Novel Anti-TM4SF1 Antibody–Drug Conjugates with Activity against Tumor Cells and Tumor Vasculature

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Abstract

Antibody–drug conjugates (ADC) represent a promising therapeutic modality for managing cancer. Here, we report a novel humanized ADC that targets the tetraspanin-like protein TM4SF1. TM4SF1 is highly expressed on the plasma membranes of many human cancer cells and also on the endothelial cells lining tumor blood vessels. TM4SF1 is internalized upon interaction with antibodies. We hypothesized that an ADC against TM4SF1 would inhibit cancer growth directly by killing cancer cells and indirectly by attacking the tumor vasculature. We generated a humanized anti-human TM4SF1 monoclonal antibody, v1.10, and armed it with an auristatin cytotoxic agent LP2 (chemical name mc-3377). v1.10-LP2 selectively killed cultured human tumor cell lines and human endothelial cells that express TM4SF1. Acting as a single agent, v1.10-LP2 induced complete regression of several TM4SF1-expressing tumor xenografts in nude mice, including non–small cell lung cancer and pancreas, prostate, and colon cancers. As v1.10 did not react with mouse TM4SF1, it could not target the mouse tumor vasculature. Therefore, we generated a surrogate anti-mouse TM4SF1 antibody, 2A7A, and conjugated it to LP2. At 3 mpk, 2A7A-LP2 regressed several tumor xenografts without noticeable toxicity. Combination therapy with v1.10-LP2 and 2A7A-LP2 together was more effective than either ADC alone. These data provide proof-of-concept that TM4SF1-targeting ADCs have potential as anticancer agents with dual action against tumor cells and the tumor vasculature. Such agents could offer exceptional therapeutic value and warrant further investigation. Mol Cancer Ther; 14(8): 1868–76. ©2015 AACR.

Introduction

Judah Folkman envisioned that targeting the “tumor angiogenic factor” responsible for initiating tumor angiogenesis would have clinical benefit by preventing the formation of the new blood vessels that tumors require if they are to survive and grow beyond minimal size (1). VEGF is now recognized as the primary tumor angiogenic factor, and antibodies directed against it can prevent the growth of many rapidly growing mouse tumors (2, 3). However, in the clinic, targeting VEGF or its primary angiogenic receptor, KDR (VEGFR-2), has met with only limited success (4–6). Antibodies or “traps” directed against VEGF, or tyrosine kinase inhibitors that target VEGF receptors, are effective for a time as monotherapies in renal cell carcinoma and in some patients with glioblastoma multiforme. When combined with chemotherapy, they delay recurrence, and, in some instances, prolong patient survival, but are not curative. If antivascular therapy is to become more effective and achieve its full potential, additional targets beyond the VEGF–VEGFR-2 axis are required.

Transmembrane-4 I. Six Family member 1 (TM4SF1) was discovered in 1986 as a tumor cell antigen recognized by the mouse monoclonal antibody L6 (7, 8). TM4SF1 is an integral membrane glycoprotein structurally related to tetraspanins (8, 9). It is abundantly expressed on many cancer cells (7, 10), on endothelial cells lining human cancer blood vessels (11), and on the endothelial cells of angiogenic blood vessels induced in mice with retinopathy of prematurity (12) or by an adenovirus expressing VEGF-A (11). It is also weakly expressed on the endothelial cells of many normal organs and tissues (13, 14). TM4SF1 regulates cell motility and intercellular adhesion in both
we hypothesized that conjugation of a cytotoxic agent to anti-TM4SF1 would provide a dual anticancer mechanism: killing tumor cells that were dependent on that network (20). Together, these data indicated that naked anti-TM4SF1 antibodies were able to kill TM4SF1-expressing cells in both the tumor and vascular compartments (20). Another murine anti-TM4SF1 monoclonal antibody, L6 (IgG2a–kappa; refs. 7, 10), had been used to treat human tumor cell lines in immunocompromised mice (10). L6, and its mouse/human chimeric variant, chL6, were well tolerated and produced objective responses in patients with several different cancers that expressed TM4SF1 (10, 13, 21–24).

