruction of complementary mRNA molecules. It has emerged as a powerful strategy for cancer therapy, especially MDR cancer. Frustratingly, delivery of these therapies to specific tumor tissues and cells followed by intracellular release from endosomal/lysosomal compartments into the cytoplasm still remains a major hurdle. Naked RNAi-based therapy is unfavorable because of the charge, size, and instability of siRNAs. Although viral vectors are efficient tools for gene delivery, concerns regarding their toxicity have limited their clinical applications, necessitating development of safer alternatives. Nonviral/polymeric nanoparticle (NP)–based delivery platforms are becoming viable alternatives for delivery of RNAi-based therapies. Indeed, siRNA against PKM-2 has been successfully delivered by NPs to induce cell apoptosis and tumor regression (7, 10). Goldberg and colleagues had previously reported the delivery of M2 isoform-specific siPKM-2 in lipidoid formulations in HepG2 and SKOV-3 tumor bearing mice. The siRNA NPs showed a 76% and 85% decrease in tumor volume relative to the controls (7).

In our experience, encapsulation of siMDR-1 and paclitaxel in polymeric NPs has provided MDR-1 gene silencing and subsequent enhancement in cytotoxicity attributed to an increase in intracellular drug concentration in SKOV-3 TR cells (11). Goldberg and colleagues (17). For this study, the GE11 peptide with a spacer sequence of GGGGC was synthesized at Tufts University Core Facility (Boston, MA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and grown at 37°C, 5% CO2.

Human ovarian adenocarcinoma cell line SKOV-3 was obtained from ATCC. A paclitaxel-resistant version of this cell line (SKOV-3TR) was obtained from Massachusetts General Hospital (Boston, MA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and grown at 37°C, 5% CO2. SKOV-3TR cells were cultured in 20 mmol/L paclitaxel to maintain their drug-resistant phenotype. The resistant phenotype of these cells was regularly assessed by cytotoxicity analysis to determine IC50 values.

**Formation and characterization of siRNA-loaded HA NPs**: determination of size, charge, morphology, siRNA encapsulation, and siRNA release

We have previously published information regarding preparing combinatorial designed HA formulations (16). The HA-PEG conjugates were prepared using this combinatorial approach (16). For the synthesis of HA-PEG-EFG, 50 mg of maleimide-PEG-amine was added to EDC/NHS activated HA. Following synthesis of HA-PEG-maleimide, an EGR-specific peptide, YHWYGTPQNVI designated as GE11 was used for conjugation with maleimide. The GE11 peptide was originally synthesized and screened as an EGR-specific peptide by Li and colleagues (17). For this study, the GE11 peptide with a spacer sequence of GGGGC was synthesized at Tufts University Core Facility (Boston, MA). We have previously successfully prepared EGR-targeted polymeric NPs using this peptide sequence (18). The carboxyl group of terminal cysteine of the peptide was reacted with the maleimide of maleimide-PEG-HA in HEPES buffer (pH 7.4) at 1:1 molar ratio while mixing under N2 at 4°C for 24 hours. The peptide conjugate was then purified by dialysis (3.5 kDa), lyophilized, and characterized by nuclear magnetic resonance spectroscopy. Similarly, for the preparation of HA-rhodamine 123, 50 mg of rhodamine 123 was added to the EDC/NHS activated HA, the solution stirred overnight, dialyzed, and lyophilized. The HA-PEI, HA-PEG solutions (3 mg/mL) were prepared by dissolving the polymer in PBS. NP size and charge were determined on a Malvern Nano ZS (Malvern Instruments). Transmission electron microscopy (JEOL, JEM-1000) was performed to assess the formation of siRNA-loaded NPs. Uranil acetate ribonucleic acid stain was used to demarcate siRNA from the polymer. The dark staining of siRNA by heavy metals such as uranyl acetate nucleic acid stain was used to demarcate siRNA from the polymer.

