Molecular Cancer Therapeutics

Targeting the Somatostatin Receptor 2 with the Miniaturized Drug Conjugate, PEN-221: A Potent and Novel Therapeutic for the Treatment of Small Cell Lung Cancer

Kerry A. Whalen, Brian H. White, James M. Quinn, Kristina Kriksciukaite, Rossitza Alargova, Tsun P. Au Yeung, Patrick Bazinet, Adam Brockman, Michelle M. DuPont, Haley Oller, James Gifford, Charles-Andre Lemelin, Patrick Lim Soo, Samantha Perino, Benoît Moreau, Gitanjali Sharma, Rajesh Shinde, Beata Sweryda-Krawiec, Mark T. Bilodeau, and Richard Wooster

Abstract

Small cell lung cancer (SCLC) is an aggressive neuroendocrine carcinoma with a 95% mortality rate with no improvement to treatment in decades, and new therapies are desperately needed. PEN-221 is a miniaturized peptide–drug conjugate (~2 kDa) designed to target SCLC via a Somatostatin Receptor 2 (SSTR2)–targeting ligand and to overcome the high proliferation rate characteristic of this disease by using the potent cytotoxic payload, DM1. SSTR2 is an ideal target for a drug conjugate, as it is overexpressed in SCLC with limited normal tissue expression. *In vitro*, PEN-221 treatment of SSTR2-positive cells resulted in PEN-221 internalization and receptor-dependent inhibition of cellular proliferation. *In vivo*, PEN-221 exhibited rapid accumulation in SSTR2-positive SCLC xenograft tumors with quick clearance from plasma. Tumor accumulation was sustained, resulting in durable pharmacodynamic changes throughout the tumor, as evidenced by increases in the mitotic marker of G_2–M arrest, phosphohistone H3, and increases in the apoptotic marker, cleaved caspase-3. PEN-221 treatment resulted in significant antitumor activity, including complete regressions in SSTR2-positive SCLC xenograft mouse models. Treatment was effective using a variety of dosing schedules and at doses below the MTD, suggesting flexibility of dosing schedule and potential for a large therapeutic window in the clinic. The unique attributes of the miniaturized drug conjugate allowed for deep tumor penetration and limited plasma exposure that may enable long-term dosing, resulting in durable tumor control. Collectively, these data suggest potential for antitumor activity of PEN-221 in patients with SSTR2-positive SCLC.

Introduction

Small cell lung cancer (SCLC) accounts for approximately 13% of all lung cancers in the United States with few new recent therapeutic options available despite over 60 agents that have been investigated in clinical trials (1, 2). SCLC is an aggressive disease that spreads quickly such that most patients with SCLC experience rapid disease progression before symptoms appear, resulting in few (about 7%) surviving 5 years (3). In addition to the lack of early symptoms, the lack of early detection reduces the chances for an optimal therapeutic response. When combined, these facts present a bleak outlook for patients and a need for effective therapies designed to target the characteristics of SCLC.

Many patients with SCLC have a history of heavy smoking, and because of the carcinogenic nature of tobacco smoke it is not surprising that SCLC genomes have a high incidence of somatic mutations (4). Despite the high level of mutations present, they are nearly all loss of function mutations in tumor suppressors that are not currently targetable such as TP53 and RB1 with less than 5% of patients with SCLC having mutations in known oncogenic drivers that can be targeted with inhibitors. Although a small proportion of patients may benefit from pathway-specific–targeted therapeutics, there is an overwhelming need to target the defects in cell-cycle regulation and the pervasive phenotype of rapid cell division to prolong the lives of patients with SCLC.

The current SCLC treatment paradigm has changed little in the last few decades; first and second line treatment includes platinum-based chemotherapy in combination or as single agent, with the addition of immunotherapy in second line. Surgery is another potential therapeutic option, but it requires the patient to present with early stage, non-metastatic disease, which is rarely the case (5–7).

The paucity of oncogenic mutations in patients with SCLC requires newer strategies to selectively target the tumor cells while minimizing deleterious effects on normal cells and tissues. There

Tarveda Therapeutics Inc., Watertown, Massachusetts.

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Corresponding Author: Kerry A. Whalen, Tarveda Therapeutics Inc., 134 Coolidge Avenue, Watertown, MA 02472. Phone: 617-639-1849; E-mail: kwhalen@tarvedatx.com

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are a number of new therapeutics being investigated in these patients that target cancer cells with overexpressed cell surface receptors. These include antibody–drug conjugates (ADC) such as rovalpituzumab tesirine, a delta-like 3 (DLL3) receptor targeting ADC, and sacituzumab govitecan, a tumor-associated calcium signal transducer 2 protein (TROP2) receptor–directed ADC. There have been disappointing Phase 2 results with either rovalpituzumab tesirine investigated in the third-line setting or sacituzumab govitecan investigated in patients with a median of two prior treatments with objective response rates (ORR) of 16% and 17%, respectively (8, 9). Antibody-based therapies have potential limitations that include the slow speed and inadequate depth of penetration as the drug moves from blood vessels into the solid tumor, potentially restricting efficacy in the solid tumor setting (10). In addition, the long plasma half-life of ADCs can lead to prolonged release of payload outside of the tumor causing potential systemic toxicities (11). Given the constraints of the antibody-based therapies, a miniature drug conjugate targeted to a cell surface receptor that is overexpressed on SCLC cells has the potential to target and rapidly accumulate potent payloads in tumor cells deep in the tumor, distant from the vasculature.

Somatostatin receptor 2 (SSTR2) is a novel target for treatment of SCLC. This receptor is one of five 7-transmembrane G-protein–coupled receptors (SSTR1 to SSTR5) that bind to the cyclopeptide hormone, somatostatin (12). SSTR2 may be an important target for treating SCLC as there are numerous observations that SSTR2 is overexpressed in cancer tissue with limited expression in normal tissue, thus providing an ideal cell surface target for a drug conjugate (13–16). Patients with SSTR2 expressing SCLC have been identified using a routinely available imaging diagnostic technique that consists of a somatostatin analog linked to ^{68}Gallium (17). This provides an easy and practical approach to identify patients who may benefit from an SSTR2-targeted therapeutic. In addition to clinical expression data, in nonclinical studies, SSTR2 signaling promotes SCLC tumor growth/survival (18). Finally, in the clinical setting SSTR2 expression was shown to correlate with worse survival in SCLC, further establishing its value as a potential target in patients with SCLC (17).

Under normal physiological conditions, the interaction of SSTR2 with ligand triggers rapid receptor internalization, leading to the inhibition of the release of secretory proteins, including many hormones. The process is tightly regulated, including extremely quick turnover (<15 minutes) for the natural somatostatin peptides, somatostatin-14 and 28, thus limiting their utility for use as therapeutic agents (19). Therefore, a number of somatostatin analogs have been developed that are less metabolically labile in plasma. The resulting longer plasma half-life of the analogs prolongs the plasma exposure that has resulted in approval of octreotide acetate for the treatment of patients with metastatic carcinoids or acromegaly (20–26).

The development of the novel therapeutic, PEN-221, for patients with SCLC combines the ability to target SSTR2 with a miniaturized conjugate containing a potent cytotoxic payload. DM1 is a potent microtubule-targeted cytotoxic agent and is effective when targeted to tumor cells as a payload of the ADC ado-trastuzumab emtansine previously approved for treatment of patients with cancer (27, 28). DM1 is an ideal payload for a miniature drug conjugate targeting SCLC cells given the highly proliferative nature of these cells.

The miniature drug conjugate PEN-221, incorporates both a derivative of the somatostatin analog Tyr^3-octreotate, and the potent cytotoxic payload, DM1, linked via a disulfide linker through the C-terminus of the peptide (Fig. 1). PEN-221 was rationally synthesized through a series of experiments that explored different SSTR2-targeting ligands, conjugation sites, linkers, and payloads. PEN-221 was selected on the basis of its potent *in vitro* and *in vivo* activity, including the ability to cause tumor regressions *in vivo* (29). In the present article, we report on the properties of PEN-221 that include high-affinity binding specifically to SSTR2 and rapid internalization of the receptor resulting in SCLC tumor-specific accumulation of DM1. The tumor-specific accumulation of the DM1 payload causes cell-cycle arrest and apoptosis resulting in tumor regressions in multiple SCLC SSTR2–expressing human xenograft models.

