Urokinase Plasminogen Activator System–Targeted Delivery of Nanobins as a Novel Ovarian Cancer Therapy


Abstract
The urokinase system is overexpressed in epithelial ovarian cancer cells and is expressed at low levels in normal cells. To develop a platform for intracellular and targeted delivery of therapeutics in ovarian cancer, we conjugated urokinase plasminogen activator (uPA) antibodies to liposomal nanobins. The arsenic trioxide–loaded nanobins had favorable physicochemical properties and the ability to bind specifically to uPA. Confocal microscopy showed that the uPA-targeted nanobins were internalized by ovarian cancer cells, whereas both inductively coupled plasma optical mass spectrometry (ICP-MS) and fluorescence-activated cell sorting (FACS) analyses confirmed more than four-fold higher uptake of targeted nanobins when compared with untargeted nanobins. In a coculture assay, the targeted nanobins showed efficient uptake in ovarian cancer cells but not in the normal primary omental mesothelial cells. Moreover, this uptake could be blocked by either downregulating uPA receptor expression in the ovarian cancer cells using short-hairpin RNA (shRNA) or by competition with free uPA or uPA antibody. In proof-of-concept experiments, mice bearing orthotopic ovarian tumors showed a greater reduction in tumor burden when treated with targeted nanobins than with untargeted nanobins (47% vs. 27%; \(P < 0.001\)). The targeted nanobins more effectively inhibited tumor cell growth both in vitro and in vivo compared with untargeted nanobins, inducing caspase-mediated apoptosis and impairing stem cell marker, aldehyde dehydrogenase-1A1 (ALDH1A1), expression. Ex vivo fluorescence imaging of tumors and organs corroborated these results, showing preferential localization of the targeted nanobins to the tumor. These findings suggest that uPA-targeted nanobins capable of specifically and efficiently delivering payloads to cancer cells could serve as the foundation for a new targeted cancer therapy using protease receptors.

Introduction
Ovarian cancer, which usually presents at an advanced clinical stage, is the most aggressive gynecologic malignancy, accounting for 6% of all cancer-related deaths in women in the United States (1). The most common sites of ovarian cancer metastasis are the other ovary, the peritoneum, and the omentum, whereas distant, extra-abdominal metastases are rare (2, 3). The current standard of care includes debulking surgery and subsequent adjuvant chemotherapy with taxane and platinum-containing drugs. This chemotherapy combination clearly prolongs the lives of patients with ovarian cancer; however, almost all tumors will eventually become chemoresistant (1).

Unfortunately, no new drugs have been approved for the treatment of ovarian cancer since 1999, when liposomal doxorubicin (Doxil) was introduced for the treatment of recurrent disease. Doxil, when compared with free doxorubicin, has a longer half-life due to its encapsulation in a polyethylene glycol (PEG)–coated liposome (4). The small size of the Doxil particles (≈100 nm) increases drug accumulation by escape through fenestrated tumor vasculature. The favorable clinical pharmacokinetics and toxicity profile of Doxil, which causes less cardiotoxicity and nausea than doxorubicin (5), has contributed to its clinical efficacy in recurrent ovarian cancer. Doxil represents one of the first examples of the successful transfer of nanotechnology into the clinical setting based on a rationale developed using a human ovarian cancer tumor xenograft model (4, 6) and sets a precedent for liposomal delivery as a means to deliver cancer drugs with more efficacy and fewer side effects (7, 8).

For nanoparticles to reach their full potential in cancer therapy, they should be designed with reduced off-target toxicity and increased circulation half-life for improved passive tumor uptake. These particles should ideally use a
cancer cell–targeted delivery method to specifically deliver large doses of a therapeutic payload directly into tumor cells. Indeed, nanoparticles can be modified for tumor cell-specific delivery with cancer-targeting ligands such as antibodies (9), aptamers (10), peptides (11), or folate (12) that recognize receptors or antigens overexpressed on the surface of cancer cells. There are particular advantages of using antibodies for targeting. These include ease of conjugation, high target specificity, and the potential for antibody-directed cellular cytotoxicity (13).

In our search for new treatments of women’s cancer, we had previously encapsulated arsenic trioxide (As$_2$O$_3$, As), an U.S. Food and Drug Administration (FDA)–approved drug for acute promyelocytic leukemia, into nanoparticles. This novel formulation strategy allowed encapsulation of a high concentration of As in a stable precipitate with nickel acetate (Ni) that we designated as a nanobin (Ni$_2$As; refs. 12, 14, 15). We showed that these nanobins significantly inhibited tumor cell growth in an orthotopic tumor xenograft model (14). However, the poor pharmacokinetic profile of As$_2$O$_3$, including a short plasma half-life and dose-limiting toxicity, had prevented its development for solid tumor indications (16, 17). The nanobin formulation of As$_2$O$_3$ resolved these issues, resulting in increased tumor accumulation, activity, plasma half-life, and improved tolerability when compared with free As$_2$O$_3$ (12, 14).

