Supplementary Files

Supplementary Materials and Methods

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Reagents

Collagen type I (rat-tail) was purchased from BD Bioscience (Bedford, MA). CellTracker Green CMFDA and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA). CD31 (M-20) antibody was obtained from Santa Cruz (Santa Cruz, CA). F4/80 antibody (A3-1) to detect mouse macrophages was from AbD Serotec (Raleigh, NC). Ki-67 antibody (SP6) was from Thermo Fisher Scientific (Fremont, CA).

Preparation of arsenic trioxide loaded nanobins

Arsenic loaded nanobins NB(Ni,As) and control nanobins NB(NaCl) were prepared as previously described (1, 2). Briefly, a mixture of cholesterol, DSPC, and DSPE-PEG_{2000} (45/51/4 mol%) was dissolved in chloroform and evaporated to a thin film at 60°C under vacuum for 24h to remove any residual solvent. The dry lipid films were hydrated at 60°C for 1h with either 300 mM nickel acetate for NB(Ni,As), or 20 mM HEPES, 150 mM NaCl, pH 7.4 for the NB(NaCl). The hydrated films were then subjected to 10 freeze-thaw cycles. The hydrated lipids were then sequentially downsized to 100 nm using a Lipex extruder operated at 55°C using 200 nm and 100 nm Nuclepore Track-Etch polycarbonate membranes at 55°C. After that, the unencapsulated nickel acetate was removed by diafiltration (50 nm) with a Krossflow II tangential flow filtration apparatus (Spectrum Laboratories) against 20 mM HEPES, 150 mM NaCl, pH 7.4. Nanobins loaded with nickel acetate were then incubated with an As_2O_3 solution at 60°C for 4 h. Unencapsulated As_2O_3 was removed by diafiltration. The molar concentrations of arsenic, nickel and phospholipid were measured on an inductively coupled plasma optical emission spectrometer (ICP-OES, Vista MPX).

Preparation of azide-functional Alexafluor 647-labeled ATN-291

ATN-291 monoclonal antibody was incubated with a hetero-bifunctional crosslinker containing an amine-reactive n-hydroxysuccinimidyl (NHS) ester linked to an azide functional group by a four-repeat unit polyethylene glycol chain (NHS-PEG-N_3) (Quanta BioDesign). The NHS-PEG-N_3 crosslinker was incubated in 5 fold molar excess compared to antibody for 3h at 37°C. The crosslinker-modified ATN-291 was then reacted with 1.2-fold molar excess amine reactive AlexaFluor647 (NHS-AF647, Invitrogen) for a further 3h at 37°C. The unconjugated AF647 and NHS-PEG-N_3 were removed by size exclusion chromatography using a Zeba Spin Column (7k MWCO, Thermo Scientific) equilibrated with 20 mM HEPES 150 mM NaCl, pH 7.4. AF647 and antibody concentrations were determined by UV/Vis spectroscopy on a Lambda 650 spectrophotometer (Perkin Elmer) measuring absorbance at 650 nm and 280 nm, respectively.

Flow cytometry

ES-2 OvCa cells were pre-treated with or without 50 nM of single chain uPA (scuPA) for 2h before incubation with 25 μM of ATN-291-NB(Calcein) for 24 h. After incubation, cells were washed, detached, resuspended in PBS, fixed by 2% paraformaldehyde, and stored at 4˚C until analysis. To evaluate early apoptosis, mitochondrial membrane potential ($\Delta\Psi m$) was measured after treating cells with 50 μM nanobins (4h). Cells were detached, washed,

incubated with JC-1 (2 µM, 30 min), a fluorescent dye that undergoes a fluorescence emission shift if cells lose ΔΨm. A total of 10,000 events were collected for each sample using a LSRFortessa cell analyzer with the following parameter settings: 488/515 nm for Calcein, 641/670 nm for Alexa647; 488/515 nm for JC-1 monomer; 488/576 nm for JC-1 aggregate. Data were analyzed by FlowJo software (TreeStar Inc).

