Published in final edited form as:

Biomaterials. 2010 January ; 31(2): 358–365. doi:10.1016/j.biomaterials.2009.09.048.

THE USE OF NANOPARTICLE-MEDIATED TARGETED GENE SILENCING AND DRUG DELIVERY TO OVERCOME TUMOR DRUG RESISTANCE

Yogesh Patil^{1,2}, Suresh Swaminathan², Tanmoy Sadhukha², Linan Ma³, and Jayanth Panyam^{2,3,*}

¹ Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI 48201

² Department of Pharmaceutics, University of Minnesota, Minneapolis, MN 55455

³ Masonic Cancer Center, Minneapolis, MN 55455

Abstract

Overexpression of drug efflux transporters such as P-glycoprotein (P-gp) enables cancer cells to develop resistance to multiple anticancer drugs. Functional inhibitors of P-gp have shown promising efficacy in early clinical trials, but their long-term safety is yet to be established. A novel approach to overcome drug resistance is to use siRNA-mediated RNA interference to silence the expression of the efflux transporter. Because P-gp plays an important role in the physiological regulation of endogenous and xenobiotic compounds in the body, it is important to deliver P-gp targeted siRNA and anticancer drug specifically to tumor cells. Further, for optimal synergy, both the drug and siRNA may need to be temporally colocalized in the tumor cells. In the current study, we investigated the effectiveness of simultaneous and targeted delivery of anticancer drug, paclitaxel, along with P-gp targeted siRNA, using poly(D,L-lactide-co-glycolide) nanoparticles to overcome tumor drug resistance. Nanoparticles were surface functionalized with biotin for active tumor targeting. Dual agent nanoparticles encapsulating the combination of paclitaxel and P-gp targeted siRNA showed significantly higher cytotoxicity in vitro than nanoparticles loaded with paclitaxel alone. Enhanced therapeutic efficacy of dual agent nanoparticles could be correlated with effective silencing of the MDR1 gene that encodes for P-gp and with increased accumulation of paclitaxel in drug-resistant tumor cells. In vivo studies in a mouse model of drug-resistant tumor demonstrated significantly greater inhibition of tumor growth following treatment with biotin-functionalized nanoparticles encapsulating both paclitaxel and P-gp targeted siRNA at a paclitaxel dose that was ineffective in the absence of gene silencing. These results suggest that the combination of P-gp gene silencing and cytotoxic drug delivery using targeted nanoparticles can overcome tumor drug resistance.

Keywords

Targeted delivery; chemotherapy; drug efflux; RNA interference; polymeric systems; drug resistance

*Author for correspondence Department of Pharmaceutics, College of Pharmacy, University of Minnesota, 308 Harvard Street SE, Minneapolis, MN 55455, Phone: 612-624-0951, Fax: 612-626-2215, jpanyam@umn.edu.

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.

1. Introduction

Multidrug resistance (MDR) remains a major barrier to the success of anticancer chemotherapy [1]. MDR is often mediated by drug efflux transporters such as P-glycoprotein (P-gp), which are overexpressed in cancer cells [2]. For example, it is estimated that nearly 40–50% of breast cancer patients demonstrate P-gp overexpression [3]. Simultaneous delivery of a drug efflux inhibitor along with the anticancer drug has the potential to overcome MDR [4,5]. Several small molecules that inhibit the efflux activity of drug transporters are currently being investigated for sensitizing tumor cells to chemotherapy [6].

An important issue with functional inhibitors is their non-specificity. For example, first-generation P-gp inhibitors (e.g., quinidine, cyclosporine-A, verapamil) were limited by unacceptable side effects [7,8], whereas second-generation agents (e.g., PSC833, VX-710) had better tolerability but were limited by unpredictable pharmacokinetic interactions with the anticancer drug and interactions with other transport proteins [9,10]. Third-generation inhibitors (tariquidar, zosuquidar, laniquidar, and ONT-093) are more specific and potent, but their long-term safety is yet to be established.

Rather than using a functional inhibitor, drug resistance could potentially be overcome by silencing the expression of the efflux transporter through RNA interference. In this approach, gene silencing is triggered by using small interfering RNA (siRNA) molecules, that are about 20–25 base pairs (bp) long. Upon introduction into cells, siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands guide the RISCs to complementary RNA molecules, where they cleave and destroy the target RNA [11]. Previous studies have shown that silencing the *MDR1* gene that encodes for P-gp using siRNA can sensitize resistant tumor cells to chemotherapy [12–17].

A major concern with P-gp inhibitors (functional inhibitors or siRNA) is that these agents may increase the side effects of chemotherapy by blocking physiological anticancer drug efflux from normal cells [18]. P-gp plays an important role in limiting the transport of endogenous and xenobiotic compounds through various critical barriers in the body [19–21]. It is important, therefore, to limit the exposure of normal cells to the inhibitor and the anticancer drug. Further, differences in physico-chemical properties of a small molecular weight anticancer drug and a much larger siRNA molecule may result in differences in the biodistribution and tumor accumulation of the two agents. For maximal synergy, both the drug and siRNA may need to be temporally colocalized in the tumor cells. Finally, because siRNA is a charged hydrophilic macromolecule, it does not penetrate the cellular membrane efficiently [22]. A carrier is needed to efficiently deliver siRNA into tumor cells. All those objectives, viz. minimal exposure of the normal tissues to the drug-siRNA combination, temporal colocalization of the combination in tumors, and efficient intracellular siRNA delivery, can be achieved by using a targeted delivery system co-encapsulating the two agents.

We have previously shown that nanoparticles formulated using poly(D,L-lactide-co-glycolide) (PLGA) and polyethyleneimine (PEI) result in sustained siRNA delivery and efficient gene silencing [23]. In the current study, we investigated the use of PLGA-PEI nanoparticles to encapsulate a combination of P-gp targeted siRNA and paclitaxel. Although a recent study reported a similar dual siRNA and drug delivery approach using block copolymer nanoparticles [15], those nanoparticles were not designed for active tumor targeting and only *in vitro* results were presented. Previous studies have shown that incorporation of targeting ligands enables greater tumor accumulation of nanoparticles and significantly improved therapeutic efficacy *in vivo* [4,24]. We used biotin as a targeting ligand in this study, because cancer cells, specifically breast cancer cells, overexpress biotin receptors [25]. Using the Interfacial Activity

Assisted Surface Functionalization (IAASF) technique [24], we engineered nanoparticles loaded with paclitaxel and P-gp targeted siRNA and functionalized with biotin, and evaluated their anticancer efficacy in both in vitro and in vivo models of tumor drug-resistance.