To further improve antitumor activity, we conjugated an antibody against the urokinase plasminogen activator (uPA; ATN-291) to the arsenic nanobins (18). Both uPA and its receptor, u-PAR, are highly expressed in ovarian cancer (19) including ovarian cancer, where it is detected in as many as 90% of clinical ovarian cancer specimens but not in untransformed ovarian/tubal epithelium [summarized in Kenny and colleagues (20)]. The antibody, ATN-291, binds to the kringle domain of uPA, even when it is bound to u-PAR, and the entire complex is internalized (18). Because uPA is internalized as a complex (21, 22), we reasoned that an antibody against uPA conjugated to nanobins would bind to tumor cells displaying receptor-bound or soluble uPA and, by leading to the internalization of the nanobin/uPA/u-PAR complex, results in intracellular delivery of a large arsenic payload.

In this study, we explore the hypothesis that targeting a nanobin formulation of As$_2$O$_3$ to the uPA system that is highly expressed in ovarian cancer will improve the drug’s antitumor activity. The targeted nanobin formulation may revive the development of a promising cancer drug by combining specific delivery with improved pharmacokinetics. We report here that the targeted nanobins internalize into u-PAR–expressing cancer cells, and effectively impair ovarian cancer tumor growth.

Materials and Methods

Reagents and cell lines

Antibodies against u-PAR (ATN-658 for immunoblot, and ATN-615 for immunohistochemistry) and the uPA antibody ATN-291 were manufactured by SDIX (18), whereas antibody (UK-1) for immunoblot was from American Diagnostica Inc. aldehyde dehydrogenase-1A1 (ALDH1A1; B-5) antibody was obtained from Santa Cruz Biotechnology. Cleaved caspase-3 (Asp175) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 14C10) antibodies were from Cell Signaling Technology, and matrix metalloproteinase (MMP)-2 (IM33) antibody was from Millipore. Hoechst 33342 and JC-1 dye were purchased from Invitrogen. Collagen type I (rat-tail) was purchased from BD Bioscience and 1,2-distearyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[(amino(polyethyleneglycol)-2000)] (DSPE-PEG$_{2000}$) were from Avanti Polar Lipids. Cell culture grade endotoxin-free water was from Thermo Scientific. As$_2$O$_3$, nickel (II) acetate, cholesterol, and other chemical reagents were purchased from Sigma-Aldrich unless otherwise indicated.

The CaOV3 and ES-2 human ovarian cancer cell lines were from American Type Culture Collection (ATCC). SKOV3ip1 and HeyA8 cells were provided by Dr. Gordon Mills (MD Anderson Cancer Center, Houston, TX). All human ovarian cancer cell lines (HeyA8, SKOV3ip1, and ES-2, CaOV3) and the primary ovarian cancer clone MONTY-1 were maintained in Dulbecco’s modified Eagle medium (DMEM, Invitrogen). MONTY-1 was isolated from human omental metastases and used at an early passage (23). All cell lines were validated by short-tandem repeat (STR) DNA fingerprinting using the AmpF/STR Identifier Kit (Applied Biosystems). The STR profiles were compared with known ATCC fingerprints, to the Cell Line Integrated Molecular Authentication database (CLIMA), and to the MD Anderson fingerprint database.

Preparation of alkyne-functional nanobins

Arsenic/nickel complex–loaded nanobins, NB(Ni, As), and empty nanobin controls NB(NaCl) were prepared and described in the Supplementary Materials and Methods as previously reported (14, 18). DSPE-PEG$_{3400}$-NH$_2$ (Laysan Bio) was reacted with 4-pentyloxy acetic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and n-NHS in chloroform at 60°C overnight. The resulting alkyne-functional lipid (DSPE-PEG$_{3400}$-Alkyne) was dried, dissolved in deionized water, and lyophilized. The DSPE-PEG$_{3400}$-alkyne lipid was then incubated with preformed nanobins at a DSPE-PEG$_{3400}$-alkyne to nanobin phospholipid molar ratio of 0.01 for 1 hour at 60°C, allowing it to insert into the lipid bilayer. Meanwhile, the total arsenic content of the nanobin solution was adjusted to 25 mmol/L to ensure that the drug loading does not change during heating. The alkyne functional nanobins were purified by diafiltration against 20 mmol/L HEPES and 150 mmol/L NaCl, pH 7.4. The phospholipid, arsenic, and nickel molar concentrations were then measured by inductively coupled plasma optical emission spectrometry (ICP-OES).
Preparation of urokinase antibody-conjugated nanobins

The uPA antibody (ATN-291) was conjugated to the surface of the nanobins using a copper (I)-catalyzed azide-alkyne cycloaddition reaction (24). The alkyne-functionalized nanobins described above were incubated with the azide-functionalized Alexa Fluor 647 (AF647)-labeled ATN-291 at a molar ratio of 3 × 10⁻⁴ moles antibody per mole of phospholipid. The preparation of azide-functionalized Alexa Fluor 647–labeled ATN-291 was described in the Supplementary Materials and Methods. Copper (II) sulfate, tris(3-hydroxypropyltriazolylmethyl) amine, and sodium ascorbate were added to the reaction mixture to a final concentration of 200 μmol/L, 1 and 2 mmol/L, respectively. The reaction proceeded for 90 minutes at 37°C, when fresh sodium ascorbate was added, bringing the total concentration to 3 mmol/L. The reaction continued for an additional 90 minutes at 37°C. The unconjugated antibody was removed from the solution using diafiltration against 20 mmol/L HEPES and 150 mmol/L NaCl, pH 7.4.

