“OA02” Peptide Facilitates the Precise Targeting of Paclitaxel-Loaded Micellar Nanoparticles to Ovarian Cancer In Vivo

Kai Xiao, 1,7,# Yuanpei Li, 1,# Joyce S. Lee, 1,3 Abby M. Gonik, 4 Tiffany Dong, 1 Gabriel Fung, 1 Eduardo Sanchez, 1 Li Xing, 5 Holland R. Cheng, 5 Juntao Luo, 6,* and Kit S. Lam 1,2,*

1Department of Biochemistry & Molecular Medicine, 2Division of Hematology and Oncology, Department of Internal Medicine, 3Department of Pharmacy, 4Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, 5Department of Molecular and Cellular Biology, University of California Davis, Sacramento, CA 95817, USA, 6Department of Pharmacology, SUNY Upstate Cancer Research Institute, SUNY Upstate Medical University, Syracuse, NY 13210, USA, and 7National Chengdu Center for Safety Evaluation of Drugs, West China Hospital, Sichuan University, Chengdu 610041, China. #These authors contributed equally to this work.

Running title: A novel targeted nanotherapeutics against ovarian cancer

Keywords: OA02 peptide, polymeric micelles, drug delivery, targeted therapy, ovarian cancer.

Disclosure of Potential Conflicts of Interest: Kit S. Lam is the founding scientist of LamnoTherapeutics which plan to develop the nanotherapeutics described in the manuscript.

*Corresponding Authors: Kit S. Lam, Department of Biochemistry & Molecular Medicine, University of California at Davis, Suite2301, 2700 Stockton Blvd, Sacramento, CA 95817, USA. E-mail: kit.lam@ucdmc.ucdavis.edu; Juntao Luo, Department of Pharmacology, SUNY Upstate Cancer Research Institute, SUNY Upstate Medical University, WHA 6299, 750 East Adams Street, Syracuse, NY 13210, USA E-mail: luoj@upstate.edu.

Word count (excluding references): 4905 Total number of figures and tables: 7
Abstract

Micellar nanoparticles (NPs) based on linear polyethylene glycol (PEG)-block-dendritic cholic acids (CA) copolymers (telodendrimers), for the targeted delivery of chemotherapeutic drugs in the treatment of cancers, are reported. The micellar NPs have been decorated with a high-affinity "OA02" peptide against alpha-3 integrin receptor to improve the tumor targeting specificity which is overexpressed on the surface of ovarian cancer cells. "Click chemistry" was used to conjugate alkyne-containing OA02 peptide to the azide group at the distal terminus of the PEG chain in a representative PEG5k-CA8 telodendrimer (micelle forming unit). The conjugation of OA02 peptide had negligible influence on the physicochemical properties of PEG5k-CA8 NPs and as hypothesized, OA02 peptide dramatically enhanced the uptake efficiency of PEG5k-CA8 NPs in SKOV-3 and ES-2 ovarian cancer cells via receptor-mediated endocytosis, but not in alpha-3 integrin negative K562 leukemia cells. When loaded with paclitaxel (PTX), OA02-NPs had significantly higher in vitro cytotoxicity against both SKOV-3 and ES-2 ovarian cancer cells as compared with non-targeted NPs. Furthermore, the in vivo biodistribution study demonstrated OA02 peptide greatly facilitated tumor localization and the intracellular uptake of PEG5k-CA8 NPs into ovarian cancer cells as validated in SKOV3-luc tumor bearing mice. Finally, PTX-loaded OA02-NPs exhibited superior anti-tumor efficacy and lower systemic toxicity profile in nude mice bearing SKOV-3 tumor xenografts, when compared with equivalent doses of non-targeted PTX-NPs as well as clinical PTX formulation (Taxol®). Therefore, OA02 targeted telodendrimers loaded with PTX have great potential as a new therapeutic approach for ovarian cancer patients.
Introduction

Ovarian cancer is the ninth most common cancer, with an estimated 21,990 new cases in 2011, but is the fifth most deadly, with an estimated 15,460 deaths in 2011 (1). The standard treatment for patients with advanced-stage disease usually involves surgical staging and debulking followed by adjuvant chemotherapy, typically with platinum and paclitaxel (PTX). However, the more extensive use of chemotherapeutic drugs such as PTX is often limited by its severe side effects, including hypersensitivity reactions, myelosuppression and neurotoxicity, which may be attributed to their non-specific systemic organ distribution and inadequate intra-tumor concentrations, resulting in suboptimal efficacy (2, 3). Despite the intensive chemotherapy, over 70% of ovarian cancer patients will suffer from disease relapse or recurrence, and ultimately die of this disease. Therefore, there is a tremendous incentive to refine existing treatment modalities to avoid or delay the recurrence and to treat recurrent ovarian cancer more effectively. Optimization of chemotherapeutic drug delivery is among the critical approaches to improve the therapeutic index of cytotoxic agents.

