Preclinical Development of an Anti-NaPi2b
(SLC34A2) Antibody–Drug Conjugate as a Therapeutic for Non-Small Cell Lung and Ovarian Cancers
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Abstract

**Purpose:** Antibody–drug conjugates (ADC) selectively deliver a cytotoxic drug to cells expressing an accessible antigenic target. Here, we have appended monomethyl auristatin E (MMAE) to an antibody recognizing the SLC34A2 gene product NaPi2b, the type II sodium–phosphate cotransporter, which is highly expressed on tumor surfaces of the lung, ovary, and thyroid as well as on normal lung pneumocytes. This study evaluated its efficacy and safety in preclinical studies.

**Experimental Design:** The efficacy of anti-NaPi2b ADC was evaluated in mouse ovarian and non–small cell lung cancer (NSCLC) tumor xenograft models, and its toxicity was assessed in rats and cynomolgus monkeys.

**Results:** We show here that an anti-NaPi2b ADC is effective in mouse ovarian and NSCLC tumor xenograft models and well-tolerated in rats and cynomolgus monkeys at levels in excess of therapeutic doses. Despite high levels of expression in normal lung of non-human primate, the cross-reactive ADC exhibited an acceptable safety profile with a dose-limiting toxicity unrelated to normal tissue target expression. The nonproliferative nature of normal pneumocytes, together with the antiproliferative mechanism of MMAE, likely mitigates the potential liability of this normal tissue expression.

**Conclusions:** Overall, our preclinical results suggest that the ADC targeting NaPi2b provides an effective new therapy for the treatment of NSCLC and ovarian cancer and is currently undergoing clinical developments.

Introduction

In the United States, non–small cell lung cancer (NSCLC) is the most common cause of cancer death and ovarian cancer is the fifth most common cause of cancer mortality in women (1). Despite the success of targeted therapies, such as erlotinib (Tarceva) (2) and bevacizumab (Avastin) (3) in lung cancer, and sensitivity to platinum-based therapy in ovarian cancer, recurrence or progression following initial treatment occurs in the majority of patients in both diseases. In addition, standard-of-care therapies are associated with significant systemic toxicity. Treatments that provide meaningful clinical benefit with acceptable safety profiles remain a significant unmet need for NSCLC and ovarian cancers.

Antibody–drug conjugates (ADC) offer the potential to deliver highly potent cytotoxic agents to cells expressing a predefined cell-surface target. The recent success with ADCs targeting erbB2 in breast cancer (trastuzumab emtansine, T–DM1) and CD30 in lymphoma (brentuximab vedotin) have validated this overall approach in oncology applications (4–6). The availability of high-throughput gene expression data has enabled the identification of new potential ADC targets that are highly expressed on cancer cells but exhibits restricted distribution in normal tissues.

In our search for potential ADC targets for the treatment of NSCLC, we identified SLC34A2, which codes for the type II Na/Pi cotransporter, NaPi2b. NaPi2b is a multitransmembrane, sodium-dependent phosphate transporter (7), which is expressed in human lung, ovarian, and thyroid cancers (8, 9). As a member of the SLC34 solute carrier protein family (10), it is responsible for transcellular inorganic phosphate absorption and maintenance of phosphate homeostasis (7, 11, 12) and has been associated with cell differentiation (13) and tumorigenesis (14). The differential expression in tumor relative to most normal tissues, cell-surface localization, and endocytosis makes it a promising target for ADC therapeutics.

In contrast to an ideal ADC target with negligible normal tissue expression, NaPi2b is expressed at a detectable level in normal lung tissues, where it takes part in the maintenance of local Pi concentration (7, 10, 15–19). Expression of NaPi2b in normal tissues could represent a potential safety concern for ADC therapy. For example, expression of the target glycoprotein nonmetastatic melanoma protein B (GPMB) on normal human skin resulted in adverse dermatologic events in clinical trials of an anti-GPMB antibody armed with the anti-mitotic monomethyl auristatin E (MMAE) (20). Targeting normal tissues, particularly those with basal regenerative and proliferative activities, with microtubule-disrupting chemotherapies, should be avoided.
cells, however, in the absence of injury, are not highly proliferative and therefore less susceptible to antimitotic agents such as MMAE, which largely relies upon cell division to elicit cytotoxic effects. This consideration, together with the retention of high levels of NaPi2b on lung cancer cells, as well as its overexpression on ovarian cancers, compelled us to assess it as an ADC target.

Anti-NaPi2b–vc–MMAE is an ADC composed of a humanized IgG1 anti-NaPi2b monoclonal antibody (mAb) and MMAE, linked through a protease sensitive valine–citrulline peptide. The linker is designed for cleavage by cathepsins to release drugs following endocytotic uptake into lysosomes (21–23). The drug, MMAE, which is a synthetic analog of the antibiotic agent dolostatin 10, binds to and blocks the polymerization of tubulin. Here, we describe the generation and preclinical characterization of anti-NaPi2b–vc–MMAE and demonstrate its potential as a treatment for NSCLC and ovarian cancers.

