AGS16F is a Novel Antibody Drug Conjugate Directed Against ENPP3 for the Treatment of Renal Cell Carcinoma


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ABSTRACT

Purpose: New cancer-specific antigens are required for the design of novel antibody drug conjugates (ADC) that deliver tumor-specific and highly potent cytotoxic therapy.

Experimental Design: Suppression subtractive hybridization identified ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3 or CD203c) as a potential human cancer-specific antigen. Antibodies targeting the extracellular domain of human ENPP3 were produced and selected for specific binding to ENPP3. Expression of ENPP3 in normal and cancer tissue specimens was evaluated by immunohistochemistry (IHC). ADC comprising anti-ENPP3 Ab conjugated with maleimidocaproyl monomethyl auristatin F via a non-cleavable linker (mcMMAF) were selected for therapeutic potential using binding and internalization assays, cytotoxicity assays, and tumor growth inhibition in mouse xenograft models. Pharmacodynamic markers were evaluated by IHC in tissues and ELISA in blood.

Results: ENPP3 was highly expressed in clear cell renal cell carcinoma (ccRCC): 92.3% of samples were positive and 83.9% showed high expression. By contrast, expression was negligible in normal tissues examined, with the exception of the kidney. High expression was less frequent in papillary renal cell carcinomas (prCC) and hepatocellular carcinomas (HCC) samples. AGS16F, an anti-ENPP3 antibody-mcMMAF conjugate, inhibited tumor growth in three different RCC xenograft models. AGS16F localized to tumors, formed the active metabolite Cys-mcMMAF, induced cell cycle arrest and apoptosis, and increased blood levels of caspase-cleaved cytokeratin-18, a marker of epithelial cell death.

Conclusions: AGS16F is a promising new therapeutic option for patients with RCC and is currently being evaluated in a Phase I clinical trial.
TRANSLATIONAL RELEVANCE

Antibody-drug conjugates (ADC) represent a new therapeutic modality for patients with cancer. ADC against a tumor-specific antigen offers targeted delivery of highly active cytotoxic agents specifically to tumors, while sparing normal tissue. Herein we report that ENPP3 is such a target for renal cell carcinoma (RCC). In vitro and in vivo studies show that AGS16F (an ADC comprising a monoclonal antibody against human ENPP3 conjugated to the microtubule-disrupting agent monomethyl auristatin F [MMAF] via the linker maleimidocaproyl (mc), is internalized and rapidly trafficked to lysosomes, allowing generation of the active drug metabolite Cys-mcMMAF. AGS16F showed potent anti-tumor activity in 3 animal models of RCC and the mechanism of action was ascertained. We also identified an early biomarker for anti-tumor activity in blood. These findings indicate that AGS16F is a potential therapeutic alternative warranting clinical investigation for the treatment of RCC.
INTRODUCTION

The goal of an antibody-drug conjugate (ADC) as an anti-cancer therapeutic is to deliver a highly effective cytotoxic agent specifically to the tumor, while sparing normal tissue, thereby effecting clinically meaningful responses in patients. The specificity of an ADC arises from the antibody component, which binds an antigen that ideally, is highly expressed in tumor cells and minimally or not expressed elsewhere (1-4).

The first ADC approved by the United States (US) Food and Drug Administration (FDA), gemtuzumab ozogamicin (Mylotarg®; Wyeth/Pfizer) targeted CD33 using calicheamicin as the payload and was indicated for the treatment of acute myelogenous leukemia (5). However, Mylotarg was withdrawn from the US market due to inadequate clinical activity and considerable toxicity. More recently, brentuximab vedotin (ADCETRIS®; Seattle Genetics) (6), an anti-CD30-auristatin conjugate, was approved for the treatment of relapsed systemic anaplastic large cell lymphoma and Hodgkin’s lymphoma, and trastuzumab emtansine (KADCYLA®; Genentech) (7), which comprises an anti-HER2 antibody conjugated to the maytansine derivative DM1, was approved for the treatment of advanced Her-2 positive breast cancer. Currently more than 30 ADCs are in clinical development (8).

Herein, we describe a novel ADC (AGS16F) that targets an antigen largely unknown in oncology, ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3; also known as CD203c). ENPP3 is a basophil activation marker and is one of 7 members of a family of cell-surface ectonucleotide pyrophosphatases/phosphodiesterases (9-14). The extracellular domain of ENPP3, a type II transmembrane protein, comprises a nuclease-like domain, a catalytic domain and a somatomedin B-like domain (including an RGD integrin-binding motif). ENPP3 hydrolyzes extracellular pyrophosphate or phosphodiester bonds (12, 15) as well as intracellular nucleotides involved in glycosyltransferase activity (16). Expression of ENPP3 has been reported in neoplastic mast cells in the context of mastocytosis (17, 18), in acute basophilic leukemia (19), in some colon cancer samples (20), and in neoplastic cells of the bile duct (21). However, a functional role for ENPP3 in cancer has not been elucidated.

The current report demonstrates that ENPP3 is highly expressed in renal cell carcinoma (RCC), but has restricted expression in normal tissues, with the exception of the kidney. We also show
that an ADC targeting ENPP3 is rapidly internalized and trafficked to lysosomes, and has significant anti-tumor activity. Thus, an ENPP3-targeted ADC is a promising therapeutic agent for the treatment of renal and other cancers that show high expression of ENPP3.
Materials and Methods

Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) was carried out with the PCR-Select cDNA Subtraction Kit (Clontech; Palo Alto, CA) using a cDNA library generated from tumor specimens from patients with RCC and a cDNA library generated from 9 normal tissues (stomach, skeletal muscle, lung, brain, liver, kidney, pancreas, small intestine and heart).