Materials and Methods

In vitro internalization assay

The PathHunter eXpress SSTR2 activated GPCR Internalization Assay from DiscoverX was used to measure the effects of PEN-221 binding on SSTR2 internalization (Catalog #93-0676E2CP0S). The eXpress kit provides Chinese Hamster Ovary (CHO) cells stably expressing human SSTR2. The cells are engineered to co-express an untagged SSTR2, Enzyme Acceptor (EA) tagged β-Arrestin, and a ProLink (PK) tag localized to the endosomes. Activation of SSTR2 induces β-Arrestin recruitment, followed by internalization of the Receptor/Arrestin-EA complex in PK-tagged endosomes. This forces complementation of the two β-galactosidase enzyme fragments (EA and PK), forming a functional enzyme that hydrolyzes substrate to generate a chemiluminescent signal. The cells were handled in accordance with the manufacturer's instructions. PEN-221, BT-984, and Somatostatin-28 powders were dissolved in DMSO and then diluted in media to a concentration of 3.3 μmol/L with a final concentration range of 300 to 0.005 nmol/L. Synthesis of BT-984 is described in the Supplementary Materials and Methods. Treated cells were incubated for 4 hours at 37°C in 5% CO_2. Plates were placed at room temperature and PathHunter detection reagents were added in accordance with the manufacturer's instructions. After one hour the chemiluminescent signal was measured using a GloMax plate reader (Promega, Catalog # E8032).

In vitro cellular proliferation and phosphohistone H3 staining

NCI-H524 10,000 cells per well and 5,000 cells per well for NCI-H69 and HCC-33 were plated into 96 well plates and 24 hours later were pre-treated with 100 μmol/L of octreotide (Chemprep Inc., Catalog #401501) for one hour, where appropriate, then treated with PEN-221 for a total of two (phosphohistone H3, PHH3) or six (proliferation) hours. Duplicate treatments starting at a dose of 20 μmol/L for PEN-221 at 3-fold serial dilutions in cell media. The cells were washed with cell media, and further incubated. To assess proliferation, Cell-titer Glo (Promega, Catalog# G7572) was used following the manufacturer's protocol 70 hours after PEN-221 treatment using the Glo Max Plate reader.

Percent proliferation inhibition was calculated using the following formula:

$$\%\text{Inhibition}=(1-(\text{treatment}-\text{time}=0)/(\text{control}-\text{time}=0)\times 100.$$

Control was vehicle alone (0.25% DMSO). Time = 0 is defined as the average of the measurements taken on the day the treatment

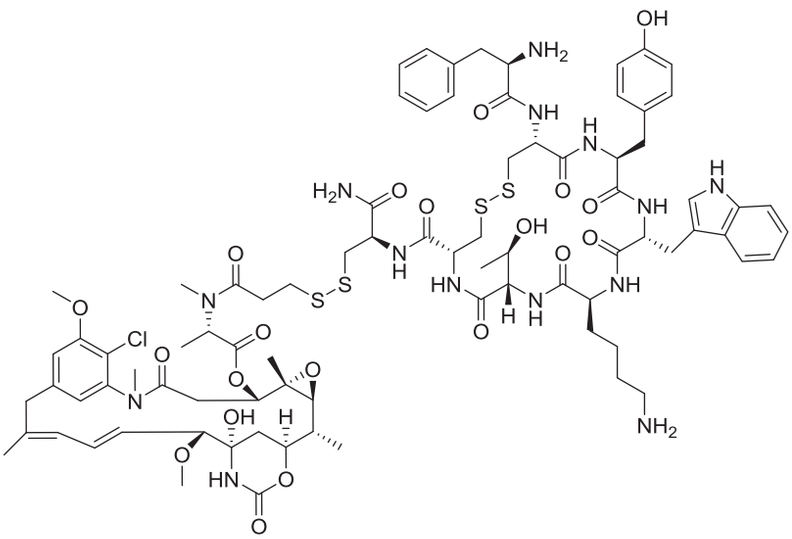

Figure 1.
PEN-221 miniature drug conjugate. PEN-221 is comprised of SSTR2 agonist [Tyr^3,Cys^8]octreotate amide linked to the microtubule agent DM1 through a disulfide bond.

was performed. IC_{50} curves were generated using the nonlinear regression analysis (four-parameter) with GraphPad Prism 6.

For PHH3 determination, 22 hours after drug treatment, cells were centrifuged, fixed with formaldehyde, washed and permeabilized (Abcam, Catalog # ab111542 kit, 100X Triton X-100 solution). Plates were washed with blocking buffer (Thermo Fisher Scientific Catalog # 8408500) and incubated in blocking buffer for 2 hours at room temperature. Anti-PHH3 primary antibody (Thermo Fisher Scientific, Catalog # PA517869) was diluted in blocking buffer (×200) and incubated overnight at 4 °C. Plates were washed with blocking buffer and FITC labeled secondary antibody (Thermo Fisher Scientific, Catalog # A16125; ×1,000) and Hoechst dye (Thermo Fisher Scientific, Catalog # PI62249; ×7,000) were diluted in blocking buffer, added and plates were protected from light and incubated for one hour at room temperature. Plates were washed with blocking buffer and then twice with PBS. Levels of PHH3 were determined by a high content screen of the plates using a Thermo Fisher Insight (Model IC903000).

The PHH3 response was calculated by measuring the percentage of cells that were both in the G_2–M phase of the cell cycle and had high levels of PHH3. EC_{50} curves were generated using nonlinear regression analysis (four-parameter) with GraphPad Prism 6.

In vivo studies

All studies were conducted in accordance with the Tarveda Therapeutics Institutional Animal Care and Use Committee (IACUC). All mice were treated in accordance with the OLAW Public Health Service Policy on Human Care and Use of Laboratory Animals and the ILAR Guide for the Care and Use of Laboratory Animals. Female athymic nude mice (CrTac:NCr-*Foxn1*^{nu}) were purchased from Taconic (IMSR Cat# TAC:ncrnu, RRID:IMSR_TAC:ncrnu). All cell lines were purchased from the ATCC except for HCC-33, which was purchased from DSMZ and grown *in vitro* culture with the recommended media. Infectious disease testing on the cell lines was done by Charles River Laboratories by PCR using the Mouse Essential Clear Panel, within 3 years of cell line use. Cells were only used if they had been passaged a total of 25 times or less. Mice were 8 to 11 weeks old and weighed approximately 20 grams at the time of dosing. PEN-221, Cisplatin, BT-984 and octreotide were dosed intravenously, and Etoposide was dosed intraperitoneally. At the end of each study, the animals were euthanized via CO_2 inhalation followed by cervical dislocation to ensure death.

Efficacy studies

Cells were harvested from tissue culture and made into a final suspension by mixing with Matrigel (BD Biosciences, Catalog #CB-40234; 1:1 ratio). All cells were implanted subcutaneously in the right flank of mice. NCI-H69 (ATCC Catalog# HTB-119, RRID: CVCL_1579) and Calu-6 (ATCC Catalog# HTB-56, RRID: CVCL_0236) cells were implanted at a concentration of 2.5×10^6 cells per mouse, HCC-33 cells (DSMZ Catalog# ACC-487, RRID:CVCL_2058) at a concentration of 3.0×10^6 cells per mouse and NCI-H524 (ATCC Catalog# CRL-5831, RRID:CVCL_1568) cells were implanted at a concentration of 5.0×10^6 cells per mouse. Tumor growth was monitored throughout the study and measured twice weekly. Tumor volume was measured in two dimensions using calipers and volume was calculated using the formula: $(w^2 \times l)/2 = \text{mm}^3$, assuming 1 mg is equivalent to 1 mm^3 of tumor volume. The health of the mice was monitored, and noteworthy clinical observations were recorded. Acceptable toxicity was defined as group mean body weight (BW) loss of less than 20% during the study and not more than one treatment-related death among 10 treated animals. Mice were randomized into treatment groups of 10 animals per group and therapy begun when tumor volumes were approximately 200 mm^3 usually 20–40 days post implantation. All doses were scaled to the BW of the individual animals at a dose volume of 10 mL/kg. Treatment regimens and dosages in each experiment are described in the Results and in the figure Legends. In the NCI-H524 and NCI-H69 studies PEN-221 was reconstituted in 10% PG (Fischer Scientific, Catalog #P355-1)/5 mmol/L Citrate buffer (Teknova, Catalog # Q2444), pH 4/WFI (Thermo fisher scientific, Catalog # CH30154.01). In the HCC-33 and Calu-6 studies PEN-221 and BT-984 were reconstituted in 0.1% Solutol HS 15 (Sigma Aldrich, Lot# 35809356PO) and 5% mannitol (Pearlitol PF; Roquette, Lot# E 747 C) The vehicle used in each study corresponded to the vehicle used to prepare PEN-221. Cisplatin (Cisplatin Injection,

