

# Combination of Dendrimer-Nanovector-Mediated Small Interfering RNA Delivery to Target Akt with the Clinical Anticancer Drug Paclitaxel for Effective and Potent Anticancer Activity in Treating Ovarian Cancer

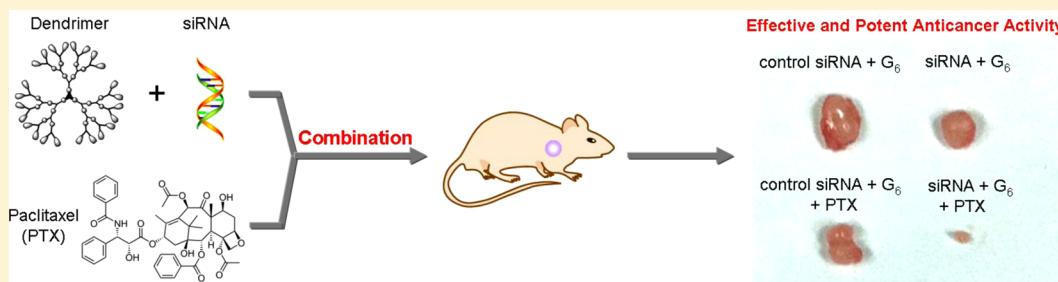
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**ABSTRACT:** The recently discovered small interfering RNA (siRNA) holds great promise in cancer therapy. However, efficient and safe delivery systems are required for the development of new therapeutic paradigms. Ovarian cancer has the highest mortality of all gynecologic tumors, and there is an urgent need for specific and effective therapies. The phosphatidylinositol 3-kinase/Akt pathway, which is strongly implicated in the biology of ovarian cancer, constitutes an attractive therapeutic target. In this study, we describe a triethanolamine-core poly(amidoamine) dendrimer which forms stable nanoparticles with the Akt siRNA, protects siRNA against RNase digestion, and is highly effective for initiating Akt target-gene silencing both in vitro and in vivo, while being minimally toxic. Most importantly, it could potentiate the antitumor effect of the anticancer drug paclitaxel. These results represent the proof-of-concept, demonstrating that dendrimer-mediated Akt siRNA delivery, in combination with a chemotherapeutic regimen, may constitute a promising nanomedicine approach in cancer therapy.

## INTRODUCTION

Ovarian cancer is the leading cause of death of all gynecologic tumors. Although surgical resection could cure early-stage ovarian cancer, most (~70%) patients present with advanced disease at diagnosis. Chemotherapy is the mainstay of treatment for metastatic ovarian cancer. However, commonly used cytotoxic chemotherapeutic agents often have narrow therapeutic indices due to highly nonspecific cytotoxicity and undesirable side effects. Furthermore, their applications are limited by both intrinsic and acquired chemoresistance, in most making the disease incurable (5 year survival <25%).<sup>1</sup> Thus, there is a great urge for the development of new and effective therapeutic strategies.<sup>2</sup>

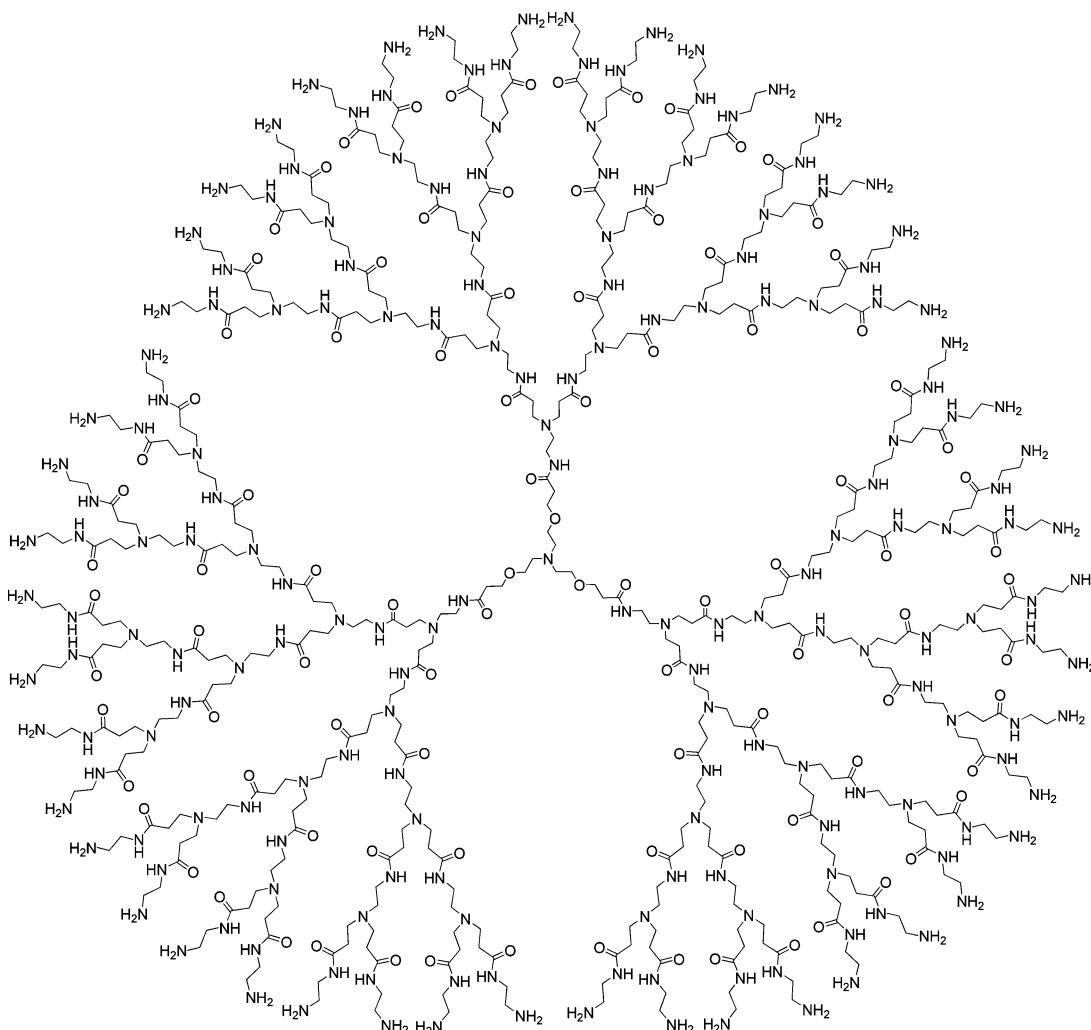
The phosphatidylinositol 3-kinase/Akt pathway has been implicated as a major driver in the development of ovarian cancer. PI3CA, the gene that encodes the p110 $\alpha$  catalytic