Cytotoxicity assay

The cytotoxicity of NB(Ni,As) and ATN-291-NB(Ni,As) was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HeyA8 and CaOV3 cells seeded onto 96-well plates were treated with increasing concentrations of NB(Ni,As) or ATN-291-NB(Ni,As) for 48h. MTT (20 µL, 5mg/mL) was added to the wells and incubated with cells for 2h. The absorbance of dissolved formazan in dimethyl sulfoxide was measure at 560 nm with a Synergy HT plate reader (Bio-Tek, Winooski, VT). IC_{50} (half maximal inhibitory concentration) values were calculated using Graphpad Prism Version 5.04 (La Jolla, CA). The experiment was repeated three times.

Confocal microscopy

For competition assay, wild type or u-PAR down-regulated ES-2 cells were seeded in chamber slides (Nunc) for 3 days (~40-50% confluent) before treatment with 25 µM of ATN-291_{AF647}-NB(Ni,As) for 24 h. After Hoechst 33342 staining, the cells were fixed, mounted prior to imaging. 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 AF647 (633/650 nm), and Hoechst (360/450 nm).

Gelatin zymography

The gelatin zymography was performed as described (3). HeyA8 cells were treated with ATN-291-NB(Ni,As), NB(Ni,As), ATN-291-NB(NaCl), ATN-291, and NB(Ni,As) with ATN-291 for 24h. The concentration of conjugated or free ATN-291 was 8 nM in all treatments. The conditioned medium was subjected to non-reducing gelatin gel zymogram gel (BioRad). The gel was rinsed twice with 2.5% Trion X-100 for 30 min, and subsequently incubated with buffer (Tris•HCl 50mM, NaCl 0.2 M, $CaCl_2$ 5mM, BriJ 35, 0.02%) at 37˚C overnight. The gel was stained with Coomassie R-250 followed by destaining with methanol. Proteolyzed gelatin appears as a white zone on a dark background at ~92 kDa and 72 kDa which corresponds to latent MMP-9 and MMP-2 respectively.

Plasminogen zymography

Plasminogen zymography was performed as previously described (4). Conditioned medium (CM) was collected from HeyA8 cells upon one-day treatment with 6.25 nM and 25 nM of uPA antibody or uPA antibody conjugated NB(NaCl) (ATN-291-NB(NaCl)) followed by electrophoresis in 10% SDS-PAGE containing 5 µg/mL of plasminogen (Calbiochem). The gel was rinsed twice with 2.5% Trion X-100 for 30 min, and subsequently incubated with 0.1 M of glycerin at 37˚C overnight. The gel was stained with amido black solution (BioRad) and

destained in a solution containing 30% methanol and 10% acetic acid. Urokinase-dependent proteolysis of plasminogen in the gel appeared as a white zone on a dark background at ~33 kDa.

Invasion assay

The invasion assays were performed as described (5)**.** Transwell inserts (8 µm, BD Bioscience) for a 24-well plate were coated with 10 µg of collagen type I in serum-free media and dried overnight. The inserts were re-hydrated in serum-free media for 1 h and the bottom chamber was filled with media. HeyA8 cells (2 X 10^4) were seeded on top of the inserts in serum-free media with 1 or 10 µM of arsenic trioxide or NB(Ni,As), and allowed to invade for 48 h. The cells on top of the inserts were removed with cotton swabs. The cells on the bottom were fixed in 4% paraformaldehyde, stained with Giemsa, imaged through an Axio observer microscope (Zeiss), and quantified by counting 5 random fields with 100x magnifications. The experiments were repeated 3 times.

For the 3-dimensional invasion assay, transwell inserts were coated with collagen I (10 µg) overnight followed by addition of primary human primary mesothelial cells. HeyA8-GFP cells were plated on the 3D culture for 16 h and then treated with free or NB(Ni,As) conjugated uPA antibody ATN-291 or combination ([ATN-291] = 8 nM) for 16h ([As] = 20 µM). The HeyA8-GFP cells that invaded through the layers of human primary mesothelial cells and collagen I to the bottom of the chamber were quantified using a fluorescence plate reader. The experiment was repeated twice.

