Published in final edited form as:

Pharm Res. 2015 June ; 32(6): 2097–2109. doi:10.1007/s11095-014-1602-1.

Cluster of Differentiation 44 Targeted Hyaluronic Acid Based Nanoparticles for *MDR1* siRNA Delivery to Overcome Drug Resistance in Ovarian Cancer

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Abstract

Purpose—Approaches for the synthesis of biomaterials to facilitate the delivery of “*biologics*” is a major area of research in cancer therapy. Here we designed and characterized a hyaluronic acid (HA) based self-assembling nanoparticles that can target CD44 receptors overexpressed on multidrug resistance (MDR) ovarian cancer. The nanoparticle system is composed of HA-poly(ethyleneimine)/HA-poly(ethylene glycol) (HA-PEI/HA-PEG) designed to deliver *MDR1* siRNA for the treatment of MDR in an ovarian cancer model.

Methods—HA-PEI/HA-PEG nanoparticles were synthesized and characterized, then the cellular uptake and knockdown efficiency of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles was further determined. A human xenograft MDR ovarian cancer model was established to evaluate the effects of the combination of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles and paclitaxel on MDR tumor growth.

Results—Our results demonstrated that HA-PEI/HA-PEG nanoparticles successfully targeted CD44 and delivered *MDR1* siRNA into OVCAR8TR (established paclitaxel resistant) tumors. Additionally, HA-PEI/HA-PEG nanoparticles loaded with *MDR1* siRNA efficiently down-regulated the expression of *MDR1* and P-glycoprotein (Pgp), inhibited the functional activity of Pgp, and subsequently increased cell sensitivity to paclitaxel. HA-PEI/HA-PEG/*MDR1* siRNA nanoparticle therapy followed by paclitaxel treatment inhibited tumor growth in MDR ovarian cancer mouse models.

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Conclusions—These findings suggest that this CD44 targeted HA-PEI/HA-PEG nanoparticle platform may be a clinically relevant gene delivery system for systemic siRNA-based anticancer therapeutics for the treatment of MDR cancers.

Keywords

HA-PEI/HA-PEG nanoparticle; multidrug resistance; siRNA delivery; ovarian cancer; CD44 targeting

INTRODUCTION

The synthesis of biodegradable and nontoxic materials for targeted and controlled drug delivery is an important area of research for designing effective therapy against challenging human diseases such as cancer. Classical, drugs are small chemical entities but in recent years, biological macromolecules have been applied as therapeutics and a new class of drugs has emerged; “biologics”. Biologics include small interfering RNA (siRNA), micro RNA (miRNA), antibodies and proteins; challenges with delivery of these highly effective but labile molecules has led to a complete shift in the design objectives of advanced drug delivery systems. Polymeric materials have been extensively explored for drug delivery applications due to advantages such as biocompatibility, tunable chain length and properties, better control over release kinetics and ability to impart stimuli-responsive characteristics (1, 2). The traditional approach of material synthesis for delivery applications involves considering numerous factors such as polymer properties, synthesis parameters and *in vivo* interactions, which often cumbersome, time consuming, expensive and sometimes non-productive. Taking cue from conventional drug discovery paradigms, a combinatorial approach of materials synthesis on a precursor molecule to develop a library of derivatives with varying physicochemical properties and biological behaviors has been accepted as a rationalized way for designing and optimizing delivery vectors. Researchers employing combinatorial design for material synthesis for drug delivery applications seek promising polymeric backbones that can be exploited to design functionally variant building blocks of the delivery system such that the final product gives the desired properties and performance. An ideal precursor polymer selected as a combinatorial backbone should have multiple qualities such as biocompatibility, non-immunogenicity, non-inflammatory behavior, ease of chemical modification, availability of multiple moieties for chemical reaction and if possible, an inherent capability for targeting specific cell types. It is due to these stringent criteria that very few promising candidates have been selected for further studies (3).

In this regard, hyaluronic acid (HA) is a promising high molecular weight anionic biopolymer composed of β 1,3 N-acetyl glucosaminyl- β 1,4 glucuronide repeats units, that is present in the human body primarily in the extracellular matrix. It has a diverse biological role such as tissue support, metabolite transport, cell migration and differentiation (4). There is contrasting evidence on role of naturally occurring high molecular weight HA in cancer, but the low-molecular weight derivatives have been extensively studied for different biomedical applications including drug delivery and tissue engineering primarily due to their biodegradability, non-immunogenicity and non-toxic profile (5, 6). Additionally, HA polymers have abundant functional sites such as –COOH and –OH groups that allow for the

synthesis of engineered derivatives that could be amenable for self-assembly, with the potential to efficiently encapsulate and deliver siRNAs (7). Most importantly, HA has been studied as a targeting moiety on the surface of nanoparticles because it can be efficiently taken up by cells through cluster of differentiation 44 (CD44)-mediated endocytosis (8, 9) (10). CD44, a family of cell-surface glycoproteins, contains a HA binding site in their extracellular domain, and therefore serves as the key cell surface receptor for HA. Coincidentally, it has been reported that CD44 is highly expressed in ovarian cancer among other cancers, and its expression level was correlated with drug resistance during ovarian cancer metastasis (11-13). Co-overexpression of CD44 and Pgp has been proven to associate with ovarian cancer progression (12). Therefore, in the present study, we chose ovarian cancer as the model disease for a HA based self-assembling nanoparticle system targeting CD44 receptors.

