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A Novel Nanoparticulate Formulation of Arsenic Trioxide with Enhanced Therapeutic Efficacy in a Murine Model of Breast Cancer

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Abstract

Purpose—The clinical success of arsenic trioxide (As₂O₃) in hematological malignancies has not been replicated in solid tumors due to poor pharmacokinetics and dose-limiting toxicity. We have developed a novel nanoparticulate formulation of As₂O₃ encapsulated in liposomal vesicles or “nanobins” [(NB(Ni,As))] to overcome these hurdles. We postulated that nanobin encapsulation of As₂O₃ would improve its therapeutic index against clinically aggressive solid tumors, such as triple negative breast carcinomas.

Experimental Design—The cytotoxicity of NB(Ni,As), the empty nanobin, and free As₂O₃ was evaluated against a panel of human breast cancer cell lines. The plasma pharmacokinetics of NB(Ni,As) and free As₂O₃ were compared in rats to measure drug exposure. In addition, the antitumor activity of these agents was evaluated in an orthotopic model of human triple negative breast cancer.

Results—The NB(Ni,As) agent was much less cytotoxic *in vitro* against a panel of human breast cancer cell lines than free As₂O₃. In contrast, NB(Ni,As) dramatically potentiated the therapeutic efficacy of As₂O₃ *in vivo* in an orthotopic model of triple negative breast cancer. Reduced plasma clearance, enhanced tumor uptake, and induction of tumor cell apoptosis were observed for NB(Ni,As).

Conclusions—Nanobin encapsulation of As₂O₃ improves the pharmacokinetics and antitumor efficacy of this cytotoxic agent *in vivo*. Our findings demonstrate the therapeutic potential of this nanoscale agent and provide a foundation for future clinical studies in breast cancer and other solid tumors.

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Keywords

arsenic trioxide; liposome; basal-like breast cancer; nanotechnology; drug delivery

Introduction

Breast cancer is the second leading cause of cancer mortality for women in the United States (1). Although preventive agents and targeted therapies directed at the estrogen-receptor (ER), progesterone-receptor (PR) and human epidermal growth factor 2 receptor (HER2/neu) have resulted in improved clinical outcomes for many women with breast cancer, formidable challenges remain in treating tumors that do not express these molecular targets. These “triple negative” breast carcinomas represent 15% of newly diagnosed breast cancer cases and often exhibit a basal epithelial or basal-like gene expression profile that is associated with poor survival (2–4). Consistent with its aggressive nature, triple negative breast cancer is characterized by high rates of distant recurrence, particularly in the lung and brain, within the first five years after diagnosis despite adjuvant chemotherapy (5,6). Hence, development of new therapeutic agents for these clinically intractable tumors is highly desirable.

Arsenic trioxide (As_2O_3) is an FDA-approved treatment for refractory acute promyelocytic leukemia (APL) and has shown preliminary activity in patients with relapsed/refractory multiple myeloma (7–10). Several mechanisms of action have been proposed for As_2O_3 activity including induction of apoptosis mediated by reactive oxygen species, promotion of cellular differentiation and inhibition of angiogenesis (9,11–13). As_2O_3 has also been shown to reduce migration and invasion of cervical and ovarian cancer cells *in vitro* (14,15). Preclinical studies of As_2O_3 have demonstrated antitumor activity in murine solid tumor models including breast, brain, liver, gastric, prostate, renal and bladder cancer (16,17). Unfortunately, little or no efficacy has been observed in clinical trials of As_2O_3 when evaluated in solid tumors (7). Two factors appear to have limited the utility of As_2O_3 in the clinic: rapid renal clearance, which limits tumor uptake, and dose-limiting toxicity (18–20).

Nanoscale drug delivery vehicles have been reported to increase the therapeutic index of cytotoxic drugs by prolonging their serum half-lives, increasing tumor accumulation and reducing systemic toxicity (21–23). Liposomes are one such nanoscale delivery vehicle that can be used to deliver cytotoxic payloads (24,25). Two technical advances have greatly improved the clinical utility of nano-liposomes: 1) passivation of liposomes with polyethylene glycol (pegylation) to reduce opsonization and prolong serum half-life; and 2) nanoscale (100 nm diameter) synthesis of liposomes to enhance tumor accumulation via extravasation of nanoliposomes through fenestrated tumor vasculature. The latter property of nanoscale drug delivery vehicles is known as the enhanced permeability and retention effect (EPR) and enables passive targeting of tumors (22,26).

