

Potent cytotoxicity of an auristatin-containing antibody-drug conjugate targeting melanoma cells expressing melanotransferrin/p97

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Abstract

Identifying factors that determine the sensitivity or resistance of cancer cells to cytotoxicity by antibody-drug conjugates is essential in the development of such conjugates for therapy. Here the monoclonal antibody L49 is used to target melanotransferrin, a glycosylphosphatidylinositol-anchored glycoprotein first identified as p97, a cell-surface marker in melanomas. L49 was conjugated via a proteolytically cleavable valine-citrulline linker to the antimetabolic drug, monomethylauristatin F (vcMMAF). Effective drug release from L49-vcMMAF likely requires cellular proteases most commonly located in endosomes and lysosomes. Melanoma cell lines with the highest surface p97 expression (80,000–280,000 sites per cell) were sensitive to L49-vcMMAF whereas most other cancer cell lines with lower p97 expression were resistant, as were normal cells with low copy numbers ($\leq 20,000$ sites per cell). Cell line sensitivity to L49-vcMMAF was found by immunofluorescence microscopy to correlate with intracellular fate of the conjugate. Specifically, L49-vcMMAF colocalized with the lysosomal marker CD107a within sensitive cell lines such as SK-MEL-5 and A2058. In contrast, in resistant cells expressing lower p97 levels (H3677; 72,000 sites per cell), L49-vcMMAF colocalized with caveolin-1, a protein prominent in caveolae, but not with CD107a. Thus, for antibody-drug conjugates targeting p97, antigen level and trafficking to the lysosomes are important factors for achieving robust *in vitro* cytotoxicity against cancer cells. Immunohistochemical analysis with L49 revealed that 62% of metastatic melanoma tumors had strong staining for p97. Overexpression of p97 in melanoma as compared

with normal tissue, in conjunction with the greater sensitivity of tumor cells to L49-vcMMAF, supports further evaluation of antibody-drug conjugates for targeting p97-overexpressing tumors. [Mol Cancer Ther 2006;5(6):1474–82]

Introduction

Malignant melanoma is responsible for ~79% of deaths from skin cancer (~7,900 deaths estimated in the United States in 2006; ref. 1). Surgery is often curative for early stage melanoma but is not a treatment option for disease that has metastasized to distant organs such as lung or brain. Available treatments are limited to use of immunotherapy (α -IFN or interleukin-2) with or without combination chemotherapy drugs to delay recurrence of disease. Chemotherapy and radiation therapy for stage IV melanoma patients are not curative but mostly used to relieve symptoms or extend the life of patients. The 5-year relative survival rate of stage III melanoma patients is poor (45%) and significantly worse in stage IV disease (10%). Unquestionably, there is an unmet need for a more effective treatment for malignant melanoma.

Melanotransferrin or p97 was first identified as a 97-kDa cell-surface marker for malignant melanoma cells (2) and has extensively been studied (3, 4). The expression of p97 is selective for, but not absolutely tumor restricted (4), as observed with many tumor-associated differentiation antigens (5). The p97 protein is highly expressed in neoplastic cells and fetal tissues, including umbilical cord, intestine, and sweat gland ducts, and is also present in some normal tissues such as salivary glands, sweat glands, brain endothelial cells, smooth muscle, and liver sinusoidal cells (3, 6–10). This antigen belongs to the transferrin family of iron-binding proteins and shows 37% to 39% sequence homology with human serum transferrin, lactoferrin, and ovotransferrin (11, 12). There are two forms of the p97 antigen: membrane-bound and soluble secreted protein. The membrane-bound form, which is attached to the cell membrane by a glycosylphosphatidylinositol anchor (7, 13), binds iron and is internalized into cells independent of transferrin/transferrin receptor endocytosis. Recently, membrane-bound p97 has been reported to be a potential cell-surface plasminogen receptor and may play a key role in activation of cell invasion (14). The soluble p97 protein is found at very low levels (3.2 ng/mL) in the plasma of normal individuals and is actively secreted into the blood and cerebrospinal fluid in patients with Alzheimer's disease (median 15 ng/mL; ref. 15). Soluble p97 does not effectively donate iron to the brain and erythropoietic tissue (16) and its function remains unknown.

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L49 is a murine immunoglobulin G1 (IgG1) monoclonal antibody (mAb) against human p97, which was derived from BALB/c mice immunized with lung carcinoma (H2981 and CH3) and melanoma (W56) cell lines (17). Earlier studies showed that p97 is stably expressed on the cell surface and, on binding of mAb, does not detectably internalize (17, 18). In one of these studies, a single-chain Fv fragment fused to β -lactamase was used for antibody-dependent enzyme-mediated prodrug therapy (17). More recently, another mAb to p97, hup97, was used as a non-internalizing control in combination with a cross-linking secondary antibody conjugated to a highly potent antitubulin drug, monomethyl auristatin E (MMAE; ref. 19). The anti-p97 antibody plus cross-linking antibody-drug conjugate showed significant cytotoxicity only at 100- to 1,000-fold higher concentration than for antibodies to other antigens known to be efficiently internalized. As for previous studies, it was concluded that the p97 antigen is not readily internalized by antibodies (19).