Nanotechnology is an emerging field that has shown great promise in the development of novel diagnostic and therapeutic agents for a variety of diseases, including cancers (4). As the vasculature in tumors is known to be leaky, and the tumor lymphatic system is also deficient, nanoparticles (NPs) can preferentially accumulate in the tumor site via the enhanced permeability and retention (EPR) effects (5). Polymeric micelles represent one of the most promising nanocarriers due to their unique core-shell structure formed by amphiphilic block copolymers, which could facilitate the solublization of poorly soluble drugs and protect the drugs from degradation and metabolism. We have recently developed a series of novel linear-dendritic block copolymers (telodendrimers) comprising polyethylene glycol (PEG) and dendritic cholic acids (CA), which can encapsulate high concentrations of hydrophobic drugs such as PTX and self-assemble to form stable core-shell micelles under aqueous condition (6-12). The representative PEG5k-CA8 micellar NPs possess the ideal properties for drug delivery, including high drug loading capacity, optimal particle size (20-60 nm), outstanding stability (over 6 months at 4 °C), and sustainable drug release profile. PTX-loaded PEG5k-CA8 NPs have been demonstrated to exhibit...
superior anti-tumor efficacy and toxicity profile compared to free drug (Taxol®) and PTX/human serum albumin nanoaggregate (Abraxane®) at equivalent PTX doses, in nude mice bearing human ovarian cancer (SKOV-3) xenografts (6).

To further facilitate the residence, penetration, and cancer cell uptake of delivered drugs within the tumor sites for more efficient cancer treatment, an attractive approach is to decorate the NPs surface with targeting ligands that specifically recognize receptors on cancer cells (active targeting). Active targeting might result in higher retention of NPs drugs at tumor sites (i.e., by reducing passive transport away from tumor) and enhanced uptake of the drugs by cancer cells via receptor-mediated endocytosis (13-15). Furthermore, actively targeted NPs have also shown the potential to overcome multidrug resistance (MDR) via bypassing of P-glycoprotein mediated drug efflux (16). Combining passive and active targeting in a single platform will further improve the therapeutic index of nanocarrier delivered drugs (17, 18). A wide variety of targeting ligands, including antibodies and single-chain Fv fragment (19, 20), peptides (21, 22), small molecules (23), and aptamers (24, 25) have been used with varying degrees of success to functionalize NPs for their potential application in targeted cancer therapy. Although antibodies or antibody fragments are effective as targeting agents, there are some innate problems such as decreased receptor affinity as a result of conjugation methods, potential immunogenicity, nonspecific uptake by reticuloendothelial system (RES) and relative poor stability (17). In contrast, peptides or peptidomimetics with high binding affinity and specificity to cancer cells may have many favorable characteristics, including deep tumor penetration due to the smaller size, lack of immunogenicity, easy synthesis and scale-up, and good stability especially if D-configuration and unnatural amino acids are used (26).

Integrins are a family of heterodimeric transmembrane glycoproteins involved in a wide range of cell-to-extracellular matrix (ECM) and cell-to-cell interactions (27, 28). It has been found that integrins are overexpressed on various cell types such as angiogenic endothelial cells and certain cancer cells. For example, alpha-3 integrin is overexpressed in several types of cancers, especially ovarian cancer, breast cancer, and melanoma (29). The overexpression of alpha-3 integrin on these cancer cells has been
exploited as a promising pharmacological target for the selective drug delivery in the treatment of these cancers. Additionally, during the cell locomotion and migration, integrins can undergo endocytosis after the activation with anchoring ligands, which may facilitate the intracellular delivery of NPs drugs into cancer cells, when these NPs are decorated with integrin-targeting ligands. A high-affinity alpha-3 integrin targeting peptide “OA02” has been identified in our laboratory through screening one-bead one-compound (OBOC) combinatorial peptide libraries (30). This “OA02” peptide has been shown to bind strongly to alpha-3 integrin overexpressing ovarian cancer cells and specifically target ovarian cancer xenografts (ES-2) in nude mice when conjugated to near-infrared fluorescence (NIRF) dyes (30).

In the present study, we hypothesize that the incorporation of “OA02” peptide ligand onto our newly developed micellar NPs, will facilitate the precise homing of drug payload to alpha-3 integrin overexpressing ovarian cancer cells. First, the alkyne-modified “OA02” peptide was synthesized and conjugated to the azide-functionalized PEG$^{5k}$-CA$_8$ telodendrimer via copper-catalyzed cycloaddition (“click chemistry”). Then, the binding specificity, uptake efficiency and \textit{in vivo} tumor targeting property of fluorescence-labeled OA02-NPs were evaluated in human ovarian cancer cells and xenograft mouse model, respectively. Finally, the anti-tumor effect of PTX-loaded OA02-NPs against ovarian cancer was studied both \textit{in vitro} and \textit{in vivo}.

\textbf{Materials and Methods}

\textbf{Materials.} Diamino polyethylene glycol was purchased from Rapp Polymere (Tübingen, Germany). Cy5.5 Mono NHS ester was purchased from Amersham Biosciences (Piscataway, NJ). Hydrophobic fluorescence dye DiD (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate, D-307), 4’, 6-diamidino-2-phenylindole (DAPI) and LysoTracker® Red were purchased from Invitrogen. Paclitaxel was purchased from AK Scientific Inc. (Mountain View, CA). Taxol® (Mayne Pharma, Paramus, NJ) was obtained from the UC Davis Cancer Center Pharmacy. Cholic acid, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromid] and fluorescein isothiocyanate (FITC) and all other chemicals were purchased from Sigma-Aldrich.
Synthesis of OA02 conjugated PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer. Boc-NH-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer was first synthesized as described previously (6). N<sub>3</sub>-PEG<sup>5k</sup>-CA<sub>8</sub> was obtained by the coupling of 4-azidobutyric acid NHS ester to the terminus of PEG after deprotecting Boc group of Boc-NH-PEG<sup>5k</sup>-CA<sub>8</sub> with 50% (v/v) trifluoroacetic acid (TFA) in dichloromethane (DCM). The telodendrimer was then dialyzed and finally lyophilized.

