GRIFFITH UNIVERSITY

Systemic Delivery of E6/7 SiRNA Using Novel Lipidic Particles and Its Application with Cisplatin in Cervical Cancer Mouse Models

Author

Wu, SY, Singhania, A, Burgess, M, Putral, LN, Kirkpatrick, C, Davies, NM, McMillan, NAJ

Published

2011

Journal Title

Gene Therapy

Version

Accepted Manuscript (AM)

DOI

10.1038/gt.2010.113

Rights statement

© 2011 Nature Publishing Group. This is the author-manuscript version of this paper. Reproduced in accordance with the copyright policy of the publisher. Please refer to the journal website for access to the definitive, published version.

Downloaded from

http://hdl.handle.net/10072/173247

Griffith Research Online

https://research-repository.griffith.edu.au

Systemic delivery of E6/7 siRNA using novel lipidic particles and its application with cisplatin in cervical cancer mouse models

Sherry Y. Wu[a,b] Ph.D., Akul Singhania[a], Melinda Burgess[a] BSc(Hons), Lisa N. Putral[a] Ph.D., Carl Kirkpatrick[b] Ph.D., Nigel M. Davies[b] Ph.D. and Nigel A.J. McMillan[a,*] Ph.D.

[a] *Diamantina Institute for Cancer, Immunology and Metabolic Medicine, University of Queensland, Princess Alexandra Hospital, Ipswich Rd, Buranda, QLD 4102, Australia.*

[b] *School of Pharmacy, University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia.*

*** Corresponding author.**

A/Prof Nigel McMillan

Tel.:+61 7 3240 5392; Fax: +61 7 3240 5946

E-mail address: n.mcmillan@uq.edu.au

Postal address:

Level 4, R-Wing, Princess Alexandra Hospital, Ipswich Rd, Buranda, QLD 4102, Australia

Running heading: Use of E6/7 siRNA For Cervical Cancer Treatment

Word count: 5353

Abstract

Small interfering RNA (siRNA) shows great promise in cancer therapy but its effectiveness *in vivo* still remains a crucial issue for its transition into the clinics. While the successful use of PEGylated lipidic delivery systems have already been reported, most of the formulation procedures employed are labour intensive and also result in unstable end products. We have previously developed a simple yet efficient Hydration-of-Freeze-Dried-Matrix (HFDM) method to entrap siRNA within lipid particles where the products exhibited superior stability. Here, we show that these HFDM-formulated particles are stable in the presence of serum and can deliver siRNA efficiently to tumours following intravenous administration. Using these particles, around 50% knockdown of the target gene expression was observed in tumours. With the use of siRNA targeting the E6/7 oncogenes expressed in cervical cancer, we showed a 50% reduction in tumour size. This level of tumour growth suppression was comparable to that achieved from cisplatin at the clinically used dose. Overall, our results demonstrate the feasibility of using HFDM-formulated particles to systematically administer E6/7-targeted siRNA for cervical cancer treatment. The simplicity of preparation procedure along with superior product stability obtained from our method offers an innovative approach for the *in vivo* delivery of siRNA.

Key words: RNA interference, siRNA Delivery, Cancer, Liposome, Chemotherapy

Introduction

Cancer formation is the result of the accumulation of numerous genetic and epigenetic changes giving rise to cells with enhanced growth and survival characteristics. Typically these changes result in the over-expression of oncogenes, making the selective targeting of these genes an attractive means to treat cancer. This can be achieved by using small interfering RNA (siRNA), 21-27 base-pair (bp), double-stranded RNAs, that once introduced into the cytoplasm of cells are highly efficient at gene knockdown at very low concentrations [1]. To date, numerous siRNA targets have been identified in cancer (reviewed in [2]) and the down-regulation of these genes *in vitro* using cationic lipidic vectors often results in reduced cancer cell proliferation [1,3].

Despite the success *in vitro*, the *in vivo* delivery of siRNA has been the major obstacle to its progression into the clinic. Much effort has therefore been devoted to the development of suitable *in vivo* siRNA delivery systems for systemic use. Of these, the PEGylated lipidic systems show promise, with early reports demonstrating their effectiveness in various cancer models [4-6]. The formulation procedures employed in those studies, however, are labour-intensive and require specialised equipments and skills. The resulting end products, being in aqueous states, are also not suitable for

long-term storage. Prompted by these limitations, we recently reported on the development of a simple, yet efficient, Hydration-of-Freeze-Dried-Matrix (HFDM) method to formulate a vector that can protect siRNA from nuclease degradation and can accumulate in tumours after intravenous (i.v.) administration [7]. In addition to the simplicity of the preparation procedure, we also showed that these freeze-dried, lipid-based, formulations are highly stable even after storage at room temperatures for one month.

Here we aim to further characterise these HFDM-formulated siRNA-loaded lipid particles and investigate their biological activities in cervical cancer mouse models. The recently developed prophylactic vaccines, though effective, will not decrease the number of new cervical cancer cases for another 15-20 years [8]. Therefore, there is still an urgent unmet clinical need for new treatments owing to the side effects and low response rate of current treatment strategies [9]. Moreover, the unique nature of the disease makes this an ideal model system for testing siRNA-based cancer therapies, as almost 100% of cases are caused by Human Papilloma Virus (HPV) infection, thereby permitting exclusive targeting of cancer cell while leaving normal healthy tissue alone. This is due to the presence of two essential viral oncogenes, E6 and E7, in cervical cancer cells. These oncogenes disrupt p53 and pRB tumour

suppression pathways in cells, and we and others have shown that targeting E6 and E7 by siRNA *in vitro* results in either cell senescence [1,10,11] or apoptosis [12,13]. To date, the *in vivo* application has been limited to intratumoural injections with significant inhibition of tumour growth reported after single or multiple administrations [14-18]. Though these findings demonstrated the feasibility of using E6/7-targeted siRNA for cervical cancer treatment, the delivery strategies employed in these studies have limited clinical application. We therefore aim to develop a suitable siRNA delivery system for the systemic administration of E6/7-targeted siRNA.

In this report, we first characterised the HFDM-formulated siRNA-loaded lipid particles in terms of stability and circulatory half-life. Importantly, we found that our designed particles are able to effectively deliver siRNA to tumours following intravenous administration, resulting in sequence-specific target gene knockdown. Using these particles, we subsequently investigated the effect of E6/7-targted siRNA in cervical cancer mouse models. The combinational use of siRNA and cisplatin was also examined both *in vitro* and *in vivo*. Overall, our results demonstrated the feasibility of using these HFDM-formulated carriers to systematically administer E6/7-targeted siRNA for the treatment of cervical cancer.

Results

E6/7 siRNA can efficiently reduce cell growth in E6/7-expressing TC-1 cells

As the use of siRNA to reduce E6/7 gene expression in TC-1 cells has not been previously demonstrated, we first examined the effect of E6/7 knockdown in TC-1 cells using cationic DOTAP/DOPE liposomes. TC-1 cells were used in this experiment as they are murine HPV16 E6/7-expressing cells, which allowed us to perform our subsequent animal experiments in immune-competent mice. Similar to our previously published data for HPV 16 CaSki cells [1], E6/7 siRNA treatment resulted in significant knockdown of E6/7 mRNA in TC-1 cells, as measured by quantitative PCR (Figure 1 A). As shown in Figure 1 B, treatment with E6/7 siRNA, but not control siRNA, resulted in a significant decrease in cell viability ($p<0.05$). This is expected as continuous expression of E6/7 is required for the survival of TC-1 cells.