In this study, L49 was directly conjugated to the auristatin drugs MMAE (20), monomethylauristatin F (MMAF; ref. 21), and auristatin F phenylenediamine (AFP; ref. 22), using linkers that contain or lack a protease-cleavable dipeptide, valine-citrulline (20, 21). As previously reported, internalization of unconjugated L49 was not observed. However, in contrast to earlier studies, L49-drug conjugates showed efficient internalization in cell lines that express high levels of p97. One of the goals of this study was to understand the mechanism of drug delivery via antibody-drug conjugates to p97, a glycosylphosphatidyl inositol-linked cell-surface target. Previous studies have shown that most glycosylphosphatidyl inositol-linked proteins are associated with caveolin-1 and internalized via the caveolae pathway (23, 24). Another goal was to determine the factors necessary for selective and efficient killing of malignant melanoma cells. Briefly, tumor cell lines and normal cells were analyzed for p97 antigen expression by quantitative fluorescence-activated cell sorting (FACS) and then compared for their sensitivity to L49-vcMMAF. Antibody-drug conjugate internalization by sensitive and resistant lines was then studied by immunofluorescence microscopy, including colocalization with subcellular markers. In addition, immunohistochemistry was used for semiquantitative assessment of the p97 expression in metastatic melanoma tissue microarrays as compared with p97-positive melanoma cell lines.

Materials and Methods

Cell Lines and Culture

The following melanoma cell lines used in this study were obtained from American Type Culture Collection (Manassas, VA): SK-MEL-5, SK-MEL-28, A2058, Malme3M, WM266/4, G361, and WM115. Colo853-Mel was obtained from the European Collection of Animal Cell Culture (Wiltshire, United Kingdom) and IGR37 and IGR39 were from DSMZ (German National Resource Centre for

Biological Materials, Braunschweig, Germany). H3677 cells were from Dr. K.E. Hellström (University of Washington, Seattle, WA) and A375-M and A375-P cell lines were from Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX). Cell lines were cultured at 37°C with 5% CO₂ in appropriate media. Melanoma cell lines were grown in RPMI 1640 with 10% fetal bovine serum supplemented with 2 mmol/L L-glutamine. Normal human endothelial cells, smooth muscle cells, and hepatocytes were obtained from Cambrex (Walkersville, MD) and grown in media recommended by the vendor.

Antibodies

Hybridoma cell lines producing the two murine anti-p97 IgG1 mAbs, L235 (American Type Culture Collection) and L49 (17), were grown as recommended and media collected for antibody purification. Antibodies were purified with MabSelect Protein A column (Amersham, Piscataway, NJ). cAC10, a chimeric mAb against CD30 (25), and MOPC21, a mouse IgG1 (Sigma, St. Louis, MO), were used as negative controls in the cytotoxicity assays.

Immunohistochemistry

Frozen tissues on slides and frozen melanoma tissue microarrays (TriStar, Rockville, MD) were air-dried then fixed in 4% paraformaldehyde for 15 minutes at room temperature. Endogenous peroxidase activity was blocked with 0.6% H₂O₂ for 15 minutes whereas endogenous biotin was blocked with an Avidin-Biotin Blocking kit (Vector Laboratories, Burlingame, CA). For primary antibodies, mAbs against p97 (L49-biotinylated and L235) and control mouse IgG were used at 2 μ g/mL for 1-hour incubation at room temperature. Biotinylated secondary antimouse antibody was used for L235 (VectaStain Elite Kit, Vector Laboratories), followed by avidin conjugated to horseradish peroxidase. 3,3'-Diaminobenzidine was used as substrate for horseradish peroxidase. Tissues were counterstained with hematoxylin and slides were dehydrated and coverslipped. Two separate stainings were done for each mAb and slides were then scored manually using a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Thornwood, NY) or analyzed using a Genovision automated scanner (Biogenex, San Ramon, CA). Melanoma cell lines expressing p97 grown in chamber slides were used as positive controls for staining. Representative images were acquired with a 63 \times oil objective.

Quantitative FACS

Cell-surface p97 expression levels were quantified with a DAKO QIFIKIT flow cytometric indirect immunofluorescence assay (DAKO A/S, Glostrup, Denmark) with L49 or L235 as the primary antibody. Briefly, cells were detached with versene or trypsin and washed once with complete medium and then with PBS. Cells (5×10^5 per sample) were incubated with a saturating concentration (10 μ g/mL) of primary antibody for 60 minutes at 4°C. After washes, FITC-conjugated secondary antibody (1:50 dilution) was added for 45 minutes at 4°C. QIFIKIT standard beads were simultaneously labeled with the secondary antibody. Binding of antibodies was analyzed by flow cytometry

and specific antigen density was calculated by subtracting background antibody equivalent from antibody-binding capacity based on a standard curve of log mean fluorescence intensity versus log antigen binding capacity.

Conjugation of Antibodies

MAb L49 in 50 mmol/L sodium borate, 50 mmol/L NaCl, and 1 mmol/L diethylenetriaminepentaacetic acid (pH 8.0) was partially reduced with 3.25 equivalents of tris(2-carboxyethyl)phosphine hydrochloride at 37°C for 2.5 hours to yield ~6 thiols per antibody. The mixture was cooled to 0°C and alkylated with 1.1 equivalents of maleimidocaproyl-valine-citrulline-*p*-aminobenzoyl-MMAF (vcMMAF) per thiol for 30 minutes. Excess vcMMAF was quenched with 1 mmol/L cysteine and the entire mixture purified on a size-exclusion chromatography column (PD10, GE Healthcare, Piscataway, NJ) equilibrated with PBS. A similar protocol was used for conjugation of mAb L49 with related auristatin analogues vcAFP (22), mcAFP (26), and vcMMAE (20).