Teva Pharmaceuticals, 1 mg/mL, Lot No. 14F19LA) and Etoposide (Etoposide Injection, Accord Healthcare Inc 20 mg/mL, Lot No. R12112) were diluted on each day of dosing with sterile saline (Hospira, Part # 00409488810). Animals were euthanized once tumors reached a maximum of 2,000 mm^3 or at the end of study, whichever came first. Percentage of tumor growth inhibition (%TGI) was defined as the difference between the mean tumor volume (MTV) of the vehicle and the MTV of the drug treated group, expressed as a percentage of the MTV of the vehicle group. Statistical analysis was done using one-way ANOVA and Tukey's multiple comparisons in GraphPad statistical software, GraphPad Prism 6. Correlation coefficients were calculated using Microsoft Excel for Mac 2011 ver. 14.5.0, built-in statistical functions.

Body weight measurements

Animals were weighed on the days indicated in the graphs (Supplementary Fig. S4A–S4D). For each treatment group, weights were averaged and compared with the average weight of the group on first day of treatment to obtain the percentage of change in weight. Statistical analysis was done using one-way ANOVA and Tukey's multiple comparisons in GraphPad statistical software, GraphPad Prism 6.

Pharmacodynamic studies

Mice were implanted subcutaneously in the right flank with 5×10^6 NCI-H524 cells per mouse for the SSTR2 internalization studies and 2.5×10^6 NCI-H69 cells per mouse for the pharmacodynamics markers studies. Animals were stratified to an $n = 3$ per treatment group. PEN-221, BT-984 and octreotide were formulated in 0.1% Solutol HS15 and 5% mannitol. Time points and dose levels are described in the Results and Figures. Tumor collection and processing are described below. For the pharmacodynamics studies, the tumors were split in half, for either DM1 tumor PK analysis or IHC analysis.

Excised tumors were fixed in neutral-buffered formalin (NBF; Thermo Fisher Scientific, Catalog #5700TS), after 24 hours the tumor was put into a labeled cassette and added to fresh NBF and processed within 48 hours. Samples were paraffin embedded using standard techniques.

An automated stainer, Tissue-Tek DRS Slide Stainer, was used to process samples for H&E and a standard staining protocol was used.

For the analysis of SSTR2, PHH3 and Cleaved Caspase-3 (CC3) paraffin embedded xenograft tissue was sectioned at 4-μm-thick on to positively charged slides. Slides were then dried for 30 minutes at 65°C, deparaffinized and hydrated using xylene (Avantik Biogroup, Catalog # RS4050), 100% ethanol, 95% ethanol and deionized water (Sigma Aldrich). Slides were placed in working dilution Declere (Cell Marque, Catalog #921P-04) antigen retrieval solution in a pressure cooker on steam setting for: 15 min/1 basket, 20 min/2 baskets. Slides were cooled at room temperature for 20 minutes and rinsed in deionized water, treated with 3% hydrogen peroxide (Sigma Aldrich, Catalog #216763) for five minutes twice and rinsed deionized water. Sample was circled with a pap pen and covered with PBS. Working dilution background block (Biogenix Protein/Casein Blocker) was applied for 20 minutes. Biogenix I6000 used to remove blocker and apply primary anti-SSTR2 antibody (Epitomics SSTR2 Catalog # AC-0162RUO at 1:100 dilution), PHH3 (Cell Signaling) at 1:50 dilution in Diamond Antibody Diluent (Cell Marque, Catalog #921P-04) or the primary CC3(Cell Signaling Technology Catalog# 9661, RRID:AB_2341188) antibody at a 1:300 dilution in Diamond Antibody Diluent which was incubated for 30 minutes at room temperature. Slides were washed with PBS and goat anti-rabbit secondary (ThermoFisher Scientific, Catalog # 31460) was applied at a 1:500 dilution for SSTR2 and for PHH3 and CC3 goat anti-rabbit secondary (Jackson ImmunoResearch Labs Cat# 111-035-144, RRID:AB_2307391) applied at 1:100 dilution in Diamond Antibody Diluent and incubated for 30 minutes. Slides were washed five times with PBS and working solution DAB chromagen (Cell Marque DAB, Catalog # 957D-20) was applied for eight minutes at room temperature. Slides were rinsed with PBS, removed from Biogenix I6000 and rinsed three times in tap water. Slides were counterstained 10 times in Hematoxylin I (Avantik Biogroup, Catalog #RS4020-B) and then rinsed in water three times. Slides were dipped 10 times in ammonia water (bluing reagent) and rinsed three times in water and then dehydrated with two changes each of 95% ethanol, 100% ethanol and xylene and a cover slip was added. SSTR2 slides were scored for IRS score as described in Specht and colleagues (30). Briefly, the IRS score is determined by multiplying the staining intensity in four gradations with the percentage of positive cells in five gradations.

SSTR2 internalization staining was measured in a semiquantitative fashion by assessing the percentage SSTR2-positive cells upon scanning a total of ten microscopic fields for each sample. Evidence for SSTR2 receptor internalization was scored in the following fashion to reflect degree of internalization ranging from membrane-bound to cytoplasmic: 0: Sharp membranous; 1: Hazy membrane, some cytoplasm; 2: Mix of membrane/cytoplasm; 3: Mostly cytoplasmic. Statistical analysis was carried out by one-way ANOVA (non-parametric) and Dunnett's multiple comparison *P* values are reported. Analysis was carried out using Graph Pad Prism 6 Software.

PHH3 and CC3 were scored by estimating the percent positive cells in five fields per sample and averaged (excluding necrotic areas). Statistical analysis was carried out by using a Student *t* test assuming unequal variance in Microsoft Excel and *P* values are reported.

PEN-221 and DM1 analysis in plasma

Three animals per each time-point were anesthetized using CO_2 and approximately 1.0 mL of blood sample was collected via cardiac puncture using a needle (BD Biosciences, Catalog# 14-826-88). When the animals were still anesthetized, the mice were euthanized via cervical dislocation. Blood was transferred to K_2EDTA sample collection tube (BD Biosciences, Catalog #02-669-33), inverted four times and centrifuged for 10 minutes at 10,000 x *g*, plasma harvested, transferred into labeled 96-well plate, and stored at −80° C. An LC-MS/MS method was used for PEN-221 and DM1 analysis. Detailed sample preparation described in Supplementary Materials and Methods. A Leap technologies HTS PAL auto-sampler was used to introduce 5 μL of sample to an ABSciex 4000 series tandem mass spectrometer coupled with a Shimadzu LC10AD VP LC system. An Agilent Poroshell 120 ECC18 2.7 μmol/L, 3.0 × 50 mm column (PN699975-302) was used to chromatographically separate analytes. PEN-221 and DM1 m/z 893.90/547.2 were monitored and the peak area ratio of analyte to Internal Standard (IS) was used for quantitation. Quantitation was performed using Analyst v.1.6.2 (ABSciex 2013). The analyte to IS area ratio was used to fit a five-point PEN-221 standard curve with linear 1/x weighted fit. The peak area ratio of analyte to IS was used for DM1 quantitation.

