Cancer Research

Antibody-Maytansinoid Conjugates Designed to Bypass Multidrug Resistance

Yelena V. Kovtun, Charlene A. Audette, Michele F. Mayo, Gregory E. Jones, Heather Doherty, Erin K. Maloney, Hans K. Erickson, Xiuxia Sun, Sharon Wilhelm, Olga Ab, Katharine C. Lai, Wayne C. Widdison, Brenda Kellogg, Holly Johnson, Jan Pinkas, Robert J. Lutz, Rajeeva Singh, Victor S. Goldmacher, and Ravi V.J. Chari

Abstract

Conjugation of cytotoxic compounds to antibodies that bind to cancer-specific antigens makes these drugs selective in killing cancer cells. However, many of the compounds used in such antibody-drug conjugates (ADC) are substrates for the multidrug transporter MDR1. To evade the MDR1-mediated resistance, we conjugated the highly cytotoxic maytansinoid DM1 to antibodies via the maleimidyl-based hydrophilic linker PEG_4Mal. Following uptake into target cells, conjugates made with the PEG_4Mal linker were processed to a cytotoxic metabolite that was retained by MDR1-expressing cells better than a metabolite of similar conjugates prepared with the nonpolar linker *N*-succinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (SMCC). In accord, PEG_4Mal-linked conjugates were more potent in killing MDR1-expressing cells in culture. In addition, PEG_4Mal-linked conjugates were markedly more effective in eradicating MDR1-expressing human xenograft tumors than SMCC-linked conjugates while being tolerated similarly, thus showing an improved therapeutic index. This study points the way to the development of ADCs that bypass multidrug resistance. *Cancer Res; 70(6); 2528–37. ©2010 AACR.*

Introduction

Treatment of cancer patients with chemotherapeutic drugs often leads to the emergence of tumors with a multidrug-resistant (MDR) phenotype (1, 2). Many mechanisms of MDR have been described, such as the overexpression of MDR1 (also known as P-glycoprotein or ABCB1) or other ATP-dependent transporters that pump drugs out of the cancer cells, amplification of drug-inactivating enzymes, mutations or modifications of drug targets, alterations in DNA repair machinery, and increased resistance to apoptosis (1–3). The MDR1-mediated efflux of anticancer drugs is the most commonly observed MDR phenotype clinically, and correlations between the transporter expression and poor response to chemotherapy have been documented for many cancer types (4, 5). To overcome MDR1-mediated resistance, numerous MDR inhibitors or modulators have been developed and tested in clinical trials in combination with approved anticancer drugs. However, high systemic toxicity was observed in these trials, possibly because these inhibitors also affect the MDR1 transporter in normal tissue, giving rise to increased accumulation of cytotoxic anticancer drugs in normal tissue as well as in tumors (1, 4).

A promising approach to achieve targeted delivery of cytotoxic drugs to tumor cells, thereby reducing their systemic toxicity, is to use antibodies as specific delivery vehicles. Several highly potent cytotoxic compounds have been conjugated to tumor-targeting antibodies. A number of such antibody-drug conjugates (ADC) are currently undergoing clinical trials, and one conjugate, gemtuzumab ozogamicin, has been approved for the treatment of acute myeloid leukemia (6, 7). However, the majority of cytotoxic compounds that have been used in ADCs, such as calicheamicin (8–10), doxorubicin, taxanes (1, 4), maytansinoids (11), analogues of dolastatin 10 (12), and CC-1065 (13, 14), are substrates for the MDR1 transporter, and the activity of many ADCs is poor in MDR1-expressing cells (8–11, 15, 16).

MDR1 is believed to confer resistance to cytotoxic compounds via two mechanisms: first, by mediating the efflux of drugs that diffuse into the plasma membrane from the extracellular space, thereby preventing the compound from reaching the cytoplasm, and second, by effluxing any compound that does enter the cytoplasm back to the outside of the cell (3, 17). ADCs deliver their cytotoxic payload inside cells via antigen-mediated endocytosis, thus bypassing the first MDR1 mechanism (16, 18). Upon intracellular processing, conjugates are processed into cytotoxic metabolites, whose structures depend on the chemistry of the linker between the cytotoxic compound and the antibody (19, 20). These metabolites may be MDR1 substrates and therefore susceptible to the second resistance mechanism, MDR1-mediated efflux from the cytoplasm to the exterior of the cell.

Authors' Affiliation: ImmunoGen, Inc., Waltham, Massachusetts

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Yelena V. Kovtun, ImmunoGen, Inc., 830 Winter Street, Waltham, MA 02451. Phone: 781-895-0600; Fax: 781-895-0615; E-mail: yelena.kovtun@immunogen.com.

doi: 10.1158/0008-5472.CAN-09-3546

Maytansinoids, highly potent antimicrotubule agents, are the payload of several ADCs in clinical development (7, 21). A maytansine-resistant phenotype, linked to the expression of MDR1, has been previously described (11, 22, 23). Building on this observation, we first confirmed that MDR1-expressing cells were resistant to killing by maytansinoids and maytansinoid conjugates. We then reengineered the antibody-maytansinoid conjugates to bypass this MDR1-mediated resistance. Although MDR1 has been shown to recognize and transport a great variety of compounds sharing little if any structural similarities, most of the reported MDR1 substrates are hydrophobic (24). We hypothesized that if maytansinoids were attached to an antibody via a hydrophilic linker, the resulting metabolite would be hydrophilic and, therefore, might be a poor substrate of MDR1, thus avoiding MDR1-mediated resistance. In this report, we describe novel antibody-maytansinoid conjugates that, upon internalization and processing, generate a cytotoxic metabolite that seems to be a poor MDR1 substrate. We found that these conjugates bypass MDR1-mediated resistance both in cultured human cell lines and in xenograft tumors in immunodeficient mice, resulting in an increase in the therapeutic index of the conjugates.

Materials and Methods

Antibody-maytansinoid conjugates. The anti–epidermal growth factor receptor (EGFR) antibody (rat IgG2a developed at ImmunoGen, Inc.), the anti-EpCAM monoclonal antibody B38.1 [murine IgG1, HB 8110; American Type Culture Collection (ATCC)], and the anti-CanAg antibody huC242 (humanized IgG1; refs. 25, 26) were modified with either *N*-succinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Sigma-Aldrich) or Mal-dPEG4-NHS (QuantaBiodesign) before conjugation with DM1 (23) as described previously (27). The conjugates used in this study contained, on average, 3.5 molecules of DM1 per antibody molecule.

Generation of COLO 205^{MDR} cell line. The coding sequence of the human *MDR1* (*ABCB1*) gene was cloned into pQCXIN plasmid (BD Biosciences) and packaged using the pantropic retroviral expression system (BD Biosciences). COLO 205 cells (ATCC CCL-222) were transduced with *MDR1* using this vector in accordance with the manufacturer's recommendations, and a COLO 205 clone expressing a functional MDR1 pump, denoted COLO 205^{MDR}, was selected by culturing the cells in medium containing paclitaxel (5 nmol/L) with subsequent subcloning.

Binding studies. Binding of antibodies and antibody-maytansinoid conjugates to cells was evaluated by indirect immunofluorescence using flow cytometry as described previously (28).