RT-PCR expression analysis

First strand cDNAs were generated using SuperScript® reverse transcriptase (Invitrogen) and Oligo (dT)12-18 priming according to manufacturer's protocol. cDNAs were normalized using GAPDH primers. To determine expression levels of ENPP3, 5µl of normalized cDNA obtained from a panel of normal tissues and patient cancer specimens were amplified using ENPP3 specific primers. PCR products were analyzed by agarose gel electrophoresis and signal quantitated by densitometry.

Generation of recombinant proteins

To express the extracellular domains (ECD) of human ENPP3, its closest family members (ENPP1 and ENPP2) and its cynomolgus monkey, rat and mouse orthologs, cDNA was amplified from normal tissues, fused to Myc/His tag followed by a stop codon and cloned into pAPtag-5 mammalian expression vector (GenHunter Corporation, Nashville, TN). Constructs were transfected into 293T cells and recombinant proteins were purified from culture supernatant on Ni-NTA His-Bind column (Novagen).

Antibody generation

Anti-human ENPP3 monoclonal antibodies were generated using standard hybridoma technology in the genetically engineered Xenomouse™ (Amgen, Inc.) (22) following immunization of mice with the human ENPP3 ECD (amino acids 48 to 875). The variable heavy chain and kappa light chains of selected antibodies were sequenced from the corresponding hybridoma and cloned into the pEE12.4 mammalian cell expression vector (Lonza; Wayne, PA). The plasmid was transfected into Chinese hamster ovary CHOK1SV host cells (Lonza).
Conjugation of Antibodies and Cytotoxic Agents

Purified antibodies were conjugated with monomethyl auristatin E (MMAE) via the cleavable linker maleimidocaproylvaline-citrulline-p-aminobenzyloxycarbonyl (vc) or to monomethyl auristatin F (MMAF) via the linker maleimidocaproyl (mc) as previously described (licensed from Seattle Genetics, Inc.) (23, 24). The drug to antibody ratio was approximately 4:1. The anti-ENPP3 IgG2 antibody AGS16-7.8 produced from the hybridoma or CHO cells was conjugated to mcMMAF to produce AGS-16M8F or AGS-16C3F, respectively.

Immunohistochemistry

A mouse anti-ENPP3 monoclonal antibody (M16-48(4)29.1.1.1) was generated by immunizing Balb/c mice with the ENPP3 ECD. Sections were stained in a Biogenex i6000 autostainer (Biogenex, San Ramon, CA). Antigen retrieval was carried out using proteinase K (Dako, Carpenteria, CA). M16-48(4)29.1.1 or MOPC21 (negative control), both at 5 μg/mL, were applied to the sections and incubated for 1 hour at room temperature. ENPP3 was visualized using the Biogenex Super Sensitive Polymer-horseradish peroxidase IHC detection kit with 3,3'-diaminobenzidine (DAB) as the chromogen.

Cytotoxicity assay

KU812 basophilic leukemia cells (ATCC, Manassas, VA) were incubated with serially diluted ADC or isotype-control ADC at 37 °C in a CO2 incubator. Cell viability was measured after 5 to 7 days using the AlamarBlue® reagent (Life Technologies, Carlsbad, CA). Percent survival was calculated as the number of live cells in treated wells compared to the number of live cells in untreated control wells. The IC50 was derived from the survival curve by Sigmoid-E\textsubscript{max} non-linear regression analysis using GraphPad Prism (GraphPad, San Diego, CA).

In vivo studies using xenografted tumor models

UG-K3 (Agensys, patient derived ccRCC xenograft), SKRC-01 (25), RXF-393 (26) and HepG2 (ATCC), were examined. All experimental protocols were approved by Agensys’ Institutional Animal Care and Use Committee (IACUC). For subcutaneous models, viable cells (UG-K3, 1.5 x 10^6; RXF393, 0.5 x 10^6; SKRC01, 0.8 x 10^6, or HepG2, 4 x 10^6) were implanted into male SCID
mice. When tumors reached a size of approximately 100 to 200 mm$^3$, animals were randomized to receive either single or multiple doses of ADC, non-conjugated native antibody (AGS16-7.8) or vehicle control administered by intravenous (i.v.) injection, as detailed in the Figure Legends. Tumor size was measured every 3 to 4 days using calipers.

For the orthotopic model, 1.5 million of UG-K3 viable cells were surgically implanted into the kidneys of male SCID mice. The tumors were allowed to grow for 7 days at which time animals were randomized to different groups (n= 10 per group) and treated, q4dx4. At the end of the study (day 41), the animals were sacrificed and the right and left kidneys weighed. The tumor weights were the weight of the tumor-bearing right kidney minus the weight of the tumor-free contralateral kidney.

Statistical analysis of the tumor volume data for the last day before animal sacrifice was performed using the Kruskal-Wallis test. The implementation of the Kruskal-Wallis test was carried out using the parametric ANOVA F-test on the ranks of the data. Pairwise comparisons were made using the Tukey-Kramer method (2-sided).