**Cell lines**

Human ovarian adenocarcinoma cell line SKOV-3 was obtained from ATCC. A paclitaxel-resistant version of this cell line (SKOV-3TR) was obtained from Massachusetts General Hospital (Boston, MA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and grown at 37°C, 5% CO2. SKOV-3TR cells were cultured in 20 mmol/L paclitaxel to maintain their drug-resistant phenotype. The resistant phenotype of these cells was regularly assessed by cytotoxicity analysis to determine IC50 values.

**Materials and Methods**

**Materials**

Sodium hyaluronate (HA) with an average molecular weight of 20 kDa was obtained from Lifecore Biomedical Co. Poly(ethylene imine; PEI MW = 20,000 Da) was obtained from Polysciences Inc. Monofunctional poly(ethylene glycol)-amine (PEG2K-NH2, MW = 2,000 Da) was purchased from Creative PEG Works, Inc. Paclitaxel was purchased from Fisher Scientific.
Assessment of HA-siRNA uptake by confocal microscopy
For the purpose of uptake studies, fluorescein-labeled scrambled siRNA was formulated with HA-PEI conjugated with rhodamine dye. Particles containing 10 nmol/L siRNA were incubated with SKOV-3 WT and SKOV-3 TR cells at 37 °C and cell uptake was assessed after 5, 15, or 30 minutes.

Transfection of siRNA against MDR-1 and PKM-2 to assess silencing of target mRNA expression
SKOV-3 cells were transfected with siRNA-loaded NPs (100 nmol/L siRNA) incubated for 72 or 96 hours. The cells were then harvested, and RNA was extracted and used to run quantitative PCR to assess mRNA levels. mRNA knockdown was determined by normalizing the expression of MDR-1 or PKM-2 to β-actin levels.

Cytotoxicity assay for single and combination siRNA therapy followed by paclitaxel
SKOV-3 WT and SKOV-3 TR cells were initially transfected with siMDR-1 and/or siPKM-2. After 72 hours, paclitaxel was added to assess the effect of cosensitization therapy. Cellular cytotoxicity was assessed by MTT assay 72 hours after paclitaxel treatment and expressed as the percentage of viable cells.

Establishment of subcutaneous SKOV-3 WT tumors
Animal procedures were performed according to a protocol approved by Northeastern University, Institutional Animal Care and Use Committee (NIU-IACUC). The tumor models for this study were developed in nude mice obtained from Charles River Laboratories (Wilmington, MA). Five- to 6-week-old nude mice were injected subcutaneously with SKOV-3 WT tumor cells (5 × 10⁶ cells + Matrigel) on the right flank. Tumor sizes were measured at least once or twice a week to monitor tumor growth.

Quantitative biodistribution studies of HA NPs using a near infrared (NIR)-encapsulated dye
The biodistribution of HA-PEI/PEG and dual-targeted HA NPs was assessed by encapsulating indocyanine green (ICG) into the NPs. Tumor bearing mice were inoculated as described above, and the study commenced when the tumors reached an average size of ~200 mm³. The particles were then injected intravenously into mice bearing SKOV-3 WT or SKOV-3 TR tumors. Mice were imaged at 1, 2, 6, and 24 hours after the injection to monitor the distribution of the particles using an Xenogen IVIS Imaging System (Xenogen Corporation; Ex: 785 nm, Em: 820 nm).

Quantitative biodistribution and pharmacokinetic studies using siPKM-2 encapsulated in HA NPs
siRNA duplexes silencing PKM-2 was encapsulated in HA-PEI/HA-PEG NPs. These particles were injected into mice (n = 6/group) bearing ovarian tumors (SKOV-3 WT) at 0.5 mg/kg twice a day on days 1, 3, and 5. Blood samples, major organs (liver, spleen, lung, heart, kidney), and tumors were collected 1, 5, 10, or 24 hours following the last injection. The organs were then homogenized using Qiagen Tissue Lyser (Qiagen) to prepare tissue lysates. The homogenized tissue lysates were subsequently diluted 1:1,000, cDNA was generated, and qRT-PCR was performed. The PKM-2 siRNA sequence, reverse primer, forward primer, and an antiprimer sequence are listed below.

