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Large Molecule Therapeutics

Molecular Cancer Therapeutics

Development of a Novel Antibody–Drug Conjugate for the Potential Treatment of Ovarian, Lung, and Renal Cell Carcinoma Expressing TIM-1

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Abstract

T-cell immunoglobulin and mucin domain 1 (TIM-1) is a type I transmembrane protein that was originally described as kidney injury molecule 1 (KIM-1) due to its elevated expression in kidney and urine after renal injury. TIM-1 expression is also upregulated in several human cancers, most notably in renal and ovarian carcinomas, but has very restricted expression in healthy tissues, thus representing a promising target for antibody-mediated therapy. To this end, we have developed a fully human monoclonal IgG1 antibody specific for the extracellular domain of TIM-1. This antibody was shown to bind purified recombinant chimeric TIM-1-Fc protein and TIM-1 expressed on a variety of transformed cell lines, including Caki-1 (human renal clear cell carcinoma), IGROV-1 (human ovarian adenocarcinoma), and A549 (human lung carcinoma). Internalization studies using confocal microscopy revealed the antibody was rapidly internalized by cells *in vitro*, and internalization was confirmed by quantitative imaging flow cytometry. An antibody–drug conjugate (ADC) was produced with the anti-TIM-1 antibody covalently linked to the potent cytotoxin, monomethyl auristatin E (MMAE), and designated CDX-014. The ADC was shown to exhibit *in vitro* cytostatic or cytotoxic activity against a variety of TIM-1–expressing cell lines, but not on TIM-1–negative cell lines. Using the Caki-1, IGROV-1, and A549 xenograft mouse models, CDX-014 showed significant antitumor activity in a clinically relevant dose range. Safety evaluation in nonhuman primates has demonstrated a good profile and led to the initiation of clinical studies of CDX-014 in renal cell carcinoma and potentially other TIM-1–expressing tumors. *Mol Cancer Ther; 15(12); 2946–54. ©2016 AACR.*

Introduction

T-cell immunoglobulin and mucin domain–containing protein 1 (TIM-1) is a type I transmembrane-containing glycoprotein with an IgV-set domain and a mucin domain with O-linked glycosylation. TIM-1 has been separately discovered and investigated from three different perspectives. TIM-1 can be expressed on activated T cells, preferentially on Th2 cells, where costimulation with T-cell receptor activation led to T-cell proliferation and IL4 production and played an immunoregulatory role in atopy in mouse studies (1). In addition, TIM-1 expression on human T cells is associated with the regulation of immune responses (2). TIM-4 expressed on antigen-presenting cells (3) and phosphatidylserine (4) have been reported as ligands for TIM-1 that potentially mediate these activities. TIM-1 has also been reported to be functional on regulatory B cells and dendritic cells in mice (5–7). However, the effects of TIM-1 on immune responses remain unclear, sometimes contradictory, and potentially dependent on the specificity and affinity of the various agonist and antagonist antibodies and soluble TIM-1 proteins used in these studies (8).

The TIM-1 glycoprotein was reported to be human hepatitis A virus cellular receptor-1 (HAVcr-1; ref. 9), and a polymorphism in TIM-1 is associated with susceptibility to severe hepatitis A virus infection in humans (10). TIM-1 was also identified as a receptor for Ebola virus and Marburg virus on certain epithelial cells with evidence of specific binding to the viral glycoprotein of Ebola virus (11), although subsequent evidence implicated phosphatidylserine binding in the attachment and entry of these and other enveloped viruses (12). At this time, it appears multiple mechanisms may contribute to the role of TIM-1 in virus entry into cells.

The TIM-1 glycoprotein has also been described as kidney injury molecule-1 (KIM-1), which is absent in normal kidney and urine but upregulated on proximal tubular epithelial cells and shed into urine in various renal injuries, including postischemic injury, nephrotoxicant renal injury, including drug-related toxicities, acute tubular necrosis, diabetic nephropathy, IgA nephropathy, membranoproliferative glomerulonephritis, systemic lupus erythematosus, polycystic kidney disease, acute and chronic allograft rejection, and others (13, 14). TIM-1 has been shown to be a biomarker for human and animal renal proximal tubule injury (15), and because the ectodomain of TIM-1 is shed into the urine following proximal tubule injury, the molecule has found favor as

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a biomarker of renal injury in many species. As a phosphatidylserine receptor, TIM-1 has been reported to mediate phagocytosis of apoptotic cells by injured kidney epithelial cells (4). Chronic conditional expression of TIM-1 in mouse renal epithelial cells was shown to cause spontaneous and progressive kidney inflammation with fibrosis, analogous to progressive kidney disease in humans (16). On the other hand, using mice expressing TIM-1 mutants that lack the mucin domain and thus exhibiting impaired phagocytosis, it was shown that phagocytosis mediated by TIM-1 actually reduced acute kidney injury (17).

TIM-1 expression is upregulated in several human cancers, most notably in renal cell carcinoma (RCC), including metastatic RCC, and ovarian clear cell carcinoma (15, 18). Furthermore, TIM-1 expression is associated with a more malignant phenotype of RCC, and shedding of the ectodomain has been associated with tumor progression (19, 20). Overexpression of TIM-1 in clear cell RCC (ccRCC) lines activated the IL6/STAT-3/HIF-1A pathway apparently dependent on TIM-1 shedding, and phosphorylation of serine 727 of STAT-3 in ccRCC patients was correlated with clinical outcome (21).