Quantification of nanobin-conjugated antibodies

The number of uPA antibodies conjugated per nanobin was determined by UV/Vis after proteolysis. Urokinase antibody–conjugated nanobins were incubated with 4 mg/mL of pronase for 2 hours at 37°C. Digested antibody was then separated from intact nanobins using an Amicon Ultra spin filter (50 k MWCO; Millipore). The concentration of Alexa Fluor 647–labeled ATN-291 was determined by measuring absorbance at 650 nm with a spectrophotometer (PerkinElmer) and was used as a proxy for ATN-291 concentration.

Transmission electron microscopy

Nanobins were visualized using the negative staining technique via a TECNAI F30 transmission electron microscope (FEI Company). The nanobin suspension was dropped onto a glow-discharged carbon-coated copper grid and stained with 1% uranyl acetate. Images were examined under 300 KV with Gatan CCD digital microscope and a magnification from five random fields (25).

Dynamic light scattering and zeta potential

The measurement of hydrodynamic size and polydispersity index (PDI) of nanobins was performed using a 384-well microplate reader using a DynaPro Nanostar (Wyatt Technology Corporation). The zeta potential assessment was carried out in a clear disposable zeta cell (Wyatt Technology Corporation). Dynamic light scattering (DLS) and zeta potential were performed in PBS at 25°C, and the results were the average of at least 10 measurements from three independent batches.

Inductively coupled plasma-optical emission spectroscopy and -mass spectrometry

For ICP-OES, all nanobin samples and standards were prepared in a matrix composed of deionized water, 3% nitric acid, 2% acetic acid, 0.1% Triton X-100, and 0.5 parts per million (ppm) indium internal standards. Known quantitative standards were prepared at concentrations ranging from 0.50 to 10 ppm arsenic, nickel, and phosphorous. A matrix only standard was also included.

For inductively coupled plasma optical mass spectrometry (ICP-MS), the tissues were rinsed with PBS and frozen at −80°C until analysis. For elemental biodistribution, frozen tissues were submerged in concentrated nitric acid, and digested in a Milestone EthosEZ microwave digestion system. Samples and standards were then diluted into an ultrapure deionized water matrix containing 3% nitric acid, 2% acetic acid, 0.1% Triton X-100, containing 5 parts per billion (ppb) indium as an internal standard.

Immunohistochemistry

Slides containing human ovarian cancer tissue were deparaffinized. The u-PAR was detected with a primary antibody (ATN-615, 1:200) and imaged through an avidin-biotin–free detection system (Dako Envision; refs. 20, 26). For HeyA8 xenograft tumors, a representative tumor area was first confirmed by a gynecologic pathologist (K. Gwin). Immunohistochemical staining of mouse tumor sections were performed to assess markers of apoptosis (cleaved caspase-3, Asp175; 1:20), and ovarian cancer stem cell markers (ALDH1A1, B5; 1:400). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore). The stained cells were imaged at ×100 and ×400 magnification from five random fields (25), and quantified by NIH ImageJ software. Immunohistochemical staining of microvessel density (CD31, M-20; 1:50), proliferation (Ki67, SP6; 1:300) and the mouse macrophage (F4/80, A3-1; 1:500) was described in the Supplementary Materials and Methods.

Immunoblot

Immunoblot was performed as previously described (20, 26). The following antibodies were used: uPA (UK-1; 1:1,000), u-PAR (ATN-658; 1:2,000), cleaved caspase-3 (Asp175; 1:1,000), ALDH1A1 (B-5; 1:250), MMP-2 (IM33; 1:1,000), and GAPDH (14C10; 1:1,000). For cytotoxicity studies, the HeyA8 cells were treated for 1 day with NB (Ni,As; 200 μmol/L of As), ATN-291-NB(Ni,As; 200 μmol/L of As), As₂O₃ (20 μmol/L of As), or ATN-291-NB(NaCl).

Confocal microscopy

Cells were cultured for 3 days and then treated with nanobins (25 μmol/L, lipid concentration). To evaluate internalization, Z-stack scanning was performed by adjusting the focal plane from the bottom to the top of the cells, followed by orthogonal three-dimensional (3D) projection. For the ovarian cancer cell–targeted delivery study, primary human mesothelial cells isolated from omentum of patients (27) were cocultured with HeyA8-GFP cells and were treated with ATN-291(A_pb)⁻NB(Ni,As)
for 24 hours. For apoptosis assessment, HeyA8 cells were treated with 25 μmol/L of As₂O₃, NB(Ni,As), or ATN-291-NB(Ni,As) for 2 hours to measure mitochondrial membrane potential with the JC-1 dye, or for 18 hours to measure DNA fragmentation with Hoechst. Staining was done with 2 μmol/L of JC-1 or 4 μmol/L of Hoechst 33342 dyes for 30 minutes, respectively. The images were acquired with a Leica SP5 confocal laser scanning microscope (oil lens-63X/1.4N). The excitation and emission wavelengths were set for the detection of JC-1 aggregates (488/514 nm), JC-1 monomer (561/592 nm), Alexa Fluor 647 (633/650 nm), and Hoechst (360/450 nm).