E6/7 siRNA-loaded liposomes prolonged survival of mice bearing lung metastasis tumours

To assess the ability of E6/7 siRNA to inhibit TC-1 tumour growth *in vivo*, we administered cationic liposome-bound E6/7 siRNA intravenously to mice bearing

cervical cancer lung metastasis [19]. We chose to use cationic liposomes to deliver siRNA in this experiment as they have previously been shown to accumulate efficiently in lung tissues following intravenous administration [20]. In this experiment, mice were treated with lipoplexes containing 24µg (1.2 mg/kg) of E6/7 siRNA on days 1, 4 and 7 following tumour cell inoculation. We observed that the mice receiving the E6/7 siRNA treatment had a delayed onset of illness compared to those treated with vehicle only or with control siRNA (Figure 2 A). However, there was no survival advantage for E6/7 siRNA-treated mice compared to mice in the control groups. In contrast, when the treatment dosage was increased to 80µg (4 mg/kg) siRNA per mouse, E6/7 siRNA-treated mice had a significantly prolonged survival ($p<0.005$), with a median survival of 31 days compared to 19, 23.5, or 21 days for vehicle only, control siRNA or empty liposomes treated groups, respectively (Figure 2 B). It must be noted, however, that while this demonstrated the feasibility of using E6/7 siRNA to treat cervical cancer metastasis, the application of this non-PEGylated liposomal delivery system likely limits to the first-pass organs and is not useful for solid tumours on other locations. We therefore subsequently evaluated the feasibility of delivering siRNA to solid cervical cancer tumours using our previously reported HFDM-formulated PEGylated lipidic system [7].

Serum stability and pharamacokinetic (PK) profiles of HFDM-formulated nucleic acid-loaded lipid particles

We have previously established the ability of HFDM-formulated lipid particles to accumulate in subcutaneous tumours following systemic administration [7]. Here we aimed to characterise these particles further via assessing their serum stability as well as their pharmacokinetic profile. As shown in Figure 3 A, in contrast to the non-PEGylated lipoplexes where large aggregates (>3μm) formed immediately after the addition of serum, the size of the HFDM-formulated particles remained constant, even after prolonged incubation with serum at 37°C (Figure 3 B). At the end of 24 hours, the average size of HFDM-formulated particles was less than 200nm, clearly demonstrating the suitability of these particles for systemic applications.

To investigate the pharmacokinetic profile of these HFDM-formulated particles, we monitored serum concentrations of entrapped FITC-labelled oligonucleotides following i.v. administration over time. A rapid distribution phase was observed with the serum concentration dropping to 10% of the initial value within 2 hours, indicating the efficient delivery of these oligonucleotides to various tissues (Figure 4 A). The concentration remained steady after 4 hours and the elimination half-life of our particles was 44 hours. At 24 hours, around 1.9% of the particles were still circulating in the bloodstream. The non-compartmental analysis showed an Area

Under Curve $(AUC)_{0\text{-infinity}}$ of 11.5 hr.ng/µL, Clearance (CL) of 3.4 mL/hr, Steady State Volume of Distribution (Vss) of 219.3 mL and Mean Residence Time (MRT) of 52 hrs.

In vivo gene knockdown efficiency and liver toxicity profile of HFDM-formulated lipid particles

We next examined the tumour delivery efficiency of our siRNA-loaded HFDM-formulated lipid particles by assessing the level of target gene knockdown in tumours following i.v. administration. GFP, instead of E6/7, was chosen as the target gene as its expression level does not interfere with tumour cell survival, thereby allowing a more accurate determination of the knockdown efficiency of our delivery system. As shown in Figure 4 B, GFP messenger RNA (mRNA) level was reduced by almost 50% in GFP-expressing tumours when mice were treated with two doses of particles loaded with 40µg of siRNA directed against GFP. This knockdown was sequence-specific as the lipid particles loaded with control siRNA did not result in a reduction in GFP mRNA.

While this demonstrated the potential use of these particles in cancer therapy, one must also consider their safety profiles *in vivo*. As a significant amount of these

particles accumulated in the liver following i.v. administration [7], we performed an Alanine Transaminase (ALT) enzyme test to detect any obvious adverse effect of these particles in the liver. The result showed that the administration of either 20µg or 40µg of siRNA-entrapped HFDM-formulated lipid particles gave no elevation of ALT enzyme activity at 24 hours compared to vehicle-treated mice (Figure 4 C).

HFDM-formulated E6/7 siRNA-loaded lipid particles are comparable to cisplatin for tumour reduction

Having established that our siRNA-loaded PEGylated lipid particles can accumulate in subcutaneous tumours after i.v. administration and result in sequence-specific gene silencing, we next examined the effect of E6/7-targeted siRNA on tumour burden. We observed a 50% reduction of tumour growth rate for mice which have been treated with three doses of HFDM-formulated E6/7 siRNA-loaded lipid particles compared to control groups ($p < 0.01$) (Figure 5 A). This effect was not observed using the control siRNA, demonstrating the specificity of our siRNA. Importantly, this anti-tumour effect of E6/7 siRNA was found to be highly dependent on the use of our delivery system as naked E6/7 siRNA did not result in any reduction in tumour growth rate (Figure 5 B). Isotonic sucrose solution, used as the vehicle in our formulation, also did not have any effect on tumour growth compared to mock saline

treatment (Figure 5 B). Overall, these results demonstrated the crucial role of our formulation in the successful systemic delivery of siRNA to tumours.

In order to compare the anti-tumour effect mediated by E6/7 siRNA with that of a standard chemotherapy drug commonly used in cervical cancer treatment, we treated mice bearing solid tumours with cisplatin. As shown in Figure 5 C, both cisplatin and E6/7 siRNA resulted in around 50% reduction in tumour size on day 14 after 2-3 treatments. This result was promising as the cisplatin dose used in the experiment ($50 mg/m^2$, assuming body surface area of $36\ cm^2$/mouse) is comparable to current clinical dosing.

Combination treatment of E6/7 siRNA and cisplatin *in vitro* and *in vivo*

As monotherapy is often inadequate in cancer treatment, we investigated the potential combined use of E6/7 siRNA-entrapped lipid particles and cisplatin in cervical cancer treatment. We hypothesised that the combined treatment will have an additive effect in reducing tumour cell growth thus producing a more beneficial outcome. To test our hypothesis, we first examined the effect of this combined treatment *in vitro* with cells receiving E6/7 siRNA treatment first followed by cisplatin (Figure 6 A).

Consistent with our finding in Figure 1 B, treatment of cells with E6/7 siRNA resulted in around 50% reduction in cell viability. As expected, cell viability decreases with the increasing dose of cisplatin, with an IC_{50} around 1.5 μM. There was, however, no obvious added benefit to combined cisplatin/siRNA treatment with similar doses of cisplatin required to produce more than 60% reduction in cell viability. As a few research groups have previously reported that either high E6/7 or low p53 status could render cells more sensitive to cisplatin treatment [21,22], we wondered if treating cells with cisplatin first (to induce DNA damage), followed by E6/7 siRNA (to release p53), would produce a better outcome. However, this reversed treatment order resulted in no improvement in terms of overall reduction in cell viability as shown in Figure 6 B.

These *in vitro* data suggested that treating E6/7-expressing cells with both E6/7 siRNA and cisplatin may not have a greater therapeutic benefit than either treatment alone. We subsequently confirmed this finding *in vivo* where we treated the mice with three doses of E6/7 siRNA and two doses of cisplatin. We found that mice which received both treatments had similar levels of tumour growth inhibition compared to mice which received E6/7 siRNA treatment alone (Figure 6 C). This suggested that while the anti-tumour effect of E6/7 siRNA *in vivo* is comparable to

that of cisplatin (Figure 5 C), there may be limited clinical value in this combined therapy.

Discussion

SiRNAs have enormous potential in cancer treatment but the development of an appropriate delivery system remains a crucial issue for their transition into the clinic. Owing to the complexity of currently available formulation procedures along with the product instability, we have developed a novel yet simple HFDM method to formulate siRNA-loaded particles where the end-products showed superior stability compared to the traditional formulations[7]. Results presented here clearly showed that the HFDM-formulated PEGylated lipidic particles can deliver siRNA to tumours efficiently after systemic administration and subsequently lead to sequence-specific anti-tumour effects in a cervical cancer mouse model. To our knowledge, this is the first paper which describes the systemic use of E6/7-targeted siRNA for treatment of cervical cancer.