Expression of TIM-1 in nonmalignant tissues appears quite limited in humans. Expression of TIM-1 has been reported on activated human T cells (2, 22), as well as on the apical surface of trachea and the basal layer of cornea and conjunctiva (11). TIM-1 shedding and expression on proximal tubular epithelial cells associated with various types of kidney injury, along with its absence in healthy renal tissues, is currently perhaps the most fully characterized expression of the human protein in nonmalignant tissues. Thus, TIM-1 may serve as an attractive target for antibody-mediated therapy in certain cancers.

In recent years, antibody–drug conjugates (ADCs) have been validated as a valuable drug class with the recent approvals of trastuzumab emtansine (Genentech) for HER2-positive breast cancer and brentuximab vedotin (Seattle Genetics) for Hodgkin lymphoma. To potentially exploit the strong association of TIM-1 expression with certain clear cell carcinomas and other malignancies, we have constructed an ADC using a fully human mAb IgG1κ (clone 2.70.2) specific for TIM-1 covalently linked to the enzyme-cleavable valine-citrulline-p-aminobenzylcarbamate-monomethylauristatin-E (vcMMAE), a potent microtubule inhibitor when cleaved (Supplementary Fig. S1). The studies reported here describe the characterization, including *in vitro* cytotoxicity and *in vivo* tumor models of the anti-TIM-1 mAb 2.70.2 IgG1κ-vcMMAE (designated CDX-014).

Materials and Methods

Generation of CR014, human anti-TIM-1 mAb clone 2.70.2

Fully human mAbs directed against the TIM-1 extracellular domain were generated by immunization of human Ig–expressing mice (XenoMouse) as described previously (23). Briefly, the human IgG4–bearing XenoMouse strain was immunized twice weekly with 10 μg of recombinant extracellular domain of human TIM-1 genetically fused with a V5 His-Tag (designated TIM-1-ECD). Hybridomas were generated by electro-cell fusion. Cell line supernatants were screened for antibodies reactive with TIM-1-ECD by ELISA, and positive hybridomas were cloned. Antibodies were purified from hybridoma supernatant by protein A chromatography. Several high-affinity hybridomas were selected for subcloning. Purified mAbs were evaluated for affinity by binding to TIM-1-ECD–coated plates by ELISA.

Generation of the IgG1 version of mAb 2.70.2 and manufacture of the ADC

From the panel of human anti-TIM-1 mAbs generated, the mAb clone 2.70.2 was chosen for further development, sequenced, and an IgG1κ mAb was generated. The variable light and heavy chain regions from anti-TIM-1 mAb clone 2.70.2 were synthesized (GenScript) with appropriate restriction sites for cloning into expression vectors pEE12.4 and pEE6.4 (Lonza, GS System). The variable light chain was cloned, in frame, into expression vector pEE12.4 containing the human kappa light chain constant region and the variable heavy chain was cloned, in frame, into expression vector pEE6.4 containing the human IgG1 heavy chain constant region. Unique restriction sites present in each expression vector (Not I and Pvu I) were utilized to clone the light chain and heavy chain expressing operons and create a double gene expression vector. A fully human IgG1 antibody was prepared using this expression vector and designated CDX-014 mAb.

The CDX-014 mAb intermediate (2.70.2 IgG1κ, also referred to as CR014), used for the manufacture of the ADC, was produced by Celldex under GMPs. CDX-014 mAb was expressed in recombinant Chinese hamster ovary cells (clone 6F2) and purified using standard methods. The CDX-014 ADC drug substance (or simply "CDX-014") was manufactured by Lonza AG under GMPs as follows. The sulfhydryl-reactive maleimidolcaproyl-valine-citrulline-p-aminobenzyloxycarbonyl-monomethyl auristatin E (mc-vcMMAE; CAS number 646502-53-6) drug cross-linker combination was manufactured by chemical synthesis by SAFC. The CDX-014 mAb was partially reduced with tris-(2-carboxyethyl-phosphine) hydrochloride, followed by reaction with mc-vcMMAE to yield the ADC. The drug-to-antibody (DAR) molar ratio was approximately 4.5.

Cell lines

Caki-1 (ATCC HTB-46, received 2010; human renal clear cell carcinoma), SK-MEL-2 (ATCC HTB-68, received 2010; human melanoma), and A549 (ATCC CRM-CCL-185, received 2013; human lung carcinoma) cells were obtained from the ATCC. IGROV-1 (human ovarian adenocarcinoma) cells were obtained from the NCI Frederick Cancer DCTD Tumor/Cell Line Repository (received 2012). A cell bank was produced and cell cultures were passaged for less than 6 months before reculturing from banked vials.

Binding affinity determination

The determination of binding affinity of CDX-014 mAb to the human peptide epitope (PMPLPRQNHE) and to the TIM-1 sequence variants found in nonhuman primates (PMPLPMQNHE and PMPLPTQNHE) utilized synthetic peptides with these various sequences, each with an appended N-terminal biotin-SGSG sequence and C-terminal amide. A streptavidin-coated ELISA plate was coated with biotinylated human and monkey TIM-1 peptides at 1 μg/mL. CDX-014 mAb was serially diluted and added to the wells with the TIM-1 peptides. As a negative control, a nonspecific human IgG1 antibody (directed to CD27) was serially diluted in wells coated with the human TIM-1 peptide. After incubation, the assay plates were washed with PBS/0.1% Tween and a goat anti-human IgG HRP-conjugated detection antibody (Jackson ImmunoResearch Inc.) was added. Following a wash, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added for color development. The CDX-014 mAb-binding

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curves were plotted using a 4-parameter fit log equation with fixed weighting.