Immunohistochemistry

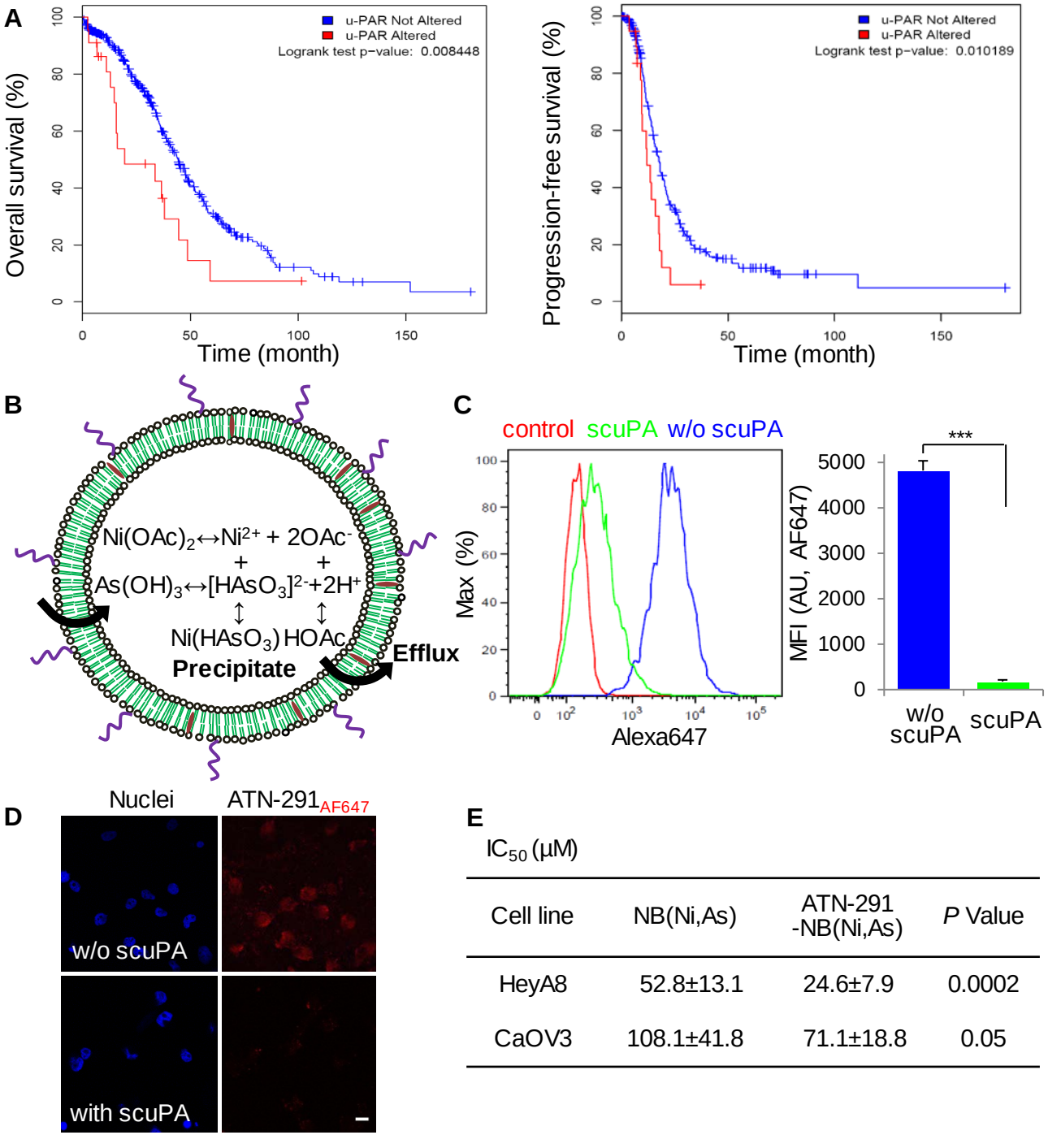
Slides containing human OvCa and xenografts were stained with hematoxylin & eosin. A representative tumor area was confirmed by a gynecologic pathologist (KG). Immunohistochemical staining of tumor sections were performed to assess markers of microvessel density (CD31, M-20, 1:50), proliferation (Ki67, SP6, 1:300) and the mouse macrophage (F4/80, A3-1, 1:500). The stained cells were imaged at 100x and 400x magnification, and quantified by counting five random fields (6).