**Detection and quantitation of Cys-mcMMAF in vivo**

Mice bearing 150 mm$^3$ UG-K3 xenografted tumors were treated with AGS16F (10 mg/kg) or AGS16-7.8 (9.5 mg/kg) by iv injection (day 0). Tumors were harvested 1, 3 and 5 days later, lysed and the internal standard $[^{13}\text{C}]$Cysteine-mcMMAF (M+7) (Seattle Genetics; Bothell, Washington) was added immediately prior to homogenization to yield a final concentration of 25 nM. The organic solvent was evaporated and the contents re-dissolved in 20% aqueous acetonitrile, 5% formic acid. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was used to detect and quantify the Cys-mcMMAF metabolite relative to the heavy Cys-MMAF internal standard.

**Internalization studies**

KU812 cells were incubated with 10 µg/mL AGS16F for 30 minutes at 4°C, 30 minutes at 37°C, or 1 hour at 37°C. Cells were then washed, fixed, permeabilized and non-specific labeling was blocked by incubation in PBS and 10% normal goat serum. Cell surface (non-permeabilized cells) and internalized AGS16F were visualized by an Alexa Fluor® 488-labeled goat anti-human
IgG (Invitrogen, Life Technologies, Grand Island, NY). Early endosomes were visualized with early endosome antigen 1 (EEA1) (Cell Signaling Technology; Danvers, Massachusetts). Lysosomes were visualized by staining with antibody directed against the lysosome-associated membrane protein 1 (LAMP1; Cell Signaling Technology) and a secondary antibody, Alexa Fluor® 568-labeled goat anti-rabbit IgG (Invitrogen). Nuclei were visualized with TOPRO®-3 Iodide (Invitrogen). Laser confocal image sections were acquired using a Leica TCS SP5-II).

**Binding and degranulation studies with basophils**

F(ab’)2 fragments were generated by digestion with pepsin. The fragments were biotinylated using E-Z-Link Sulfo-NHS-LC-Biotin (Thermo Scientific). Normal donor human and cynomolgus monkey fresh whole blood samples were obtained from Bioreclamation (Baltimore, MD). Goat-anti-hIgE antibody (Invitrogen) or PBS was added to human or cynomolgus monkey whole blood (1 µg/mL) and incubated at 37 °C for 15 minutes. Next, triplicate sample of blood cells were washed and incubated with the F(ab’)2 fragments to a final concentration of 1 µg/mL and incubated at 4 °C for 1.5 hours, washed, stained with anti-hIgE-FITC (Invitrogen) and Streptavidin-PE (Jackson Immunoresearch, West Grove, PA), and analyzed in a FACScan (Beckton Dickenson).

For degranulation studies, blood samples (Bioservices, LLC, Reseda, CA) were incubated with AGS16F or anti-hIgE (Invitrogen) and histamine release was analyzed by ELISA according to manufacturer’s protocol (Immunotech). Any response from a test substance greater than 5% of total histamine (after subtraction of spontaneous release values) was regarded as positive (17).

**Mechanism of action and biomarker studies**

Single dose of AGS16F or control ADC at 1 or 5 mg/kg (n= 4 per group) were administered by iv injection to mice bearing subcutaneous UG-K3 tumors (size ~ 350 mm³). Tumors and blood were collected at the indicated times. Caspase-cleaved human cytokeratin-18 (ccCK18) in mouse serum was detected by ELISA (Peviva, Bromma, Sweden). Apoptosis and tumor cell arrest were detected by IHC with an antibody against cleaved poly(ADP-ribose) polymerase 1 (PARP-1; Epitomics; Burlingame, CA) and an antibody against phospho-histone H3 (PPH3; EMD Millipore; Billerica, Massachusetts), respectively. Quantitation of IHC–stained slides was
performed using Aperio Scan Scope CS software (Leica, Vista, CA). Localization of AGS16F was detected with the anti-MMAE/F antibody (SG15-22) (Agensys, Santa Monica, CA).

RESULTS

ENPP3 target identification and expression in normal tissues and cancer

SSH showed that ENPP3 mRNA was differentially expressed in tumor samples from patients with kidney cancer and normal tissues. Highest levels of mRNA for ENPP3 were confirmed by qPCR in several tumor specimens from patients with kidney cancer, while expression was minimal in normal tissues.

IHC studies using an anti-human ENPP3 antibody showed moderate to strong staining in a subset of tubules in the kidney cortex (Figure 1A and B). Low level of staining was also observed in the epithelium of the fallopian tube, and in a subset of cells within gastrointestinal tract epithelium (Figure 1A), as well as in uterine endometrium, parotid gland and the cortex of the thymus (data not shown). Limited expression was observed in epithelium of the prostate, breast and adrenal gland (data not shown).

RCC, HCC and colon cancer, tumor types positive for ENPP3 mRNA by qPCR, were investigated by IHC. High expression was detected in clear cell renal cell carcinoma (ccRCC; Figure 1C), and lower intensity expression in papillary renal cell carcinoma (prCC; Figure 1D) and hepatocellular carcinoma (HCC; Figure 1E). Quantification of expression by H-scores for TMAs and individual tumor tissue sections from RCC, HCC and colon cancer are shown in Supplemental Table 1. Results showed that ccRCC had the strongest and most frequent expression, with 267/285 (93.7.3%) tumor TMAs positive for ENPP3 and 239 (83.9%) showing high expression (H-score ≥200). High expression in prCC was less frequent than in ccRCC and differed somewhat between TMA samples and individual FFPE specimens, albeit with an overall smaller sample size. Among 25 prCC tumor TMAs, 60% expressed ENPP3: 24% had high expression and 16% had moderate expression (40% moderate to high expression). Among 14 individual prCC tumor samples, 85.7% were positive for ENPP3 expression: 14.3% had high expression and 50% had moderate expression (64.3% moderate to high expression). High expression of ENPP3 also was less frequent in HCC than in ccRCC, and as observed for prCC,
variation was observed between the HCC TMAs and individual HCC specimens (note that the number of individual samples was markedly lower than the number of TMA samples). In HCC TMAs, 65/293 (22.2%) samples were positive for ENPP3 and 2.7% had high expression, whereas in individual HCC FFPE, 20/27 (74.1%) were positive and 18.5% had high expression. In colon cancer specimens, expression of ENPP3 was observed in 10/32 (31.3%) primary tumor TMA samples and 3/21 (14.3%) metastatic TMA samples and nearly all of the tissues had low level expression. Likewise, the expression in ovarian endometrioid cancer was low. This pattern of expression provides a strong rationale for use of an ENPP3 targeted ADC for treatment of ccRCC and subsets of prCC and HCC.

