A FZD7-specific Antibody–Drug Conjugate Induces Ovarian Tumor Regression in Preclinical Models

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ABSTRACT

Although WNT signaling is frequently dysregulated in solid tumors, drugging this pathway has been challenging due to off-tumor effects. Current clinical pan-WNT inhibitors are nonspecific and lead to adverse effects, highlighting the urgent need for more specific WNT pathway–targeting strategies. We identified elevated expression of the WNT receptor Frizzled class receptor 7 (FZD7) in multiple solid cancers in The Cancer Genome Atlas, particularly in the mesenchymal and proliferative subtypes of ovarian serous cystadenocarcinoma, which correlate with poorer median patient survival. Moreover, we observed increased FZD7 protein expression in ovarian tumors compared with normal ovarian tissue, indicating that FZD7 may be a tumor-specific antigen. We therefore developed a novel antibody–drug conjugate, seputuximab vedotin (F7-ADC), which is composed of a chimeric human–mouse antibody to human FZD7 conjugated to the microtubule-inhibiting drug monomethyl auristatin E (MMAE). F7-ADC selectively binds human FZD7, potently kills ovarian cancer cells in vitro, and induces regression of ovarian tumor xenografts in murine models. To evaluate F7-ADC toxicity in vivo, we generated mice harboring a modified Fzd7 gene where the resulting Fzd7 protein is reactive with the human-targeting F7-ADC. F7-ADC treatment of these mice did not induce acute toxicities, indicating a potentially favorable safety profile in patients. Overall, our data suggest that the antibody–drug conjugate approach may be a powerful strategy to combat FZD7-expressing ovarian cancers in the clinic.

Introduction

The mammalian genome encodes 19 WNT proteins and numerous WNT receptors [including Frizzled (FZD)1–10; LRP5,6; ROR1,2; RYK, and PTK7; ref. 1], and WNT/β-catenin signaling is regulated by the intracellular β-catenin destruction complex, which includes APC (2) and AXIN (3). WNT signaling plays critical roles in embryonic development and adult tissue homeostasis by regulating stem cell renewal and differentiation (reviewed in ref. 4). Consistent with these roles, dysregulation of WNT signaling is frequently observed in human cancers. Examples of mutated or hyperactive WNT pathway components in cancer include, but are not limited to APC and AXIN; transcription factor, β-catenin (5); WNT signaling agonists, RSPO2,3 (6); and E3 ubiquitin ligase, RNF43 (7). RSPOs, RNF43, and ZNRF3, the latter also an E3 ubiquitin ligase, act by stabilizing the abundance of cell-surface receptors that mediate WNT signals. These receptors include FZD1–10, many of which have been implicated in multiple cancer histologies (reviewed in refs. 1, 8).

Pharmacologic efforts to target the WNT pathway in solid tumors have primarily focused on inhibiting WNT receptors or WNT proteins (reviewed in ref. 9). In particular, the WNT receptors FZD1, 2, 5, 7, and 8 were targeted in pancreatic, metastatic breast, and various other untreated solid cancers by OMP-18R5 (vantictumab), a human IgG2 antibody (refs. 10–13; NIH clinical trial numbers: NCT01345201, NCT01957007, NCT01973309, and NCT02005315). Antibody–drug conjugates (ADC) have also been developed to FZD10 (OTS1A101–DTPA; trial NCT04176016; ref. 14) and alternate WNT receptors, ROR1 (VLS-101, NCT03833180, NCT04504916; ref. 15), and PTK7 (PF-06647020; trial NCT03243331; ref. 16). Inhibition of pan-WNT signaling in the clinic has been achieved by two methods: using FZD8 decoy receptor, OMP-54F28 (iparcitept), to sequester extracellular WNT proteins (17–19), or by blocking Porcupine (PORCN), a protein critical to WNT secretion. Iparcitept was used in combination therapies to treat hepatocellular, liver, ovarian, and pancreatic cancers (NCT01608867, NCT02050178, NCT02069145, and NCT02092363). Many small-molecule inhibitors have been developed to inhibit PORCN, thereby inhibiting WNT secretion from cells, including WNT97/LGK974 (NCT01351103, NCT02278133, and NCT02649530), ETC-192159 (ETC-159; NCT02521844), RXC004 (NCT03447470), and CGX1321 (NCT02675946 and NCT03507998). These PORCN inhibitors potently disrupt growth of RSPO3-translocated cancers (20), which have been observed in 10% of colon cancers (6).