Blood toxicity studies

Hey8 (5x10^5) cells were injected intraperitoneally (i.p.) into 5-6 week-old female nude mice. Ten days later, ATN-291-NB(Ni,As) (As, 4mg/kg) or PBS was injected i.p. and mice were sacrificed 24 h later. Blood serum was collected and sent to IDEXX laboratories (North Grafton, MA) to test alkaline phosphatase (ALP), alanine aminotransferase (ALT) aspartate aminotransferase (AST), blood urea nitrogen (BUN), and serum creatinine.

Supplementary Figures

Supplementary Figure S1.



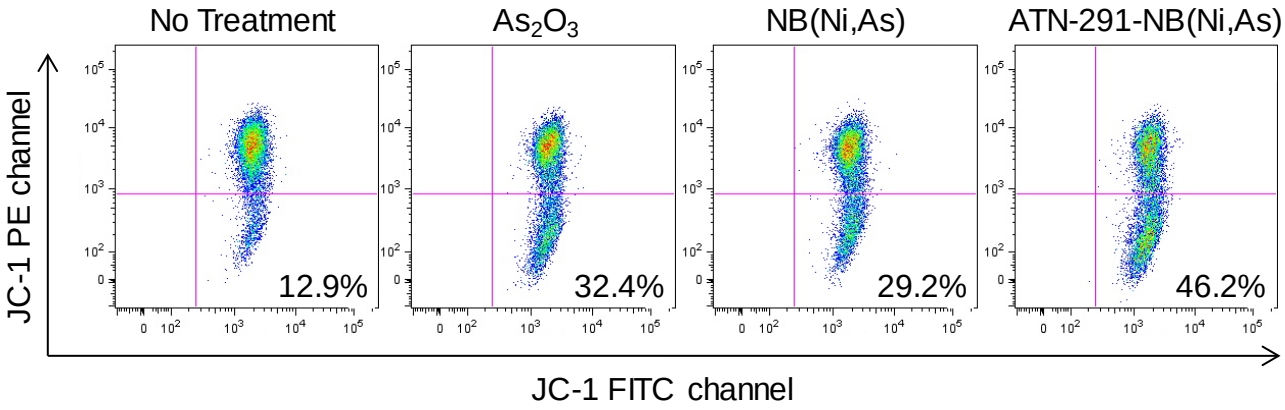
E

IC_{50} (μM)

Cell line	NB(Ni,As)	ATN-291 -NB(Ni,As)	*P* Value
HeyA8	52.8±13.1	24.6±7.9	0.0002
CaOV3	108.1±41.8	71.1±18.8	0.05