subunit of phosphatidylinositol 3-kinase, is increased in copy number in 40% of ovarian cancers.<sup>3</sup> Akt is activated in ~36% of primary tumors, with the activation being associated with high-grade tumor and aggressive clinical behavior.<sup>4,5</sup> Indeed, Akt contributes to tumorigenesis in several processes which are considered as hallmarks of cancer, including survival, proliferation, invasion, angiogenesis, and metastasis.<sup>6,7</sup> Akt has also been reported to protect ovarian cancer cells from drug-induced apoptosis,<sup>8,9</sup> suggesting that its activation may have a role in drug resistance and relapse. Small-molecule inhibitors of Akt are being investigated in various clinical settings, but with mixed success at the levels of efficacy and toxicity.<sup>10</sup> In recent years, small interfering RNA (siRNA), a powerful technology

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**Figure 1.** Structure of the TEA-core PAMAM dendrimer (generation 4 as an example).

for gene silencing with high efficiency, high specificity, and low toxicity, has been widely used for silencing malignant genes. This technique provides great promise in the field of cancer therapy. However, the lack of a safe and efficient delivery method makes its clinical application difficult. This is because siRNA molecules are intrinsically unstable against various enzymes, and they are also highly charged to readily cross cell membranes. Delivery systems that can favorably protect siRNA from degradation and mask the high negative charge of siRNA to promote cell uptake as well as alter the biodistribution and pharmacokinetics of siRNA to prolong the siRNA half-lives in plasma are essential.<sup>11</sup>

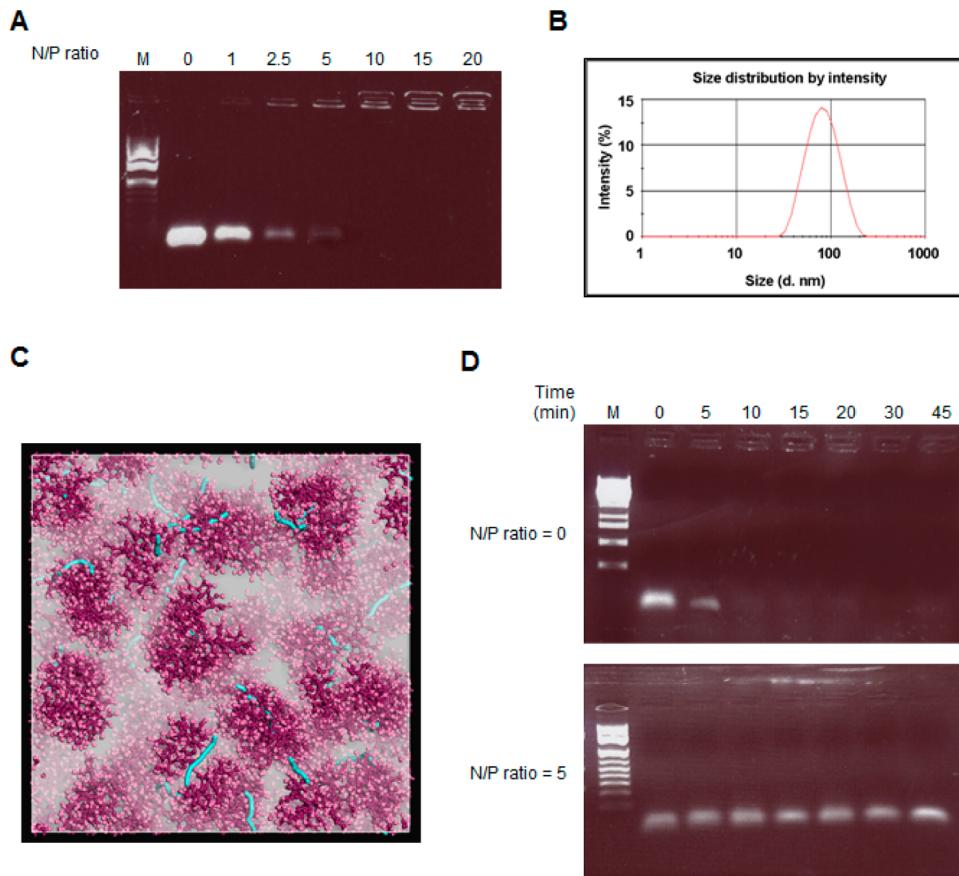
Although viral vectors have been considered to be promising nucleic acid delivery carriers, the immunogenicity and toxicity of these vectors in humans at therapeutic doses urge the development of alternative nonviral vectors, which are noninfectious and elicit only weak humoral immune responses.<sup>12</sup> Among the nonviral vectors, we are particularly interested in developing dendrimers for siRNA delivery, because dendrimers are a special class of macromolecules with uniquely well-defined spherical architecture having multivalency and cooperativity confined within a nanoscale volume.<sup>13,14</sup> We have recently developed a series of structurally flexible triethanolamine (TEA)-core poly(amidoamine) (PAMAM) dendrimers,<sup>15</sup> which bear primary amines at the

dendrimer surface and tertiary amines at the branching units inside (Figure 1). These dendrimers self-assemble with siRNA into compact, uniform, and nanoscale particles via electrostatic interactions, which protect siRNA from degradation and facilitate binding with the negatively charged cell membrane and hence promote efficient cell uptake, resulting in high transfection efficiency and potent gene silencing in various cells and disease models.<sup>16–23</sup>