Many of these WNT-targeting drugs demonstrated antitumor efficacy in patients but also induced bone-related adverse effects, likely due to the requirement for WNT signaling in bone development and homeostasis (21, 22). Trials using vantictumab (10), iparcitept (19), and ETC-159 (23, 24) were terminated due to concerns over bone-related safety or required concurrent bone-protective therapies. Such...
bone toxicity may be side-stepped through the administration of bone-protective agents, as has been demonstrated for the PORCN inhibitor ETC-159; simultaneous treatment with alendronate, a clinically approved inhibitor of bone resorption, significantly reduced loss of bone mass (25).

An alternative strategy to alleviate bone toxicity is through more specific WNT pathway–targeting strategies. The WNT receptor Frizzled-7 (FZD7) is a strong candidate for such precise targeting due to its restricted expression pattern. FZD7 expression is largely restricted to embryonic development, with absent to modest expression in most normal adult tissues. In contrast, overexpression of FZD7 has been observed in a large number of cancer types, indicating that FZD7-specific targeting strategies may offer opportunities to treat multiple cancer types, including cancers of the stomach (26, 27), colon (28, 29), liver (30, 31), ovary (32, 33), breast (34–36), cervix (37), skin (38), and more (reviewed in refs. 39, 40).

Here, we report the development and therapeutic evaluation of a novel FZD7-targeting ADC, septuximab vedotin (F7-ADC), which consists of a chimeric human–mouse IgG1 antibody conjugated to antimitotic payload drug, monomethyl auristatin E (MMAE). We demonstrate that F7-ADC selectively and potently kills ovarian cancer cells that express high levels of FZD7 in vitro, as well as FZD7-high ovarian tumor xenografts in vivo. Importantly, we did not observe toxicities following F7-ADC treatment in mice expressing a Fzd7 gene that has been modified to render the Fzd7 protein reactive with the human-targeting F7-ADC. Our data suggest that an ADC approach may be a powerful strategy to therapeutically attack FZD7-expressing ovarian cancers in the clinic.

Materials and Methods

Cell lines and culture conditions

All cell lines used in these studies were routinely tested to confirm absence of Mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza). MA-148 (kindly provided by Prof. Sundaram Ramakrishnan, University of Miami, Miami, FL; RRID:CVCL_AK47) and MA-148 FZD7-KO were cultured in RPMI1640, 10% FBS, and 1% penicillin/streptomycin. PA-1 were obtained from ATCC (CRL-1572, RRID:CVCL_0479) and cultured in DMEM, 20% FBS, Glutamax, nonessential amino acids (NEAA), and penicillin/streptomycin. OVCAR-3 (RRID:CVCL_0465) and OVCAR-3 FZD7 were cultured in RPMI1640, 20% FBS, Glutamax, NEAA, and penicillin/streptomycin. OVCAR-3 cell line was obtained from ATCC (HTB-161). HEK293T FZD1,2,7-KO were kindly provided by Dr. Michael Boutros (Harvard University, Boston, MA). OVCAR-3 FZD7 overexpression (OE) cells were generated by transfecting OVCAR-3 cells with pPB–EF1α:FZD7–IRES:H2B-mCherry–BlastR and pCS2-PiggyBac–Transposase.

gRNA sequence #1 for FZD7-KO: GGGCGCTCCGCTTTGCT-CCC
gRNA sequence #2 for FZD7-KO: GGGCATGAGAAGGGGAA-GG

Expression vectors and cloning

7TGC (RRID:Addgene_24304); Cas9-EGFP and pGRNA (kindly provided by Dr. Chad Cowan, Harvard University, Boston, MA; pcDNA3.4-TOPO FZD7 IgG1 antibody (F7-Ab) heavy chain; pcDNA3.4-TOPO F7-Ab light chain; pcPS2-PiggyBac–Transposase; pLVX-Luciferase-PuroR (kindly provided by Drs. Ian Huggins and Steve Dowdy, UC San Diego, San Diego, CA); PBP–EF1α:FZD7–IRES: H2B-mCherry–BlastR.