Total DM1 analysis in tumors

The tumors were weighed and 0.1 mol/L of ammonium bicarbonate buffer pH 8.0 (Sigma-Aldrich cat# 09830) was added at the ratio of 1:4 (w/v). Samples were kept on ice and homogenized for 20 to 30 seconds each. DM1 stock solution in methanol (Fisher Scientific, Catalog #A452-1) was used to prepare standards and assay controls in blank NCI-H69 tumor homogenate. Detailed sample preparation described in Supplementary Materials and Methods.

An LC-MS/MS method was used for total DM1 analysis. A Leap technologies HTS PAL autosampler was used to introduce 5 μL of sample to an ABSciex 4000 series tandem mass spectrometer coupled with a Shimadzu LC10AD VP LC system. An Agilent Poroshell 120 EC-C18 2.7 μmol/L, 3.0 × 50 mm column (PN699975-302) was used to chromatographically separate analytes. Total DM1 was measured as DM1–NEM conjugate and DM1-maleimidohexnoic acid was used as IS. The peak area ratio of analyte to IS was used for quantitation.

Results

PEN-221 stimulates SSTR2 receptor internalization *in vitro*

PEN-221 consists of a peptide targeting ligand joined through a cleavable linker to the payload DM1 (Fig. 1). PEN-221 binding to SSTR2 is the first step in a cascade of events required for PEN-221 antitumor activity. PEN-221 binds with high affinity (K_i of 51 pmol/L) and selectivity to SSTR2 (>420-fold higher than the other 4 SSTR isoforms; ref. 29). The second step is the internalization and accumulation of the PEN-221 receptor complex within the intracellular compartment of the tumor cell. This is especially important due to the low membrane permeability of PEN-221, limiting the passive uptake of PEN-221 (29). In an *in vitro* assay using CHO cells that overexpress the human SSTR2 receptor, PEN-221 triggered SSTR2-dependent internalization equivalent to the natural ligand, somatostatin-28, with an EC_{50} of 0.51 nmol/L ($n = 3$) and 1.08 nmol/L ($n = 3$), respectively (Fig. 2A). There is limited internalization of the non-binding SSTR2 scrambled control conjugate (BT-984) where minimal activity was observed at the highest concentration (300 nmol/L) tested and the EC_{50} could not be calculated ($n = 3$).

PEN-221 *in vitro* cellular activity is dependent upon binding to SSTR2 on cancer cells

The pharmacological consequence of PEN-221 internalization into human cancer cells was assessed *in vitro* using the SCLC SSTR2-expressing NCI-H524, NCI-H69 and HCC-33 cells. The selection of the cell lines used for this *in vitro* work as well as the *in vivo* work was based on positive SSTR2 protein and mRNA expression (Supplementary Fig. S1A and S1B). To determine the extent of PEN-221 DM1-induced cell-cycle effects following its intracellular accumulation, assays were developed to measure the inhibition of cellular proliferation and phosphorylation of the mitotic arrest marker, PHH3. In addition, to investigate the receptor dependence of PEN-221, experiments were implemented in the presence or in the absence of excess SSTR2 ligand, octreotide.

PEN-221 inhibited *in vitro* cellular proliferation in a dose-dependent fashion and this activity could be diminished when cells were pre-incubated with excess octreotide illustrating the receptor dependence of the PEN-221 antiproliferative effect (Table 1; Supplementary Fig. S2A). PEN-221 receptor dependent activity *in vitro* varied from strong (NCI-H524) to modest (HCC-33) but was consistent ($N = 3$) and correlated with *in vivo* activity (described below). Octreotide alone did not have an effect on cellular growth. These cell lines also demonstrated sensitivity to the cytotoxic payload, DM1, with sub-micromolar IC_{50}'s after a 6-hour exposure and 70-hour additional incubation period (Supplementary Fig. S2B). Lower receptor expression seen in the NCI-H69 cells and decreased sensitivity to DM1 seen with the HCC-33 cell line most likely contributed to the NCI-H524 cells being the most sensitive to PEN-221. The on-target effects of the mitotic inhibitor DM1 were also evidenced by the dose-dependent increases in PHH3 levels in the NCI-H524 cell line. PHH3 is a marker of cells that are in the G_2–M phase of the cell cycle, and DM1 induces cell-cycle arrest at this stage; therefore, an increase in PHH3 indicates an effect on the cells by DM1. The activity of PEN-221 after a 2-hour incubation was 2.4 times higher when dosed alone, compared with when cells pre-treated with octreotide (Fig. 2B). These *in vitro* results are clear evidence that PEN-221 affects cellular growth *in vitro* and this activity is dependent upon binding to SSTR2.

PEN-221 stimulates internalization of SSTR2 *in vivo*

A series of pharmacodynamic studies were performed to characterize the internalization of SSTR2 triggered by PEN-221 and to measure the pharmacological changes as a result of PEN-221 treatment.

A receptor internalization study was performed to determine the ability of PEN-221 to stimulate internalization of the SSTR2 receptor *in vivo*. The NCI-H524 model was selected due to the high level of SSTR2 expressed on the cellular membrane of the tumor cells. The intracellular localization of SSTR2 was compared in xenograft tumors of NCI-H524 in mice that were administered either PEN-221, octreotide, BT-984 (the non-binding SSTR2 scrambled control conjugate) or vehicle. Representative images of scoring are shown in Fig. 3A. A single dose of PEN-221 at 2.0 mg/kg resulted in significant increases in SSTR2 receptor internalization compared with changes observed following doses of BT-984 at 2.0 mg/kg at 1, 4, and 72 hours ($P < 0.05$; Fig. 3B). In addition, PEN-221 treatment at 2.0 mg/kg also resulted in a statistically significant increase in SSTR2 receptor internalization when compared with the increases observed an equimolar amount of octreotide (1.4 mg/kg) after 72 hours ($P < 0.05$). No SSTR2 receptor internalization was observed in the vehicle or BT-984–treated mice. These results demonstrated that PEN-221 rapidly triggers SSTR2 internalization *in vivo* and are consistent with the *in vitro* binding and internalization studies.

Rapid and durable tumor accumulation of PEN-221

A short plasma half-life ($t^1/_2$) of PEN-221 has benefits when considering potential normal tissue toxicity. Rapid tumor accumulation and durable tumor exposure of PEN-221 is a critical component for productive *in vivo* tumor activity. Plasma levels of PEN-221 were determined after an intravenous dose to nude mice. In addition to directly measuring PEN-221 in plasma, an assay was developed to measure the total quantity of circulating DM1. This method includes a chemical step to reduce any disulfide forms of DM1 and trap the resulting free thiol form of DM1. This indirectly quantitates all DM1 containing entities in plasma, including PEN-221, free DM1 and all other DM1 disulfide metabolites. The DM1 released from PEN-221 was then determined by subtracting the PEN-221 concentration from the