PKM-2 siRNA:
Sense: 5'-CCA1AAU1UGC1U1C1C1CC1AC1C1AA-3'
Antisense: 5'-U1H1UG1UG1AG1GG1AC1G1H1A1U1GG1-3'
Primer:
Reverse: GGA1G1GGC1G1AC1C1C1C1AA1TCC1G1AT1T1T1T1T1G1
Forward: /5FAM/ACT1C1CC1TC1C1CG1AT1T1T1G1T1GAG1G1GAC1GA

Antiprimer: AA1TCC1G1G1G1G1G1G1G1G1G1G1G1GG1C1G1G1G1C1/1BHQ1/1
siRNA was denatured and annealed to the RT primer (6 µL diluted siRNA and 18 µL reverse primer, 100 nmol/L). siRNA was denatured by incubating at 95 °C for 5 minutes. Primers were then annealed by 2 minutes of incubation at 80, 70, 60, and 45 °C with 4°C hold. Then the RT reaction was carried out as follows. A master mix was made by mixing the following components: RT-PCR buffer (6.25 µL), forward primer (10 µmol/L, 0.12 µL), reverse primer (10 µmol/L, 0.12 µL), anti-primer (100 µmol/L, 0.12 µL), 25 U RT-PCR enzyme (0.5 µL), and water (1.5 µL).

A total of 8.5 µL of this master mix was then mixed with 3.5 µL of sample and run on the PCR at the following listed conditions: 50°C (10 minutes), 90°C (10 minutes); 40 cycles of 95°C (15 s), 45°C (60 s). The amplified siRNAs were finally detected and quantitated by running a standard curve using lysate from untreated mouse tissue spiked with known concentrations of siRNA.

Evaluation of target gene knockdown in established subcutaneous SKOV-3 tumors
When subcutaneous tumors were ~150 and 200 mm³ in size, the animals were randomized into groups such that each group had similar tumor size. The animals were given an siRNA dose of 0.5 mg/kg twice a day on days 1, 3, and 5. Twenty-four after the last dose, the tumors were excised. RNA was extracted, and qRT-PCR was performed to determine the extent of MDR-1 and PKM-2 silencing.

Efficacy studies and toxicity analysis to determine safety of siRNA-encapsulated NPs in SKOV-3 WT tumor-bearing mice
Animals bearing SKOV-3 WT tumors were initially given an siRNA dose of 0.5 mg/kg twice a day every alternate days 1, 3, and 5. Paclitaxel was administered at a dose of 20 mg/kg once per week for 2 weeks. Tumor volumes were measured using the following formula to monitor the tumor growth inhibition:

\[ \text{tumor volume} = \left( \text{length} \times \text{width} \times \text{depth} \right) / 2 \]

The mice were weighed the day the treatments commenced and every day during the dosing period. To measure liver enzyme levels, blood was collected from all groups, and alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels were assessed. Liver, kidney, and spleen samples from mice were also collected for histopathological analysis at the end of the study.

In vivo-induced paclitaxel resistance development, efficacy studies, and safety analysis for combination therapy of siRNA with paclitaxel
For development of the in vivo-induced paclitaxel-resistant mouse model, the mice were inoculated subcutaneously with SKOV-3 WT cells. When subcutaneous tumors were ~200 mm³ in size, the animals were treated with paclitaxel solution at a dose of 20 mg/kg every alternate day for 10 doses. One week
following completion of paclitaxel therapy, the animals were randomized to different treatment groups, and tumor volume was measured to monitor for tumor growth inhibition. Body weight measurements were conducted throughout the entire period of efficacy studies. Liver, kidney, and spleen toxicity studies were conducted as described in section "Efficacy studies and toxicity analysis to determine safety of HA-encapsulated NPs."

Results
Formation of siRNA-loaded HA NPs determination of size, charge, morphology, siRNA encapsulation, and release by polyanion competition by gel retardation assay
Figure 1A provides a schematic representation of negatively charged siMDR-1/siPKM-2 encapsulation in HA-PEI/PEG particles. The self-assembling NPs showed a spherical morphology in TEM (Fig. 1B) with particles in the size range of 106 to 125 nm, polydispersity index (PDI) of 0.1 to 0.3, and surface charge in the range of −25 to −28 mV (Table 1). Electrophoretic retardation analysis of siRNA binding by HA-PEI with the release of intact siRNA by PAA showed 92% to 95% siRNA encapsulation efficiency.