ADC synthesis

F7-Ab-MMAE drug conjugate was synthesized using methods previously described (41, 42). A solution (2 mL, 10.2 mg/mL) of F7-Ab was treated with sodium bicarbonate buffer (200 μL, 1 mol/L pH 8.3) and sodium diethylentriaminepentaacetic acid (20 μL, 100 mmol/L pH 7). Following reduction with 4 equivalents of tris(carboxyethyl)phosphine (TCEP) at 37°C for 2 hours, the solution was added to four equivalents of maleimidocaproyl-valine-citrulline-PABC-MMAE (MC-VC-PABC-MMAE, Levena Biopharma). After 30 minutes at room temperature, Cy5-maleimide (2 equivalents) was added and after another 30 minutes, gel-filtered (Sephadex G25, 1.0 g) eluting with phosphate buffered saline (PBS). Following centrifugal concentration (Centricon 30 kDa MWCO) to about 500 μL, the concentrations of antibody and Cy5 were determined by absorbance using extinction coefficients of 251,000 M⁻¹ cm⁻¹ (F7-Ab) at 280 nm and 12,500 M⁻¹ cm⁻¹ and 250,000 M⁻¹ cm⁻¹ at 280 nm and 650 nm respectively, for Cy5. Drug loading was measured by denaturing reverse-phase HPLC of the reaction mix prior to addition of Cy5 maleimide, following reduction of any remaining intersubunit disulfides with 50 mM/L DTT for 30 minutes. Peaks corresponding to light or heavy chains with 0–3 MMAE were identified by electro-spray mass spectroscopy and peak areas at 280 nm were integrated and weighted to calculate the drug loading. Modified light chain (L1) and unmodified H chain (H0) were not resolved so MMAE loading is an underestimate. No free MC-VC-PABC-MMAE was detected by HPLC following gel filtration.

Flow cytometry

Cells were dissociated using Accutase (Innovative Cell Technologies, RRID:AB_2869384), pelleted, resuspended in FACS buffer (PBS, 5 mmol/L EDTA, and 2% FBS), and passed through a 40-μm cell strainer (Corning). 1×10⁵ cells were incubated on ice for 30 minutes with the indicated concentrations of F7-ADC or F7-Ab-Alexa Fluor 647. Cells were washed with 3 mL of FACS buffer, pelleted, and resuspended in FACS buffer with 0.5 μg/mL DAPI (Cell Signaling Technology). Cells were analyzed on an LSRFortessa (BD Biosciences) and FSC files were processed using FlowJo (BD Biosciences).

qRT–PCR

qRT–PCR methods and primer sequences (FZD7, RPL13A) were used as previously described (43).

Confocal live imaging

Imaging of ADC internalization was performed as previously described (41). In brief, cells were seeded on a glass-bottom plate for imaging. Cells were washed with PBS, then treated with a vehicle control (PBS) or F7-ADC in 1% PBS media at 37°C for 3 hours. Cells were washed with PBS and replenished with regular 10% FBS media and 50 mmol/L Lysotracker Green (Invitrogen). Live imaging was performed for 1 hour after media replenishment on a Nikon Eclipse Ti2-E or Nikon CSU-W1 microscope with a Plan Apo Lambda 60 × 1.4NA objective.
Cytotoxicity assay

Cells were seeded in a 96-well plate the day before drug treatment. MA-148, PA-1, and MA-148 FZD7-KO were incubated with the indicated drugs for 3 days. OVCAR-3 and OVCAR-3 FZD7 were incubated for 5 days due to slower doubling time compared with the other three cell lines. Drug-containing media were then removed, replaced with 100 µL RPMI1640 and 100 µL of CellTiter-Glo2.0 per well, and incubated in the dark for 25 minutes. CellTiter-Glo2.0 luciferase readouts were performed on a Promega GloMax Discover Microplate Reader.

Fzd7<sup>WT/Ft</sup> mouse line generation

Fzd7<sup>WT/Ft</sup> mice were generated by standard CRISPR/Cas9 methods by Transgenic Service at UC San Diego (UCSD, San Diego, CA) and in compliance with UCSD Institutional Animal Use and Care Committee (IACUC). C57BL/6/NHsd mouse embryos were injected with a Fzd7 repair oligo, Fzd7 guide RNA (gRNA), and Cas9 mRNA (Invitrogen). The repair oligo was synthesized by Integrated DNA Technologies. The in vitro transcribed gRNA was generated using a MEGAscript T7 Transcription Kit (Thermo Fisher).