Flow cytometry

Ovarian cancer cells were treated with 25 μmol/L (lipid concentration) of ATN-291-NB(Calcein) or NB(Calcein). For time-course studies, HeyA8 cells were monitored over 1 to 48 hour. In competition assays, HeyA8 cells were pretreated with either ATN-291 or single chain uPA (scuPA) with the indicated concentrations for 2 hours before further incubation with nanobins for 24 hours. In receptor-competition assays, ES-2 wild-type (WT) and knockdown cells were incubated with nanobins for 24 hours. After incubation, cells were washed, detached, resuspended in PBS, fixed by 2% paraformaldehyde, and stored at 4°C until analysis. A total of 10,000 events were collected for each sample using an LSRFortessa cell analyzer (calcein: 488/515 nm, Alexa Fluor 647: 641/670 nm). Data were analyzed by FlowJo software (TreeStar Inc.). The analysis of JC-1 stained cells was described in the Supplementary Materials and Methods.

Animal studies

Hey8-GFP (5 × 10⁵) cells (28) were injected intraperitoneally into 5- to 6-week-old athymic female nude mice. Four days after inoculation, the mice were randomized into five groups (5 mice/group): 200 μL of As₂O₃, NB(Ni,As), ATN-291-NB(Ni,As), PBS, or ATN-291-NB(NaCl) were injected intraperitoneally and injections were repeated five times every other day at arsenic concentration of 4 mg/kg (14). The mice were dissected 2 days after the last treatment. All animal experiments described were approved by Institutional Animal Care and Use Committee (IACUC). To study nanobin biodistribution, organs (liver, heart, lung, spleen, and kidney) and the tumor were dissected, rinsed with PBS, and imaged using an Olympus fluorescence molecular imaging system. Blood toxicity studies were described in the Supplementary Materials and Methods.

Cell fractionation

HeyA8 cells were treated with nanobins for 24 hours followed by fractionation using the Qproteome Cell Compartment Kit (Qiagen). The cell compartments were isolated according to the manufacturer’s instructions. The cytosolic, membrane/organelle, and nuclear compartments were verified by immunoblot before ICP-MS analysis for arsenic content.

Statistical analysis

Results were analyzed as mean ± SEM. Statistical analyses were performed using two-tailed t test or one-way ANOVA at P < 0.05 level of significance with GraphPad Prism version 5.04. The Cancer Genome Atlas (TCGA) database was queried for u-PAR expression in 580 patients with ovarian cancer (29) and analyzed using the cBio cancer genomics portal (http://www.cbioportal.org/public-portal/index.do; ref. 30).

Results

Synthesis and characterization of urokinase antibody-conjugated nanobins loaded with As₂O₃

Epithelial ovarian cancer cells homogeneously express elevated levels of u-PAR in the primary tumor and all abdominal metastasis (Fig. 1A). Analysis of the TCGA ovarian cancer database (29) at the cBio cancer genomics portal (30) showed that patients with ovarian cancer with u-PAR gene expression alterations had a significantly worse overall (19.5 vs. 44.3 months) and disease-free survival (12.0 vs. 17.5 months; Supplementary Fig. S1A). And our previous tissue microarray studies demonstrated that u-PAR is highly expressed in epithelial tumors (19) including more than 90% of clinical ovarian cancer specimens (20). This raises the possibility that high u-PAR expression might not only function as a marker of aggressiveness for epithelial ovarian cancer cells but could also be used to specifically deliver a therapeutic payload to those cells. For the experiments described below, we identified ovarian cancer cell lines with high (HeyA8, ES-2) and with low (SKOV3ip1, MONTY-1, and CaOV3) u-PAR expression, all of which also express uPA (Fig. 1B). Because the uPA system is highly expressed in ovarian cancer (18, 20, 31), we sought to develop novel nanobins that would actively target uPA/u-PAR–expressing ovarian cancer cells. A monoclonal antibody raised against the kringle domain of uPA in mice (ATN-291) was chosen as a targeting ligand as it binds tightly to human uPA with a Kd ≈ 0.5 nmol/L and does not disrupt uPA/u-PAR binding (18). The nanobins were assembled from cholesterol, DSPC, and DSPE-PEG, and loaded with an arsenic/nickel coprecipitate (Fig. 1C; Supplementary Fig. S1B; refs. 12, 14, 15). Next, DSPE-PEG-alkyne was postinserted into the bilayer to facilitate the conjugation of the azide-functionalized uPA antibody using click chemistry. This method ensures that the targeting antibodies are not exposed to potentially denaturing high temperatures (Fig. 1C; ref. 32). To facilitate characterization of antibody-nanobin conjugates, the antibody was labeled with Alexa Fluor 647 before click chemistry. UV/Vis analysis of Alexa Fluor 647 after proteolysis showed that the arsenic-loaded nanobins were decorated with an average of 8.5 antibodies per nanobin (Table 1). We also found that, after conjugation, the ATN-291–targeted nanoparticle still bound uPA with nanomolar binding affinity (data not shown).