Previous attempts at encapsulating As_2O_3 in liposomes have been reported; however, these formulations were not stable, resulting in rapid leakage of the active agent (27). The predominant form of As_2O_3 in aqueous solution at physiological pH is arsenous acid [$\text{As}(\text{OH})_3$] and it rapidly crosses lipid bilayers, so passive encapsulation of the drug in these previous formulations resulted in rapid loss of $\text{As}(\text{OH})_3$ from the liposomal vesicles (27–29). We have developed a novel nanoparticulate formulation of As_2O_3 , in which the As_2O_3 is stabilized as a nanoscale precipitate inside a pegylated 100 nm liposome (Fig.1A). These arsenic nanobins [NB(Ni,As)] are characterized by a nanoparticulate core containing extremely high densities of arsenic (>270 mmol/L) and Ni^{2+} cations that both stabilize and potentiate drug activity (30,31). NB(Ni,As) is stable for at least 12 months at 4°C with less

than 10% leakage of free As₂O₃. The release of arsenic from NB(Ni,As) is triggered by exposure to low pH environments (5.0–6.5), such as the acidic tumor milieu and inside endocytotic vesicles in tumor cells and tumor macrophages. We have previously demonstrated that sequestration of As₂O₃ inside the nanobins attenuates *in vitro* cytotoxicity compared with free As₂O₃ because the As₂O₃ inside the vesicle is not bioactive until released (30–32). These results led us to postulate that the nanobin formulation may also reduce the systemic toxicity of As₂O₃ by shielding normal tissues from the cytotoxic agent, which may overcome some of the limitations observed with free As₂O₃ in clinical studies.

In this study, we evaluated the *in vitro* and *in vivo* activity of our novel NB(Ni,As) using breast cancer cells and a mouse model of triple negative breast cancer. We hypothesized that NB(Ni,As) would enhance the antitumor activity of As₂O₃ by improving its pharmacokinetics *in vivo*, promoting tumor uptake of As₂O₃ via the EPR effect, and reducing systemic toxicity by shielding non-tumor tissues from drug exposure. We demonstrate here that NB(Ni,As) exhibits improved pharmacokinetic characteristics and enhanced therapeutic efficacy in an orthotopic model of triple negative breast cancer compared to the free drug. Our findings provide the first proof of principle evidence for NB(Ni,As) antitumor efficacy *in vivo* and suggest that NB(Ni,As) may represent a novel therapeutic agent for triple negative breast cancer as well as other solid tumors.

Materials and Methods

Materials

1,2 -distearoyl-glycero-3-phosphocholine (DSPC) and 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N[Methoxy(Polyethylene glycol)-2000] (ammonium salt) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), arsenic trioxide, nickel(II) acetate, sodium chloride, 2-[4-2-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid (HEPES), Blastocidin-S, arsenic trioxide, and Sephadex G-50 were obtained from Sigma (St. Louis, MO), and were the highest grade available. Nickel(II) acetate was also purchased from Strem Chemical (Newburyport, MA). The As₂O₃ used in the pharmacokinetic study was Trisenox® (Cephalon, Frazer, PA). Trace metal grade nitric acid (69%) for ICP-MS was purchased from Fisher Scientific (Waltham, MA).

Preparation of arsenic trioxide-loaded nanobins

Arsenic trioxide-loaded nanobins were prepared from a modified procedure developed in our laboratory (31). Briefly, a thin lipid film consisting of DSPC/Chol/DSPE-PEG₂₀₀₀ = 51/4/45 mol % was prepared by dissolving the lipids in chloroform followed by rotary evaporation to dryness in a round bottom flask. The resulting lipid film was placed under high vacuum overnight to remove any residual solvent. The dry lipid films were hydrated with either: (a) 300 mM Ni(OAc)₂ for arsenic trioxide loaded nanobins [NB(Ni,As)] or (b) 20 mM HEPES, 150 mM NaCl, pH 7.4 buffer for vehicle control nanobins [NB(NaCl)] at 60°C for 1 h. The hydrated lipid suspensions were subjected to 10 freeze-thaw-cycles (alternating between an ethanol/dry-ice bath and 60°C water bath). The hydrated lipids were downsized to 100 nm using a Lipex Extruder (Northern Lipids, Burnaby, BC, Canada) operated at 60°C sequentially through 200 and 100 nm polycarbonate filters (Whatman International, Maidstone, United Kingdom). Buffer exchange to remove unencapsulated Ni(OAc)₂ was performed using a Sephadex G-50 column equilibrated with 20 mM HEPES, 150 mM NaCl, pH 6.8 or with continuous diafiltration with tangential flow filtration (TFF) using Minimate TFF cartridges (100 kD MWCO) or Spectrum Labs Hollow Fiber Cartridges (500 kD MWCO) with 20 mM HEPES, 150 mM NaCl pH 6.8. The resulting 100 nm vesicles were either (a) incubated with a As₂O₃ solution for 2 h at 60°C to generate NB(Ni,As), or (b) pH adjusted to 7.4 in the case of NB(NaCl). Extraliposomal arsenic

trioxide was removed from NB(Ni,As) using a Sephadex G-50 column preequilibrated with 20 mM HEPES, 150 mM NaCl, pH 7.4 or with TFF as described above into 20 mM HEPES, 150 mM NaCl, pH 7.4. The molar ratios of As/P (phospholipid) were measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Vista MPX, Varian, USA). The size and stability (pH, thermal) of the nanobins was determined by transmission electron microscopy (TEM) on a Hitachi HF2000 electron microscope and by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

In vitro drug release assay

NB(Ni,As) was mixed with FBS in a volume/volume ratio of 2:8 (80% serum), resulting in a final lipid concentration of 1 mM, pH 7.4 or pH 5.5, 37°C. At various time points, aliquots were applied to a Sephadex G-50 column to remove the arsenic and nickel species that had leaked out of the nanobins. The excluded nanobin fractions were evaporated prior to digestion with concentrated trace-metal free grade HNO₃ (69%) before analysis with ICP-OES to determine the drug-to-lipid molar ratios. The drug release percentage (%) was calculated as $[(r_0 - r_t)/r_0] \times 100\%$, where r_0 is the initial As(Ni)-to-lipid molar ratio and r_t the As(Ni)-to-lipid molar ratio at a specific time point (30).