In this study, we show for the first time that the TEA-core PAMAM dendrimer of generations 6, denoted as G<sub>6</sub>, exhibits high efficacy and low cytotoxicity in delivering Akt siRNA into ovarian cancer cells both in culture and in a mouse model. We also report a strategy to suppress Akt via dendrimer-delivered siRNA as a novel mechanism to enhance apoptosis induced by paclitaxel, the front-line anticancer agent used in ovarian cancer therapy, leading to a preferential killing of the cancer cells. This combination therapeutic approach shows higher levels of anticancer efficacy than monotherapy and may constitute a valuable and promising nanomedicine approach for the treatment of ovarian and other life-threatening cancer forms for which there are currently no effective therapies.

## RESULTS

**Dendrimers G<sub>6</sub> Form Stable Complexes with Akt siRNA and Protect siRNA from Degradation.** We first

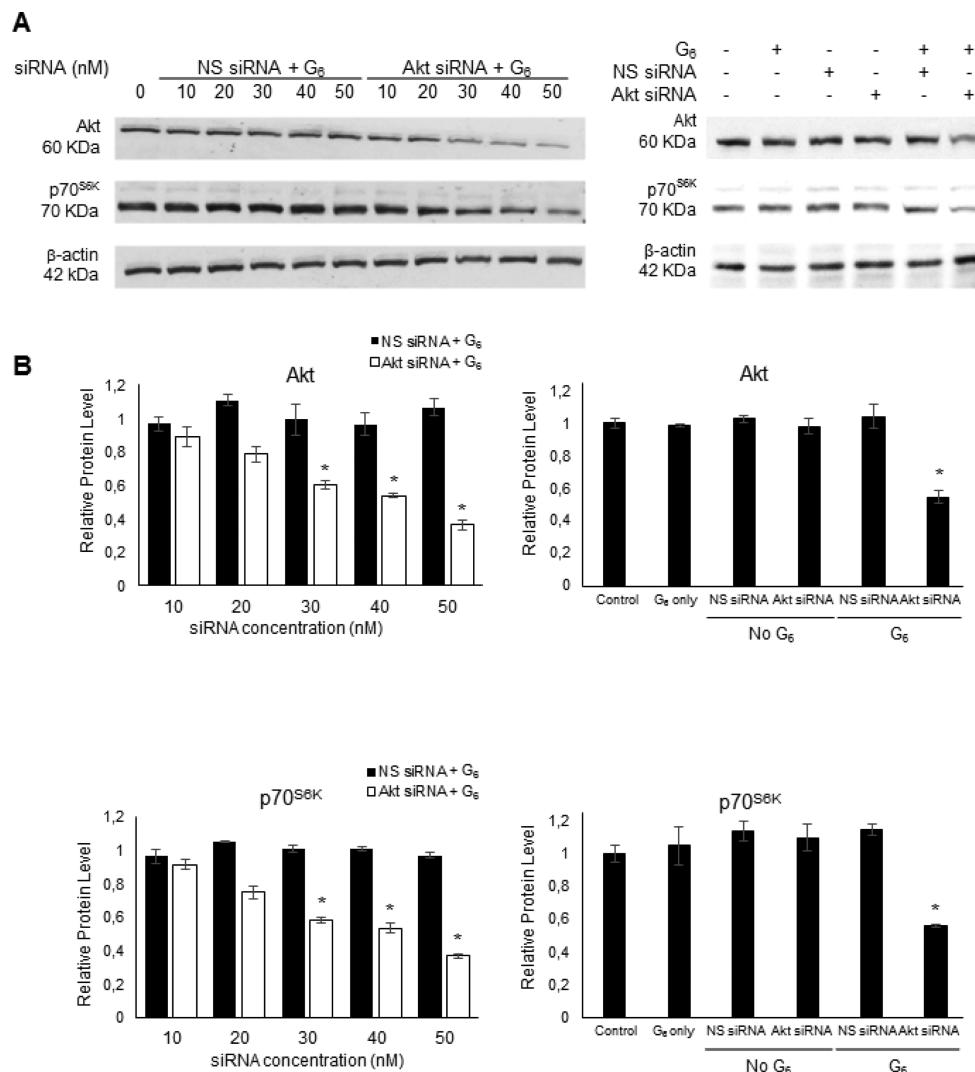


**Figure 2.** Characterization of Akt siRNA dendriplexes. (A) The binding abilities of G<sub>6</sub> dendrimer with siRNA (0.5  $\mu$ g) in DEPC-treated water at N/P ratios varying from 1 to 20 were tested using agarose gel electrophoresis. Naked Akt siRNA was used as an internal control. (B) Size distribution of the Akt siRNA/G<sub>6</sub> complexes using 1  $\mu$ M siRNA and G<sub>6</sub> at an N/P ratio of 5 at 37 °C measured by DLS. (C) Zoomed view of the mesoscopic simulation of G<sub>6</sub> TEA-core PAMAM dendrimers in complex with Akt siRNA at N/P = 5. The dendrimers are portrayed as wine-colored spheres, with charged amine groups depicted in pink. siRNA molecules are shown as turquoise sticks. A transparent gray field is used to represent the solvent environment. (D) RNase A protection assay. Complexes prepared in the specified way were incubated with 0.01  $\mu$ g/ $\mu$ L RNase A at room temperature for 0–45 min and then incubated with 0.42% SDS at 4 °C.