Ovarian cancer is the fifth most lethal cancer and the leading cause of gynecological cancer deaths among women in the United States. The combination of paclitaxel and carboplatin chemotherapy following aggressive surgical cytoreduction currently defines the standard treatment for this malignancy. Although this therapy generates desirable tumor cytoreduction, remission rates, and multi-year survival statistics, cure of this malignancy remains uncommon (14, 15). A majority of tumors initially are sensitive to chemotherapy agents, but develop resistance to the same or a variety of functionally and structurally unrelated anticancer drugs (14). Hence, the development of multidrug resistance (MDR) is a fundamental obstacle in ovarian cancer chemotherapy (16, 17). The mechanisms of the development of MDR have been widely studied for several decades since the initial use of chemotherapy to treat cancer (18). Overexpression of ATP-binding cassette (ABC) transporter P-glycoprotein (Pgp) is one of the best-known MDR mechanisms (19, 20). Pgp is the protein product of multidrug resistance gene 1 (*MDR1*), and overexpression of *MDR1* and corresponding Pgp has been found in numerous MDR ovarian cancer cell lines and MDR ovarian cancer tissues (21, 22).

Strategies for reversing MDR have been studied extensively in recent years in order to improve the outcome of chemotherapy. In this regard, one of the innovative approaches is to inhibit *MDR1* mRNA expression by *MDR1* siRNA using RNA interference (RNAi) technology. Previously, we and others found that knocking down the expression level of Pgp by transfection of *MDR1* siRNA with lipofectmine or shRNA with a lentivirus restores sensitivity to paclitaxel and other chemotherapy agents in MDR cells (23-26). However, there are multiple barriers to the effective systemic delivery of functional siRNA. Unmodified siRNA cannot readily cross biomembranes, which leads to poor intracellular uptake. Moreover, siRNA also has limited stability in the bloodstream and can be immunogenic (27). Although viral vectors can serve as an efficient siRNA delivery material, safety concerns, apparent toxicity and other negative public perceptions of these vectors currently limit their further development in clinic trials (28). Therefore, to allow siRNA as a clinically-viable therapeutic for treating cancer patients, more effective, efficient and safe systemic delivery of siRNA into target tissues (such as tumors) and organs is needed (29). In recent years, nanoparticles have been shown to be one of the promising siRNA delivery vehicles based on their unique physical and biological properties (28, 30, 31). Moreover,

nanoparticles have been proven to be a potential platform to overcome MDR by delivery of multiple payloads such as siRNAs, chemotherapeutic agents, ABC transport regulators either alone or in combination with a classical or experimental drug (siRNA/drug combination) into cells (32-35).

We report inherently CD44 targeting, self-assembling polyethyleneimine and polyethylene glycol (HA-PEI and HA-PEG respectively) derivatives of HA as polymeric delivery vectors for siRNA. The primary objective of this study was to deliver *MDR1* siRNA and therefore block *MDR1* gene expression and further study the system in combination with paclitaxel treatment in MDR ovarian cancer *in vitro* as well as *in vivo*.

MATERIALS AND METHODS

Materials

HA with an average molecular weight of 20 kDa was purchased from Lifecore Biomedical Co. (Chaska, MN). PEI (MW10 kDa) was purchased from Polysciences Inc. (Warrington, PA). Mono-functional PEG amine (mPEG2k-NH₂, MW 2 kDa) was obtained from Creative PEG Works (Winston Salem, NC). All the fatty amines and other reagents were purchased from Acros Organics (Thermo Fisher, Pittsburgh, PA) or Sigma Aldrich (Milwaukee, WI) at high purity (>99%) and were used without further purification.

Synthesis of HA-PEI and HA-PEG Polymers

HA was chemically conjugated with PEI by using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). For the preparation of HA-PEG polymer, HA was chemically modified with poly(ethylene glycol amine) (PEG₂₀₀₀-NH₂) using EDC and *N*-hydroxy sulfo succinimide (sulfo-NHS). Detailed synthesis and purification protocols have been included in the Supplementary Materials. 3 mg of the purified and lyophilized HA-PEI or HA-PEG was dissolved in 600 μ L of D₂O and characterized by 400 MHz ¹H NMR spectroscopy (Varian Inc., CA) for determining the percent modification of the HA backbone.

HA-PEI/HA-PEG/*MDR1* siRNA Nanoparticle Formulation and Characteristics

The purified HA-PEI and HA-PEG conjugates (1:1 w/w) were incubated at room temperature for 5 min to form self-assembled nanoparticles (HA-PEI/HA-PEG). We followed a protocol based on our previous research that demonstrated that highest gene silencing efficiency was observed when siRNA was encapsulated in HA-PEI/HA-PEG at a mass ratio of 54:1 (polymer : siRNA). Briefly, *MDR1* siRNA was encapsulated in HA-PEI/HA-PEG at a mass ratio of 54:1 (polymer : siRNA) and incubated for another 15 min at room temperature (7). The particle size and zeta potential measurements of the *MDR1* siRNA loaded HA nanoparticles were performed with a Zetasizer Nano S Instrument (Malvern Instruments Ltd, Worcestershire, UK). Transmission electron micrographs (TEM) of nanoparticles were assessed using a JEOL JEM-1000 instrument (JEOL Ltd, Tokyo, Japan).