For conjugation to a fluorophore, L49 or L49-vcMMAF in 50 mmol/L sodium borate, 50 mmol/L NaCl (pH 8.0) was reacted with 50 equivalents of Alexa Fluor 488 *N*-hydroxysuccinimide ester, 15 equivalents of Alexa Fluor 568, or 40 equivalents of Alexa Fluor 594 *N*-hydroxysuccinimide ester (Invitrogen, Carlsbad, CA) at 25°C for 1 hour. The mixture was purified on a PD-10 column equilibrated with PBS. The fluorophore loading per antibody was typically 12, 4, and 7 equivalents of Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 594, respectively. These fluorophore loadings were optimized to give the maximum fluorescent signal and prevent self-quenching from decreasing the overall fluorescent signal.

Cytotoxicity Assay

Cytotoxicity was measured with resazurin (Sigma) dye reduction assay (27). Briefly, cells were plated at 5,000 to 7,500 per well in 96-well plates, allowed to attach to plates for 18 hours before addition of fresh media with or without antibody-drug conjugates or antibody with or without cross-linking secondary antibody (2-fold excess). After 96 hours of exposure to antibody or antibody-drug conjugates, resazurin was added to cells to a final concentration of 50 µmol/L. Cells were incubated for 2 to 6 hours depending on dye conversion of cell lines, and dye reduction was measured on a Fusion HT fluorescent plate reader (Packard Instruments, Meriden, CT) with excitation and emission wavelengths of 530 and 590 nm, respectively. The IC₅₀ value is defined here as the drug concentration that results in 50% reduction in growth or viability as compared with untreated control cultures.

Immunofluorescence

Cells were grown in coverslip-bottom chamber slides to ~75% confluence. Antibody-drug conjugates directly conjugated to Alexa Fluor 594 or Alexa Fluor 568 were added to the cells at 1 µg/mL and immunofluorescence was done using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss Microimaging). After 2, 24, 46, or 96 hours, cells were fixed and permeabilized with paraformaldehyde/saponin as provided in the Cytotfix/

Cytoperm kit (BD Biosciences, San Jose, CA) and then stained with anti-CD107a-FITC (lysosomal marker; BD Biosciences) or rabbit polyclonal anti-caveolin-1 (BD Biosciences). Nuclei were stained with 4',6-diamidino-2-phenylindole (Roche, Applied Sciences, Indianapolis, IN). Cells were mounted with DABCO antifade medium containing 25 mg/mL 1,4-dizobicyclometric grade glycerol and 10% 1× PBS. Images were obtained using the Zeiss Axiovert 200M and Leitz Orthoplan epifluorescence microscopes (Leitz, Wetzlar, Germany).

Results

Quantification of p97 Expression on Tumor Cell Lines and Normal Cells

Human melanoma cell lines were analyzed for cell-surface expression of p97 by quantitative FACS with mAb L49 (Table 1). All 13 melanoma cell lines were positive for p97 expression including 7 (54%) cell lines with high p97 levels (52,000–280,000 sites per cell). Three of the

Table 1. p97 expression and sensitivity to L49-vcMMAF in melanoma and normal cells

	L49 sites/ cell* (×10 ³)	L235 sites/ cell* (×10 ³)	L49-vcMMAF IC ₅₀ , ng/mL
Melanoma cell lines			
SK-MEL-5	280	320	1
SK-MEL-28 [†]	130	150	1,000
A2058	130	145	8
Malme3M	110	100	26
Colo853-Mel	80	94	7
H3677	72	66	>10,000
WM266/4	52	nd	>10,000
G361	40	nd	18
A375M [†]	36	33	>10,000
A375P [†]	25	nd	>10,000
IGR37	20	nd	4
IGR39	16	16	>10,000
WM115	3.6	2	>10,000
Normal human cells			
Neonatal melanocytes	64	66	2,100
Aortic smooth muscle	20	nd	>10,000
Uterine MVEC	6.9	6.9	8,700
Neonatal dermal MVEC	4.9	5.2	>10,000
Uterine smooth muscle	4.3	nd	>10,000
Pulmonary EC	3.6	3.1	>10,000
Hepatocytes	3.3	nd	>10,000
Lung MVEC	1.5	2.6	4,400
Adult dermal MVEC	0.95	nd	>10,000
Aortic endothelial cells	0.9	0.9	>10,000

NOTE: The sensitivity of the cells and cell lines to L49-vcMMAF is shown as IC₅₀ values in *in vitro* cytotoxicity assays.

Abbreviations: EC, endothelial cells; MVEC, microvascular endothelial cells; nd, not determined.

*The number of L49 and L235 binding sites per cell was estimated by quantitative FACS.

[†]Both p97-positive and p97-negative populations of cells were observed by FACS analysis for these cell lines; thus, the sites per cell were estimated for the antigen-positive cells.

melanoma cell lines (SK-MEL-28, A375P, and A375M) were found by flow cytometry to have p97-positive and p97-negative subpopulations. The estimate of p97 binding sites was made for the p97-positive subpopulation and thus is not affected by the presence of antigen-negative cells. In contrast, p97-negative cells did affect cytotoxicity assays as described below. Concordant quantitative FACS data for p97 were obtained with a second mAb, L235 (Table 1).

Beyond melanoma, p97 levels were also analyzed on various normal cells including endothelial cells, melanocytes, aortic and uterine smooth muscle cells, and hepatocytes. Only the human neonatal melanocytes were found to express high levels of p97 (64,000 sites per cell). The other neonatal tissue evaluated, dermal microvascular endothelial cells, has low levels of p97 (4,900 sites per cell). As for normal adult tissues, only aortic smooth muscle had moderate levels of p97 (20,000 sites per cell) whereas all other tissues tested had only low levels of p97 ($\leq 6,900$ sites per cell).