Antibody development and selection of ADC platform

Fully human antibodies were generated in XenoMouse® transgenic mice following immunization with the ENPP3 ECD protein. Anti-ENPP3 antibodies were selected based on affinity and epitope specificity, as well as internalization and cross-reactivity to cynomolagus monkey ENPP3. Selected antibodies produced in hybridoma cells were conjugated to either vcMMAE or to mcMMAF and tested for in vitro cytotoxicity against KU812 cells, a basophilic leukemia line which expresses endogenous ENPP3 (11).

Among all ADC tested, those comprising Abs AGS16-7.8 and AGS16-9.69 conjugated to either vcMMAE or to mcMMAF showed the highest cytotoxic potency in vitro (Figure 2A), with IC$_{50}$ ranging from 0.06 to 0.12 nM (Figure 2A inset). Therefore, these ADCs were selected for testing in the ENPP3 positive, UG-K3 xenograft model of a patient-derived ccRCC.

In Vivo Studies

As shown in Figure 2B (inset), ENPP3 expression is high and homogenous in xenografted UG-K3 tumors. Treatment with ADC on days 0, 3, 7 and 10 comprising AGS16-9.69 or AGS16-7.8 conjugated to mcMMAF at 3 mg/kg effectively inhibited tumor growth for up to 50 days after the first dose, while ADCs conjugated to the cleavable vcMMAE did not (Figure 2B). The levels of AGS16-7.8 conjugated to mcMMAF or vcMMAE in blood two days after last dose were 75±10.8 and 65±9.5 μg/ml (average, STD) respectively, ruling out different exposure as a reason for the difference in activity. While AGS16-9.69 and AGS16-7.8 conjugated to
mcMMAF had similar anti-tumor activity, the affinity of AGS16-9.69 for cynomolgus monkey ENPP3 was not considered optimal for future nonclinical studies (data not shown) and AGS16-7.8 conjugated to mcMMAF was selected for further development.

Initially, AGS16-7.8–mcMMAF ADC was produced in hybridoma cells (AGS-16M8F) and later in CHO cells (AGS-16C3F). Both were found to be comparable and used during the Phase I clinical trial having similar abilities to a) bind to the ENPP3 ECD, b) bind to KU812 cells, c) induce cytotoxicity \textit{in vitro}, and d) inhibit tumor growth \textit{in vivo} (\textit{Supplemental Table 2}). Therefore, in the remainder of this manuscript, both the hybridoma and CHO-derived ADC will be referred to as AGS16F.

**Biological Characterization of AGS16F**

**Binding and cytotoxicity of AGS16F and its active metabolite**

ADCs engineered with mcMMAF are internalized and catabolized to yield the active metabolite Cys-mcMMAF (24). The IC\textsubscript{50} for of AGS16F and Cys-mc-MMAF against KU812 cells were 0.12 nM and 113 nM. Thus, when delivered extracellularly, the parent ADC has approximately 1000-fold higher potency than the active metabolite. These findings demonstrate the intracellular targeting capability of the antibody and poor bystander effect of the active metabolite, consistent with previous reports showing poor membrane permeability mainly due to the charged C-terminal phenylalanine residue in MMAF (24).

**Cross-reactivity with other ENPP family members and other species**

The sequence homologies of ENPP3 with ENPP1 and ENPP2 are 53% and 44%, respectively, while homologies with other ENPP family members range from 15% to 18%. Therefore, the ability of AGS16-7.8 to recognize the ECD of ENPP1 and ENPP2 was examined by ELISA using the corresponding extracellular domains. ELISA binding assays showed strong binding of AGS16-7.8 to the ENPP3 ECD, but no binding to the ECD of ENPP1 or ENPP2 (\textit{Supplemental Figure 1A-C}).

AGS16-7.8 and AGS16F were able to bind to cells expressing cynomolgus monkey ENPP3 with K\textsubscript{d} of 0.30 and 1.1 nM, respectively. These binding data compare favourably (~ 4-fold lower) to
the affinity for human ENPP3 in KU812 cells, Kd= 0.08 and 0.25 nM respectively. Despite high homology of human ENPP3 with rat or mouse ENPP3 (87%), in vitro testing showed negligible binding to ENPP3 from both species (data not shown).

**Effect of AGS16-7.8 on ENPP3 enzymatic activity**

The potential effect of AGS16-7.8 on the enzymatic activity of ENPP3 was investigated using a purified ENPP3 ECD fragment and pNP-TMP as a substrate. As shown in *Supplemental Figure* 2, AGS16-7.8 does not inhibit the phosphodiesterase activity of purified ENPP3.