The lipid composition of the nanobins was optimized to ensure that the nanobin surface was neutral at physiologic pH and to minimize nonspecific cellular binding. The
conjugation of ATN-291 to the nanobin minimally affected the neutral zeta potential of the lipid membrane (Fig. 1D and Table 1). DLS was applied to assess the hydrodynamic diameter and polydispersity of the nanoparticles before and after antibody conjugation. The average hydrodynamic diameter of the arsenic loaded ATN-291-NB(Ni,As) was 104.4 nm, an increase of 10.5% when compared with NB(Ni,As; 94.4 nm), which was expected given the addition of antibodies. Although the conjugation of the uPA-antibody to NB(Ni,As) may increase the heterogeneity of the nanoparticle, the PDI of ATN-291-NB(Ni,As) remained low (Fig. 1E and Table 1). Transmission electron microscopy also showed that the antibody-conjugated nanobins are homogenous, spherical particles (Fig. 1F).

Table 1. Nanobin concentrations, size, PDI, and zeta potential

<table>
<thead>
<tr>
<th>Nanobin</th>
<th>Concentration (mmol/L)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
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<tr>
<td></td>
<td>[As]a</td>
<td>[Ni]a</td>
<td>[Lipid]</td>
<td>Antibodyb</td>
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<tr>
<td>NB(Ni,As)</td>
<td>8.8 ± 0.6</td>
<td>10.4 ± 0.7</td>
<td>18.2 ± 1.3</td>
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<td>ATN-291-NB(Ni,As)</td>
<td>8.5 ± 0.3</td>
<td>10.7 ± 0.2</td>
<td>18.7 ± 1.4</td>
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<tr>
<th>Concentration (nmol/L)</th>
<th>NB(Ni,As)</th>
<th>ATN-291-NB(Ni,As)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
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<td></td>
<td>8.8 ± 0.6</td>
<td>8.5 ± 0.3</td>
<td>104.5 ± 3.1</td>
<td>0.10 ± 0.02</td>
<td>-3</td>
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aMeasured by ICP-OES.
bQuantification of cleaved Alexa Fluor 647-labeled urokinase antibody using a fluorescence spectrophotometer.

Cellular uptake of the urokinase antibody–conjugated nanobins is urokinase and urokinase receptor dependent

Binding of uPA to u-PAR triggers internalization (21). To determine whether the antibody-conjugated nanobins...
are internalized we made: (i) calcein-encapsulated nanobins to visualize whether the nanobin payload becomes internalized into the cytoplasm (Fig. 2A and B), and (ii) ATN-291_{AF647} nanobins, with the antibody labeled with Alexa Fluor 647, to evaluate whether the antibody is internalized together with the nanobins (Fig. 2C–E).

Confocal microscopy showed strong calcein fluorescence in the cytoplasm of HeyA8 cells treated with ATN-291-NB(Calcein) at both 3- and 24-hour time points, whereas the uptake of untargeted NB(Calcein) was much lower (Fig. 2A). This was confirmed in flow cytometry studies in which, after 24 hour, HeyA8 cells treated with ATN-291-NB(Calcein) showed a 4-fold higher mean fluorescence intensity (MFI) compared with NB(Calcein), confirming effective cellular uptake of nanobins targeted to uPA (Fig. 2B). The uptake of fluorescently labeled ATN-291_{AF647}-NB(Ni,As) was increased in HeyA8 cells in a time-dependent manner with maximal signal after incubation for 24 hours (Fig. 2B and C). Confocal laser scanning microscopy showed cytoplasmic localization of ATN-291_{AF647}-NB(Ni,As) when single micron optical sections were reassembled in 3D and the reconstruction shown from the side to visualize the entire Z-axis (Fig. 2D). After only 6 hours, most of the ATN-291_{AF647}-NB

![Figure 2](image_url)

**Figure 2.** Fluorescence analysis of nanobin uptake in ovarian cancer cells. A, confocal microscopy of HeyA8 ovarian cancer cells treated with ATN-291-conjugated and -unconjugated NB(Calcein) for 3 and 24 hours. Scale bar, 20 μm. B, flow cytometry. Comparison of cellular uptake of ATN-291-conjugated (green triangle) or unconjugated (red square) NB(Calcein) in HeyA8 cells over time (1–48 hours). Bars represent mean ± SEM from three independent experiments. Statistical analyses were performed using two-way ANOVA. C and D, confocal microscopy analyses of HeyA8 ovarian cancer cells treated with Alexa Fluor 647-labeled ATN-291-NB(Ni,As). C, time course (0–24 hours). Merged pictures of Alexa Fluor 647 (red), Hoechst (nucleus; blue) and differential interfering contrast images are shown. D, Z-axis (0–9 μm) reconstruction of optical sections with orthogonal views (right) after 6 hours of treatment. Scale bar, 20 μm. E, tumor cell-targeted nanobin delivery. Human primary omental mesothelial cells were cocultured with GFP-labeled HeyA8 and treated with ATN-291_{AF647}-NB(Ni,As) for 24 hours before confocal imaging. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 20 μm. Immunoblot analysis of u-PAR expression in human primary mesothelial cells from 3 patients with ovarian cancer (right). u-PAR–expressing ES-2 cells and ES-2 cells stably transfected with a shRNA targeting u-PAR serve as positive and negative controls, respectively.
(Ni,As) was detected in a perinuclear position, shown previously to be caused by u-PAR internalization (22). Specificity of the targeted nanobins could also be shown by coculturing primary human mesothelial cells with GFP-expressing ovarian cancer cells (Fig. 2E). The Alexa Fluor 647–labeled antibody (red) colocalized with the high u-PAR–expressing ovarian cancer cells (green) but was not detected in the u-PAR-negative mesothelial cells (Fig. 2E; refs. 20, 31).

