

Design of Cationic Multi-Walled Carbon Nanotubes as Efficient siRNA Vectors for Lung Cancer Xenograft EradicationChang Guo, Wafa T Al-Jamal, Francesca M Toma, Alberto Bianco,
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Design of Cationic Multi-Walled Carbon Nanotubes as Efficient siRNA Vectors for Lung Cancer Xenograft Eradication

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ABSTRACT

Polo-Like Kinase (PLK1) has been identified as a potential target in cancer gene therapy via chemical or genetic inhibitory approaches. The biomedical applications of chemically functionalized carbon nanotubes (*f*-CNTs) in cancer therapy have been studied due to their ability to efficiently deliver siRNA intracellularly. In this study, we established the capacity of cationic MWNT-NH₃⁺ to deliver the apoptotic siRNA against PLK1 (siPLK1) in Calu6 tumor xenografts by direct intratumoural injections. A direct comparison with cationic liposomes was made. This study validates the PLK1 gene as a potential target in cancer gene therapy including lung cancer, as demonstrated by the therapeutic efficacy of siPLK1:MWNT-NH₃⁺ complexes and their ability to significantly improve animal survival. Biological analysis of the siPLK1:MWNT-NH₃⁺ treated tumors by RT-PCR and Western blot, in addition to TUNEL staining confirmed the biological functionality of the siRNA intratumorally, suggesting that tumor eradication was due to PLK1 knockdown. Furthermore, by using a fluorescently labeled, non-coding siRNA sequence complexed with MWNT-NH₃⁺, we established for the first time that the improved therapeutic efficacy observed in *f*-CNT-based siRNA delivery is directly proportional to the enhanced siRNA retention in the solid tumor and subsequent uptake by tumor cells after local administration *in vivo*.

INTRODUCTION

Over the past decade, there has been an exponential growth in research combining nanotechnology tools and cancer therapy. One of nanomaterials are carbon nanotubes (CNTs) which based on their structure are classified into two categories, single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) made of single or several graphene layers rolled up into cylindrical structures, respectively. The capability of surface modification of CNTs offers an improved aqueous dispersibility hence broadening their biomedical applications, including their use as a delivery vector for various molecules in cancer therapy. Functionalized CNT (*f*-CNT) have been explored in almost every single modality used in cancer therapy including photothermal therapy, chemotherapy and gene therapy.¹

In comparison to traditional chemotherapy or radiotherapy, gene therapy offers higher specificity to regulate diseases of disordered genes origin, including cancer. The induction of apoptosis has demonstrated great benefits for cancer therapy.^{2,3} One of the cancer-related genes, polo-like kinase 1 (PLK1) has been attracting increased attention in terms of cancer therapy. These highly conserved serine/threonine kinases are the principal kinase proteins which serve as regulatory enzymes for mitotic events by performing phosphorylation at nuclear protein B23.^{4,5} They play an important role in G2-M transition and show an over-expression in a variety of tumors analyzed to date.^{6,7} Therefore, inhibition of PLK1 is predicted to block the reproduction of cancer cells, thus indicating its potential as a promising cancer drug target. Studies carried out so far have been exploring both chemical and genetic inhibition approaches. A chemical approach using a stilbazole compound, HMN-176, and its orally bioavailable prodrug HMN-214, have both shown potent cytotoxic activity against several tumor cell lines.⁸ Garland and co-workers believe that these compounds interact with PLK1 by interference with its normal subcellular spatial distribution at centrosomes and along the cytoskeletal structure, but not *via* direct inhibition. An alternative genetic approach whereby silencing PLK1 by siRNA induces apoptosis via inactivation of cyclin-dependent mitotic arrest has already shown some promising results.^{9,10} Strebhardt's group has revealed an elevated number of apoptotic nuclei, an increased sub-2N DNA content, and up to 5-fold increases in G2-M compared with controls, followed by apoptosis after depletion of PLK1 in cancer cells.^{11,12} Subsequent studies combining PLK1 gene silencing with chemotherapeutics such as paclitaxel, carboplatin or Herceptin showed improved sensitivity of breast cancer cells against drug therapy both *in vitro* and *in vivo*.^{13,14}

f-CNTs have been employed as non-viral gene delivery vectors offering several advantages compared to alternative non-viral vectors including liposomes, cationic polymers and cell-penetrating peptides. Advantages include efficient membrane translocation and *in vivo* stability.^{15,16} *f*-CNTs are able to deliver nucleic acids such as plasmid DNA, siRNA or aptamers intracellularly. In these studies, siRNA:*f*-CNT complexation was achieved by (i) chemical conjugation of siRNA to the vector *via* cleavable disulfide linkage, (ii) adsorption of siRNA on the PEG-phospholipid coat adsorbed onto the SWNTs, or (iii) by non-covalent complexation between the negatively charged siRNA and the cationic *f*-CNTs. Effective gene silencing was observed in all of these studies thereby indicating the potential of CNT-based delivery of siRNA in mammalian cells. Furthermore, both SWNTs¹⁷⁻²¹ and MWNTs²²⁻²⁴ have shown promise as siRNA transporters *in vitro* and *in vivo* in the field of cancer therapy. In a study performed by Wang et al., *f*-CNT-based transfection of cyclinA siRNA was found to successfully suppress cyclinA expression, resulting in cell growth inhibition and apoptosis induction.¹⁹ Moreover, several reports have shown the therapeutic effect and suppression of tumor growth when *f*-CNTs were employed as siRNA delivery systems *in vivo*.^{18,22,25} Bartholomeusz *et al.*, observed biological effects after exposing a wide variety of

1 cancer cell types *in vitro* to siRNA:SWNT complexes. The same study showed that intratumoral
2 administration of HIF-1 α siRNA:SWNT complexes resulted in inhibition of HIF-1 α activity in a
3 xenograft mouse tumor model.²⁵ Furthermore, another study demonstrated that when positively
4 charged SWNT-CO(CH₂)₆NH₃⁺ are employed for siRNA delivery, suppression of telomerase
5 reverse transcriptase (TERT) expression is observed both *in vitro* and *in vivo*, resulting in inhibition
6 of the tumor growth in both murine and human tumor models.¹⁸ We have also reported antitumor
7 activity in the human lung carcinoma (Calu6) xenograft model and prolonged animal survival by
8 delivering a proprietary toxic siRNA sequence (siTOX \square) using amino-functionalized multi-walled
9 carbon nanotubes (MWNT-NH₃⁺),²² which has been also utilized in this study.