**Epitope mapping**

The location of the epitope for AGS16-7.8 was analyzed using various His-tagged fragments of the ENPP3 ECD (*see Supplemental Figure 3A*): 1) ENPP3 full length ECD (amino acids 46 to 875); 2) ENPP3 ECD fragment 2, which includes the somatomedin-B-like domains (amino acids 46 to 157); 3) ENPP3 ECD fragment 3, comprising the catalytic domain (amino acids 157-558); and 4) ENPP3 ECD fragment 4, comprising the catalytic and nuclease domains (amino acids 157-875). AGS16-7.8 was able to bind to fragments 1 and 4, but not to fragments 2 and 3 (*Supplemental Figure 3B*), suggesting that the binding epitope may reside within residues 558 to 875, in a region encompassing the nuclease domain. Binding of AGS16-7.8 to this region is consistent with the lack of inhibition of the enzymatic activity of purified ENPP3 by AGS16-7.8.

**AGS16F Internalization Studies**

KU812 cells incubated at 4°C to prevent internalization (*Figure 3 A-C*) showed distinct cell surface localization for AGS16F, with minimal evidence of internalization. Lysosomes in cells maintained at 4°C appeared as discrete cytosolic aggregates; (LAMP1, red fluorescence in *Figure 3 B and C*). After incubation at 37°C for either 30 minutes or 1 hour, surface membrane localization of AGS16F was dramatically reduced and the predominant distribution of the ADC appeared in discrete intracellular aggregates throughout the cytosol (green fluorescence in *Figure 3 D-F and G-I*). The aggregates of intracellular AGS16F co-localized with LAMP1 (yellow fluorescence in *Figure 3 F and I*), suggesting that the AGS16F ADC had trafficked to the lysosomes and was targeted for subsequent hydrolysis within 30 minutes of incubation with KU812 cells. Co-localization of cytosolic AGS16F with the early endosome marker EEA1 was
also observed in cells incubated at 37°C for either 30 minutes or 1 hour (data not shown). Altogether, the data indicate that AGS16F is internalized and co-localized to lysosomes as early as 30 minutes after exposure to ENPP3-expressing cells.

**AGS16F Binds to Human and Cynomolgus Monkey Blood Basophils**

ENPP3 is expressed on the surface of human basophils and mast cells and expression is rapidly and dramatically up-regulated after crosslinking of FcεRI or stimulation with anti-IgE antibodies (27, 28). These observations raised the concern that AGS16F could induce degranulation of basophils/mast cells. To address this issue, the binding of AGS16-7.8 and AGS16F to human and cynomolgus monkey basophils in whole blood were examined. F(ab)_2 fragments were used to avoid interactions with Fc receptors in basophils. As shown in Table 1, small populations of 0.66% and 0.75% of IgE^+ cells (basophils) were stained with AGS16-7.8 and AGS16F, respectively. Binding of AGS16F and AGS16-7.8 was increased 2.1- and 2.8-fold, respectively, after stimulation of cells with an anti-hIgE antibody. Further, the percentage of IgE^+/ENPP3^+ cells increased 1.2-fold upon stimulation by anti-hIgE antibody, consistent with published observations (25). Similar results were obtained with blood from cynomolgus monkeys. These data indicate that AGS16F binds to ENPP3 present in whole blood basophils from both humans and cynomolgus monkeys.

The potential effect of AGS16-7.8 and AGS16F on basophil degranulation and histamine release in human blood was investigated. After subtraction of spontaneous release, the positive control (anti-hIgE) should induce release of more than 5% of the total histamine content in 80-90% of donors (29). Results showed that anti-hIgE treatment, but not AGS16F up to 100 µg/mL (67 nM), induced release of 5.7% to 52.1% of total histamine from the blood samples of 5 different donors. Therefore, AGS16F does not trigger histamine release from whole blood basophils in vitro.

**In vivo studies of AGS16F**

The pharmacokinetics of AGS16F in mice was examined in male ICR SCID non-tumor bearing mice (Supplemental Figure 4). The terminal half-life for the ADC and total antibody was 11.9 days and 14.8 days respectively. The degree on deconjugation (ADC Vs total antibody) based on the calculated Area-under-the-curve (AUC) analysis was approximately 32%.
The effect of AGS16F on inhibition of tumor growth was determined in several studies using three different kidney cancer models and a hepatocellular carcinoma xenograft model positive for ENPP3 expression. AGS16F, but not the naked antibody, showed consistent and profound anti-tumor activity in all RCC models at different doses and schedules as seen in Fig 4. However, AGS16F did not show statistically significant antitumor activity in the HepG2 HCC model at doses and schedules which had shown anti-tumor activity in RCC models. The ENPP3 expression in the HepG2 model by IHC showed strong to moderate punctate staining of ENPP-3, similar to Fig 1E.

**Figure 4A** and **Supplemental Table 2** shows a dose titration of AGS16F in the UG-K3 model demonstrating a sharp dose activity relationship. Similarly, AGS16F (3 mg/kg) inhibited the growth of the UG-K3 tumor cells when implanted orthotopically in the kidney of mice (2.3 g in control compared to 0.02 g in treated, p<0.001).