Fzd7 repair oligo (underlined: new BgiII site; bolded: changed nucleotides):

\[
\text{GAGCGTCCGGGGGCGGCAGTCCCACCGCCTTACCTTTCA-} \\
\text{TCCCTTCTCACGATCGAGTCTACCTTTCACTGGATGTCCGCGC-
\text{CTGCGATGTCCCCTCAGATGGCAGAGGCCGTCTTGTTTCTCCTTCTGCTGCGCCGC}
\]

Fzd7 guide RNA (Underlined: PAM sequence): GGGGACATCG-TACCTTTCA-TCCCTTCTCACGATCGAGTCTACCTTTCACTGGATGTCCGCGC-

MEF generation

All animal studies have been conducted in accordance with, and with the approval of, an IACUC (protocol number S05387, PI: K. Willert). MEFs were generated from decapitated E13.5 embryos. 7–8 embryos per genotype were minced in warm trypsin for 10 minutes, washed with trypsin, and pipetted up and down. DNase (Promega) was added to the embryo suspension, pipetted up and down three times, and incubated at 37°C for 5 minutes. MEF medium was added to the suspension, pipetted up and down, and centrifuged at 1,000 × g. Supernatant was removed and the dissociated embryo pellet was resuspended in MEF medium and plated for cell expansion.

Immunoblot

Immunoblot methods and antibody dilutions were used as previously described (43).

Wnt reporter (TOP-GFPP) assay

Primary MEFs at passage 3 (P3) were transduced with third-generation lentivirus containing 7TGC (Addgene 24304) and then passaged. P4 MEFs were treated with Wnt3a storage buffer (PBS, 1% CHAPS, 1 mol/L NaCl, 5 mmol/L Wnt3a, or 5 mmol/L F7L6. Cells were dissociated using Accutase, pelleted, resuspended in FACS buffer with 0.5 µg/mL DAPI (Cell Signaling Technology), and passed through a 40-µm cell strainer (Corning). Cells were analyzed on an LSRFortessa (BD Biosciences) and FSC files were processed using FlowJo (BD Biosciences).

In vivo tumor xenograft response studies

All animal studies have been conducted in accordance with, and with the approval of, an IACUC (protocol number S05387, PI: K. Willert). Six-week-old female nude mice were purchased from the UC San Diego Animal Care Program breeding colony. MA-148-Luciferase (Luc), PA-1-Luciferase, or MA-148 FZD7-KO-Luciferase cells were injected in the bilateral upper thigh to establish subcutaneous tumors. For MA-148-Luciferase or MA-148 FZD7-KO-Luciferase tumors, 3 × 10<sup>6</sup> cells were suspended in 100 µL of a 1:1 mix of Matrigel and 10% FBS media. For PA-1-Luciferase tumors, 5 × 10<sup>6</sup> cells were suspended in 100 µL of a 1:1 mix of Matrigel (BD Biosciences) and 10% FBS media. Tumor bioluminescence was measured by an IVIS Spectrum (Perkin Elmer) after intraperitoneal injection of Xenolight D-luciferin (Perkin Elmer; 200 µL of a 15 mg/mL stock). Tumor bioluminescence was normalized to background bioluminescence of a non–tumor-bearing mouse injected with luciferin. Mice were randomized into groups once normalized tumor bioluminescence (radiance) reached at least 1 × 10<sup>7</sup> photons/second. In Vivo Imaging System (IVIS) analyses were performed using Living Image software (Perkin Elmer). Treatments were delivered twice per week by bilateral tail vein injection. To prevent unnecessary morbidity, mice were euthanized if tumor length exceeded 15 mm by caliper measurement.

In vivo toxicity study

All animal studies have been conducted in accordance with, and with the approval of, an IACUC (protocol number S05387, PI: K. Willert). Twelve-week-old Fzd7<sup>WT/Ft</sup> and Fzd7<sup>WT/Ft</sup> mice were treated with F7-ADC (10 mg/kg) by bilateral tail vein injection. Seven days after injection, mice were euthanized and tissues collected. All tissues were fixed in 4% paraformaldehyde (PFA) for 24 hours at 4°C. Brains were transferred to a 30% sucrose solution and all other tissues were transferred to 70% ethanol (EtOH) and stored at 4°C. Blood samples were analyzed by ELISA as described below.

Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining was done by UCSD Tissue Technology Shared Resource (TTSR) on a Thermo Gemini AS Stainer. Tissues were baked at 60°C for 1 hour and transferred to solutions in the following order: Clearite-3 (Thermo Fisher), 100% EtOH, 95% EtOH, diH<sub>2</sub>O, hematoxylin (Thermo Fisher), diH<sub>2</sub>O, Clarifier 1 (Thermo Fisher), diH<sub>2</sub>O, Bluing Solution (Thermo Fisher), diH<sub>2</sub>O, 70% EtOH, Eosin-Y (Thermo Fisher), 70% EtOH, 95% EtOH, 100% EtOH, Clearite-3, mounting medium (VWR).