As a proof-of-concept experiment, we first examined the effect of E6/7-targeted siRNA using cationic liposomes in a lung metastasis cervical cancer mouse model. Unlike the effect observed at low dosage (1.2 mg/kg/dose) (Figure 2 A), treatment of mice with 80μg of E6/7 siRNA (4mg/kg) for 4 doses resulted in an average of 50% increase in their median survival compared to mice in the control groups (Figure 2 B). While this is encouraging, it must be noted that the application site of this cationic

delivery system would be restricted to first pass organs, such as lungs or liver [23-26]. Its application for treatment of tumours located elsewhere in the body would thus be limited unless extremely high doses were used, as is the case for the studies performed using cationic cardiolipin-based liposomes in subcutaneous breast or prostate cancer mouse models (15 mg/kg/day for 5 days) [27,28]. This, along with the possibility of embolism occurring after administration, would be likely to limit their clinical applications.

In contrast to these non-PEGylated lipid particles, our HFDM-formulated PEGylated particles exhibit much more favourable characteristics for systemic application (Figure 3). The circulatory half life of these HFDM-formulated particles ($T_{1/2}$ $\lambda z >$ 40 Hrs, Figure 4 A) was also found to be much longer than other formulations such as galactosylated cationic liposomes ($T_{1/2}$ $\lambda z <$ 1 Hr) [23] or PEGylated polyplexes ($T_{1/2}$ λz = 1.5 Hrs) [29]. Using these HFDM-formulated particles, we demonstrated a 50% knockdown of the GFP target gene in tumours following systemic administration. This level of knockdown is comparable to many other studies using similar dosage [5,30,31], although some have reported higher gene-silencing efficiency [4,32]. However, it must be noted that the autograft TC-1 tumours employed in our study, as opposed to the xenograft tumour models used in many other studies[31-34], would result in an

underestimation of gene knockdown efficiency. This is due to the fact that the β-actin normalising gene used in our assays would measure expression in all murine cells in the tumour and some of these would be host-derived, support cells that that do not express GFP.

For siRNA to be used successfully as a cancer therapeutic agent, a fine balance between long-circulatory characteristic and the gene-silencing efficiency of the delivery particle is required. The importance of this was demonstrated in a recently published study on neutral lipid-coated wrapsome formulation[6]. While their particles exhibited excellent PK profile, with 20% of the particles still circulating in the bloodstream at 24 hours after administration, it was found that frequent administration of relatively high dose of siRNA (2.5 – 7.5mg/kg, 7-10 doses) was required for effective gene-silencing and anti-tumour effect. In contrast to this, we required much lower doses of siRNA to produce a comparable outcome from our HFDM-formulated particles (2 mg/kg, 3 doses) (Figure 5 A). Indeed, our dosing is comparable to other PEGylated cationic systems such as SNALP (Stable nucleic acid-lipid particles; 2mg/kg, 6 doses) [4] or LPD particles (1.2mg/kg, 3 doses) [5]. As shown in Figure 5 A, with three doses of treatments, there was a 50% decrease in tumour size for E6/7 siRNA-treated mice compared to mice in the control groups.

This result is encouraging as it was not only comparable to other studies [4,35,36], but the anti-tumour effect was also similar to that achieved from cisplatin treatments using a clinically effective dosage (Figure 5 C). However, it is difficult to assess the knockdown of E6/7 in these tumours at the termination of the experiment as the tumour cells which had taken up the E6/7 siRNA would likely have undergone apoptosis during the course of the experiment. Nevertheless, given the successful knockdown of the GFP target gene in tumours with the use of siGFP (Figure 4 B), it is reasonable to conclude that the anti-tumour effect observed following E6/7 siRNA treatments was at least partially contributed to by the specific knockdown of E6 and E7 in tumours. It must be noted, however, that siRNAs were administered into mice when tumour burden was small in our study. Although similar approaches have been employed in other studies [6,32], it is important in the future to establish the optimal treatment time points and dosing schedule for our HFDM-formulated particles in this TC-1 tumour model. This can be achieved via performing a detailed pharmacokinetic and pharmacodynamic study on these particles which will in turn improve our understanding of the clinical applicability of these siRNA-entrapped HFDM-formulated particles.

While our work presents an advance in bringing siRNA forward as a cervical cancer

therapeutic agent, monotherapy in cancer treatment is rarely sufficient to completely eradicate tumour cells in the body. This was evident from our tumour growth data where mice treated with either E6/7 siRNA or cisplatin still have tumours of significant size at the end-point (Figure 5 C). Although increasing the dose of treatment may produce a better outcome, the combination approach presents as a much more attractive treatment option. However, contrary to our previously published data using E6/7 short hairpin RNA (shRNA) and cisplatin treatment in HeLa cells[1], we found no added benefit for this combined approach with siRNA, regardless of the sequence of treatment (Figures 6 A and B). Although one may attribute this finding to the difference in duration of action between siRNA and shRNA, we also showed that several doses of siRNA treatment *in vivo* produced the same outcome (Figure 6 C). At this time, the exact mechanism behind this discrepancy between these two studies is not well understood. Despite this, our results, along with other *in vitro* studies performed using either HPV16 [21] or HPV18-expressing cell lines [22,37], suggest that the knockdown of E6/7 may render these cancer cells more resistant to cisplatin treatment, possibly through p53 pathways [21,22]. While the combination therapy with other forms of chemotherapeutic agent, such as paclitaxel, may be beneficial [21,37], the study performed by Liu and colleagues also indicated that the target sequence of the E6/7 siRNA is crucial for a successful

outcome[37]. Co-administration of siRNAs which target other genes that are also over-expressed in cervical cancer cells, such as epidermal growth factor receptor (EGFR) [5,38] or Bcl-2 [39,40], may also be beneficial. Overall, the choice of siRNA target site, type and dose of chemotherapeutic agent used as well as treatment time points should all be taken into consideration when designing a better treatment regimen. Our development of effective and easy-to-prepare delivery particles for siRNA delivery will allow faster screening for *in vivo* application and enable rapid clinical translation.

In conclusion, this is the first paper describing the systemic use of E6/7 siRNA for treatment of cervical cancer. We showed for the first time that the Hydration of Freeze-Dried Matrix (HFDM) method is a feasible way to formulate stable and efficient siRNA delivery particles which result in knockdown of the target gene in tumours following systemic administration. Using these particles, we demonstrated a sequence-specific anti-tumour effect in a subcutaneous cervical cancer model. While further studies are required to fully investigate the pharmacokinetic/pharmacodynamic profiles of our lipidic particles as well as their potential to induce cytokines following administration, the simplicity of preparation and the superior product stability are major advancements for the *in vivo* application

of siRNA. Our technology offers an innovative approach for formulating siRNA as effective cancer therapeutics and can also be applied to other forms of cancer.

Materials and Methods

Materials

Dioleoyl trimethylammonium propane (DOTAP) and cholesterol were purchased from Sigma (St Louis, MO). Polyethylene Glycol $(PEG)_{2000}$-C16Ceramide conjugate was from Avanti Polar Lipids (Alabaster, AL) and dioleoylphosphatidylethanolamine (DOPE) was from Northern Lipids (Vancouver, Canada).

Control oligodeoxynucleotides (ODN) with sense sequence of 5'-GTCAGAAATAGAAACTGGTCATC-3' and antisense sequence of 5'-GATGACCAGTTTCTATTTCTGAC-3' were obtained from Invitrogen (Carlbad, CA). SiRNA which targets both E6 and E7 (E6 and E7 are produced by the same transcriptional unit) (5'-GCAACAGUUACUGCGACGUUU-3'; 5'-ACGUCGCAGUAACUGUUGCUU-3') was obtained from Sigma-Aldrich (St Louis, MO) in annealed form. Similarly, Green fluorescent protein (GFP) targeted siRNA (5'-GCACGACUUCUUCAAGUCCUU-3'; 5'-GGACUUGAAGAAGUCGUGCUU-3') and control siRNA (5'-UUAUGCCGAUCGCGUCACAUU-3'; 5'-UGUGACGCGAUCGGCAUAAUU-3') were also purchased from Sigma-Aldrich.