2. Materials and Methods

2.1 Materials

Murine P-gp targeted siRNA (SMARTpool; 21 bp), non-targeted scrambled siRNA having four mismatched base pairs (siCONTROL) and siRNA transfection reagent (DharmaFECT®) were obtained from Dharmacon (Lafayette, CO). PEI (average Mw 25,000 Da) was obtained from Sigma (St. Louis, MO). Leibovitz L-15 medium was obtained from American Type Culture Collection (Manassas, VA). Penicillin/streptomycin, fetal bovine serum, Quant-iT™ picogreen dsDNA reagent, RPMI 1640 and Trypsin-EDTA solution were obtained from Invitrogen Corporation (Carlsbad, CA). Cell culture lysis reagent, CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS) reagent powder, RQ1 RNase-free DNase and Tris-EDTA (1X) buffer were purchased from Promega (Madison, WI). RNeasy plus Mini kit was from Qiagen (Valencia, CA). RevertAid first Strand cDNA Synthesis Kit and Maxima SYBR Green qPCR Mastermix was purchased from Fermentas (Glen Burnie, MD).

2.2 Methods

2.2.1 Preparation of biotin-functionalized dual agent nanoparticles—Paclitaxel (6 mg), PEI (100 µg) and PLGA (30 mg) were dissolved in 1 ml of chloroform. A water-in-oil (W/O) emulsion was initially formed by emulsifying an aqueous phase (0.2 ml) containing acetylated bovine serum albumin (4 mg) and siRNA (245 µg) in the above chloroform solution by using a probe sonicator (Sonicator® XL, Misonix, NY) for 30 seconds over an ice bath. The W/O emulsion was then added into 6 ml of 2.5% w/v aqueous PVA solution and sonicated for 3 min over ice bath. PLA-PEG-biotin conjugate was synthesized as reported earlier [26] and was dissolved in chloroform (4 mg in 100 µl). This solution was added to the above emulsion with stirring. The emulsion was further stirred for 18 hrs at ambient conditions, followed by for 2 hrs under vacuum to remove chloroform. Nanoparticles were recovered by ultracentrifugation ($148,000 \times g$ for 35 min at 4 °C, Optima™ LE-80 K, Beckman, Palo Alta, CA), and washed three times with sterile, deionized water. Nanoparticle suspension was then lyophilized (−80 °C and <10 µm mercury pressure, Labconco, FreeZone 4.5, Kansas City, MO).

2.2.2 Encapsulation efficiency—The amount of siRNA loaded in nanoparticles was determined as described previously [23]. The amount of untrapped siRNA recovered in the nanoparticle wash solutions obtained during the formulation step was quantified using the Quant-iT™ picogreen reagent. Fluorescence generated by the binding of picogreen to double stranded siRNA was measured using FLx-800 microplate reader (BIO-TEK Instruments, Winooski, VT). siRNA loading in nanoparticles was determined by subtracting the amount of siRNA recovered in the wash solutions from the initial amount of siRNA added. Encapsulation efficiency was defined as the percent of siRNA initially added that was encapsulated in nanoparticles. Paclitaxel loading in nanoparticles was determined by extracting nanoparticles with methanol and analyzing the methanol extract by HPLC as described previously [24]. In brief, a C-18 column (4.6 mm × 25 cm) with 5 µm packing (Beckmann Instruments, Fullerton, CA) was used. A mixture of ammonium acetate (10 mM, pH 4.0) and acetonitrile in 45:55 ratio was used as the mobile phase at a flow rate of 1 ml/min. Paclitaxel was quantified by UV detection at 228 nm (System Gold 168 detector).

2.2.3 Particle size and zeta potential—Hydrodynamic diameter and zeta potential of nanoparticles were determined using ZetaPlus particle size and zeta potential analyzer

(Brookhaven Instruments, Holtsville, NY) equipped with a 35 mW solid state laser (658 nm). Mean hydrodynamic diameters were calculated for size distribution by weight, assuming a lognormal distribution, and the results were expressed as mean $\pm$ S.E.M. of five runs. Zeta potential values were calculated from measured velocities using Smoluchowski's equation, and the results were expressed as mean $\pm$ S.E.M. of five runs. Particle size and zeta potential of nanoparticle formulations were determined in distilled water.

2.2.4 Characterization by scanning electron microscopy (SEM)—The surface morphology of nanoparticles was characterized using a Hitachi S-900 Cold Field Emission Gun SEM (Hitachi, Germany) operating at 3 kV. Initially, samples were prepared by air-drying a drop of nanoparticle suspension in water (2 mg/ml) on 300-mesh copper grid. The samples were then coated with platinum under vacuum for 6 min (VCR Gr. Inc, CA). During microscopic examination, sample chamber temperature was maintained at -70°C by circulating liquid nitrogen around the chamber.

2.2.5 *In vitro* release of siRNA and paclitaxel—About 1 mg of siRNA loaded nanoparticles was incubated with 1 ml of release medium (Tris-EDTA buffer, pH 7.4) in a rotary shaker at 100 rpm and 37°C . Samples were taken in triplicate for each time point. At predetermined intervals, the nanoparticle suspension was centrifuged at 7,500 rpm and 4°C for 10 min. The amount of oligo or siRNA in the supernatant was determined by picogreen assay. Paclitaxel release was determined in TE buffer containing 0.5% w/v Tween 80 at 37°C [4]. Nanoparticle suspension (2 mg/ml, 0.5 ml) was placed in Float-A-Lyzer® dialysis tube (molecular weight cut-off 10,000 Da, Pierce), and the dialysis tube was immersed in 10 ml of the release buffer in a 15-ml centrifuge tube. The centrifuge tubes containing dialysis tubes were placed in an incubator shaker set at 100 rpm and 37°C . At predetermined time intervals, 1 ml of the release buffer was removed from the tube and was replaced with fresh release buffer. The collected buffer samples were lyophilized for 48 hrs and reconstituted with mobile phase (0.2 ml/sample) and paclitaxel concentration was determined by HPLC as described earlier.

2.2.6 Biotin quantitation assay—Number of biotin molecules present per particle was determined by using the FluoReporter biotin quantitation kit (Invitrogen) as described [24]. In brief, the assay utilizes avidin molecules labeled with a fluorescent dye and with a fluorescence-quenching ligand occupying the biotin binding sites in the avidin molecule. The ligand quenches the fluorescence of the label through fluorescence resonance energy transfer. When biotin is added to this reagent, the quencher ligand is displaced from the biotin binding sites, yielding fluorescence proportional to the amount of added biotin. Nanoparticles without biotin were used as negative control while biotinylated goat anti-mouse IgG was used as positive control. Based on the particle size and density of the polymer, 1 mg of nanoparticles was estimated to contain $\sim 10^{12}$ particles. The results were expressed as the average ($\pm$ S.D.) number of biotin molecules present on each particle.