IHC

OV801a ovarian cancer tissue array with matched or unmatched adjacent normal tissue was purchased from US Biomax. IHC was done by UCSD TTSR. Tumor samples were fixed in 4% PFA at 4°C, and transferred to 70% EtOH. Tissues were baked at 60°C for 1 hour, then cleared and rehydrated through successive solutions: xylene, 100% EtOH, 95% EtOH, 70% EtOH, diH<sub>2</sub>O, hematoxylin (Thermo Fisher), diH<sub>2</sub>O, Clarifier 1 (Thermo Fisher), diH<sub>2</sub>O, Bluing Solution (Thermo Fisher), diH<sub>2</sub>O, 70% EtOH, Eosin-Y (Thermo Fisher), 70% EtOH, 95% EtOH, 100% EtOH, Clearite-3, mounting medium (VWR).
Mayer Hematoxylin (Sigma) for 5 minutes, TBST washes, diH₂O washes, cleared, and mounted in xylene-based mounting media.

ELISA
Detection of human IgG in mouse serum samples was performed using the IgG (Total) Human Uncoated ELISA Kit (Invitrogen) according to manufacturer’s protocol.

Statistical analyses
Details on statistical tests used for each experiment are provided in corresponding figure legends.

The Cancer Genome Atlas analyses
RNA-sequencing (RNA-seq) counts [transcripts per million (TPM) normalized] were obtained from ref. 44. The median TPM value of each gene in each cancer type was computed and log₂-normalized. The median TPM value of FZD7 RNA and protein expression are elevated in ovarian carcinomas.

To evaluate expression of FZD7 across human cancers, we interrogated bulk RNA-seq patient datasets in The Cancer Genome Atlas (TCGA; Fig. 1A). We found elevated median FZD7 expression in breast invasive carcinoma (BRCA), glioblastoma multiforme (GBM), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), prostate adenocarcinoma (PRAD), and uterine cancer (UCS) studies. We then further analyzed TCGA OV and BRCA datasets. We separated OV samples into four subtypes defined by “Classification of Ovarian Cancer” (CLOVAR), a prognostic model for high-grade serous ovarian carcinoma (45). Median FZD7 expression was higher in the mesenchymal and proliferative subtypes (Fig. 1B), which correlated with a median survival of ~36 months in patients (45). In contrast, median survival for the differentiated and immunoactive subtypes was approximately 48 months (45). We also separated BRCA samples based on reported histopathology status and identified higher FZD7 expression in triple-negative breast cancer (Supplementary Fig. S1), which also has been associated with poor prognosis (that is, decreased disease-free survival and overall survival; ref. 46). In addition, we analyzed FZD7 protein expression by IHC and observed higher levels in ovarian carcinomas compared with normal ovarian tissues (Fig. 1C), indicating that FZD7 may represent a tumor-specific antigen. By mining TCGA data and performing histologic analyses, we confirmed that FZD7 is a viable candidate for targeted cancer therapies against the Wnt pathway and importantly for clinically aggressive subtypes of ovarian and breast cancer.

ADC septuximab vedotin binds FZD7
To target human FZD7, we developed an ADC, septuximab vedotin, hereafter referred to as F7-ADC. We previously demonstrated that the chimeric human–mouse IgG1 antibody to FZD7 (F7-Ab) binds FZD7 and does not cross-react with the other nine human FZD receptors, FZD1(−6, 8–10) (43, 47). F7-ADC consists of F7-Ab conjugated to the antimitic drug MMAE, by cleavable MC-VC-PABC linkers (Fig. 2A). An average of four MMAE molecules were conjugated to cysteine residues in the antibody hinge region, following reduction of disulfides (41, 42). For noninvasive F7-ADC tracking, we also conjugated a Cy5 fluorophore to a hinge region disulfide via a noncleavable maleimide linker. MMAE drug loading was measured by denaturing reverse-phase HPLC and electro-spray mass spectroscopy (Supplementary Fig. S2). By flow cytometry, we confirmed that F7-ADC bound to FZD7-high (MA-148, PA-1, and OVCAR-3 FZD7) and FZD7-low (OVCAR-3) human cancer cell lines, but did not bind MA-148 FZD7-knockout (KO) cells (Fig. 2B and C). FZD7 RNA levels in these cell lines directly correlated with protein levels (Fig. 2D). These data demonstrate that F7-ADC specifically bound human cancer cells expressing FZD7.