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14 Herein, we validated PLK1 as a target in cancer gene therapy and demonstrated by combining
15 PLK1 siRNA (siPLK1, a siRNA sequence specific for PLK1) with nanotechnology tools (*f*-CNT
16 delivery vectors) an improved therapeutic efficacy and observed prolonged animal survival
17 Moreover, we explored for the first time the correlation between siRNA retention within the solid
18 tumor together with their cellular uptake in tumor cells *in vivo* and the resultant therapeutic
19 outcome. Improved tumor retention and enhanced cellular uptake of siRNA:MWNT-NH₃⁺
20 complexes appear to play an essential role in improving the therapeutic outcome after local
21 administration of siRNA *in vivo*.
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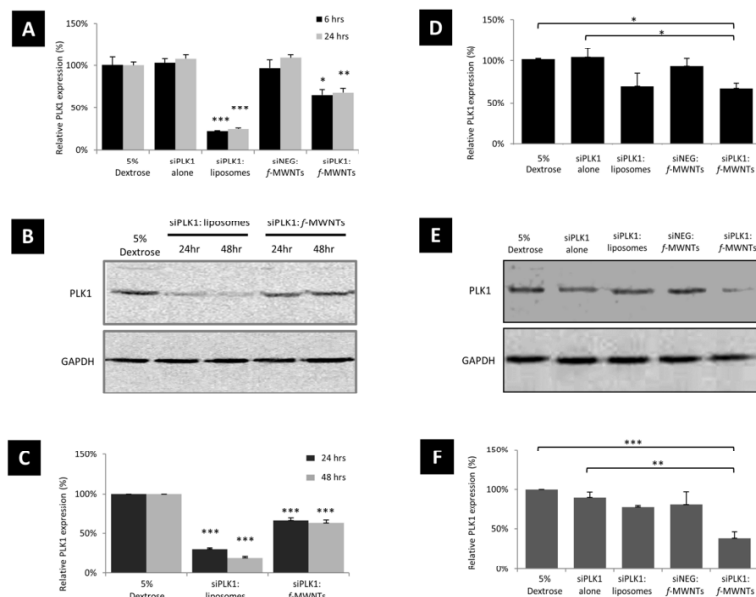
27 RESULTS

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30 **siPLK1 expression in siPLK1:*f*-MWNT complexes treated group.** The chemical structure of *f*-
31 MWNT (MWNT-NH₃⁺) studied here is shown in **Fig. S1**. To validate the PLK1 siRNA (siPLK1)
32 sequence *in vitro*, Calu6 cells were incubated with siPLK1:*f*-MWNT complexes (1:8 mass ratio) for
33 24 or 48 hours. The level of PLK1 expression in Calu6 cells was assessed by real-time PCR and
34 Western blotting (**Fig. 1A, B & C**). GAPDH was used as a housekeeping gene for real-time PCR
35 and as a control for Western blotting. Lipoplexes formed by complexation of siPLK1 and cationic
36 liposomes (DOTAP:cholesterol) were included in the study as a benchmark transfecting reagent.
37 There was a clear difference in gene silencing efficacy between the two vectors *in vitro*, where
38 lipoplexes exhibited more efficient PLK1 gene knockdown than siPLK1:*f*-MWNT complexes as
39 measured by PLK1 protein level (**Fig. 1A&B**). Protein expression was reduced to 30% \pm 1% and
40 20% \pm 2% after 24 and 48 hours, respectively, in case of the lipoplex transfection compared to only
41 66% \pm 3% and 63% \pm 4% in case of transfection using siPLK1:*f*-MWNT complexes after the same
42 incubation times (**Fig. 1B**). **Figure 1C** shows that incubation of Calu6 cells with siRNA:*f*-MWNT
43 complexes decreased the number of mRNA copies of the PLK1 to 66% \pm 9% and 68% \pm 4% at 6
44 and 24 hours, respectively, as shown by real-time PCR. In comparison, incubation of Calu6 cells
45 with lipoplexes attenuated relative PLK1 mRNA level to 22% \pm 1% and 25% \pm 1% at 6 and 24
46 hours, respectively. Hence, we concluded that more potent gene silencing could be achieved using
47 the lipoplexes than siRNA:*f*-MWNT complexes *in vitro*.
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52 We then investigated if siPLK1 is still active under biological conditions *in vivo*. siPLK1 was
53 complexed with either *f*-MWNTs or cationic liposomes and directly introduced into Calu6 tumors by
54 intratumoral injection. PLK1 mRNA transcripts and protein were extracted from injected tumors
55 and PLK1 expression was evaluated by real-time PCR and Western blotting (**Fig. 1D, E&F**) in all
56 groups. Negative siRNA siNEG:*f*-MWNTs treated group was included to eliminate any non-specific
57 toxicity of the siRNA or the *f*-MWNTs. Both techniques demonstrated a significant reduction in
58 PLK1 mRNA transcripts and protein levels in the tumors treated with siPLK1:*f*-MWNT complexes
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1 compared to the injected tumours of control groups (5% dextrose dextrose and siPLK1 alone)
 2 tumors. Intratumoral injection of siPLK1:*f*-MWNT complexes reduced PLK1 mRNA levels to 67% ±
 3 6% and PLK1 protein levels to 38% ± 8% relative to expression of the housekeeping gene GAPDH
 4 (Fig. 1D, E&F). Lipoplexes and siNEG: *f*-MWNT treated groups exhibited different effects in PLK1
 5 silencing both in vitro and in vivo. Such results proposed that siPLK1:*f*-MWNT complexes as
 6 promising vectors for RNAi *in vivo*. Furthermore, the delivery capacity of well-established delivery
 7 vectors such as liposomes which have shown to be potent *in vitro* can be compromised when
 8 administered locally within tumor environment *in vivo*.
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40 **Figure 1: PLK1 gene silencing analysis in Calu6 cell lines and tumor xenografts. (A-C) *In vitro* gene silencing.**
 41 Calu6 monolayers were treated with siPLK1 at 100 nM final concentration in serum free media for 4 hours followed by
 42 incubation in complete media for another 6 hours, 24 hours or 48 hours. GAPDH gene was used as a control
 43 housekeeping gene. Treatment groups include 5% dextrose, siPLK1 alone, lipoplexes (siPLK1 in complex with
 44 DOTAP:cholesterol liposomes, (4:1 N/P)), siNEG:*f*-MWNT complexes (1:8 mass ratio) and siPLK1:*f*-MWNT complexes
 45 (1:8 mass ratio). (A) mRNA expression levels of PLK1 analyzed in Calu6 monolayers lysates by real time PCR (n=3) at 6
 46 hours or 24 hours post-incubation. (B&C) Western blots of cell lysates to determine PLK1 expression at 24 hours or 48
 47 hours post-incubation. Both protein and mRNA levels of PLK1 were reduced more efficiently in case of lipoplexes
 48 compared to siPLK1:*f*-MWNT complexes. Results were analyzed using *t*-test between treated groups and control groups
 49 (5% Dextrose) at different time points respectively. (D-F) *In vivo* gene silencing. Calu6 xenograft tumors were
 50 administered with siPLK1 at a dose of 4 µg per tumor in 5% dextrose on days 17, 20, 23, 27 and 30. GAPDH gene was
 51 used as a control housekeeping gene. Treatment groups include 5% dextrose, siPLK1 alone, lipoplexes, siNEG:*f*-MWNT
 52 complexes and siPLK1:*f*-MWNT complexes. (D) mRNA expression levels of PLK1 analyzed in Calu6 tumor lysates by
 53 real time PCR (n=3) post-administration. (E&F) Western blots of cell lysates to determine PLK1 expression in the
 54 lysates. Both protein and mRNA levels of PLK1 were reduced more efficiently in case of siPLK1: *f*-MWNT complexes
 55 compared to lipoplexes. Results were analyzed using *t*-test between group of siPLK1:*f*-MWNT complexes and other
 56 groups (p values = * <0.05 ; ** <0.01 ; *** <0.001).