Materials and Methods

mRNA tissue expression analysis

The analysis of NaPi2b mRNA expression in multiple human tumor and normal biopsy samples was conducted with microarray data from Gene Logic Inc. The analysis shown for probe set ID 204124 was conducted using the GeneChip Human Genome U133 Plus 2.0 array on 3,879 normal human tissue samples, containing 1,605 human cancer tissue samples (1,291 primary and 314 metastatic), and 3,872 human noncancer disease tissue samples. Microarray data were normalized using the Affymetrix Microarray Suite version 5.0 software with sample expression values scaled to a trimmed mean of 500.

NaPi2b expression in malignant and normal tissues determined by immunohistochemistry

The reactivity of the mouse anti-NaPi2b antibody on human lung carcinomas (31 adenocarcinomas and 25 squamous cell carcinomas), ovarian carcinomas (67 ovarian tumors), and thyroid carcinomas (56 thyroid carcinomas, adenomas, and thyroid hyperplasias) was assessed by immunohistochemical (IHC) analysis. NaPi2b expression in normal human and monkey tissues was also evaluated using a normal human tissue microarray or normal monkey tissues stained with the mouse anti-NaPi2b antibody. IHC was performed on 5-μm-thick formalin-fixed, paraffin-embedded tissue sections mounted on glass slides. Slides were deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Slides were pretreated with Trilogy Pretreatment Solution (Cell Marque) for 1 hour at 99 °C and then treated with blocking solution (Kierkegaard and Perry Laboratories) and avidin/biotin block (Vector Laboratories) respectively. Nonspecific IgG binding was blocked using TBS with Tween-20 containing 1% BSA (Roche) and 10% normal horse serum (Life Technologies Corp.). Primary antibody, mouse anti-human NaPi2b, was incubated on slides at 10 μg/mL for 60 minutes at room temperature. Slides were rinsed and then incubated with horse anti-mouse biotinylated secondary followed by incubation in Vectastain ABC Elite reagent (Vector Laboratories). Slides were then incubated in Pierce metal enhanced diaminobenzidine (Thermo Fisher Scientific), counterstained, dehydrated, and cover-slipped.

NaPi2b cloning

A human NaPi2b sequence (Gene ID 204124 was subcloned into pRK5tkneo (Gene Logic Inc) cytomegalovirus mammalian expression, and a stable cell line was generated in human embryonic kidney (HEK) 293 under 200 μg/mL neomycin selection (293 NaPi2b B8).

mAb and ADC production

Mouse mAbs to NaPi2b were generated by immunizing BALB/c mice with recombinant human NaPi2b poly-His–tagged protein (amino acids 250–361). Immunization, hybridoma selection, antibody humanization, and purification processes were done by the same methodology as described previously for MUC16 (24). The 2 control antibodies, anti-6-transmembrane epithelial antigen of prostate 1 (anti-STEAP1) and anti-glycoprotein (anti-gD) mAbs, were constructed as previously described (25, 26). All antibodies were conjugated with MMAE via a protease labile linker, MC-VC-PAB as described previously (22). The average drug-to-antibody ratio is 3.5 for anti-Napi2b conjugate and other control conjugates.

Cell lines

HEK 293 cells (CRL-1573) were transfected with pRK5tkneo cytomegalovirus mammalian expression vector (Genentech) containing human NaPi2b, and a stable cell line (B8) was generated in 50:50 DMEM and Ham F-12 media (Genentech) supplemented with 10% FBS, 2 mmol/L l-glutamine, 1 × penicillin–streptomycin, and 200 μg/mL G418, at 37 °C in 5% CO₂. The OVCAR3 ovarian cancer mammary fat pad (MFP) transplant xenograft tumor model, C93-071405, was developed using the OVCAR3 ovarian adenocarcinoma cell line, which was obtained from ATCC. The OVCAR3-X2.1 cell line was derived from an OVCAR3 human ovarian adenocarcinoma MFP transplant line (OVCAR-3 MFP No. 4382-061404) developed from the OVCAR3 (HTB-161) cell line from the ATCC. The NCI-H441 (HTB-174), a human lung adenocarcinoma cell line, was obtained from ATCC; Igrov-1, a human ovarian adenocarcinoma cell line, was obtained from the NCI cell collection.

Binding affinity by biacore and in vitro cellular staining by immunoblotting

Binding activity of the anti-NaPi2b mAb was determined in multiple NaPi2b-expressing cell lines. Anti-NaPi2b immunoblot...
of cell lysates from PC3 cells, expressing the SLC34A CDNA or vector control, and 2 ovarian cancer cell lines (OvCAR3 and Igrov1) expressing the endogenous SLC34A gene were pretreated with or without deglycosylase (−/+) to investigate the impact of glycosylation on binding to antibody.