Anti-p97-Drug Conjugates Have Potent Cytotoxicity Activity against SK-MEL-5 Cells

We selected the melanoma cell line with the most abundant p97 expression (i.e., SK-MEL-5; 280,000 copies/cell) for an initial assessment of p97 as a potential target for antibody-drug conjugates. The L49 mAb was conjugated to several different auristatin drugs, including vcMMAE (20), vcMMAF (21), mcAFP (26), and vcAFP (22). All L49 antibody-drug conjugates were potently cytotoxic against SK-MEL-5 cells with IC_{50} values ranging from 0.7 to 7.1 ng/mL (Fig. 1A). In contrast, a negative control antibody-drug conjugate (cAC10-vcMMAF) directed against an irrelevant antigen (CD30) was sparingly active against SK-MEL-5 cells (IC_{50} , 1,000 ng/mL; Fig. 1B). Normal microvascular endothelial cells from lung and neonatal dermis with very low levels of p97 antigen ($< 5,000$ sites per cell) were resistant (IC_{50} , 4,400 and $> 10,000$ ng/mL, respectively) to the cytotoxic effects of all the anti-p97 antibody-drug conjugates tested, including L49-vcMMAF (Fig. 1B; Table 1). Unconjugated L49, in the presence or absence of a cross-linking mAb, did not perturb the growth of SK-MEL-5 cells (Fig. 1C), indicating that antibody alone did not have growth inhibitory activity.

Correlation of p97 Expression and Sensitivity to L49-vcMMAF in Melanoma and Normal Cell Lines

L49-vcMMAF was the conjugate with the most potent activity against SK-MEL-5 cells (IC_{50} , 0.7 ng/mL) and was selected for screening of the other melanoma cell lines and normal cells. Six of 13 melanoma cell lines studied were highly sensitive to L49-vcMMAF, with IC_{50} values ranging from 1 to 26 ng/mL, and were p97 positive, with 20,000 (IGR37) to 280,000 binding sites per cell (SK-MEL-5; Table 1). Four of these six melanoma cell lines have high levels of p97 ($\geq 80,000$ binding sites per cell). SK-MEL-28 cells, previously used in other p97 studies (18, 28), have low sensitivity to L49-vcMMAF (IC_{50} , 1,000 ng/mL), which likely reflects loss of p97 expression because antigen-positive and antigen-negative subpopulations were observed (Table 1). The A375M and A375P cell lines are

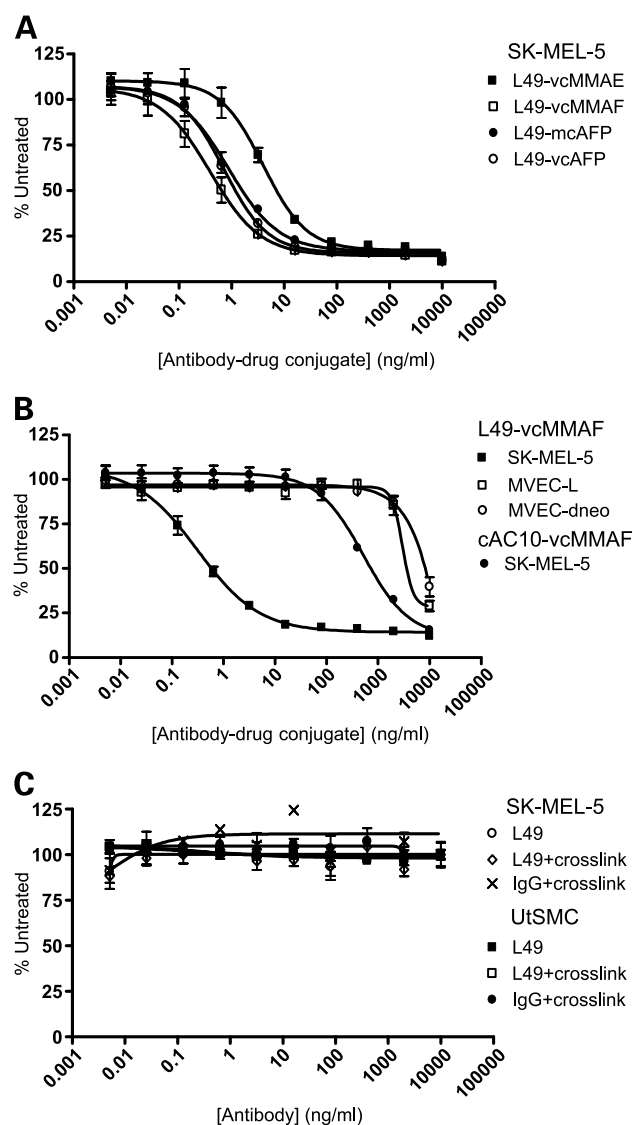


Figure 1. Antibody-drug conjugates targeting p97 have potent cytotoxic activity against antigen-positive melanoma cells, SK-MEL-5, while sparing p97-negative cells (normal endothelial cells). **A**, cells were grown in 96-well plates and anti-p97 antibody-drug conjugates were added for 96 h. Cell viability was measured by resazurin dye conversion. **B**, normal human microvascular endothelial cells from neonatal dermis (*MVEC-dneo*) and lung (*MVEC-L*) and SK-MEL-5 were treated with either L49-vcMMAF or a control antibody-drug conjugate (cAC10-vcMMAF) for 96 h. **C**, unconjugated L49 did not have cytotoxic activity in SK-MEL-5 and normal uterine smooth muscle cells. Cells were incubated with unconjugated L49 in the absence or presence of a 2-fold molar excess of a cross-linking secondary antibody and cell viability was measured after 96 h. MAb MOPC21 was used as an isotype-matched IgG control antibody.