2.2.7 Cell culture studies—For all the cell culture studies, JC cells were used as a model drug-resistant tumor cell line. The JC cell line was established from a spontaneous primary mammary adenocarcinoma. The original tumor was passaged once in female BALB/c mice, and the resulting tumors were harvested and passaged *in vitro* [27]. JC cells overexpress P-gp and exhibit drug-resistant phenotype *in vitro* and *in vivo* when grown in BALB/c mice [28].

2.2.7.1 Cytotoxicity in drug-resistant cells: Cells were seeded in 96-well plates at a seeding density of 1×10^4 cells/well. Following attachment, cells were treated with dual agent nanoparticles containing paclitaxel and P-gp targeted siRNA, paclitaxel and siRNA in solution along with the transfection reagent, siRNA with the transfection reagent, nanoparticles containing only paclitaxel, or paclitaxel in solution. Paclitaxel was used in the concentration

range of 0 to 1000 nM. Untreated cells were used as controls. The dose of siRNA in solution groups was based on siRNA loading in the dual agent nanoparticle formulations. After 24 hrs, treatments were removed, and fresh growth medium was added. Cell viability was determined using MTS assay at the end of 1 and 5 days. This study was repeated in MCF-7 cells (drug-sensitive breast adenocarcinoma) to demonstrate the specificity of siRNA for silencing P-gp expression.

2.2.7.2 Evaluation of gene silencing by real time polymerase chain reaction (RT-PCR):

Cells were seeded in 6-well plates at a density of 1×10^5 cells/well. After attachment, cells were incubated with dual agent nanoparticles and other treatments as described in the cytotoxicity study at a paclitaxel dose of 100 nM. After incubation with treatments for 72 hrs, cells were washed twice with PBS, and mRNA was isolated by using RNeasy plus Mini kit (Qiagen) according to manufacturer's guidelines. In order to avoid genomic DNA contamination, RNA samples were subjected to RQ1 RNase-free DNase treatment at 37° C for 30 min. The reaction was terminated by adding the supplied stop solution and incubating at 65° C for 10 min. One half of the sample was used for cDNA synthesis. The other half was used to generate –RT controls. cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit in a S1000 thermocycler (Biorad). Briefly, 1 µl of random hexamer primer (0.2 µg/µl) was added to the RNA samples and the volume was made up to 12 µl with DEPC water. Samples were incubated at 70° C for 5 min, chilled on ice and collected by centrifugation. To each of these, a mixture containing 4 µl of 5X reaction buffer, 1 µl of RiboLock ribonuclease inhibitor (20 µg/µl), and 2 µl of 10 mM dNTP mix was added, mixed well and incubated at 25° C for 5 min. 1 µl of reverse transcriptase (RT) was added to +RT samples alone. The mixture was incubated at 25° C for 10 min and finally at 42° C for 90 min. The reaction was stopped by heating at 70° C for 10 min. The cDNA samples were diluted with DEPC water. To 5 µl of the diluted sample in microAmp Optical 96 well reaction plate (Applied Biosystems), 12.5 µl of 2X Maxima SYBR Green qPCR Mastermix and 1 µl of each of the gene specific primer (10 picomole/µl) was added. Total volume was made up to 25 µl. The plate was then covered with MicroAmp optical adhesive film. Gene expression was analyzed by employing the absolute quantification method using ABI 7900HT system (Applied Biosystems). Standard curves for each gene analyzed were generated using cDNA obtained from untreated JC cells. The gene expression data were analyzed using SDS software (Applied Biosystems). –RT samples did not show any gene expression (data not shown). Primers employed in these studies were based on a previously published study [29] and are described in Table 1.

2.2.7.3 Paclitaxel accumulation: Cells were seeded in 6-well plates at a density of 2×10^6 cells/well. After attachment, cells were incubated with dual agent nanoparticles and other treatments as described in the cytotoxicity study at a paclitaxel dose of 100 nM. After incubation with treatments for 24 hrs, cells were washed with PBS twice and lysed using Triton X-100 in PBS. Cell lysates were lyophilized and paclitaxel in the lyophilized samples was determined by LC-MS/MS [24]. In brief, a capillary HPLC system (Agilent, CA) coupled to a TSQ Quantum discovery Max triple-quadrupole mass spectrometric detector (Thermo Finnigan) and equipped with an electrospray ionization source was used. Analytes were eluted at a flow rate of 0.1 ml/min, using isocratic mobile phase composed of 10 mM ammonium acetate (pH 4.0) and acetonitrile (70:30). The mass spectrometer was run in positive electrospray mode and the source conditions employed for creation of daughter ions were as follows: capillary voltage of 3 kV; spray voltage of 3900 V; capillary temperature of 385° C; sheath gas pressure of 55 L/h; source CID (collision energy) of 5 and collision gas pressure of 1 mTorr. Under these conditions, the 854.5 → 286.2 transition was used to generate a standard curve. Paclitaxel concentration in the cell lysates was determined using the standard curve and was normalized to the cell protein determined using Pierce protein assay kit.

2.2.8 Efficacy of targeted dual agent nanoparticles *in vivo*—All the procedures performed in animals were approved by the institutional animal use and investigation committee at the University of Minnesota, Minneapolis. Female BALB/c mice (Charles river, MA, USA), about 6–8 weeks old, bearing JC tumors were used in this experiment. About 1×10^6 cells were suspended in PBS and injected subcutaneously to induce tumors. When the tumor volume reached $\sim 100 \text{ mm}^3$, animals received a single intravenous dose of biotin functionalized dual agent nanoparticles, dual agent nanoparticles without biotin functionalization, biotin functionalized nanoparticles containing paclitaxel alone, paclitaxel nanoparticles without surface functionalization, a combination of paclitaxel and siRNA in solution (along with DharmaFECT[®]), paclitaxel in cremophor EL[®] solution, nanoparticles containing scrambled siRNA (non-targeted) or just the vehicle (cremophor). Each animal received 20 mg/kg dose of paclitaxel and $\sim 20 \mu\text{g}/\text{animal}$ siRNA. The nanoparticle dose in the different treatment groups was adjusted based on the loading of paclitaxel and siRNA. Following treatment administration, animals were monitored every other day for tumor growth and survival. Tumor volumes were calculated by measuring two perpendicular diameters with Vernier calipers and using the formula $(L \times W^2)/2$, where L is the longest diameter and W is perpendicular to L. Tumor volume on the day of treatment was normalized to 100% for all groups.