Alkyne modified OA02 peptide (cdG-HoCit-GPQc-Ebes-K-alkyne) was synthesized via solid-phase synthesis on Fmoc-Rink Amide MBHA Resins using the standard Fmoc chemistry as described previously (30). 5-hexynoic acid was coupled onto the ε-amino group of lysine on the peptide. Alkyne modified OA02 peptide was conjugated to the N<sub>3</sub>-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer via Cu<sup>1</sup> catalyzed cycloaddition (21). The conjugation was confirmed by the amino acid analysis (AAA). The molecular structure and molecular weight of OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer were measured by <sup>1</sup>H-NMR and MALDI-TOF mass spectrometry (MS), respectively.

FITC or Cy5.5 labeled telodendrimers were synthesized by coupling FITC or Cy5.5 NHS ester to the amino group of the proximal lysine between PEG and cholic acid after the removal of Dde protecting group by 2% (v/v) hydrazine in DMF.

Preparation and characterization of PTX-loaded OA02-NPs. PTX-loaded OA02-NPs (PTX-OA02-NPs) were prepared using the mixture (1:1) of blank PEG<sup>5k</sup>-CA<sub>8</sub> and OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimers via a dry-down (evaporation) method as described previously (6). To determine the amount of PTX loaded in the NPs, PTX-loaded NPs were dissolved in DMSO (1:9, v/v), and measured by HPLC. The encapsulation efficiency (EE) was calculated according to the following formula:

\[ EE(\%) = \frac{\text{mass of PTX encapsulated in NPs}}{\text{mass of PTX added}} \times 100\% \]

The morphology, particle size distribution and zeta potential of PTX-OA02-NPs were characterized by cryo-transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS, Microtrac), respectively. The in vitro drug release kinetics from PTX-OA02-NPs was measured by the dialysis method. Briefly, aliquots of PTX-OA02-NPs solution were injected into dialysis cartridges with the MWCO of 3.5 kDa. The cartridges were dialyzed against 1 L PBS and shaken at 37 °C at 100 rpm
with activated charcoal to create a sink condition. The concentration of PTX remained in the dialysis cartridge at different time points were measured by HPLC.

**Cell culture and animals.** SKOV-3, ES-2, and K562 cells were purchased from American Type Culture Collection. SKOV3-luc cells were obtained from Caliper Life Sciences (Hopkinton, MA). All these cancer cell lines were authenticated by the suppliers, and passaged in the laboratory for fewer than six months after resuscitation. Cells were maintained in a 37°C/5% CO₂ humidified chamber in McCoy's 5A (SKOV-3, ES-2 and SKOV3-luc) or RPMI-1640 (K562) media supplemented with 10% FBS.

Female nude mice, 6-8 weeks age, were purchased from Harlan Laboratories. All animal protocols were approved by the Institutional Animal Care and Use Committee. Ovarian cancer xenograft mouse model was established by subcutaneously injecting 5×10⁶ SKOV-3/SKOV3-luc cells in a 100 µL of mixture of PBS and Matrigel (1:1, v/v) at the right flank in female nude mice.

**Confocal microscopy.** SKOV-3 and ES-2 cells were seeded in 8-well chamber slides. When the cells were almost confluent, cells were incubated with 2 µM FITC fluorescent-labeled NPs and OA02-NPs for 2 h at 37 °C with 5% CO₂, respectively. Then, cells were washed three times with cold PBS, fixed with 4% paraformaldehyde for 10 min, and the nuclei were counterstained by DAPI. The slides were mounted with coverslips and observed by Olympus FV1000 confocal microscopy. In another set of experiment, excess amount of alpha-3 integrin antibody or free OA02 peptide (200 µM) were added into the medium 30 min prior to the incubation of 2 µM FITC-labeled OA02-NPs with cells, followed by the same procedure as above.

**Flow cytometry.** To demonstrate the overexpression of alpha-3 integrin, SKOV-3 and ES-2 cells were incubated with Alex Fluor 488 conjugated alpha-3 integrin Ab (Chemicon International, 1:500) for 30 min at 4 °C, followed by PBS wash twice, and then resuspended in PBS for the flow cytometric analysis.

SKOV-3, ES-2 and K562 (alpha-3 integrin negative) cells were incubated with 2 µM FITC-labeled NPs or OA02-NPs for 2 h at 37 °C, respectively. Then the cells were washed with PBS three times and
resuspended in PBS for the flow cytometric analysis. 10,000 events were collected for each sample. For peptide inhibition experiments, free OA02 peptides with the final concentration from 2 µM to 200 µM were added into the medium 30 min prior to the incubation of 2 µM FITC-labeled NPs or OA02-NPs with cells.