Rhodamine-123 accumulation and retention assays. Cultures of HCT-15 (ATCC CCL-225), UO-31 (National Cancer Institute-Frederick Cancer Research & Development Center, Frederick, MD), COLO 205, and COLO 205^{MDR} were maintained in RPMI 1640 (Invitrogen, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 50 μg/mL gentamicin sulfate (Invitrogen) at 37°C in 6% CO_2. Cell samples (2.5×10^5 cells/mL) were treated with rhodamine-123 (1.3 μmol/L; Sigma-Aldrich) in the presence or absence of cyclosporin A (5 μmol/L; Sigma-Aldrich) for 90 min at 37°C. The cells were then washed twice with HBSS containing 10% (v/v) fetal bovine serum and analyzed by flow cytometry. The cells evaluated for rhodamine-123 retention were resuspended in culture medium with or without cyclosporin A (5 μmol/L), incubated for an additional 90 min at 37°C, washed, and analyzed by flow cytometry.

In vitro *cytotoxicity assays.* Cells in 96-well plates (2×10^3 per well) were treated with test compounds at various concentrations in appropriate cell culture medium (total volume, 0.2 mL). Control wells containing cells and the medium but lacking test compounds and wells containing medium only were included in each assay plate. The plates were incubated for 4 to 6 d at 37°C in a humidified atmosphere containing 6% CO_2. WST-8 reagent (10%, v/v; Dojindo Molecular Technologies) was then added to the wells, and the plates were incubated at 37°C for 2 to 6 h depending on the cell line. Then, the absorbance was measured on a plate reader spectrophotometer in the dual-wavelength mode 450 nm/650 nm, and the absorbance at the 650 nm (nonspecific light scattering by cells) was subtracted. The apparent surviving fraction of cells in each well was calculated by first correcting for the medium background absorbance and then dividing each value by the average of the values in the control wells (nontreated cells). Each cytotoxicity experiment was repeated at least twice. The data shown in the figures represent a typical experiment.

Intracellular metabolism. COLO 205^{MDR} cells (2×10^6/mL) were incubated in culture medium containing a conjugate (10^{-8} mol/L) for 24 h at 37°C. Cells were then washed in medium, and metabolites were extracted with acetone and analyzed by reverse-phase high-performance liquid chromatography (HPLC) and by electrospray mass spectrometry as described previously (19).

In vivo *efficacy study.* COLO 205, COLO 205^{MDR}, or HCT-15 cells (1×10^7 per animal) were injected s.c. in the area under the right shoulder of female CB.17 severe combined immunodeficient (SCID) mice (Charles River Laboratories). When the tumor volume reached average size of 130 to 140 mm^3, mice were randomized into treatment groups with similar mean tumor volumes ($n = 6$ animals per group). Groups were treated with a single i.v. bolus injection of PBS (vehicle control) or an antibody-maytansinoid conjugate. Tumor growth was monitored and tumor volume was calculated by the following formula: length × width × height × 1/2.

Cell cycle analysis. Cells (2.5×10^5/mL) were exposed to conjugate (4 nmol/L of conjugated DM1) for 3 h at 37°C, washed, and incubated in conjugate-free medium for an additional 18 h at 37°C. Cells were then fixed with 1 mL of 70% ethanol, and DNA content was determined after staining with propidium iodide by flow cytometry as previously described (19).

Quantification of maytansinoid metabolites. The cells were collected by centrifugation and resuspended in 50 μL of 0.05% Tween 20 in TBS (pH 7.5). Cold acetone (200 μL) was added to each sample, and the samples were then incubated on ice for 30 min, which led to protein precipitation.

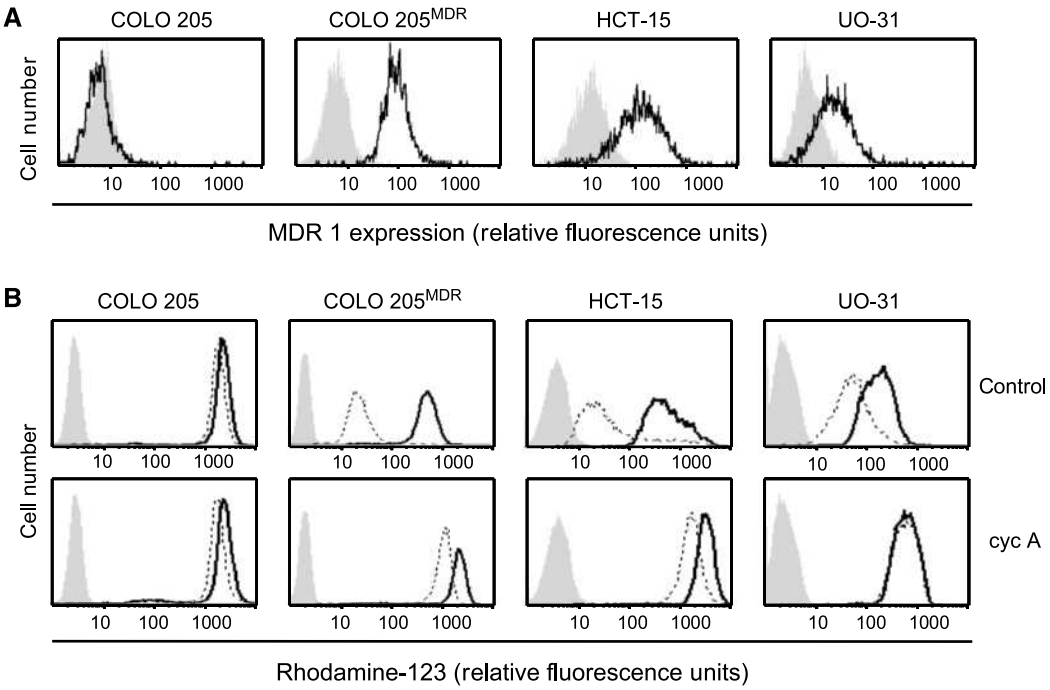

Figure 1. COLO 205^{MDR}, HCT-15, and UO-31 cells express functionally active MDR1 protein on their surface. A, cell surface expression of MDR1. Cells were incubated with the anti-MDR1 antibody (solid lines) or binding buffer (filled histograms) and then with anti-IgG–FITC and analyzed on a flow cytometer. B, uptake (solid lines) and retention (dotted lines) of rhodamine-123 in the absence (Control) or the presence of cyclosporin A (cyc A). Filled histograms show the cell autofluorescence.

The samples were then centrifuged (16,100 × *g*, 30 min) and ^{3}H levels of supernatants were determined using a liquid scintillation analyzer.

Results

Human tumor cell lines that express active MDR1 show decreased sensitivity to maytansine and to some antibody-maytansinoid conjugates. Three MDR human cell lines were used in this study to evaluate the effect of the MDR1 transporter on maytansinoid-dependent cytotoxicity: a clone of the colon adenocarcinoma cell line COLO 205 that has been retrovirally transduced with MDR1 (COLO 205^{MDR}) and two naturally evolved MDR cell lines (the colon adenocarcinoma HCT-15 and the renal adenocarcinoma UO-31). The expression of MDR1 on the plasma membrane of these cells was confirmed by immunostaining with an anti-MDR1 antibody (Fig. 1A). The parental MDR1-negative COLO 205 cells were used as a negative control (Fig. 1A). The functional integrity of the MDR1 pump was examined by the ability of the cells to accumulate and retain the fluorescent dye rhodamine-123, a well-established MDR1 substrate (29). The exposure of COLO 205^{MDR} cells to rhodamine-123 resulted in a 4-fold lower accumulation of the dye compared with that in the parental COLO 205 cells (mean fluorescence of 700 versus 2,800) and a 100-fold reduction in the amount of cell-associated dye retained by the cells after incubation in dye-free medium (mean fluorescence of 28 versus 2,800; Fig. 1B). Importantly, in the presence of an inhibitor of MDR1, cyclosporin A (1, 3), the COLO 205^{MDR} cells accumulated and retained as much rhodamine-123 as COLO 205 cells (Fig. 1B), indicating that the reduced accumulation and retention in COLO 205^{MDR} cells was due to the activity of the MDR1 pump. Similar results were obtained with other MDR1-expressing cell lines, HCT-15 and UO-31, where cyclosporin A caused a substantial increase in the accumulation and retention of rhodamine-123 (Fig. 1B). These results show that COLO 205^{MDR}, HCT-15, and UO-31 cells express active MDR1 transporter on their plasma membrane.