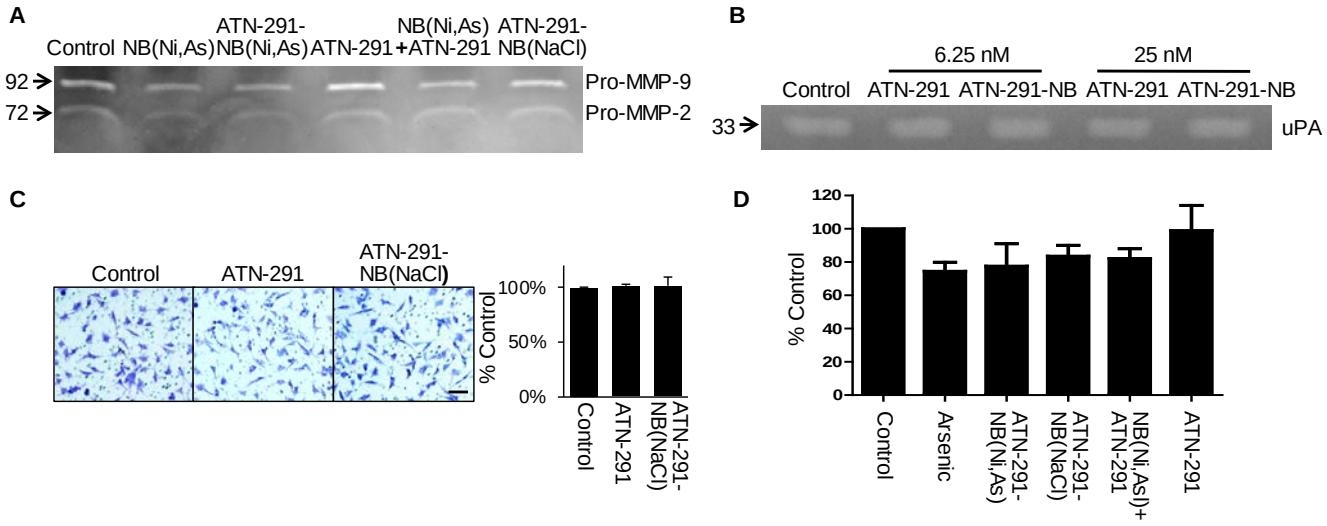
Supplementary Figure S1: Specific uptake of ATN-291-NB(Ni,As) in ovarian cancer cells.
A, Survival analyses. Overall (left) and progression-free survival (right) of ovarian cancer patients (n=580) with or without u-PAR gene alterations from the TCGA database. Statistical analyses were performed using a Logrank test.
B, Chemical reaction describing how arsenic was loaded into liposomes**.** Liposomes were first loaded with nickel(II) acetate and then arsenic acid was added, leading to the formation of precipitates ($Ni(HAsO_3)$) and efflux of acetic acid. See also Fig. 1C.
C – D, ES-2 cells expressing high u-PAR were treated with 25 µM of Alexa 647 labeled ATN-291-NB(Ni,As) with and without (w/o) 50 nM of single chain urokinase (scuPA).
C, Flow cytometry analysis. A representative histogram plot and the quantification of mean fluorescence intensity (MFI) of Alexa 647 associated with nanobins are shown. Bars represent means ± SEM from 3 independent experiments. ***, p value < 0.001, Statistical analyses were performed using a two-tailed *t*-test.
D, Confocal images of ATN-291_{AF647}-NB(Ni,As) (Alexa647, red) and nuclei (Hoechst, blue) are displayed. Scale bar, 10 µm.
E, IC_{50} values of ATN-291-NB(Ni,As) and NB(Ni,As) in HeyA8 and CaOV3 cells. Statistical analyses were performed using a two-tailed *t*-test.

Supplementary Figure S2.



Supplementary Figure S2: Confocal studies of early apoptosis in HeyA8 cells treated with nanobins.
Flow cytometry analysis of mitochondrial membrane potential ($\Delta\Psi_m$) using JC-1 dye. HeyA8 cells were treated with arsenic trioxide (As_2O_3), NB(Ni,As), or ATN-291-NB(Ni,As) for 4h ([As] = 50 µM), and stained with JC-1 for 0.5h (2 µM) before detection. JC-1 undergoes an emission shift from red (590 nm) to green (529 nm) fluorescence (excitation 488 nm) when cells lose ΔΨm, an indication of early apoptosis.

Supplementary Figure S3.



Supplementary Figure S3: Targeted delivery using a urokinase antibody (ATN-291) does not inhibit uPA enzymatic activity *in vitro*.