Septuximab vedotin selectively targets and kills FZD7-expressing cells in vitro
ADCs constructed with MC-VC-PABC linkers function by binding cell membrane receptors which are then internalized into lysosomes of target-expressing cells. Within lysosomes, cathepsin B cleaves the valine-citrulline dipeptide of the MC-VC-PABC linker followed by PABC self-immolation and release of the drug (48). To confirm that F7-ADC internalized to the lysosomes of FZD7-expressing cancer cells, we treated FZD7-positive and FZD7-negative cells with a vehicle control (PBS) or F7-ADC and performed confocal live imaging. We observed colocalization of F7-ADC and lysosomes specifically in FZD7-high MA-148, PA-1, and OVCAR-3 FZD7 cells, but not in the MA-148 FZD7-KO cells (Fig. 3A).

Next, we tested F7-ADC-specific cytotoxicity in vitro (Fig. 3B). A single dose of F7-ADC killed MA-148 and PA-1 cells at an IC₅₀ of 5 nmol/L (0.76 µg/mL), and OVCAR-3 FZD7 cells at an IC₅₀ of 0.025 nmol/L (3.9 ng/mL). The IC₅₀ for OVCAR-3 and MA-148 FZD7-KO cells were 25 nmol/L (3.9 µg/mL) and 60 nmol/L (9.6 µg/mL), respectively. At concentrations tested, treatment with unconjugated F7-Ab did not affect the viability of cells, suggesting that the naked F7-Ab does not interfere with FZD7 functions critical to cell survival. These results established a therapeutic window in which F7-ADC specifically internalized and killed FZD7-expressing cells in vitro.

Septuximab vedotin induces regression of FZD7 tumors in vivo
To test in vivo efficacy of the human-targeting F7-ADC, we utilized xenograft tumor models. We established MA-148, PA-1, or MA-148 FZD7-KO subcutaneous tumors expressing a luciferase reporter, in athymic nude mice. Tumor volume was measured by bioluminescence detection using an IVIS. Mice were treated twice per week with a vehicle control or F7-ADC (3 mg/kg; ~0.5 nmol/L) by intravenous (tail vein) injection. In MA-148-Luciferase and PA-1-Luciferase tumors, F7-ADC treatment resulted in significant tumor regression (Fig. 4A, B, and D). In contrast, F7-ADC had no therapeutic effect on MA-148 FZD7-KO-Luciferase tumor growth because they continued
to grow exponentially (Fig. 4C), similarly to the vehicle-treated tumors. IHC at the conclusion of the study showed a marked reduction in staining of the proliferation marker Ki67 in MA-148 and PA-1 tumors, but not in MA-148 FZD7-KO tumors (Supplementary Fig. S3).

Generation of Fzd7hf7/hF7 mice

We previously identified the extracellular epitope on human FZD7 to which our antibody (F7-Ab) binds (43), mapping to the linker region between the ligand binding cysteine rich domain and the first transmembrane domain of FZD7. Moreover, we found that a single amino acid change (proline to leucine at position 188, P188L) in mouse Fzd7 rendered Fzd7 reactive with F7-Ab. We used CRISPR-Cas9 to engineer the P188L mutation in mouse Fzd7 (Fig. 5A and B). The resulting mouse line, Fzd7hF7/hF7, expresses Fzd7P188L receptors that are recognized by F7-Ab (Fig. 5C). Fzd7P188L mutant mice are viable, fertile, and do not exhibit any of the phenotypic abnormalities previously described in Fzd7 knockout mice, including tail truncation and kinking (49), suggesting that the Fzd7P188L allele produces a functional Fzd7P188L protein.

To establish that Fzd7P188L is functional, we tested its ability to transduce Wnt signaling in vitro. We derived MEFs from E13.5 Fzd7+/+ (wild type) and Fzd7hF7/hF7 embryos and transduced them with Wnt reporter, 7TGC (TOP-GFP:SV40-mCherry; ref. 50). To selectively activate Fzd7P188L, we used a previously described WNT mimetic, F7L6 (43), which only interacts with human FZD7. MEFs carrying the 7TGC reporter were treated with a Wnt buffer control, F7L6, or Wnt3a. We quantified reporter GFP activity from mCherry-expressing MEFs using flow cytometry and confirmed that Fzd7hF7/hF7 mouse line expressing Fzd7P188L receptors retain Wnt signaling capability.