Building on our mouse experiments with 8G4, and the clinical experience with L6 and chL6 anti-TM4SF1 antibodies, we hypothesized that conjugation of a cytotoxic agent to anti-TM4SF1 antibodies would significantly amplify their anticancer activity (25). To test this hypothesis, we humanized L6, generating a new antibody v1.10; L6 and v1.10, unlike 8G4, cross-reacted with cynomolgus monkey TM4SF1 (10). We then armed v1.10 with LP2 (chemical name mc-3377), a synthetic analog of dolastatin-10 (26); dolastatin-10 and synthetic analogs, termed auristatins, inhibit tubulin polymerization, and ultimately induce C2–M cell-cycle arrest and cell death at low picomolar intracellular concentrations (27). Tubulin inhibitors have been extensively investigated as vascular targeting agents and seem to be preferentially toxic against tumor and tumor vascular endothelial cells with high proliferation rates, while sparing the nondividing endothelial cells of normal tissues (28–30).

Because TM4SF1 is highly expressed by both tumor cells and tumor vascular endothelium, an ideal therapeutic would target TM4SF1 on both cell types. Unfortunately, none of the monoclonal antibodies we and others have raised against human TM4SF1 cross-react with mouse TM4SF1 (7, 10, 20). Consequently, v1.10-LP2 would be expected to exert a direct effect on human tumor cells implanted in nude mice, but would not be expected to have an indirect effect on the xenograft’s mouse vasculature. We therefore generated a humanized anti-mouse TM4SF1 antibody, 2A7A, and conjugated it to LP2 to test the therapeutic potential of targeting the mouse tumor vasculature. We now report that both v1.10 and 2A7A antibody–drug conjugates (ADC) are highly effective as single agents against tumor xenografts that express TM4SF1 and are still more effective when combined so as to target both human tumor cells and the mouse tumor vasculature.

Materials and Methods

Cell lines and reagents

Tumor cell lines were purchased from ATCC and maintained according to vendor recommended conditions (RPMI/10% FBS media, supplemented with sodium pyruvate and nonessential amino acids for Calu-3 and SK-Mes-1, at 37°C and 5% CO₂). Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured according to the supplier’s protocols, and used at passages 3–6. Endothelial cell purity was confirmed by flow cytometry (FACS) which yielded 100% double positive TM4SF1/CD31 cells. 293 stably expressing human or mouse TM4SF1 were generated by lentiviral transduction using a lentiviral expression vector from BioSettia, and packaged using ViraPower (Life Technologies). Human and mouse TM4SF1–transfected 293 cells expressed 994 and 875 TM4SF1 mRNA copies/cell, respectively. Mouse anti-rabbit IgG was from Southern Biotechnologies.

Linker/payload LP2

The structure of LP2 (chemical name mc-3377) is shown in Supplementary Fig. S1A. LP2 is comprised of a synthetic dolastatin-10 analog that resembles in its structure momomethyl auristatin F (MMAF, red color in Supplementary Fig. S1A; ref. 27). However, a key difference of MMAF is the presence of a N-methylated α,α-dimethyl amino acid (Aib) that replaces the N-methyl valine on the N-terminus of MMAF. This structural modification of dolastatin-10 analogs has recently been described by Madera and colleagues and provides synthetic analogs with excellent potencies and differentiated ADME properties (27). The conjugation to the antibody is accomplished with the maleimidocaproyl linker (mc, blue color in Supplementary Fig. S1A) that is attached to the cytotoxin via an amide bond. After ADC catabolism in lysosomes, ADCs generated with this linker/payload are expected to produce Cys-capped-mc-3377 (26). The mc linker is termed "noncleavable" because the release of the cytotoxic payload requires ADC catabolism in the lysosome.