To evaluate the effect of MDR1 expression on maytansinoid-induced cell killing, we compared the sensitivities of COLO 205^{MDR} cells and the parental cell line COLO 205 to maytansine and its derivative DM1SMe (23). We also tested the activity of two other antimicrotubule cytotoxic compounds, paclitaxel and vinblastine, which have previously been shown to be MDR1 substrates (1, 3). As expected, paclitaxel and vinblastine were less potent in killing COLO 205^{MDR} cells than in killing COLO 205 cells (13- and 17-fold, respectively; Table 1). Similarly, sensitivity of cells to maytansine and DM1SMe was also affected by MDR1 expression: COLO 205 ^{MDR} cells were 8-fold less sensitive to the maytansinoids than COLO 205 cells. We also compared the cytotoxicity of

Table 1. *In vitro* cytotoxicity of paclitaxel, vinblastine, maytansine, and DM1SMe for COLO 205, COLO 205^{MDR}, HCT-15, and UO-31 cells in the presence or absence of 1 μmol/L cyclosporin A

Cytotoxic agent	COLO 205^{MDR} IC_{50} (nmol/L)	COLO 205 IC_{50} (nmol/L)	IC_{50} COLO 205^{MDR}/IC_{50} COLO 205	HCT-15			UO-31		
				IC_{50} (nmol/L)		IC_{50} (−cycA)/ IC_{50} (+cycA)	IC_{50} (nmol/L)		IC_{50} (−cycA)/ IC_{50} (+cycA)
				−cycA	+cycA		−cycA	+cycA	
Paclitaxel	53 ± 5	4.0 ± 1.6	13	70 ± 29	4 ± 2	18	105 ± 40	6 ± 3	18
Vinblastine	24 ± 11	1.4 ± 0.4	17	5 ± 2	0.5 ± 0.2	10	65 ± 21	6.5 ± 2.1	10
Maytansine	0.6 ± 0.1	0.08 ± 0.01	8	0.4 ± 0.2	0.05 ± 0.03	8	0.7 ± 0.2	0.12 ± 0.06	6
DM1SMe	0.42	0.05	8	0.14	0.02	7	0.4	0.07	6

NOTE: The data are presented as the mean IC_{50} ± SEM (n = 3).
Abbreviation: cycA, cyclosporin A.

maytansine, DM1SMe, paclitaxel, and vinblastine to MDR1-positive HCT-15 and UO-31 cells in the presence or absence of the MDR1 inhibitor cyclosporin A. Maytansinoid-induced cytotoxicity against HCT-15 and UO-31 cells was enhanced 6- to 8-fold in the presence cyclosporin A, and that of paclitaxel and vinblastine was enhanced 10- and 18-fold, respectively (Table 1). Cyclosporin A alone at 1 μmol/L was not cytotoxic to the cells (data not shown). These results indicate that MDR1 activity limits the potency of these cytotoxic compounds.

To evaluate whether expression of MDR1 affects the cytotoxicity of an antibody-maytansinoid conjugate, we evaluated the activity of an anti-EpCAM antibody-maytansinoid conjugate against the EpCAM-positive cell lines HCT-15, COLO 205^{MDR}, and COLO 205 (Supplementary Fig. S1; Supplementary Table S1). The conjugate anti-EpCAM–SMCC–DM1 was composed of the anti-EpCAM antibody B38.1 conjugated with the maytansinoid DM1 using the SMCC linker (Fig. 2A). The conjugation did not affect the ability of the antibody to bind to the EpCAM-positive cells (Supplementary Fig. S2). The anti-EpCAM–SMCC–DM1 conjugate showed antigen-dependent cytotoxicity against the MDR1-positive HCT-15 and COLO 205^{MDR} cells, and the MDR1-negative COLO 205 cells, as shown by the ability of excess unconjugated anti-EpCAM antibody to block the cell-killing activity (Fig. 3A, top row). Cyclosporin A (Fig. 3A, top row) or another MDR1-specific inhibitor, PGP4008 (data not shown; ref. 30), significantly enhanced the potency of anti-EpCAM–SMCC–DM1 against the MDR1-positive HCT-15 and COLO 205^{MDR} cells, but not against the MDR1-negative COLO 205 cells, indicating that MDR1 activity limits the potency of this conjugate.

In addition to the MDR1 transporter, we evaluated expression of two other ATP-dependent transporters that are

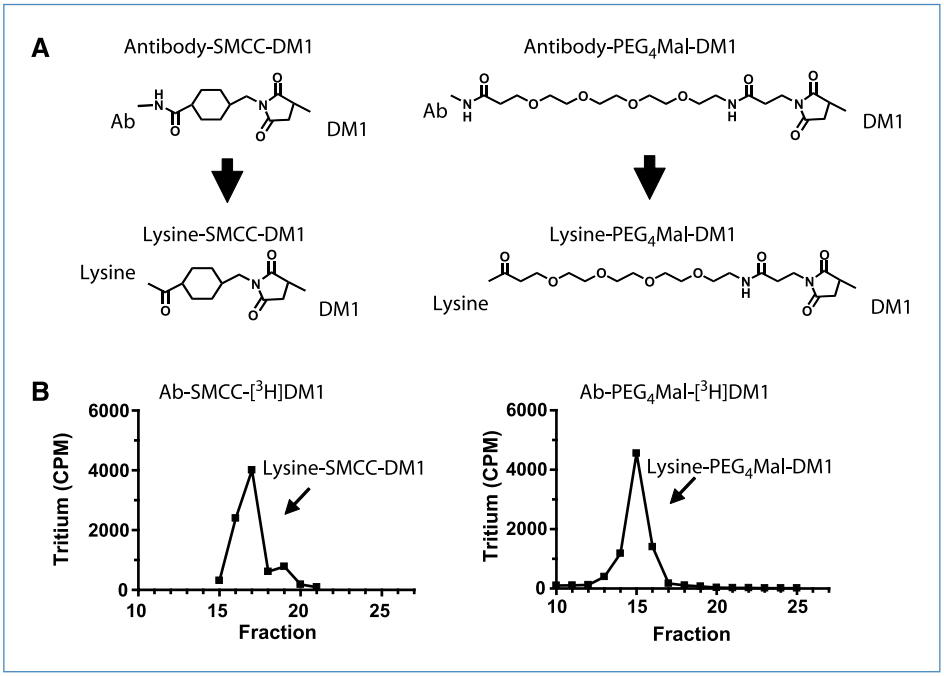

Figure 2. Metabolism of antibody-maytansinoid conjugates. A, chemical structures of antibody (Ab)-maytansinoid conjugates and their metabolites. B, HPLC analysis of [^{3}H]maytansinoid metabolites of the anti-EpCAM–DM1conjugates. COLO 205^{MDR} cells were exposed to anti-EpCAM–SMCC–[^{3}H]DM1 and anti-EpCAM–PEG$_4$Mal–[^{3}H] DM1. The [^{3}H]maytansinoid metabolites were extracted from the cells with acetone as described in Materials and Methods and analyzed by reverse-phase HPLC and liquid scintillation counting.