**Intracellular tracking of OA02-NPs in live ovarian cancer cells.** In order to simultaneously track the payload and carrier of OA02-NPs, DiD dyes were encapsulated as drug surrogates into FITC conjugated OA02-NPs. SKOV-3 ovarian cancer cells were seeded in the coverglass chamber slides. After reaching 80% confluence, cells were incubated with DiD/FITC dual labeled OA02-NPs. After 1.5 h, LysoTracker Red (50 nM) was added in the medium and the cells were further incubated for another 30 min (31). Then, the live cells were observed under the Olympus FV1000 confocal microscopy.

**MTT assay.** MTT assay was used to evaluate the *in vitro* cytotoxicity of blank/PTX-loaded non-targeted NPs and OA02-NPs against ovarian cancer cells (32). Cells were treated with blank/PTX-loaded NPs and OA02-NPs, respectively. After 2 h of treatment, cells were washed with PBS three times, and fresh media were replaced in the plates. At 72 h, MTT was added to each well and further incubated for another 4 h. The absorbance at 570 nm with a reference wavelength of 660 nm was detected using a microplate reader. Untreated cells served as a control. Results were shown as the average cell viability \[
\frac{(OD_{treat} - OD_{blank})}{(OD_{control} - OD_{blank})} \times 100\%
\] of triplicate wells.

**In Vivo and Ex Vivo NIRF optical imaging.** Nude mice bearing subcutaneous SKOV3-luc tumors were intravenously injected with 4 nM Cy5.5 fluorescent-labeled NPs and OA02-NPs, respectively. At different time point (0.5, 2, 4, 8 and 24 h) post-injection, mice were scanned with Kodak imaging system IS2000MM. At 24 h, tumors and major organs were excised for *ex vivo* imaging. For the microscopic analysis, excised tumors were frozen in O.C.T. medium at 80 °C. The corresponding slices (10 µm) were prepared, air-dried for 10 min and fixed with 4% paraformaldehyde for 10 min. The alpha-3 integrin expression in the tumor section was stained by Alex Fluor 488 conjugated alpha-3 integrin antibody (1:500) for 1 h at room temperature. The blood vessel was stained by rat anti-mouse
CD31 primary antibody (Millipore, 1:100) for 1 h, and Cy3 conjugated goat anti-rat IgG secondary antibody (Millipore, 1:1500) for 1 h at room temperature.

**Therapeutic study.** The anti-tumor efficacy and toxicity profiles of different PTX formulations were evaluated in the subcutaneous xenograft mouse model of SKOV-3 ovarian cancer. The treatment was initiated when tumor volume reached 100 ~ 200 mm³ and this day was designated as day 0. The maximum tolerated dose (MTD) of Taxol® in mice is approximately 10 mg/kg (6, 33), and the micellar formulations of PTX were expected to be better tolerated than Taxol® according to our previous report (6). Mice were administrated intravenously with PBS, Taxol® (10 mg/kg), PTX-NPs (10, 30 mg/kg) and PTX-OA02-NPs (10, 30 mg/kg), respectively (n = 8 ~ 10). The dosage was given every three day for a total of 6 doses. Tumor sizes were measured with a digital caliper twice per week. Tumor volume was calculated by the formula (L*W²)/2, where L is the longest, and W is the shortest in tumor diameters (mm). Relative tumor volume (RTV) equals the tumor volume at given timepoint divided by the tumor volume prior to initial treatment. For humane reasons, animals were sacrificed when the implanted tumor volume reached 1500 mm³, which was considered as the end point of survival data. Survival rate was analyzed using a Kaplan-Meier plot. The potential toxicities after treatment were monitored by the animal behavior observation and the body weight measurement twice per week.

**Statistical analysis.** Statistical analysis was performed by Student’s t-test for comparison of two groups, and one-way analysis of variance (ANOVA) for multiple groups, followed by Newman-Keuls test if overall P < 0.05.

**Results and Discussion**

**Synthesis of OA02-PEG⁵k-CA₈ telodendrimer.** N₃-PEG⁵k-CA₈ telodendrimer was first synthesized via stepwise solution-phase condensation reactions as reported previously (6). “Click chemistry” was used to covalently conjugate alkyne-containing OA02 peptide onto the azide group at the PEG terminus of N₃-PEG⁵k-CA₈ telodendrimer, resulting in OA02-PEG⁵k-CA₈ telodendrimer (Figure 1A). The conjugation of OA02 peptide onto PEG⁵k-CA₈ telodendrimer was confirmed by AAA (Figure S-1).
Each amino acid of OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer was hydrolyzed and quantitatively measured by HPLC. As summarized in Table S-1, the determined numbers of each amino acid by AAA were almost identical to their corresponding theoretical values in the molecular formula of OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer, indicating the successful conjugation of OA02 peptide to the telodendrimer. The molar ratio of OA02 peptide to telodendrimer was almost 1:1, which meant that there was approximately one targeting peptide molecule per telodendrimer monomer. The molecular weight of OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer was measured with MALDI-TOF MS. The mono-dispersed mass trace was detected, and its molecular weight from MALDI-TOF MS was almost identical to the theoretical value (Figure S-2A). The chemical structure of OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer was also determined by <sup>1</sup>H-NMR spectrometry. As shown in Figure S-2B, the signals at 0.6-1.3 ppm and 3.5-3.7 ppm could be assigned to cholic acids and PEG chains, respectively. The <sup>1</sup>H-NMR signals of OA02 peptide were overlapped with the signals from the telodendrimers and no distinguishable signal was observed.