Flow cytometry

The expression of TIM-1 by three human cancer cell lines used in subsequent *in vitro* and *in vivo* studies was confirmed using flow cytometry. CDX-014 mAb was added to the cells, incubated for 20 minutes at room temperature, washed, and phycoerythrin-labeled goat anti-human IgG (Fc specific) reagent was added to detect bound antibody. An anti-gpNMB antibody (CDX-011 mAb) was used as a negative control. After washing, the level of fluorescence was determined on a Becton Dickinson FACSCanto II flow cytometer.

The number of antigen-binding receptors on each cell line was determined using Quantum Simply Cellular beads from Bangs Laboratories (catalog no. 814). Briefly, the beads were labeled with the same antibody used to stain the cells, anti-TIM-1 FITC, and then analyzed on a FACSCanto II flow cytometer. Using QuickCal Data Analysis Program, a standard curve was generated and the number of surface receptors was calculated.

Receptor internalization

ACHN and IGROV-1 cell lines were pelleted, washed with HBSS containing 25 mmol/L HEPES (HHBSS), and enumerated using a hemocytometer. Cell suspensions were adjusted to 1×10^7 cells/mL, and 100 μL of suspension was distributed to polystyrene tubes. The labeling tubes were prechilled on ice and incubated for 1 hour with either 0.5 μg of FITC-conjugated CDX-014 mAb or FITC-conjugated IgG1 isotype control. After incubation, labeled cells were washed with HHBSS, suspended in 10% RPMI, and incubated at 37°C and 5% CO_2 for up to 80 minutes to allow for the internalization of antibody-labeled receptors.

Labeled cells were washed with cold HHBSS and labeled with 0.5 μg Alexa Fluor 647–conjugated anti-MHC class I antibody (Clone W6/32, BioLegend). After incubation, labeled cells were washed and fixed with 4% methanol-free formaldehyde (Polysciences) for 10 minutes at room temperature. Fixed cells were washed, stained with 500 μL 4′, 6-diamidino-2-phenylindole (DAPI), and washed again. A portion of each cell suspension was stabilized with 15 μL ProLong Gold anti-fade reagent (Thermo Fisher Scientific), prior to mounting on cover-slipped glass slides for acquisition of images by confocal microscopy. Labeled cells were imaged using a Leica SP2 confocal microscope fitted with 405, 488, 594, and 633 nm lasers as excitation sources.

Immunohistochemistry

A study was conducted to evaluate the expression of TIM-1 in a commercially available kidney cancer tissue array. An array of human kidney cancer tissue from 94 cases as well as normal kidney tissue from 10 individuals (catalog no. KD2085) was purchased from US Biomax. Sections of the formalin-fixed, paraffin-embedded positive and negative control cells were cut at approximately 5 μm to produce slides for immunohistochemical staining. Fluoresceinated CDX-014 mAb or a fluoresceinated nonspecific negative control monoclonal human IgG1 antibody (EMD Millipore) was applied at a concentration of 5 μg/mL and visualized using a mouse anti-fluorescein conjugate. Slides were visualized with light microscopy (Olympus BX45 microscope for microscopic evaluation and an Olympus DP12 camera to capture images) and evaluated by a trained pathologist (Charles River Laboratories).

A GLP-compliant IHC study was conducted to determine the potential cross reactivity of CDX-014 with cryosections of a full panel of tissues of human and cynomolgus monkey tissues, consistent with current regulatory guidance. Precomplexed CDX-014 or a nonspecific human IgG1 antibody (EMD Millipore) was applied to cryosections of tissues at two concentrations (5 and 1 μg/mL). Tissues from at least three donors per species were evaluated. Slides were evaluated by light microscopy by a trained pathologist (Charles River Laboratories). Each stained cell type or tissue element was identified, the subcellular (or extracellular) location of the staining was recorded, and the intensity (strength) of staining was assigned. Frequency of cell type staining was also assigned to provide the approximate percentage of cells of that particular cell type or tissue element with staining.

In vitro cytotoxicity of anti-TIM-1-vcMMAE

Cells expressing TIM-1 (Caki-1, IGROV-1, and A549) or TIM-1$^-$ cells (SK-MEL-2) were plated at 2×10^3 cells per well and allowed to adhere overnight in a 96-well tissue culture plate. The next day, serial dilutions of the antibody or ADCs were added. Additional wells of colchicine or media were added as positive and negative killing controls, respectively. After incubating for 72 hours, 20 μL of Alamar blue was added to each well, and relative fluorescence units were measured on a PerkinElmer Victor plate reader. The percent viability was calculated as follows: (mean sample RFU−mean colchicine RFU)/(mean media RFU−mean colchicine RFU) × 100.

Tumor challenge studies

Three TIM-1–expressing xenograft models were established to demonstrate the efficacy of CDX-014 *in vivo* against human tumors. In all models, cells were grown in culture, harvested and suspended in saline, and then injected subcutaneously into the flanks of immunocompromised mice. Tumors were measured using calipers, and tumor volume was determined by the equation "volume = (length × width^2)/2." When tumors reached an average of approximately 0.3 cm^3, mice were randomized into groups and treated intraperitoneally with CDX-014 ADC or saline. Treatment was given every 4 days for a total of 4 injections, or in three cycles for a total of 12 injections. Mice were euthanized according to predefined health criteria. All methods were approved by Celldex's IACUC.