2.2.9 Statistical analyses—Generalized linear mixed effect model (mixed model) following natural log transformation of tumor volumes was used to analyze tumor growth inhibition data [24]. The differences in the slopes of the tumor growth curves were tested by Bonferroni adjustment. One-way ANOVA was used to analyze differences in other experiments. A probability level of $P < 0.05$ was considered significant.

3.0 Results

3.1 Characterization of surface functionalized dual agent nanoparticles

Dual agent nanoparticles with and without biotin surface functionalization were characterized for morphology, size, drug and siRNA loading and *in vitro* release. SEM studies showed that nanoparticles were spherical and had a smooth surface (figure 1). Dynamic light scattering studies demonstrated an average particle size (hydrodynamic diameter) of about 200–250 nm (Table 2). Nanoparticles had a net negative surface charge; presence of biotin appeared to reduce the magnitude of zeta potential ($-12.1 \pm 0.3 \text{ mV}$) compared to non-functionalized nanoparticles ($-26.0 \pm 0.2 \text{ mV}$; Table 2). Both paclitaxel and siRNA were encapsulated efficiently in both single agent and dual agent nanoparticle formulations (Table 3). The number of biotin molecules on the surface was similar (~ 30) for both dual agent and single agent nanoparticle formulations (Table 3). *In vitro* release studies demonstrated that dual agent nanoparticles sustained the release of paclitaxel and siRNA (figure 2). A burst release was seen for both siRNA and paclitaxel (15–20% in the first 24 hrs; figures 2A and B). A more sustained and continuous release was observed over the next 10 to 20 days (for siRNA and paclitaxel, respectively).

3.2 Cytotoxicity of dual agent (paclitaxel-siRNA) in drug resistant cells

Cytotoxicity of paclitaxel and the effect of P-gp targeted siRNA on paclitaxel-induced cytotoxicity were investigated in the drug-resistant JC tumor cell line. In order to isolate the effect of P-gp inhibition and to reduce the number of treatment groups, this study was performed with nanoparticles that were not functionalized with biotin. Paclitaxel, either in solution or encapsulated in nanoparticles, was largely ineffective in inducing cytotoxicity (figures 3A and B). Significant cytotoxicity was observed only at doses $> 100 \text{ nM}$. Addition of P-gp-targeted siRNA significantly improved the cytotoxicity for both nanoparticles and solution treatments. A significant difference in cytotoxicity ($P < 0.05$) was observed at lower

paclitaxel doses (10 nM to 100 nM) in the case of dual agent nanoparticles as well as the combination treatment in solution compared to paclitaxel nanoparticles or paclitaxel solution, respectively. There was not a significant difference between the effectiveness of solution and nanoparticle treatments. The enhanced cytotoxicity of the combination treatment was observed over 5 days (figure 3B). At higher paclitaxel doses (1000 nM), differences in cytotoxicity between combination treatments and paclitaxel-only treatments appeared to diminish. This could be attributed to the saturation of P-gp and consequently increased sensitivity to paclitaxel-induced cytotoxicity at high doses. Treatment with siRNA in the absence of paclitaxel had no effect on cytotoxicity. Paclitaxel, both free in solution and encapsulated in nanoparticles, was effective against drug-sensitive MCF-7 cells (not shown; also described in [4]). Addition of P-gp targeted siRNA had no significant effect on paclitaxel-induced cytotoxicity in MCF-7 cells.

3.3 P-gp gene silencing

In order to determine the mechanism of efficacy of dual agent nanoparticles in drug-resistant cells, we determined P-gp gene expression using real time RT-PCR. JC cells were treated with siRNA in nanoparticles or complexed with the commercial transfection reagent. As can be seen in figure 4, treatment with P-gp targeted siRNA in nanoparticles, either alone or in combination with paclitaxel, resulted in about 50% decrease in relative expression levels on day 3 when compared with control treatments. Interestingly, free paclitaxel in solution reduced P-gp gene expression slightly; this effect appeared to increase the gene silencing effectiveness of the commercial transfection reagent.

3.4 Paclitaxel accumulation in drug resistant cells

To further confirm the mechanism of enhanced cytotoxicity observed with the combination therapy, we evaluated the effect of dual agent nanoparticles on paclitaxel accumulation in JC cells. As can be seen in figure 5, inclusion of P-gp targeted siRNA along with paclitaxel significantly increased paclitaxel accumulation in JC cells ($P < 0.05$). Also, the combination treatment resulted in higher cellular accumulation of paclitaxel when encapsulated in nanoparticles than when in solution. It has to be noted, however, that in the case of nanoparticle-treated cells, paclitaxel concentration measured includes both free (released) and nanoparticle-encapsulated paclitaxel.

3.5 Efficacy of dual agent nanoparticles *in vivo*

Based on effective cytotoxicity in drug resistant cells *in vitro*, we next evaluated the efficacy of dual agent nanoparticles, surface functionalized with biotin, in the JC tumor model. Mice bearing tumors of $\sim 100 \text{ mm}^3$ were injected with a single dose of the different treatments through the tail vein. Doses of siRNA ($\sim 20 \text{ }\mu\text{g/animal}$) and paclitaxel (20 mg/kg) were equivalent across all the treatment groups. The study showed that a single intravenous dose of tumor-targeted dual agent nanoparticles resulted in significantly greater tumor growth inhibition than non-targeted dual agent nanoparticles and other controls ($P < 0.05$; figure 6). Other treatments like vehicle, scrambled siRNA nanoparticles, paclitaxel solution, paclitaxel nanoparticles, and biotin conjugated paclitaxel nanoparticles did not show significant tumor growth inhibition. All the animals in some of the control groups (vehicle, paclitaxel-scrambled siRNA nanoparticles, and paclitaxel-siRNA combination in solution) reached the pre-set end point (defined as tumor load $> 1000 \text{ mm}^3$ or body weight loss $> 20\%$) and tumors became ulcerated within the first 10 days. Animals that reached the pre-set end points were euthanized. Correlating with the greater tumor growth inhibition, biotin functionalized dual agent nanoparticle treatment resulted in the highest survival rate (20% survival over 26 days; not shown). In the non-targeted dual agent nanoparticle treatment group, 25% of animals survived over 16 days.