Assessment of siRNA encapsulated HA NP uptake by confocal microscopy
Figure 1D shows cell uptake studies in SKOV-3WT and SKOV-3TR model following administration of nano-assemblies formed with rhodamine 123 tagged HA and fluorescein tagged scrambled siRNA for 5, 15, and 30 minutes. The cells showed uptake within 15 minutes of incubation in the wild-type (WT) and resistant SKOV-3 cell lines. The dual-targeted system showed higher fluorescence signal compared with the HA-PEI/PEG system in both cell lines.

Transfection of MDR-1– and PKM-2–targeted siRNA to assess silencing of target gene expression
Transfection studies with siMDR-1 and siPKM-2 were conducted in SKOV-3WT (Fig. 2A) and SKOV-3TR cells (Fig. 2B) for 72 and 96 hours. Earlier and later time points were also assessed in preliminary studies, however, as these time points did not indicate a significant downregulation in gene expression these were not assessed further. Likewise, concentrations ranging from 50 and 200 nmol/L were assessed for activity. A concentration dependent downregulation of gene expression was observed and a concentration of 100 nmol/L was selected (lowest concentration with effective gene downregulation). Also, in our previous work with HA-PEI–based delivery systems we had observed, 100 nmol/L siRNA encapsulated in the HA-based systems provided effective downregulation with minimal cellular toxicity from PEI. Figure 2A measures the transfection efficiency of siMDR-1 and siPKM-2 in SKOV-3WT cells. A significant downregulation of MDR-1 was not observed even with Lipofectamine, as the basal levels of MDR-1 expression in SKOV-3 WT cells is fairly low; siPKM-2, however, showed an approximately 80% downregulation with Lipofectamine and both the NPs. Figure 2B reports the transfection efficiency of the delivery systems in SKOV-3TR cells. Both Lipofectamine and the NPs showed 80% to 85% gene expression downregulation with the NPs showing greater efficiency after longer periods of incubation compared with transfection achieved following use of Lipofectamine.

Table 1. NP size range, PDI, surface charge, and encapsulation efficiency

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>ζ-potential (mV)</th>
<th>Encapsulation efficiency</th>
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<tbody>
<tr>
<td>HA-PEI/PEG blank</td>
<td>106 ± 16</td>
<td>0.124</td>
<td>−27 ± 1.7</td>
<td>−</td>
</tr>
<tr>
<td>HA-PEI/PEG/MDR-1</td>
<td>117 ± 9</td>
<td>0.327</td>
<td>−28 ± 1.6</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>HA-PEI/PEG/PKM-2</td>
<td>125 ± 26</td>
<td>0.286</td>
<td>−25 ± 0.8</td>
<td>92 ± 8</td>
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</table>

Cytotoxicity assay for single and combination siRNA therapy in combination with paclitaxel
Figure 2 shows the effect of single and combination therapy on paclitaxel IC_{50} in SKOV-3WT and SKOV-3TR cell lines. In SKOV-3WT cells, both the single and combination therapy did not show a significant change in paclitaxel IC_{50} values (P > 0.05). In SKOV-3TR cells, single therapy with siPKM-2 showed a significant decrease in paclitaxel IC_{50} values (P < 0.05). A 1.6-fold difference in the IC_{50} value was observed between siPKM2 encapsulated in dual-targeted NPs compared with HA-PEI/PEG NPs. Between single and combination therapy, combination therapy showed
a 1.3-fold decrease in IC50 value (P < 0.05) compared with siMDR-1 therapy.