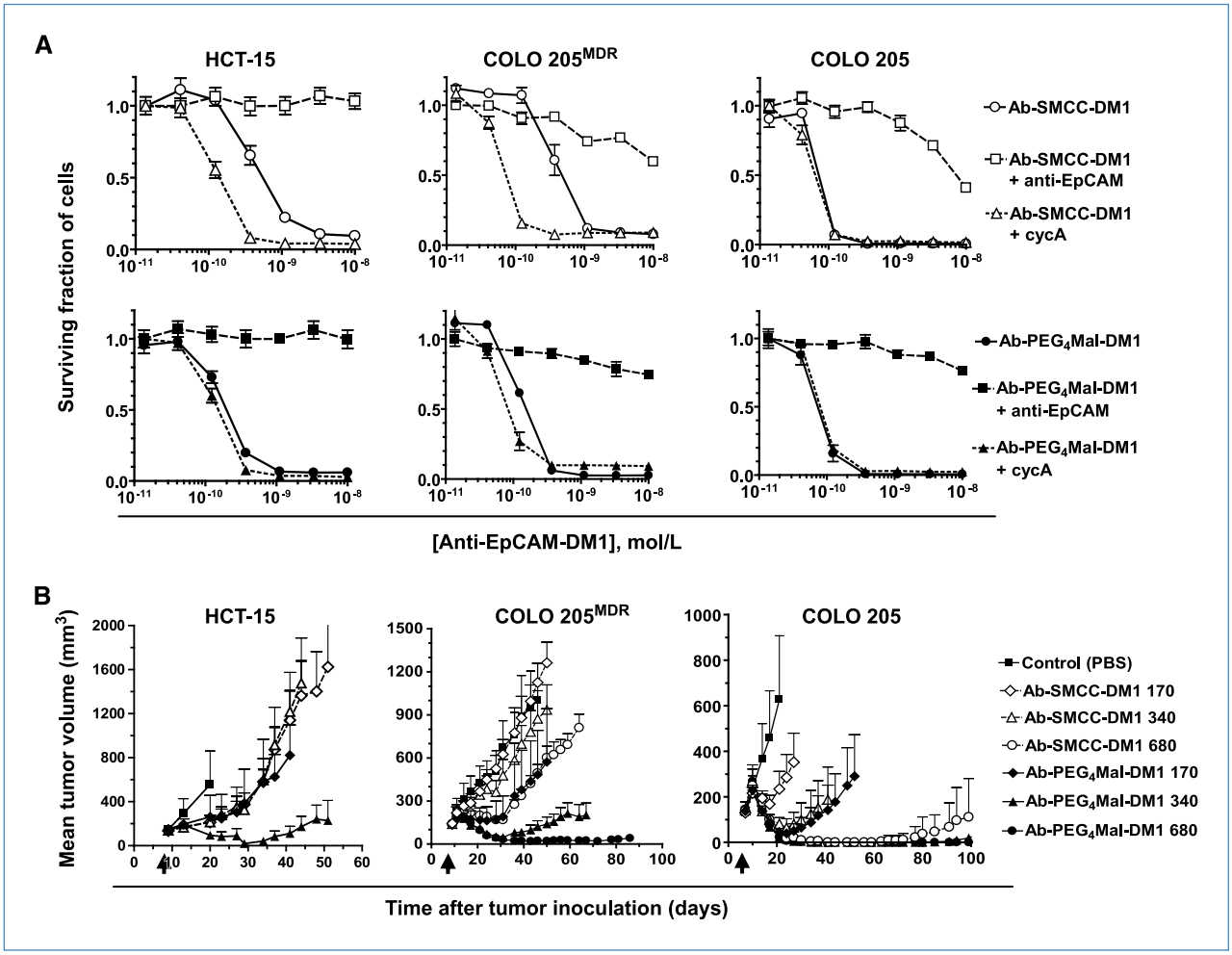

Figure 3. The anti-EpCAM–DM1 conjugate with PEG_4Mal linker shows improved potency against MDR1-positive cells and tumors. A, *in vitro* cytotoxicity of anti-EpCAM–SMCC–DM1 (top row) and anti-EpCAM–PEG_4Mal–DM1 (bottom row) toward HCT-15, COLO 205^{MDR}, or COLO 205 cells in the presence of cyclosporin A (1 μmol/L; Δ and ▲), unconjugated anti-EpCAM antibody (1 μmol/L; □ and ■), or neither (○ and ●). The data are presented as the mean ± SEM (n = 3). B, *in vivo* efficacy of anti-EpCAM–DM1 conjugate in subcutaneous xenograft tumor models. Established xenografts of human colon carcinoma HCT-15, COLO 205^{MDR}, and COLO 205 cells were treated with a single i.v. dose (arrow) of anti-EpCAM–SMCC–DM1 or anti-EpCAM–PEG_4Mal–DM1 conjugate (170, 340, 680 μg/kg of conjugated DM1). Data are presented as the mean ± SEM of tumor volume (n = 6).

commonly expressed in tumors: multidrug resistance protein 1 (MRP1; also known as ABCC1) and breast cancer resistance protein (BCRP; also known as ABCG2; refs. 1–3, 5). COLO 205, COLO 205^{MDR}, HCT-15, and UO-31 cells seem to express significant amounts of the MRP1 transporter but very little of the BCRP transporter (Supplementary Fig. S3). To evaluate whether MRP1 reduced the sensitivity of the cells to maytansinoids and antibody-maytansinoid conjugates, we tested if MK571, a selective MRP inhibitor (31), would enhance the cytotoxicity of DM1SMe and anti-EpCAM–SMCC–DM1 toward COLO 205 cells. Although the inhibitor enhanced the cytotoxicity of the known MRP1 substrate doxorubicin (1, 3), it did not alter the sensitivity of the cells to DM1SMe or anti-EpCAM–SMCC–DM1 (Supplementary Table S2), suggesting that maytansinoids are not MRP1 substrates. To test if BCRP could reduce the cytotoxicity of maytansinoids, we used RPMI-8226 cells, which express significant amounts of the transporter (Supplementary Fig. S3; ref. 32) as well as EpCAM (Supplementary Fig. S1; Supplementary Table S1). A specific inhibitor of BCRP transporter, fumitremorgin C (33), enhanced the cytotoxicity of a known BCRP substrate mitoxantrone (1, 3), but not of DM1SMe or anti-EpCAM–SMCC–DM1 (Supplementary Table S2), indicating that maytansinoids and antibody-maytansinoid conjugates are not BCRP substrates. These observations agreed with a recent report that MDR1, but not MRP1 and BCRP, mediates resistance to maytansinoids and antibody-maytansinoid conjugates (11).