tested the ability of the dendrimer G<sub>6</sub> to form stable complexes with Akt siRNA using gel mobility shift assays. PAMAM dendrimers do not migrate toward the cathode during gel electrophoresis because of their positive charge and large size, whereas dendrimer binding to siRNA retards siRNA movement into the gel, and thus, the degree of complex formation can be inferred. Compared to the naked siRNA control, Akt siRNA in complex with dendrimer G<sub>6</sub> showed a significant retardation of migration at a N/P ratio of 2.5 or above (Figure 2A). The formed Akt siRNA/G<sub>6</sub> complexes were further analyzed for their size and size distribution using dynamic light scattering (DLS). As we can see in Figure 2B, G<sub>6</sub> and the Akt siRNA readily formed stable and uniform nanoparticles with an average size of 85 nm in diameter at N/P = 5. This size of the dendriplexes, less than 100 nm, is known to be advantageous for cellular uptake and delivery efficacy. Moreover,  $\zeta$ -potential measurements gave positive values of 32 mV for these nanoparticles, implying that the Akt siRNA dendriplexes are stable colloids. Figure 2C shows a zoomed view of the morphology of G<sub>6</sub>/siRNA complexes within the nanoparticles as obtained from mesoscopic simulations based on constraints provided by the DLS data. As can be seen in Figure 2C, there is a well-defined tendency for the siRNA duplexes to wrap themselves around the dendrimer molecules. Moreover, the siRNA strands are uniformly distributed and better encased

within the dendrimer network, indicative of an efficient protection from the external environment. Consistently, the siRNA/G<sub>6</sub> complexes were able to protect siRNA from degradation (lower panel in Figure 2D), whereas naked siRNA was rapidly degraded upon RNase digestion (upper panel in Figure 2D). These results demonstrate that dendrimer G<sub>6</sub> forms stable nanosized complexes with the Akt siRNA and protects it from degradation, highlighting its potential use for further *in vitro* and *in vivo* experiments.

**G<sub>6</sub>-Mediated Effective siRNA Delivery and Potent and Specific Gene Silencing of Akt.** We next assessed the effect of the Akt gene-silencing effect with dendrimer G<sub>6</sub>-mediated delivery in the SKOV-3 cell line, which is a model for advanced, drug-resistant ovarian cancer, where Akt plays a key role in tumor cell proliferation and survival.<sup>24</sup> Indeed, G<sub>6</sub>-mediated delivery of Akt siRNA was effective and led to the reduction of Akt at the protein expression level (left panel in Figure 3A), which is the direct consequence of siRNA-mediated gene silencing, as no notable effect was observed with the nonspecific siRNA (NS siRNA) in the presence of G<sub>6</sub>. Neither G<sub>6</sub> alone nor siRNA alone (right panel in Figure 3) had any effect, consistent with previous observations.<sup>25–27</sup> Our results showed that the effect of gene silencing was dependent upon the siRNA concentration, in which significant gene silencing was already obtained at an siRNA concentration of 30 nM, where 50%



**Figure 3.** Specific inhibition of Akt and its downstream effector p70<sup>S6K</sup> by Akt siRNA delivered via dendrimer G<sub>6</sub>. (A) SKOV-3 cells were transfected with NS siRNA or Akt siRNA by PAMAM dendrimer G<sub>6</sub> at a N/P ratio of 5. Akt and p70<sup>S6K</sup> expression levels were analyzed by Western blotting 72 h after treatment. β-Actin was a loading control. (B) The levels of proteins were quantified by densitometric analysis and are expressed as the fold change normalized to β-actin. Bars represent the mean ± SD. An asterisk indicates  $P < 0.05$  vs NS siRNA.

inhibition was observed (Figure 3B). Akt suppression also affected its downstream signaling protein p70<sup>S6K</sup> (Figure 3), which is involved in cell survival. Neither Akt nor p70<sup>S6K</sup> expression was decreased by NS siRNA (Figure 3). This suggests that the Akt protein was successfully suppressed by the Akt siRNA after G<sub>6</sub>-mediated delivery into SKOV-3 cells.

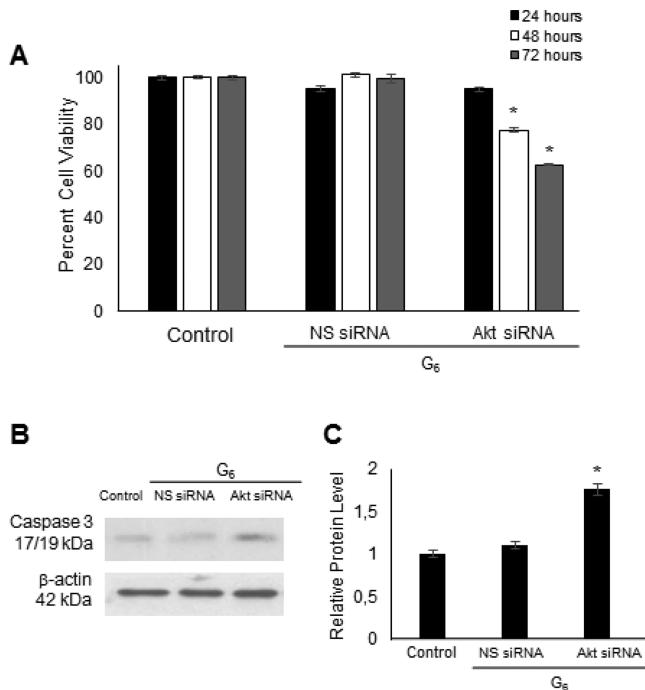
**Akt Knockdown Reduces Proliferation and Induces Apoptosis.** To evaluate cytotoxicity and cell proliferation, we used MTT assays. As shown, an efficient inhibition of cell proliferation and/or viability was observed after treatment with Akt siRNA/G<sub>6</sub> for 24 h and subsequent incubation of up to 72 h, relative to the NS siRNA control as well as nontreatment control (Figure 4A). The inhibition of cell growth was evident by 48 h after treatment with Akt siRNA (Figure 4A). In contrast, NS siRNA did not affect cell growth (Figure 4A). To assess whether apoptosis accounted for the loss of viability, we examined the expression of active (cleaved) caspase 3 by Western blot analysis. As shown in Figure 4B,C, Akt siRNA treatment displayed a significantly enhanced expression of active caspase 3 compared with the control or NS siRNA dendriplexes. The delivery step was relatively safe for the cells

with no toxicity observed with NS siRNA/G<sub>6</sub> (Figure 4A), further demonstrating its great potential for in vivo application.