Anti-TM4SF1 antibodies and ADCs

Mouse anti-human TM4SF1 antibodies 8G4 (20), and L6 and chL6 (31), were described previously. L6 was humanized by Complementarity Determining Regions grafting and the humanized L6 VH and VL were joined to the human IgG1 and human Kappa constant regions, respectively, with proprietary expression vectors. On the basis of structure modeling (32), we generated a series of humanized L6 variants modified by the introduction of back mutations from the parental mouse antibody to restore binding efficiency and antibody stability. The humanized L6 variant chosen for further preclinical development, v1.10, outperformed the chL6 antibody as an ADC in a PC3 xenograft model. Recombinant humanized v1.10 antibody was produced at different scales in CHO or 293 cells. Preparation and characterization of the rabbit/human chimeric anti-mouse/rat TM4SF1 antibody, 2A7A, are presented in Supplementary Materials and Methods.

Generation of anti-TM4SF1 ADCs

ADCs were prepared by partial reduction of the antibodies with tris(2-carboxyethyl)phosphine followed by coupling to maleimidocaproyl-auristatin (mc3377; ref. 33). Excess of N-ethylmaleimide and L-Cys were added in sequence to cap the unreacted thiols and quench any unreacted linker-payload. After overnight dialysis in PBS, pH 7.4, the antibodies were purified by size exclusion chromatography. Protein concentrations were
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Cytotoxicity assays
Target cells were plated at densities of 500–2,000 cells/100 μL culture medium per well in 96-well plates. After overnight incubation at 37°C, 100 μL of culture media containing serial dilutions of ADCs were added. Ninety-six hours later, 50 μL of CellTiter-Glo (Promega) were added to each well and the plates were read for luminescence. Data are expressed as % viability compared with that of control untreated cells.

Gene expression analysis
TM4SF1 gene expression levels were estimated from the Cancer Cell Line Encyclopedia (CCLE, http://www.broadinstitution.org/ccle/home). RNA expression was quantified with the Affymetrix microarray platform. Normalization of gene expression was done by applying the Robust Multi-array Average algorithm. For validation of the microarray data, we employed multi-gene transcriptional profiling (MGTP) to quantify mRNA copies per cell by normalization to 18S-rRNA, assuming that, on average, cells express approximately 10^6 copies of 18S-rRNA (35, 36).

FACS analysis
A total of 1×10^5 cells were reacted with the indicated antibody or ADCs in 100 μL of PBS/2% FBS for 60 minutes on ice and washed twice. In some instances, antibodies were conjugated to a fluorophore; otherwise, an Alexa 647-labeled secondary anti-human Fc (Life Technologies) was added and cells were incubated for an additional 60 minutes on ice. After washing in PBS/2% FBS, fluorescence was quantified by FACS (BD LSR Fortessa; 10,000 events collected/point). To determine the relative number of TM4SF1 copies on the cell surface, we stained cells with saturating amounts (30–100 μg/mL) of Alexa488 (Life Technologies) conjugated anti-TM4SF1 antibodies (chL6 or 2A7A) or isotype control antibodies. The degree of labeling (DOL) for each antibody was determined using a Nanodrop spectrophotometer [moles dye/molecule = absorbance_{494}/(71,000 × protein concentration (mol/L)]. Calibration curves were generated using the Quantum Alexa488 MESF kit (Bangs Laboratories, Inc.). The geometric mean fluorescence values obtained from the test samples were then divided by the DOL and the background (isotype control-stained cells) subtracted. TM4SF1 receptor numbers were calculated from a calibration curve.

Results
Expression of TM4SF1 in human cancers
We used immunohistochemistry to confirm literature reports (10) that TM4SF1 protein was strongly expressed on the tumor and vascular cells of cancers resected from patients. Collaborating with Indivumed, we found strong tumor cell staining in 10 of 13 liver, 16 of 20 non–small cell lung (NSCLC), 4 of 20 breast, and 3 of 20 colon cancer patient samples (Supplementary Fig. S2), in agreement with previous literature reports (29, 30).