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3 **Tumor growth and animal survival *in vivo* after intratumoral administration of siPLK1:*f*-**
4 **MWNT complexes.** We further studied if gene silencing by siPLK1:*f*-MWNT complexes is sufficient
5 to delay tumor growth and improve animal survival. For this purpose, treatments were injected
6 intratumorally in an established human lung Calu6 xenograft tumor model. Tumor growth was
7 monitored by measuring the tumor size three times per week with Vernier calipers over a period of
8 30 days. **Figure 2A** showed a significant inhibition in tumor growth observed in siPLK1:*f*-MWNT
9 complexes treated group. Reductions in tumor volume were noticeable after the second
10 therapeutic injection. Other treatment groups (siPLK1 alone, siPLK1:liposomes, siNEG:*f*-MWNT
11 complexes) showed no difference in volume compared to the 5% dextrose treated group. To
12 compare the efficiency of different types of therapies used, quadrupling time was calculated and
13 expressed in **Figure 2B**. Quadrupling time of siPLK1:*f*-MWNTs treated group was 20.5 ± 2.3 days
14 ($n=9$) compared to only 11.3 ± 0.4 days ($n=10$) in the case of 5% dextrose treated group. siPLK1:*f*-
15 MWNT group exhibited significant increase in quadrupling time compared to all other treated
16 groups. Animal survival analysis is shown in **Fig. 2C**. siPLK1:*f*-MWNT complexes treated group
17 showed significant increase in animal survival compared to all other groups (5% dextrose, siPLK1
18 alone, siPLK1:liposomes, siNEG:*f*-MWNT complexes). There was no significant improvement in
19 animal survival for those treated with siPLK1:liposomes compared to 5% dextrose or siPLK1 alone.
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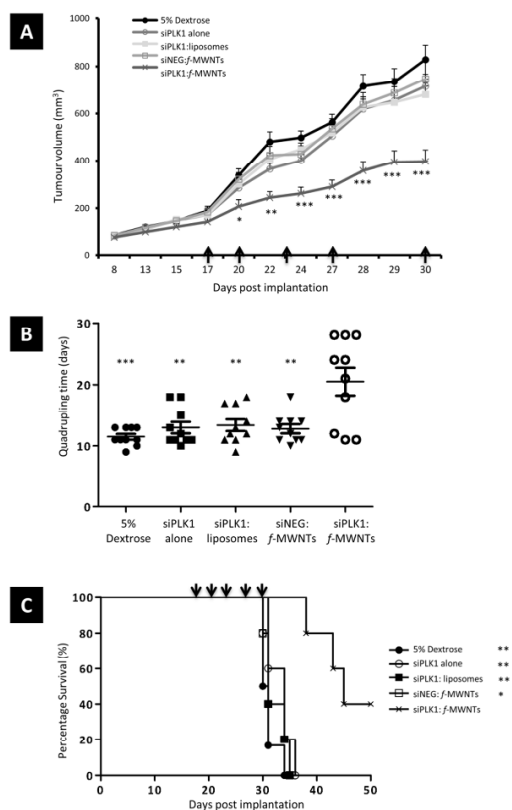
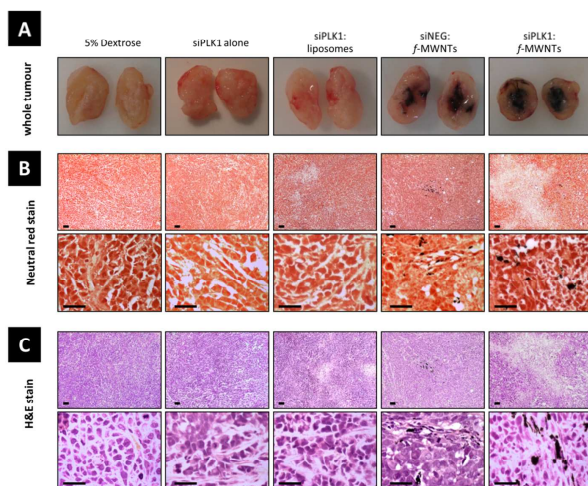