Cell culture, Reagents and Human Ovarian Cancer Tissues

The MDR ovarian cancer cell line OVCAR8TR was established previously (26, 36) and was routinely cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies) in 5% CO₂-95% air atmosphere at 37°C. The fluorescent labeled 3'-AlexaFluor488 (AF488)-*MDR1* siRNA (target sequences: 5'-GAGCTTAACACCCGACTTACATT-3', green color) was purchased from QIAGEN GmbH (Germany). The unlabeled *MDR1* siRNA, non-specific siRNA and Lipofectamine® RNAiMAX (Invitrogen) were purchased from Life Technologies Corp (Carlsbad, CA). The mouse anti-human Pgp monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO). The mouse anti-human CD44 monoclonal antibody was acquired from Cell Signaling Technology (Beverly, MA). Paclitaxel was obtained from the pharmacy at the Massachusetts General Hospital. Ovarian cancer tissue samples (OST1–OST6) were acquired from the Massachusetts General Hospital ovarian cancer tissue bank (Boston, MA) and were used in accordance with the policies of the institutional review board of the hospital. The diagnoses were confirmed histologically.

Determination of HA-PEI/HA-PEG/*MDR1* siRNA Nanoparticles Cellular Uptake

OVCAR8TR cells were seeded at a density of 6×10^4 cells/well in 24 well plates. On the following day, the transfection was performed. Briefly, the same mass of HA-PEI and HA-PEG were incubated together at room temperature for 5 min, and *MDR1* siRNA was then encapsulated in HA-PEI/HA-PEG at a mass ratio of 54:1 (polymer : siRNA). After incubation for another 15 min at room temperature, HA-PEI/HA-PEG/*MDR1* siRNA were forward transfected into OVCAR8TR cells. 90 nM AF488-*MDR1* siRNA encapsulated in HA-based nanoparticles or mixed with Lipofectamine®RNAiMAX were added into each well respectively. The cells treated with 90 nM AF488-*MDR1* siRNA alone were employed as a negative control. The efficacy of cellular uptake of *MDR1* siRNA was examined at 6h post transfection. Prior to microscopy, the nuclei were stained with 1 μ g/mL Hoechst 33342 (Life Technologies) for 1 min. Then the cells were washed with PBS and visualized on a Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments, Inc, Melville, NY) equipped with a SPOT RT™ digital camera (Diagnostic Instruments, Inc, Sterling Heights, MI).

Real-time PCR Assay

Real-time PCR was adapted to verify the expression level of *MDR1* mRNA in OVCAR8TR cells transfected with *MDR1* siRNA. The full details are presented in the Supplementary Materials. The Comparative CT method was used to analyze the relative *mdr1* mRNA expression level. Expression of β -actin was used as an internal control.

Western Blot Analysis

To evaluate the expression level of Pgp and CD44 in OVCAR8TR cells, total proteins were extracted with 1X RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA). After assessing proteins concentration using the DC Protein Assay (Bio-Rad, Hercules, USA), western blot assay was performed. The specific full details are in the Supplementary Materials.

Immunofluorescence Assay

OVCAR8TR cells transfected with *MDR1* siRNA were grown in 24-well plate and an immunofluorescence (IF) assay was conducted 48 h post transfection as previously described (37); complete details are described in the Supplementary Materials.

Calcein AM Retention Assay

The Vybrant™ multi-drug resistance assay kit (Invitrogen/Molecular Probes) was used to measure the accumulation level of Calcein AM, which is a substrate of Pgp. The experimental details are supplied in the Supplementary Materials.

Cytotoxicity Assay

Cytotoxicity of paclitaxel in OVCAR8TR cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA was assessed by MTT (methyl thiazolyl tetrazolium) assay. The complete experimental details are described in the Supplementary Materials.

Tumor Xenograft Model and Treatment

To demonstrate the potential of *MDR1* siRNA delivered by HA-PEI/HA-PEG nanoparticles to knock down gene expression and inhibit tumor growth *in vivo*, animal procedures were carried out according to the protocol approved by Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC). The Crl:SHO-Prkdc^{SCID}Hr^{hr} nude female mice at approximately 3–4 weeks of age were purchased from the Charles River Laboratories (Ann Arbor, MI). For the human xenograft MDR ovarian cancer model, 5×10^6 OVCAR8TR cells in log phase were suspended in 1:1 (v/v) mixture of culture medium and Matrigel (BD Biosciences, San Jose, CA), and then injected subcutaneously into nude female mice. When the ovarian tumors were approximately 200 mm³ in size, they were randomized into 4 groups to start treatment designated as day 1 treatment. Group one received HA-PEI/HA-PEG nanoparticles encapsulated with *MDR1* siRNA by tail vein injection at a dose of 0.5 mg/kg every day for 3 consecutive days, group two was treated with nonspecific siRNA encapsulated in HA-PEI/HA-PEG nanoparticles at 0.5 mg/kg for 3 consecutive days, group three was treated with *MDR1* siRNA alone, and group four received saline alone for 3 consecutive days. All four groups were followed with intraperitoneal paclitaxel (20 mg/kg) treatment twice a week for two weeks commencing on the fifth day. The treatment scheme is shown in Fig. 6a. Tumor volumes were measured twice a week with a digital caliper. Tumor volume (mm³) was calculated as $(W^2 \times L)/2$, where W is width and L is length. The relative tumor volume used in this study was calculated for each single tumor by dividing the tumor volume on day X by that on day 1 (time of treatment starting).