highly resistant to L49-vcMMAF, and, like SK-MEL-28, the presence of p97-positive and p97-negative subpopulations suggests a potential mechanism for resistance. The melanoma cell lines H3677 (72,000 sites per cell) and WM266/4 (52,000 sites per cell) are both uniformly p97 positive; thus, antigen loss is not a likely explanation for their resistance to

L49-vcMMAF (IC_{50} , >10,000 ng/mL). The normal cells tested were resistant to L49-vcMMAF (IC_{50} , 2,100 to >10,000 ng/mL), which may reflect, in part, their low-level expression of the p97 antigen.

p97 Expression in Primary and Metastatic Melanoma by Immunohistochemistry

To further define the significance of the antigen expression levels in primary and metastatic melanomas, we did a survey of p97 expression in patient tumors with more samples of metastatic cases than previous studies (29, 30). A frozen tissue array of melanoma consisting of 9 cases of primary disease, 24 metastatic sites (lymph nodes, soft tissue, lung, and spleen), and 10 normal skin samples was used to determine the frequency of expression of p97 by immunohistochemistry with two different primary antibodies for staining, L49 and L235. Slides were scored (0–3+ scale) on the percentage and intensity of tumor cell staining: 0, no detectable staining; 1+, $\leq 25\%$ cells positive and low intensity; 2+, 25% to 75% cells positive and low to moderate intensity; and 3+, $\geq 75\%$ cells positive with intense staining as exemplified in Fig. 2A. Concordant staining data were obtained for the two different primary antibodies (Table 2) for primary tumors whereas there was slightly more reactivity of L49 observed in metastatic tumors than for L235. In metastatic melanoma, 15 of 24 (62%) of the tumors analyzed had moderate (2+) to high (3+) levels of p97 expression (L49 staining; Table 2). In addition, in primary tumors, 56% of cases showed 1+ to 3+ staining with four of nine cases having moderate to high percentage (2–3+) of cells positive for p97 with moderate to high intensity of staining. About 36% of the melanoma cases analyzed had no detectable p97 expression by immunohistochemistry. Similarly, p97 expression was not detectable on normal adult melanocytes in the skin. Melanoma cell lines with varying levels of p97 expression, as judged by quantitative FACS (Table 1), were grown in chamber slides, fixed, and used as controls for the staining. Comparison of p97 immunohistochemistry staining in positive melanoma cell lines shows that the intensity of staining correlate with the number of antigen binding sites per cell (Fig. 2B).

Subcellular Colocalization of Anti-p97-Drug Conjugate with Lysosomal Marker CD107a

Immunofluorescence microscopy was used to investigate whether differences in internalization route or kinetics might provide a mechanistic basis for the observation that some melanoma cell lines expressing relatively high levels of p97 (e.g., H3677 and WM266/4) are resistant to the cytotoxic effects of L49-vcMMAF whereas many other melanoma cell lines are highly sensitive (Table 1). Specifically, the internalization of L49-vcMMAF fluorescently tagged with Alexa Fluor 594 (red signal) was studied in both sensitive (SK-MEL-5) and resistant (H3677) cell lines by immunofluorescence microscopy. A lysosome marker, FITC-labeled anti-CD107a, was also used for staining fixed and permeabilized cells. The L49-vcMMAF-Alexa Fluor 594 conjugate is detected intracellularly for both sensitive and resistant cell lines within 2 hours of