Next, we sought to verify that the antibody-conjugated nanobins are specific for u-PAR–expressing cells. To this end, the ES-2 ovarian cancer cell line, which has high u-PAR expression (Fig. 1B), was infected with short-hairpin RNAs (shRNA) against u-PAR. We found that shRNAs effectively knocked down u-PAR expression in ES-2 cells, and that the inhibition was preserved over several passages (Fig. 3A). Flow cytometry studies showed that uptake of ATN-291-NB(Calcein) was significantly lower in the u-PAR knockdown cells as compared with the WT cells (Fig. 3B), which was confirmed using confocal microscopy (Fig. 3C). Moreover, addition of either the uPA antibody, ATN-291, or recombinant scuPA was able to dose-dependently compete out the cellular uptake of ATN-291-NB(Calcein) but did not affect the internalization of unconjugated NB(Calcein), either in HeyA8 (Fig. 3D) or ES-2 cells (Supplementary Fig. 5C and 5D) both of which express high uPA/u-PAR. These findings suggest that the delivery of ATN-291-nanobins is uPA- and u-PAR–dependent.

Urokinase-targeted arsenic-loaded nanobins inhibit tumor growth

Next, we evaluated the efficacy of the ATN-291 decorated, As$_2$O$_3$-loaded nanobins in vitro (Fig. 4) and in vivo (Fig. 5) to ascertain whether targeting nanobins with an antibody affects the delivery efficiency of the loaded...
arsenic. Cytotoxicity assays using HeyA8 cells were performed to determine whether improved delivery would impact cell survival. At equivalent doses, actively targeted ATN-291-NB(Ni,As) were more effective in suppressing cell survival of high uPA/u-PAR–expressing HeyA8 cells than the unconjugated NB(Ni,As), as reflected by a significantly different IC₅₀ (25 vs. 53 μmol/L; P = 0.0002). In contrast, the low uPA/u-PAR–expressing CaOV3 cells had a much higher IC₅₀ (Supplementary Fig. S1E). In addition, ICP-MS analysis of cell fractions (cytosol, membrane/organelle, and nucleus) showed that targeted treatment with ATN-291 increased the intracellular delivery of arsenic by an average of 5.5-fold (Fig. 4A). More importantly, as a concern with nanobins has been that they can be trapped in endosomes and lysosomes (7, 8), only 13.9% of the arsenic was detected in the membrane/organelle fraction of ATN-291-NB(Ni,As). These results are consistent with the punctate, perinuclear fluorescence observed with ATN-291 nanobins (Fig. 2C and D).

Because As₂O₃ has been shown to induce apoptosis (33, 34), we first confirmed that free arsenic, NB(Ni,As), and ATN-291-NB(Ni,As) stimulate apoptosis by assessing nuclear fragmentation (Fig. 4B). ATN-291-NB(Ni,As) triggered early apoptosis, as evidenced by a loss in mitochondrial membrane potential (Δψₘ) which was confirmed both by confocal microscopy (Fig. 4C) and fluorescence-activated cell sorting (FACS) analysis (Supplementary Fig. S2). We then assessed the effects of our nanobin preparations on the generation of cleaved caspase-3, a marker of caspase-dependent apoptosis. Figure 4D shows that both types of arsenic-containing nanobins, as well as arsenic, induced caspase-3 cleavage in HeyA8 cells. The nanobin formulation significantly inhibited the expression of ALDH1A1, a marker of cancer stem cells (Fig. 4D; refs. 35, 36). In contrast, conjugation with the antibody induced protein expression of both latent and active forms of the MMP-2. This, however, did not affect MMP-2 activity of cancer cells treated with targeted or untargeted nanobins (Supplementary Fig. S3A). Given that uPA can convert plasminogen to plasmin, thereby generating proteolytic activity (19), we investigated the possibility that the targeted antibody might have activating functions. However, the targeted nanobins had no effect on plasminogen activation, invasion through collagen, or invasion in a 3D culture (27) of human omentum (Supplementary Fig. S3B–S3D).