Statistics

Statistical analysis was done using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Data were given as the mean $\pm$ SEM. Student's t-test was used to compare between groups. $P < 0.05$ value was considered as statistically significant.

RESULTS

Characterization of HA-PEI/HA-PEG Nanoparticle Encapsulated with *MDR1* siRNA

The HA-PEI and HA-PEG derivatives were characterized by ^1H NMR to confirm 11% substitution of the carboxyl group with PEI in HA-PEI and 7.6% substitution with m-PEG₂₀₀₀ in HA-PEG (Fig. S1). A detailed discussion of the NMR calculations can be found in the supporting information. The HA-PEI and HA-PEG were blended in a 1:1 (w/w) ratio to allow self-assembly in the presence of *MDR1* siRNA for nanoparticle formation. The particle size and zeta potentials of HA-PEI/HA-PEG encapsulated with *MDR1* siRNA were measured using a Zetasizer Nano-S® instrument. The Z-average particle size of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles was 173.3 ± 13.7 nm (Fig. 1a and d). Despite the presence of positively charged PEI species, the PEI/HA-PEG/*MDR1* siRNA nanoparticle surface was negatively charged. The zeta potential of nanoparticles was -22.5 ± 0.44 mV (Fig. 1b and d). Interestingly, the particle sizes of HA-PEI/HA-PEG nanoparticle decreased once the *MDR1* siRNA was loaded, which is due to the siRNA complexation with PEI resulting in tighter self-assembly and thus a more compact nanostructure (Fig. 1d). As shown in Fig. 1c, there was high contrast in the core of nanoparticles, which may be attributed to the uranyl acetate stained *MDR1* siRNA loaded in the core of the HA-PEI/HA-PEG nanoparticles. TEM visualization confirms that HA-PEI/HA-PEG systems self-assemble like a core shell structure where siRNA concentrates in the core of the particles and is therefore well protected from the outside environment.

The safety of nanocarriers is one of the major issues in clinical application. Therefore, the cytotoxicity of different polymers conjugates in OVCAR8TR cells was evaluated by the methyl MTT assay prior to gene silencing tests. To maintain an incubation time consistent with transfection, the cells were seeded in 96-well plates and incubated with polymers for 6 h on the following day. Subsequently, the cells were washed and incubated with fresh media for another 48 h and the MTT assay was conducted. The results suggested that the HA-PEG polymer had significantly less cytotoxicity than HA-PEI, and the cytotoxicity of HA-PEI declined when mixed with HA-PEG. Most importantly, the cytotoxicity of HA-PEI/HA-PEG further decreased once the *MDR1* siRNA was encapsulated where 90% cells remained viable after HA-PEI/HA-PEG/*MDR1* siRNA treatment at the concentration of 600 $\mu\text{g/mL}$. However, the highest concentration of HA-PEI/HA-PEG used for transfection in the current study was 170 $\mu\text{g/mL}$. This finding indicated that the cells could tolerate HA-PEI/HA-PEG/*MDR1* siRNA well during transfection (Supplementary Fig. S2).

Expression Level of CD44 and Pgp in Ovarian Cancer

CD44 is known to be involved in a variety of biological activities in ovarian cancer such as receptor redistribution, cell invasion and migration, tumor metastasis and the development of drug resistance (13). HA has a high affinity and specificity for CD44 receptors, which makes it a promising candidate for the intracellular delivery of various therapeutic agents and nucleic acid. Therefore, to effectively deliver *MDR1* siRNA to the tumor site using HA based nanoparticles, it is pertinent that the target tumor cells express CD44. So we first determined the expression level of CD44 in parental and MDR ovarian cancer cell lines. The western blot result demonstrated that CD44 could be detected in both sensitive human

ovarian cancer OVCAR8 cells, and paclitaxel-resistant OVCAR8TR cells (Fig. 2a). Relative expression levels of CD44 were analyzed by densitometry and the results were shown in Fig. 2b. No statistical difference in CD44 expression was observed between the sensitive and paclitaxel-resistant cells. CD44 expression exhibiting in ovarian cancer cells was further demonstrated by IF. CD44+ staining could be observed in both sensitive and resistant cells with no obvious differences (Fig. 2c). We found that CD44 was highly expressed in both drug sensitive cells and drug resistant cells. In addition, we also evaluated the CD44 expression in human ovarian cancer tissues derived from patients. As shown in Fig. 2d, human ovarian cancer samples stained positive for CD44 expression confirming the overexpression of CD44 on their surface. Additionally, consistent with previous studies that report overexpression of Pgp as one of the mechanisms of MDR (26), only paclitaxel-resistant OVCAR8TR cells show high expression of Pgp as determined by western blot as well as IF (Fig. 2a and c).