**Preparation and characterization of PTX-loaded OA02-PEG<sup>5k</sup>-CA<sub>8</sub> NPs.** The critical micelle concentration (CMC) of OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer was found to be comparable with that of blank PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer, with the range of 6 to 12 μM (Table 1 and Figure S-3), indicating its excellent micelle-forming property. Using the dry-down method, hydrophobic drugs such as PTX can be readily encapsulated into the core of micellar NPs. When the feeding ratio of drug PTX/telodendrimer (w/w) was 1:4, the EE of PEG<sup>5k</sup>-CA<sub>8</sub> NPs and OA02-PEG<sup>5k</sup>-CA<sub>8</sub> NPs were approximately 92%, and 96%, respectively. To enable the developed NPs to possess both antibiofouling (“stealth”) and cell-specific targeting properties (34), OA02-PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer was mixed with blank PEG<sup>5k</sup>-CA<sub>8</sub> telodendrimer (1:1, w/w) to prepare PTX-OA02-NPs for the subsequent cell and animal studies (Figure 1B). The cryo-TEM image (Figure 2A) demonstrated that PTX-OA02-NPs were spherical, with uniform particle sizes of around 50 nm in diameter, which was similar with the result obtained from DLS measurement (Figure 2B). The zeta potential of PTX-OA02-NPs in PBS was almost neutral (-0.64 mV). The stability of PTX-OA02-NPs was evaluated by measuring the changes of particle sizes over time at different conditions. PTX-OA02-NPs were found to be very stable at 4 °C for
over 3 months, and also stable at 37 °C for at least 72 h when incubated with 50% FBS (data not shown), which indicated that they will likely be able to maintain their stability and integrity during their in vivo applications. The drug release pattern from PTX-OA02-NPs was similar with that from PTX-NPs, which both were biphasic, with the initial rapid release of PTX during the first 4 h, followed by the slow linear release over the subsequent few days (Figure 2C). In summary, the decoration of OA02 peptide had negligible impact on the physicochemical properties of PEG\textsuperscript{Sk}-CA\textsubscript{8} NPs, including CMC, morphology, particle size, drug loading capacity, encapsulation efficiency, stability and drug release profile. This is probably because the OA02 peptide conjugation occurs at the distal end of PEG chain which is located on the shell of self-assembled micellar NPs without interfering with the drug-holding hydrophobic core unit.

**Cellular uptake studies.** Alpha-3 integrin was demonstrated to be overexpressed on both SKOV-3 and ES-2 cells, as measured by the flow cytometric analysis (Figure S-4). The uptake profiles of FITC-labeled OA02-NPs in ovarian cancer cells were first qualitatively observed by the confocal microscopy. Non-targeted NPs had minimal non-specific cellular uptake after 2-h incubation, whereas the decoration of OA02 peptide greatly increased the extent of NPs uptake in both SKOV-3 (Figure 3A) and ES-2 cells (Figure S-5A). Most FTIC-labeled NPs (green) distributed around the perinuclear region, which meant that these NPs were internalized into the cytoplasm. More importantly, the uptake of OA02-NPs in both ovarian cancer cells was able to be remarkably inhibited by excess amount of alpha-3 integrin antibody or free OA02 peptide, suggesting the alpha-3 integrin receptor-dependent internalization of OA02-NPs.

The uptake efficiencies of FITC-labeled OA02-NPs in ovarian cancer cells and K562 leukemia cells (alpha-3 integrin negative) were further quantitatively measured by the flow cytometric analysis. Both non-targeted NPs and OA02-NPs had similar low nonspecific uptake in K562 cells (Figure 3B). However, OA02-NPs exhibited significantly higher uptake than the non-targeted NPs in both ovarian cancer cells (P < 0.05), with almost 6-fold higher uptake in SKOV-3 cells (Figure 3C) and 4-fold higher uptake in ES-2 cells (Figure S-5B), respectively. The addition of free OA02 peptide was able to inhibit the uptake of OA02-NPs in SKOV-3 cells (Figure 3D) and ES-2 cells (Figure S-5C) in a dose-
dependent manner, further confirming the alpha-3 integrin targeting specificity of OA02-NPs. It should be noted that significant higher concentration of free peptide (10 to 100-folds) was required to inhibit the cellular uptake of OA02-NPs, while the equal concentration of free peptide did not produce obvious inhibition effect. This could be possibly explained by the increased binding affinity of OA02 peptides presented on the NPs surface due to the multivalency effects (35).