AGS16F as a single dose was also effective against SKRC-01 and RXF-393 ccRCC xenografts, which are ENPP3 positive (insets, **Figure 4**). Tumor growth of SKRC-01 RCC xenografts was inhibited by 79% at day 11 after treatment with AGS16F at either 5 mg/kg or 10 mg/kg (p < 0.0001 for both comparisons; **Figure 4B**). Tumor growth of RXF-393 at day 21 was inhibited by 85% and 93% after treatment with AGS16F at 3 or 10 mg/kg, respectively (p< 0.0001 for both doses versus control ADC; **Figure 4C**). The levels of Cys-mcMMAF at different time points were measured from UG-K3 renal cancer xenograft tumors treated with a single dose of 10 mg/kg AGS16F or unconjugated AGS16-7.8 antibody control. The concentration of Cys-mcMMAF extracted from AGS16F treated tumors (n= 2) was 16.1 nM (average of 15.6 and 16.6 nM) after 24 h and 14.75 nM (average of 18.2 and 11.3 nM) after 72 h, decreasing to 5.05 nM (average of 6.0 and 4.1 nM) on day 5. Cys-mcMMAF was not detected in the AGS16-7.8 treated animals as expected. Since the *in vitro* IC_{50} for AGS16F cytotoxicity is 0.1 nM, which corresponds to 0.4 nM of Cys-mcMMAF (DAR ~ 4), the levels are ~ 40-fold higher during days 1 to 3, decreasing to ~ 12-fold higher at day 5. Thus, high levels of the active metabolite can persist for at least 5 days after dosing.

**Pharmacodynamics and Potential Biomarkers for AGS16F Effects**
To better understand the mechanism of action of AGS16F and to evaluate potential pharmacodynamic (PD) markers for clinical trials, we next investigated molecular changes induced in the tumor and in blood upon administration of AGS16F to UG-K3 tumor bearing mice.

One possible PD biomarker is the caspase cleaved form of cytokeratin-18 (ccCK18), which is formed upon epithelial cell death (30, 31). Levels of ccCK18 in serum in cancer patients have been used as an early indicator of tumor cell death in clinical trials (32). Levels of human ccCK18 in blood of UG-K3 tumor bearing mice were measured at 6, 24, 48, 72 h and 7 days after a single dose administration of AGS16F at 3 or 5 mg/kg (Fig 5A). The results showed a dose dependent increase in ccCK18 peaking at 48 h post AGS16F administration and declining to similar levels than those of control treated mice at 7 days. Tumor volume was also measured showing a decrease in tumor volume after 7 days for the AGS16F treated mice (Fig 5B). These data established an association between increased levels of ccCK18 in serum and decreased tumor volume upon AGS16F treatment of tumor bearing mice.

The tumors from this study were also investigated for changes in possible downstream markers of AGS16F activity, changes in morphology, and changes in ADC localization and ENPP3 expression. Like other microtubule disrupting agents, auristatins block cell cycle progression, which may result in increases in phospho-histone H3 (PPH3) and cleaved PARP-1 (33). As expected, levels of cleaved PARP-1 and PPH3 increased with time after AGS16F administration, reaching a peak at approximately 48 h (Figure 5C,D and Supplemental Figure 5) mirroring the increases observed for ccCK18 (Figure 5A). H&E staining of the tumors revealed changes in cell morphology (cellular atypia and polymorphism) in the AGS16F treated group after 7 days (Fig 5E) consistent with the changes in cPARP1 and PPH3. The levels of ENPP3 in the tumors did not change with treatment at 6 to 72 hours post dosing. However, an overall lowered ENPP3 staining intensity was observed after 7 days. This is likely due to the fact that cell death is more apparent at day 7 day than at earlier times. Representative images at 24 h and 7 days after dosing are shown in Figure 5F. When dosed at 1 or 5 mg/kg, dose-dependent staining of AGS16F was detected in UG-K3 tumors. Levels of staining were similar from 6 to 72 hours, decreasing at the 7 day time point (Figure 5G).
Taken together, these data suggest maximal activity of AGS16F in the tumor at around 48-72 hours decreasing sharply to approximately pre-dose levels 7 days after dosing. This is despite a long terminal half-life of 11.9 to 14.8 days (Supplemental Figure 4).

DISCUSSION

Data presented herein show for the first time that ENPP3 is highly and uniformly expressed in RCC, especially in ccRCC, and that an ADC targeting ENPP3 has profound anti-tumor activity in three different ccRCC models. However, AGS16F lacked anti-tumor activity in a HCC xenograft model tested possibly because the expression of ENPP3 is mostly focal which may be a problem for a MMAF containing ADC having minimal bystander effect. ENPP3 expression in normal tissues is minimal with the exception of normal human kidney and in activated human basophils and mast cells (27, 28). In vitro experiments showed no histamine release upon incubation of human blood with AGS16F. Moreover, there were no signs of allergic reactions to AGS16F in cynomolgus monkeys treated with doses up to 6 mg/kg/week for 4 weeks (Agensys, data on file). Likewise, no overt kidney toxicities were observed (Agensys, data on file). ENPP3 expression in activated basophils, mast cells and a few related cancer cell lines such as KU812 may support testing of AGS16F in the very rare diseases of chronic basophilic leukemia and chronic mast cell leukemia (18, 19).

The antitumor activity of some ADC arises not only from the delivery of a highly potent toxin to tumor cells, but also from the modulation of the biology of the target and by contributions of the immune system mediated by the Fc region of the antibody (34). These two potential attributes of an ADC can also increase its toxicity by damaging normal tissue in which the target is expressed. We have shown that the unconjugated antibody AGS16-7.8 did not have significant anti-tumor activity. Further, AGS16-7.8 is a fully human IgG2 which elicits a much lower effector function than an IgG1. This attribute may allow good tolerability by limiting toxicity against normal tissues that might result from IgG effector functions.