Cell culture

Unless indicated otherwise, human cell lines were purchased from ATCC (Manassas, VA) and were grown in media containing 4 mM L-glutamine and 100 units/mL of penicillin/streptomycin. All sera, media and additives were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. MDA-MB-231 and BT-20 breast cancer cells were grown in Minimum Essential Media (MEM) supplemented with 10% FBS (ATCC, Manassas, VA). MDA-MB-231-mCherry cells were kindly provided by Dr. Jennifer Koblinski (Northwestern University) and were grown in Dulbecco's Modified Eagle Medium and Ham's F12 (DMEM/F12) supplemented with 1 µg/ml Blasticidin S (Sigma) and 5 % heat inactivated FBS. SK-BR-3 breast cancer cells were grown in McCoy's 5A media supplemented with 10% FBS (ATCC). MCF-10A cells were grown in phenol red-free DMEM/F12 with 5% heat-inactivated horse serum, 10 µg/mL insulin, 20 ng/mL EGF, 100 ng/ml cholera toxin, and 0.5 µg/ml hydrocortisone.

Viability assays

The cytotoxicity of As₂O₃ and NB(Ni,As) was assessed by MTS assay using the CellTiter 96® AQueous MTS (Promega, Madison, WI). Cells (2.4×10^4 cells/well) were plated in half-area 96-well plates (Greiner Bio-One, Monroe, NC) overnight in phenol red-free DMEM supplemented with 10% FBS. Serial dilutions of As₂O₃-loaded nanobins (NB(Ni,As)) and empty nanobins (NB(NaCl)) in phenol-red free media were transferred to the cells. After 96 h, MTS solution was added to the 96-well plates and the absorbance was measured at 495 nm 2 h later. The normalized viability of cells was plotted versus the log concentration of elemental arsenic and a sigmoidal dose response was fit (Prism, Graphpad, San Diego, CA). The IC₅₀ values are based on two to three independent experiments.

Apoptosis assay

Apoptosis was measured by fluorescence-activated cell sorting (FACS) using the Annexin V-PE Apoptosis Detection Kit I (BD Bioscience, San Jose, CA) following the manufacturer's protocol. Cells were treated with 50 µM As₂O₃, NB(Ni,As) or media with or without 50 µM Z-VAD-FMK (Promega, Madison, WI) for 48 h and analyzed by flow cytometry. Z-VAD-FMK, a pan-caspase inhibitor, was used to test whether cell death was caspase-dependent.

Migration and Invasion Assays

MDA-MB-231-mCherry were grown in serum-free media for 24 h. Cell migration and invasion were measured as described (33) with modifications. Briefly, cell suspensions (500 μ L, 2.5×10^4 cells) were seeded on the top of uncoated (migration assay) and Matrigel™-coated (invasion assay) transwells (8- μ m pore diameter; BD Biosciences). Serum-free cell suspensions (500 μ L) containing NB(NaCl), As₂O₃ (0.1, 1.0, 10 μ M) or NB(Ni,As) (0.1, 1.0, 10 μ M) were added to the top chamber of the transwell. The lower chambers contained drug-free, complete media (750 μ L) supplemented with 5% serum. Cells invading the lower chamber were stained with 0.5% crystal violet (60% PBS, 40% EtOH), photographed at 5 $\times$ magnification with an inverted microscope and counted. The same cell suspensions (100 μ L, 5.0×10^3 cells) were plated in uncoated (migration) and Matrigel™-coated (invasion assay) 96 well plates. After addition, 100 μ L of complete media was added to the wells, and the viability after 24 h (migration) or 48 h (invasion) was determined by MTS assay. The results from at least 2 independent experiments in triplicate are presented.

Pharmacokinetic studies

Double jugular catheterized 10-week-old female Sprague Dawley (SD) rats (approx. weight of 220 grams) were purchased from Charles River Laboratories (Raleigh, N.C.). Rats (n=5) were treated intravenously by the left jugular catheter with 4 mg As equivalents/kg of either the NB(Ni,As) or Trisenox® (As₂O₃). Blood samples were collected in lithium-heparin coated tubes from the right jugular catheter at 15, 30, 60, 120, 240, 480 and 1440 min. The blood samples were centrifuged (2000 g $\times$ 5 min), and plasma was collected and stored at 4°C. Plasma samples were analyzed for As and Ni concentration by inductively coupled plasma mass spectrometry (ICP-MS) (methods are detailed in Supporting Information).