Toxicology study

A GLP-compliant toxicology study was conducted at Charles River Laboratories. Purpose-bred, naïve cynomolgus macaque monkeys (*Macaca fascicularis*), shown to be positive for TIM-1 alleles that bind the CDX-014 mAb, were dosed via an intravenous (slow bolus) injection on days 1, 22, and 43, and necropsies occurred on days 49 and 86. Clinical observations included body weights, food consumption, ophthalmic examinations, electrocardiology, and neurologic examinations. Laboratory evaluations included clinical pathology (hematology, coagulation, clinical chemistry, and urinalysis), bone marrow smear evaluation, bioanalytical evaluation by ELISA for toxicokinetic analysis, and antidrug antibody analysis. Free MMAE concentrations were determined using solid-phase extraction followed by analysis using HPLC followed by tandem mass spectrometric detection (LC/MS-MS). Terminal procedures included a necropsy and a full panel of tissues collected for histology.

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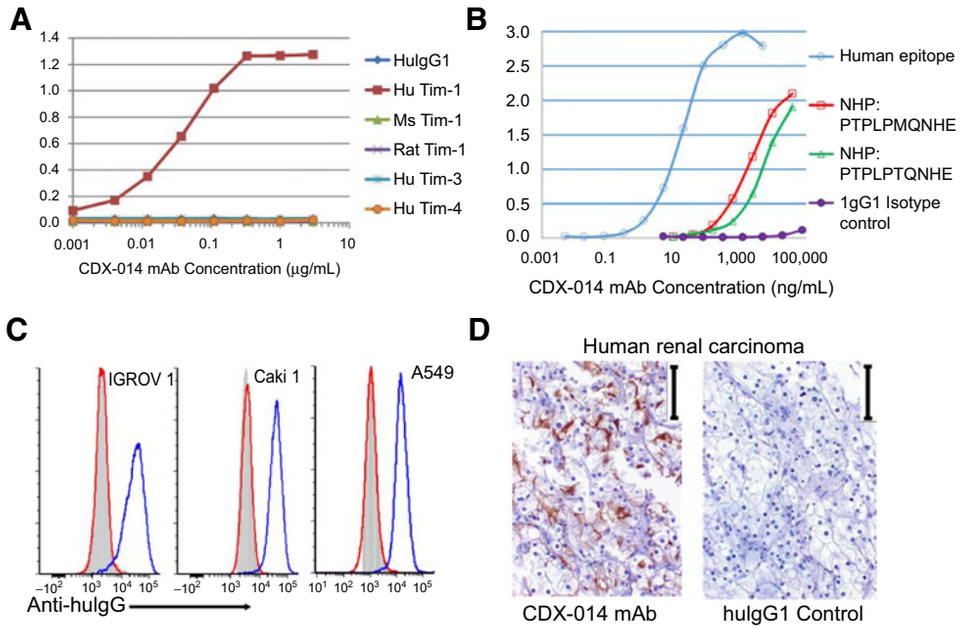

Figure 1.

Binding of CDX-014 mAb to TIM-1. **A,** Binding by ELISA of CDX-014 mAb or isotype control IgG1 to human, mouse, or rat TIM-1 and to human TIM-3 or TIM-4. **B,** Binding by ELISA of CDX-014 mAb to amino acids 209–218 of human TIM-1 PMPLPRQNHE (○), to the TIM-1 sequence variants found in cynomolgus macaques PTPLPMQNHE (□) and PTPLPTQNHE (△), or of an isotype control IgG1 to the human peptide epitope (●). **C,** Flow cytometry histograms (fluorescence intensity vs. counts) for the binding of the CR014 mAb (CDX-014 mAb, blue), CR011 mAb (anti-gpNMB CDX-011 mAb, red), or HulgG mAb (nonspecific human IgG1 control, shaded) to three TIM-1^{+} gpNMB^{-} human tumor cell lines, namely IGROV-1, Caki-1, and A549. **D,** Binding of CDX-014 mAb or a human IgG1 isotype control to representative clear cell carcinoma tissue samples from a kidney cancer tissue array (bar, 100 μm).

Results

Development and characterization of a fully human anti-TIM-1 mAb

A panel of human antibodies to TIM-1 was generated by immunization of human Ig transgenic mice with the extracellular domain of human TIM-1. Hybridomas were made from splenocytes from immunized mice and screened for TIM-1 reactivity. Antibodies with strong reactivity to TIM-1 were further evaluated for binding by flow cytometry, affinity, and epitope specificity. On the basis of its specificity and high affinity for TIM-1 (K_d of 2.71×10^{-9} M, estimated by ELISA), the hybridoma clone 2.70.2 was chosen for further development. The immunoglobulin variable regions from the hybridoma 2.70.2 were sequenced and cloned into an expression vector that encoded the constant domain regions of human IgG1k and transfected and expressed in CHO cells to yield the CDX-014 mAb.