**Intracellular tracking of dual fluorescent-labeled OA02-NPs in live cells.** After 2-h incubation, both FITC-labeled telodendrimer carrier (green) and DiD dye payload (red) were simultaneously internalized into the SKOV-3 cells with co-localized dot-shape fluorescent foci in the perinuclear region of the cytoplasm (Figure 4A, bottom left), indicating that the intact OA02-NPs were taken up into the cytoplasm. These fluorescent foci were generated as a result of the accumulation of OA02-NPs in the endocytic vesicles (i.e. endosomes and lysosomes). This was evidenced by the partial co-localization of internalized FITC-labeled OA02-NPs (green) with lysosomal compartment (red), producing yellow fluorescence in the merge images (Figure 4A, bottom right). Similar observation was also reported by Frank and coworkers in the LNCaP prostate cancer cells incubated with A10 aptamer targeted PLGA-b-PEG-b-Apt NPs (34).

**In vitro cytotoxicity study.** PTX-OA02-NPs were found to be significantly more cytotoxic against both SKOV-3 (Figure 4B) and ES-2 cells (Figure S-6), when compared to non-targeted PTX-NPs at the equivalent PTX concentration (P < 0.05). The enhanced cytotoxicity of PTX-OA02-NPs is likely related to the capability of OA02 peptide to facilitate the uptake of PTX-OA02-NPs into ovarian cancer cells, thus increasing the intracellular PTX concentration. Furthermore, there was no observable cytotoxic effect associated with both blank non-targeted NPs and OA02-NPs at the equivalent NPs concentration, which eliminated the possibility of OA02 peptides or NPs induced cytotoxic activity.

**Biodistribution and tumor targeting specificity in vivo.** NIRF optical imaging is an important tool for visualizing molecular processes in vivo, as NIRF dyes with deep penetration, low autofluorescence, and low tissue absorption and scattering enable the high-resolution tissue imaging (26). Both Cy5.5 fluorescent-labeled NPs and OA02-NPs distributed throughout the body of the mice immediately after
the intravenous injection, and gradually accumulated into the SKOV3-luc tumor. However, the uptake rate of OA02-NPs in the tumor site was faster than that of non-targeted NPs. Substantial contrast between tumors and background in the mice injected with OA02-NPs was observed at around 4 h post-injection and these NPs were able to be retained in the tumor throughout the 24-h period, whereas the accumulation of non-targeted NPs in the tumor was not apparent until 8 h post-injection (Figure 5A). Ex vivo images at 24 h demonstrated that both non-targeted NPs and OA02-NPs exhibited relatively high uptake in the SKOV3-luc tumor compared to normal organs except the liver (macrophage uptake), as the result of EPR effect (Figure 5B). However, the median fluorescence intensity (MFI) of tumors for OA02-NPs was approximately 1.7-fold higher than that for non-targeted NPs (Figure 5C, P < 0.05). The histological distribution of NPs in the tumor tissue was further observed under the confocal microscopy. As shown in Figure 5D, the majority of Cy5.5-labeled non-targeted NPs (red) were mainly distributed in the perivascular region, which is in concordance with previous histological observations of passive accumulation of liposomes and micelles in tumor tissues (14). In contrast, OA02-NPs were able to extravasate from the tumor vasculature, penetrate deep into the interstitial space of the tumor, bind to alpha-3 integrin overexpressing tumor cells and eventually become internalized. Similar observations were also reported by Kirpotin and colleagues using PEGylated liposome targeted by an anti-HER-2 antibody (36). The enhanced tumor localization and intracellular uptake of OA02-targeted NPs into ovarian cancer cells is probably attributed to the specific interaction between the OA02 peptide and alpha-3 integrin, thus facilitating the homing of targeted NPs to ovarian cancer, and the receptor-mediated endocytosis.

**Therapeutic study.** The anti-tumor effect of PTX-OA02-NPs was evaluated in the subcutaneous SKOV-3 ovarian cancer xenograft mouse model when compared with non-targeted PTX-NPs and PTX clinical formation (Taxol®). As shown in Figure 6A, all the PTX formulations significantly inhibited the tumor growth, when compared with the control group (P < 0.05). However, both PTX-NPs and PTX-OA02-NPs exhibited better tumor growth inhibition than Taxol®, at equivalent PTX dose of 10 mg/kg. The superior tumor growth inhibition of PTX micellar formulations might be attributed to the larger
amount of PTX delivered to the tumor site via the EPR effect. More importantly, mice treated with PTX-OA02-NPs had significantly slower tumor growth rate than those treated with non-targeted PTX-NPs at the equivalent PTX doses (P < 0.05). The survival rate of mice in each group is presented by the Kaplan-Meier survival curve, respectively (Figure 6B). In general, compared to PBS control, all the PTX formulations significantly prolonged the survival rates of tumor bearing mice. However, mice treated with 30 mg/kg PTX-OA02-NPs exhibited the longest survival time among these treatment groups. The median survival time of mice in the group of PBS control, Taxol®, PTX-NPs (10, 30 mg/kg), and PTX-OA02-NPs (10, 30 mg/kg) were 20, 27, 29, 69, 32, and 95 days, respectively. It was also noted that complete response (CR), defined as a complete disappearance of palpable tumor nodule, was achieved in 5 of 10 mice (50%) in the 30 mg/kg PTX-OA02-NPs group, and 2 of 8 mice (25%) in the 30 mg/kg PTX-NPs group. The better tumor growth inhibition, prolonged survival time and higher complete tumor response rate observed in the PTX-OA02-NPs group are likely due to the more efficient and specific delivery of PTX to the ovarian cancer cells by the unique ligand-targeted nanoformulation, as mentioned earlier. Several independent investigations have also demonstrated that some other targeted NPs such as antibody-targeted liposomes (37), transferrin-targeted polymeric NPs (38), and folate-targeted PAMAM polymers (39), can significantly enhance the anti-tumor effects as compared with their non-targeted counterparts.