**Determination of in vitro binding by flow-cytometry assay**

Binding of anti-NaPi2b mAb and ADC to OVCAR3-X2.1, 293 NaPi2b B8, and a nonexpressing cell line was analyzed by flow cytometry as previously described (24). The FACS buffer was prepared with PBS plus 1% FBS and 2 mmol/L EDTA.

The OVCAR3-X2.1 tumor tissues were harvested, chopped, and macerated with cell dissociation buffer (Gibco-BRL; catalog No. 13151-014; Invitrogen). Dissociated tumors were incubated at 37°C for 15 minutes and then poured and washed through the strainer with FACS buffer. All subsequent steps were carried out at 4°C. Cells were spun down and resuspended with 20 mL of ammonium chloride-potassium red blood cell lysis buffer (Genentech; 163 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L ammonium chloride-potassium red blood cell lysis buffer (Genentech; 163 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4) to lyse the red blood cells. Samples were spun down at 4°C and resuspended in FACS buffer and incubated for 1 hour each with 3 µg/mL of primary antibodies and then stained with the phycoerythrin-labeled anti-human IgG Fc-specific secondary antibody at 2 µg/mL.

All samples were stained with propidium iodide at 0.5 µg/mL and then analyzed using a FACS flow cytometer (FACSCalibur; BD Biosciences). Cytometric data were processed using the FlowJo software application (version 8.4.5) and selection for viable cells was made on the basis of propidium iodide exclusion forward-scatter values. Geometric mean fluorescence intensity values for each gated populations were transferred to Excel (version 11.5.6; Microsoft) for graphic display.

**Internalization of anti-NaPi2b mAb and ADC by fluorescence microscopy**

NaPi2b-negative 293 cells, NaPi2b-positive 293 NaPi2b B8 cells, and OVCAR3-X2.1 cells were seeded onto BD BioCoat poly-d-lysine–treated cell culture chamber slides, and the slides were cultured at 37°C in a humidified incubator charged with 5% CO₂. Twenty-four hours later, cell culture media were replaced with fresh growth medium containing 3 µg/mL of anti-NaPi2b mAb or ADC plus 50 µg/mL leupeptin and 5 µg/mL, peptatin to inhibit lysosomal degradation. After 20-hour incubation, cells were washed and then fixed with 4% formaldehyde in PBS. After fixation, cells were washed with PBS and permeabilized with 0.05% (w/v) saponin in PBS and then incubated with 1% BSA in PBS to block nonspecific bindings. Lysosomes were then labeled with 2 µg/mL mouse anti-lysosomal–associated membrane protein 1 (LAMP1) mAb (BD Biosciences), followed by the addition of 2 µg/mL Alexa488 goat anti-mouse IgG antibody for anti-LAMP1 antibody detection and 2 µg/mL Cy3 goat anti-human IgG antibody for anti-NaPi2b ADC detection. After washing, chamber inserts were removed to expose the glass slides and slides were mounted by applying VectaShield with 4′,6-diamidino-2-phenylindole to label nuclei (Vector Laboratories). The slides were placed under a glass coverslip and sealed with clear nail polish. Images were acquired on a Nikon TE-300 inverted microscope equipped with a 60× magnification 1.4 NA infinity-corrected Plan Apo oil objective (Technical Instruments) and a Retiga EX-cooled CCD camera (Q Imaging), using the QCapture (version 3.1.1) software application (Q Imaging). Image analysis was performed using Photoshop CS software (version 8.0; Adobe Systems).

**Inhibition of in vitro cell proliferation**

The effects of anti-NaPi2b mAb and ADC and free MMAE on tumor cell viability were assessed using Cell Titer-Glo assay (Promega Corp.) as previously described (27). Human NaPi2b-positive 293-B8 and NaPi2b-negative 293 cells were seeded at 5,000 cells, and OVCAR3-X2.1 cells were seeded at 2,000 cells per well with 50 µL culture medium in 96-well culture plates. Anti-NaPi2b ADC was serially diluted and added into the wells. After 4 days, Cell Titer-Glo reagent was added into the plate. The luminescent signal was measured using a Wallac Victor2-V 1420 Multilabel HTS Counter (PerkinElmer) and the data were analyzed using Excel (version 11.3.7; Microsoft Corp.). Fifty percent inhibitory concentration (IC₅₀) values were determined on the basis of cell viability values calculated as percentage of untreated control using KaleidaGraph 3.6 (Synergy Software) 4-parameter sigmoidal fit. The same procedure was used in the analysis of a nontarget-specific control, anti-gD ADC at 2.4 mg/mL to address the target specificity in antiproliferation activities.