Figure 1. v1.10 and v1.10-LP2 (mc-3377) reactivity with, and cytotoxicity against, cultured human endothelial cells. A, FACS analysis of reactivity of v1.10 and v1.10-LP2 on HUVEC and HLEC. Geometric mean fluorescence (MFI) values are plotted versus antibody concentration. Apparent K_d values (EC_{50}) were generated using GraphPad software. B, FACS analysis demonstrates that v1.10 interacts strongly with 293 cells stably transduced with human TM4SF1 (293^{TM4SF1}, clear profile), but not with 293 cells (gray profile) or with 293 cells stably transduced with mouse TM4SF1 (293^{TM4SF1}, black profile). C, cytotoxicity of HVEC and HLEC treated with varying amounts of v1.10-LP2 and a control ADC, 8.8-LP2. Cell viability was assessed at 96 hours by luminometry.
all cases the tumor vasculature and the vasculature of adjacent normal tissues also stained.

Binding properties and cytotoxic effects of v1.10 and v1.10-LP2 on cultured endothelial cells

8G4 and L6 interact with different epitopes on external loop 2 of human TM4SF1. Because L6 reacts with cynomolgus TM4SF1 (7, 10), whereas 8G4 does not (20), we selected L6 for further studies and prepared v1.10, a humanized clone of L6. We analyzed v1.10’s reactivity with cultured human endothelial cells that express high endogenous levels of TM4SF1, that is, approximately 100 copies of TM4SF1 mRNA/cell (11, 20) and 4–5 × 10^5 surface protein molecules/cell. As shown in Fig. 1A, v1.10 bound to cultured HUVECs and human lung microvascular endothelial cells (HLEC) with an apparent \( K_d \) of 2 to 5 nmol/L (the antibody concentration that gave 50% of the maximal binding by FACS analysis). v1.10 was then conjugated to an auristatin-like payload LP2 (chemical name mc-3377) to an average DAR of 4 (Supplementary Fig. S1B). The resulting anti-human TM4SF1 ADC (v1.10-LP2) bound to HUVECs and HLECs in a manner similar to v1.10 (Fig. 1A). Neither v1.10 nor v1.10-LP2 interacted with cells that did not express human TM4SF1, for example, 293 cells or 293 cells that were stably transfected with mouse TM4SF1 (Fig. 1B).

To achieve cytotoxicity, the antibody–toxin conjugate must be internalized and trafficked to lysosomes, where the antibody is catalyzed and the toxic payload is released. HUVEC internalized substantial amounts of v1.10 by 30 minutes and there was essentially complete colocalization of antibody with LAMP1-positive vesicles (late lysosomes) by 4 hours (Supplementary Fig. S3). v1.10-LP2 ADC was highly effective in killing HUVECs and HLECs (Fig. 1C). For both cell lines, the IC_{50} of v1.10-LP2 killing was in the low nanomolar range (1.7 nmol/L for HUVECs and 2.2 nmol/L for HLECs), while a control nontargeting human IgG1, antibody, 8.8-LP2, did not kill endothelial cell at concentrations below 100 nmol/L.

Selection of TM4SF1-expressing tumor cell lines

Tumor cell lines expressing high levels of TM4SF1 were identified with an in silico survey of public and proprietary gene expression databases (CCLE, Oncomine, Gene Logic, and OTP) and from prior publications (7, 8, 16, 37). As a group, NSCLC tumor cell lines expressed the highest levels of TM4SF1 among solid tumors (Fig. 2A). However, many other cancer cell lines also ranked high, including those originating in ovary, liver, pancreas, prostate, colon, and breast (data not shown). TM4SF1 expression levels gave similar rank order whether measured as mRNA copy numbers and surface protein levels, respectively, by MGTP and FACS analysis on five NSCLC and on the breast cancer cell line MCF-7. Mean fluorescence values were obtained using saturating amounts of Alexa488-labeled chimeric L6, and were converted to receptor numbers using calibration beads (Bangs Laboratories Inc.). Data represent the mean of three independent determinations.