Figure 2: Tumor growth and survival curves in Calu6 xenografts bearing mice. (A) Growth curves analysis of Calu6 xenografts. Female Swiss nude mice Calu6 xenograft tumors were administered with siPLK1 (4 μ g) in complex with either *f*-MWNTs (1:8 mass ratio) or DOTAP:cholesterol liposomes (N/P=1/4; 4 μ g siRNA). siNEG:*f*-MWNT (1:8 mass ratio) complexes and siPLK1 alone were injected as negative controls. Arrowheads indicate injections on days 17, 20, 23, 27 and 30. Results were presented as mean \pm SEM ($n=9-10$). (B) Tumor volume quadrupling times (TVQT, in days) for treatment and control groups. (C) Survival analysis of Calu6 xenograft bearing mice. Formulations: 5% dextrose as control (circle); siPLK1 alone (4 μ g) (open circle); lipoplexes (N/P=1/4; 4 μ g siRNA) (square); siNEG:*f*-MWNT complexes (mass ratio=1:8; 4 μ g siRNA) (open square); siPLK1:*f*-MWNT complexes (mass ratio=1:8; 4 μ g siRNA) (star). Results were analyzed using *t*-test between group of siPLK1:*f*-MWNT complexes and other groups (p values = * <0.05 ; ** <0.01 ; *** <0.001).

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3 **Observation of siPLK1:*f*-MWNT complexes in tumor mass and the toxicity of *f*-MWNT vehicle to tumor cells.** Tumors were excised when the tumors reached their maximum growth volume allowed by the UK Home Office. **Figure 3** shows a representative cross section image of the whole tumor from each treatment group. The presence of *f*-MWNTs in siNEG: *f*-MWNTs and siPLK1:*f*-MWNTs treated tumors was confirmed by the greyish color in the core of these tumors (at 100X magnification). Tumors were fixed in formalin and processed for either neutral red or H&E staining in order to perform histological examination of the tumors at the microscopic level. Neutral red staining (at 400X magnification) confirmed the presence of *f*-MWNTs in tumor cells. Moreover, H&E staining revealed extended necrosis in the areas around the CNTs only in the siPLK1:*f*-MWNT treated group. Tumors treated with siNEG:*f*-MWNT complexes lacked areas of necrotic tissues that were apparent in siPLK1:*f*-MWNTs group, indicating that the toxicity seen was due to siPLK silencing after being internalized into the cells. Major organs such as heart, spleen, lung, liver and kidney were also dissected and sectioned for neutral red and H&E staining (**Supplementary Fig. S2**). However, *f*-MWNTs could not be detected in these organs indicating there was retention of the vectors in tumor tissue after intratumoral injection.



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42 **Figure 3: Histological analysis of Calu6 tumor xenografts.** (A) Photographs of whole tumors excised once reaching 800 mm³ volume. Histological examination of fixed tumor sections processed for routine histology with (B) neutral red stain or (C) and hematoxylin and eosin (H&E) stain. Tumors treated with siPLK1:*f*-MWNT complexes showed significant tissue necrosis compared to all other treated groups.

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47 **Apoptosis induction in tumor mass by PLK1 silencing.** To confirm if apoptosis was the mechanism of cell death as a result of PLK1 silencing, TUNEL staining was performed and all nuclei were counterstained by using DAPI stain (**Fig. 4**). Only apoptotic cells exhibited green fluorescence (TUNEL positive). siPLK1:*f*-MWNT treated tumors were TUNEL positive in the same regions where *f*-MWNTs were abundant as confirmed by DIC images. Similar tumor regions exposed to siNEG:*f*-MWNT complexes were TUNEL negative. This result further confirms that apoptosis may be attributable to cell death induced by PLK1 gene silencing. The groups treated with 5% dextrose and lipoplexes showed predominantly healthy appearance with scarce areas of TUNEL positive cells which could be due to a natural process of cell death during tumor growth.