The mechanism of action of AGS16F was confirmed in the UG-K3 RCC xenograft model. First, the levels of Cys-mcMMAF, the active metabolite, in tumor-bearing mice following a single injection of AGS16F at 10 mg/kg were approximately 40-fold higher than the in vitro IC50 during the first 72 hours after dosing, and declined to approximately a level 12-fold higher at day 5.
Additionally, IHC analysis demonstrated that AGS16F was localized within the tumor by 6 hours, and the level was maintained until 72 hours, decreasing after 7 days. The tumors presented with increases in the levels of PPH3 and cleaved PARP-1 with time peaking at approximately 48 h and then returned to basal levels at 7 days. These data suggest that AGS16F localization, generation of active metabolite and the appearance of markers of anti-tumor activity have similar kinetics and that maximal activity occurs for up to 3 days, decreasing at 5 days (lower Cys-mcMMAF) with a further decline to approximately pre-dose levels at seven days. These data also suggest that to maximize activity, a once weekly or more frequent dosing may be needed despite AGS16F having a terminal half-life of approximately 12 days. Increases in the apoptotic marker ccCK18 in mouse blood were observed with similar kinetics to the intra-tumor markers of activity. This finding suggests that serial sampling of ccCK18 in blood may be a convenient early biomarker of activity of AGS16F in clinical trials (32).

Tumor targeting of highly potent chemotherapy by ADCs represents a new therapeutical modality for RCC and four ADCs besides AGS16F, all against CD70, are at different stages of development (4, 35, 36). The data presented herein identify ENPP3 as a novel target against RCC and AGS16F as a potential ADC for the treatment of patients with RCC.
Figure Legends

Figure 1. **ENPP3 expression in normal and tumor tissues.** Representative images of ENPP3 staining in FFPE of normal and tumor tissues. A) normal kidney cortex, fallopian tube, stomach, small intestine, and colon. B) higher magnification of normal kidney cortex with apical ENPP3 expression in proximal tubules; C) ccRCC; D) prCC; E) HCC. High ENPP3 expression was observed in a high proportion of ccRCC samples, but was less frequent in prCC and HCC (see Supplemental Table 1).

Figure 2. **In vitro and in vivo effects of anti-ENPP3 ADC.** A) KU812 cells were incubated for 6 days with increasing concentrations of anti-ENPP3 or isotype control Ab conjugated to vcMMAE or mcMMAF and cell viability was determined. The inset table shows drug-antibody ratios (DAR) and IC$_{50}$ values for each ADC. B) Mice bearing 100 to 150 mm$^3$ UG-K3 RCC xenografted tumors were treated with AGS16-7.8 or AGS16-9.69 conjugated to vcMMAE or mcMMAF at 3 mg/kg by iv injection on days 0, 3, 7 and 10. Data show mean ± SEM tumor volume for each group. The insert figure shows a representative image of ENPP3 expression by IHC in a UG-K3 tumor section.

Figure 3. **Internalization of AGS16F by KU812 cells.** AGS16F was incubated with KU812 cells, then fixed and stained as indicated. Samples were imaged using laser scanning confocal fluorescence microscopy: AGS16F appears in green; LAMP-1 (a marker of lysosomes) appears in red; and the nuclei are shown in blue in the 3-channel Z-stack overlay in the last column. Z-stack overlay images show single plane cross sections through horizontal plane (XY), sagittal plane (YZ), and coronal plane (XZ). Panel A-C) 30 minutes at 4 ºC (inhibit internalization); Panel D-F) 30 minutes at 37 ºC; and Panel G-I) 60 minutes at 37 ºC.

Figure 4. **AGS16F showed potent antitumor activity in RCC xenograft models.** Data are shown as mean ± SEM tumor volume for each group over time. A) Mice (N=10) bearing subcutaneous UG-K3 tumor xenografts (size ~ 200 mm$^3$) were treated with AGS16F by iv injection at doses of 0.125, 0.25 and 0.5 mg/kg, q4dx4; B) Mice bearing subcutaneous SKRC-01 cells tumor xenografts (size ~ 200 mm$^3$) were treated with single 5 or 10 mg/kg doses of AGS16F by iv injection; C) Mice bearing subcutaneous RXF-393 cells tumors xenografts (size ~ 100 mm$^3$) were treated with single 3 or 10 mg/kg doses of AGS16F by iv injection.
Figure 5. Mechanism of action of AGS16F and pharmacodynamic markers. Mice bearing subcutaneous UG-K3 tumors (~ 350 mm$^3$) were treated as indicated and biomarkers, ADC localization and tumor volume were measured at 6, 24, 48, 72 h and 7 days. A) Level of ccCK18 in serum over time. B) Mean (± SEM) tumor volume for each group over time. C) Percentage of FFPE tumor specimens positive for cleaved PARP-1 over time. D) Percentage of FFPE tumor specimens positive for PPH3 over time. E) Representative images of H&E staining of FFPE tumors at 24 h and 7 days after dosing, with arrows showing cellular atypia and polymorphism. F) Representative images of ENPP3 expression at 24 h and 7 days after dosing. G) Representative images of localization of AGS16F and control ADC in FFPE tumor samples using an antibody against MMAF at 24 h and 7 days after dosing.
REFERENCES