An anti-EpCAM–DM1 conjugate linked via PEG_4Mal shows improved potency against MDR1-expressing cells and tumors. Previously, it was shown that an antibody-SMCC-DM1 conjugate is processed in the lysosomes of target cells to yield a sole cytotoxic metabolite, lysine-SMCC-DM1 (Fig. 2A; ref. 19). Therefore, we speculated that

an antibody-maytansinoid conjugate comprising a more polar linker moiety might yield a hydrophilic maytansinoid metabolite that would be a poor MDR1 substrate and thereby would show improved potency against MDR1-expressing cells. To test this hypothesis, we prepared an anti-EpCAM–DM1 conjugate using a PEG_4Mal linker (Fig. 2A). We expected that incubation of this conjugate with EpCAM-expressing cells would result in the formation of a hydrophilic metabolite, lysine-PEG_4Mal-DM1 (Fig. 2A). Indeed, incubation of EpCAM-positive COLO 205^{MDR} cells with either anti-EpCAM–SMCC–[^{3}H]DM1 or anti-EpCAM–PEG_4Mal–[^{3}H]DM1 for 24 hours yielded acetone-extractable radiolabeled compounds with retention times on HPLC that were identical to those of synthetic lysine-SMCC-DM1 or lysine-PEG_4Mal-DM1, respectively (Fig. 2B). The identities of the metabolites as lysine-SMCC-DM1 and lysine-PEG_4Mal-DM1 were confirmed in a separate experiment by electrospray mass spectrometry.

The anti-EpCAM–PEG_4Mal–DM1 conjugate showed antigen-selective cell killing *in vitro* (Fig. 3A, bottom row), and although its potency toward COLO 205 cells was equal to that of the SMCC-linked conjugate (Fig. 3A, top row), it was significantly higher on two MDR1-expressing cell lines: HCT-15 and COLO 205^{MDR}. The MDR1 inhibitor cyclosporin A had only a marginal effect on the potency of anti-EpCAM–PEG_4Mal–DM1 against the MDR1-expressing cell lines (Fig. 3A, bottom row) but significantly enhanced the potency of SMCC-linked conjugate (Fig. 3A, top row), suggesting that MDR1 activity limits the potency of the SMCC-linked but not of the PEG_4Mal-linked conjugate.

The improved antitumor activity of the PEG_4Mal-linked conjugate against MDR1-positive tumor cells was also apparent in human tumor xenograft studies in mice. SCID mice bearing established HCT-15, COLO 205^{MDR}, and COLO 205 xenografts were treated with a single i.v. injection of either anti-EpCAM–PEG_4Mal–DM1 or anti-EpCAM–SMCC–DM1 at 170, 340, or 680 μg/kg of conjugated DM1. Both conjugates showed antigen-dependent antitumor activity in the HCT-15 tumor xenograft model (Fig. 3B) because control conjugates that do not bind to HCT-15 cells did not affect the tumor growth (Supplementary Fig. S4). The anti-EpCAM–PEG_4Mal–DM1 conjugate was ~2-fold more active than the SMCC-linked conjugate (in Fig. 3B, compare the antitumor activity of the anti-EpCAM–PEG_4Mal–DM1 conjugate dosed at 340 μg/kg DM1 with that of the anti-EpCAM–SMCC–DM1 conjugate dosed at 680 μg/kg DM1). The difference in the activity of the two conjugates was even more pronounced in the COLO 205^{MDR} tumor xenograft model, with the anti-EpCAM–PEG_4Mal–DM1 conjugate being ~4-fold more efficacious than the anti-EpCAM–SMCC–DM1 conjugate, as the activity of the 170 μg/kg DM1 dose of the PEG_4Mal conjugate was comparable with that of the 680 μg/kg DM1 dose of the SMCC conjugate (Fig. 3B). In addition, a single treatment with anti-EpCAM–PEG_4Mal–DM1 at 680 μg/kg DM1 resulted in complete regressions in all five treated mice in this MDR1-positive tumor model, whereas no complete regressions were observed at this dose with anti-EpCAM–SMCC–DM1. These data agree with the *in vitro* cytotoxicity data showing that anti-EpCAM–PEG_4Mal–DM1 is more active than anti-EpCAM–SMCC–DM1 against MDR1-positive HCT-15 and COLO 205^{MDR} tumor cell lines. The anti-EpCAM–PEG_4Mal–DM1 conjugate was also more efficacious than the conjugate with SMCC linker in the MDR1-negative COLO 205 model (Fig. 3B), albeit the advantage of the PEG_4Mal linker was greater in COLO 205^{MDR} model (4-fold versus 2-fold).

The improved cell-killing activity of PEG_4Mal-linked conjugates toward MDR1-expressing cells is not limited to anti-EpCAM. To test whether the enhanced activity of anti-EpCAM–PEG_4Mal–DM1 against MDR1-expressing cells is a general property of antibody-PEG_4Mal-DM1 conjugates and not limited to the anti-EpCAM antibody and/or target antigen, we constructed PEG_4Mal- and SMCC-linked DM1 conjugates with antibodies targeting EGFR and CanAg. The human renal adenocarcinoma cell line UO-31, a MDR cell line that expresses both MDR1 transporter (Fig. 1A) and EGFR (Supplementary Fig. S1; Supplementary Table S1), was used to test the activity of the anti-EGFR conjugates *in vitro*. Although the two conjugates bound to the UO-31 cells with similar affinity (Supplementary Fig. S2), anti-EGFR–PEG_4Mal–DM1 was 7-fold more potent in killing UO-31 cells than anti-EGFR–SMCC–DM1 (Fig. 4A). Cyclosporin A (Fig. 4A) or another MDR1-specific inhibitor, PGP4008 (data not shown), restored the potency of the SMCC-linked conjugate to the level of the PEG_4Mal-linked conjugate but failed to enhance the already high cytotoxicity of PEG_4Mal-linked conjugate, suggesting that the latter is capable of evading the MDR1-mediated resistance of these cells.

COLO 205 and COLO 205^{MDR} cells, which express similar levels of CanAg (Supplementary Fig. S1; Supplementary Table S1), were used to test the cytotoxicity of anti-CanAg–DM1 conjugates linked via PEG_4Mal and SMCC. Although the two conjugates were equally potent toward COLO 205 cells (Fig. 4B), the PEG_4Mal-linked conjugate was 5-fold more potent toward COLO 205^{MDR} cells (Fig. 4B). Consistent with the *in vitro* results, anti-CanAg–PEG_4Mal–DM1 was more efficacious than anti-CanAg–SMCC–DM1 against COLO 205^{MDR} tumors (Fig. 4C).

The maximum tolerated dose for a PEG_4Mal-linked conjugate is similar to that of an analogous SMCC-linked conjugate in mice. The tolerability of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 was tested in CD1 mice. This antibody has no cross-reactivity with mouse tissues. Toxicity was monitored by frequent body weight measurements in groups of mice treated with a single i.v. bolus of either conjugate at approximately 1.8 and 2.7 mg/kg of conjugated DM1. Similar changes in body weight compared with control were observed for each conjugate, with some mortality observed in the high-dose group for both conjugates, suggesting that the maximum tolerated doses for the two conjugates are similar, ~2.2 mg/kg (Fig. 4D).