TC-1 cells (murine C57B/6 lung epithelial cells transformed with HPV 16 E6/7 and ras oncogenes) were obtained from TC Wu [41]. TC-1 GFP^{+ve} cells were prepared according to the protocol described in Gu *et al* [42]. All cells were cultured and maintained in Dulbecco's Modified Eagle Media (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bovoge, Keilor East, Australia), 0.2% primocin (Invivogen, San Diego, CA) and 2mM L-glutamine (Invitrogen). Primocin was included in the media in order to prevent mycoplasma infection of the cells.

All other chemicals and solvents used were of at least analytical grade.

Methods

Liposome Formulations

Non-PEGylated Lipoplexes

DOTAP/DOPE (1:1 molar ratio) liposomes were prepared using the hydration of lipid film method as described previously [43]. Briefly, the required amount of lipids were dissolved in chloroform in a round-bottomed flask. Chloroform was then removed under low pressure using a rotary evaporator and a lyophiliser (ALPHA 1-2 LDplus,

Martin Christ, Germany). Dried lipid film was hydrated using sterile 5% dextrose solution with the final liposome concentration being 5mM. Liposomes were subsequently extruded through 800nm x 5, 400nm x 5, 200nm x 5, 100nm x 5 and 50nm x 5 Nucleopore™ track-etched membranes using Lipex™ extruder under nitrogen pressure of up to 500psi. Extruded liposomes were left at room temperature for an hour before being stored at 4°C. Liposomes were then complexed with the required amount of nucleic acids at an Nitrogen:Phosphate (N:P) ratio of 4 immediately before *in vitro* or *in vivo* studies.

HFDM-formulated Nucleic acids-Entrapped PEGylated lipid particles

Nucleic acid-entrapped PEGylated lipid particles were prepared as previously described [7]. Briefly, required amounts of DOTAP, cholesterol, and PEG_{2000}-C16Ceramide with or without DOPE were mixed with oligonucleotides or siRNA at an N:P ratio of 4 in a sucrose-containing water/*tert*-butanol (1:1 *v*/*v*) co-solvent system. DOTAP, cholesterol, DOPE and PEG_{2000}-C16Ceramide with a molar ratio of 50:35:5:10 was used. The mixture was then snap-frozen and freeze-dried overnight. Freeze-dried matrix was then hydrated with sterile water immediately before use so that the final product contained 40µg oligonucleotides or siRNA in 300µL of isotonic sucrose solution.

In Vitro Studies

siRNA Transfection Study and Gene-knockdown Analysis

TC-1 cells were seeded the day before the transfection experiment at a density of 100 000 cells/well in a 6-well plate. DOTAP/DOPE liposomes were used to complex with HPV 16 E6/7 siRNA and control siRNA at an N:P ratio of 4. One mL of 40nM liposome-entrapped siRNA suspended in antibiotic-free complete Dulbecco's Modified Eagle Media (DMEM) was then added to each well. SiRNAs were left on cells for 8 hours and cells were then incubated in primocin-containing DMEM culture media overnight at 37°C.

After overnight incubation, total RNA was isolated from the cells using Trizol reagent (Invitrogen) according to manufacturer's protocol. RNA (2μg) was subsequently reverse-transcribed using Omniscript RT kit (Qiagen, Victoria, Australia). Real-time Polymerase Chain Reaction (PCR) was performed on a Corbett Rotor-Gene 3000 (Qiagen) using 0.1μL of complementary DNA (cDNA) for each sample or the comparable amount of RNA extract with no addition of reverse transcriptase. A standard curve was also generated using 0.002-0.1 μL of cDNA per reaction and all reactions were performed in 20μL in triplicates. SYBR green (Applied Biosystems,

Warrington, UK) was used to detect products and 200nM of the following primers were used: E6/7 forward 5'-AAGCAACAGTTACTGCGACGTG-3', E6/7 reverse 5'-GCCCATTAACAGGTCTTCCAAA-3', β-actin forward 5'-GCTACAGCTTCACCACCACA-3' and β-actin reverse 5'-TCTCCAGGGAGGAAGAGGAT-3'. PCR was set at 94°C initially for 10 minutes, followed by 40 cycles of 95°C x 10s, 60°C x 15s and 72°C x 20s. Results were analysed using Rotor-Gene 6000 series software (Qiagen). The relative amount of cDNA in each sample was normalised using β-actin and melt curve was used to verify specificity.

Cell Viability Assay

TC-1 cells were seeded in a 48-well plate at a seeding density of 3000 cells/well the day before the experiment. HPV16 E6/7 siRNA or control siRNA was complexed with DOTAP/DOPE liposomes at an N:P ratio of 4. One hundred μL of 40nM siRNA-containing lipoplexes or empty liposomes suspended in OptiMem™ was then applied to each well. After 4 hours of incubation, cells were then washed with phosphate-buffered saline (PBS) and were incubated in primocin-containing complete DMEM media for 4 days. All treatment was performed in triplicate (n=3). Cell

Titre Glo™ cell viability assay was subsequently performed according to manufacturer's instructions.

For cisplatin experiments, cells were treated with siRNA and then cisplatin, or in the reverse order. SiRNA was applied to cells as described above and cells were treated with cisplatin (Sigma) at various concentrations from 0 to 50µM in primocin-containing DMEM complete media (250µL/well) for 24 hours. Cell Titre Glo™ viability assay was performed at the end of day 4 and each treatment condition was performed in quadruplicate (n=4).

Serum Stability Study

Non-PEGylated and HFDM-formulated PEGylated liposome formulations (100 µL) which contained 8µg of siRNA were incubated with 60µL of non-heat inactivated fetal bovine serum (FBS) at 37°C. At various time points, samples were mixed with 4 mL of filtered distilled water and the particle size was measured by photon correlation spectroscopy using a Zetasizer 3000™ (Malvern Instruments, Malvern, UK). All measurements were carried out at room temperature and three samples were measured at each time point (n=3).

Animal Studies

All animal experiments were approved by the University of Queensland Animal Ethics Committee and two-month-old female C57B/6 mice (Perth, ARC) were used in all studies.

Pharmacokinetics (PK) Study of HFDM-formulated Particles

Mice were injected with HFDM-formulated lipid particles loaded with 40µg of fluorescein (FITC)-labelled oligonucleotides. At different time points, around 50µL of blood was collected from mice and serum was isolated as previously described [44]. FITC-oligonucleotdies were extracted from liposomes using the method described previously [5] and the fluorescence intensity of the samples was measured using a Fluostar® plate reader at excitation and emission wavelengths of 485nm and 520nm, respectively. FITC-oligonucleotide concentration in each sample was calculated from a standard curve. Four to five mice were used in this experiment (n = 4-5) and the PK profile was analysed using non-compartmental methods using WinNonlin Professional (version 5.2). The key PK parameters provided from this analysis were: area under curve (AUC), clearance (CL), mean residence time (MRT), and steady state volume of distribution (Vss), and terminal phase half life was extrapolated from the last five time points.

In Vivo Gene-silencing Experiment

Subcutaneous TC-1 GFP^{+ve} tumours were established in mice as described above. On day 13 and 14 post tumour cell inoculation, mice were treated intravenously with either saline, HFDM-formulated lipid particles containing 40μg of either GFP targeted siRNA, or control siRNA. Tumours were subsequently harvested on day 15 and RNA was isolated using Trizol™ reagent (Invitrogen) according to manufacturer's protocol.

RNA (2μg) isolated from the tumours was reverse-transcribed using an Omniscript RT kit (Qiagen, Victoria, Australia). Real-time PCR analysis was performed as described earlier. The following primers were used: GFP forward 5'-ACGTAAACGGCCACAAGTTC-3', GFP reverse 5'-GGTCTTGTAGTTGCCGTCGT-3', β-actin forward 5'-GCTACAGCTTCACCACCACA-3' and β-actin reverse 5'-TCTCCAGGGAGGAAGAGGAT-3'.