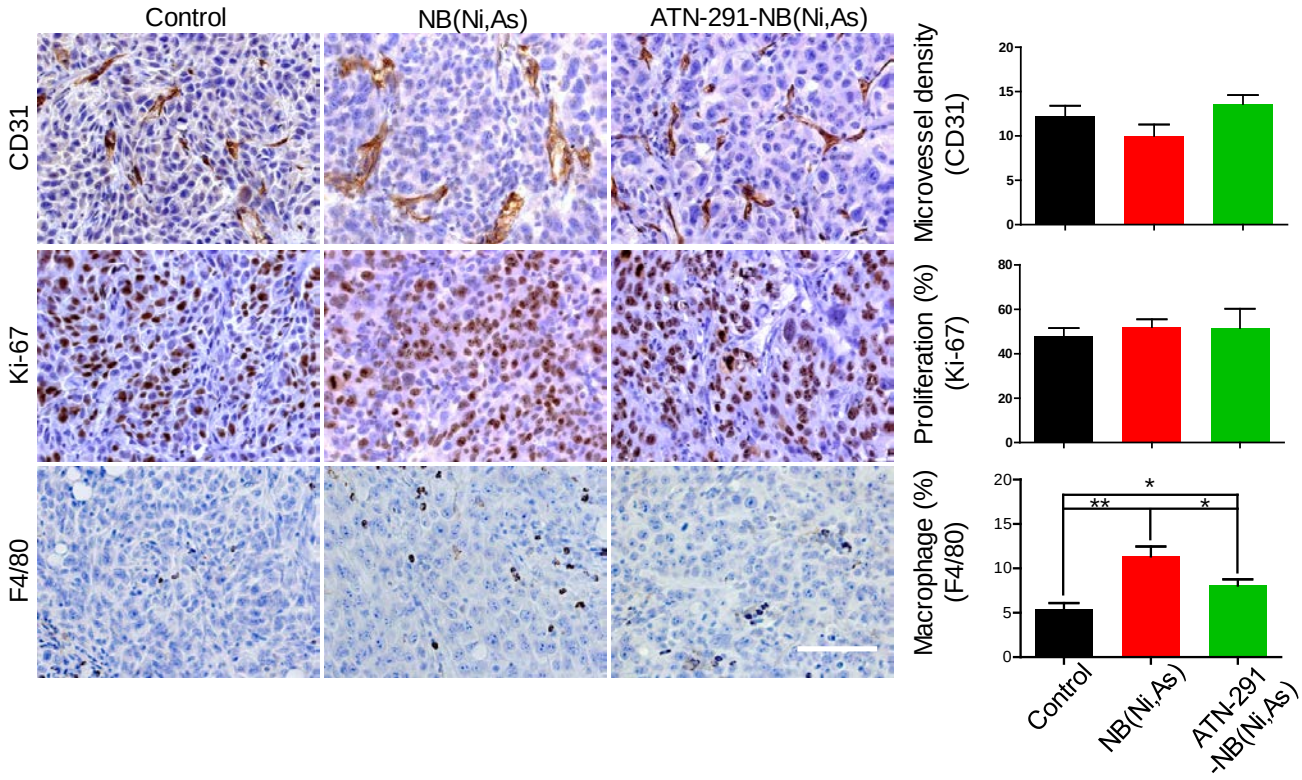
A, Gelatin zymogram. HeyA8 cells were treated with ATN-291-NB(Ni,As), NB(Ni,As), ATN-291-NB(NaCl), ATN-291, and NB(Ni,As) with ATN-291 for 24 h ([ATN-291] = 8 nM) . The conditioned medium was collected, concentrated and subjected to non-reducing gelatin gel zymogram gel. Control, no treatment.

B, Plasminogen zymogram. HeyA8 cells were incubated with either free ATN-291 or ATN-291-NB(NaCl) for 24 h ([ATN-291] = 6.25 or 25 nM). CM was collected, concentrated, and subjected to plasminogen zymogram gel.

C, Invasion assay. HeyA8 cells were treated with uPA antibody ATN-291 or ATN-291-NB(NaCl) ([ATN-291] = 8 nM) for 2 days. The cells invaded through the collagen I (10 µg) layer onto the bottom of the transwell inserts were imaged and quantified. The representative images from two independent experiments were shown. Scale bar, 100 µm. Bars represent means ± SEM.