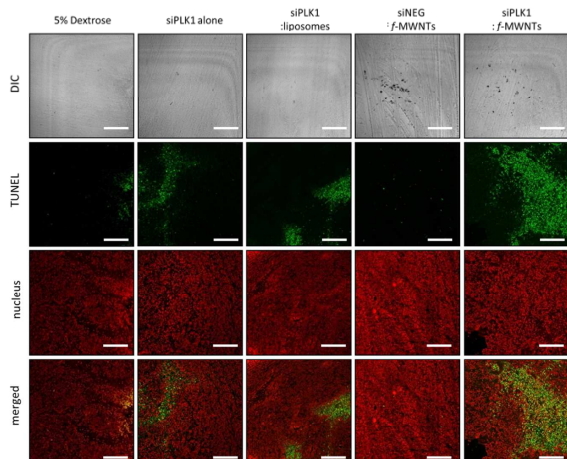


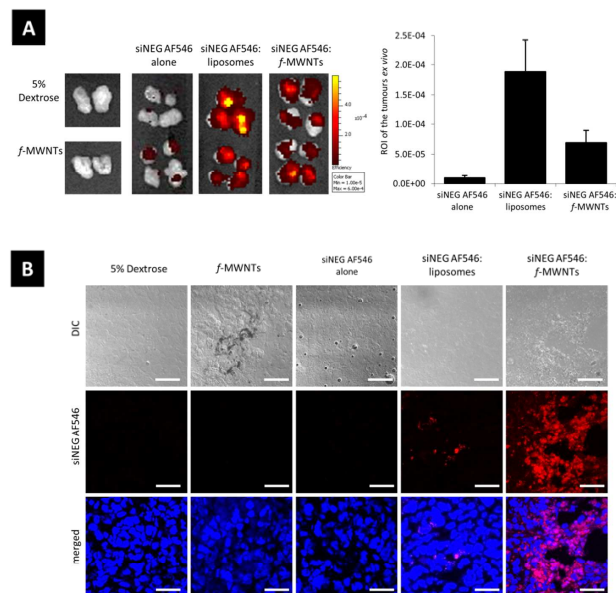
Figure 4: TUNEL analysis of Calu6 tumor xenografts. Tumor sections were deparaffinised and rehydrated through graded ethanol then TUNEL stained to identify apoptotic (green) cells from counterstained nuclei (red). Phase contrast images of corresponding fields of view shown to indicate MWNT-NH₃⁺ localization in tumor tissues. Tumors treated with siPLK1:f-MWNT complexes showed significant apoptosis of the tumor cells. Scale bar is 200 μ m.

siRNA retention in tumor mass after local administration of siRNA: f-MWNT complexes. In order to investigate the kinetics of siRNA clearance from the tumor mass and more importantly to learn about the interaction between siRNA and tumor cells, we have substituted the siPLK1 with fluorescently labeled negative siRNA with AlexaFluor AF546 (siNEG AF546). Whole body imaging in addition to imaging of excised tumors (*ex vivo*) was carried out after single intratumoural injections (**Supplementary Fig. S3 and Fig. 5**) using the Xenogen IVIS imaging system to visualize the fluorescence signal representing siNEG AF546 complexed with both f-MWNT and liposomes. It was clear from whole body imaging that the lipoplex group exhibited the long-lasting retention in the tumor mass up to 24 h (47.5% ID \pm 3.0% ID after 1 h and 21.3% ID \pm 4.6% ID after 4 h, n=6). On the contrary, siNEG AF546 alone cleared rapidly from the tumor mass with 26.6% ID \pm 3.8% ID (n=6) and 8.9% ID \pm 2.0% ID detected in the tumors after 1 and 4 h, respectively (**Supplementary Fig. S3**). Interestingly, the siNEG AF546:f-MWNT treated group exhibited lower signals compared to the lipoplex formulation by whole body imaging technique, with 62.3% ID \pm 6.3% ID and 15.2% ID \pm 1.5% ID (n=6) remaining within the tumor mass at 1 and 4 h post-injection, respectively.

Imaging of tumors (*ex vivo*) was then performed to account for problems associated with deep tissue penetration of fluorescence signals (**Fig. 5**). Tumors were dissected 24 hours after intratumoural administration of free or complexed siNEG AF546 and observed under Xenogen IVIS imaging system (**Figure 5A**). Similar tumor retention profiles were obtained *ex vivo* as those obtained by whole body imaging. To confirm whether the fluorescence detected by IVIS was associated with tumor cells or within the interstitial space, tumor masses were excised, cryo-sectioned, post-fixed, and examined by confocal laser scanning microscopy (CLSM) (**Figure 5B**). During the fixation and subsequent washing steps, free non-associated siNEG AF546 was thus

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1
2 eliminated before imaging by CLSM. At 24 h post-intratumoral administration, siNEG AF546
3 treated group showed no fluorescence signals indicating that siRNA alone was not able to
4 internalize into cancer cells without the aid of a delivery system. siNEG AF546:*f*-MWNT complexes
5 treated tumors showed intense red fluorescence signals confirming intracellular delivery of siNEG
6 AF546 by means of *f*-MWNTs. Moreover, fluorescence signals in the latter group were extended
7 throughout the cells including the nucleus. Such results were compatible with the results we
8 previously obtained in cancerous cell monolayer studies.^{26, 27} Surprisingly and contrary to whole
9 tumor imaging studies (**Figure 5A**), intracellular delivery of siNEG AF546 by lipoplexes appeared
10 to be less intense than the *f*-MWNT-mediated delivery. Such results were in line with the reduced
11 gene silencing efficiency obtained in case of the cationic liposome-mediated delivery compared to
12 the nanotube-mediated delivery *in vivo*.
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Figure 5: Uptake of siNEG AF546 in tumor xenografts. (A) *Ex vivo* imaging (left) and quantification of the fluorescence signals (right) detected from the dissected tumors by Xenogen IVIS imaging system. Tumors were dissected 24 hours after intratumoral administration of siNEG AF546 in free or complexed forms. **(B)** Intracellular uptake of siNEG AF546 in the dissected tumors by CLSM. Scale bars, 50 μ m.

DISCUSSION

We have previously reported the most comprehensive and promising study to date describing tumor apoptosis, delayed tumor growth and prolonged survival of human lung xenograft bearing mice after intratumoral administration of siRNA:*f*-MWNT complexes.²² In this previously reported study, we observed significant improvements in the therapeutic outcome in the group treated with siRNA:*f*-MWNT complexes compared to the lipoplexes treated group. That study utilized the proprietary sequence siTOX□ to induce tumor apoptosis and death, whilst ammonium functionalized MWNT and cationic liposomes were used as delivery vectors for the siRNA molecules. Although it was essential to investigate the reason behind the drastically improved performance of the ammonium functionalized MWNTs over cationic liposome vectors for siRNA delivery *in vivo*, due to the unknown nature of siTOX□, it was difficult to perform a direct comparison between the two delivery systems at the molecular level (e.g. mRNA and protein synthesis level). Therefore, it was deemed essential to carry out further studies to correlate the pharmacokinetic profiles with bioactivity of the siRNA delivered by means of the two fundamentally different delivery systems. In this study the siTOX□ was substituted with a siRNA sequence specific for PLK1, a gene known to play an important role in cancer cell replication and proliferation, so successful gene silencing could lead to tumor growth arrest. Furthermore, we performed mechanistic studies substituting functional siRNA with fluorescently labeled non-coding siRNA (siNEG) in order to determine the siRNA uptake into tumor mass *in vivo* and its retention in the tumor following intratumoral administration.