A PEG_4Mal-linked conjugate is more effective than an analogous SMCC-linked conjugate in arresting COLO 205^{MDR} cells in the G_2-M phase. Maytansinoids and antibody-maytansinoid conjugates arrest dividing cells in the G_2-M phase of the cell cycle (19), an event that ultimately leads to cell death. We compared the antimitotic activities

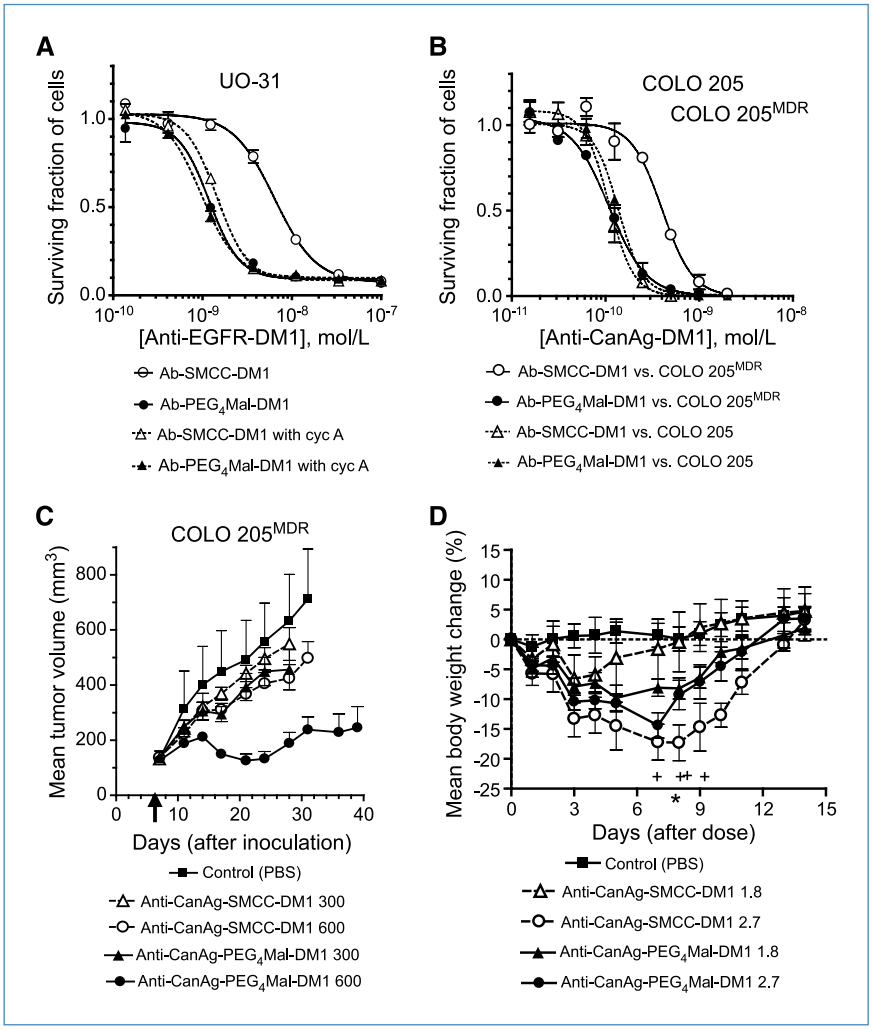

Figure 4. PEG_4Mal-linked conjugates of anti-EGFR and anti-CanAg display improved cytotoxicity over the SMCC-linked versions while being tolerated similarly. A, cytotoxicity of anti-EGFR–SMCC–DM1 (○ and Δ) and anti-EGFR–PEG_4Mal–DM1 (● and ▲) to UO-31 cells in the presence (Δ and ▲) or absence (○ and ●) of cyclosporin A (1 μmol/L). B, cytotoxicity of anti-CanAg–SMCC–DM1 (○ and Δ) and anti-CanAg–PEG_4Mal–DM1 (● and ▲) to COLO 205 (Δ and ▲) and COLO 205^{MDR} cells (○ and ●). C, *in vivo* efficacy of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 conjugates (300 and 600 μg/kg of conjugated DM1) in established subcutaneous COLO 205^{MDR} xenograft tumor models. Data are presented as the mean ± SEM of tumor volume ($n = 6$). D, tolerability assessment of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 conjugates in mice. CD1 mice were randomized based on body weight and treated on day 0 with a single i.v. dose of a conjugate (~1.8 and ~2.7 mg/kg of conjugated DM1). Body weights are represented as the change from a pretreatment measurement ($n = 8$). Four animals treated with the higher dose of anti-CanAg–SMCC–DM1 (+) and one animal treated with anti-CanAg–PEG_4Mal–DM1 (*) were sacrificed as indicated due to >20% body weight loss.

of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 in COLO 205 and COLO 205^{MDR} cells. The cells were incubated with either conjugate for 3 hours, then washed to remove unbound conjugate, and incubated for an additional 18 hours in the conjugate-free medium. The percentage of cells in the G_2-M phase was then determined by flow cytometry. COLO 205 cells were equally sensitive to the two conjugates, with an increase in cells in the G_2-M phase after treatment from 27% to 85% and 88% (Fig. 5A). In contrast, COLO 205^{MDR} cells were less sensitive to anti-CanAg–SMCC–DM1 than to the PEG_4Mal-linked conjugate. The percentage of cells in the G_2-M phase went up from 15% to 37% in the anti-CanAg–SMCC–DM1–treated culture and from 15% to 67% in the anti-CanAg–PEG_4Mal–DM1–treated culture (Fig. 5A).

MDR1-expressing cells accumulate a higher level of DM1-containing metabolite when treated with a PEG_4-Mal-linked conjugate than with an analogous SMCC-linked conjugate. To determine whether the enhanced potency of PEG_4Mal-linked conjugates against MDR1-positive cells could be attributed to increased retention of active metabolites within the cells, COLO 205 and COLO 205^{MDR} cells were incubated with either anti-CanAg–PEG_4Mal–[^{3}H]DM1 or anti-CanAg–SMCC–[^{3}H]DM1 under conditions identical to those in the cell cycle experiments. The metabolites lysine-PEG_4Mal-[^{3}H]DM1 and lysine-SMCC-[^{3}H]DM1 produced during the incubation and retained in the cells were then extracted as in Fig. 2B, and their amounts were determined by liquid scintillation counting. Similar levels of metabolites were detected in COLO 205 cells treated with either conjugate. However, the level of maytansinoid metabolites in COLO 205^{MDR} cells incubated with the SMCC-linked conjugate was ~50% lower than that in the cells treated with the PEG_4Mal-linked conjugate (Fig. 5B). Therefore, the enhanced cytotoxicity and cell cycle arrest activity of the PEG_4-Mal-linked conjugates against MDR1-positive cells can be explained by increased intracellular retention of the active maytansinoid metabolite.

MDR1 transporter activity is not affected in the cells treated with PEG_4Mal-linked conjugates. It has been reported previously that polyethylene glycol can inhibit the transporter activity of MDR1 *in vitro* (34). To test whether the metabolite of PEG_4Mal-linked conjugates, lysine-PEG_4-

Mal-DM1, inhibits the transporter activity, COLO 205^{MDR} cells were incubated with either anti-EpCAM–PEG$_4$Mal–DM1 or anti-EpCAM–SMCC–DM1 for 3 hours at a concentration that can cause substantial killing followed by 18 hours in conjugate-free medium to allow for accumulation of the metabolites within the cells. The activity of MDR1 transporter was then tested using the rhodamine-123 accumulation and retention assay as described in Fig. 1. In contrast to that observed upon treatment with cyclosporin A (Fig. 1B), the treatment of cells with either conjugate did not alter the levels of rhodamine-123 accumulation and retention (Fig. 5C). In addition, incubation of the cells with lysine-PEG$_4$Mal-DM1 directly did not inhibit MDR1 activity (data not shown). Therefore, inhibition of the transporter by the PEG$_4$Mal-containing metabolite is unlikely.