Cellular Uptake of *MDR1* siRNA Loaded HA-PEI/HA-PEG Nanoparticles

To evaluate the delivery efficacy of *MDR1* siRNA using HA-based nanoparticles, the cellular uptake of HA-PEI/HA-PEG nanoparticles loaded with *MDR1* siRNA was measured. *MDR1* siRNA was labeled with Alexa-AF488 and was used to study intracellular uptake characteristics of the nanoparticles. The results show that HA-PEI/HA-PEG nanoparticles could efficiently deliver AF488-*MDR1* siRNA into OVCAR8TR cells confirming the high transfection efficiency of the nanoparticles (Fig. 3).

MDR1 mRNA Expression in Cells Transfected with HA-PEI/HA-PEG/*MDR1* siRNA Nanoparticles

After we confirmed that *MDR1* siRNA can be efficiently delivered to OVCAR8TR cells, the expression level of *MDR1* gene was further evaluated to determine whether the delivered *MDR1* siRNA was successfully released from the nanoparticles and was able to retain its functional activity of knocking down the expression of *MDR1* *in vitro*. The results of real-time PCR revealed that the expression level of *MDR1* significantly declined in OVCAR8TR cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles. On the other hand, the cells incubated either with *MDR1* siRNA alone or HA-PEI/HA-PEG loaded with non-specific siRNA did not change the expression levels of *MDR1*. Moreover, the decrease in *MDR1* expression levels was correlated with the dosage of *MDR1* siRNA loaded nanoparticles (Fig. 4a). Assessment of gene knockdown at different time points indicated that *MDR1* siRNA delivered by HA-PEI/HA-PEG nanoparticles in OVCAR8TR cells achieved maximum activity at 24 h post-transfection, and this effect could be maintained for at least 120 h (Fig. 4b).

Suppression Efficacy of Pgp Using *MDR1* siRNA Loaded HA-PEI/HA-PEG Nanoparticles

Pgp is the protein product of *MDR1* gene and inhibiting the overexpression of Pgp by *MDR1* siRNA has been demonstrated as a promising approach to overcome MDR in ovarian cancer (24). In the present study, HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles transfected cells showed a significant decrease in Pgp expression and western blot analysis revealed that this inhibition was dose-dependent (Fig. 5a). There was no change in Pgp expression level of

cells transfected with HA-PEI/HA-PEG/non-specific siRNA nanoparticles and with *MDR1* siRNA alone. Moreover, evaluation of Pgp expression levels at different time points demonstrated that HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles achieved the suppression of Pgp at 24 h after transfection and were able to suppress Pgp expression for at least 120 h (Fig. 5b). Thus, by evaluating both the mRNA and protein level of the target gene *in vitro*, the present work further reinforces the potential utility of HA-PEI/HA-PEG nanoparticles for effective siRNA delivery.

Others work (including our own) has previously demonstrated that HA-PEI could successfully deliver siRNA into cells (38, 39), therefore the difference in transfection efficiency between HA-PEI and HA-PEI/HA-PEG was evaluated. *MDR1* siRNA delivered by HA-PEI nanoparticles alone also down-regulated Pgp expression. However, HA-PEI/HA-PEG could deliver siRNA more efficiently and displayed higher Pgp knockdown compared to HA-PEI/*MDR1* siRNA nanoparticles in OVCAR8TR cells. It is interesting to note that HA-PEI showed gene silencing at the mass ratio of 54:1 (polymer: siRNA), but failed to demonstrate activity at the ratio of 27:1, which is consistence with previous results(7) (Fig. 5c).

It is well-known that Pgp acts as an energy-dependent drug efflux pump to expel structurally unrelated chemotherapeutic agents from tumor cells and the overexpression of Pgp causes decreased drug uptake. Reversal of MDR is usually the results of an increased intracellular accumulation of chemotherapeutics, and can be achieved by regulating Pgp-mediated drug uptake/efflux. Therefore, the effects of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles on the uptake and efflux of the Pgp substrate, calcein AM, was determined. Cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA showed significantly increased calcein AM retention (Fig. 5d). Increased intracellular retention of chemotherapeutics can increase cell sensitivity to chemotherapeutics agents and overcome drug resistance. Thus, the IC₅₀ of paclitaxel was next assessed to further identify the effects of HA-PEI/PEG/*MDR1* siRNA nanoparticles on drug resistance. The MTT assay results indicated that the IC₅₀ of paclitaxel in OVCAR8TR cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles was dramatically decreased compared with that of untreated cells ($1.276 \pm 0.057 \mu\text{M/mL}$ vs. $0.288 \pm 0.008 \mu\text{M/mL}$, $P < 0.05$, Fig. 5e). In addition, compared with untreated cells, the IC₅₀ of paclitaxel in OVCAR8TR cells transfected with HA-PEI/HA-PEG/non-specific siRNA nanoparticles and *MDR1* siRNA alone were unchanged ($1.144 \pm 0.041 \mu\text{M/mL}$ and $1.128 \pm 0.082 \mu\text{M/mL}$, respectively, Fig. 5e).