Expression of TM4SF1 in human tumor xenografts

Seven different tumor xenografts (Supplementary Fig. S5A) were grown as xenografts, either at Crown Bioscience Inc. in Balb/c nude mice or at BIDMC in athymic 490 nude mice. SK-MES-1, A549, SW620, MiaPaCa2, and PC3 tumors expressed more than 50 TM4SF1 mRNA copies/10^5 18S copies as determined by MGTTP (35, 36), whereas NCI-H460 and Calu6 tumors expressed fewer than 50 copies. Tumor xenografts that were grown in athymic 490 nude mice at BIDMC expressed substantially higher levels of mouse TM4SF1, as well as the specific endothelial cell markers CD144 and VEGFR-2, than tumors grown in Balb/c nude mice at Crown Bioscience Inc. (Supplementary Fig. S5B): TM4SF1 mRNA assays of both Crown Bioscience and BIDMC xenograft tumors were performed at BIDMC. These data suggest that tumor xenografts grown in different nude mouse strains generate different levels of vascular density.

Effect of v1.10-LP2 ADCs on NSCLCs and other tumor xenografts

To assess the therapeutic potential of anti-TM4SF1 ADCs in vivo, select NSCLC tumor cell lines (horizontal arrows...
in Fig. 2A) were implanted subcutaneously in nude mice. When tumors had grown to approximately 200 mm$^3$ (300 mm$^3$ for A549 and SK-MES-1 cells), mice received intraperitoneal injections of 100 μL of PBS, or PBS containing 3 or 10 μpk of v1.10-LP2, or 10 μpk of control 8.8-LP2. Treatments were administered at 4-day intervals for four cycles (q4d/C24).

v1.10-LP2 induced complete tumor regression, defined as nonpalpable tumors, in nearly all mice bearing NSCLC xenografts that expressed high levels of TM4SF1 (Fig. 3A). Calu-6 cells, which express low levels of TM4SF1 and were insensitive to v1.10-LP2 in culture, were also poorly sensitive to v1.10-LP2 in vivo.

Efficacy of v1.10-LP2 was also evaluated in human cancer xenografts originating in colon (SW620), prostate (PC3), and pancreas (MiaPaca2, Capan-1, and Panc-1). Similar results were obtained whether drugs were administered intravenously or intraperitoneally, and whether tumors were, or were not, implanted in Matrigel (Fig. 3B). Complete regressions were achieved in the majority of the tumors tested at 3 mpk, and, at 10 mpk, in all of the models except for SW620 at the Pfizer Oncology site where tumors did not regress but remained growth static for approximately 50 days.

v1.10-LP2 was well tolerated at all three study sites. Mice did not exhibit signs of toxicity such as changes in grooming habits or weight loss at either 3 or 10 mpk. On the basis of the A549, MiaPaCa2 and PC3 regression profiles and the pharmacokinetics of v1.10-LP2 in nude mice, we calculated a serum effective concentration range of 0.5 to 16 μg ADC/mL (Supplementary Fig. S6A).

2A7A, a humanized rabbit anti-mouse TM4SF1 monoclonal antibody

v1.10 does not recognize mouse TM4SF1. This is not surprising in that the amino acid sequences of human and mouse TM4SF1 extracellular loop 2 differ significantly (20), and neither we nor others have been able to develop an antibody that reacts with both human and mouse TM4SF1. Therefore, to target the mouse TM4SF1 expressed on endothelial cell lining the tumor xenograft vasculature, we developed a humanized rabbit surrogate monoclonal antibody, 2A7A. 2A7A recognized mouse TM4SF1, but did not react with human TM4SF1. As shown in Fig. 4A, 2A7A bound to 293 cells that had been stably transduced with mouse TM4SF1 (293mTM4SF1) and to the immortalized mouse microvascular endothelial cell line, MS1. However, 2A7A did not bind to untransfected parental 293 cells or to cells expressing human TM4SF1 such as HUVEC (Fig. 4B).