Septuximab vedotin does not induce toxicity in Fzd7hF7/hF7 mice

To evaluate for potential toxicities induced by human-FZD7-targeting F7-ADC, we tested F7-ADC in Fzd7hF7/hF7 mice. On day 0, we gave a single tail vein injection of F7-ADC (10 mg/kg) in 12-week-old females and males of Fzd7hF7/hF7 and Fzd7+/+ genotype. Uninjected control mice for each sex and genotype were also
included (Table 1). Fzd7+/− mice were used as additional negative controls since F7-ADC does not recognize wild-type mouse Fzd7. Neither F7-ADC–treated Fzd7+/− nor Fzd7+/+ mice exhibited changes in activity levels or grooming behavior 2 hours postinjection and over the following 7 days, compared with uninjected control mice (Table 1; Supplementary Fig. S4). In addition, we did not observe diarrhea or concerning changes in weight between days 0 and 7. To determine whether the high dose of F7-ADC induced damage at the tissue level, we euthanized the mice on day 7 postinjection at which time brain, eye, heart, kidney, liver, ovary or

Figure 2.

ADC septuximab vedotin binds FZD7. A, Schematic of ADC, septuximab vedotin (F7-ADC). MC-VC-PABC-MMAE and Cy5 are conjugated to the IgG1 antibody at cysteine (cys) residues. B, F7-ADC selectively binds FZD7-expressing human cancer cell lines, MA-148, PA-1, OVCAR-3, and OVCAR-3 FZD7. F7-ADC does not bind MA-148 FZD7-KO. Live cells were stained with F7-ADC at the indicated concentrations and analyzed by flow cytometry. C, FZD7 protein expression represented as mean fluorescence intensity (MFI). Live cells were stained with F7-Ab and analyzed by flow cytometry. D, FZD7 expression by qRT-PCR and normalized to HEK293T (HEK) FZD1,2,7-KO. Data represented as mean ± SEM for three technical replicates.
testis, skin, small intestine, and spleen were harvested. Sections of the organs from Fzd7+/+ mice were H&E stained and reviewed digitally from 2× to 40× magnification. No histopathologic abnormalities were noted in any organs, specifically no areas of necrosis, fibrosis, calcification, increased inflammatory infiltrates, edema, dysplastic changes or carcinoma were detected (representative images of the intestine, ovary, skin, and liver in Supplementary Fig. S4). Importantly, we did not observe damage to the crypt or villi structures within the small intestine, where Fzd7 is expressed in Lgr5+ stem cells (51, 52). Furthermore, presence of human IgG in serum from all mice at the time of euthanasia was confirmed using quantitative ELISAs (Supplementary Fig. S5).
Discussion

Although aberrant WNT signaling is observed in many solid tumor histologies, pharmacologically drugging the WNT pathway for therapeutic gain has proved to be a clinical challenge. Most WNT-targeting therapies aimed to globally inhibit WNT signaling, frequently resulting in bone-related adverse effects in patients. In an effort to mitigate toxicities associated with WNT inhibition, we developed an ADC, septuximab vedotin (F7-ADC), to target and kill human cancer cells expressing WNT receptor, FZD7. In bulk RNA-seq analyses of TCGA...
datasets, we found elevated median FZD7 expression in BRCA, GBM, LUSC, OV, prostate adenocarcinoma (PRAD), and uterine carcinosarcoma (UCS) studies. Upon closer inspection of TCGA BRCA and OV samples, we identified higher FZD7 expression in triple-negative breast cancer and mesenchymal and proliferative ovarian cancer subtypes, all of which correlate with poorer overall survival. In addition, we observed high FZD7 protein expression in ovarian carcinomas and low FZD7 in normal ovary tissues by IHC, indicating
FZD7 could serve as a tumor-specific antigen. By mining TCGA data and performing histologic analyses, we validated that FZD7 is a viable candidate for targeted cancer therapies against the WNT pathway.

F7-ADC is a chimeric human–mouse IgG1 antibody conjugated by MC-VC-PABC linkers to four MMAE molecules, on average. We confirmed that F7-ADC selectively binds FZD7 on human cancer cells, then internalized to the lysosomes of target cells, where cathepsin B cleaves the linkers and releases the payload drug, MMAE. F7-ADC treatment of FZD7-high ovarian cancer cells, such as MA-148 and PA-1, induced direct cytotoxicity. F7-ADC treatment of FZD7-overexpressing Fzd7hF7/hF7 mice, which we engineered to express Fzd7 receptors carrying the P188L mutation necessary for F7-ADC binding. The lack of toxicity even in tissues known to express Fzd7, such as the small intestine (51), is possibly due to low expression levels, because tumor cell lines with low FZD7 levels (e.g., OVCAR-3) tolerated high F7-ADC concentrations in vitro and tumor xenograft regression in nude mouse models. Furthermore, high-dose F7-ADC treatment did not result in toxicities at the organism or tissue level in Fzd7hF7/hF7 mice, which we engineered to express Fzd7 receptors carrying the P188L mutation necessary for F7-ADC binding. In a previous study, we showed that an antigen binding fragment (Fab) of F7-Ab reduced Wnt3a signaling in human embryonic stem cells only when cells were pre-incubated with the Fab (47). Simultaneous addition of Wnt3a and Fab had no detectable effect on Wnt signal transduction, indicating that Fab binding to the linker region did not interfere with Wnt3a signaling. Vantictumab, on the other hand, binds the CRD and effectively blocks WNT binding and signaling (11). Additionally, vantictumab’s tumor inhibitory effects may be attributable to its ability to bind multiple FZD7 receptors (FZD1, 2, 5, 7 and 8) that singly or collectively may play crucial roles in tumorigenesis.