Qualitative biodistribution studies using NIR-encapsulated dye in the HA nanoassemblies

The whole body NP distribution in live animals was evaluated using an NIR dye, ICG encapsulated in HA NPs. ICG was loaded into HA-PEI/PEG NPs using a similar technique as used to encapsulate siRNA. These ICG loaded NPs had similar characteristics as siRNA encapsulated HA-PEI/PEG NPs (size range of ~200 nm, surface charge −15 mV). The ICG loaded NPs were intravenously injected in SKOV-3WT (Fig. 3A) and SKOV-3TR (Fig. 3B) tumor bearing mice at a dose equivalent to the dose used in efficacy studies. The NPs were stable during circulation following intravenous injection and the NIR signal was measured at different time points to capture the distribution pattern in live mice. Strong NIR signal was observed throughout the whole body 1 hour after administration with

**Figure 2.**

HA-PEI/siRNA mediated MDR-1 and PKM-2 gene silencing in SKOV-3WT (A) and SKOV-3TR (B). Cells were treated with scramble, naked siRNA, siRNA formulated in Lipofectamine, and siRNA formulated in HA-PEI/PEG NPs. MDR-1 and PKM-2 expression was measured by qPCR. Data represented as a mean SD (n = 3). C, cytotoxicity assay following single and combination therapy in SKOV-3WT (left) and SKOV-3TR cells (right). PTX, paclitaxel.
both the HA-PEI/PEG and dual-targeted NPs. A gradual decrease in signal intensity was observed over 6 hours with majority of the signal coming from liver and tumor regions 24 hours after ICG NP administration. Figure 3C shows ex vivo images of liver, kidney, spleen, heart, lungs, and tumors 24 hours after ICG administration. Among these tissues, liver showed the highest level of signal intensity, followed by the tumor, kidney, and spleen. We have previously observed that intravenous injection of free ICG in healthy mice shows a signal that is detectable in the liver as early as 3 minutes after injection, reaching a peak level between 5 and 10 minutes, indicative of a rapid hepatic clearance from the systemic circulation. The relatively short circulation time of free ICG in vivo could be attributed to fluorescence quenching of free ICG in physiological environments.

Quantitative biodistribution and pharmacokinetic studies using siPKM-2 encapsulated in HA nanoassemblies

After six intravenous injections of 0.5 mg/kg siRNA doses (in HA NP) into tumor bearing mice, blood and tissue samples were collected for siRNA quantitation at 1, 6, and 24 hours after last-administered dose. Accurate siRNA quantitation was studied using the antiprimer quenching based real-time PCR method. A fluorescently labeled PCR primer was designed to anneal to the template RNA and to a universal antiprimer. Following initial PCR, the temperature was lowered to allow the antiprimer to bind to unincorporated primer to quench the fluorescence. As double stranded PCR product would not bind with the antiprimer, an increase in fluorescent signal would enable siRNA quantitation. The siRNA was quantitated in each tissue and the % input dose per whole organ was calculated based on the starting siRNA
dose. Figure 3D shows the biodistribution profile of siPKM-2 in HA-PEI/PEG NPs, whereas Fig. 3E indicates the distribution of the dual-targeted particles. With both the delivery systems, 30% to 40% of the siRNAs are detected in the liver, kidney, and spleen within 1 hour of administration. A very small proportion of the siRNA was detected in the plasma whereas 3% to 9% of the siPKM-2 was detected in the tumor tissues.

Evaluating target gene knockdown in SKOV-3 tumor-bearing mice

Figure 4A depicts in vivo gene downregulation following administration of siMDR-1 and siPKM-2 in HA-PEI/PEG NPs. With both the delivery systems, 30% to 40% of the siRNAs are detected in the liver, kidney, and spleen within 1 hour of administration. A very small proportion of the siRNA was detected in the plasma whereas 3% to 9% of the siPKM-2 was detected in the tumor tissues.

Efficacy studies and toxicity analysis to determine safety of HA-encapsulated NPs