**In vivo efficacy**

All efficacy studies were conducted in accordance with the Guide of the Care and Use of Laboratory Animals (28). For the OVCAR3-X2.1 efficacy study, the cell line was derived from an OVCAR3 ovarian adenocarcinoma transplant line obtained from ATCC (24). OVCAR3 cells were injected intraperitoneally into female C.B-17 severe combined immunodeficient (SCID) beige mice. A donor tumor was excised from a mouse bearing intraperitoneal tumors, minced, and surgically implanted into the right thoracic mammary fat pad (MFP) of female C.B-17 SCID beige recipient mice. The tumors were serially passaged into the right thoracic MFP of recipient mice to maintain the transplant line. Female SCID or beige with x-linked immunodeficient mice from Charles River Laboratories were inoculated in the 2 of 3 MFP with 50 × 10⁶ OVCAR3-X2.1 or 25 × 10⁶ Igrov1 cells, respectively, suspended in Hank balanced salt solution with Matrigel. The NCI-H441 efficacy model was done with an injection of 50 × 10⁶ NCI-H441 cells into the right dorsal flank in C.B-17 SCID beige mice, suspended in Hank balanced salt solution with Matrigel. The mice were 6- to 12 weeks old and weighed approximately 20 to 25 g each. When tumors reached a volume range of 100 to 300 mm³, animals were randomized into groups of 6 to 10 each and dosed with vehicle control or anti-NaPi2b ADC at a dose range of 3 to 24 mg/kg, MMAE conjugated to anti-gD antibody was also included as a control.

Tumors were measured using UltraCal-IV calipers (Model 54-10-11, Fred V. Fowler Company) according to the following formula: tumor volume (mm³) = (length × width²) × 0.5. Mice were euthanized before tumor volume reached 3,000 mm³ or when tumors showed signs of impending ulceration.

**In vivo pharmacokinetic studies**

All studies were conducted in accordance with the Guide of the Care and Use of Laboratory Animals (28). To fully characterize the pharmacokinetic profiles of anti-NaPi2b ADC, single dose studies were conducted in OVCAR3-X2.1 tumor–bearing and nontumor female SCID beige mice from Charles River Laboratories, Sprague-Dawley (SD) rats from Charles River Laboratories,
and cynomolgus monkeys (*Macaca fascicularis*) from SNBL USA Ltd. The mice were 6 to 8 weeks old and weighed approximately 16 to 28 g; the rats were 6 to 13 weeks old and weighed approximately 160 to 450 g; and the monkeys were 3 to 8 years old and weighed approximately 2 to 7 kg. Anti-NaPi2b ADC was administered intravenously in tumor-bearing mice (1, 3, and 6 mg/kg), non—tumor-bearing mice (0.5 and 5 mg/kg), rats (0.5 and 5 mg/kg), and monkeys (0.3 and 1 mg/kg). Blood samples were collected and analyzed for total antibody concentrations by ELISA. Pharmacokinetic parameters were estimated using a 2 compartmental model with WinNonlin (version 5.2.1, Pharsight Corporation).

**ELISA for total antibody concentration measurement**

Multiple ELISA formats were used to analyze total antibody concentrations in different matrices. A bridging format ELISA was developed for samples from mice (both tumor-bearing and non-tumor-bearing) and rats; the assay used a biotinylated recombinant human NaPi2b extracellular domain protein and a goat anti-human IgG conjugated to horseradish peroxidase (HRP; Bethyl Laboratories, Inc.) to capture anti-NaPi2b antibodies. These reagents were coincubated with diluted samples, standards, or assay controls and then added to a NeutrAvidin-coated plate for capturing the reagent-analyte complex. The lower limit of quantification (LLOQ) for the assay is <0.313 ng/mL with a minimum 1:100 dilution. The monkey serum samples were analyzed with a similar bridging format by using a sheep anti-human IgG conjugated to biotin and HRP (The Binding Site Inc.) to capture anti-NaPi3b antibodies. The reagent-analyte complex is captured in streptavidin-coated plates followed by the color development reaction. The assay has a LLOQ of 0.100 µg/mL (0.660 nmol/L) in cynomolgus monkey serum.

![Figure 1.](image_url)

Expression of NaPi2b by transcript in human tissues. A, analysis of mRNA transcript. Measurements were carried out on the Affymetrix U133P chip and are expressed as scaled average difference. Each dot represents a normal (green), tumor (red), or diseased nontumor (blue) human tissue specimen. Rectangles encompass the 25th to 75th percentile range for each distribution. WBC, white blood cells. B, IHC detection of NaPi2b protein in the indicated tumor samples representing increasing intensity of staining.
Toxicity studies in rat and monkey

The safety profile of anti-NaPi2b ADC was evaluated in naive SD rats (Charles River Laboratories) and cynomolgus monkeys (M. fascicularis, Covance Research Products Inc.). Male and female rats (250–450 g) were given intravenous injections of vehicle or anti-NaPi2b ADC at 2, 6, or 12 mg/kg once weekly for 4 weeks followed by a 6-week recovery period. Thirty rats (15 male and 15 female) per group were designated for toxicity evaluations at the end of dosing (day 26, 10/sex/group) and recovery period (day 64, 5/sex/group) necropsies. Toxicological assessments included evaluation of mortality, clinical signs, body weights, food consumption, ophthalmic examinations, functional observation battery, motor activity, micronuclei in bone marrow, clinical pathology, and anatomic pathology.