Noncompartmental pharmacokinetic parameters were determined by the following methods, using WinNonlin Version 4.1 software (Pharsight Corp., Mountain View, CA, USA). The area under the time concentration curve (AUC_∞) was calculated using the linear trapezoidal rule with extrapolation to time infinity. Clearance (CL) was calculated from dose/AUC_∞. Apparent volume of distribution (V_d) was calculated from dose/Co (concentration at time zero calculated from extrapolation of the plasma time curve). Plasma half-life (t_{1/2}) was calculated from 0.693/slope of the terminal elimination phase (λ). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service *Policy for the Care and Use of Laboratory Animals*. Animal care was provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals* (34).

Orthotopic breast cancer model

Human triple negative MDA-MB-231-mCherry breast cancer cells (1×10^6) suspended in 100 μ L of chilled Matrigel (BD Biosciences, Bedford, MA, USA) were injected bilaterally into the lactiferous ducts of the 4th mammary gland of 5–6 week old female athymic nu/nu mice (Harlan Sprague-Dawley, Indianapolis, IN). Twelve days post-inoculation, mice were randomized into 4 treatment groups (8 mice per group): PBS alone, empty vehicle nanobins (NB(NaCl)), As₂O₃ (4 mg/kg), and NB(Ni,As) (4 mg/kg). Stock solutions of As₂O₃ were prepared by dissolving solid As₂O₃ in 5 M NaOH, then the concentrated stock solution was diluted into PBS and the pH was readjusted to 7.4. Stock solutions of NB(NaCl) and NB(Ni,As) were diluted with PBS. Each group was treated twice weekly for three weeks (7 total treatments) by intraperitoneal injection. Tumors were measured with digital calipers and tumor volumes were calculated using the equation $V_{\text{Tumor}} = (w^2 \times l \times \pi)/6$. Mice in which tumors did not take were excluded from analysis (3 from PBS, 1 from NB(NaCl) and 1 from As₂O₃). Mice were weighed twice weekly.

In a second study tissue samples for arsenic biodistribution analysis and histology were obtained from mice (n =3) treated with PBS, NB(NaCl), As₂O₃ (4 mg/kg), and NB(Ni,As) (4 mg/kg) for 3 doses or 5 doses in the same schedule described above and sacrificed 48 h after the last treatment. Tumors (n=6) were also harvested, with one half fixed in 10% formalin and embedded in paraffin for sectioning and the other half frozen for arsenic determination by ICP-MS. Immunohistochemical (IHC) staining for active cleaved caspase-3 was performed by the R. H. Lurie Cancer Center Pathology Core Facility using an active caspase-3 Ab (Biocare Medical, CP 229, Concord, CA) and standard IHC methods. Three randomly selected fields from each tumor were scored by an individual who was blinded to the treatment status. Cleaved caspase-3 values were normalized to the vehicle NB(NaCl) control group. A third study in which the effect of arsenic therapy on organ function was measured in which mice were treated with PBS, NB(NaCl), As₂O₃ (4 mg/kg) and NB(Ni,As) (4 mg/kg) on the schedule of the efficacy study. At the termination of the study, blood was collected from euthanized mice and a peripheral smear and metabolic profile was performed by RADIL (Colombia, MO). All animal experiments were conducted under protocols approved by the Animal Care and Use Committee of Northwestern University.

Inductively coupled plasma–mass spectrometry

Tumor and tissue arsenic levels were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using a Thermo XSeries II ICP-MS (Thermo-Fisher, Waltham, MA). Samples were prepared by digesting tissues in 500 µL of concentrated trace metal free grade nitric acid (65–70%) in capped, metal-free falcon tubes for two h at 60°C. At 20-minute intervals during the digestion, the sample tubes were vortexed and vented in a fume hood. After two hours, the digests were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into a fresh metal-free falcon tube. For ICP-MS analysis, a portion of the filtered digest was diluted with ultra-pure laboratory grade water (18 MΩ-cm) and an internal standard mixture of Sc, Tb, Y, In, Bi (CPI International, Santa Rosa, CA) was added. Standards between 0 and 90 ppb were made using a custom mixed element solution (CPI International). The final ICP-MS samples and elemental standards were prepared in a matrix of 2% nitric acid.

Statistical analyses

Statistical analysis described in experimental sections was performed with GraphPad Prism. Statistical significance was determined by one-way ANOVA, followed by Tukey's post-hoc analysis for tumor volumes, animal weights and arsenic levels or Bonferroni posttest for the invasion and migration assays. Statistical significance was determined by pairwise t-tests for cleaved caspase-3 staining. $P < 0.05$ was considered significant.