The main finding was that complexation of siPLK1 with MWNT-NH₃⁺ facilitated its internalization by tumor cells in solid tumor mass *in vivo*, resulting in significant PLK1 knockdown compared to the equivalent lipoplex formulation and all other treated groups. We didn't observe significant inhibition in tumor growth when treated with lipoplexes in our study although the lipoplexes worked *in vitro* (Fig. 1A-C). This could be due to the retention of lipoplexes into the tumor after intratumoral injection was not good and they were washed quickly while the CNT complexes were retained within the tumor so were longer acting, as observed from the 24 hours retention of fluorescence based siNEG AF546 in the free or complexed forms after intratumoral administration (Fig. 5B). Pharmacokinetic studies suggested that the bioavailability of siRNA within tumor cells after complexation with carbon nanotubes compared to the cationic liposomal formulation could be the rationale behind the superior bioactivity of the former delivery system. Whole tumor imaging by IVIS imaging system showed high fluorescence signals in tumors injected with the cationic liposomal formulation which was surprising and contradicting the bioactivity results. However, further inspection of tumor masses microscopically by CLSM confirmed that the majority of the siRNA was not delivered inside the cell and rather was retained in the interstitial space of the tumor in the case of the cationic liposome-based vectors. Non-internalized siRNA was most likely eliminated during the process of washing and fixation prior to imaging by CLSM, whilst internalized siRNA was resilient to washing and was therefore visualized inside the cells by CLSM as was the case for *f*-MWNT-based carriers.

Cationic liposome based siRNA nanocarriers have been widely used for cancer therapy in different tumor models. These complexes were usually injected systemically²⁸ that may explain the less therapy effects in our case. Previous studies have shown that liposomal systems exhibit broad interstitial diffusion within tumor mass after intratumoral injection, with slow clearance from the interstitial space.²⁹ Prolonged accumulation of liposomes and the therapeutics within tumor mass does not always lead to enhanced intra-cellular delivery as it is necessary to establish if binding to the extra-cellular matrix does not hinder intra-cellular uptake into tumor cells. In conclusion of this

1 study, carbon nanotube-mediated delivery exhibited the highest uptake in tumor cells as obtained
2 by CLSM which conflicts with, conflicting the results obtained by whole body IVIS imaging which
3 showed higher fluorescence signals of tumors injected with the lipoplexes. It is evident that detailed
4 mechanistic studies utilizing multiple techniques are necessary for expressing the delivery
5 efficiency of various delivery systems. Results obtained in this study demonstrated lack of toxicity
6 of *f*-MWNTs and their efficiency as cytosolic siRNA carriers in addition to the bioactivity of the
7 delivered siRNA as demonstrated by efficient gene silencing *in vivo* superseding the efficiency of
8 cationic liposomal vectors as previously reported in other studies using stroke and cancer disease
9 models.^{15, 22}

10 Interestingly, amino functional groups on the MWNT-NH₃⁺ sidewalls can be further modified such
11 as building dendritic structures as reported previously.^{26, 27} Engineering the degree and type of
12 dendritic branching is expected to affect the degree of siRNA association and gene silencing
13 efficiency. We concluded from previous *in vitro* studies^{26, 27} that increasing the degree of the
14 dendritic branching on the *f*-MWNT side walls improved their water dispersibility, individualization
15 and siRNA complexation. Future work should focus on comparing the bioactivity and
16 pharmacokinetic profiles of other types of dendritic *f*-MWNT generations for siRNA delivery *in vivo*.
17 In conclusion, we reformulated MWNT-NH₃⁺ by complexing with siPLK1 or siNEG AF546, to
18 elucidate and understand of the efficient siRNA delivery in xenograft tumors. We proved that
19 cationic multi-walled carbon nanotubes are efficient siRNA vectors for tumor eradication *in vivo*.

20 This work is a proof-of-principle study demonstrating that biocompatible *f*-MWNTs are effective
21 intra-cellular transporters for siRNA in cancer gene therapy *in vivo*. So far, only a limited number of
22 studies have reported *f*-CNTs as siRNA delivery systems for *in vivo* applications for diseases other
23 than cancer. Lanner et al. presented successful CNT-based transfection of TRPC3 siRNA in adult
24 skeletal muscle cells. The TRPC3 protein expression was knocked down, with functional effects
25 seen in the inhibition of DAG-induced Ca²⁺ and decreases in the insulin-mediated glucose
26 uptake.³⁰ This validated TRP3 as a target for treatment of insulin-resistant conditions. Moreover,
27 evidence has also shown that *f*-CNTs can effectively aid the transfection of generally difficult to
28 transfect cells help the gene delivery, such as T cells and primary cells, including neurons and
29 cardiomyocytes.³¹⁻³⁴ Very recently, we reported MWNT-NH₃⁺ as transporters for Caspase-3 siRNA
30 for the prevention of apoptosis following stroke induction in a murine Endothelin-1 stroke model.
31 Functional recovery was achieved to peri-lesional levels after stereotactic administration of the
32 therapy in both pre- and post-lesioned animals.¹⁵ Overall, although *f*-CNT-mediated gene silencing
33 is at an early pre-clinical stage, recent studies have shown that *f*-CNT offer significant benefits in
34 the area of gene therapy and more specifically gene silencing.