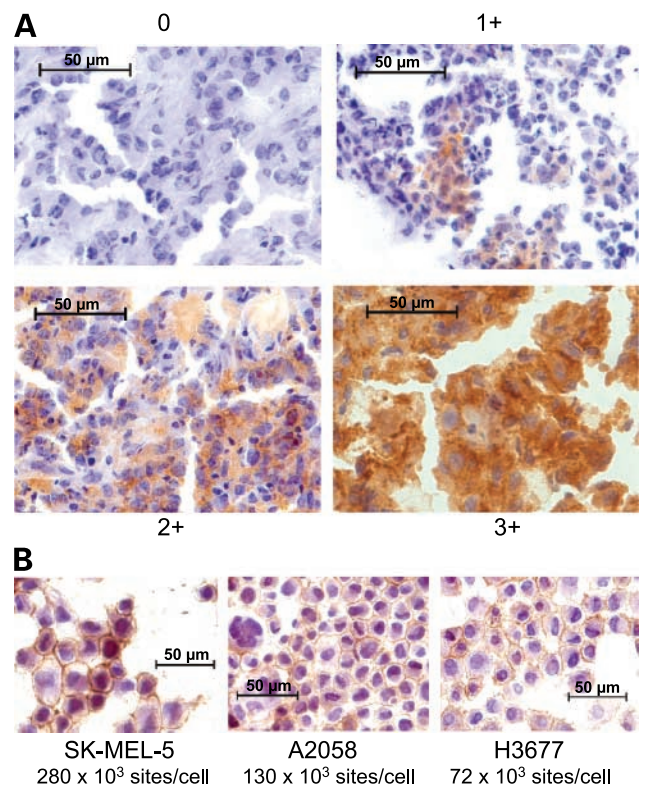


Figure 2. Immunohistochemical analysis of p97 expression in metastatic melanoma by tissue microarrays. **A**, immunohistochemical staining in metastatic melanomas showing relative intensity of staining for p97. Biotinylated L49 was used as primary antibody for detection of p97. Brown staining indicates relative p97 protein expression ($\times 630$ magnification). Slides were scored on the percentage and intensity of tumor cell staining using a 0 to 3+ scale: 0, no detectable staining; 1+, $\leq 25\%$ cells positive and low intensity; 2+, 25% to 75% cells positive and low to moderate intensity; and 3+, $\geq 75\%$ cells positive with intense staining. **B**, melanoma cell lines were grown in chamber slides, fixed, and used as controls for immunohistochemistry. Biotinylated L49 and L235 mAbs were used as primary antibodies. Staining shown is for biotinylated L49 mAb using a $63\times$ oil immersion objective. Bar, 50 μm .

incubation (Fig. 3A and E). In contrast, L49-Alexa Fluor that was not conjugated to vcMMAF did not effectively internalize and was mostly observed on the cell surface (Fig. 4B). On merging of the images of lysosomal marker CD107a (green) with the antibody-drug conjugate (red), there was good overlap of the signals (yellow) for the antibody-drug conjugate and CD107a in SK-MEL-5 (sensitive cells), indicating colocalization in the lysosomes (Fig. 3A–D). Similar colocalization was observed with A2058 cells (data not shown). In contrast, there was differential localization of L49-vcMMAF and CD107a in H3677 cells that were insensitive to the antibody-drug conjugate (Fig. 3E–H). These data strongly suggest that on internalization of L49-vcMMAF by H3677 cells, most of the antibody-drug conjugate failed to reach the lysosome for effective release of the drug. This was also noted in resistant SK-MEL-28 cells that had survived 96-hour exposure to L49-vcMMAF (Fig. 4D–F). Additional immunofluorescence

Table 2. p97 expression in primary and metastatic melanoma

Melanoma	Sample number	Immunohistochemistry score: L49, L235					
		0	1+	2+	3+	1–3+ (%)	2–3+ (%)
Primary	9	4, 4	1, 1	2, 3	2, 1	56, 56	44, 44
Metastatic	24	8, 9	1, 3	8, 7	7, 5	67, 62	62, 50

NOTE: Slides were scored on the percentage and intensity of tumor cell staining using a 0 to 3+ scale: 0, no detectable staining; 1+, $\leq 25\%$ cells positive and low intensity; 2+, 25% to 75% cells positive and low to moderate intensity; and 3+, $\geq 75\%$ cells positive with intense staining (see Fig. 2A).

studies with anti-caveolin-1 antibody and L49-vcMMAF-Alexa Fluor 594 showed colocalization of L49-vcMMAF with caveolin-1, a protein highly expressed in caveolae (Fig. 3I–L). Unconjugated L49 also colocalizes with caveolin-1 on the cell surface of H3677 (Fig. 4A–C).

Discussion

There are several reasons why the melanotransferrin/p97 antigen holds significant promise as a target for antibody-based therapeutics for malignant melanoma. As previously reported and as shown here, a significant proportion of primary and metastatic melanomas express p97 at high

levels (2, 6, 29) and only a few normal tissues express the antigen at low levels. As reported here for the first time, p97-expressing melanoma cell lines can be potentially killed by antibody-drug conjugates (e.g., L49-vcMMAF). Importantly, L49-vcMMAF has differential *in vitro* cytotoxicity, efficiently killing cancer cells that express high levels of p97 while sparing normal cells expressing p97 at low levels.

In this study, the level of expression of p97 in both melanoma cell lines and normal cells was determined by quantitative FACS. In addition, primary melanoma and metastatic melanoma tumors were analyzed by immunohistochemistry to determine the level of p97 expression in patient tumors. Overall, 62% of tumors analyzed had detectable p97 expression (1–3+). Forty-four percent of primary melanomas and 62% of metastatic tumors showed intense staining and a high percentage of cells were positive for p97. Using melanoma cell lines with various p97 levels as controls for immunohistochemistry staining, the intensity of staining observed correlated with quantified levels of antigen binding sites per cell. Notably, the observed intensity for the highest-expressing melanoma line, SK-MEL-5, was comparatively lower than what was observed in patient tumors, suggesting that primary and metastatic tumors express p97 at a level that can be effectively targeted by anti-p97 antibody-drug conjugate compared with normal tissues. About 36% of melanomas