Results

Preparation of- arsenic trioxide-loaded nanobins

Previous protocols for producing NB(Ni,As) and NB(NaCl) were scaled up and modified to produce sufficient material for xenograft studies (30,31). Encapsulation of arsenic trioxide in nanobins is represented schematically in Fig. 1A. To measure loading efficiency, we determined the arsenic to phospholipid ratio by ICP-OES (As:P) to be 0.45–0.50 which is consistent with our previous work (30,31). Characterization of NB(Ni,As) by transmission electron microscopy revealed intact 100 nm particles (Fig. 1B). Dynamic light scattering established the average diameter of the NB(Ni,As) particles to be 95 nm for both preparations. A low polydispersity index (PDI = 0.06–0.09) revealed highly homogenous particle size. The hydrodynamic radius of the NB(Ni,As) was also measured in water, 10 mM NaCl and in PBS at multiple dilutions, and the size was not dependent on concentration

or the dispersing media (Fig. S1, Table S1 in Supplementary Information). The thermal stability of the NB(Ni,As) was evaluated from 20–60°C in 5°C increments, revealing no significant change in diameter (Fig. S2 and Table S2). The effect of pH (2.3–11.3) on the hydrodynamic size of the nanobins was evaluated by titration, and the pH did not affect the diameter in the pH range tested (Fig. S3 and Table S3). Thus, the arsenic-loaded nanobins exhibited remarkable size stability under these conditions. Control empty nanobins NB(NaCl) were similar in size (102 nm diameter) and polydispersity (PDI = 0.06).

The stability of the NB(Ni,As) was assessed by measuring the release of As₂O₃ in 80% fetal bovine serum at 37°C, pH 7.4 and at pH 5.5 (Fig. 1C). Over 48 h, 22% of the total As₂O₃ was released from the nanobins at physiological pH, confirming that the majority of the As₂O₃ is sequestered within the nanobins. At the acidic pH encountered in the acidic tumor interstitium and in the endocytic pathway of tumor macrophages, the NB(Ni,As) undergoes a triggered-release in which > 50% of the arsenic is released over 48 h. Long term stability measurements show that the NB(Ni,As) does not increase in size over time or release arsenic (Fig. S4 and Tables S4 and S5).

Nanobin encapsulation of arsenic trioxide attenuates its cytotoxicity in vitro

The cytotoxicity of As₂O₃, NB(Ni,As) and NB(NaCl) was evaluated using a panel of human breast cancer cell lines and an immortalized mammary epithelial cell line (MCF-10A) by MTS cell viability assay. These human cancer cell lines represent the major subgroups of breast cancer, including ER-positive (MCF-7), HER2/Neu-positive (SK-BR-3), and triple negative/basal-like (MDA-MB-231 and BT-20) (35). Dose response curves for As₂O₃, NB(Ni,As) and NB(NaCl) revealed that nanobin encapsulation of As₂O₃ resulted in a three to four-fold increase in the IC₅₀ compared with free As₂O₃ in all cell lines tested (Fig. 2 and Table 1A). These results corroborate findings in other cancer cell lines (30,31) and underscore the attenuated cytotoxicity of nanobin encapsulated As₂O₃. We next examined the cytotoxic mechanism of NB(Ni,As) and free As₂O₃ *in vitro* using quantitative flow cytometric analysis of apoptosis by Annexin V labeling (Fig. 3A). MDA-MB-231-mCherry triple negative breast cancer cells were treated with these agents (50 μM) for 48 h with or without a 1 h preincubation with the pan-caspase inhibitor Z-VAD-FMK (50 μM). Consistent with the MTS assay results, NB(Ni,As) was less potent than free As₂O₃ at inducing Annexin V-positive cells. Moreover, Z-VAD-FMK partially rescued cell death induced by As₂O₃ or NB(Ni,As). These findings indicate that caspase-mediated apoptosis contributes to the cell death induced by NB(Ni,As) and As₂O₃.

The effect of NB(Ni,As) and free As₂O₃ on the migration and invasion of MDA-MB-231-mCherry cells was determined in transwell migration and Matrigel™ invasion assays. Both NB(Ni,As) and free As₂O₃ inhibited migration (Fig. 3B) and invasion (Fig. 3C) in a dose-dependent manner. Under the conditions tested, cell viability was not significantly affected during migration (24 h) or invasion (48 h) (Fig. S6), which suggests that these agents have anti-migratory and anti-invasive activity.

Intravenous administration of NB(Ni,As) leads to increased plasma arsenic and decreased tissue biodistribution compared with free drug

The plasma pharmacokinetics of As₂O₃ and NB(Ni,As) were measured in double jugular catheterized, 10 week old, female SD rats. Intravenous administration of NB(Ni,As) resulted in a dramatically altered plasma concentration profile compared with that of free As₂O₃ measured by ICP-MS (Fig. 4). The NB(Ni,As) exhibited a monophasic decay of arsenic and nickel components, while the free As₂O₃ showed a biphasic decay. Selected pharmacokinetic parameters from non-compartmental are shown (Table 1B). The volume of distribution (V_d) of the NB(Ni,As) was approximately 50 ml/kg which is roughly equal to

the plasma volume of the rat, indicating minimal distribution of NB(Ni,As) outside of the plasma compartment. The peak (C_0) arsenic concentration of the NB(Ni,As) was approximately 100-times greater than that of the free drug, while clearance (Cl) was decreased ~300-fold and total exposure, area under curve (AUC_{∞}), was increased ~300-fold. Simultaneous measurement of plasma nickel revealed that the nickel levels paralleled plasma arsenic, and had similar C_0 , V_d , AUC_{∞} and Cl. Since the nickel is released very slowly from the NB(Ni,As) (Fig. 1), the parallel serum levels of As and Ni suggest that the NB(Ni,As) is a robust delivery platform that is stable *in vivo*. The stability and low RES sequestration of the NB(Ni,As) platform appear to contribute to the favorable pharmacokinetic profile in comparison to free As_2O_3 .