Effects of the Combination of HA-PEI/HA-PEG/*MDR1* siRNA Treatment and Paclitaxel on the Growth of Resistant Ovarian Cancer Tumors

To further support the biological significance of the findings *in vitro* and evaluate the antitumor efficacy of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles *in vivo*, MDR ovarian cancer cells OVAR8TR were grown as xenografts in nude mice. The treatment scheme is shown in Fig. 6a. The results revealed that saline alone, *MDR1* siRNA alone or HA-PEI/HA-PEG/non-specific siRNA nanoparticles followed with paclitaxel treatment had no obvious beneficial effects on MDR ovarian cancer suppression in xenograft model. In contrast, injection with HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles followed by

paclitaxel treatment produced a significant inhibitory effect on the resistant tumor growth as compared with control groups (Fig. 6b). Representative images of tumors excised from one mouse in each group were shown in Fig. 6c. Additionally, on the basis of animal weight and mortality, no considerable toxicity was observed and the animals appeared to have tolerated HA-PEI/HA-PEG nanoparticles well in all the treatments regimens (Fig. 6d).

DISCUSSION

In the current study, we demonstrated that HA-PEI/HA-PEG nanoparticles can successfully delivery *MDR1* siRNA into MDR ovarian cancer cells. The cells incubated with HA-PEI/HA-PEG/*MDR1* siRNA displayed significantly decreased *MDR1* mRNA and corresponding declined Pgp expression level in a dose dependent manner. The increased intercellular enhancement of Pgp substrate and tumor cell sensitivity to paclitaxel further confirmed efficient delivery of *MDR1* siRNA into cells using the HA-PEI/HA-PEG nanosystem. Importantly, the current study revealed that administering the combination of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles and paclitaxel produced a significant inhibitory effect on MDR tumor growth in MDR ovarian cancer mouse models. We demonstrated for the first time the impact of siRNA loaded HA-PEI/HA-PEG nanoparticles in overcoming MDR in ovarian cancer.

RNAi based antitumor therapeutics holds great promise for treating MDR cancers in the clinic. However, there are very few non-viral vectors that are currently being investigated for systemic delivery. The aim of this work was to develop a safe and effective translatable nanotechnology based system for delivering *MDR1* siRNA in conjunction with paclitaxel chemotherapy for systemic use. There are several groups that report the use of targeting moieties decorated on nanoparticle surface that can engage specific markers on tumor cells and tissues. We designed modified HA derivatives that not only have the inherent ability to target CD44 receptor expressing tumors but also perform the function of encapsulating siRNA payloads and delivering them to solid tumors (7). siRNA delivered by HA-PEI/HA-PEG nanoparticles may cause higher targeted gene and protein down-regulation efficiency *in vivo* than conventional Lipofectamine. Several publications have shown that siRNA loaded with HA-PEI/HA-PEG nanoparticles could successfully lead to decreased target mRNA expression *in vivo* (6, 7). Lipofectamine is a common transfection reagent used in molecular and cellular biology. Although Lipofectamine has been successfully used to deliver siRNA *in vitro*, however, Lipofectamine alone will not mediate high down-regulation *in vivo* experiments like that in the cultured cells, and Lipofectamine-mediated cellular toxicity has prevented its use *in vivo*. It has been proved that they induced oxygen free radical-mediated toxicity and some Lipofectamine formulations elicit inadvertent gene expression and enhance the immune response to siRNA *in vitro* (40). Therefore, more recently, modified Lipofectamine, InvivoFectamine® 2.0 Reagent has been manufactured as the latest innovation for *in vivo* siRNA delivery from Life Technologies.

HA can innately recognize the specific cellular receptor CD44 that is overexpressed on numerous cancer cells; increasing studies have investigated the targeted delivery of drugs and nucleic acid to specific sites by HA based carriers for the development of anticancer therapeutics. It has been validated that HA-based nanosystems have the potential to target

siRNA and chemotherapeutics agents when applied as a delivery vehicle (5, 9, 13, 41). Report suggested doxorubicin loaded HA-PEG-PCL nanoparticles showed significant higher concentrations of doxorubicin at tumor sites and significantly inhibited tumor growth in comparison with pure doxorubicin and non-HA-containing doxorubicin-loaded MPEG-PCL nanoparticles (5). siRNA/PEI-HA complexes could be successfully taken up by B16F cells, and a PGL3-Luc gene silencing effect can be achieved when cells were transfected with anti-PGL-Luc siRNA/PEI-HA complexes (38, 39). Cyclooxygenase-2 (COX-2) siRNA encapsulated in hydrophobized HA-spermine conjugate (HHSC) could be taken up by gastric cancer SGC-7901 cells and subsequently down-regulated COX-2 gene expression (42). HA-decorated PLGA-PEG nanoparticles were constructed for the targeted delivery of SN-38 to ovarian cancer (9). PEG conjugated HA nanoparticles, P-HA-NPs, were used to deliver doxorubicin into breast cancer MDA-MBP231 cells (41). The present study provided another ideal HA-based nanosystem HA-PEI/HA-PEG to delivery siRNA.