#### Akt Knockdown Potentiates Paclitaxel-Induced Death.

The role of siRNA to inhibit Akt in overcoming chemoresistance in ovarian cancer cells is of particular interest, since current chemotherapeutic agents have limited efficacy in patients with advanced ovarian cancer.<sup>28</sup> To this end, we examined the combined use of Akt siRNA/G<sub>6</sub> with paclitaxel. Paclitaxel is the first-line chemotherapeutic drug used for treating ovarian cancer in the clinic, and one of the major modes of its action is via the induction of apoptosis. Upon combined treatment of Akt siRNA dendriplexes and paclitaxel, we found a significant inhibition of growth of SKOV-3 cells (Figure 5A), concomitant with an increase in paclitaxel-induced apoptosis compared to treatment with either Akt siRNA dendriplexes alone or paclitaxel alone (Figure 5B,C). No additional inhibition was observed for paclitaxel in combination with NS siRNA dendriplex (Figure 5).

**Reduced Tumorigenicity after Akt Knockdown.** To assess whether Akt silencing could inhibit tumor outgrowth in vivo in a setting relevant to cancer treatment, we administered

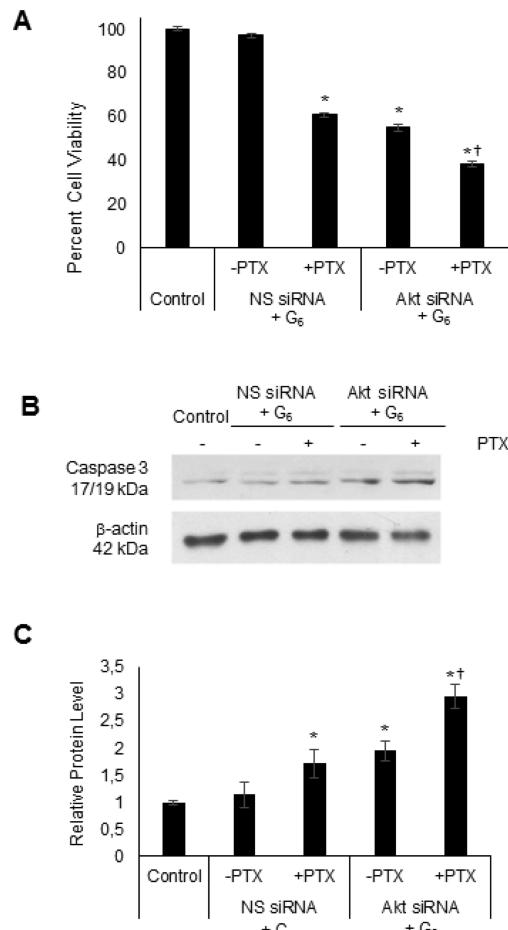


**Figure 4.** Cell growth inhibition induced by dendrimer-delivered Akt siRNA. (A) SKOV-3 cells were transfected with NS siRNA or Akt siRNA by PAMAM dendrimer G<sub>6</sub> at a N/P ratio of 5. Cell viability was measured with the MTT assay. Data from three independent experiments run in duplicate wells are shown. (B) Apoptosis was assessed by cleaved (active) caspase 3 staining.  $\beta$ -Actin was a loading control. (C) The levels of proteins were quantified by densitometric analysis and are expressed as the fold change normalized to  $\beta$ -actin. Bars represent the mean  $\pm$  SD. An asterisk indicates  $P < 0.05$  vs the control.

Akt siRNA dendriplexes to tumor-xenografted mice. SKOV-3 subcutaneous tumors were allowed to grow to  $\sim 30$  mm<sup>3</sup> and then treated by direct intratumoral injection of dendrimer-delivered NS siRNA or Akt siRNA. As shown, tumor outgrowth was significantly reduced in the Akt siRNA/G<sub>6</sub>-treated mice compared to the NS siRNA/G<sub>6</sub>-treated mice (Figure 6A). Indeed, tumors of the treated mice were approximately half the size of the control tumors at the time of sacrifice (Figure 6A,B). The reduction of Akt expression in tumor xenografts injected with Akt siRNA dendriplexes was confirmed by immunohistochemical staining (right panel in Figure 6C). In addition, the Akt siRNA-treated tumors showed signs of necrosis (left panel in Figure 6C). Furthermore, Akt siRNA dendriplex and paclitaxel combined treatment showed an effective sensitization to chemotherapy, reducing tumor growth by 85% compared to the treatment with either paclitaxel alone or Akt siRNA dendriplexes alone (Figure 6D,E). G<sub>6</sub> alone or siRNA alone had no effect (Figure 6F). Therefore, the dendrimer efficiently delivered siRNA into xenografts of tumors to induce a substantial antitumor and chemosensitization effect *in vivo*.