CDX-014 mAb binds well to recombinant human TIM-1-Fc but not to other human TIM family members, nor to TIM-1 derived from rat or mouse sequences (Fig. 1A). Epitope mapping using overlapping TIM-1 fragments indicated the epitope to be within the amino acid sequence 209–222 found in the extracellular mucin domain region of the glycoprotein. The peptide corresponding to amino acids 209–222 was synthesized and coated to plates to demonstrate direct binding by CDX-014 mAb (Supplementary Table S1). As shown in Fig. 1B, the epitope sequence for the CDX-014 mAb was further refined to be within the amino acids 209–218 (PMPLPRQNHE). Binding was also observed to the corresponding sequences from two allelic versions from cynomolgus macaques (PTPLPMQNHE, PTPLPTQNHE). Further ELISA peptide binding studies using shorter sequences showed the minimum human epitope to be amino acids 212–217 (LPRQNH; Supplementary Table S1). Importantly, the CDX-014 mAb bound specifically to naturally expressed TIM-1 on human tumor cell lines derived from ovarian, renal, and lung carcinomas (Fig. 1C) and to human renal carcinoma tissue (Fig. 1D). We found a similar level of TIM-1 expression on these cell lines when quantified using Quantum Simply Cellular beads: IGROV-1 - 135,000, A549 - 103,000, and Caki-1 - 286,000 average molecules per cell.

Efficient internalization is required for the efficacy of ADCs and was demonstrated using confocal microscopy (Fig. 2). Compared with an isotype control mAb, the CDX-014 mAb was rapidly internalized by IGROV-1 cells with significant intracellular accumulation of the mAb within 40 minutes at 37°C. Similar internalization was observed using other TIM-1–expressing cell lines (not shown). These data supported the CDX-014 mAb as a good candidate for ADC development.

Development and characterization of the *in vitro* cytotoxicity of anti-TIM-1-vcMMAE (CDX-014)

On the basis of our prior experience with an ADC-targeting glycoprotein NMB (gpNMB), designated CDX-011 or glembatumumab vedotin (24), which also utilized the microtubule-disrupting agent vcMMAE, we used a similar approach for developing the anti-TIM-1 ADC. The ADC, named CDX-014, was generated by conventional methods that included the limited reduction of the mAb followed by maleimide-based conjugation of vcMMAE (25). Size exclusion chromatography revealed an

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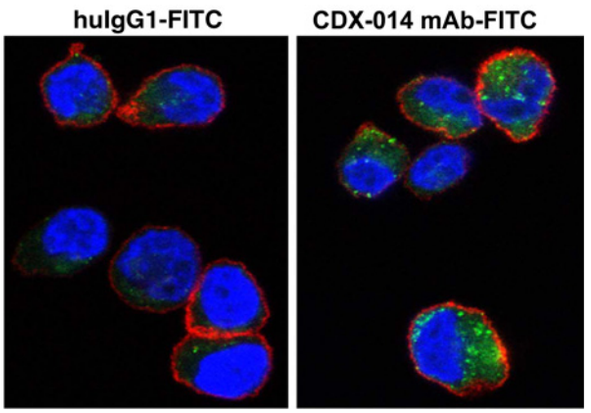

Figure 2.

Internalization of fluoresceinated CDX-014 mAb. Internalization of the isotype-matched IgG1-FITC control (left) or CDX-014 mAb-FITC (right) after incubation of IGROV-1 cells with the labeled antibodies (green) for 40 minutes at 37°C. The cells were counterstained with anti-MHC class I (red) to elucidate cell membranes and DAPI (4′,6-diamidino-2-phenylindole, blue) to stain DNA before analysis by confocal microscopy.

efficient conjugation with less than 5% free mAb and a DAR of approximately 4.5. The conjugation did not significantly change the binding to TIM-1 when the ADC was compared with the mAb in ELISA assays (50% max binding concentration of 0.052 μg/mL for the mAb vs. 0.082 μg/mL for the ADC).

To establish that binding and internalization leads to effective and selective killing of TIM-1–expressing human cancer lines, we used *in vitro* cell viability assays to determine the effect of CDX-014. Figure 3 shows that treatment of the three TIM-1–expressing cell lines (IGROV-1, Caki-1, and A549) with CDX-014 resulted in a dose-dependent decrease in cell viability at concentrations where CDX-011, a nontargeting ADC (IGROV-1, Caki-1, and A549 do not express gpNMB), had little effect. The specificity of CDX-014 was shown by comparing the activity of the ADCs on SKmel-2, a TIM-1–negative melanoma cell line that expresses gpNMB. These data confirm that CDX-014 is appropriately internalized into compartments for efficient cleavage of the linker and release of MMAE and also exemplify the variability in the concentration dependence of the cytotoxicity among these cell lines, which may be due to multiple factors.

Efficacy of CDX-014 in xenograft models of TIM-1–expressing cancers

The *in vivo* efficacy of CDX-014 was assessed with human TIM-1–expressing tumor cell lines representing ovarian, renal, and lung cancer. In each of these therapeutic mouse models, tumors were implanted and allowed to develop to significant size (an average of approximately 0.3 cm^3) before initiating dosing with CDX-014. Figure 4 demonstrates the effects of a single cycle of four doses of CDX-014 on tumor volume and survival in three tumor models. The unconjugated CDX-014 mAb at similar dose regimens provided no significant improvement in survival antitumor effects in these xenograft models (data not shown). However, we observed significant improvement in survival at the 50, 75, 150, 300 μg doses of CDX-014, and tumor regressions were observed at early time points following dosing at the 300 μg dose in all three models. In these challenging therapeutic models, however, the 4-dose regimen given over 12 days was not sufficient to provide long-term inhibition of tumor growth, and the majority of mice eventually succumbed to the tumor. We found that the tumor