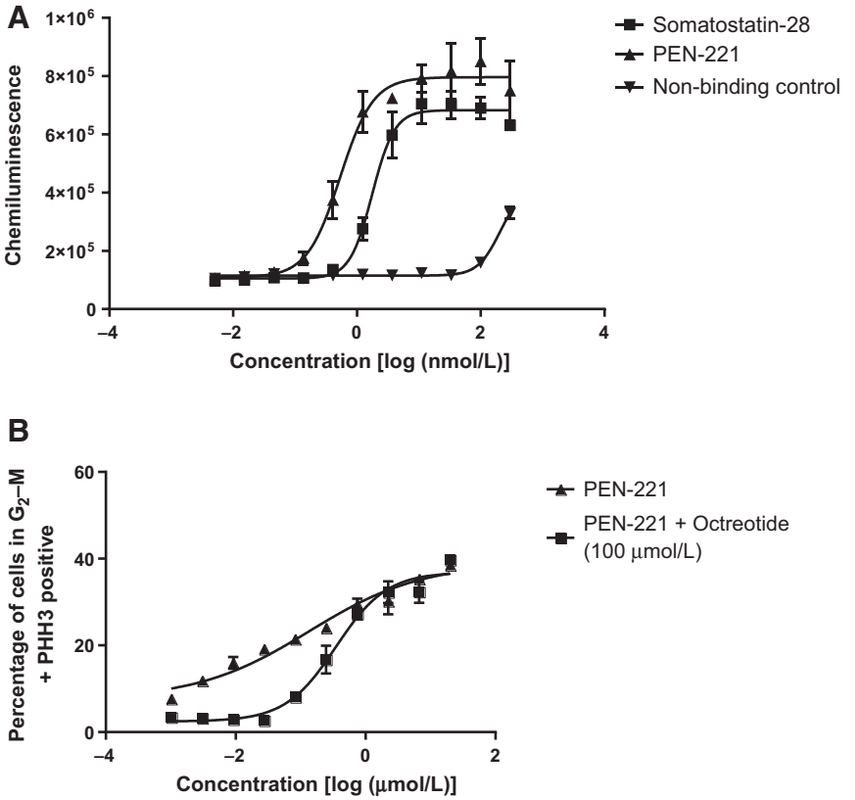

Figure 2.
PEN-221 stimulates the internalization of SSTR2 *in vitro* and inhibits the *in vitro* phosphorylation of Histone H3 (PHH3) in SSTR2-expressing cells. **A,** Measurement of receptor internalization *in vitro* was performed using CHO cells expressing SSTR2 that were treated with either PEN-221, Somatostatin-28 (positive control), or BT-984 (negative control). Chemiluminescent signal is indicative of internalization of the receptor. Error bars represent standard deviation of duplicate samples. **B,** NCI-H524 cells were treated with PEN-221 for 2 hours, in the absence or presence of 100 μmol/L of the SSTR2 agonist, octreotide. The cells were washed after treatment and incubated for an additional 22 hours before measuring G_2–M and PHH3-positive cells. Error bars represent standard deviation of duplicate samples.

total DM1 levels. Although PEN-221 was detected in plasma out to 24 hours post administration when given intravenously to CrTac:NCr-*Foxn1*^{nu} nude mice, the resulting mean $t^1/_2$ of 12.2 hours is significantly less than the days long half-lives observed for most ADC's (Supplementary Fig. S3). The concentration–time profiles show very similar plasma levels for both PEN-221 and total DM1, indicating that the majority of the total DM1 measured was a result of the PEN-221 reduction during the sample treatment and minimal DM1 was released into the circulation of the mice. The speed of tumor accumulation of PEN-221 and the duration of intra-tumoral exposure was assessed in tumor xenografts of the SSTR2-expressing SCLC cell line NCI-H69. By 2 hours, rapid tumor accumulation of DM1, the active component of PEN-221, was observed with a peak concentration of (Fig. 3C) 542 nmol/L at 8 hours. Five days after a single dose of PEN-221, an average of 88% (477 nmol/L) of the DM1 remained in the tumor (Fig. 3D). This DM1 retention in the tumor was much higher than circulating PEN-221 with only 32 nmol/L remaining at 8 hours compared with the initial maximum concentration of 3,660 nmol/L at 5 minutes. These data highlight the rapid and durable accumulation of DM1 from PEN-221 in tumors with the potential for limited exposure to normal tissues because of the short PEN-221 plasma half-life.

Table 1. PEN-221 activity in SCLC lines

	Inhibition of PROLIFERATION IC_{50}	
Cell line	**Without octreotide (μmol/L)**	**With octreotide (μmol/L)**
NCI-H524	0.060	0.333
NCI-H69	0.108	0.258
HCC-33	0.658	0.83

NOTE: PEN-221 inhibits the *in vitro* cellular proliferation in SSTR2-expressing SCLC cells. Cells were treated with PEN-221 for 6 hours in the absence or presence of 100 μmol/L of the SSTR2 agonist octreotide ($N = 3$). The cells were washed after treatment and incubated for an additional 70 hours before measurement of proliferation.

PEN-221 treatment results in cell-cycle arrest and tumor cell death *in vivo*

A pharmacodynamic study was performed in the mouse NCI-H69 xenograft model to determine the biological effects of PEN-221 treatment on the tumors and to confirm the *in vitro* findings described above. In this study, mitotic arrest was measured by quantitating PHH3, and apoptosis was measured by quantitating levels of cleaved caspase-3 (CC3). In parallel, tumor concentrations of DM1 were measured as described above. PEN-221 at 2.0 mg/kg or vehicle was given as a single dose to NCI-H69 xenografted animals and tumors were collected at 24, 72, and 168 hours after dosing. After a single dose of PEN-221 was administered, statistically significant increases in PHH3 were observed in tumors in treated mice at 72 hours and coincident with this effect, PEN-221 induced apoptosis, as quantified by the increase in CC3 (Fig. 4A–D). In addition, the peak concentration of DM1 in the tumor was observed 24 hours post dose, concordant with the temporal cascade of the pharmacodynamic changes (Fig. 3D).

In addition to the rapid and sustained accumulation of PEN-221, the small size of the conjugate has the potential advantage of penetrating deeply into the tumor as compared with the much larger sized ADC. In support of this hypothesis, pharmacodynamic study samples were also analyzed across the whole tumor

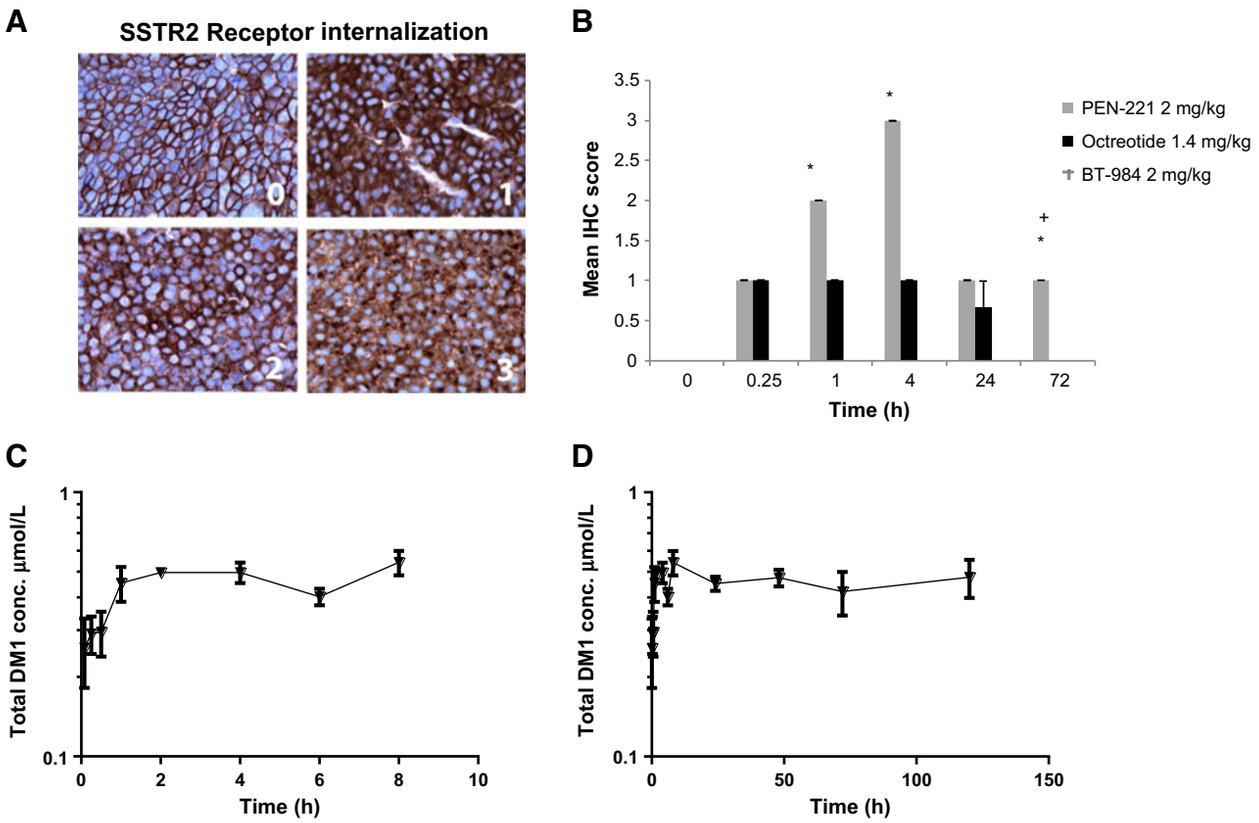

Figure 3.