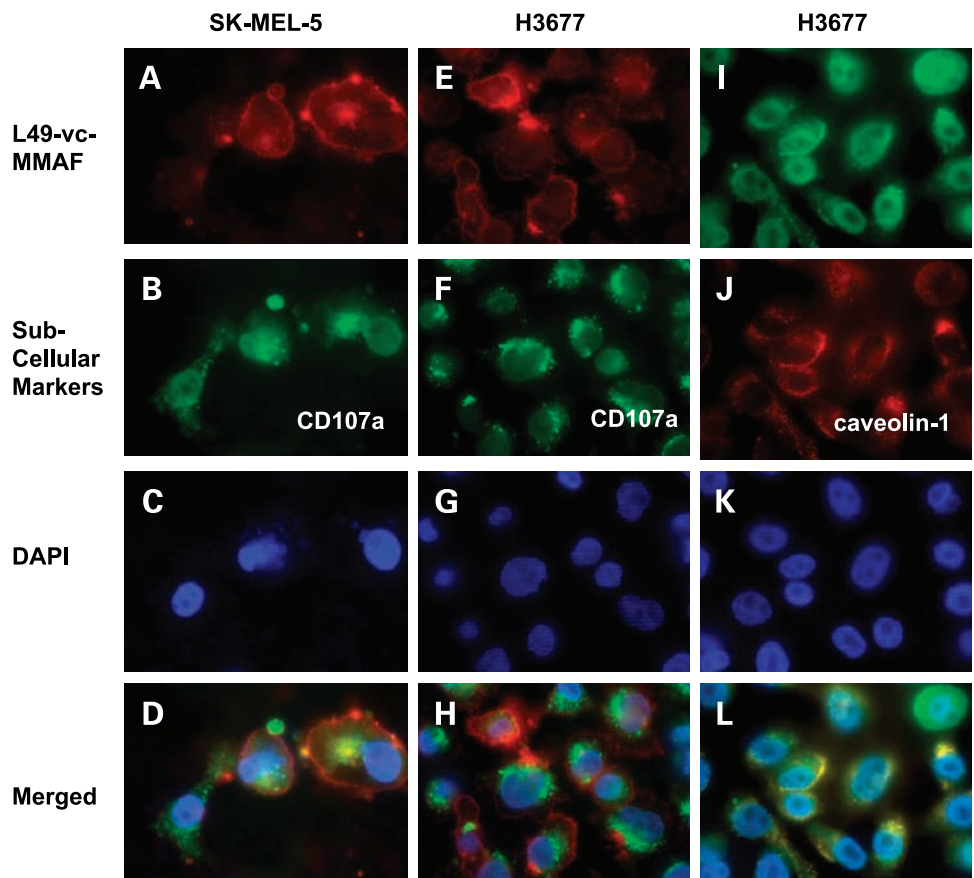


Figure 3. The anti-p97 antibody-drug conjugate, L49-vcMMAF, colocalizes (yellow) with the lysosomal marker, CD107a, in a sensitive cell line (SK-MEL-5; D) but not in a resistant cell line (H3677; H) in which it colocalizes with caveolin-1 (L). Subcellular localization of L49-vcMMAF (red) and CD107a (green) in SK-MEL-5 (A–D) and H3677 (E–H) cells after 96-h incubation with the antibody-drug conjugate. Subcellular localization of L49-vcMMAF (green) and caveolin-1 (red) in H3677 cells after 96-h incubation with the antibody-drug conjugate (I–L). Nuclei were stained blue with 4',6-diamidino-2-phenylindole (C, G, and K). Images were acquired with a 63 \times oil immersion objective.

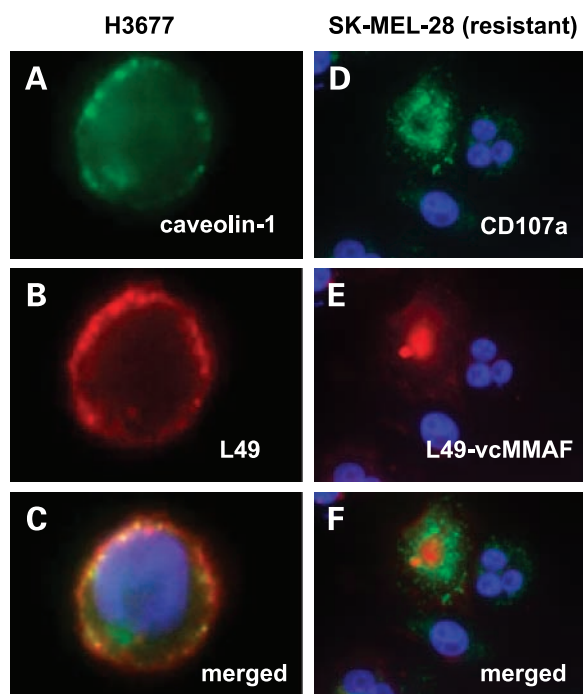


Figure 4. Subcellular localization of unconjugated L49 and L49-vcMMAF in resistant melanoma cell lines. **A to C**, H3677 cells were incubated with unconjugated L49 for 96 h. L49 was detected with Alexa Fluor 568 – labeled goat anti-mouse IgG (red). Rabbit polyclonal antibody against caveolin-1 and Alexa Fluor 488 – labeled goat anti-rabbit IgG (green) were used for colocalization with caveolin-1. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Representative images were taken using a Leitz epifluorescence microscope (100 \times oil immersion objective). **D to F**, SK-MEL-28 cells were incubated with L49-vcMMAF-Alexa Fluor 568 (red) for 96 h. Cells that survived the antibody-drug conjugate treatment were fixed and anti-CD107a (lysosomal marker) conjugated with FITC (green) was used for colocalization to lysosome. Images were acquired using a Leitz epifluorescence microscope (100 \times oil objective).

do not have detectable p97 expression by immunohistochemistry, indicating a need for a diagnostic tool to identify patients that might potentially benefit from an antibody-drug conjugate targeting p97.