Arsenic trioxide-loaded nanobins inhibit tumor growth in an orthotopic model of triple negative breast cancer

The antitumor efficacy of NB(Ni,As), empty nanobins and free As_2O_3 were evaluated in female athymic nude mice bearing orthotopic human MDA-MB-231 triple negative mammary tumors. We determined that a well tolerated dose of NB(Ni,As) was 4 mg/kg (twice weekly) (data not shown). Mice were treated with vehicle, empty nanobins (NB(NaCl)), NB(Ni,As) (4 mg As_2O_3 /kg) or free As_2O_3 (4 mg As_2O_3 /kg) twice weekly by intraperitoneal (ip) injection. Although free As_2O_3 had no effect in the study, the corresponding arsenic concentration-equivalent of NB(Ni,As) robustly inhibited MDA-MB-231 tumor growth (Fig. 5A). Moreover, a higher dose of free As_2O_3 (8 mg/kg ip given on the same dosing schedule) also did not inhibit mammary tumor growth in this model (data not shown). Consistent with the enhanced antitumor efficacy of NB(Ni,As), the concentration of elemental As was 3–5-fold higher in MDA-MB-231 tumors treated with NB(Ni,As) than in tumors treated with free As_2O_3 (Fig. 5B). Mammary tumors from mice treated with NB(Ni,As) had increased caspase-3 activation as determined by cleaved caspase-3 immunostaining compared with vehicle or empty nanobin treated mice (Fig. 5C). The observation that free As_2O_3 induces apoptosis in tumor cells, at least transiently, but does not suppress mammary tumor growth in mice suggests that the improved pharmacokinetics and tumor delivery of nanobin encapsulated As_2O_3 are responsible for its antitumor efficacy *in vivo*.

Importantly, treatment of mice with NB(Ni,As) (4 mg/kg, twice weekly for three weeks) did not cause significant weight loss during the study period (Fig. 5D). To further assess toxicity, we obtained completed blood counts, liver function, kidney function and serum chemistries (Table S8). An isolated elevation of blood urea nitrogen was observed in mice treated with NB(Ni,As); however, electrolytes (sodium, potassium, calcium, phosphorus and chloride) and creatinine were normal. Moreover, histological analysis of H&E stained tissues (skin, kidneys, liver, heart and lungs) from NB(Ni,As)-treated mice did not reveal any histopathology (Fig. S10). Measurement of total arsenic in the liver, kidney and the heart and lungs after five treatments revealed modest accumulation of arsenic in the liver and kidney (Fig. S11). Taken together, our findings indicate that NB(Ni,As) is an effective and well tolerated therapeutic agent in this murine model of breast cancer.

Discussion

We observed that encapsulation of As_2O_3 in a novel nanobin, NB(Ni,As), led to increased *in vivo* antitumor efficacy of As_2O_3 in the MDA-MB-231 orthotopic model of human triple negative breast cancer. NB(Ni,As) inhibited mammary tumor growth at doses (4 mg/kg twice weekly) that are significantly lower than the anticipated efficacious dose of the parent drug based on published reports in other preclinical models of solid tumors (36,37). Indeed, two times the equivalent dose of free As_2O_3 had no effect on tumor growth in our study (data not shown). We attribute the enhanced antitumor efficacy *in vivo* of NB(Ni,As) to the

reduced plasma clearance and the increased tumor accumulation of arsenic via the EPR effect of the nanobin platform. Specifically, we observed that the area under the curve (AUC_{∞}), an estimate of drug exposure, was increased approximately 300-fold for NB(Ni,As) compared with free As_2O_3 . Moreover, the tumor arsenic concentrations were 3–5 fold higher in NB(Ni,As) treated mice. The As_2O_3 that is delivered to tumors by NB(Ni,As) appears to be released over an extended time period, leading to metronomic-like dosing compared to free As_2O_3 and resulting in prolonged antitumor activity even after treatment was stopped (Fig. 5A). In contrast, free As_2O_3 had no anti-tumor activity at the dose and schedule used and may have caused a “rebound” tumor growth. The latter phenomena has been observed in maximal tolerated dose (MTD)-based regimens where neovascularization and tumor growth resumes during the necessary recovery periods from drug-induced toxicity (38). The NB(Ni,As) may block this tumor rebound effect by loading a tumor with drug that is continuously released and does not follow the peak-trough kinetics typically associated with MTD-based chemotherapy. To that end, NB(Ni,As) may behave like a metronomically dosed or depot agent, although that aspect of NB(Ni,As) pharmacology remains to be explored.