## ■ DISCUSSION AND CONCLUSION

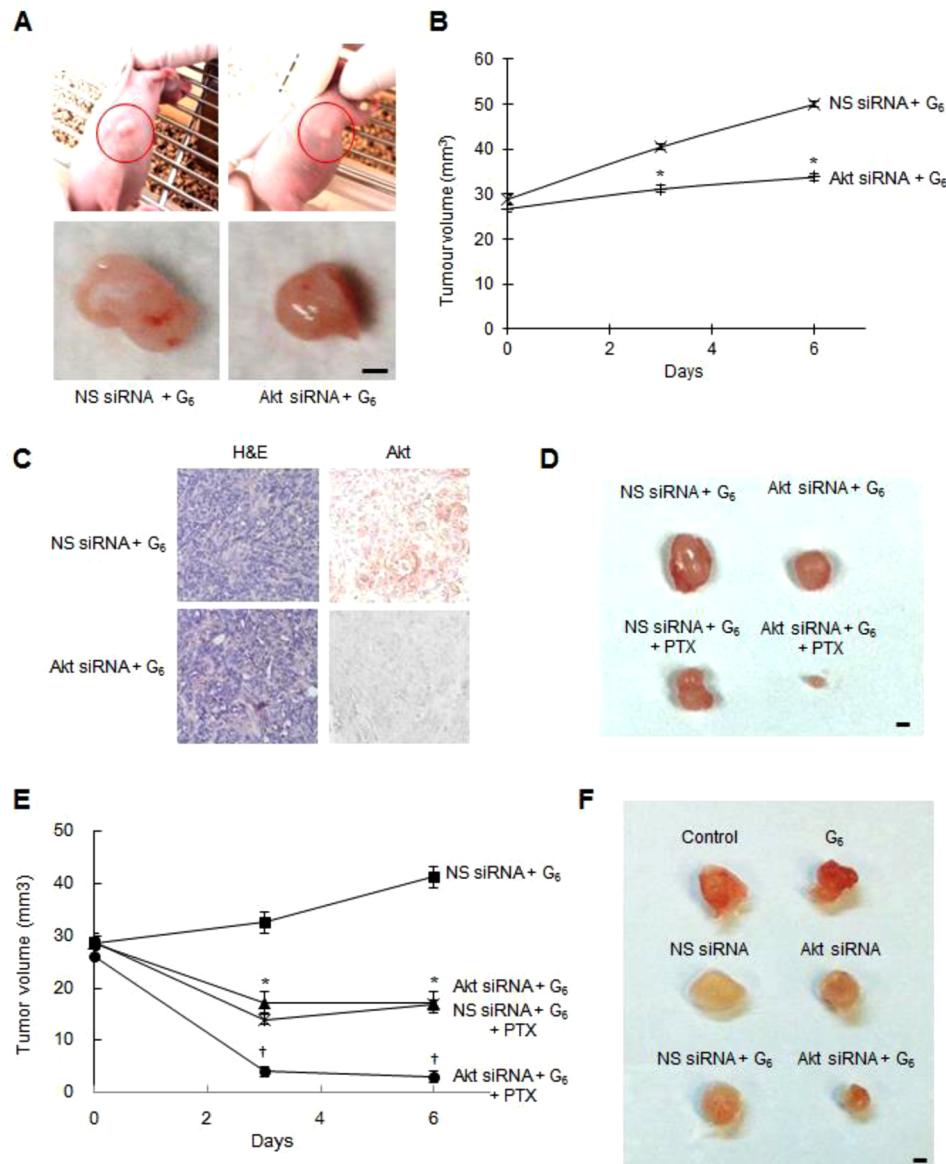
Since the discovery of RNA interference (RNAi), siRNA molecules are emerging as a novel class of therapeutics in cancer therapy. Unlike traditional chemotherapeutic drugs of small molecular weight that are often hydrophobic and can easily pass through the plasma membrane, siRNAs are highly negatively charged macromolecules and are not able to freely



**Figure 5.** Akt siRNA potentiates paclitaxel-induced death in SKOV-3. (A) SKOV-3 cells were transfected with NS siRNA or Akt siRNA by PAMAM dendrimer G<sub>6</sub> at a N/P ratio of 5 alone or in combination with paclitaxel (PTX; 100 nM). Cell viability was measured with the MTT assay. Data from three independent experiments run in duplicate wells are shown. (B) Apoptosis was assessed by cleaved (active) caspase 3 staining.  $\beta$ -Actin was a loading control. (C) The levels of proteins were quantified by densitometric analysis and are expressed as the fold change normalized to  $\beta$ -actin. Bars represent the mean  $\pm$  SD. An asterisk indicates  $P < 0.05$  vs control. The symbol “†” indicates  $P < 0.05$  vs NS siRNA + PTX.

cross the membrane. In addition, siRNAs are intrinsically unstable (half-life  $\sim 3$  min) and can be rapidly degraded by endogenous circulating enzymes. Thus, delivery of siRNAs into target cells is a major impediment to the clinical translation of RNAi in cancer therapy. While previously PAMAM dendrimers have been intensively investigated to act as nanovectors for DNA delivery,<sup>29–31</sup> only a few recent studies have reported their use in siRNA-based therapies.<sup>13</sup> In this study, we have demonstrated that the TEA-core PAMAM dendrimer G<sub>6</sub> is an effective vector for delivering Akt siRNA into chemoresistant ovarian cancer cells, resulting in potent gene silencing and producing a promising anticancer effect both *in vitro* and *in vivo*.

Indeed, TEA-core PAMAM dendrimers are particularly well-suited for the delivery of siRNA because of their open flexible conformation with void spaces in the interior.<sup>16</sup> In our previous studies,<sup>32</sup> we have shown that these structural and flexibility characteristics render TEA-core dendrimers more efficient in siRNA delivery with respect to other PAMAM-based molecules



**Figure 6.** RNAi-mediated Akt downregulation inhibits ovarian cancer cell growth in nude mice. (A) SKOV-3 cells were subcutaneously injected into nude mice and allowed to grow to  $\sim 30 \text{ mm}^3$ . Xenografts were then treated with intratumoral injection of NS siRNA or Akt siRNA dendriplexes. Comparison of representative xenograft tumors is shown. The bar represents 2 mm. (B) Tumor volumes were measured by calipers. Data are expressed as the mean  $\pm$  SD. (C) H&E staining of xenografts treated with Akt siRNA showed necrosis compared with that of xenografts treated with NS siRNA. The expression of Akt was determined by immunohistochemistry. All sections are shown at 400 $\times$  magnification. (D) The same assay was performed along with paclitaxel (PTX; 2 mg/kg/week) administered to the mice by intraperitoneal injection. Comparison of representative xenograft tumor sizes is shown. (E) Tumor sizes ( $\text{mm}^3$ ) are reported. (F) The representative tumors from xenograft mice treated with PBS control, G<sub>6</sub> alone, NS siRNA alone, Akt siRNA alone, NS siRNA with G<sub>6</sub>, and Akt siRNA with G<sub>6</sub> are shown. The bar represents 2 mm. An asterisk indicates  $P < 0.05$  vs NS siRNA. The symbol “†” indicates  $P < 0.05$  vs NS siRNA + PTX.