Discussion

Several antibody-maytansinoid conjugates are presently in clinical trials for patients with multiple myeloma, non–Hodgkin's lymphoma, breast cancer, gastric cancer, and other solid tumors (6, 7, 35, 36). Patients with these cancers often develop MDR1-mediated multidrug resistance. Similar to other cytotoxic compounds used in ADCs, such as calicheamicin, dolastatin 10, and CC-1065 analogues (4, 8, 10, 12–14), maytansine is a substrate for the MDR1 transporter, and consequently, the transporter can reduce the potency of some antibody-maytansinoid conjugates. Because preferred substrates for the MDR1 transporter are hydrophobic compounds (24), we reasoned that the potency of antibody-maytansinoid conjugates toward MDR1-positive cells could be improved by increasing the hydrophilicity of the maytansinoid metabolites released from conjugates inside the cell, thereby avoiding MDR1-mediated efflux. To achieve this, we used an ethylene glycol tetramer (PEG$_4$)–containing linker. The observed metabolite, lysine-PEG$_4$Mal-DM1, was retained inside MDR1-expressing cells better than the lysine-SMCC-DM1 metabolite from an analogous SMCC-linked conjugate. The increased retention of the metabolite of PEG$_4$Mal-linked conjugate correlated with the greater antimitotic and cytotoxic potency of the conjugate toward MDR1-expressing cells *in vitro* and with its increased efficacy against MDR1-positive xenograft tumors.

We showed that PEG$_4$Mal linkers can bestow the ability to bypass MDR1-mediated resistance to any antibody-maytansinoid conjugate. Antibody-maytansinoid conjugates with this

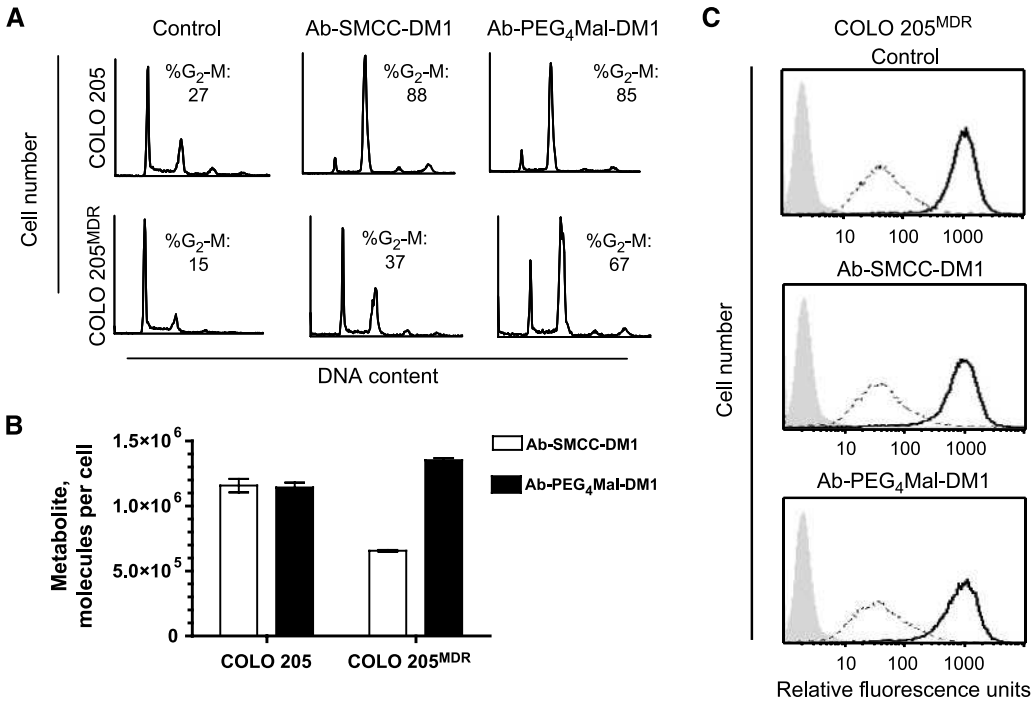

Figure 5. The enhanced potency of PEG$_4$Mal-linked conjugate for MDR cells correlates with increased intracellular retention of maytansinoid metabolite. A, cell cycle distribution in COLO 205 (top row) and COLO 205^{MDR} (bottom row) cell populations exposed to either anti-CanAg–SMCC–DM1 or anti-CanAg–PEG$_4$Mal–DM1 compared with nontreated exponentially growing cells (Control). Cells were exposed to each conjugate for 3 h at 37°C, washed, and incubated in conjugate-free medium for an additional 18 h at 37°C. The DNA content of the cells was evaluated by flow cytometry as described in Materials and Methods. B, intracellular concentrations of metabolites retained by COLO 205 and COLO 205^{MDR} cells. The cells were exposed to anti-CanAg–SMCC–[^{3}H]DM1 or anti-CanAg–PEG$_4$Mal–[^{3}H]DM1 as described in A. The metabolites were then extracted from the cells and quantified as described in Materials and Methods. Data are presented as the mean ± SEM (n = 3). C, rhodamine-123 uptake (solid lines) and retention (dotted lines) by COLO 205^{MDR} cells preexposed to either anti-EpCAM–SMCC–DM1 or anti-EpCAM–PEG$_4$Mal–DM1 compared with that of the nontreated cells (Control). Filled histograms show the cell autofluorescence.

linker were as cytotoxic to MDR1-expressing cells as they were to MDR1-negative cells or to MDR cells whose MDR1 activity was inhibited, suggesting that the PEG_4Mal-linked conjugates were capable of evading the MDR1-mediated resistance. Significantly, the advantage of the linker was observed for the conjugates of antibodies that recognize three different antigens: anti-EpCAM, anti-EGFR, and anti-CanAg. Although the PEG_4Mal-linked conjugate was more efficacious than the SMCC-linked conjugate in MDR tumor models, the two conjugates were equally well tolerated in mice. Thus, incorporation of a short PEG oligomer within the conjugate linker improved the therapeutic index of the antibody-maytansinoid conjugate against the MDR1-expressing tumors.

There are several possible explanations for the enhanced potency of the PEG_4Mal-linked conjugates in MDR1-expressing cells. First, PEG_4Mal-linked conjugates or their metabolites might inhibit MDR1-mediated efflux. The unaltered activity of the transporter in the cells pretreated with cytotoxic concentrations of a PEG_4Mal-linked conjugate (Fig. 5C) or its cellular metabolite, lysine-PEG_4Mal-DM1, argues against this hypothesis. Second, the resulting lysine-PEG_4Mal-DM1 metabolite may bind tighter to its intracellular target, β-tubulin, than maytansine or lysine-SMCC-DM1, thereby reducing the amount of active metabolite needed to kill the cell and/or reducing the intracellular free metabolite pool available to interact with the MDR1 pump. Although we cannot rule out this possibility at present, the similar potencies of the conjugates with the PEG_4Mal and SMCC linkers in non-MDR cells suggest that this hypothesis is unlikely. Another possible explanation is that the lysine-PEG_4Mal-DM1 metabolite may be a poor MDR1 substrate due to its low affinity for the transporter. Finally, the PEG_4Mal-containing metabolite may simply have limited accessibility to the transporter. According to several reports, the binding of a substrate to MDR1 occurs only after diffusion of the substrate into the lipid bilayer of the plasma membrane (37–39). The increased hydrophilicity of the PEG_4Mal-containing metabolite may diminish its partitioning into the plasma membrane and, therefore, prevent interaction with the transporter. Indeed, lysine-PEG_4Mal-DM1 is ~8-fold less cytotoxic than lysine-SMCC-DM1 (data not shown), suggesting that the former has limited ability to penetrate the plasma membrane.