Consistent with its binding pattern, 2A7A-LP2, was highly cytotoxic to MS1 cells and 293mTM4SF1 with IC50 values of 0.015 and 0.003 nmol/L, respectively (Fig. 4C). The number of mouse TM4SF1 molecules on the surface of 293mTM4SF1 or MS1 cells was calculated to be 5.2 × 10$^5$ and 5.7 × 10$^5$ copies/cell, respectively; that is, levels comparable with those of human TM4SF1 expressed by HUVEC (~4 × 10$^5$ copies/cell) and HLEC (~5 × 10$^5$ copies/cell). However, the potency of the 2A7A-LP2 ADC toward cultured rodent endothelial cells was
about two orders of magnitude higher than that of v1.10-LP2 against human HUVEC and HLEC, possibly due to the 10-fold higher binding affinity of 2A7A for mouse TM4SF1 (0.5 nmol/L) versus that of v1.10 for human TM4SF1 (~5 nmol/L).

**Distribution and antitumor activity of 2A7A-LP2**

2A7A-LP2 injected intraperitoneally into mice bearing PC3 xenografts bound strongly to the mouse tumor vasculature, and also to a lesser extent to the vasculature of nearby normal tissues, but did not bind tumor cells which expressed human TM4SF1 (Fig. 5A). In contrast, v1.10-LP2, similarly injected intraperitoneally into mice bearing PC3 tumors, localized almost entirely to the tumor cells which express human TM4SF1 and was not detected in tumor or adjacent normal tissue blood vessels that express mouse TM4SF1 (Fig. 5B). A nontargeting isotype-matched control antibody, 8.8-LP2, did not bind either tumor or normal tissue blood vessels. These results suggest that the antibody binds to both the TM4SF1 protein and the tumor vasculature, which may be a strategy to block tumor growth.

**Figure 4.**

2A7A and 2A7A-LP2 (mc-3377) reactivity with and cytotoxicity against different cultured cells. A, FACS analysis demonstrates that 2A7A-Alexa488 conjugate reacts strongly with 293 cells that stably express human TM4SF1 (293mTM4SF1) and with M1 immortalized mouse endothelial cell, but not with untransfected parental 293 cells. B, M1 cells (top) stained with 2A7A-Alexa488, whereas HUVEC (bottom) did not. C, cytotoxicity assays. 293 cells were not susceptible to either 2A7A-LP2 or 8.8-LP2 (top), 2A7A-LP2 efficiently killed 293mTM4SF1 (middle), and M1 cells (bottom), whereas control 8.8-LP2 was not toxic to any of the cells at the concentrations used. Percent cell viability was determined at 96 hours using the CellTiter-Glo kit. Results are from two independent experiments which gave similar results.

**Figure 5.**

Distribution of 2A7A-LP2 (mc-3377) and v1.10-LP2 (mc-3377) in PC3 tumor xenografts and the effect of 2A7A-LP2 on tumor xenografts. PC3 tumor cells were implanted in athymic nude mice and allowed to grow to 300 mm³. Mice were then injected intraperitoneally with 3 mpk 2A7A-LP2 (A) or v1.10-LP2 (B). Forty-eight hours later, tumors were harvested after intravenous injections of FITC-dextran to visualize blood vessels. Fresh frozen sections were stained with Alexa594 conjugated anti-human IgG antibodies to visualize 2A7A-LP2 (A) or v1.10-LP2 (B). Dashed white lines indicate tumor-host interface. 2A7A-LP2 was localized to tumor (white arrows) and host (yellow arrows) blood vessels, largely obscuring green FITC-dextran staining (A). In contrast, v1.10-LP2 was localized to tumor cells (B) and green FITC-dextran stained vessels are clearly seen. C, effects of 2A7A-LP2 (red lines) or control 8.8-LP2 (black lines) on A549, MiaPaCa2, PC3, and SW620 human tumor xenografts. Treatments (3 mpk, q4d, vertical dotted lines) were started when tumors had reached a size of approximately 300 mm³. Experiments were performed at BIDMC, 5 mice per group.
not localize to the tumor or to the host vasculature (not shown).