Experimental

Materials. MWNTs were purchased from Nanostructured & Amorphous Materials Inc., Huston, TX; Stock #: 1240XH, 95%, OD 20-30 nm. MWNT-NH₃⁺ were prepared as previously described.²⁷ Chemicals and solvents were obtained from Sigma-Aldrich (USA) and they were used as received. Liposomes were prepared by lipid film hydration, followed by filtration. Briefly, DOTAP:cholesterol (2:1 molar ratio) was dissolved in chloroform:methanol (4:1 v/v), the organic solvent was evaporated in a rotary evaporator (Buchi, Switzerland) under vacuum at 40 °C for 30 min and then flushed with a N₂ stream to remove any residual traces of organic solvent. The dried lipid film was hydrated with 1 mL of 5% dextrose, sonicated and extruded twice through 0.1 μm filter under sterile conditions. The final lipid concentration was 2 mM. AlexaFluor 546-labelled siNEG (siNEG AF546) (5' → 3': UGCGCUACGAUCGACGAUG) and siPLK1 (5' → 3': CCUUGAUGAAGAAGAUAC) were purchased from Eurogentec (UK). Advanced RPMI, fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffered saline (PBS) were from Gibco, Invitrogen (UK). Human lung carcinoma Calu6 (HTB-55) were from ATCC (UK). BCA Protein assay kit, paraformaldehyde (PFA), Restore Plus Western Blot Stripping Buffer and ECL detection system were purchased from Thermo Scientific Ltd (USA). Mouse monoclonal GAPDH antibody were obtained from Ambion (Austin, USA) and HRP-linked anti-mouse IgG was obtained from Jackson ImmunoResearch (West Grove, MA, USA). Mouse monoclonal PLK1 antibody was purchased from Abcam (Cambridge, UK) and HRP-linked anti-rabbit IgG was obtained from Cell Signaling (MA, USA). Vectashield mounting media with DAPI (H1200) was purchased from Vector Laboratories (USA). Hybond ECL nitrocellulose membranes were obtained from GE Healthcare (UK). Complete Protease Inhibitor cocktail is from Roche. Unless otherwise stated, all others reagents were obtained from Sigma.

Liposome: siRNA and siRNA:MWNT-NH₃⁺ complexation for biological studies. siRNA complex preparations for *in vivo* experiments were carried out by diluting the appropriate volume of liposome (1.4 mg/mL) or MWNT-NH₃⁺ (1.28 mg/mL) dispersion to a total volume of 25 μL in 5% dextrose/dextrose, to achieve siRNA:liposomes N/P=4 or siRNA:MWNT-NH₃⁺ mass ratio=8. siRNA initial stock was prepared at 20 μM. An equal volume of 160 μg/mL siRNA in 5% dextrose was then added to the liposome or MWNT-NH₃⁺ aliquots and mixed by rapid pipetting, yielding a final siRNA concentration of 80 μg/mL. Fifty μL of the complexes were injected per animal. A similar procedure was used to prepare complexes for *in vitro* experiments except that complexation was carried out initially in a total volume of 100 μL then diluted 5X with serum-free media yielding siRNA final concentration of 100 nM.

***In vitro* PLK1 gene silencing in Calu6 cells and in vivo PLK1 gene silencing in Calu6 xenograft tumors.** Calu6 cells (30,000 cells / well) were seeded in 6-well plates. 24 hours later, cells were transfected with siPLK1 in different formulations. After 6 hours, 24 hours or 48 hours post-transfection, cells were washed twice with ice-cold PBS before lysis. Tumor tissues were harvested and snap-frozen immediately in liquid-nitrogen-cooled isopentane. Samples were stored at -80 °C prior to lysis. The tumors were placed in liquid nitrogen and ground thoroughly with a pestle. Decanted tissue powder was weighed in Eppendorfs before lysis.

Gene silencing by Western blot. Cells or decanted tissue powder were collected directly into lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40 and 0.5% sodium deoxycholate). The tumor tissue lysate was sonicated and then left on ice for 30 min. Samples were cleared by centrifugation at 15000 rpm for 15 min at 4 °C and the supernatant was collected. Total protein concentration was assessed using BCA Protein assay kit and 10 μg of total protein was taken from each supernatant and resolved on 10% SDS-PAGE gels and transferred to Hybond ECL nitrocellulose membranes. After blocking in 3% BSA for overnight, the blots were incubated with rabbit monoclonal PLK1 antibody at 1:1000 dilution, followed by horseradish peroxidase (HRP)-linked anti-mouse IgG at 1:10000 dilution used as secondary antibody. The specific bands were detected with enhanced chemiluminescence (ECL) detection system. Band intensities of the PLK1 were quantified by Image J. GAPDH was used as an internal reference (house-keeping) gene. Membranes were stripped, blocked and rehybridized with mouse anti-GAPDH antibody (1:4000 dilution), followed by incubation with HRP-conjugated antibody.

Gene silencing by real-time PCR Analysis. Total RNA was extracted with an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The concentration of total RNA was determined by measuring the optical density at 260 nm and the purity was checked as the 260 nm/280 nm ratio with expected values between 1.8 and 2.0. First strand cDNA was prepared from 1 μg RNA in a total volume of 20 μL using the QuantiTect Reverse Transcription Kit (QIAGEN). Real-time PCR was performed using the CFX96™ Real-Time PCR Detection System (BioRad). The reactions contained 1X Fast SYBR® Green Master Mix (BioRad), each primer (h-PLK1 sense 5' ACA GTA TTC CCA AGC ACA TC3'; h-PLK1 antisense 5' AAT GGT CAG GCA GGT GAT 3'; h-GAPDH sense 5' GGT CGG AGT CAA CGG ATT 3'; h-GAPDH antisense 5' ATC GCC

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CCA CTT GAT TTTG 3') at 200 nM and 1 μ L of cDNA from reverse transcription PCR in a 25 μ L reaction. After an initial denaturation step at 95 $^{\circ}$ C for 10 min, amplification was carried out with 40 cycles of denaturation at 95 $^{\circ}$ C for 10 sec and annealing at 60 $^{\circ}$ C for 30 sec. Amplification was followed by a melting curve analysis to confirm PCR product specificity. No signals were detected in no-template controls. All samples were run in triplicates and the mean value of each triplicate was used for further calculations. Relative gene expressions were calculated using the $\Delta\Delta C_T$ method. The quantity of GAPDH (housekeeping) transcript in each sample was used to normalize the amount of PLK1 transcripts, then the normalized value was compared to the normalized expression in naïve cells to calculate a fold change value.