Figure 4C shows the effect of single and combination therapy of siRNA and paclitaxel in SKOV3WT tumor bearing mice. Tumor bearing mice were initially administered siMDR-1 and siPKM-2 as single or combination therapy twice a day every alternate day for 3 days. Twenty-four hours after the last dose, the animals were
injected with intravenous dose of paclitaxel at 20 mg/kg. This treatment regimen was repeated over 2 weeks and tumor volume measurements were measured every alternate day for 4 weeks. siRNA therapy in combination with paclitaxel showed significant improvement in efficacy. The siMDR-1 NPs showed a tumor growth profile similar to paclitaxel solution indicating that combination of siMDR-1 and paclitaxel has minimal effect on tumor growth. This effect could be correlated to low level of MDR1 expression in SKOV-3WT tumor bearing mice. Treatment with siPKM2 in both the NP systems showed slightly delayed tumor growth compared with paclitaxel solution \((P > 0.05)\). Dual siRNA therapy showed a significant decrease in tumor volume compared with control animals. Table 2 shows the tumor growth inhibition (TGI) and tumor volume doubling (TVD) time for all the treatment groups. Single siRNA therapy with paclitaxel showed 50% to 60% tumor inhibition \((P < 0.05)\) compared with saline group. In comparison, combination therapy of dual siRNA and paclitaxel showed 72% to 84% tumor growth inhibition \((P < 0.05)\). Similarly, this combination therapy showed almost a 2-fold increase in tumor volume doubling time. Compared with paclitaxel solution, a significant increase in TGI and TVD time was not observed. Safety and toxicity analysis showed minimal effect on body weight, ALT, and AST levels.

### Discussion

The aim of our current investigation was to study the effectiveness of combination therapy with siMDR-1 and siPKM-2 in SKOV-3WT and SKOV-3TR human ovarian adenocarcinoma cell lines and xenograft models. Previously, combination of MDR-1 gene silencing and chemotherapy administration has been assessed using polymeric NPs (11, 12, 19). These delivery systems have provided improved cytotoxicity attributed to an increased intracellular accumulation of anticancer agents upon MDR-1 gene silencing. Similarly, for PKM-2–based therapy, several nanocarrier-based delivery approaches have been reported in literature for anticancer activity. Goldberg and colleagues screened a library of siPKM-2 and identified a specific PKM-2 siRNA (si156), which when encapsulated in lipidoid NPs induced cell apoptosis showing tumor regression in HepG2 and SKOV3 xenograft tumor models (7). Another report showed that short hairpin RNA (shRNA) loaded in liposomal formulation showed effective PKM-2 silencing in human A549 lung cancer xenograft model and an increased efficacy in combination with cisplatin as an anticancer drug (10). PKM-2 silencing by shRNA loaded in Lipofectamine has also shown increased efficacy of docetaxel in a similar model. Thus, use of drug delivery systems to deliver nucleic acid to silence PKM-2 provides novel prospects of treating cancer. We have previously reported the development of CD44 targeting HA-based NPs for the delivery of siRNA to A549 non–small lung cancer cells and tumors (20–22). In this study, we wanted to extend the capability of our delivery system to assess the synergistic activity following the administration of combination of siRNA (targeting MDR-1 and PKM-2 expression) with paclitaxel in the treatment of ovarian cancer. As SKOV-3 cells have a high surface EGFR expression, we also examined the effect of actively targeted NPs in the delivery of these therapeutic agents (15). While testing these delivery systems in vivo, we explored the possibility of developing an in vivo paclitaxel resistance model, which would provide a realistic recapitulation of paclitaxel resistance development. Traditionally, in xenograft models paclitaxel resistance is developed by subcutaneous injection of cancer cells, which have been established by the reverse transformation technique, where the sublines are established in vitro by repeated or prolonged exposure to increasing doses of antineoplastic agents (23). However, these cells usually show strongly suppressed metastatic and/or tumorigenic properties in vivo, which makes

<table>
<thead>
<tr>
<th>Therapeutic groups</th>
<th>Tumor growth inhibition (%)</th>
<th>Tumor volume doubling time (days)</th>
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<tbody>
<tr>
<td>PBS</td>
<td>7.4 ± 4.8</td>
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<tr>
<td>Paclitaxel</td>
<td>10.9 ± 13.7</td>
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</tr>
<tr>
<td>Naked siMDR1</td>
<td>7.7 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>Naked siPKM2</td>
<td>7.6 ± 3.4</td>
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</tr>
<tr>
<td>Scrambled HA-PEI/PEG NP</td>
<td>7.6 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>Scrambled dual-targeted NP</td>
<td>7.8 ± 5.2</td>
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</tr>
<tr>
<td>NP-MDR1 HA-PEI/PEG NP</td>
<td>11.1 ± 5.6</td>
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<td>NP-MDR1 dual-targeted NP</td>
<td>10.0 ± 3.9</td>
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<tr>
<td>NP-PK2M2 HA-PEI/PEG NP</td>
<td>10.5 ± 6.5</td>
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<td>NP-MDR1-PK2M2 dual-targeted NP</td>
<td>17.9 ± 8.8</td>
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Abbreviation: PTX, paclitaxel.