PEN-221 stimulates the internalization of SSTR2 in NCI-H524 xenograft tumors and results in rapid accumulation and sustained retention of PEN-221 in the SSTR2-expressing NCI-H69 xenograft tumors. **A,** Representative images of SSTR2 receptor internalization in xenograft tumors. The cellular location of SSTR2 was quantitated by the analysis of tumor xenograft sections stained with an anti-SSTR2 antibody. The scoring is dependent upon the localization of the staining and is independent of the intensity: 0: Sharp membranous (no internalization); 1: Hazy membrane, some cytoplasm (minimal internalization); 2: Mix of membrane/cytoplasm (some internalization); 3: Mostly cytoplasmic (extensive internalization), with the score of either 1 or 2 representing intermediate internalization. **B,** A single dose of each agent was given a time zero and tumors collected and analyzed for SSTR2 location at multiple time points using the scoring system outlined above. PEN-221 compared with BT-984 (negative control) or vehicle *, $P < 0.05$, PEN-221 compared with octreotide $P < 0.05$ (+), both using Dunn's multiple comparisons. Error bars represent SEM ($n = 3$ per arm). BT-984 score was zero throughout the study and octreotide score at 72 hours was zero; therefore, these scores are not visible on the graph. **C** and **D,** PEN-221 was dosed at 2 mg/kg and total DM1 was measured in tumors post-dosing at the time points indicated ($n = 5$ per time point). Error bars represent SD.

for both PHH3 and CC3. A consistent pattern of staining for both markers across the tumor sections, including the tumor core, indicates the broad penetration of PEN-221 across the whole-tumor xenograft (Fig. 4E).

PEN-221 treatment of SSTR2-positive SCLC xenograft tumors results in potent and durable tumor inhibition superior to standard of care

The antitumor activity of PEN-221 was determined in a series of efficacy studies. Initial experiments defined the MTD of PEN-221 at 2.0 mg/kg given once weekly and were used to help guide dose level selection. In support of the MTD data, the BWs for all efficacy studies are shown in Supplementary Fig. S4A–S4D and demonstrate tolerability of the compound at doses up to 2 mg/kg given once weekly. These studies explored the relationship of PEN-221 activity to dose level, schedule, and receptor expression. In addition, PEN-221 was compared head-to-head in the same experiment as the currently used standard-of-care (SOC) treatment for SCLC.

The SSTR2-expressing SCLC xenograft NCI-H524 mouse model was used to evaluate the antitumor activity of PEN-221 given as a single dose (Fig. 5A). This study allowed for the evaluation of the relationship between dose level and efficacy of PEN-221. PEN-221 at 0.5, 1.0, or 2.0 mg/kg was administered to animals bearing NCI-H524 tumors and resulted in tumor growth inhibition of 6.0% (P = NS), 59% ($P < 0.05$), and 96% ($P < 0.0001$), respectively, when compared with vehicle control. The relationship between the dose level of PEN-221 and the inhibition of tumor growth was high ($r^2 = 0.92$). Importantly, these data also demonstrated that a single dose of PEN-221 is effective in an SSTR2-expressing xenograft model of SCLC.

Antitumor activity of PEN-221 was investigated at varying dose levels and with differing dosing schedules in the SSTR2-positive SCLC HCC-33 cell line in mice (Fig. 5B). Treatments were given either once or twice weekly for the duration of the study. Once weekly treatment of HCC-33 tumor-bearing mice with PEN-221 dose at 1.0 or 0.5 mg/kg resulted in greater than 95% efficacy, with the high-dose treatment resulting in complete regressions in all

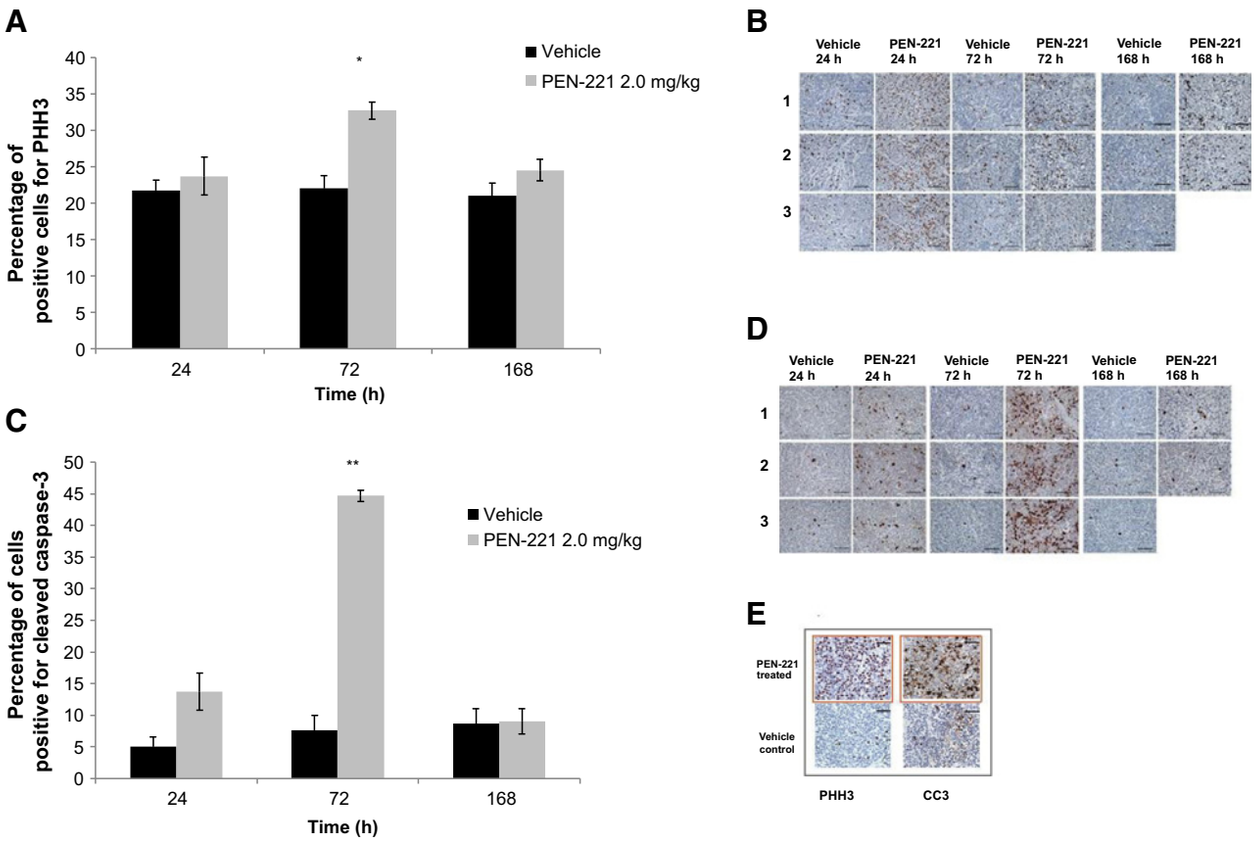

Figure 4.

PEN-221 treatment of mice bearing NCI-H69 xenografts results in increased levels of the mitotic arrest marker, pHH3, and the apoptosis marker, CC3, in tumor cells, deep in the core of the xenograft tumors. **A,** Semiquantitative histological analysis of PHH3 staining of NCI-H69 tumors from treated animals at the indicated post-dose time points. *, $P = 0.01$ for PEN-221 compared with vehicle (Student *t* test assuming unequal variance). Error bars represent SEM. ($n = 3$ per arm except $n = 2$ for PEN-221 at 168 hours). **B,** Representative pictures taken at the indicated time points of PHH3 staining of NCI-H69 tumors; brown stain is PHH3-positive staining. **C,** Semiquantitative histological analysis of CC3 staining of NCI-H69 tumors from treated animals at the indicated post-dose time points. **, $P = 0.001$ for PEN-221 compared with vehicle treatment (Student *t* test assuming unequal variance). Error bars represent SEM. ($n = 3$ per arm except $n = 2$ for PEN-221 at 168 hours). **D,** Representative pictures taken at the indicated time points of CC3 staining of NCI-H69 tumors; brown stain is positive CC3 staining. **E,** Representative staining of tumor samples from the central region of the tumor, for both PHH3 and CC3 from PEN-221 and vehicle-treated NCI-H69 tumor-bearing animals.

animals treated. In addition, statistically significant antitumor activity was demonstrated with a dose as low as once weekly 0.33 mg/kg. There was a clear relationship ($r^2 = 0.81$) between dose and antitumor activity with increased dose level resulting in increased efficacy, consistent with the NCI-H524 results described above. Treatments were also administered twice weekly, with PEN-221 given at doses of 0.5 and 0.25 mg/kg resulting in greater than 95% efficacy and tumor regressions in both treatment groups.