Male and female monkeys (2.0–6.5 kg) were given intravenous injections of vehicle or anti-NaPi2b ADC at 1, 3, or 6 mg/kg once every 3 weeks for 5 doses followed by a 6-week recovery period. Five monkeys per sex per group were designated for toxicity evaluations at the end of dosing (day 92, 3/sex/group) and recovery period (day 127, 2/sex/group) necropsies, respectively. Two additional monkeys were included in each group for cardiovascular evaluations via telemetry. Toxicologic assessments included evaluation of mortality, clinical signs, body weights, food consumption, neurologic and physical examinations, respiration rates, ophthalmic and electrocardiograms (external or telemetry, or both) examinations, blood pressure measurements (external), telemetry hemodynamics, intraabdominal body temperature, clinical pathology, and anatomic pathology.

Blood samples were also collected for toxicokinetics (TK) analysis of anti-NaPi2b ADC total antibody (conjugated and unconjugated), free MMAE, and anti-therapeutic antibody (ATA) levels.

Results

NaPi2b is highly expressed in lung and ovarian cancers

To identify potential targets for ADC therapy in lung cancer, we searched a large mRNA transcript database generated by oligonucleotide microarray analysis of more than 5,000 human cancer and normal tissue samples. We failed to identify any transcripts coding for cell-surface proteins highly overexpressed in lung cancer relative to normal lung while maintaining low expression in other normal vital tissues. On the basis of the assumption that an antimitotic drug would spare nonproliferating cells, we expanded the search to include expression in normal lung tissues. Accordingly, we identified NaPi2b mRNA, which was expressed at high levels in normal and cancerous lung tissues, as well as in ovarian and thyroid tumor samples relative to the vast majority of all other normal and cancer tissues analyzed (Fig. 1A). IHC analysis, using mouse anti-NaPi2b antibodies, demonstrated a high frequency of NaPi2b expression in human nonsquamous, NSCLC, nonmucinous ovarian cancer, and papillary thyroid carcinomas (Fig. 1B and Table 1). Similar staining frequency and intensity were found in matched primary and metastatic carcinoma samples of lung and ovarian cancers. IHC staining in normal human tissues revealed staining in the epithelial compartments of several tissues including lung, bronchus, and kidney (data not shown).

Antigen-5

Anti-NaPi2b antibody and its ADC show specific and comparable binding affinities to human and nonhuman primate

NaPi2b is a cell-surface transporter containing 12 transmembrane domains with both the amino and carboxyl termini localized to the cytosol (10). As an initial step toward generating an ADC reactive with NaPi2b on live cells, we immunized mice with a recombinant fusion protein containing amino acid sequence present in the large extracellular loop separating transmembrane domains 4 and 5. We selected one mAb, hereafter designated as anti-NaPi2b, based on its affinity and cross-reactivity with NaPi2b homolog expressed by the nonhuman primate cynomolgus monkey. By immunoblotting, anti-NaPi2b recognized a 100-kDa protein expressed by PC3 cells transfected with the NaPi2b cDNA (PC3-SLC34A) but not with vector control (Fig. 2A). Ovarian cancer cells OVCAR3 and IGROV1 also expressed a similarly sized endogenous protein reactive with the antibody. Following enzymatic deglycosylation, the mobility of the NaPi2b protein increased but remained reactive with anti-NaPi2b. Anti-NaPi2b also reacted with all 3 positive cell lines as determined by flow cytometry and immunofluorescent detection on live cells (Fig. 2B) but not with the vector control PC3 cell line (data not shown). Thus, anti-NaPi2b recognizes epitopes within the extracellular domain of NaPi2b independent of glycosylation.

Using a series of protein fragments derived from the 111-amino acid extracellular loop, the reactive epitope was delineated to

Table 1. NaPi2b IHC in lung NSCLC, ovarian carcinoma, and thyroid carcinoma (prevalence %)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Negative</th>
<th>1⁺</th>
<th>2⁺</th>
<th>3⁺</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung NSCLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>13%</td>
<td>10%</td>
<td>35%</td>
<td>42%</td>
<td>31</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>80%</td>
<td>12%</td>
<td>0%</td>
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<td>25</td>
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<tr>
<td>Ovarian carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous adenocarcinoma</td>
<td>4%</td>
<td>4%</td>
<td>69%</td>
<td>23%</td>
<td>26</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>60%</td>
<td>30%</td>
<td>10%</td>
<td>10%</td>
<td>10</td>
</tr>
<tr>
<td>Endometrioid carcinoma</td>
<td>5%</td>
<td>70%</td>
<td>25%</td>
<td></td>
<td>20</td>
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<tr>
<td>Clear cell carcinoma</td>
<td>0%</td>
<td>18%</td>
<td>64%</td>
<td>18%</td>
<td>11</td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>9%</td>
<td>12%</td>
<td>0%</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Medullary carcinoma</td>
<td>91%</td>
<td>0%</td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular adenocarcinoma</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Adenoma</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