Figure 1

A

B

C

D

E

Kidney Cortex  Fallopian Tube  Stomach  Small Intestine  Colon

Normal kidney cortex, higher magnification

Renal clear cell carcinoma

Renal papillary cell carcinoma

Hepatocellular carcinoma
Figure 2A

![Graph showing % Survival vs ADC (ng/ml)]

<table>
<thead>
<tr>
<th>ADC</th>
<th>DAR</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS16-7.8-vcE</td>
<td>4.7</td>
<td>0.11</td>
</tr>
<tr>
<td>AGS16-7.8-mcF</td>
<td>4.3</td>
<td>0.06</td>
</tr>
<tr>
<td>AGS16-9.69-vcE</td>
<td>4.2</td>
<td>0.12</td>
</tr>
<tr>
<td>AGS16-9.69-mcF</td>
<td>4.0</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Figure 2B

![Graph showing Mean tumor volume (mm³) vs Time (Days)]

- PBS, one dose, iv
- AGS16-7.8-vcE
- AGS16-7.8-mcF
- AGS16-9.69-vcE
- AGS16-9.69-mcF

U937-K3
Figure 3

A AGS16C3F
B LAMP1
C Z-stack Overlay

30 minutes - 4°C

D AGS16C3F
E LAMP1
F Z-stack Overlay

30 minutes - 37°C

G AGS16C3F
H LAMP1
I Z-stack Overlay

1 hour - 37°C
Figure 4A

Mean tumor volume (mm$^3$) +/- sem

- Control ADC-mcMMAF, 0.5mg/kg
- AGS16F, 0.125mg/kg
- AGS16F, 0.25mg/kg
- AGS16F, 0.5mg/kg

Time (Days)

Figure 4B

Mean tumor volume (mm$^3$) +/- sem

- 5% Dextrose, n=4
- Control ADC-mcF 5mg/kg, n=6
- AGS16-7.8 5mg/kg, n=4
- AGS16F 5mg/kg, n=10
- AGS16F 10mg/kg, n=10

Time (Days)
Figure 4C

Mean tumor volume (mm$^3$) ± sem

- PBS n=10
- AGS16F 10mg/kg n=6
- AGS16F 3mg/kg n=6

Time (Days)
Table 1. Binding of AGS16F and unconjugated AGS16-7.8 to Human and Cynomolgus Monkey Basophils.

<table>
<thead>
<tr>
<th>Sample and Category</th>
<th>AGS16F</th>
<th>AGS16-7.8 Ab</th>
<th>Control Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human basophils (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFI, mean (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without hIgE stimulation</td>
<td>94 ± 1.2</td>
<td>46 ± 4.3</td>
<td>8 ± 0.8</td>
</tr>
<tr>
<td>With hIgE stimulation</td>
<td>196 ± 19.7</td>
<td>130 ± 4.6</td>
<td>9 ± 0.6</td>
</tr>
<tr>
<td>% IgE⁺/ENPP3⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without hIgE stimulation</td>
<td>0.75 ± 0.1</td>
<td>0.66 ± 0.1</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>With hIgE stimulation</td>
<td>0.81 ± 0.04</td>
<td>0.84 ± 0.1</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Monkey basophils (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFIR, mean (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without hIgE stimulation</td>
<td>15 ± 0.88</td>
<td>12 ± 0.67</td>
<td>9 ± 0.58</td>
</tr>
<tr>
<td>With hIgE stimulation</td>
<td>19 ± 3.28</td>
<td>17 ± 0.0</td>
<td>9 ± 0.33</td>
</tr>
<tr>
<td>% IgE⁺/ENPP3⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without hIgE stimulation</td>
<td>0.26 ± 0.04</td>
<td>0.22 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>With hIgE stimulation</td>
<td>0.22 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 5A-D

(A) ccCK18 (U/L) vs. Time (hours)

- AGS16F 5 mg/kg
- AGS16F 1 mg/kg
- Control ADC-mcMMAF 5 mg/kg
- Control ADC-mcMMAF 1 mg/kg
- 5% Dextrose

(B) Tumor volume (cm³) vs. Time (hours)

- AGS16F 5 mg/kg
- AGS16F 1 mg/kg
- Control ADC-mcMMAF 5 mg/kg
- Control ADC-mcMMAF 1 mg/kg
- 5% Dextrose

(C) PARP-1 (% positive cells) vs. Time (hours)

- 5% Dextrose
- Control ADC-mcMMAF 1 mg/kg
- Control ADC-mcMMAF 5 mg/kg
- AGS16F 1 mg/kg
- AGS16F 5 mg/kg

(D) PHH3 (% positive cells) vs. Time (hours)

- 5% Dextrose
- Control ADC-mcMMAF 1 mg/kg
- Control ADC-mcMMAF 5 mg/kg
- AGS16F 1 mg/kg
- AGS16F 5 mg/kg
Figure 5E-H

H&E

ENPP3 Expression

AGS16F Localization

Dextrose - 24hrs
Dextrose - 7 Days

AGS16F 5mg/kg - 24hrs
AGS16F 5mg/kg - 7 Days

Control ADC 1mg/kg - 24hrs
Control ADC 1mg/kg - 7 Days

AGS16F 1mg/kg - 24hrs
AGS16F 5mg/kg - 24hrs

AGS16F 5mg/kg - 7 Days
AGS16F 5mg/kg - 7 Days
AGS16F is a Novel Antibody Drug Conjugate Directed Against ENPP3 for the Treatment of Renal Cell Carcinoma

Fernando Donate, Arthur Raitano, Kendall Morrison, et al.

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