of comparable generation. For example, the enhanced mobility of the TEA-core dendrimer branches with respect to diethyldiamine (DEA)-core PAMAMs becomes manifest through a higher density of the dendrimer charged terminal groups at the dendrimer periphery.<sup>32,33</sup> This, in turn, results in increased enthalpy gain and reduced entropy penalties paid by the more flexible dendrimers in enwrapping the siRNA molecules and thus a more efficient siRNA complexation. Furthermore, the open and flexible dendrimer structure allows water molecules to pervade the dendrimer interior more easily, consequently increasing the availability of the inner tertiary amines for protonation and resulting in enhanced buffering capacity via a

proton sponge effect, ultimately leading to more efficient nucleic acid release and hence a better delivery efficiency.<sup>16</sup>

It is important to note that the dendrimer-mediated Akt siRNA delivery is effective in suppressing tumor cell growth in ovarian cancer. This finding has clinical significance because ovarian cancer is an especially aggressive form of cancer. While most ovarian cancer patients initially respond to first-line chemotherapy, almost all relapse, leading to chemoresistant ovarian cancer for which current therapies are ineffective. These intriguing findings are also relevant to a large number of ovarian carcinomas which constitutively overexpress Akt compared with normal cells and tissues. Furthermore, the relevance of the PI3K/Akt in ovarian cancer was supported by the clinical

observation that ovarian cancer patients show lower survival and tend to have a worse outcome when expressing higher p-Akt levels.<sup>34</sup>

Akt is known to be frequently activated in human cancers, resulting in enhanced proliferation and tumor progression and therefore can be a promising therapeutic target. siRNA-based therapies provide several advantages over traditional therapeutic approaches such as small-molecule inhibitors. For example, unlike small-molecule inhibitors, RNAi-based therapeutics can selectively and potently target any gene with known sequence, even those previously considered undruggable. Moreover, on the basis of the mRNA sequence for the target gene, siRNAs require simple strategies to design, optimize, and implement compared to the time needed to synthesize and screen conventional small-molecule drugs and thus may constitute an attractive approach for personalized therapy.<sup>35</sup>

A promising strategy to abrogate tumor cells is the induction of apoptosis either alone or in synergy with chemotherapeutic drugs. Paclitaxel is one of the most widely used anticancer drugs in the clinical treatment of ovarian cancer. In addition to the dose-limiting toxicity, this agent induces drug resistance, which still poses a major problem in its clinical use.<sup>28</sup> In this study we found that Akt siRNA dendriplexes specifically exert an enhancing role on ovarian cancer cell apoptosis induced by chemotherapeutic treatment. Importantly, these changes in chemotherapy sensitivity occurred at pharmacologically attainable drug levels.<sup>34</sup> On the basis of our knowledge, this is the first report that the delivery of Akt siRNA mediated by dendrimers can potentiate the antitumor effect of the anticancer drug paclitaxel both in vitro and in vivo in ovarian cancer models, suggesting the potential of clinical application. Moreover, low concentrations of paclitaxel with Akt siRNA can also induce an enhanced cytotoxic response in the cancer cells (data not shown), suggesting potential utility to improve clinical efficacy.

Compared to the siRNA concentration for the cancer cells treated with PAMAM dendrimer nanovectors reported in the literature, the TEA-core PAMAM dendrimer formulation (30 nM in vitro and 0.625 mg/kg in vivo) required ~50% less siRNA in this study. It is known that high concentrations of siRNA are accompanied by a high frequency of off-target effects.<sup>35</sup> Moreover, our preliminary experiments have also shown that similar gene silencing was observed in our TEA-core PAMAM dendrimers and commercial cationic lipids (Lipofectamine 2000) (data not shown). This again proves the TEA-core PAMAM dendrimer G<sub>6</sub> delivery system is highly effective in initiating siRNA-based therapeutic applications.

In summary, these results demonstrate that TEA-core PAMAM dendrimers represent promising siRNA delivery systems which will provide the nanocarrier platform delivering siRNA therapeutics. In addition, dendrimer-mediated Akt siRNA delivery, in combination with a chemotherapeutic regimen, may constitute a promising nanomedicine approach for drug development in cancer therapy. We are actively pursuing this direction.

## EXPERIMENTAL SECTION

**Dendrimer/siRNA Complexation.** The dendrimer used was TEA-core PAMAM of generations 6 ( $M_w = 43648$  g/mol, 192 amine end groups). It was dissolved in deionized, autoclaved, 0.22  $\mu\text{m}$  filtered water at a concentration of 500  $\mu\text{M}$  and were freshly dissolved to 10  $\mu\text{M}$  for every transfection. Human Akt siRNA (NM\_005163) and NS siRNA (D-001210-02-20) were obtained from Dharmacon (Lafayette,

CO). The N/P ratio was calculated as the ratio of available surface amines of the dendrimer to the number of phosphates in an siRNA duplex molecule. Dendriplexes were prepared by diluting appropriate amounts of siRNA and dendrimer in the appropriate solution, mixed by pipetting, and allowed to complex at room temperature for 30 min.