The inhibition of NB(Ni,As)- and free As_2O_3 -induced cell death by Z-VAD-FMK, and the enhanced caspase-3 activation in mammary tumors observed in mice treated with these cytotoxic agents, indicate that the antitumor effects of NB(Ni,As) are at least partly mediated by a caspase-dependent apoptotic mechanism. Although we observed similar rates of mammary tumors apoptosis in the NB(Ni,As) and free As_2O_3 groups 48 h after treatment, only NB(Ni,As) reduced tumor burden *in vivo*. This seeming paradox likely reflects the rapid clearance of free As_2O_3 from the circulation, leading to transient induction of apoptosis in the mammary tumors and potential “rebound” growth (36). In contrast, NB(Ni,As) prolongs the pharmacokinetics of As_2O_3 and increases tumor uptake, resulting in sustained antitumor effects. Intriguingly, free As_2O_3 and NB(Ni,As) inhibit migration and invasion at concentrations well below their IC_{50} values, suggesting that these agents may have anti-metastatic activity in addition to their cytotoxicity.

Although As_2O_3 is currently approved for use in acute promyelocytic leukemia (APL), several clinical trials in patients with solid tumors have failed to demonstrate a clinical benefit of As_2O_3 at doses in the 0.25–0.35 mg/kg/d range (39,40). APL patients receive 0.16 mg/kg/d of As_2O_3 , and this dose is associated with Grade 3/4 toxicities such as peripheral neuropathy, hepatic and cardiac toxicity (10,20). These dose-limiting toxicities have limited further dose escalation of As_2O_3 in other malignancies. Conversion between mouse and human dose levels by body weight (mg/kg) can be estimated by dividing the mouse dose by 12.3 (41). Thus, a 4 mg/kg dose in the mouse is estimated to be equivalent to a human dose of 0.32 mg/kg, which results in a comparable weekly dose of free As_2O_3 used in APL patients because the nanobin is given twice weekly rather than daily. Hence, clinically efficacious concentrations of NB(Ni,As) are associated with minimal systemic toxicity in this preclinical model.

As noted, we observed that NB(Ni,As) is more effective at suppressing tumor growth *in vivo* than the parent drug As_2O_3 ; however, free As_2O_3 is much more cytotoxic *in vitro*. This disparity suggests that standard *in vitro* cytotoxicity assays of nanoparticle-encapsulated drugs may be a poor predictor of *in vivo* antitumor activity because they fail to capture the effects of drug encapsulation on pharmacokinetics and biodistribution *in vivo*. Hence, early *in vivo* testing of nanoparticle encapsulated cytotoxic agents in animal models of cancer is of paramount importance and an important component of the development of this class of drugs.

Many potentially effective cancer drugs have been abandoned due to unacceptable systemic toxicity, poor pharmacokinetics and/or biodistribution (22,42). We have devised a nanoparticulate platform (nanobin) in which the nanoscale size leads to the concentration and retention of drug in the tumor, while the nanobin encapsulation improves the pharmacokinetic characteristics and potentially the toxicological properties of the encapsulated drug. This platform has been applied to the encapsulation and reformulation of a highly cytotoxic drug, As₂O₃, which is currently limited in its use clinically to APL and other hematologic malignancies. The reformulated As₂O₃ nanobin, NB(Ni,As), differs substantially from parent drug in both its pharmacological and efficacy profile. These results provide a foundation for additional preclinical development and future clinical interventions in breast cancer and other solid tumors.

Statement of Translational Relevance

Arsenic trioxide (As₂O₃) is a highly effective therapy for acute promyelocytic leukemia and has antitumor activity in preclinical models of solid tumors; however, clinical trials of As₂O₃ in several solid tumors reveal a narrow therapeutic window that limits wider application. Nanoscale drug carriers have increased the therapeutic index of several cytotoxic agents by increasing tumor drug delivery, enhancing antitumor efficacy and attenuating systemic toxicity. We have developed a novel high-density nanoparticulate formulation of As₂O₃ that is encapsulated in 100 nm liposomal vesicles or nanobins. Nanobin encapsulation of As₂O₃ [NB(Ni,As)] dramatically improves pharmacokinetic properties of the active agent and leads to greater therapeutic efficacy compared to free As₂O₃ in an orthotopic model of triple negative breast cancer. Moreover, we show that NB(Ni,As) is well tolerated in this model, suggesting that this nanoscale platform may have the potential to expand the clinical utility of As₂O₃ as a cancer therapeutic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

NB(Ni,As) is a novel formulation of As_2O_3 in which the active drug is encapsulated as a nanoparticulate inside a lipid bilayer. *A*, schematic representation of transition metal triggered precipitation of As_2O_3 inside the nanobin. *B*, transmission electron micrograph of NB(Ni,As) stained with 4% uranyl acetate shows homogenous particles ~100 nm in size (Scale Bar, 100 nm). *C*, kinetics of arsenic release in 80% FBS at 37°C are accelerated at pH 5.5 compared with pH 7.4. Points, mean; bars, $\pm$ SEM.

Figure 2.