4.0 Discussion

The goal of current studies was to develop a paclitaxel-based therapeutic approach that is effective against drug resistant tumors. The natural product paclitaxel, currently available as Taxol® (solubilized in cremophor/ethanol solution) or as Abraxane® (human albumin-stabilized nanoparticle formulation), is a first-line chemotherapeutic agent against various cancers [30–32]. However, many cancer cells overexpress the drug efflux transporter P-gp and develop resistance to paclitaxel, resulting in therapy failure and cancer recurrence [33,34]. Studies from several groups, including ours, have shown that encapsulation in specific nanoparticulate formulations can improve the effectiveness of anticancer drugs in drug-resistant cells [35–38]. Mechanisms of such improvements have included shielding of the drug from the transporter, direct inhibition of ATPase activity, or through indirect depletion of cellular ATP. While such formulations are promising, the long-term safety and efficacy of the excipients used in those formulations are yet to be established.

Nanoparticles formulated using PLGA, a polymer that is biodegradable, biocompatible and approved for clinical use [39,40], have been widely investigated for targeted drug delivery [41–44]. Several studies have shown that targeted PLGA nanoparticles encapsulating paclitaxel or other chemotherapeutics result in improved anticancer efficacy [4,24,43,45]. Such studies, however, predominantly utilized drug-sensitive tumor models. We have previously shown that paclitaxel encapsulated in PLGA nanoparticles is susceptible to P-gp-mediated drug efflux [46], and that the addition of a P-gp inhibitor like verapamil or tariquidar can effectively overcome resistance to nanoparticle-encapsulated paclitaxel [4,46]. We further showed that targeting of nanoparticles to tumor is essential for maximizing the therapeutic efficacy of the drug-inhibitor combination *in vivo* [4].

While functional inhibitors such as tariquidar are highly effective in inhibiting drug efflux, their lack of specificity is a concern. To address this issue, we used siRNA-mediated RNA interference to reduce P-gp expression in this study. Gene silencing using siRNA is widely as a highly specific approach to reduce the expression of a target gene [47]. Unlike that of small molecular weight inhibitors, however, formulation of siRNA poses additional challenges. In our previous study, we observed that siRNA-loaded nanoparticles formulated using PLGA alone resulted in poor gene silencing effectiveness [23]. This was attributed to inefficient siRNA encapsulation in nanoparticles and incomplete release of siRNA over a two-week period. Our studies further showed that addition of a small amount of PEI, a cationic polymer, to the polymer matrix significantly increased siRNA encapsulation, improved siRNA release profile and the gene silencing effectiveness. Based on these prior results, we used PLGA-PEI nanoparticles in the current study for simultaneous delivery of P-gp-targeted siRNA and paclitaxel.

The physical characterization studies confirmed that both biotin-functionalized and non-functionalized dual agent nanoparticle formulations investigated in this study were similar in particle size, morphology, and siRNA/drug loading. *In vitro* release studies demonstrated sustained and near-complete release of both paclitaxel and siRNA from dual agent nanoparticles. Interestingly, the larger molecular weight siRNA was released faster than paclitaxel (80% in 15 days Vs 70–90% in 30 days, respectively). This may be due to the differences in the localization of the two molecules within nanoparticles and in the mechanism of drug release. Paclitaxel was likely present as a molecular dispersion in the polymer matrix, while siRNA was probably present in the pores in the matrix. Paclitaxel release from particles is expected to be through diffusion in the polymer while siRNA release was likely by diffusion in aqueous channels created in the polymer matrix through a combination of polymer degradation and water diffusion [48,49]. The faster siRNA release and relatively slower paclitaxel release observed from dual agent nanoparticles was well suited for the current study,

as these release profiles would allow downregulation of P-gp expression to occur prior to the complete release of paclitaxel. Further, slow release observed for both paclitaxel and siRNA probably enabled sustained tumor growth inhibition *in vivo* even with a single dose of dual agent nanoparticles.

In vitro cytotoxicity studies demonstrated the therapeutic efficacy of dual agent nanoparticles in drug resistant cells, and further confirmed that paclitaxel alone, either free or encapsulated in nanoparticles, was not effective. Dual agent nanoparticles sustained cytotoxicity over five days in these cells; these results are comparable to our previous *in vitro* cytotoxicity studies with tariquidar-paclitaxel combination [4] and are in agreement with sustained gene silencing observed with nanoparticles containing luciferase targeted siRNA [23]. The maximal cytotoxicity observed was in the range of 40–70%. Interestingly, similar incomplete inhibition has been observed by others. For example, Yadav et al observed ~60% inhibition following simultaneous delivery of siRNA targeted to P-gp and 1000 nM paclitaxel in drug-resistant ovarian adenocarcinoma (SKOV3) cells [15]. Similarly, about 60–70% inhibition was observed in *MDR1*-transfected Caco-2 cells that were treated with minicells containing P-gp targeted shRNA and a cytotoxic drug (5-fluorouracil or irinotecan) [50]. The reason for such incomplete inhibition is not clear. It is possible that downregulation of P-gp expression using siRNA is not complete. Indeed, our RT-PCR studies confirm that. In addition, it is possible that there are other minor drug-resistance mechanisms that enable these tumor cells to survive high concentrations of the anticancer drug [1].

RT-PCR studies demonstrated *MDR1* gene silencing by dual agent nanoparticles as well as nanoparticles containing only siRNA. Drug accumulation studies demonstrated enhanced paclitaxel accumulation with dual agent nanoparticles. Taken together, these results provide evidence for the proposed mechanism of efficacy with dual agent nanoparticles. Dual agent nanoparticles slowly release both siRNA and paclitaxel; slow release of siRNA enables sustained inhibition of P-gp expression, which sensitizes resistant tumor cells to paclitaxel released from nanoparticles. Higher paclitaxel accumulation with dual agent nanoparticles than that with paclitaxel-siRNA combination in solution suggests greater cellular uptake of nanoparticle-encapsulated paclitaxel and siRNA by endocytosis [51].

Previous studies have shown that JC cells overexpress biotin receptors on the cell membrane [52]. We used IAASF technique to functionalize nanoparticle surface with biotin as a model tumor targeting ligand. We have previously performed extensive quantitative and qualitative characterization studies to demonstrate the presence of targeting ligands such as biotin on nanoparticle surface [24]. In the current study, we confirmed through a quantitative assay that both dual agent and single agent nanoparticles had similar number of biotin molecules. *In vivo* tumor growth inhibition studies showed that paclitaxel, either free in solution or encapsulated in nanoparticles, was not effective in inhibiting the growth of JC tumors. This confirms our *in vitro* results, which show that encapsulation of paclitaxel in nanoparticles is by itself not enough to overcome drug efflux. Similarly, biotin functionalization had no effect on the therapeutic efficacy of nanoparticle-encapsulated paclitaxel. Biotin-functionalized dual agent nanoparticles containing paclitaxel-siRNA combination resulted in the greatest tumor growth inhibition but dual agent nanoparticles without biotin also had improved therapeutic efficacy compared to paclitaxel-alone treatments. This is probably due to the passive targeting of dual agent nanoparticles to the tumor tissue through the enhanced permeability and retention effect [53–55].