Tumor xenograft animal model and survival studies. All animal experiments were performed in compliance with the UK Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Six to eight-weeks old female Swiss nude mice (Charles River Laboratories, UK) were caged in individually vented cages (IVC; Allentown, USA) in groups of four with free access to food and water. A temperature of 19–22 $^{\circ}$ C was maintained, with a relative humidity of 45–65%, and a 12 hours light/dark cycle. An established human lung Calu6 xenograft tumor model was applied as described previously.²² Mice were inoculated subcutaneously with 1×10^6 Calu6 human epithelial lung carcinoma cells mixed 1:1 with Matrigel (Becton Dickinson, UK) in 100 μ L on the both flanks subcutaneously. Intratumoral injections were performed when the tumor volume reached 0.2–0.4 cm^3 and were carried out on days 17, 20, 23 27 and 30 post-tumor implantation in this study. For intratumoral administration and tissue analysis, mice were anesthetized using isoflurane and injected with the siRNA alone or the complexed prepared in 5% dextrose. The needle was inserted in the longitudinal direction from the tumor edge into the center of the tumor, 50 μ L of the dispersion was administered slowly over 1 minute, and the needle was left in the tumor for another 5 minutes to prevent sample leakage.

Tumor growth was monitored by measuring the tumor size three times per week with Vernier calipers over a period of 30 days. The tumor volume was estimated by bilateral Vernier caliper measurement three per week and calculated using the formula (width \times width) \times (length) \times ($\pi/6$), where length was taken to be the longest diameter across the tumor. Implantation with 5% dextrose (naïve), carbon nanotubes alone and siRNA in different formulations were carried out twice weekly for all groups. Quadrupling time is used to compare the efficiency of different types of therapies used and is defined as the time required for a tumor to reach four times of its initial volume at the start of the therapy.^{35, 36} Mice were sacrificed by cervical dislocation when tumor area reached 800–1000 mm^3 .

Neutral red and Hematoxylin/Eosin (H&E) tissue histology. For histological analysis, tumors samples and the major organs (heart, lung, liver, spleen and kidneys) were fixed in 10% buffered formalin and processed for routine histology with neutral red and hematoxylin and eosin (H&E) stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London, UK). Microscopic observation of tissues was carried out with Nikon Microphot-FXA microscope coupled with Infinity 2 digital camera.

TUNEL labeling. Tissue sections were deparaffinized in Histoclear and rehydrated through graded ethanol. The DeadEnd™ Fluorometric TUNEL System (Promega, UK) was used to label nicked DNA through incorporation of fluorescein-12-dUTP. Samples were incubated with recombinant Terminal Deoxynucleotidyl Transferase (rTdT) as per manufacturer's instructions and fluorescein labeling was visualized. Slices were visualized under 10X lens using CLSM. DAPI was used to counterstain nuclei. DAPI and fluorescein were excited at 4% of 365 and 2% of 488 laser power, respectively.

In vivo and ex vivo optical imaging study. Optical imaging studies were performed using an IVIS spectrum small-animal *in vivo* imaging system (Caliper Lifescience, Hopkinton, MA). Mice bearing Calu6 tumors were intratumorally/intratumorally injected when the tumors reached 500 mm^3 , and imaged at 5 minutes, 15minutesmin, 15 min, 30 minutes, 1hourmin, 1 h, 2 hours, 3 hours, 4 hours and 24 hours post-administration. Isoflurane (2%) was used for anesthesia during imaging. The fluorescence images were obtained with the following settings: exposure time (1 s), f/stop (2 s), binning (4 s) and field of view (24 s). Excitation filters were 535 nm. All images were background subtracted using Living Image software. Tumors and different organs were dissected 24 hours post-injection. Tumors were cross-sectioned with the inside above and imaged immediately. The mean fluorescence intensity of the tumors was calculated using Living Image software.

Tissue analysis. 24 hours post-injection of siNEG AF546 in different formulations, the mice were killed and tumors samples were collected. Samples were stored at -80 $^{\circ}$ C prior to frozen sectioning. Frozen tumors were embedded into OCT and plunged into a liquid-nitrogen bath for at least 30 seconds. Samples were retrieved from the bath then sectioned using the cryostat at -18 $^{\circ}$ C into 10 μ m-thick sections. The sections were mounted on a superfrost slide and left to dry at room temperature for 15–30 minutes. For tumor

1
2 visualization, the sections were fixed for 3 minutes in cold acetone at -20 °C, rinsed with PBS for 15 minutes
3 at room temperature and mounted with Vectashield as a mounting medium. Nuclei were Nucleus
4 counterstained with DAPI. The slices were visualized under 40X lens using CLSM (LSM 710, Zeiss UK).
5 DAPI and AlexaFluor546 were excited at 4% of 365 and 5% of 514 laser power, respectively.
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Supporting Information description

Table of contents

Supplementary Figure 1	Chemical structures and TEM images of positively charged f -MWNT (MWNT-NH ₃ ⁺).
Supplementary Figure 2	Histological examination of the major organs in tumor-bearing mice.
Supplementary Figure 3	Quantification of the fluorescence signals detected in the tumor region in xenograft-bearing mice after intratumoral administration.

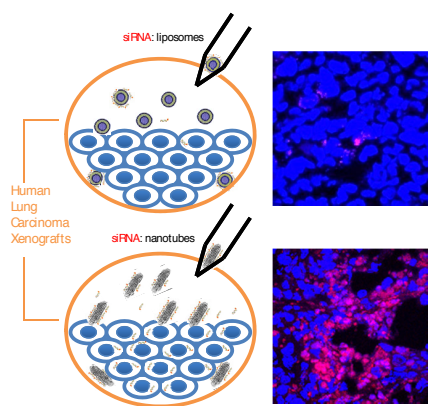
Keywords

Gene silencing; polo-like kinase (PLK1); siRNA; xenograft; lung cancer; carbon nanotubes (CNTs)

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