### Complex Formation Analysis by Agarose Gel Retardation.

An appropriate amount of G<sub>6</sub> dendrimer (diluted in 50 mM Tris-HCl buffer, pH 7.4) and 0.5  $\mu\text{g}$  of siRNA were complexed in DEPC-treated water and incubated at 37 °C for 30 min. Each sample was then loaded onto a 1.2% agarose gel containing 0.10% ethidium bromide and run for 10 min at 100 V in TBE buffer, pH 7.0. The bands were then visualized using a UV transilluminator, and the stability of the complexes was gauged by observing the position of RNA bands on the gel or in the wells. Naked siRNA subjected to the same treatment was used as a control.

**Size and  $\zeta$ -Potential Measurement of Akt siRNA/G<sub>6</sub> Complexes.** The Akt siRNA solution was mixed with the indicated amount of dendrimer solution at a N/P ratio 5. The final concentration of siRNA was 1  $\mu\text{M}$ . After incubation at 37 °C for 30 min, size and  $\zeta$ -potential measurements were performed using a Zetasizer Nano-ZS (Malvern, Ltd., Malvern, U.K.) with a He-Ne ion laser of 633 nm.

**RNase A Protection Assay.** siRNA/dendrimer complexes were incubated with 0.01  $\mu\text{g}/\mu\text{L}$  RNase A for 45 min at 37 °C. A 4  $\mu\text{L}$  volume of this solution was aliquoted at the indicated time points and then added to 3  $\mu\text{L}$  of 1% SDS solution at 4 °C to release the siRNA. These solutions were then loaded onto a 1.2% agarose gel containing ethidium bromide and run at 100 V in standard TBE buffer. The bands were then visualized using a UV transilluminator, and the protective ability of the dendrimer G<sub>6</sub> was then gauged by the presence of RNA bands in the gel. Naked siRNA was used as a control.

**Cell Culture and Transfection.** SKOV-3 cells (ATCC, Manassas, VA) were cultured as adherent monolayers and maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>/95% air. The cells were seeded at a density of 2 × 10<sup>4</sup> cells per well and grown in the medium for 24 h at 37 °C. The cells were then treated with dendrimer/siRNA complexes for the indicated time. In some cases, paclitaxel (Calbiochem, San Diego, CA) at 100 nM was added to the medium.

**Western Blot Analysis.** Cells were lysed using 1× SDS lysis buffer containing 260 mM Tris/HCl, pH 6.8, 0.8% SDS, and 40% glycerol. The protein concentration was determined by the Bio-Rad DC protein assay (Hercules, CA). Equal amounts of protein (10  $\mu\text{g}$ ) were run on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was then incubated with primary antibodies for Akt, p70 S6 kinase (p70<sup>S6K</sup>), caspase 3 (Cell Signaling, Austin, TX) (1:1000), and  $\beta$ -actin (Sigma, St. Louis, MO) (1:5000) overnight at 4 °C. The membranes were then incubated with HRP-conjugated antirabbit secondary antibodies, visualized by chemiluminescence (Perkin-Elmer, Waltham, MA), and quantified by image densitometry using ImageJ (NIH, Bethesda, MD).

**Cytotoxicity and Cell Proliferation Analysis by MTT Assay.** The medium was removed, the cells were washed with PBS, and medium containing 0.1  $\mu\text{g}/\mu\text{L}$  MTT (Sigma, St. Louis, MO) was added. The cells were incubated at 37 °C for 4 h, washed with PBS, and lysed in DMSO. The absorbance was read using a Bio-Rad 550 microplate reader at 570 nm (reference 630 nm, filters from Bio-Rad). To measure the cytotoxicity of the dendrimer/siRNA treatment, this process was performed immediately following 24 h of transfection. Cell proliferation was measured at two further intervals post-transfection.

**In Vivo Tumor Model.** Female athymic nude mice were purchased from Charles River Laboratories (Wilmington, MA) and were cared for according to guidelines set forth by the University of Hong Kong. All animals were 6–8 weeks of age at the time of injection. A total of 10<sup>6</sup> cells were injected subcutaneously into the right flank of the mice. Tumor volumes were calculated as an ellipsoid volume using the formula  $(\pi/6)lw^2$ , where  $l$  = larger measurement and  $w$  = smaller measurement. When the tumors reached ~30 mm<sup>3</sup>, 12.5  $\mu\text{g}$  of Akt

siRNA and dendrimer G<sub>6</sub> at a N/P ratio of 5 were allowed to form a complex in PBS and injected intratumorally. For pathological examination, tissue sections were fixed on formalin, embedded in paraffin blocks, and hematoxylin and eosin (H&E) stained. For paclitaxel treatment, paclitaxel (2 mg/kg/week) was administered intraperitoneally in the presence or absence of Akt siRNA dendriplexes. Tumor sizes were measured for one week further, and the mice were sacrificed by cervical dislocation, after which the tumors were excised and their final length measurements taken.

**Statistical Analysis.** All the data are presented as the mean  $\pm$  SD, unless stated otherwise. The data were compared by one-way ANOVA followed by a Tukey's test among more than two groups and by unpaired Student's *t* test between two groups. The differences were considered to be statistically significant when the *P* value was less than 0.05.

**Molecular Modeling.** Mesoscopic simulations were performed in the framework of a multiscale modeling strategy developed by our group.<sup>20,32,36–40</sup> This approach is based on the systematic elimination of computationally expensive degrees of freedom while retaining implicitly their influence on the remaining degrees freedom in the mesoscopic model. At the coarse-grained (mesoscopic) level, we employed the corresponding most accurate and effective methods/simulation techniques available to investigate physical properties of each system at that level. Accordingly, using the information obtained from atomistic molecular dynamics simulations, we parametrized the coarse-grained (e.g., DPD)<sup>41</sup> models that incorporate all essential physics/phenomena observed at the finer level.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

siRNA, small interfering RNA; PAMAM, poly(amidoamine); TEA, triethanolamine; G<sub>6</sub>, generation 6 of the TEA-core PAMAM dendrimer; DLS, dynamic light scattering; SDS, sodium dodecyl sulfate; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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