While the current studies clearly show that dual agent nanoparticles encapsulating paclitaxel and P-gp targeted siRNA can significantly inhibit the growth of drug-resistant tumor, the inhibition achieved was not complete. There are several possible reasons for this. Only a single dose of dual agent nanoparticles was used in this study. It is possible that administration of

more doses could further improve the anticancer efficacy of dual agent nanoparticles. A recent study reported that multiple sequential doses (6–9 doses over a 4-week period) of bacterial minicells containing siRNA and a cytotoxic drug resulted in near-complete tumor growth inhibition [50]. A second possibility is that the dose of dual agent nanoparticle formulation used here is not optimal. The dose of siRNA and paclitaxel used in this study was based on preliminary *in vitro* studies, and it is possible that these doses may not be optimal *in vivo*. A dose-response study investigating higher doses of siRNA and paclitaxel will help identify optimal *in vivo* doses of the two molecules. Finally, to achieve complete tumor growth inhibition, paclitaxel-siRNA combination has to be delivered intracellularly to each individual tumor cell. Previous studies have shown that elevated interstitial fluid pressure in tumors reduces the transport of macromolecules, and possibly nanoparticles, through the tumor tissue [56]. This, in turn, will result in incomplete gene silencing and tumor cell kill. In future studies, we will evaluate the effect of multiple doses of dual agent nanoparticles and of decreasing the elevated tumor interstitial fluid pressure on the therapeutic effectiveness of dual agent nanoparticles.

5.0 Conclusion

We have examined the combination of targeted P-gp gene silencing and paclitaxel delivery to overcome tumor drug resistance. Using PLGA-PEI nanoparticles for siRNA and paclitaxel encapsulation, we found that inhibition of P-gp expression enhanced paclitaxel accumulation and cytotoxicity in drug-resistant cells. Biotin functionalized dual agent nanoparticles demonstrated significant inhibition of tumor growth *in vivo*. These studies suggest that the combination of P-gp gene silencing and cytotoxic drug delivery using targeted nanoparticles can overcome tumor drug resistance.

Acknowledgments

Funding from NIH (7R21CA116641-02). We thank Alice Ressler at the Characterization Facility, University of Minnesota, Minneapolis, MN, for assistance with TEM studies.

References

- Teicher BA. Acute and chronic *in vivo* therapeutic resistance. *Biochem Pharmacol* 2009;77:1665–73. [PubMed: 19428320]
- Duhem C, Ries F, Dicato M. What Does Multidrug Resistance (MDR) Expression Mean in the Clinic? *Oncologist* 1996;1:151–158. [PubMed: 10387981]
- Clarke R, Leonessa F, Trock B. Multidrug resistance/P-glycoprotein and breast cancer: review and meta-analysis. *Semin Oncol* 2005;32:S9–15. [PubMed: 16360717]
- Patil Y, Sadhukha T, Ma L, Panyam J. Nanoparticle-mediated simultaneous and targeted delivery of paclitaxel and tariquidar overcomes tumor drug resistance. *J Control Release* 2009;136:21–9. [PubMed: 19331851]
- Soma CE, Dubernet C, Bentolila D, Benita S, Couvreur P. Reversion of multidrug resistance by co-encapsulation of doxorubicin and cyclosporin A in polyalkylcyanoacrylate nanoparticles. *Biomaterials* 2000;21:1–7. [PubMed: 10619673]
- Baumert C, Hilgeroth A. Recent advances in the development of P-gp inhibitors. *Anticancer Agents Med Chem* 2009;9:415–36. [PubMed: 19442042]
- Ferry DR, Traunecker H, Kerr DJ. Clinical trials of P-glycoprotein reversal in solid tumours. *Eur J Cancer* 1996;32A:1070–81. [PubMed: 8763349]
- Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur J Pharm Sci* 2000;11:265–83. [PubMed: 11033070]
- Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48–58. [PubMed: 11902585]