Toxicities were assessed by direct observation of animal behavior and body weight monitoring. Mice treated with 10 mg/kg Taxol® showed a decline of overall activity during the first 30-min post injection, which is likely a sign of hypersensitivity reaction related to the diluent (Cremophor® EL and ethanol) (33). In addition, this group of mice was found to have significant amount of body weight loss (with a nadir of 6.7%) after receiving the first few doses, when compared to the control group (P < 0.05). In contrast, the mice in both PTX-NPs and PTX-OA02-NPs groups tolerated the regimens well. The treatments did not seem to have obvious adverse impact on their activity level and body weight (Figure 6C). Overall, the PTX nanoformulations, both PTX-NPs and PTX-OA02-NPs, demonstrated with an
improved systemic toxicity profile, which might be attributed to their Cremophor-free composition, prolonged blood retention time and sustained drug release features (40).

**Conclusion**

We have successfully developed OA02 peptide-targeted polymeric micelles system to effectively deliver chemotherapeutic drugs to ovarian cancer. High-affinity and high-specificity “OA02” peptide against alpha-3 integrin was successfully conjugated to the distal PEG terminus of PEG^{5k}-CA$_8$ telodendrimer via “click chemistry”, and displayed on the surface of self-assembled micellar NPs. OA02 peptide decoration dramatically enhanced the intracellular delivery of NP-drugs into alpha-3 integrin overexpressing ovarian cancer cells via receptor-mediated endocytosis, resulting in higher *in vitro* cytotoxicity of PTX-OA02-NPs against those cancer cells than non-targeted PTX-NPs. OA02 peptide also significantly facilitated the distribution of targeted NPs into tumor tissues and cells in SKOV3-luc tumor bearing mice. Furthermore, PTX-OA02-NPs were found to be more efficacious and less toxic than the equivalent doses of non-targeted PTX-NPs and Taxol® in ovarian cancer xenograft mouse model. Therefore, OA02 peptide-targeted NPs drug delivery system has great translational potential in the treatment of ovarian cancer patients.

**Acknowledgments**

**Grant support:** NIH/NCI R01CA140449 (Luo), R01CA115483 (Lam), R01EB012569 (Lam), and DoD Postdoctoral Fellowship Award (W81XWH-10-1-0817 Xiao).
References

Table 1. The physicochemical characteristics of PEG<sup>5K</sup>-CA<sub>8</sub> and OA02-PEG<sup>5K</sup>-CA<sub>8</sub> telodendrimers

<table>
<thead>
<tr>
<th>Telodendrimers</th>
<th>CMC (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Particle size (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PTX/Polymer ratio (w/w)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PTX loaded in micelles (mg/ml)</th>
<th>EE (%)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Particle size after PTX loading (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG&lt;sup&gt;5K&lt;/sup&gt;-CA&lt;sub&gt;8&lt;/sub&gt;</td>
<td>6.1</td>
<td>21.0 ± 2.3</td>
<td>1:4</td>
<td>4.6 ± 0.5</td>
<td>92 ± 1.8</td>
<td>46.2 ± 3.4</td>
<td>-1.62</td>
</tr>
<tr>
<td>OA02-PEG&lt;sup&gt;5K&lt;/sup&gt;-CA&lt;sub&gt;8&lt;/sub&gt;</td>
<td>11.8</td>
<td>20.5 ± 1.9</td>
<td>1:4</td>
<td>4.8 ± 0.8</td>
<td>96 ± 2.7</td>
<td>49.1 ± 2.8</td>
<td>-0.64</td>
</tr>
</tbody>
</table>

<sup>a</sup> CMC was measured by fluorescence spectrometry using pyrene (2 µM) as a probe; <sup>b</sup> Measured by DLS; <sup>c</sup> The concentrations of telodendrimers were 20 mg/ml; <sup>d</sup> Encapsulation efficiency expressed as a percentage mean of three determinations ± standard deviation of PTX weight recovered in NPs compared to theoretical loaded weight.
**Figure Legends**

**Figure 1.** Chemical structure of OA02 peptide functionalized PEG<sup>Sk</sup>-CA<sub>8</sub> telodendrimer (A) and the preparation of stealth targeted NPs by the self-assembly of blank/OA02-functionalized telodendrimers (B). Alkyne-containing OA02 peptide was conjugated to the azide group on the distal terminus of PEG chain in PEG<sup>Sk</sup>-CA<sub>8</sub> telodendrimer via “click chemistry”. The Boc-protected amino group on the proximal lysine in OA02-PEG<sup>Sk</sup>-CA<sub>8</sub> telodendrimer can be used for the conjugation of fluorescence dyes such as FITC or Cy5.5 for cell and animal imaging. The OA02 peptides presented on the surface of targeted NPs are able to specifically recognize and bind the alpha-3 integrin receptors, which are overexpressed on the cell membrane of ovarian cancer cells.