In recent years, ADCs have emerged as a powerful modality to target a variety of cancers, with an increasing number of ADCs receiving approval for oncotherapies (reviewed in (53, 54)). This current study highlights the potential for septuximab vedotin as a novel treatment of ovarian cancer. Current treatment options for ovarian cancer are limited, and standard of care involves aggressive surgical efforts to debulk the cancer and neoadjuvant chemotherapy. Critical to the development of this ADC will be the identification of suitable biomarkers for the early detection of ovarian cancer.

Our preclinical F7-ADC data demonstrates therapeutic efficacy and exquisite specificity to FZD7 in vitro and in vivo with no toxicity in the Fzd7P188L/hF7 mouse model. By targeted killing of FZD7-positive cancer cells, rather than globally inhibiting WNT signaling, we predict that the bone-related adverse events frequently associated with WNT-targeting drugs will be mitigated. Moreover, FZD7 expression has been implicated in multiple cancers with poor patient prognoses. In ovarian cancer, FZD7 expression correlated with resistance to platinum-based chemotherapy, tumor initiation, and cancer stem cell proliferation (55, 56). In additional examples, FZD7 was associated with epithelial-mesenchymal transition in triple-negative breast cancer (57), cisplatin and paclitaxel resistance in uterine cancer (58), and tumor proliferation in gastric cancer (26). As such, the F7-ADC approach may provide a powerful biomarker-driven therapy to combat a variety of aggressive solid tumors overexpressing FZD7.

Table 1. Summary of septuximab vedotin toxicity study in Fzd7hF7/hF7 mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>% Weight change</th>
<th>Brain</th>
<th>Eye</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Ovary or testis</th>
<th>Skin</th>
<th>Small intestine</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fzd7hF7/hF7</td>
<td>F</td>
<td>None</td>
<td>-1.4%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADC</td>
<td>-1.4%</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>M</td>
<td>None</td>
<td>+3.3%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>-3.2%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADC</td>
<td>-3.7%</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>M</td>
<td>None</td>
<td>-0.8%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>-2.3%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADC</td>
<td>-1.9%</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Note: Fzd7hF7/hF7 and Fzd7+/+ mice were untreated or given a single intravenous dose of F7-ADC (10 mg/kg). Mice were weighed prior to injection (day 0) and seven days after (day 7). H&E staining was performed on the indicated organs harvested on day 7. The study was blinded for histopathologic analyses and no tissue damage was detected.
Authors’ Disclosures

C. Patel reports personal fees from Roche outside the submitted work. J.P. Mesirov reports grants from NIH/NCI during the conduct of the study. D.A. Carson reports a patent for US 10,766,962 B2; issued September 8, 2020 issued. S.J. Advani reports grants from NIH during the conduct of the study. K. Willert reports grants from NIH/NIGMS and grants from The Mary Kay Foundation during the conduct of the study; in addition, K. Willert has a patent for US 10,766,962 B2 issued. No disclosures were reported by the other authors.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The authors declare no competing financial interests.

Authors’ Contributions

M. Do: Conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft. C.C.N. Wu: Data curation, investigation, methodology. P.R. Sonavane: Investigation, methodology. E.P. Iaizere: Data curation, software. S.R. Adams: Resources, methodology. J. Ross: Investigation, methodology. A. Rodriguez y Baena: Investigation. C. Patel: Data curation, formal analysis. J.P. Mesirov: Software, formal analysis, funding acquisition. D.A. Carson: Conceptualization, supervision, funding acquisition, writing—review and editing. S.J. Advani: Resources, funding acquisition, methodology, writing—review and editing. K. Willert: Conceptualization, resources, supervision, funding acquisition, investigation, project administration, writing—review and editing.

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