Since the negative charge of HA makes it is difficult to encapsulate negatively charged siRNA, the cationic polyamine polymer PEI was conjugated with HA to efficiently deliver siRNA. PEI can condense siRNA to form PEI/siRNA complexes through electrostatic interactions between phosphate groups of the negatively charged siRNA and the abundant amine residues on the polycation PEI. It is also proposed that the PEI/siRNA complexes have the ability to protect siRNA from nuclease degradation, and to allow siRNA to escape from the lysosome due to the proton-sponge-effect of PEI. PEI/siRNA complexes can also release siRNA from the carriers (43, 44). However, the cationic nature of PEI is the cause of concern in terms of its cytotoxicity and rapid clearance from the blood stream (45). Therefore, conjugating PEI with HA was undertaken, which not only reduce the cytotoxic effects of PEI through electrostatic neutralization of its positive charge by negatively charged HA but also contributes to the formation of a protective hydrophilic surface (38). The HA-PEI derivative could complex with siRNA forming nanostructures in a core-shell architecture. In addition, we synthesized and used a HA-PEG derivative in the multi-component HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles that affords non-immunogenicity and stealth characteristics to the nanoparticles along with tumor targeting ability (Supplementary Fig. S3). Our data indicated that the HA-PEG polymers in our nanosystem could decrease the cytotoxicity and enhance the transfection efficiency of nanoparticles. It has been proven that PEGylation of cationic polymers can greatly ameliorate the problems of cytotoxicity, inter-particle aggregation, non-specific protein adsorption (46, 47). Previous report showed that HA-PEI/HA-PEG could achieve higher target mRNA knockdown than HA-PEI polymer in lung cancer xenografts (7). PEG on the surface of nanoparticles effectively suppresses liver uptake of HA based nanoparticles, and enhances the circulation time in the blood, subsequently leading to targeted accumulation of nanoparticles at tumor sites (41, 47). Collectively, HA allows specifically targeting of OVCAR8TR cells overexpressing CD44 and Pgp, PEG-modification prolong circulation time in the blood, and PEI-mediated *MDR1* siRNA encapsulation may contribute to the inhibitory effect of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles on MDR ovarian cancer tumor growth.

MDR severely limits the effectiveness of chemotherapy and contributes to the poor overall long term survival rates in ovarian cancers (48). Overexpression of *MDR1* and Pgp is one of the best-characterized MDR mechanisms and Pgp may have some predictive value for the clinical outcome of patients with advanced ovarian cancer (49). CD44 makes HA as promising candidate for targeting the intracellular delivery of various therapeutic agents and nucleic acid. It has been previously reported that the expression level of CD44 mRNA is significantly increased in tissues derived from primary and metastatic ovarian cancer when compared with normal ovarian tissues of the same patient (12, 13). Moreover, it has been confirmed that co-expression of CD44 and Pgp strongly correlates with epithelial ovarian cancer progression (12). Our results also revealed that co-expressed CD44 and Pgp could be detected in both MDR ovarian cancer cells, which is one of the important theoretical foundations of using HA based nanoparticles to knockdown *MDR1* siRNA in MDR ovarian cancer. The clinical application of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles in the treatment of ovarian cancer may improve the outcome of ovarian cancer patients with poor prognosis due to the development of MDR. However, siRNA delivery is a multi-step process which needs multi-functional nanoparticles to go through each step, and systemic delivery within human is much more complex than the challenges of biologic delivery within the mouse (46, 50). To reach the standard requirements for clinical use, some other unexpected barriers may be required to overcome.

CONCLUSION

HA-PEI/HA-PEG nanoparticles can successfully delivery siRNA into ovarian cancer cells. Decreased expression level of target gene can be observed and tumor growth inhibition can be achieved in HA-PEI/HA-PEG nanoparticle treated groups. These findings suggest that this CD44 targeted HA-PEI/HA-PEG nanoparticle platform is a promising vector for systemic siRNA delivery for treating MDR cancers and may be a viable solution on the challenge of biologic drug delivery..

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS AND DISCLOSURES

We thank Dr. Meghna Talekar for measurement of the particle size and surface charge. Dr. Yang is supported by Scholarship from China Scholarship Council. This study is supported by the NIH/NCI, Cancer Nanotechnology Platform Partnership (CNPP) grants U01- CA151452.

ABBREVIATIONS

CD44	Cluster of differentiation 44
HA	Hyaluronic acid
HA-PEI	HA-poly(ethyleneimine)
HA-PEG	HA-poly(ethylene glycol)

Pgp	P-glycoprotein
siRNA	Small interfering RNA
MDR	Multidrug resistance
ABC	ATP-binding cassette
MDR1	Multidrug resistance gene 1
RNAi	RNA interference
TEM	Transmission electron micrographs
IF	Immunofluorescence
MTT	Methyl thiazolyl tetrazolium

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Fig. 1. Characteristics of HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles. (a) The particle size and (b) zeta potentials of HA-PEI/HA-PEG encapsulated with *MDR1* siRNA. (c) Representative TEM image showing HA-PEI nanoparticles loaded with *MDR1* siRNA. (d) A table showing the average size $\pm$ S.D. and average zeta potential $\pm$ S.D. of HA-PEI alone, HA-PEI/HA-PEG (1:1 w/w) and HA-PEI/HA-PEG (1:1 w/w) in the presence of *MDR1* siRNA. All measurements were performed using independent triplicates of the formulation.