**Figure 2.** The morphology (A) and particle size distribution (B) of PTX-OA02-NPs (5 mg PTX in 20 mg/ml telodendrimer) measured by cryo-TEM and DLS, respectively. In Figure 2A, white arrows point to NPs, while Mosaic Virus (TMV) was used as calibration standard (18 nm in width). The ice crystals usually have clear edge contrast. C, In vitro PTX release kinetics from PTX-NPs or PTX-OA02-NPs in PBS at 37 °C. The concentration of PTX remained in the dialysis cartridge at various time points was measured by HPLC. Error bars were obtained from triplicate samples.

**Figure 3.** The uptake of FITC-labeled OA02-NPs in SKOV-3 ovarian cancer cells and K562 leukemia cells (alpha-3 integrin negative). A, Confocal microscopic images of SKOV-3 cell incubated with FITC-labeled NPs and OA02-NPs (green) for 2 h. To demonstrate the alpha-3 integrin dependent uptake, excess amount of alpha-3 integrin antibody, or free OA02 peptide was added prior to the incubation of OA02-NPs with SKOV-3 cells. The flow cytometric analysis of OA02-NPs uptake in K562 cells (B) and SKOV-3 cells (C). The mean fluorescence intensity (MFI) of cells incubated with NPs and OA02-NPs are shown as insets. * P < 0.05. D, the addition of free OA02 peptides at concentrations from 2 to 200 μM inhibited cellular uptake of OA02-NPs in SKOV-3 cells in a dose-dependent manner.

**Figure 4.** (A) Intracellular tracking of dual fluorescent-labeled OA02-NPs upon their uptake in live SKOV-3 cells. DiD dye (red) was encapsulated as drug surrogates into FITC (green) chemically labeled OA02-NPs. SKOV-3 cells were incubated with DiD/FITC dual labeled OA02-NPs for 2 h, and the cells were stained with lysosome tracker (red) before the live-cell imaging by confocal microscopy. (B) The differential cytotoxicities of 0.5 μg/ml PTX-loaded NPs (PTX-NPs), PTX-loaded OA02-NPs (PTX-OA02-NPs), and the equivalent dose of blank NPs, OA02-NPs against SKOV-3 ovarian cancer cells measured by MTT assay. NPs were incubated with cells for 2 h, and the cells were subsequently washed and incubated in fresh media for a total of 72 h before assessing cell viability in each group. * P < 0.05.

**Figure 5.** In vivo and ex vivo NIRF optical imaging of Cy5.5-labeled NPs or OA02-NPs biodistribution after i.v. injection in subcutaneous SKOV3-luc tumor bearing mice. (A) In vivo optical images of real-time tumor targeting characteristics of NPs or OA02-NPs. Tumor bearing mice were injected via tail vein with 4 nM Cy5.5-labeled NPs or OA02-NPs, and were scanned with Kodak multimodal imaging system IS2000MM at different time point. (B) Representative ex vivo optical images of tumors and organs of SKOV3-luc bearing...
mice sacrificed at 24 h. (C) quantitative fluorescence intensities of tumors and organs from ex vivo images (n = 3). (D) Histological analysis of NPs or OA02-NPs distribution (Cy5.5, red) in tumor cryosections. The nuclei were stained by DAPI (blue), alpha-3 integrin expression on SKOV-3 tumor cells and vascular endothelial cells were visualized by anti-alpha-3 (green) and anti-CD31 (orange) staining, respectively.

**Figure 6.** *In vivo* tumor growth inhibition (A), Kaplan-Meier survival curves (B), and body weight changes (C) of SKOV-3 tumor bearing mice after the intravenous treatment of various PTX formulations. Tumor bearing mice were administered intravenously with PBS (control), Taxol® (10 mg/kg), PTX-NPs (10 mg/kg and 30 mg/kg) and OA02-PTX-NPs (10 mg/kg and 30 mg/kg), respectively, every three day on days 0, 3, 6, 9, 12 and 15 for a total of 6 doses. Data represent mean ± SEM (n = 8-10).
Figure 1

A

Click Chemistry
alkyne-OA02 reacts with N2-PEG^{5k}-CA8

OA02

Covalently conjugation of fluorescence dye (FITC or Cy5.5)

OA02-PEG^{5k}-CA8

B

Self-assemble

PEG

Cholic acid cluster

Drugs (PTX)

Fluorescence dye

Ligand (OA02 peptide)

Receptor (α3 integrin)

Ovarian Cancer Cells
Figure 2

A. Image showing TMV and ice at 200 nm scale.

B. Graph showing size distribution in nanometers.

C. Graph showing PTX remained in micelles over time with two lines: one for PTX-NPs and another for PTX-OA02-NPs.
Figure 3
Figure 4
Figure 5
Figure 6
"OA02" Peptide Facilitates the Precise Targeting of Paclitaxel-Loaded Micellar Nanoparticles to Ovarian Cancer In Vivo

Kai Xiao, Yuanpei Li, Joyce S. Lee, et al.

Cancer Res Published OnlineFirst March 6, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-3883

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/03/06/0008-5472.CAN-11-3883.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.