10. Rowinsky EK, Smith L, Wang YM, Chaturvedi P, Villalona M, Campbell E, et al. Phase I and pharmacokinetic study of paclitaxel in combination with biricodar, a novel agent that reverses multidrug resistance conferred by overexpression of both MDR1 and MRP. *J Clin Oncol* 1998;16:2964–76. [PubMed: 9738565]
11. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–8. [PubMed: 11373684]
12. Pichler A, Zelcer N, Prior JL, Kuil AJ, Piwnicka-Worms D. In vivo RNA Interference-Mediated Ablation of MDR1 P-Glycoprotein. *Clin Cancer Res* 2005;11:4487–4494. [PubMed: 15958634]
13. Xu D, McCarty D, Fernandes A, Fisher M, Samulski RJ, Juliano RL. Delivery of MDR1 Small Interfering RNA by Self-Complementary Recombinant Adeno-Associated Virus Vector. *Mol Ther* 2005;11:523–530. [PubMed: 15771955]
14. Xu D, Ye D, Fisher M, Juliano RL. Selective inhibition of P-glycoprotein expression in multidrug-resistant tumor cells by a designed transcriptional regulator. *J Pharmacol Exp Ther* 2002;302:963–71. [PubMed: 12183653]
15. Yadav S, van Vlerken LE, Little SR, Amiji MM. Evaluations of combination MDR-1 gene silencing and paclitaxel administration in biodegradable polymeric nanoparticle formulations to overcome multidrug resistance in cancer cells. *Cancer Chemother Pharmacol*. 2008
16. Yague E, Higgins CF, Raguz S. Complete reversal of multidrug resistance by stable expression of small interfering RNAs targeting MDR1. *Gene Ther* 2004;11:1170–4. [PubMed: 15164094]
17. Zhang T, Guan M, Jin HY, Lu Y. Reversal of multidrug resistance by small interfering double-stranded RNAs in ovarian cancer cells. *Gynecol Oncol* 2005;97:501–507. [PubMed: 15863151]
18. Hubensack M, Muller C, Hoehnerl P, Fellner S, Spruss T, Bernhardt G, et al. Effect of the ABCB1 modulators elacridar and tariquidar on the distribution of paclitaxel in nude mice. *J Cancer Res Clin Oncol* 2008;134:597–607. [PubMed: 17932689]
19. Bebawy M, Sze DM. Targeting P-glycoprotein for effective oral anti-cancer chemotherapeutics. *Curr Cancer Drug Targets* 2008;8:47–52. [PubMed: 18288943]
20. Linnet K, Ejlsing TB. A review on the impact of P-glycoprotein on the penetration of drugs into the brain. Focus on psychotropic drugs. *Eur Neuropsychopharmacol* 2008;18:157–69. [PubMed: 17683917]
21. van Zuylen L, Verweij J, Nooter K, Brouwer E, Stoter G, Sparreboom A. Role of intestinal P-glycoprotein in the plasma and fecal disposition of docetaxel in humans. *Clin Cancer Res* 2000;6:2598–603. [PubMed: 10914699]
22. Zhang S, Zhao B, Jiang H, Wang B, Ma B. Cationic lipids and polymers mediated vectors for delivery of siRNA. *J Control Release* 2007;123:1–10. [PubMed: 17716771]
23. Patil Y, Panyam J. Polymeric nanoparticles for siRNA delivery and gene silencing. *Int J Pharm* 2009;367:195–203. [PubMed: 18940242]
24. Patil YB, Toti US, Khadair A, Ma L, Panyam J. Single-step surface functionalization of polymeric nanoparticles for targeted drug delivery. *Biomaterials* 2009;30:859–66. [PubMed: 19019427]
25. Lee ES, Na K, Bae YH. Super pH-sensitive multifunctional polymeric micelle. *Nano Lett* 2005;5:325–9. [PubMed: 15794620]
26. Salem AK, Cannizzaro SM, Davies MC, Tendler SJ, Roberts CJ, Williams PM, et al. Synthesis and characterisation of a degradable poly(lactic acid)-poly(ethylene glycol) copolymer with biotinylated end groups. *Biomacromolecules* 2001;2:575–80. [PubMed: 11749223]
27. Capone PM, Kadohama N, Chu TM. Immunotherapy in a spontaneously developed murine mammary carcinoma with syngeneic monoclonal antibody. *Cancer Immunol Immunother* 1987;25:93–9. [PubMed: 3664534]
28. Lee BD, French KJ, Zhuang Y, Smith CD. Development of a syngeneic in vivo tumor model and its use in evaluating a novel P-glycoprotein modulator, PGP-4008. *Oncol Res* 2003;14:49–60. [PubMed: 14552591]
29. Goralski KB, Acott PD, Fraser AD, Worth D, Sinal CJ. Brain cyclosporin A levels are determined by ontogenic regulation of *mdr1a* expression. *Drug Metab Dispos* 2006;34:288–95. [PubMed: 16303871]

30. Bradley DA, Hussain M. Promising novel cytotoxic agents and combinations in metastatic prostate cancer. *Cancer J* 2008;14:15–9. [PubMed: 18303478]
31. Edelman MJ. Novel taxane formulations and microtubule-binding agents in non-small-cell lung cancer. *Clin Lung Cancer* 2009;10 (Suppl 1):S30–4. [PubMed: 19362944]
32. Morris PG, Fornier MN. Novel anti-tubulin cytotoxic agents for breast cancer. *Expert Rev Anticancer Ther* 2009;9:175–85. [PubMed: 19192956]
33. Dean M. ABC transporters, drug resistance, and cancer stem cells. *J Mammary Gland Biol Neoplasia* 2009;14:3–9. [PubMed: 19224345]
34. Turk D, Szakacs G. Relevance of multidrug resistance in the age of targeted therapy. *Curr Opin Drug Discov Devel* 2009;12:246–52.
35. Chavanpatil MD, Khadair A, Gerard B, Bachmeier C, Miller DW, Shekhar MP, et al. Surfactant-polymer nanoparticles overcome P-glycoprotein-mediated drug efflux. *Mol Pharm* 2007;4:730–8. [PubMed: 17705442]
36. De Juan BS, Von Briesen H, Gelperina SE, Kreuter J. Cytotoxicity of doxorubicin bound to poly (butyl cyanoacrylate) nanoparticles in rat glioma cell lines using different assays. *J Drug Target* 2006;14:614–22. [PubMed: 17090397]
37. de Verdiere AC, Dubernet C, Nemati F, Soma E, Appel M, Ferte J, et al. Reversion of multidrug resistance with polyalkylcyanoacrylate nanoparticles: towards a mechanism of action. *Br J Cancer* 1997;76:198–205. [PubMed: 9231919]
38. Dong X, Mattingly CA, Tseng MT, Cho MJ, Liu Y, Adams VR, et al. Doxorubicin and paclitaxel-loaded lipid-based nanoparticles overcome multidrug resistance by inhibiting P-glycoprotein and depleting ATP. *Cancer Res* 2009;69:3918–26. [PubMed: 19383919]
39. Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv Drug Deliv Rev* 2003;55:329–47. [PubMed: 12628320]
40. Wischke C, Schwendeman SP. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. *Int J Pharm* 2008;364:298–327. [PubMed: 18621492]
41. Choi SW, Kim JH. Design of surface-modified poly(D,L-lactide-co-glycolide) nanoparticles for targeted drug delivery to bone. *J Control Release* 2007;122:24–30. [PubMed: 17628158]
42. Diez S, Navarro G, de ICT. In vivo targeted gene delivery by cationic nanoparticles for treatment of hepatocellular carcinoma. *J Gene Med* 2009;11:38–45. [PubMed: 19021130]
43. Sun B, Ranganathan B, Feng SS. Multifunctional poly(D,L-lactide-co-glycolide)/montmorillonite (PLGA/MMT) nanoparticles decorated by Trastuzumab for targeted chemotherapy of breast cancer. *Biomaterials* 2008;29:475–86. [PubMed: 17953985]
44. Sundaram S, Roy SK, Ambati BK, Kompella UB. Surface-functionalized nanoparticles for targeted gene delivery across nasal respiratory epithelium. *Faseb J*. 2009
45. Esmaeili F, Ghahremani MH, Ostad SN, Atyabi F, Seyedabadi M, Malekshahi MR, et al. Folate-receptor-targeted delivery of docetaxel nanoparticles prepared by PLGA-PEG-folate conjugate. *J Drug Target* 2008;16:415–23. [PubMed: 18569286]
46. Chavanpatil MD, Patil Y, Panyam J. Susceptibility of nanoparticle-encapsulated paclitaxel to P-glycoprotein-mediated drug efflux. *Int J Pharm* 2006;320:150–6. [PubMed: 16713148]
47. Takahashi Y, Nishikawa M, Takakura Y. Nonviral vector-mediated RNA interference: its gene silencing characteristics and important factors to achieve RNAi-based gene therapy. *Adv Drug Deliv Rev* 2009;61:760–6. [PubMed: 19386274]
48. Berklund C, Pollauf E, Raman C, Silverman R, Kim K, Pack DW. Macromolecule release from monodisperse PLG microspheres: control of release rates and investigation of release mechanism. *J Pharm Sci* 2007;96:1176–91. [PubMed: 17455338]
49. Klose D, Siepmann F, Elkharras K, Siepmann J. PLGA-based drug delivery systems: importance of the type of drug and device geometry. *Int J Pharm* 2008;354:95–103. [PubMed: 18055140]
50. MacDiarmid JA, Amaro-Mugridge NB, Madrid-Weiss J, Sedliarou I, Wetzel S, Kocher K, et al. Sequential treatment of drug-resistant tumors with targeted micelles containing siRNA or a cytotoxic drug. *Nat Biotechnol* 2009;27:643–51. [PubMed: 19561595]
51. Panyam J, Labhasetwar V. Sustained cytoplasmic delivery of drugs with intracellular receptors using biodegradable nanoparticles. *Mol Pharm* 2004;1:77–84. [PubMed: 15832503]