NOTE: Staining intensity takes into account both intensity of staining and the observation of staining in >50% of the tumor cells where negative, no detectable signal; 1⁺, weak signal; 2⁺, moderate signal; and 3⁺, strong signal.
concentrations of antibody ranging from 0.1 to 10 μg/mL, cells expressing the cynomolgus monkey cDNA were positive by flow cytometry with signal intensity equivalent to that of cells expressing human cDNA (Fig. 3B). Immunoblotting of a series of human NaPi2b fragments containing single amino acid substitutions revealed a loss of reactivity upon substitution of a leucine with a tyrosine at position 327 (L327Y, Fig. 3C). This replacement reflects the corresponding residue contained in rat NaPi2b and is consistent with the lack of recognition of rat NaPi2b by our antibody. In contrast, reactivity was largely retained with the N335T mutant, which reflects the single alternation in the cynomolgus monkey sequence. Binding affinity of anti-NaPi2b antibody to human and cynomolgus monkey NaPi2b was comparable by radioligand cell-binding assays, with $K_d$ values of approximately 10.19 ± 0.74 and 8.42 ± 0.81 nmol/L, respectively. Comparable expression of NaPi2b in human and cynomolgus monkey pulmonary bronchus and alveoli was demonstrated by IHC staining of lung tissues with anti-NaPi2b (Fig. 3D).

**Anti-NaPi2b ADC is internalized effectively and displays strong in vitro activity**

The potent cytotoxic compound MMAE was conjugated to anti-NaPi2b by using a linker containing the protease-sensitive vc dipeptide (22). The resulting anti-NaPi2b ADC bound to live cells as effectively as the unconjugated antibody as determined by flow cytometry (data not shown). Following cell-surface binding, the activation of the ADC requires efficient internalization and trafficking to degradative lysosomal vesicles. We monitored the uptake and internalization of anti-NaPi2b ADC following a 20-hour incubation with live cells that were then fixed and permeabilized for immunofluorescent detection. Localization of lysosomes was determined using a mouse IgG antibody against LAMP1 and a fluorescent secondary antibody against mouse IgG. Anti-NaPi2b ADC was readily detected in LAMP1-positive vesicles in 293 cells overexpressing NaPi2b but not in the 293 vector control cell line (Fig. 4A). Uptake and trafficking to LAMP1-positive vesicles was also observed in ovarian cancer cell lines OVCAR3 and Igrov1, which expressed NaPi2b endogenously (Fig. 4B). We also tested the NCSLC cell line NCI-H441; however, the low level of endogenous NaPi2b expressed by this cell line precluded its detection in LAMP1-positive vesicles. These results demonstrate the delivery of anti-NaPi2b ADC to lysosomes where MMAE or MMAE-containing catabolites may be released to exert cytotoxicity, consistent with the proposed mechanism of action of ADCs (29).

To evaluate target-dependent cell killing with anti-NaPi2b ADC, the 293 cell line that overexpressed NaPi2b and the matched vector control cell line were treated with increasing amounts of ADC. No loss of viability was observed for the control cell line at concentrations of ADC exceeding 10 μg/mL, whereas the 293-NaPi2b cell line exhibited a dose-dependent reduction in viability, with an IC$_{50}$ of approximately 0.15 μg/mL (Fig. 5A). We next tested the cancer cell lines that expressed NaPi2b endogenously. Strong cell-surface expression of NaPi2b was observed with the ovarian cancer cell lines OVCAR3 and Igrov1, whereas expression in the NCI-H441 lung cancer cell line was significantly lower (Fig. 5B). Although comparable amounts of NaPi2b were expressed on the cell surface of the OVCAR3 and Igrov1 cell lines, the OVCAR3 appeared to be more sensitive to the ADC (Fig. 5C). Commensurate with its low level of NaPi2b expression, the NCI-H441 lung cancer cell line was far less sensitive to the ADC relative to the NCI-H441 lung cancer cell line.
to the ovarian cells. For all 3 of the cell lines, the control ADC was ineffective at concentrations below 5 μg/mL.