Liver Toxicity Study

Tumour-free mice were treated intravenously with either isotonic sucrose solution or HFDM-formulated lipid particles containing 20µg or 40 µg of siRNA. Similar to other reported studies [23,33,45,46], blood was collected from mice at 24 hours after the administration and three mice were used for each treatment group (n=3). Serum was then isolated from the samples as previously described and samples were stored at 4°C before analysis. The assay was performed using Alanine transaminase (ALT) detection kit (Bioo Scientific, Austin, TX) according to manufacturer's protocol. All samples were tested in triplicate and ALT enzyme (12.5 ng/µL, Roche Diagnostics, Mannheim, Germany) was used as a positive control to test the validity of the assay.

In vivo Tumour Growth Inhibition Study

For the lung metastasis study, 0.5 million TC-1 cells suspended in 100µL of sterile PBS were injected intravenously into each mouse. On day 1, 4, 7 and 11 after tumour cell injection, mice were treated with 5% dextrose solution, empty DOTAP/DOPE liposomes, liposome-complexed E6/7 siRNA or control siRNA. Liposome-siRNA formulations were prepared at a concentration no greater than 24µg siRNA/300µL at an N:P ratio of 4 to prevent particle aggregations. For mice receiving 24 µg of siRNA per dose, the prepared formulation was administered intravenously into each mouse without further volume reduction step. When higher

siRNA dosage was required, formulations were concentrated by centrifugation at 1000g using a 100K Ultra-free centrifugation filter (Millipore, New South Wales, Australia). The final formulation contained 80μg of siRNA in 200 μL and was administered to each mouse. Six to nine mice were used per treatment group and the health of the mice was monitored over the course of the study. All mice were sacrificed when they showed signs of illness, such as rough hair coat, hunched posture, laboured breathing, or significant weight loss.

For the subcutaneous tumour study, one million TC-1 cells suspended in 100μL of sterile PBS were injected subcutaneously into each mouse on the right abdominal side. On days 3, 7 and 10 after tumour cell inoculation, mice were treated with saline, vehicle only, naked E6/7 siRNA or HFDM-formulated lipid particles containing E6/7 siRNA or control siRNA. All formulations were prepared at an N:P ratio of 4, and 40μg of siRNA was administered intravenously per dose. For the cisplatin experiment, 90μg of cisplatin suspended in 200μL PBS was administered into each mouse via intraperitoneal injection on day 8 and 11 after tumour cell inoculation. PBS was used as a negative control. Five to six mice were used per treatment group (n=5-6) and tumour size was monitored using callipers during the course of the experiment.

Statistical Analysis

All data was analysed using GraphPad Prism™ software and the student *t*-test was performed to assess the difference between treatment and control groups. For survival studies, the Mantel-Cox log-rank test was employed.

Acknowledgements

This work was funded by National Health and Medical Research Council (NHMRC). The authors would like to thank Danielle Wilson, Claudia Cueva and Kim Woolley for their assistance in animal studies.

Conflict of interest

The authors declare no conflict of interest.

References

1. Putral LN, Bywater MJ, Gu W, Saunders NA, Gabrielli BG, Leggatt GR *et al*. RNA interference against human papillomavirus oncogenes in cervical cancer cells results in increased sensitivity to cisplatin. *Mol Pharmacol* 2005; **68:** 1311-1319.
2. Putral L, Gu W, McMillan N. RNA interference for the treatment of cancer. *Drug News Perspect* 2006; **19:** 317-324.
3. Wang W, Wang CY, Dong JH, Chen X, Zhang M, Zhao G. Identification of effective siRNA against K-ras in human pancreatic cancer cell line MiaPaCa-2 by siRNA expression cassette. *World J Gastroenterol* 2005; **11:** 2026-2031.
4. Judge AD, Robbins M, Tavakoli I, Levi J, Hu L, Fronda A *et al*. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest* 2009; **119:** 661-673.
5. Li SD, Chen YC, Hackett MJ, Huang L. Tumor-targeted delivery of siRNA by self-assembled nanoparticles. *Mol Ther* 2008; **16:** 163-169.
6. Yagi N, Manabe I, Tottori T, Ishihara A, Ogata F, Kim JH *et al*. A nanoparticle system specifically designed to deliver short interfering RNA inhibits tumor growth in vivo. *Cancer Res* 2009; **69:** 6531-6538.

7. Wu SY, Putral LN, Liang M, Chang HI, Davies NM, McMillan NA. Development of a Novel Method for Formulating Stable siRNA-Loaded Lipid Particles for In vivo Use. *Pharm Res* 2009; **26:** 512-522.

8. Wright TC, Van Damme P, Schmitt H-J, Meheus A. Chapter 14: HPV vaccine introduction in industrialized countries. *Vaccine* 2006; **24:** S122-131.

9. Moore DH. Chemotherapy for advanced, recurrent, and metastatic cervical cancer. *J Natl Compr Canc Netw* 2008; **6:** 53-57.

10. Jiang M, Milner J. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* 2002; **21:** 6041-6048.

11. Hall AHS, Alexander KA. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. *J Virol* 2003; **77:** 6066-6069.

12. Butz K, Ristriani T, Hengstermann A, Denk C, Scheffner M, Hoppe-Seyler F. siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. *Oncogene* 2003; **22:** 5938-5945.

13. Lea JS, Sunaga N, Sato M, Kalahasti G, Miller DS, Minna JD *et al*. Silencing of HPV 18 oncoproteins With RNA interference causes growth inhibition of cervical cancer cells. *Reprod Sci* 2007; **14:** 20-28.

14. Fujii T, Saito M, Iwasaki E, Ochiya T, Takei Y, Hayashi S *et al*. Intratumor injection of small interfering RNA-targeting human papillomavirus 18 E6 and E7 successfully inhibits the growth of cervical cancer. *Int J Oncol* 2006; **29:** 541-548.

15. Niu X, Peng Z, Duan W, Wang H, Wang P. Inhibition of HPV 16 E6 oncogene expression by RNA interference in vitro and in vivo. *Int J Gynecol Cancer* 2006; **16:** 743-751.

16. Viel T, Boisgard R, Kuhnast B, Jego B, Siquier-Pernet K, Hinnen F *et al*. Molecular imaging study on in vivo distribution and pharmacokinetics of modified small interfering RNAs (siRNAs). *Oligonucleotides* 2008; **18:** 201-212.

17. Jonson AL, Rogers LM, Ramakrishnan S, Downs LS, Jr. Gene silencing with siRNA targeting E6/E7 as a therapeutic intervention in a mouse model of cervical cancer. *Gynecol Oncol* 2008; **111:** 356-364.

18. Yamato K, Yamada T, Kizaki M, Ui-Tei K, Natori Y, Fujino M *et al*. New highly potent and specific E6 and E7 siRNAs for treatment of HPV16 positive cervical cancer. *Cancer Gene Ther* 2008; **15:** 140-153.

19. Ji H, Chang EY, Lin KY, Kurman RJ, Pardoll DM, Wu TC. Antigen-specific immunotherapy for murine lung metastatic tumors expressing human papillomavirus type 16 E7 oncoprotein. *Int J Cancer* 1998; **78:** 41-45.

20. Garbuzenko OB, Saad M, Betigeri S, Zhang M, Vetcher AA, Soldatenkov VA *et al*. Intratracheal versus intravenous liposomal delivery of siRNA, antisense oligonucleotides and anticancer drug. *Pharm Res* 2009; **26:** 382-394.

21. Koivusalo R, Krausz E, Helenius H, Hietanen S. Chemotherapy compounds in cervical cancer cells primed by reconstitution of p53 function after short interfering RNA-mediated degradation of human papillomavirus 18 E6 mRNA: opposite effect of siRNA in combination with different drugs. *Mol Pharmacol* 2005; **68:** 372-382.