The p97 antigen is expressed in some neonatal tissues, such as liver and melanocytes, and in adult normal tissues, including salivary glands, sweat glands, smooth muscle, and brain endothelial cells. Using a double-determinant assay, Woodbury et al. (6) reported that cells from most melanoma express 50,000 to 500,000 p97 molecules on their surface whereas the highest expression observed in normal adult tissues is \sim 8,000 molecules per cell (smooth muscle), with most normal adult cells having $<$ 4,000 p97 molecules on their surfaces. Quantitative FACS results from this study were concordant with the calculated number of p97 molecules on the cell surface of melanomas versus normal tissues. We were unable to quantify the level of p97 expression in normal human brain, detected by immunohistochemistry (31), for lack of available cultured human brain endothelial cells. Other

normal human microvascular endothelial cells and smooth muscle cells were analyzed for p97 expression and were found to have lower levels of antigen than most melanoma cell lines (\leq 20,000 molecules per cell).

With the differential levels of p97 expression observed between melanoma cell lines and normal cells, we proceeded to determine the effects of an anti-p97-drug conjugate on normal cells. Cytotoxicity assays showed that neonatal melanocytes and microvascular endothelial cells are resistant to the antibody-drug conjugate L49-vcMMAF, with IC_{50} values of \geq 2,100 ng/mL. In contrast, melanoma cells that are sensitive to L49-vcMMAF are susceptible at \sim 100- to 1,000-fold lower concentrations with IC_{50} values of 1 to 26 ng/mL.

A general concern for targeted therapy, particularly of heterogeneously expressed targets such as p97, is selection and outgrowth of antigen-negative tumor cells *in vivo*. In the case of antibody-drug conjugates, this challenge can potentially be addressed by judicious design of the cytotoxic drug and linker to facilitate the killing of antigen-negative bystander tumor cells (32). For auristatins, MMAE (20) is much more cytotoxic as a free drug than MMAF (21), and it has been proposed that this is due to cell permeability differences. On release of the drug from the antibody, MMAE can potentially diffuse into other nearby tumor cells that are antigen negative and be cytotoxic to these cells. A bystander killing effect can be both potentially beneficial, by providing potent cytotoxicity to tumor cells regardless of levels of antigen, and potentially harmful because surrounding normal cells will also be susceptible to the cytotoxic effects. Ideally, the potential benefit of effectively killing the tumor cells will outweigh the potential risk of normal cell cytotoxicity. Identifying the therapeutic window for targeting molecules is critical for a successful cancer therapy. These challenges notwithstanding, in preliminary *in vivo* studies we have observed dose-dependent efficacy of L49-vcMMAF against established (\sim 100 mm³) SK-MEL-5 xenografts in severe combined immunodeficient mice, at doses of 3 to 10 mg/kg, which are well below the maximum tolerated dose of \sim 40 mg/kg (data not shown).

The intracellular trafficking behavior of mAb L49 was found in this study to depend on both drug conjugation and antigen level. L49 had previously been characterized as a noninternalizing antibody (19). Indeed, on binding of unconjugated L49 to p97, the antibody/antigen complex remains largely on the cell surface, as judged by fluorescence microscopy (Fig. 4). In contrast, conjugation of L49 to derivatives of auristatin, such as MMAE, MMAF, and AFP, endows this antibody with ability to internalize efficiently. Similar observations have previously been made for the anti-CD20 antibodies rituximab and 1F5, following conjugation to auristatins (33), and for the anti-HER2 antibody trastuzumab, on conjugation to the drug geldanamycin (34).

Once internalized, L49-vcMMAF exerts a cytotoxic effect for cell lines that traffic this conjugate to the lysosome, but not for cell lines that traffic it away from the lysosome to

locations such as caveolae. The p97 antigen is anchored to the cell membrane via a glycosylphosphatidyl inositol linkage, adding another factor that may influence the internalization pathway. Clustering of antibody-drug conjugates on the cell surface due to drug-drug interaction is a potential mechanism. Subcellular localization of p97 in fetal intestinal cells showed apical polarization, a feature of many membrane proteins attached through glycosylphosphatidyl inositol moiety covalently bound to the COOH terminus (7). Most glycosylphosphatidyl inositol-linked proteins are associated with caveolin-1 and internalized via the caveolae pathway (23). When bound to an atom of iron, glycosylphosphatidyl inositol-anchored p97 has been proposed to internalize via an undefined endocytic pathway, possibly involving caveolae (35), although a porcine homologue of melanotransferrin has been shown to localize to the detergent-insoluble noncaveolar microdomains (36). Signaling molecules arranged along the cytoplasmic face of caveolae are likely to be crucial for preventing lysosomes from fusing with caveolar vesicles (reviewed in ref. 24). Caveolins can either directly traffic from the plasma membrane to the endoplasmic reticulum/Golgi or localize to nonendosomal vesicles called "caveosome" (24, 37).

L49-vcMMAF requires cleavage of the dipeptide linker to release the active drug inside the cell, a process that depends on exposure to lysosomal proteases (20, 38). Because p97 is glycosylphosphatidyl inositol linked and potentially associated with insoluble glycolipid rafts, it is possible that, due to differential trafficking, the antibody-drug conjugate does not reach the lysosomes and is not exposed to a proteolytic environment that would release the cytotoxic drug. Colocalization of L49-vcMMAF with a lysosomal marker, CD107a/Lamp-1, in the sensitive cell lines but not in resistant cells implies that, indeed, the antibody-drug conjugates traffic to lysosomes. Differential subcellular localization of antibody-drug conjugates in sensitive and resistant cells indicates that successful trafficking to the lysosome is an important factor for potent cytotoxic activity of anti-p97-auristatin conjugates against melanoma cell lines. The overexpression of p97 in melanoma as compared with normal tissue, in conjunction with the greater sensitivity of tumor cells to L49-vcMMAF, supports further evaluation of antibody-drug conjugates as a means to target p97-overexpressing tumors.

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