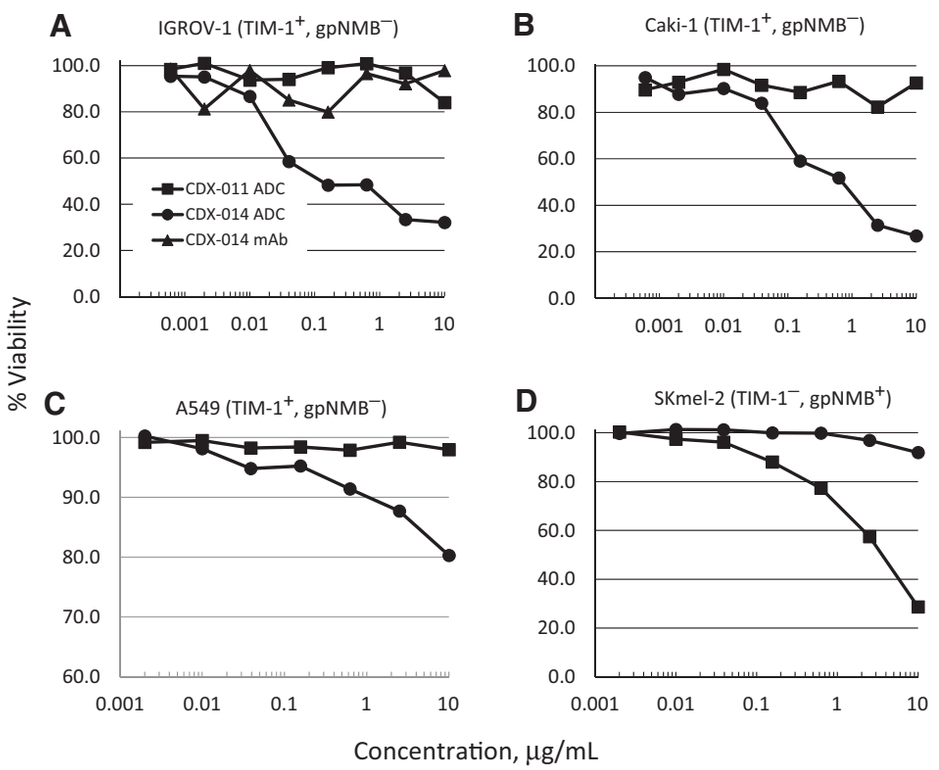

Figure 3.

A–D, *In vitro* cytotoxicity of CDX-014 ADC. Cytotoxicity of CDX-014 ADC, CDX-014 mAb, or an anti-gpNMB control ADC (CDX-011) on three TIM-1^+ cell lines (IGROV-1, **A**; Caki-1, **B**; A549, **C**) and a TIM-1^-/gpNMB$^+$ melanoma cell line (SKmel-2, **D**). The lack of cytotoxicity of the unconjugated CDX-014 mAb against the IGROV-1 line is also shown.

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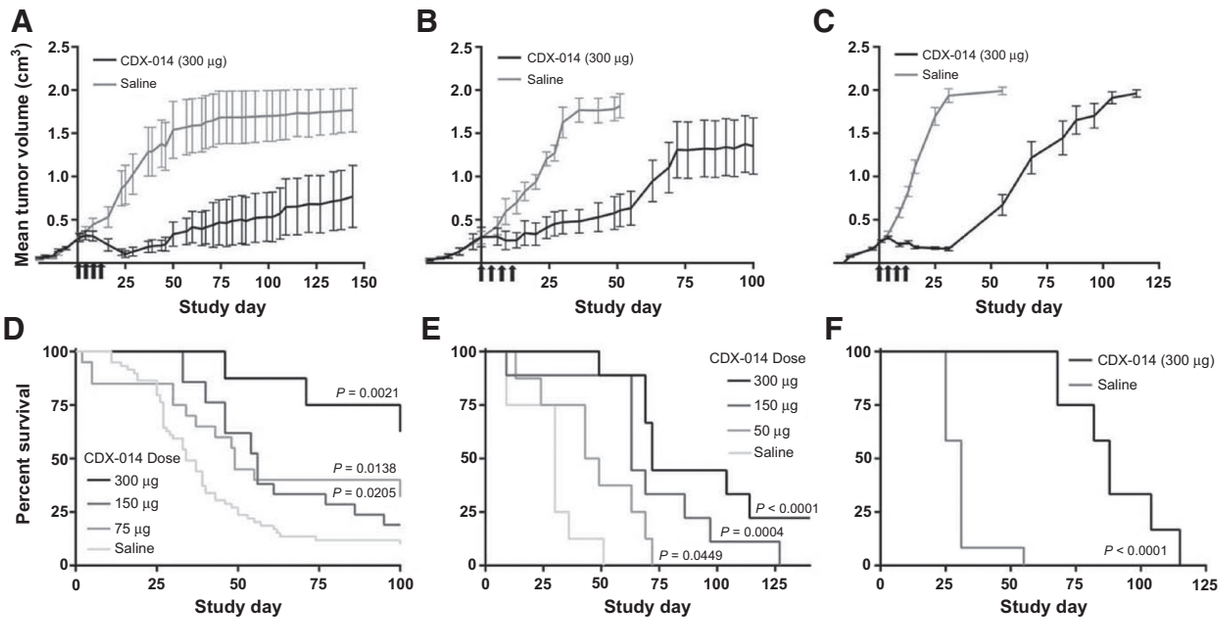

Figure 4.