52. Russell-Jones G, McTavish K, McEwan J, Rice J, Nowotnik D. Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumours. *J Inorg Biochem* 2004;98:1625–33. [PubMed: 15458825]
53. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2003;2:214–21. [PubMed: 12612647]
54. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986;46:6387–92. [PubMed: 2946403]
55. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* 2007;2:751–60. [PubMed: 18654426]
56. Kano MR, Komuta Y, Iwata C, Oka M, Shirai YT, Morishita Y, et al. Comparison of the effects of the kinase inhibitors imatinib, sorafenib, and transforming growth factor-beta receptor inhibitor on extravasation of nanoparticles from neovasculature. *Cancer Sci* 2009;100:173–80. [PubMed: 19037999]

Figure 1. SEM image of dual agent nanoparticles without surface functionalization (**A**) and with biotin functionalization (**B**). Nanoparticles were platinum-coated for visualization. Bar is 670 nm.

Figure 2.

In vitro release of (A) paclitaxel and (B) siRNA targeted to P-gp from dual agent nanoparticles. Legend: Pac-siRNA NP: nanoparticles containing paclitaxel and siRNA; BI Pac-siRNA NP: nanoparticles surface functionalized with biotin and containing paclitaxel and siRNA. Data as mean $\pm$ S.D.; n = 3.

Figure 3. Enhanced cytotoxicity of dual agent nanoparticles in JC cells. Cells were incubated with treatments for 24 hrs and the cell viability was determined by MTS assay at the end of 1 day (A) or 5 days (B) after the addition of treatments. Legend: siRNA Soln - siRNA in solution complexed with DharmaFECT; Pac-Soln - paclitaxel in solution; Pac NP - nanoparticles containing paclitaxel; Pac-siRNA Soln - Paclitaxel and siRNA in solution along with DharmaFECT; and Pac-siRNA NP - Nanoparticles containing paclitaxel and P-gp targeted siRNA. Data as mean $\pm$ S.D.; n = 10.

Figure 4.

Quantitative RT-PCR analysis of *MDR1* gene expression in JC cells. Cells were incubated with different treatments, either free in solution (Soln) or encapsulated in nanoparticles (NP). The siRNA solution treatments also contained DharmaFECT. P-gp mRNA levels were normalized to β -actin mRNA levels, and the results are presented as fold-change in the normalized values relative to untreated controls. The experiment was performed in duplicate and the values presented here are mean $\pm$ S.D. of the two replicates.

Figure 5.

Enhanced paclitaxel accumulation in the presence of P-gp targeted siRNA. JC cells were incubated with nanoparticles containing paclitaxel and siRNA (Pac-siRNA NP), paclitaxel and siRNA in solution with DharmaFECT (Pac-siRNA Soln), nanoparticles containing paclitaxel (Pac NP) or paclitaxel in solution (Pac Soln) at a paclitaxel concentration of 100 nM. Paclitaxel concentration was determined by LC-MS/MS and normalized to total cell protein. Data as mean $\pm$ S.D.; n = 3. # P<0.05 Vs Pac NP; * P<0.05 Vs Pac Soln.

Figure 6.

Incorporation of biotin on dual agent nanoparticles results in enhanced anticancer effectiveness *in vivo*. BALB/c mice bearing JC tumors of $\sim 100 \text{ mm}^3$ were injected with a single dose of the different treatments through the tail vein. Growth in tumor volume was monitored on a regular basis. Legend: Pac Soln - paclitaxel in solution; Pac NP - Nanoparticles containing paclitaxel; BI Pac NP - Nanoparticles containing paclitaxel and functionalized with biotin; Pac-siRNA Soln - paclitaxel and siRNA in solution with DharmaFECT; Pac-siRNA NP - Nanoparticles containing paclitaxel and siRNA; Pac-SsiRNA NP - Nanoparticles containing paclitaxel and scrambled siRNA and BI Pac-siRNA NP - Nanoparticles containing paclitaxel and siRNA and functionalized with biotin. Data as mean $\pm$ S.D.; $n = 5$

Table 1

Primers used in RT-PCR studies

Gene	Sequence
mMDR1a (P-gp)	GAGTGAGGCCGATAAAAGAGCCATGTT TCATCTGTGAGCCGGGTGTTGAGCTCCC
mActb (β Actin)	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT

Table 2

Particle size and zeta potential of dual agent nanoparticle formulations

Formulation ^a	Particle size (nm)	Zeta potential (mV)
Pac-siRNA NP	228 ± 22	-24.0 ± 0.5
BI Pac-siRNA NP	237 ± 16	-12.1 ± 0.3

^aLegend: see figure 6

Table 3

Characterization of dual agent nanoparticle formulations

Formulation ^a	%Encapsulation efficiency		Loading		
	siRNA	Paclitaxel	siRNA (µg/30 mg)	Paclitaxel (mg/30 mg)	Biotin/particle
Pac NP	-	80 ± 2	-	4.8 ± 0.1	-
siRNA NP	81 ± 2	-	198 ± 5	-	-
Pac-siRNA NP	78 ± 1	77 ± 4	191 ± 2	4.6 ± 0.2	-
BI Pac NP	-	79 ± 4	-	4.7 ± 0.2	32 ± 2
BI Pac-siRNA NP	80 ± 2	82 ± 4	196 ± 5	4.9 ± 0.2	30 ± 2

^a Legend: see figure 6