The effectiveness of PEN-221 was compared with SOC treatment in the SSTR2-expressing SCLC line, NCI-H69, in mice given either as a single cycle or two cycles of treatment, with each cycle being a week in length (Fig. 5C). SOC single-cycle treatment consisted of Cisplatin dosed once per week given in combination with Etoposide dosed three times per week. PEN-221 single-cycle treatment consisted of a once weekly dose. When comparing single-cycle treatments, PEN-221 was superior to SOC treatment, and treatment with two cycles of PEN-221 trended toward significance compared with SOC ($P = 0.058$). In addition, PEN-221 treatment resulted in 9 out of the 10 animals having complete regressions in the single-cycle treatment group and 8 out of 9 animals having regressions in the two-cycle treatment group. These data demonstrate in this tumor model of SCLC that PEN-221 has superior antitumor efficacy compared with first-line SOC for SCLC.

To determine the dependency of PEN-221 activity on SSTR2 expression *in vivo*, the antitumor activity of PEN-221 was compared with a non-targeting control (BT-984) in the SSTR2 non-expressing Calu-6 (NSCLC) xenograft model (Fig. 5D). PEN-221 and BT-984 had equivalent antitumor activity at the highest dose of PEN-221. These data are consistent with the *in vitro* data obtained for PEN-221 and BT-984 where no differences were seen in activity for either molecule in the presence or absence of competitive ligand, demonstrating a lack of targeting in this cell line. This activity is presumed to be due to non-specific uptake and to a much lesser extent, the cleavage of PEN-221 in the plasma with release of the untargeted DM1 payload. However, PEN-221 activity dropped off

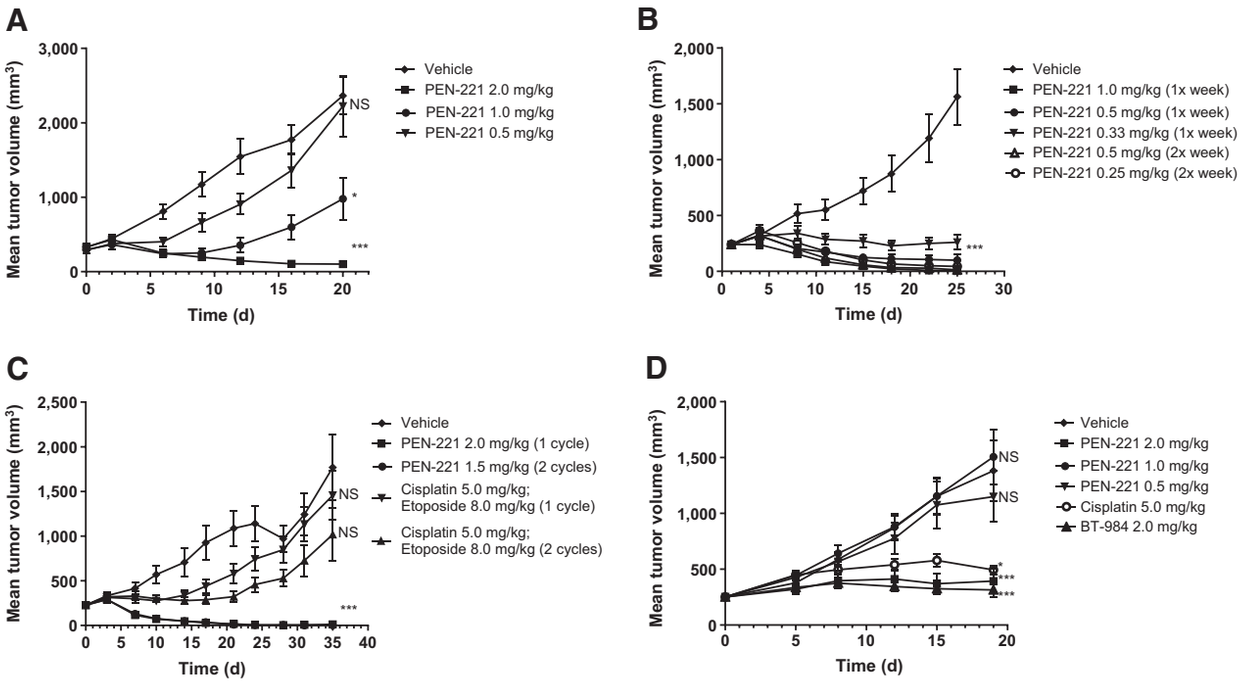

Figure 5.

PEN-221 treatment of xenograft tumors results in target-dependent, durable tumor inhibition superior to standard of care. **A,** Animals bearing SSTR2-expressing NCI-H524 SCLC xenograft tumors were given a single dose of PEN-221 (2.0, 1.0, or 0.5 mg/kg) or vehicle was administered on day 0 (*, $P < 0.05$, one-way ANOVA; ***, $P < 0.0001$, NS, not significant, one-way ANOVA). **B,** Animals bearing SSTR2-expressing HCC-33 SCLC xenograft tumors were given PEN-221 on a once weekly schedule on days 1, 8, 15, and 22, or on a twice weekly schedule on days 1, 4, 8, 11, 15, 18, and 22. Vehicle was given on a once weekly schedule (***, $P < 0.0001$, one-way ANOVA for all groups treated with PEN-221). **C,** Animals bearing SSTR2-expressing SCLC NCI-H69 xenograft tumors were given PEN-221 on day 1 (1 cycle) or on days 1 and 8 (2 cycles); treatment resulted in overlapping data not visible on the graph. Cisplatin was given on day 1 with etoposide on days 1, 2, and 3 (1 cycle), or cisplatin was given on days 1 and 8 with etoposide on days 1, 2, and 3 and days 8, 9, and 10 (2 cycles). The vehicle group was dosed on day 1 (***, $P < 0.0001$, NS, not significant, one-way ANOVA). **D,** Animals bearing non-SSTR2-expressing NSCLC Calu-6 xenograft tumors were given PEN-221 and the negative control, BT-984, on day 1 and day 9 (*, $P < 0.05$; ***, $P < 0.005$, NS, not significant, one-way ANOVA). Error bars represent SEM ($n = 10$ per group). BW data are summarized in Supplementary Fig. S4A–S4D.

dramatically in Calu-6 compared with the dose–response seen in SSTR2-expressing tumor xenograft models. In addition, the lack of antitumor activity with 1.0 mg/kg of PEN-221 in the Calu-6 model is in stark contrast with the efficacy seen at this dose level in SSTR2-expressing xenograft models (Fig. 5A and B). These data support the conclusion that SSTR2 expression is required for activity of PEN-221.

Discussion

SCLC is an aggressive neuroendocrine malignancy with a high-mortality rate that is different from non–small cell lung cancer (NSCLC). The treatment landscape for patients with SCLC has remained largely static, having not benefited from recent advances in targeted therapies and immunotherapies like what has been seen for the NSCLC patient population (2). New treatments for first-line use and for relapsed/refractory patients are urgently needed. The lack of early diagnosis and lack of oncogenic driving mutations combined with the high proliferation rate that are characteristics of this disease makes development of effective therapeutics a challenge. Although there are new therapies in development, such as rovalpituzumab tesirine and sacituzumab govitecan, that could be used in the future, the initial clinical trial results have been disappointing.