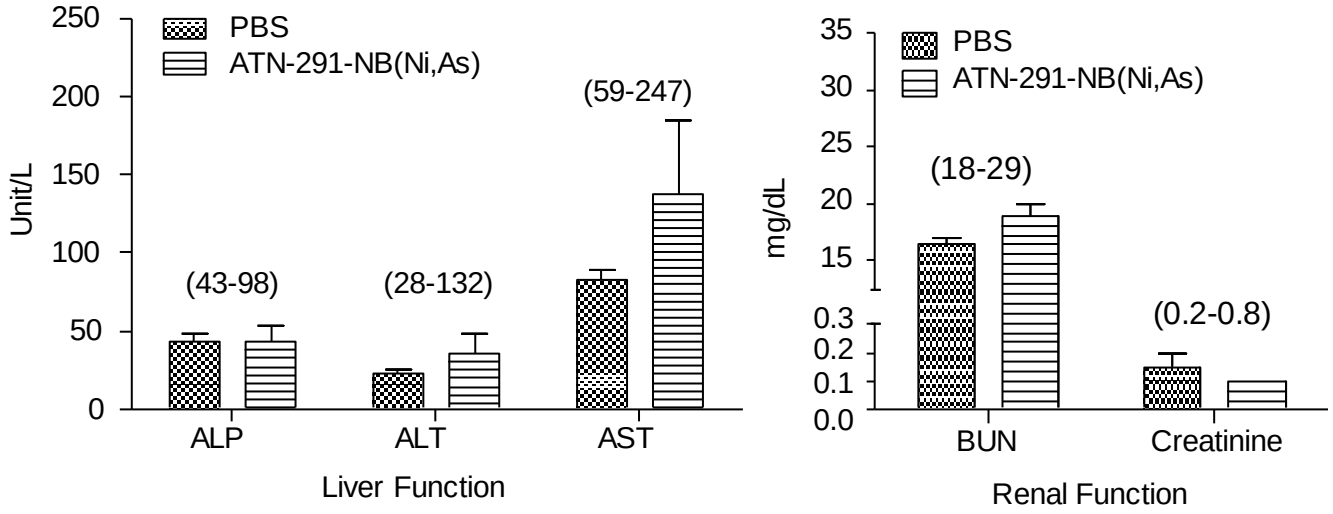
D, Three-dimensional culture invasion assay. HeyA8-GFP cells were treated with free or nanobin conjugated uPA antibody ATN-291 or combination ([ATN-291] = 8 nM) for 16 h. The fluorescent cancer cells that invaded through the primary human mesothelial cells, fibroblasts and collagen type I (10 µg) layers to the bottom of the inserts were quantified by a fluorescence plate reader. The experiment has been repeated twice. Bars represent means ± SEM.

Supplementary Figure S4.



Supplementary Figure S4: Immunohistochemical analysis of the HeyA8 xenograft tumors upon the treatments of nanobins *in vivo*.
Human HeyA8 OvCa cells were injected intraperitoneally (i.p.) into female nude mice. As outlined in Figure 5A, mice were injected i.p. with 5 doses of PBS (control), NB(Ni,As), and ATN-291-NB(Ni,As) (4mg/Kg, arsenic). Immunohistochemical staining of tumor sections for microvessel density (CD31), proliferation (Ki-67), and mouse macrophages (F4/80). Scale bar, 100 µm. The representative images are shown (left) and quantified by ImageJ (right). Bars represent (n= 6 mice) means ± SEM. *, p value < 0.05; **, p value < 0.01. Statistical analyses were performed using a two-tailed *t*-test.

Supplementary Figure S5.



Supplementary Figure S5: Organ function of mice treated by ATN-291-NB(Ni,As).
Human HeyA8 OvCa cells were injected intraperitoneally (i.p.) into female nude mice. Ten days later, ATN-291-NB(Ni,As) (As, 4 mg/kg) or PBS were injected i.p. and mice were sacrificed 24h later. Blood serum was collected and the indicated values measured.
A, Liver function analysis. Serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) aspartate aminotransferase (AST).
B, Renal function analysis. Blood urea nitrogen (BUN) and serum creatinine were assessed. Values in parentheses represent normal ranges in mice. Bars represent means (n= 2 mice) ± SD.

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