NB(Ni,As) attenuates As₂O₃ cytotoxicity *in vitro*. Breast cancer cells were treated with free As₂O₃ (■), NB(Ni,As) (▼), or NB(NaCl) (●). Cell viability was evaluated at 72 h by MTS assay. Points, mean; bars, $\pm$ SEM.

Figure 3.

As₂O₃ and NB(Ni,As) induce apoptosis and inhibit migration and invasion in MDA-MB-231 triple negative breast cancer cells *in vitro*. A, MDA-MB-231-mCherry cells were treated with As₂O₃ or NB(Ni,As) ([As] = 50 μM. [As₂O₃] = 25 μM), alone or in combination with pan-caspase inhibitor Z-VAD-FMK (50 μM). Apoptosis was measured by Annexin V labeling and membrane integrity was measured by DAPI by FACS analysis at 48 h. B, Migration was assessed by seeding MDA-MB-231-mCherry cells on the top of an uncoated transwell, and cells were treated with As₂O₃ (0.1, 1.0, 10.0 μM As), NB(Ni,As) (0.1, 1.0, 10.0 μM As) or NB(NaCl) control for 24 h. C MDA-MB-231-mCherry cells were seeded on Matrigel coated transwells, treated with As₂O₃ (0.1, 1.0, 10.0 μM As), NB(Ni,As) (0.1, 1.0, 10.0 μM As) or NB(NaCl) for 48 h, and invading cells were scored. Bars ± SEM **P* < 0.05; ***P* < 0.01, ****P* < 0.001 versus media control.

Figure 4.

The pharmacokinetic properties of NB(Ni,As) and free As₂O₃ were measured in double jugular catheterized SD Rats (n=5). Rats received a single injection of NB(Ni,As) (4 mg As/kg) or free As₂O₃ (4 mg As/kg) and plasma samples were collected over a 24 h period.

Points, mean; bars, $\pm$ SEM.

Figure 5.

NB(Ni,As) inhibits mammary tumor growth *in vivo* in an orthotopic model of breast cancer. A, twelve days after orthotopic inoculation of MDA-MB-231-mCherry cells, female athymic nude mice were randomized into 4 treatment groups (8 mice per group): PBS, empty nanobins (NB(NaCl)), free As₂O₃ (4 mg/kg), and NB(Ni,As) (4 mg/kg). Agents were delivered twice weekly for 3 weeks by intraperitoneal injection as indicated. Mammary tumor volumes were measured using calipers (*left panel*). **P* < 0.05; ***P* < 0.01 versus vehicle treated mice. mCherry fluorescence of mammary tumors at week 5.5 (*right panels*). B, enhanced arsenic accumulation in mammary tumors from mice treated with NB(Ni,As) compared to those receiving free As₂O₃. Tumor arsenic concentrations were measured by

ICP-MS analysis in mice receiving 3 (3 Tx) or 5 doses (5 Tx) of these agents as indicated in A. $**P < 0.01$ versus NB(NaCl) control. C, immunohistochemical staining for active cleaved caspase-3 in mammary tumors from mice treated with PBS, NB(NaCl) NB(Ni,As) or free As₂O₃ for 2 weeks (5 doses). $*P < 0.05$; $***P < 0.001$ versus NB(NaCl) treated mice. D, no significant weight loss was observed in any treatment group compared with vehicle treated mice.

Table 1AIC₅₀ Values for As₂O₃ and NB(Ni,As)

Cell Line	Arsenic Trioxide		NB(Ni,As)
	[As]	As ₂ O ₃	[As]
<i>MDA-MB-231</i>	7.3 ± 1.1	3.7 ± 0.5	28.1 ± 1.1
<i>MCF-7</i>	8.1 ± 1.1	4.1 ± 0.5	28.0 ± 1.1
<i>BT-20</i>	27.9 ± 1.1	14.0 ± 0.5	105.7 ± 2.1
<i>SK-BR-3</i>	21.8 ± 1.1	10.9 ± 0.5	62.2 ± 1.2
<i>MCF-10A</i>	20.6 ± 1.1	10.3 ± 0.5	71.2 ± 1.1

NB(NaCl) did not cause appreciable growth inhibition at all tested doses. IC₅₀ values were calculated from sigmoidal dose-response curves in Fig. 2 and are reported for both elemental As and As₂O₃ for free As₂O₃.

Table 1B

Selected Pharmacokinetic Parameters

Drug Treatment	$t_{1/2}$ (h)	C_0 (mM)	AUC_{∞} ($\mu\text{mol} \times \text{h/ml}$)	V_d (ml/kg)	Clearance (ml/h)
As ₂ O ₃	6.9 ± 3.3	0.01 ± 0.002	0.04 ± 0.01	5735 ± 1801	334 ± 116
NB(Ni,As) -As	8.3 ± 1.2	1.12 ± 0.14	11.8 ± 1.9	48 ± 6	1.1 ± 0.1
NB(Ni,As) -Ni	8.1 ± 1.4	1.41 ± 0.17	15.7 ± 3.0	49 ± 6	0.9 ± 0.1