22. Liu ZG, Zhao LN, Liu YW, Li TT, Fan DM, Chen JJ. Activation of Cdc2 contributes to apoptosis in HPV E6 expressing human keratinocytes in response to therapeutic agents. *J Mol Biol* 2007; **374:** 334-345.

23. Sato A, Takagi M, Shimamoto A, Kawakami S, Hashida M. Small interfering RNA delivery to the liver by intravenous administration of galactosylated cationic liposomes in mice. *Biomaterials* 2007; **28:** 1434-1442.

24. Abrams MT, Koser ML, Seitzer J, Williams SC, Dipietro MA, Wang W *et al*. Evaluation of Efficacy, Biodistribution, and Inflammation for a Potent siRNA

Nanoparticle: Effect of Dexamethasone Co-treatment. *Mol Ther* 2009; **18:** 171-180.

25. Sorensen D, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J Mol Biol* 2003; **327:** 761-766.

26. Yano J, Hirabayashi K, Nakagawa S, Yamaguchi T, Nogawa M, Kashimori I *et al*. Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. *Clin Cancer Res* 2004; **10:** 7721-7726.

27. Chien P, Wang J, Carbonaro D, Lei S, Miller B, Sheikh S *et al*. Novel cationic cardiolipin analogue-based liposome for efficient DNA and small interfering RNA delivery in vitro and in vivo. *Cancer Gene Ther* 2005; **12:** 321-328.

28. Pal A, Ahmad A, Khan S, Sakabe I, Zhang CB, Kasid UN *et al*. Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer. *Int J Oncol* 2005; **26:** 1087-1091.

29. Merkel OM, Librizzi D, Pfestroff A, Schurrat T, Buyens K, Sanders NN *et al*. Stability of siRNA polyplexes from poly(ethylenimine) and poly(ethylenimine)-g-poly(ethylene glycol) under in vivo conditions: effects on pharmacokinetics and biodistribution measured by Fluorescence

Fluctuation Spectroscopy and Single Photon Emission Computed Tomography (SPECT) imaging. *J Control Release* 2009; **138:** 148-159.

30. Santel A, Aleku M, Keil O, Endruschat J, Esche V, Durieux B *et al*. RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy. *Gene Ther* 2006; **13:** 1360-1370.

31. Bartlett DW, Su H, Hildebrandt IJ, Weber WA, Davis ME. Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging. *Proc Natl Acad Sci U S A* 2007; **104:** 15549-15554.

32. Villares GJ, Zigler M, Wang H, Melnikova VO, Wu H, Friedman R *et al*. Targeting melanoma growth and metastasis with systemic delivery of liposome-incorporated protease-activated receptor-1 small interfering RNA. *Cancer Res* 2008; **68:** 9078-9086.

33. Medarova Z, Pham W, Farrar C, Petkova V, Moore A. In vivo imaging of siRNA delivery and silencing in tumors. *Nat Med* 2007; **13:** 372-377.

34. Crombez L, Morris MC, Dufort S, Aldrian-Herrada G, Nguyen Q, Mc Master G *et al*. Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. *Nucleic Acids Res* 2009; **37:** 4559-4569.

35. Santel A, Aleku M, Keil O, Endruschat J, Esche V, Fisch G *et al*. A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene Ther* 2006; **13:** 1222-1234.

36. Sonoke S, Ueda T, Fujiwara K, Sato Y, Takagaki K, Hirabayashi K *et al*. Tumor regression in mice by delivery of Bcl-2 small interfering RNA with pegylated cationic liposomes. *Cancer Res* 2008; **68:** 8843-8851.

37. Liu WL, Green N, Seymour LW, Stevenson M. Paclitaxel combined with siRNA targeting HPV16 oncogenes improves cytotoxicity for cervical carcinoma. *Cancer Gene Ther* 2009; **16:** 764-775.

38. Bonner J, Harari P, Giralt J, Azarnia N, Cohen R, D R *et al*. Cetuximab prolongs survival in patients with locoregionally advanced squamous cell carcinoma of head and neck: A phase III study of high dose radiation therapy with or without cetuximab *J Clin Oncol* 2004; **22:** Abstract 5507.

39. Hagemann T, Bozanovic T, Hooper S, Ljubic A, Slettenaar VI, Wilson JL *et al*. Molecular profiling of cervical cancer progression. *Br J Cancer* 2007; **96:** 321-328.

40. Manavi M, Hudelist G, Fink-Retter A, Gschwandtler-Kaulich D, Pischinger K, Czerwenka K. Gene profiling in Pap-cell smears of high-risk human

papillomavirus-positive squamous cervical carcinoma. *Gynecol Oncol* 2007; **105:** 418-426.

41. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM *et al*. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996; **56:** 21-26.

42. Gu W, Putral L, Hengst K, Minto K, Saunders NA, Leggatt G *et al*. Inhibition of cervical cancer cell growth in vitro and in vivo with lentiviral-vector delivered short hairpin RNA targeting human papillomavirus E6 and E7 oncogenes. *Cancer Gene Ther* 2006; **13:** 1023-1032.

43. Lee CH, Ni YH, Chen CC, Chou CK, Chang FH. Synergistic effect of polyethylenimine and cationic liposomes in nucleic acid delivery to human cancer cells. *Biochim Biophys Acta* 2003; **1611:** 55-62.

44. Sakurai F, Nishioka T, Saito H, Baba T, Okuda A, Matsumoto O *et al*. Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. *Gene Ther* 2001; **8:** 677-686.

45. Ito Y, Kawakami S, Charoensit P, Higuchi Y, Hashida M. Evaluation of proinflammatory cytokine production and liver injury induced by plasmid

DNA/cationic liposome complexes with various mixing ratios in mice. *Eur J Pharm Biopharm* 2009; **71:** 303-309.

46. Charoensit P, Kawakami S, Higuchi Y, Hashida M. Incorporation of all-trans retinoic acid into lipoplexes inhibits nuclear factor kappaB activation mediated liver injury induced by lipoplexes in mice. *J Gene Med* 2008; **10:** 61-69.

Figure Legends

Figure 1 Effect of E6/7-targeted siRNA in HPV16 E6/7-expressing TC-1 cells. (A) E6/7 siRNA which was delivered using DOTAP/DOPE liposomes reduced E6/7 mRNA level by 70% in TC-1 cells. Real-time PCR analysis was performed at 24 hours post-treatment. **p<0.01, significantly different compared to control siRNA treatment. (B) Decreased TC-1 cell viability was observed after E6/7 siRNA treatment when delivered using DOTAP/DOPE liposomes. This decrease was not observed when cells were treated with control siRNA or empty liposomes. Tests were performed in triplicate four days after treatment using Cell Titre Glo™ assay. * p<0.05, significantly different to control siRNA treatment. All bars and error bars represent the mean value and the corresponding SEM.

Figure 2 Treatment of lung metastasis using cationic liposome complexed E6/7-targeted siRNA. (A) Mice treated with three doses of 24µg of E6/7 siRNA on day 1, 4 and 7 after TC-1 cell inoculation had delayed onset of illness but had no significant improvement in overall survival compared to mice treated with controls. Six to eight mice were used per treatment group (n=6-8). (B) In contrast, mice treated with 80µg of E6/7 siRNA for 4 doses on day 1, 4, 7 and 11 after tumour cell

inoculation showed prolonged survival compared to mice in all three control groups. **$P < 0.005$, significantly different from control groups ($n = 7\text{-}9$).

Figure 3 Characteristics of non-PEGylated and HFDM-formulated PEGylated siRNA-loaded lipid particles in the presence of serum. (A) Cationic DOTAP/DOPE lipoplexes formulated at an N:P ratio of 4 aggregated rapidly after incubation with serum at 37°C. In contrast, the size of HFDM-formulated lipid particles (N:P 4) remained constant even after 24 hours of incubation in serum (B). Three samples were prepared for each treatment condition and time point (n=3) and Z_{ave} particle size was recorded from photon correlation spectroscopy analysis. Each point and error bar represents the mean value and the corresponding SD.