Using the same q4d×4 protocol used for v1.10-LP2 (Fig. 3), 2A7A-LP2 at 3 mpk induced partial (PC3) or complete (A549, MiaPaCa2, and SW620) regressions (Fig. 5C). MiaPaCa2 regression persisted for almost 50 days, whereas A549 and SW620 tumors recurred at around 8 days after the fourth injection of 2A7A-LP2, and PC3 tumors began to recur even before the fourth injection. Treatment with 2A7A-LP2, like that with the control ADC 8.8-LP2, was well tolerated at the 3 mpk dose level with mice exhibiting no signs of systemic toxicity such as changes in grooming behavior or weight loss (Supplementary Fig. S6B). However, at higher doses of 5 to 10 mpk, significant weight loss was observed immediately after the fourth injection (data not shown).

Simultaneous targeting of TM4SF1 expressed on tumor cells and on tumor vasculature

The foregoing studies with v1.10-LP2 and 2A7A-LP2 established that targeting TM4SF1 expressed by either tumor cells or by the tumor vasculature could regress tumor xenografts. Because the two approaches are nonredundant, we hypothesized that the combined effects of targeting tumor and vasculature would be additive. To test this hypothesis, we studied Calu-6 and NCI-H460 tumors whose cells express low levels of TM4SF1 in culture (Fig. 2B and C). Also, whereas cultured NCI-H460 cells did respond to v1.10-LP2, Calu-6 did not (Supplementary Fig. S4A). Furthermore, Calu-6 xenografts were resistant to v1.10-LP2 as a single agent (Fig. 3A).

As shown in Fig. 6A, 3 mpk v1.10-LP2 or 2A7A-LP2 alone only minimally impacted the growth of Calu-6 xenografts. However, when combined, these ADCs added an additional 15 days to progression-free survival. Despite in vitro sensitivity, the NCI-H460 model was resistant to v1.10-LP2 in vivo, but responded to 2A7A-LP2, delaying tumor progression by approximately 15 days (Fig. 6B). Combination therapy delayed tumor growth for an additional 10 days.

Discussion

TM4SF1 is an important housekeeping gene that is required for the polarization and migration of cultured endothelial cell (11, 18). It is also highly expressed by many different human cancer cells (7, 10) and has important roles in cancer initiation, migration, and invasion (38). Earlier studies with L6, an antibody targeting human TM4SF1, exhibited low toxicity and gave promising results in mouse tumor xenografts and in a small number of cancer patients, presumably via mechanisms involving CDC and ADCC (21, 39). Together these findings suggested that therapy could be enhanced by targeting TM4SF1 with an ADC approach. ADCs have entered the clinic and are currently used to target tumors expressing many different antigens, including CD33 (40), Her2 (41), and CD30 (42); more than 100 additional ADCs directed against different tumor cell targets populate the preclinical and clinical pipelines (43–46). An ADC against a target expressed by both tumor cells and the tumor vasculature would be expected to offer exceptional therapeutic benefit.