Fig. 2.

The expression level of CD44 and Pgp in ovarian cancer. **(a)** Western blot analysis confirming CD44 expression in sensitive cell line OVCAR8 as well as resistant cell line OVCAR8TR. However, only resistant cell lines OVCAR8TR expressed Pgp. **(b)** Relative expression of CD44 and Pgp from a) as analyzed by densitometry. Data are presented as means $\pm$ SD and analyzed using Student's t-test. Statistical comparisons between OVCAR8TR and OVCAR8 cells are presented: *** $P < 0.001$. **(c)** The IF imaging further corroborated that CD44 staining could be observed in both sensitive and resistant cells, while Pgp staining could be detected only in resistant cells. **(d)** CD44 staining was exhibited in human ovarian cancer samples.

Fig. 3.

Intracellular uptake of *MDR1* siRNA loaded in HA-PEI/HA-PEG nanoparticles.

Fluorescence microscopic analysis revealed HA-PEI/HA-PEG nanoparticles could successfully transfer AF488-*MDR1* siRNA into an average of 96% of OVCAR8TR cells within 6 h of treatment. OVCAR8TR cells were treated with 90 nM AF488-*MDR1* siRNA alone (**a, b, c**), 90nM AF488-*MDR1* siRNA mixed with Lipofectamine® RNAiMax (**d, e, f**), or 90nM AF488-*MDR1* siRNA encapsulated with HA-PEI/HA-PEG nanoparticles (**g, h, i**).

Fig. 4.

Determination of *MDR1* gene down-regulation in OVCAR8TR cells. (a) The expression level of *MDR1* was significantly shut-off in OVCAR8TR cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles. The decrease in *MDR1* expression level correlated with the administered *MDR1* siRNA loaded nanoparticle in a dose dependent manner. (b) HA-PEI/PEG/*MDR1* siRNA achieved the suppression of *MDR1* gene 24 h post-transfection and was able to suppress *MDR1* expression for 120 h. *MDR1* siRNA delivered by Lipofectamine was used as positive control. The experiments were performed in triplicate. Data are presented as means $\pm$ SD and analyzed using Student's t-test. Comparisons between *MDR1* siRNA transfected cells and control cells, OVCAR8TR cells only, are displayed: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

Fig. 5.

Suppression efficacy of Pgp expression and functional activity using *MDR1* siRNA loaded HA-PEI/HA-PEG nanoparticles in MDR ovarian cancer cells. (a) Pgp expression showed dramatic decrease in OVCAR8TR cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA. HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles decreased the expression of Pgp in a dose dependent manner. (b) 90 nM *MDR1* siRNA was delivered by Lipofectamine® RNAiMax and HA-PEI/HA-PEG nanoparticles, respectively. The proteins were extracted at different time points and the expression level of Pgp was determined. Evaluation of Pgp expression level at different time points demonstrated that HA-PEI/HA-PEG/*MDR1* siRNA nanoparticles achieved the suppression of Pgp at 24 h after transfection and were able to suppress Pgp expression for at least 120 h. (c) *MDR1* siRNA delivered by HA-PEI alone also showed effects on suppression of Pgp expression, and considerable increased transfection efficiency was observed in the presence of HA-PEG. It is interesting to note that HA-PEI showed gene silencing at the mass ratio of 54:1 (polymer: siRNA), but failed to demonstrate activity at the ratio of 27:1. (d) Cells transfected with HA-PEI/HA-PEG/*MDR1* siRNA were shown to significantly decrease calcein AM retention. The experiments were performed in triplicate. Data are presented as means $\pm$ SD and analyzed using Student's t-test. Comparisons between *MDR1* siRNA transfected cells and control cells, OVCAR8TR cells only, are displayed: *** $P < 0.001$. (e) The IC_{50} of paclitaxel in OVCAR8TR cells

transfected with HA-PEI/PEG/*MDR1* siRNA nanoparticles was dramatically decreased compared with untreated cells. Additionally, comparing with untreated cells, the Pgp activity and IC₅₀ of paclitaxel in OVCAR8TR cells transfected with HA-PEI/HA-PEG/non-specific siRNA nanoparticles and *MDR1* siRNA alone remained unchanged. The experiments were performed in triplicate.

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Fig. 6.

Effects of the combination of HA-PEI/HA-PEG/*MDR1* siRNA treatment and paclitaxel on resistant ovarian cancer tumors. Treatment scheme in four groups is shown in (a). (b) Saline alone, *MDR1* alone and HA-PEI/HA-PEG/non-specific siRNA followed with paclitaxel treatment had no significant effects on the MDR ovarian cancer cells OVCAR8TR growth in xenograft model. In contrast, HA-PEI/HA-PEG/*MDR1* siRNA followed with paclitaxel treatment produced an inhibitory effect on the resistant tumor growth as compared with control groups. (c) Images show represented tumors extracted from one mouse in each group. (d) On the basis of animal weight and mortality, no considerable toxicity was observed and the animals appeared to have tolerated HA-PEI/HA-PEG nanoparticles well when HA-PEI/HA-PEG/*MDR1* siRNA and HA-PEI/HA-PEG/non-specific siRNA were administered systemically.