Figure 4 Characteristics and effect of nucleic acid-loaded HFDM-formulated lipid particles *in vivo*. (A) Serum concentration profile of HFDM-formulated FITC-Oligonucleotide-loaded lipid particles after i.v. administration. Points and error bars represent the mean and SEM, respectively, from samples collected from 4 to 5 mice at different time points. (B) Knockdown of GFP level was observed in tumours after i.v. administration of siGFP-loaded HFDM-formulated lipid particles to mice bearing subcutaneous GFP-expressing tumours. Treatment was performed on

days 13 and 14 after tumour cell inoculation and GFP mRNA levels were measured on day 15 using real-time PCR analysis. Tests were performed in triplicate and five to six mice were used per treatment group (n = 5-6). (C) Liver toxicity study of siRNA-loaded HFDM-formulated lipid particles. No elevation of serum ALT activity was detected at 24 hours after i.v. treatment of lipid particles containing either 20μg or 40μg of siRNA. Three mice were used per treatment group (n=3) and tests were performed in triplicate. Each bar and error bar represent the mean value and the corresponding SEM. ALT enzyme (12.5 ng/μL) was used as a positive control.

Figure 5 Inhibition of subcutaneous tumour growth in C57B/6 mice by E6/7-targeted siRNA. All treatments were administered on days 3, 7 and 10 post tumour cell inoculation and 40μg siRNA was used per dose. (A) Mice treated with E6/7 siRNA formulated in HFDM-formulated lipid particles had significant reduced tumour size compared to mice treated with saline or with control siRNA. The error bars represent the SEMs of tumour size of 5 mice. ** $p<0.01$, significantly different from saline or control siRNA treated groups. (B) Distribution of tumour size on day 16 for mice treated with saline, vehicle only or naked E6/7 siRNA. No difference in tumour size was observed between three treatment groups. (C) E6/7 siRNA formulated in lipid particles produced similar tumour growth inhibition effect

compared to two doses of cisplatin (90µg/dose/mouse) treatments on day 8 and 11. Tumour size was measured on day 14 of the experiment. *$p<0.05$, significantly different from vehicle or control siRNA treated group.

Figure 6 Combined treatment of E6/7 siRNA and cisplatin *in vitro* and *in vivo*. Treatment of TC-1 cells with E6/7 siRNA and cisplatin did not result in greater reduction of cell viability than cells treated with either treatment alone. This was irrespective of the sequence of treatment, with similar results observed when cells were treated with E6/7 siRNA first followed by cisplatin (A) or in the reverse order (B). SiRNAs (40nM) and cisplatin (0-50µM) were applied to cells for 4 hours and 24 hours, respectively. All tests were performed in quadruplicate on day 4 using Cell Titre Glo^{TM} assay and the error bars represent the SEMs (n=4). (C) No enhanced tumour growth inhibition effect was observed when mice bearing subcutaneous tumours were treated with both 40µg E6/7 siRNA and 90µg cisplatin. Mice were treated with siRNA on day 3, 7 and 10 and cisplatin on day 8 and 11 (n = 5-6). Tumour size was measured on day 14 of the experiment. * $p<0.05$, significantly different compared to vehicle-treated group.

(A)

Relative E6/7 mRNA Level

150
100
50
0

DOTAP LP

**

Control
E6/7 siRNA
Control siRNA

(B)

% Cell Viability

150
100
50
0

DOTAP LP

*

Control
E6/7 siRNA
Control siRNA
Empty LP

(A)

100
80
60
40
20
0
Percent survival
0 10 20 30
Days post i.v. inj of TC-1 cells

Vehicle
LP-E6/7 siRNA
LP-Control siRNA

i.v. siRNA inj

(B)

100
80
60
40
20
0
Percent survival
**
0 5 10 15 20 25 30 35
Days post i.v. inj of TC-1 cells

Vehicle Only
LP-E6/7 siRNA
LP-Control siRNA
Empty LP

i.v. siRNA inj

(A)

Serum

H_2O

1 min

15 mins

4000
3500
3000
2500
500
400
300
200
100
0

Particle size (nm)

Non-PEGylated LP

HFDM-formulated LP

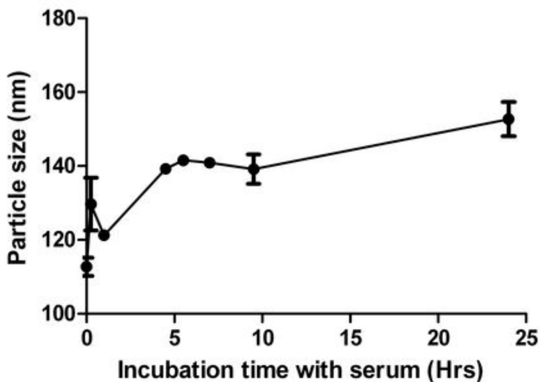
Non-PEGylated LP

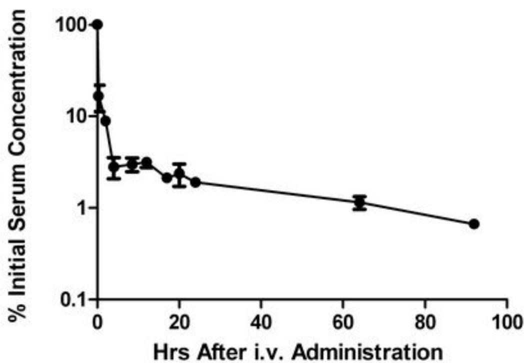
HFDM-formulated LP

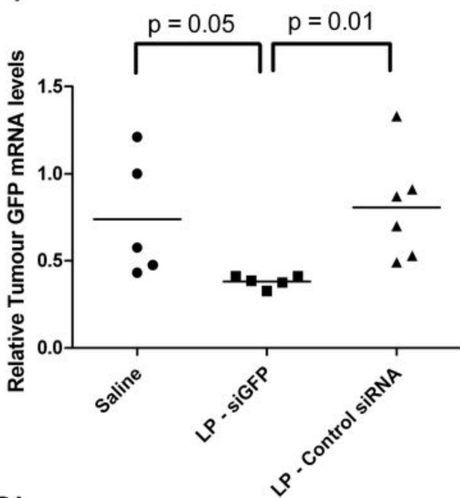
Non-PEGylated LP

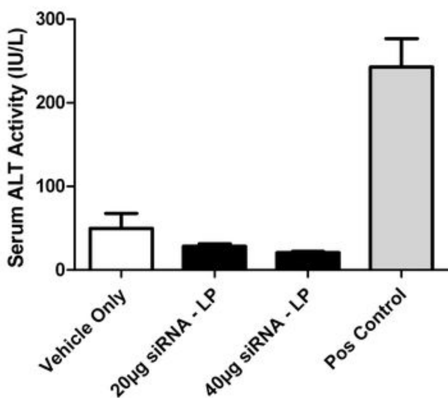
HFDM-formulated LP

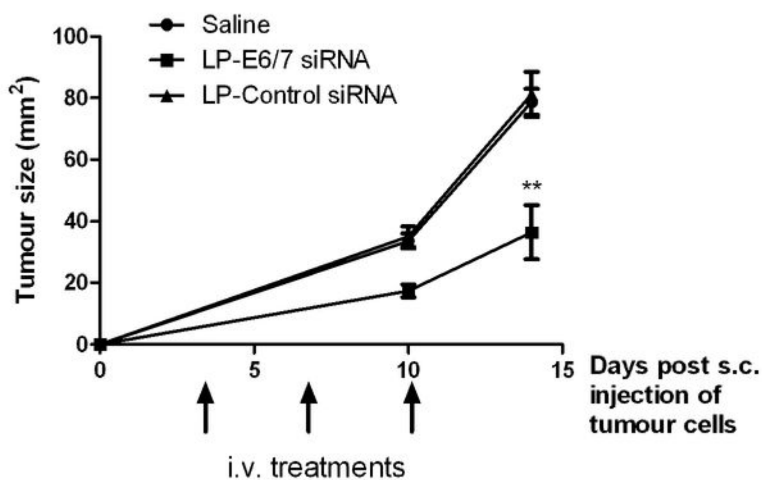
(B)