**Table 2.** Percentage tumor growth inhibition and tumor volume doubling time following treatment with single or combination therapy of siRNA and PTX in SKOV-3WT tumor-bearing mice.
them incompatible comparators to recurrent cancers, which show increased metastatic and/or tumor growth potential. Several groups have reported development of xenograft resistant models by administration of paclitaxel in a subcutaneous model followed by excision and retransplantation over 6 months and the final tumor-derived cells being established and maintained as in vivo resistance sublines (24). In this study, we aimed to develop in vivo paclitaxel resistance by repeated administration of paclitaxel to recapitulate clinical resistance development and assess the effectiveness of our therapies in these models.

The siRNA nano-assemblies were prepared by charged based encapsulation of siRNA into HA-PEI particles. HA is a naturally occurring mucopolysaccharide composed of alternating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (NAG) with β(1→4) interglycosidic linkage. It is a highly anionic biopolymer present in the extracellular matrix and synovial fluids. It is biodegradable, nontoxic, nonimmunogenic and noninflammatory, which makes it an ideal carrier polymer for systemic drug delivery applications. The HA backbone specifically recognizes CD44, an integral membrane glycoprotein overexpressed on several tumor cell surfaces including tumor-initiating stem cells. Similarly, PEI is widely used for nucleic acid delivery, including siRNA delivery where nucleic acids are condensed by electrostatic interactions to form dense particles (complexes), which protect the genetic material from enzymatic degradation and promote resistance sublines (24).

### Table 3. Percentage tumor growth inhibition and tumor volume doubling time following treatment with single or combination therapy of siRNA and PTX in SKOV3-Tx tumor-bearing mice

<table>
<thead>
<tr>
<th>Therapeutic groups</th>
<th>Tumor growth inhibition (%)</th>
<th>Tumor volume doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10.7 ± 8.6</td>
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</tr>
<tr>
<td>PTX solution</td>
<td>2.1</td>
<td>12.3 ± 7.9</td>
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<tr>
<td>Scrambled dual-targeted NP</td>
<td>16.3</td>
<td>11.0 ± 11.6</td>
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<tr>
<td>NP-MDR1 dual-targeted NP</td>
<td>57.9</td>
<td>14.7 ± 12.8</td>
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<tr>
<td>NP-PKM2 dual-targeted NP</td>
<td>75.6</td>
<td>32.0 ± 10.7</td>
</tr>
<tr>
<td>NP-MDR1+PKM2 dual-targeted NP</td>
<td>89.7</td>
<td>79.4 ± 10.2</td>
</tr>
</tbody>
</table>

Abbreviation: PTX, paclitaxel.

**Figure 5.**
In vivo paclitaxel (PTX) resistance model development: A, qPCR studies to assess MDR-1 and PKM-2 expression; B, immunofluorescence of tumor tissues from P-gp and PKM-2 expression in PBS-treated and PTX-treated animals. C, schematic representation of the dosing schedule adopted to develop the SKOV-3TR mouse model followed by NP treatment. D, effect of the combination of paclitaxel treatment and MDR1 and PKM2 silencing on growth of SKOV3-Tx tumors.
their cellular uptake by absorptive endocytosis. PEI also has an intrinsic endosomal escape mechanism known as the "proton sponge" effect, which causes osmotic swelling and rupture of the endosome membrane, triggering the release of PEI complexes into the cytosol. In this study, for the preparation of the complexes, a 54:1 polymer:siRNA ratio was adapted from our previous studies, where optimal encapsulation and release was observed with this ratio (21). At these ratios, we obtained ~92% to 95% encapsulation efficiency of siRNA. Similarly, for in vitro studies, we selected a siRNA concentration of 100 nmol/L because of effect of higher concentrations on cell viability (Supplementary Fig. S2). For the incorporation of EGFR-targeted HA, in our preliminary investigation, we altered the ratio of HA-EGF and determined the transfection efficiency (Supplementary Fig. S3). Earlier transfection periods showed increased downregulation of gene expression; however, at later time points, altering ratios did not have a significant effect on downregulation of MDR-1 expression.