Because arsenic-loaded, uPA-targeted nanobins increased cytotoxicity and apoptosis in vitro, we studied their therapeutic effect (Fig. 5A–D) and biodistribution (Fig. 5E and F) in vivo. ATN-291-NB(Ni,As) treatment was tested in a human ovarian cancer xenograft model in which HeyA8 ovarian cancer cells were injected intraperitoneally into female nude mice (20). Four days after injection, mice were randomized to five groups and treated intraperitoneally every other day with either PBS, ATN-291-NB(Ni,As), As₂O₃, or targeted ATN-291-NB(Ni,As) at an As₂O₃ concentration of 4 mg/kg (14). All treated mice continued...
to gain weight and there was no apparent toxicity in any treatment group (Fig. 5A). Free As$_2$O$_3$, empty nanobins NB (NaCl), and PBS treatment had no impact on tumor growth (Fig. 5B), as reported by us previously in a breast cancer model (14). NB(Ni,As) reduced tumor weight significantly and this effect was further enhanced with the addition of active targeting on the nanobins (Fig. 5B and C). Analysis of three independent xenograft experiments showed that,
compared with the control treatment group, tumor weight was reduced by 27% (\( P = 0.04 \)) with the nontargeted NB (Ni,As) treatment and 47% (\( P = 0.0005 \)) with the targeted ATN-291-NB(Ni,As) treatments (Fig. 5C). The additional decrease in tumor burden after treatment with ATN-291-NB(Ni,As), compared with treatment with NB(Ni,As), was statistically significant (\( P = 0.02 \)).

Pathologic review of the tumors at the end of treatment showed undifferentiated, high-grade tumors (Fig. 5D), which did not differ in angiogenesis (microvessel density, CD31), proliferation rate (Ki-67; Supplementary Fig. S4), or u-PAR expression (data not shown). Both targeted and nontargeted nanobins induced macrophage infiltration into the tumor. Untargeted nanobins recruited more macrophages than the uPA-targeted nanobins (Supplementary Fig. S4). Treatment with the untargeted arsenic nanobins induced active caspase-3 and DNA fragmentation (TUNEL stain) in the tumors. These effects were more pronounced in ATN-291-NB(Ni,As)–treated tumors (Fig. 5D), suggesting, as is consistent with the \textit{in vitro} results (Fig. 4B–D), that arsenic-loaded nanobins reduce tumor burden by inducing apoptosis rather than by inhibiting tumor growth. Also consistent with the \textit{in vitro} results is that ATN-291-NB(Ni,As) treatment reduced expression of ALDH1A1 (Fig. 4D; ref. 35). Analysis of kidney and liver function in serum showed no significant toxicity with ATN-291-NB(Ni,As) treatment (Supplementary Fig. S5). Together, these results corrobore the therapeutic effectiveness of NB(Ni,As) treatment and the improved anti-tumoral effect of targeted ATN-291-NB(Ni,As) treatment.

To study biodistribution of nanobins using a fluorescence imaging system, HeyA8-GFP ovarian cancer xenografts were established and then injected with nanobins. The ATN-291\(_{\text{AF647-NB(Ni,As)}}\) homed to the tumor, spleen, lung, and liver (Fig. 5E), common accumulation sites for liposome nanoparticles (11, 37). \textit{Ex vivo} images from ATN-291\(_{\text{AF647-NB(Ni,As)}}\)–treated animals showed that the tumors had a higher fluorescence intensity than the organs, indicating the efficient delivery of nanobins to the tumor with active targeting. The arsenic concentration in organs/tumor detected by ICP-MS showed no significant difference in the total amount of \( \text{As}_2\text{O}_3 \) delivered to the tumors (Fig. 5F).

Discussion

Ovarian cancer is generally confined to the peritoneal cavity, and intraperitoneal chemotherapy treatment is a well-established clinical treatment modality (38). Therefore, nanoparticles that efficiently target tumor cells intraperitoneally, deliver anticancer drugs potently, and minimize the exposure of normal tissue to chemotherapeutics that could lead to significant treatment improvements. Many reports show that the uPA system is consistently overexpressed in ovarian cancer, and that its expression in normal cells is low (18, 20, 31). Given that the u-PAR is internalized upon uPA binding, we developed a uPA/u-PAR–dependent targeted delivery using uPA antibody–targeted nanobins.

We found that targeted single-agent treatment with nanobins \{ATN-291-NB(Ni,As)\} suppressed tumor growth in an orthotopic ovarian cancer mouse model by almost 50%, whereas the untargeted NB(Ni,As), at equimolar arsenic doses, were half as effective (Fig. 5C). Several of our observations are consistent with the specific uptake of arsenic-loaded nanobins by ovarian cancer cells. When u-PAR expression was downregulated in cancer cells with shRNA, we observed reduced intracellular uptake of targeted nanobins (Fig. 3A–C). Consistent with this observation, confocal microscopy showed that the targeted nanobins were internalized (Fig. 2). Adding free antibody or competition with recombinant single-chain uPA to u-PAR–overexpressing cells also inhibited specific uptake of nanobins (Fig. 3D). Finally, the targeted nanobins were taken up \textit{in vivo} by the uPA/u-PAR–expressing tumor cells (Fig. 5E).

Antibodies are not the only targeting agents that can efficiently deliver intracellular payloads. We have previously shown that folate-targeted liposomes containing \( \text{As}_2\text{O}_3 \) are internalized by folate-receptor–positive tumor cells but not by tumor cells without the receptor (12). In a different study, u-PAR–targeted delivery of iron-oxide nanoparticles enhanced detection of human breast cancer using MRI (39). Targeting transferrin nanoparticles not only delivered siRNA but allowed selective imaging of subcutaneous neuroblastoma tumors using a positron emission tomography/computed tomography (PET/CT) scan (40). Conversely, targeting tumor vasculature using Arginine-glycine-aspartic acid (RGD)-linked nanogels containing docetaxel inhibited tumor growth in a breast cancer model (41). These studies, together with our study, highlight the importance of specific intracellular delivery of payloads to achieve a therapeutic and diagnostic advantage.