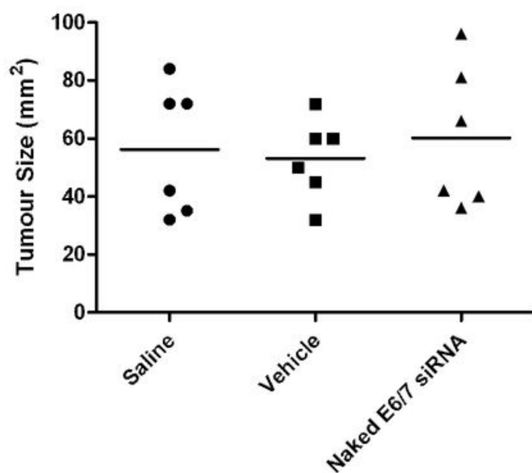


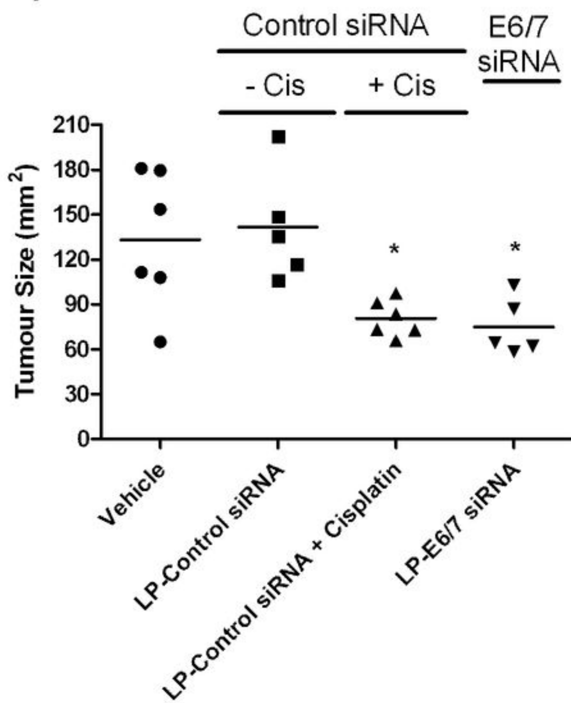

(A)
% Initial Serum Concentration
100
10
1
0.1
0
20
40
60
80
100
Hrs After i.v. Administration

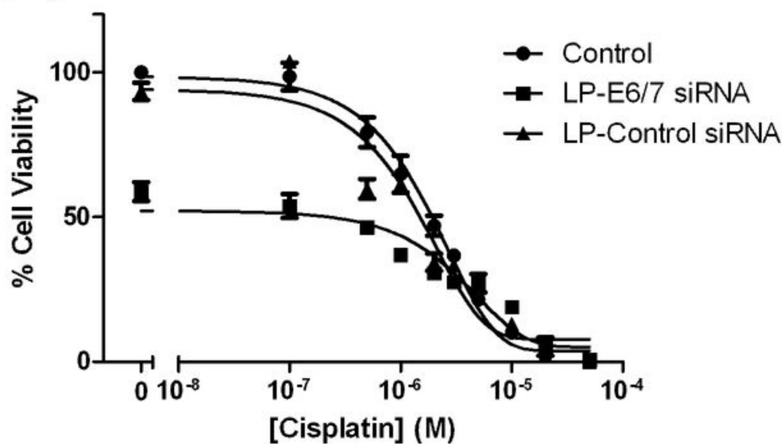

(B)
p = 0.05
p = 0.01
Relative Tumour GFP mRNA levels
1.5
1.0
0.5
0.0
Saline
LP - siGFP
LP - Control siRNA

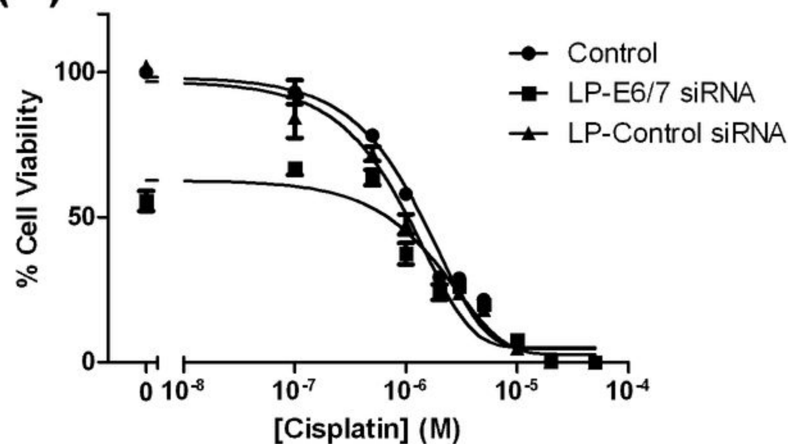

(C)
Serum ALT Activity (IU/L)
300
200
100
0
Vehicle Only
20μg siRNA - LP
40μg siRNA - LP
Pos Control

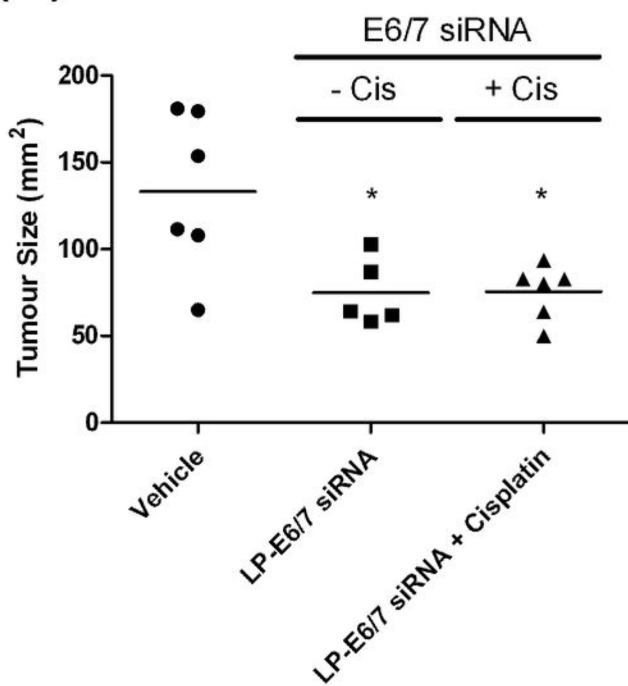

(A)
Saline
LP-E6/7 siRNA
LP-Control siRNA
Tumour size (mm2)
100
80
60
40
20
0
**
0
5
10
15
Days post s.c. injection of tumour cells
i.v. treatments


(B)
Tumour Size (mm2)
100
80
60
40
20
0
Saline
Vehicle
Naked E6/7 siRNA


(C)
Control siRNA
- Cis
+ Cis
E6/7 siRNA
Tumour Size (mm2)
210
180
150
120
90
60
30
0
*
*
Vehicle
LP-Control siRNA
LP-Control siRNA + Cisplatin
LP-E6/7 siRNA


(A)
% Cell Viability
100
50
0
0 10-8
10-7
10-6
10-5
10-4
[Cisplatin] (M)
Control
LP-E6/7 siRNA
LP-Control siRNA


(B)
% Cell Viability
100
50
0
0 10-8
10-7
10-6
10-5
10-4
[Cisplatin] (M)
Control
LP-E6/7 siRNA
LP-Control siRNA


(C)
E6/7 siRNA
- Cis
+ Cis
Tumour Size (mm2)
200
150
100
50
0
*
*
Vehicle
LP-E6/7 siRNA
LP-E6/7 siRNA + Cisplatin