**Anti-NaPi2b ADC inhibits tumor growth and causes tumor regression in animal models**

Three xenograft tumor models, OVCAR3-X2.1 and Igrov1 derived from human ovarian cancer and NCI-H441 derived from human lung adenocarcinoma epithelial cell, were used in the in vivo efficacy studies. Animals received a single intravenous injection of vehicle control, anti-NaPi2b ADC, or dose-matching ADC control at the indicated dose levels. All 3 models demonstrated specific and dose-dependent inhibitory activity compared with the vehicle control (Fig. 6). Despite the apparent lower level of antibody uptake and cell-surface expression observed in vitro (Figs. 4B and 5B), sensitivity of the NCI-H441 tumors was similar to that of OVCAR3 X2.1 model. This could be explained by the robust and comparable levels of NaPi2b detected by staining tumor sections obtained from the NCI-H441 and OVCAR3 × 2.1 models (Fig. 6 insets). However, the Igrov1 tumor expressed levels of NaPi2b similar to the other 2 models but was relatively less sensitive to the ADC, indicating that factors in addition to ADC target level contribute to the response. At the same dose level, anti-NaPi2b ADC showed a substantially greater tumor growth inhibition than the ADC control, indicating targeted antitumor activity of anti-NaPi2b ADC. These results suggest that efficacy can be achieved with appropriate dosing of anti-NaPi2b ADC in a variety of tumor xenograft models.
Anti-NaPi2b ADC exhibits linear pharmacokinetics in rodent and nonhuman primate studies

To further understand the relationship between systemic exposure and antitumor efficacy, the pharmacokinetic property of anti-NaPi2b ADC in SCID-beige mice bearing OVCAR3-X2.1 tumors were evaluated. Consistent with the in vivo efficacy studies discussed earlier, dose-dependent efficacy measured by tumor volume was observed for animals administrated with anti-NaPi2b ADC at 1, 3, and 6 mg/kg (data not shown). Pharmacokinetic analysis of total antibody, which includes both the anti-NaPi2b ADC and the unconjugated antibody, demonstrated linearity over doses of 1 to 6 mg/kg. In tumor-bearing mice dosed with anti-NaPi2b ADC at 0.5 and 5 mg/kg, the clearance ranged from 9.09 to 10.8 mL/kg/d and was comparable with the range of 8.94 to 9.13 mL/kg/d in non—tumor-bearing mice dosed identically. This indicates that antigen expressed on tumors did not impact the pharmacokinetics (Fig. 7 and Table 2).

Figure 4.
Cell internalization of NaPi2b antibody. Live cells were incubated with 2 μg/mL of anti-NaPi2b antibody for 2 hours at 37°C and then fixed and stained with antibody to LAMP1 to localize lysosomes. Anti-NaPi2b was detected with Cy3-labeled secondary antibody (red) and antibody to LAMP1 with Alex488-labeled secondary antibody (green). Only the merged images are presented in color (color overlay). A, 293 cells stably expressing the SLC34A cDNA (bottom) or vector control (top). B, cancer cell lines endogenously expressing the SLC34A gene.

Figure 5.
Inhibition of in vitro cell proliferation by anti-NaPi2b ADC. Live cells were incubated with increasing concentrations of the anti-NaPi2b ADC, and viability was assessed by the Cell Titer-Glo assay. A, 293 cells stably expressing the SLC34A cDNA or vector control. B, relative intensity of NaPi2b cell-surface expression measured as mean fluorescent intensity (MFI) on cancer cell lines endogenously expressing SLC34A by flow cytometry. C, viability of cancer cells incubated with increasing concentrations of anti-NaPi2b ADC (solid lines) or a non-target-specific control anti-gD ADC (dashed lines).
Anti-NaPi2b ADC was also tested in rats and cynomolgus monkeys to fully characterize the pharmacokinetics in preclinical species. The clearance of total antibody was dose-independent after a single intravenous administration in rats [CL, 21.2 / $C_6$ 5.21 and 15.9 ($n = 2$) mL/kg/d for 0.5 and 5 mg/kg, respectively] or cynomolgus monkeys (CL, 10.8 / $C_6$ 0.804 and 13.8 / $C_6$ 2.76 mL/kg/d for 0.3 and 1 mg/kg, respectively) at the dose range studied. This suggests that the presence of cross-reactive antigen in the monkeys had no apparent impact on the clearance (Fig. 7 and Table 2).

Anti-NaPi2b ADC was well-tolerated in rats and monkeys. The major effects of anti-NaPi2b ADC included bone marrow toxicity in rats and monkeys, and liver and testicular toxicity in rats. The observed bone marrow toxicity led to the death of...
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### Discussion

Advances in ADC technology have resulted in encouraging responses in recent clinical trials (30). The development of more potent drugs and linkers with enhanced stability has greatly improved the prospects for this approach. This has coincided with advances in high-throughput technologies, enabling the identification of highly specific cell-surface antigens expressed on tumor cells. Our gene expression analysis, in which thousands of human tumor and normal tissue samples were represented, revealed overexpression of NaPi2b mRNA in human lung, ovarian, and thyroid cancers. Therefore, we sought to target NaPi2b with therapeutic antibodies.