Like integrin and folate receptors, the uPA/u-PAR is widely expressed in epithelial cancers. However, unlike other receptors, their expression is found in tumor-associated stromal cells as well as in tumor cells (18, 19). Further clinical development of this targeted nanomedicine hinges on leveraging uPA/u-PAR expression in tumor cells with this effective delivery system. A central challenge to clinical translation of targeted nanoparticles is the ability to reliably scale up and reproducibly characterize the drug carrier. The inclusion of antibodies to the nanobin surface, using copper-catalyzed alkyne-azide cycloaddition click chemistry, has several advantages because it involves a reaction between alkyne and azide functional groups that are not otherwise found in biomolecules. Unlike other common conjugation methods using amine-coupling or thiol-coupling chemistries, this click reaction is chemically orthogonal to reactions that can occur between protein side chains, ensuring that no reactions occur that may lead to particle-particle cross-linking or nonspecifically alter the surface properties of the nanobins. In addition, the functional groups are not susceptible to hydrolysis. Click chemistry exposes only the thermally stable alkyne functional groups on the DSPE-PEG-alkyne to high temperatures, avoiding thermal deactivation of...
the antibody. Because the ATN-291 antibody is labeled with Alexa Fluor 647 at a known ratio, we can use fluorophore concentration as a proxy for antibody concentration. Visible light spectrometric measurements are subject to light-scattering interference in the presence of nanoparticles. By digesting the protein in a small sample of nanobins, then probing the liberated fluorophore after filtration of the liposome, we can quantify the precise number of antibodies per particle without light-scattering interference from the lipid particles.

A landmark article showed that As$_2$O$_3$ is a very potent drug for the treatment of patients with acute promyelocytic leukemia (42). It is currently being considered for the treatment of solid human tumors because it has demonstrated potent cytotoxic effects against epithelial tumor cell lines in vitro (16). However, development in solid tumor indications has been limited by the poor pharmacokinetics and dose-limiting toxicity of the free drug. The most commonly described mechanisms of action (reviewed in ref. 34) of As$_2$O$_3$ are inhibition of tumor angiogenesis, selective killing of cancer stem cells, inhibition of MMPs, and induction of apoptosis (14, 16, 17, 33). We found that ATN-291-NB(Ni,As) did not affect tumor cell proliferation or angiogenesis (Supplementary Fig. S4), but very efficiently induced ovarian cancer cell apoptosis (Figs. 4B–D, 5D; Supplementary Fig. S2). This explained, at least in part, the observed significant cytotoxicity of the conjugated nanobins and is consistent with the mechanisms of arsenic function elucidated in a murine model of cancer (15). ATN-291-NB(Ni,As) also demonstrated potent cytotoxic effects against epithelial tumor cell lines in vitro (16). However, development in solid tumor indications has been limited by the poor pharmacokinetics and dose-limiting toxicity of the free drug. The most commonly described mechanisms of action (reviewed in ref. 34) of As$_2$O$_3$ are inhibition of tumor angiogenesis, selective killing of cancer stem cells, inhibition of MMPs, and induction of apoptosis (14, 16, 17, 33).

We showed inhibition of the cancer stem cell marker ALDH1A1 both in vitro (Figs. 4D and 5D) and by ATN-291-NB(Ni,As). These data are consistent with a study (36) reporting that ALDH1A1 is expressed in 73% of all human ovarian cancer and that its silencing in ovarian cancer cell lines blocked tumor cell growth in vitro and in vivo. The blocking of ALDH1A1 sensitized cells to platinum and taxane-based chemotherapy (36), which suggests that ATN-291-NB(Ni,As) may have a role in combination therapy for ovarian cancer, as these tumors frequently develop chemotherapy resistance (1).

More than 60 years after the discovery of urokinase (44), the promise that tumor growth could be blocked by inhibiting specific proteases has not come to fruition. This is in part because other proteases compensate for the proteolytic function when a single protease is inhibited. However, given that most epithelial cancers strongly express uPA and u-PAR, it may be possible to harness the tumor-associated uPA system as an entry point to deliver therapeutic or diagnostic payloads specifically to cancer cells rather than as a target for antiproteolytic therapy. Although, in this report, we have focused on formulating As$_2$O$_3$ in a nanobin, our delivery system could also be loaded with other chemotherapeutics or imaging agents and used to target any cancers that express uPA and u-PAR.

Disclosure of Potential Conflicts of Interest

A.P. Mazar is employed as a Managing Member in Tactic Pharma and has ownership interest (including patents) in the same. T.V. O’Halloran has ownership interest in US Patent #8,246,985, Tactic Pharma, and Tactic Pharma. No potential conflicts of interest were disclosed by the other authors.

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References


35. Dadashzadeh S, Mirahmadi N, Babai MH, Vali AM. Peritoneal reten-
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