Here, we report the development of v1.10, a humanized version of the previously described anti-TM4SF1 mouse monoclonal antibody L6. v1.10 has low nanomolar affinity for cultured human endothelial cells and for many cancer cells (Figs. 1A and 2), and, when reacted with plasma membrane TM4SF1, was internalized (Supplementary Fig. S3), a property essential for ADC efficacy. When conjugated to a proprietary tubulin inhibitor, LP2 (chemical name mc-3377), the resulting ADC was highly cytotoxic against both cultured endothelial (Fig. 1C) and tumor cells (Supplementary Fig. S4). v1.10-LP2 induced complete regressions in five of six different NSCLC tumor xenografts (Fig. 3A) and of tumor xenografts of pancreas, prostate, and colon origin (Fig. 3B). In general, sensitivity to v1.10-LP2 correlated well with TM4SF1 expression at both the RNA and protein levels and as measured both in vitro and in vivo. Naked v1.10 antibody (3 mpk, q4d×4) had slight anti-PC3 xenograft tumor activity, presumably due to ADCC, and limited tumor growth during the four injection cycle; however, PC3 tumors did not regress and grew rapidly after treatment ceased (data not shown). Thus, the antitumor activity of v1.10-LP2 is largely implemented by ADC.

ADCs targeting TM4SF1 offer the opportunity to attack both cancer cells and cancer-associated vascular endothelium. A concern, of course, is that such ADCs will also damage the vascular endothelium of normal organs. Four cycles of 2A7A-LP2 at 3 mpk were well tolerated, despite antibody binding to normal vasculature (Fig. 5A, yellow arrows), with no changes in body weight (Supplementary Fig. S6B), animal activity, or grooming behavior. Treatments could be safely increased to 10 cycles of 3 mpk 2A7A-LP2 at 2-day intervals (data not shown). Thus, 2A7A-LP2 has a clear predilection for damaging xenograft tumor endothelial cells versus normal endothelial cell. However, at higher doses (5–10

Figure 6.

Combination treatment of refractory tumor xenografts with ADC directed against human and mouse TM4SF1. Clau-6 (A) and NCI-H460 (B) xenograft tumors were grown in Balb/c nude mice (Crown Biosciences, Inc.) to approximately 200 to 300 mm³ and were treated with v1.10-LP2 or 2A7A-LP2, alone or in combination, q4d×4 (vertical dotted lines). Mean tumor volumes ± SEM, 10 mice per group.

Days

Volume mm³ ± SEM

NCI-H460

8.8-LP2 (10 mpk)

v1.10-LP2 (3 mpk)

2A7A-LP2 (3 mpk)

Combination (3+3)

Calu-6

8.8-LP2 (10 mpk)

v1.10-LP2 (3 mpk)

2A7A-LP2 (3 mpk)

Combination (3+3)

Days

Volume mm³ ± SEM

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markers in tumors grown in different strains of nude mice may also be a factor as we observed differences in vascular tumor and vascular endothelial cells. Overall tumor vascularity is effective. The response is likely determined by a number of single agents in treating mouse tumor xenografts (Figs. 3 and 5), and that, when combined (Fig. 6), are still more effective. The response is likely determined by a number of variables, including TM4SF1 surface expression levels on tumor and vascular endothelial cells. Overall tumor vascularity may also be a factor as we observed differences in vascular markers in tumors grown in different strains of nude mice (Supplementary Fig. S5B). Unfortunately, v1.10-LP2 does not react with murine TM4SF1, and so we do not at present have a single ADC that is reactive with both human tumor and mouse vascular TM4SF1 for use in preclinical studies. Nonetheless, our data validate the potential of TM4SF1 as an attractive human cancer target and provide proof-of-concept that targeting TM4SF1 with an ADC can be exploited therapeutically to treat human cancer xenografts. v1.10-LP2 may be the first in a new class of drugs with the bifunctional capacity to target a molecule, TM4SF1, that is expressed both by tumor cells and by the tumor vasculature.

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

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Novel Anti-TM4SF1 Antibody–Drug Conjugates with Activity against Tumor Cells and Tumor Vasculature

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