CDX-014 ADC in mouse xenograft tumor models. Using a single cycle of four doses (indicated by the vertical arrows) over 12 days, CDX-014 inhibited the increase in tumor volumes relative to saline control treatment (**A–C**) in models using the IGROV-1, Caki-1, and A549 tumor lines, respectively. Decreases in tumor volumes can be seen during and immediately following the four CDX-014 doses. The reductions in tumor volumes led to increased durations of survival (**D–F**) in these same IGROV-1, Caki-1, and A549 models, respectively. A clear dose dependence in the survival curves is evident in the IGROV-1 (**D**) and Caki-1 (**E**) models. SEMs are indicated.

control and survival could be substantially increased by repeating the treatment cycles (Fig. 5). Using three cycles of CDX-014 to treat the aggressive A549 tumor, 100% of animals were still alive at the conclusion of the study, although none of the tumors completely resolved. Collectively, these preclinical models suggest that CDX-014 has the potential to inhibit the growth and viability of human tumors expressing TIM-1.

IND-enabling studies with CDX-014

Prior to human trials, we performed Investigational New Drug (IND)–enabling studies for CDX-014, including a GLP-compliant tissue cross-reactivity study and a GLP-compliant toxicology study. We selected cynomolgus macaques for the nonclinical evaluation of CDX-014 because the mAb does not bind to rat or mouse TIM-1 but does recognize TIM-1 peptides derived from the corresponding cynomolgus macaque sequence (Fig. 1). The lower affinity binding to the macaque TIM-1 peptide relative to the human peptide is recognized, but this species has been widely used for preclinical toxicology studies of ADCs, increasing the interpretability of our findings and limiting the number of animals required for our study.

A tissue cross-reactivity study, with a full panel of human and cynomolgus macaque tissues, was performed to identify potential tissue targets of CDX-014 and to support the use of cynomolgus macaques for the toxicology study. Unexpectedly, a broad range of human and monkey tissues showed reactivity with the CDX-014 mAb, including endothelium, epithelium, smooth muscle, and neurons. Most staining was analogous between the two species. However, this staining was almost entirely cytoplasmic or nuclear, and it is unclear whether it represents true tissue expression of

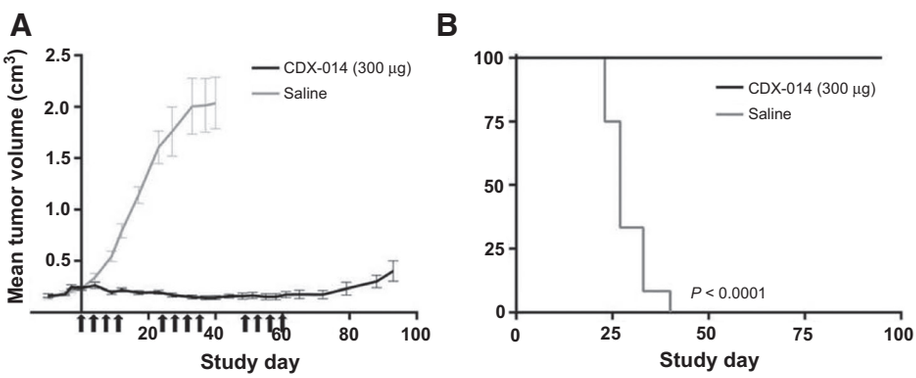

Figure 5.

Extended dosing of CDX-014 ADC in the A549 tumor model. Three cycles of four doses of CDX-014 prolong the inhibition of tumor growth (**A**) and duration of survival (**B**) *in vivo*. Arrows (**A**), treatment days. SEMs are indicated.

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Table 1. Selected toxicokinetic/pharmacokinetic data from the CDX-014 toxicology study from samples collected following the day 1 dose

Dose (mg/kg)	Analyte	Sex	$t_{1/2}$ (hr)	$AUC_{(0-72)}$ (hr × mg)/mL
0.3	ADC	Male	155	150
		Female	147	147
	Total antibody	Male	188	300
		Female	201	302
	Free MMAE	Male	—	—
		Female	—	—
1	ADC	Male	143	628
		Female	166	598
	Total antibody	Male	218	1,140
		Female	202	1,060
	Free MMAE	Male	—	1.60×10^{-6}
		Female	—	—
3	ADC	Male	126	1,570
		Female	154	1,610
	Total Antibody	Male	204	3,110
		Female	195	4,190
	Free MMAE	Male	—	5.10×10^{-6}
		Female	—	4.37×10^{-6}

NOTE: Animals that produced antidrug antibodies or that provided inadequate data (e.g., R^2 values of less than 0.800, insufficient data) were excluded from the analysis.

TIM-1 or cross-reactivity with other molecules. Nevertheless, the potential binding to intracellular targets is not likely to be of *in vivo* relevance as therapeutic antibodies do not generally reach intracellular sites (26). Plasma membrane staining was only observed in bile duct epithelium in the human liver and hepatocytes in the monkey liver.

In a GLP-complaint toxicology study evaluating CDX-014 in cynomolgus macaques, CDX-014 or the vehicle control was administered via intravenous injection on days 1, 22, and 43 at levels of 0.3, 1.0, and 3.0 mg/kg/dose. Main study and recovery animals were euthanized on day 49 and 86, respectively, and were subjected to complete necropsy examinations at those times.

Systemic exposure to CDX-014 ADC and CDX-014 total antibody (ADC and free antibody) increased with increasing dose in a dose proportional manner over the dose level range. No notable gender differences in exposure were observed for either CDX-014 ADC or CDX-014 total antibody. The average estimated $t_{1/2}$ was approximately 149 hours for CDX-014 ADC and approximately 201 hours for CDX-014 total antibody following the drug administration on day 1 (Table 1). The $AUC_{(0-72)}$ ratio of CDX-014 total antibody relative to CDX-014 ADC ranged from 2.01 to 2.30, indicating that approximately half of the antibody remained conjugated in the blood.