Targeting SSTR2 with a miniature conjugate to prolong cancer patient survival has been validated through the evaluation of ^{177}Lu-Dotatate. This conjugate consists of a radiopharmaceutical (Lutetium 177) linked to a peptide that binds to SSTR2. This therapy has been approved by Health Authorities for treatment of gastrointestinal and pancreatic neuroendocrine tumors where there is an approximately 3-fold increase in progression-free survival with ^{177}Lu-Dotatate compared with placebo (31). Although very promising, the benefits from ^{177}Lu-Dotatate could be curtailed by the limited number of doses (4 over 8 months; ref. 31). Given the rapid progression of many SCLC tumors, dosing a therapy such as ^{177}Lu-Dotatate only once every 2 months may be too infrequent to slow the tumor growth or cause regressions. Importantly, ^{177}Lu-Dotatate treatment was unable to cause complete tumor regressions in the NCI-H69 SCLC xenograft model where PEN-221 was highly effective (32).

PEN-221 was designed to circumvent the distinctive challenges of SCLC biology and the efficacy limitations of ADCs and radiotherapeutics such as ^{177}Lu-Dotatate. The unique design of PEN-221 allows for repeated dosing of a targeted tumor-selective cytotoxic agent, which results in rapid intracellular tumor accumulation of the DM1 payload in SSTR2-expressing cells in the tumor. The long retention of the DM1 payload within the tumor

triggers mitotic catastrophe to tumor cells going through the cell cycle at the time of dosing as well as tumor cells that enter the cell cycle many days after the administration of PEN-221.

The SSTR2 protein is overexpressed in SCLC and its expression correlates with worse survival. This establishes its value as a target in this patient population (17). The differential expression and the ability to be rapidly internalized makes SSTR2 an ideal target for a therapeutic delivering a potent cytotoxic payload. The advantageous qualities of the receptor combined with the specific, high affinity binding of PEN-221 to SSTR2, contribute to the successful delivery of PEN-221 selectively to and within the tumor cells. This delivery was demonstrated by PEN-221–mediated stimulation of receptor internalization both *in vitro* and *in vivo* and the sustained accumulation of the therapeutic out to 5 days in the tumor.

PEN-221 causes both cell-cycle arrest and apoptosis of cancer cells as demonstrated by both *in vitro* and *in vivo* experiments. After PEN-221 treatment, uniform distribution of the pharmacodynamic changes were observed in the tumor, including deep into the core of the tumor. Although potent payloads can be attached to ADCs, allowing for effective cell killing, they are challenged by their limited and uneven distribution in the tumor, effectively leaving some cells untargeted and their proliferation unchecked. This lack of even distribution throughout the tumor has been seen in multiple examples in the literature with antibodies against various targets, including HER2, Carbonic Anhydrase IX (CAIX) and more recently anti–PD-L1 antibodies (10, 33, 34). Direct comparison of a full-length CAIX-targeting antibody (cG250) and a smaller, Fab fragment of the same antibody demonstrated that the Fab was better able to penetrate the tumor versus full-length antibody in the preclinical setting (34). This collection of data, including the data with PEN-221, supports the hypothesis that there is an advantage in treating patients with solid tumors with therapeutics smaller than ADC's, given the ability to infiltrate into spaces larger molecules struggle to penetrate, with the possibility of translating into more extensive and durable efficacy.

The pharmacodynamic effects observed with PEN-221 treatment translated into potent antitumor activity in multiple human SCLC xenograft mouse models that express SSTR2, whereas no additional benefit was observed in a non–SSTR2 -expressing model, Calu-6. The expression of SSTR2 in SCLC patient samples varies in the literature with 20% to 40% demonstrating positive staining for SSTR2 in IHC analysis. Two IHC studies we performed have shown positive expression of SSTR2 in 28% (10/35) and 27% (12/44) of patient samples, consistent with the published literature (Supplementary Tables S1 and S2; refs. 17, 18). In addition, expression of SSTR2 that is similar to levels seen in our tumor models (IRS > 4) was observed in 14% and 11% of the primary patient samples. The dependence of receptor expression upon the activity of PEN-221 leads to the ability to select for patients most likely to benefit from treatment. Because patients with SSTR2-expressing SCLC may achieve greater benefit than those that do not express the receptor and given the variability in the expression observed for SSTR2, a biomarker to identify SSTR2-expressing SCLCs would facilitate the evaluation of PEN-221 efficacy to the appropriate SCLC patient population. Currently, there are two approved imaging agents, ^{68}Ga-DOTATATE and ^{111}In-pentotreotide available to detect SSTR2-expressing tumors, including SCLC. It has also been demonstrated that there is a high level of correlation between SSTR2 staining by IHC and with the ^{68}Ga-DOTATATE imaging (17).

We have demonstrated that PEN-221 is efficacious in various *in vitro* and *in vivo* models. The efficacy of PEN-221 was dose-dependent and was observed using different dosing schedules. In the HCC-33 efficacy study, tumor growth inhibition was observed with low doses, about one sixth (0.33 mg/kg) of the mouse MTD (2.0 mg/kg). This demonstrates a large therapeutic window. Besides inhibiting tumor growth, PEN-221 was capable of driving tumor regressions, even after a single treatment. Furthermore, when compared with a single treatment cycle of Cisplatin/Etoposide, a classic human SOC, in the NCI-H69 model, a single dose of PEN-221 had statistically significant efficacy compared with the SOC.

These data collectively support the hypothesis that PEN-221 actively targets DM1 in an SSTR2-dependent fashion into SCLC tumors, leading to significant efficacy seen in multiple SCLC models. Complete tumor control with regressions, were observed with either repeat dosing at a low dose of PEN-221 or with a single higher dose of PEN-221. The activity observed at doses well below the MTD highlight the potential for a large therapeutic window in the clinical setting. PEN-221 has the capability to penetrate the solid tumor effectively across the whole tumor, highlighting the ability of the miniature drug conjugates' capacity to penetrate the solid tumor effectively. These observations strongly suggest that PEN-221 has the potential to provide significant efficacy in patients with SCLC and especially those with SSTR2-expressing tumors.

Disclosure of Potential Conflicts of Interest

K.A. Whalen is a director at and has ownership interest (including patents) in Tarveda Therapeutics. B.H. White has ownership interest (including patents) in Tarveda Therapeutics. K. Kriksciukaite is a research leader at Tarveda Therapeutics, Inc. and has ownership interest (including patents). A. Brockman has ownership interest (including patents) in Tarveda. B. Moreau is a research leader at and has ownership interest (including patents) in Tarveda Therapeutics. R. Shinde is a scientist at, has ownership interest (including patents) in, and has provided expert testimony for Tarveda Therapeutics. M.T. Bilodeau and R. Wooster are the CSOs at and have ownership interest (including patents) in Tarveda Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: K.A. Whalen, B.H. White, R. Alargova, A. Brockman, J. Gifford, P. Lim Soo, B. Moreau, M.T. Bilodeau, R. Wooster

Development of methodology: K.A. Whalen, B.H. White, J.M. Quinn, R. Alargova, T.P. Au Yeung, A. Brockman, H. Oller, J. Gifford, C.-A. Lemelin, B. Moreau, B. Sweryda-Krawiec, M.T. Bilodeau, R. Wooster

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Whalen, J.M. Quinn, K. Kriksciukaite, T.P. Au Yeung, P. Bazinet, A. Brockman, M.M. DuPont, H. Oller, J. Gifford, S. Perino, G. Sharma, R. Shinde

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Quinn, K. Kriksciukaite, T.P. Au Yeung, P. Bazinet, A. Brockman, M.M. DuPont, H. Oller, J. Gifford, S. Perino, B. Moreau, G. Sharma, R. Shinde, B. Sweryda-Krawiec, R. Wooster

Writing, review, and/or revision of the manuscript: K.A. Whalen, B.H. White, J.M. Quinn, H. Oller, M.T. Bilodeau, R. Wooster

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.H. White, J.M. Quinn, T.P. Au Yeung, M.M. DuPont

Study supervision: K.A. Whalen, J.M. Quinn, M.M. DuPont, J. Gifford, R. Wooster

Other (formulation development of PEN-221): P. Lim Soo

Other (formulation development and formulation stability to support *in vivo* studies to optimize tumor penetration and limited plasma exposure): B. Sweryda-Krawiec

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