Following the preparation of these particles uptake and transfection, studies showed efficient uptake and transfection with both the NP systems showing similar decrease in gene expression in both SKOV-3WT and SKOV-3TR cell lines. Synergistic activity of dual siRNA therapy showed minimal effect on SKOV-3WT but had a significant effect on IC50 of paclitaxel in SKOV-3TR. In SKOV-3WT cells, as there is a low level of basal MDR-1, the cells would be sensitive to paclitaxel thus HA-siMDR-1 expression would have minimal effect on cytotoxicity. In contrast, SKOV-3TR cells have an increased MDR-1 expression; thus, single therapy with siMDR-1 would affect cytotoxicity. In addition, combination therapy with dual siRNA showed a further improvement in cytotoxicity, which could be attributed to synergistic effect of siMDR-1 on P-gp expression and PKM-2 on the glycolytic pathway. A minimal effect on cytotoxicity was observed with both the NP delivery systems, which could be attributed to similar uptake profile of these delivery systems in vitro.

In vivo biodistribution studies indicated a high degree of accumulation of the NPs in the liver, kidney, and spleen because of preferential uptake of these particles by macrophages residing in these tissues. Although a small proportion of the total siRNA dose was detected at the tumor site, in vivo knockdown studies showed a significant downregulation of gene expression. This downregulation translated to a small improvement in antitumor efficacy following combination therapy of dual siRNA and paclitaxel with minimal toxicity.

After in vivo studies in SKOV-3WT tumor-bearing models, the efficacy of the delivery systems was assessed in the in vivo paclitaxel resistant model. This model was developed over 6 weeks of therapy and MDR-1 expression was determined. As a significant difference in an increase in MDR-1 expression was not observed between 4 and 6 weeks after paclitaxel treatment, we initiated in vivo studies in TR model within 4 weeks of therapy. Post-model development and dosing with NP with a similar regimen as was adopted in the WT model there was an improvement in antitumor efficacy with siRNA-based therapy. Dual siRNA-based approach further augmented the antitumor effect possibly because of synergistic effect of siMDR-1 on P-gp expression, leading to increased cellular paclitaxel concentration and the effect of siPKM-2 on the glycolysis pathway affecting cancer cell metabolism. Thus, combination of siRNA with cytotoxic agents provided synergistic effect and enhanced antitumor efficacy because of its actions on different resistance mechanism in cancer.

Conclusions

In summary, anticancer therapeutics employing RNAi mechanism holds promise for sequence-specific silencing of target genes. However, targeted delivery to tumor sites remains a major hurdle for cancer therapy. Thus, in this study, we evaluated encapsulation and delivery of siMDR-1 and siPKM-2 in HA-PEI–based self-assembling nanosystems for the treatment of WT and resistant ovarian cancer. The nano-assemblies showed effective transfection and improved cytotoxic effect of paclitaxel in cancer cells. Although the effect was not pronounced in WT animal models, dual therapy provided action of these therapeutic agents on multiple pathways in cancer cells providing synergistic activity for cancer therapy. Although an in vivo paclitaxel resistance model was developed to assess the effectiveness of our therapies, future studies in paclitaxel resistance model development in orthotopic or genetically modified ovarian cancer mice would indicate further clinical relevance of these therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Talekar, M.S. Goldberg, M.M. Amiji
Development of methodology: M. Talekar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Talekar
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Talekar, Q. Ouyang
Writing, review, and/or revision of the manuscript: M. Talekar, Q. Ouyang, M.S. Goldberg, M.M. Amiji
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Talekar
Study supervision: M. Talekar, M.S. Goldberg, M.M. Amiji

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