Administration of CDX-014 to cynomolgus monkeys resulted in no test article–related changes in clinical observations, body weights, food consumption, ophthalmology parameters, electrocardiography, body temperature, neurologic parameters, coagulation parameters, urinalysis parameters, gross pathology, and histopathology. At the main (day 49) necropsy, CDX-014–related changes in clinical pathology parameters were limited to attenuated reticulocyte responses, minimally increased red cell distribution width, and mildly to moderately decreased neutrophils at 3.0 mg/kg/dose, and decreased monocytes and glucose at ≥0.3 mg/kg/dose. CDX-014–related changes in bone marrow cytology at the main necropsy were limited to 3.0 mg/kg/dose and included increased myeloid-to-erythroid ratio in males associated with increased myeloid cells and/or decreased erythroid cells and morphologic changes in the myeloid series in males and females. At the main necropsy, lower organ weights (mean absolute and/or relative) were observed in the kidney and liver of females given >1.0 mg/kg/dose. No microscopic correlates were observed in these tissues. All the above parameters approximated or approached control values by the end of the recovery period (day 84).

Discussion

The use of ADCs to selectively deliver highly potent toxins to cancer cells represents an exciting and challenging approach to cancer therapy. To date, there have been only a limited number of FDA approvals, but many innovative ADCs are advancing through late-stage clinical trials (27). In addition, novel mechanisms and rational combination approaches are opening new opportunities for ADCs that are likely to increase their effectiveness and utility such as in combination with checkpoint inhibitors (28).

In this study, we explored the potential of TIM-1 as an ADC target and described the development and characterization of CDX-014, an ADC composed of a human IgG1κ mAb specific for TIM-1, covalently linked to the enzyme-cleavable microtubule inhibitor vcMMAE. Several studies have documented that TIM-1 expression is selectively upregulated on the plasma membrane of renal cell and clear cell ovarian carcinomas and has been associated with poor prognosis, making TIM-1 a tractable target for an antibody-based approach (15, 18–21).

We selected a high-affinity mAb that bound a linear epitope within the mucin domain of TIM-1 that is not impacted by any of the known polymorphisms described for human TIM-1 (29). Using the CDX-014 mAb and confocal microscopy, we demonstrated that upon binding, TIM-1 was rapidly internalized, leading to significant accumulation of the mAb within intracellular compartments, thus providing the rationale for developing the ADC.

CDX-014 was characterized using three human cancer cell lines representing different histologies: Caki-1 (human renal clear cell carcinoma), IGROV-1 (human ovarian adenocarcinoma), and A549 (lung carcinoma). Each of these cell lines expressed similar levels of TIM-1 and was sensitive to the selective cytostatic or cytotoxic activity of CDX-014 *in vitro*, but to varying degrees, further emphasizing that factors in addition to surface expression level contribute to the sensitivity of cancer cells to ADCs.

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To assess the therapeutic activity of CDX-014, we treated immunodeficient mice bearing established tumors and demonstrated the CDX-014 showed a potent antitumor efficacy against each tumor line *in vivo*. This activity was dose dependent, but generally not curative in these models with a single course of therapy. However, sustained antitumor activity could be achieved with longer dosing regimens using multiple courses of therapy.

On the basis of these promising preclinical results, we performed IND-enabling studies to support the initiation of a clinical program. A tissue cross-reactivity study assessed the binding of CDX-014 to a full panel of human and monkey tissues. There was cytoplasmic staining identified in a number of tissues and more restricted membranous staining. Importantly, the majority of staining observed with CDX-014 was similarly present in the human and cynomolgus macaque tissue panels, supporting the relevance of this species in assessing safety in a toxicology study.

In a multidose toxicology study in nonhuman primates, CDX-014 was well tolerated, with the highest dose level (3 mg/kg/dose) identified as the no observed adverse effect level (NOAEL). Consistent with prior experience with MMAE-containing ADCs, CDX-014–related changes in hematology and bone marrow cytology were observed at the initial necropsy, and fully resolved during the recovery period. Overall, we observed less toxicity at the 3 mg/kg dose compared with our gpNMB-targeted CDX-011 ADC (NOAEL of 0.3 mg/kg/dose in males and 1 mg/kg/dose in females in a similarly designed study), which correlated with a slower accumulation and lower maximum concentration of free MMAE in CDX-014–treated animals compared with CDX-011–treated animals. It remains to be seen whether this will translate into a higher MTD in humans, as compared with other MMAE-containing ADCs, which are in the 1.8–2.4 mg/kg range (30).

These studies support the advancement of CDX-014 into trials with patients that have TIM-1–expressing tumors, and a phase I clinical trial has recently been opened for patients with refractory clear cell or papillary renal cell carcinoma. The study includes a dose escalation to identify the MTD and a recommended phase II dose level, followed by a phase II component to assess preliminary antitumor activity. The study will also include a retrospective analysis of TIM-1 expression in archival tissue from patients using an IHC assay.

Disclosure of Potential Conflicts of Interest

H.C. Marsh is the vice president (research) at and has ownership interest (including patents) in Celldex Therapeutics. T. Keler is an employee at and has ownership interest (including patents) in Celldex Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

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Molecular Cancer Therapeutics

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