Our selection of the anti-NaPi2b mAb for drug conjugation was based on high-affinity binding, cross-species reactivity, and a high rate of cellular internalization. When conjugated with MMAE through the vc peptide linker, anti-NaPi2b ADC exhibited selective and high-affinity binding to human and cynomolgus monkey NaPi2b and showed potent and selective inhibition of cell proliferation in NaPi2b-expressing cells in vitro. In vivo studies confirmed that anti-NaPi2b ADC inhibited growth of NaPi2b-expressing human ovarian and non-small cell lung tumor xenografts models with single doses of anti-NaPi2b ADC ranging from 3 to 24 mg/kg.

Despite high levels of expression in normal lung of nonhuman primate, the cross-reactive ADC exhibited an acceptable safety profile with a dose-limiting toxicity unrelated to normal tissue expression. Our safety study concluded that anti-NaPi2b was well-tolerated in rats up to 12 mg/kg and in monkeys up to 3 mg/kg. The slow proliferative nature of normal pneumocytes, together with the antiproliferative mechanism of MMAE, likely mitigates the potential liability of high NaPi2b expression in normal lung tissue. Overall, our preclinical results suggest that the ADC targeting NaPi2b provides a promise of an effective new therapy for the treatment of NSCLC and ovarian cancer and is currently undergoing clinical developments.

The pharmacokinetics of anti-NaPi2b ADC were fully characterized and demonstrated linearity in all the preclinical species tested, suggesting that NaPi2b expression in normal tissues in cynomolgus monkeys as a binding species or in tumor-bearing mice did not show any apparent impact on pharmacokinetics.

The MMAE-conjugated anti-NaPi2b ADC has specific antiproliferative activity in cancer cells in vitro and antitumor activity in xenograft cancer models expressing NaPi2b. Conjugation of MMAE to an anti-NaPi2b mAb improved the tolerability and widened the therapeutic window of MMAE in animal studies.

These properties provide the rationale for the selective antitumor activity of anti-NaPi2b ADC against NaPi2b-expressing tumors. IHC staining in normal human and cynomolgus monkey tissues was generally consistent and revealed congruous staining in the epithelial compartments of several tissues including lung, bronchus, and kidney tissues (data not shown). Rats, a nonbinding species for anti-NaPi2b ADC, were included in the safety

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose level,* mg/kg</th>
<th>C1, mL/d/kg</th>
<th>t1/2, days</th>
<th>Vc, mL/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing mouse</td>
<td>1 (n = 3)</td>
<td>10.8</td>
<td>6.22</td>
<td>50.3</td>
</tr>
<tr>
<td></td>
<td>3 (n = 3)</td>
<td>10.8</td>
<td>7.68</td>
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<td></td>
<td>6 (n = 3)</td>
<td>9.09</td>
<td>10.5</td>
<td>51.1</td>
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<tr>
<td>Non–tumor-bearing mouse</td>
<td>0.5 (n = 3)</td>
<td>8.94</td>
<td>12.6</td>
<td>54.8</td>
</tr>
<tr>
<td></td>
<td>5 (n = 3)</td>
<td>9.13</td>
<td>11.5</td>
<td>54.4</td>
</tr>
<tr>
<td>Rat</td>
<td>0.5 (n = 3)</td>
<td>21.2 ± 5.21</td>
<td>8.43 ± 3.39</td>
<td>50.6 ± 3.40</td>
</tr>
<tr>
<td></td>
<td>5 (n = 2)</td>
<td>15.9</td>
<td>12.3</td>
<td>51.6</td>
</tr>
<tr>
<td>Monkey</td>
<td>0.3 (n = 4)</td>
<td>10.8 ± 0.804</td>
<td>8.42 ± 1.19</td>
<td>32.5 ± 2.34</td>
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<tr>
<td></td>
<td>1 (n = 4)</td>
<td>13.8 ± 2.76</td>
<td>8.26 ± 2.15</td>
<td>38.1 ± 1.77</td>
</tr>
</tbody>
</table>

NOTE: Determined using a two-compartmental model.

Abbreviations: CL, clearance; Vc, volume of distribution at central compartment; t1/2, terminal half-life.

*Number of animals per timepoint.
evaluation to evaluate the antigen-independent toxicity of the conjugate. In normal rats and monkeys, toxicologically significant effects were consistent with the pharmacology of MMAE with the most sensitive tissues including bone marrow, liver, and testes. In summary, anti-NaPi2b ADC has demonstrated significant efficacy in nonclinical NaPi2b-expressing xenograft models and exhibited acceptable safety profiles in animal studies, suggesting that anti-NaPi2b ADC may be a promising therapeutic for the treatment of ovarian cancer and NSCLC. With its highly promising results in preclinical studies, anti-NaPi2b ADC has entered into clinical development in both ovarian and lung cancers and demonstrated early evidence of clinical benefit in patients (31).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: K. Lin, K. Xu

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Preclinical Development of NaPi2b Antibody–Drug Conjugate

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