Hydrophilic (or polar) linkers have not been used to bypass multidrug resistance previously. However, our analysis of two prior studies suggests that the polarity of a released metabolite may have enhanced the cytotoxicity of two non-maytansinoid ADCs to MDR cells. Hamann and colleagues (16) reported that substitution of a hydrazide group by an amide in the linker of an antibody-calicheamicin conjugate rendered this ADC more efficacious against MDR cells. Although the authors did not explain the mechanism of this phenomenon, we speculate that the conjugate with the pH-sensitive hydrazide linker was reduced to a hydrophobic calicheamicin, whereas the noncleavable amide-linked conjugate was processed to a polar amino acid–containing derivative that might be a poor MDR1 substrate. In another study, ADC of a polar version of the cytotoxic compound auristatin was able to kill MDR cells (20). However, it is not clear if the potency of this conjugate could be attributed to the polarity of the drug because the potency of ADC of the original non-polar auristatin was not reported.

In conclusion, our finding that the antitumor potency of antibody-maytansinoid conjugates can be improved by rational linker design suggests that this strategy may be also effective in improving the potency of immunoconjugates with other cytotoxic molecules, paving the way for a new generation of ADCs that are more active against MDR tumors.

Disclosure of Potential Conflicts of Interest

All authors are employed by and have ownership interest in ImmmunoGen, Inc.

Acknowledgments

We thank Den Tavares, Barbara Leece, and Rabih Gabriel for providing critical reagents and Thomas Chittenden and John Lambert for numerous helpful discussions and critical review of the manuscript.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/23/2009; revised 12/08/2009; accepted 01/07/2010; published OnlineFirst 03/02/2010.

References

1. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219–34.
2. Wilson TR, Longley DB, Johnston PG. Chemoresistance in solid tumours. Ann Oncol 2006;17 Suppl 10:x315–24.
3. Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics 2008;9:105–27.
4. Takara K, Sakaeda T, Okumura K. An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. Curr Pharm Des 2006;12:273–86.
5. Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. Oncologist 2003;8:411–24.
6. Carter PJ, Senter PD. Antibody-drug conjugates for cancer therapy. Cancer J 2008;14:154–69.
7. Lambert JM. Drug-conjugated monoclonal antibodies for the treatment of cancer. Curr Opin Pharmacol 2005;5:543–9.
8. Matsui H, Takeshita A, Naito K, et al. Reduced effect of gemtuzumab ozogamicin (CMA-676) on P-glycoprotein and/or CD34-positive leukemia cells and its restoration by multidrug resistance modifiers. Leukemia 2002;16:813–9.
9. Walter RB, Gooley TA, van der Velden VH, et al. CD33 expression and P-glycoprotein-mediated drug efflux inversely correlate and predict clinical outcome in patients with acute myeloid leukemia treated with gemtuzumab ozogamicin monotherapy. Blood 2007;109:4168–70.
10. Walter RB, Raden BW, Hong TC, Flowers DA, Bernstein ID, Linenberger ML. Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. Blood 2003;102:1466–73.
11. Tang R, Cohen S, Perrot JY, et al. P-gp activity is a critical resistance factor against AVE9633 and DM4 cytotoxicity in leukaemia cell lines, but not a major mechanism of chemoresistance in cells from acute myeloid leukaemia patients. BMC Cancer 2009;9:199.

12. Toppmeyer DL, Slapak CA, Croop J, Kufe DW. Role of P-glycoprotein in dolastatin 10 resistance. Biochem Pharmacol 1994;48:609–12.
13. Butryn RK, Smith KS, Adams EG, et al. V79 Chinese hamster lung cells resistant to the bis-alkylator bizelesin are multidrug-resistant. Cancer Chemother Pharmacol 1994;34:44–50.
14. Zsido TJ, Beerman TA, Meegan RL, Woynarowski JM, Baker RM. Resistance of CHO cells expressing P-glycoprotein to cyclopropylpyrroloindole (CPI) alkylating agents. Biochem Pharmacol 1992;43: 1817–22.
15. Takeshita A, Shinjo K, Yamakage N, et al. CMC-544 (inotuzumab ozogamicin) shows less effect on multidrug resistant cells: analyses in cell lines and cells from patients with B-cell chronic lymphocytic leukaemia and lymphoma. Br J Haematol 2009;146:34–43.
16. Hamann PR, Hinman LM, Beyer CF, et al. An anti-MUC1 antibody-calicheamicin conjugate for treatment of solid tumors. Choice of linker and overcoming drug resistance. Bioconjug Chem 2005;16:346–53.
17. Lehne G. P-glycoprotein as a drug target in the treatment of multidrug resistant cancer. Curr Drug Targets 2000;1:85–99.
18. Guillemard V, Uri Saragovi H. Prodrug chemotherapeutics bypass p-glycoprotein resistance and kill tumors *in vivo* with high efficacy and target-dependent selectivity. Oncogene 2004;23:3613–21.
19. Erickson HK, Park PU, Widdison WC, et al. Antibody-maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. Cancer Res 2006;66:4426–33.
20. Doronina SO, Mendelsohn BA, Bovee TD, et al. Enhanced activity of monomethylauristatin F through monoclonal antibody delivery: effects of linker technology on efficacy and toxicity. Bioconjug Chem 2006;17:114–24.
21. Goldmacher VS, Blättler WA, Lambert JM, Chari RVJ. Immunotoxins and antibody-drug conjugates for cancer treatment. In: Muzykantov V, Torchilin V, editors. Biomedical aspects of drug targeting. Boston/Dordrecht/London: Kluwer Academic Publishers; 2002, p. 291–309.
22. Kartner N, Riordan JR, Ling V. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 1983; 221:1285–8.
23. Widdison WC, Wilhelm SD, Cavanagh EE, et al. Semisynthetic maytansine analogues for the targeted treatment of cancer. J Med Chem 2006;49:4392–408.
24. Loo TW, Clarke DM. Recent progress in understanding the mechanism of P-glycoprotein-mediated drug efflux. J Membr Biol 2005; 206:173–85.
25. Liu C, Tadayoni BM, Bourret LA, et al. Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. Proc Natl Acad Sci U S A 1996;93:8618–23.
26. Roguska MA, Pedersen JT, Keddy CA, et al. Humanization of murine monoclonal antibodies through variable domain resurfacing. Proc Natl Acad Sci U S A 1994;91:969–73.
27. Chari RV, Martell BA, Gross JL, et al. Immunoconjugates containing novel maytansinoids: promising anticancer drugs. Cancer Res 1992; 52:127–31.
28. Kovtun YV, Audette CA, Ye Y, et al. Antibody-drug conjugates designed to eradicate tumors with homogeneous and heterogeneous expression of the target antigen. Cancer Res 2006;66:3214–21.
29. Pallis M, Das-Gupta E. Flow cytometric measurement of functional and phenotypic P-glycoprotein. Methods Mol Med 2005;111: 167–81.
30. Smith CD, Myers CB, Zilfou JT, Smith SN, Lawrence DS. Indoloquinoxaline compounds that selectively antagonize P-glycoprotein. Oncol Res 2000;12:219–29.
31. Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 1995; 208:345–52.
32. Imai Y, Nakane M, Kage K, et al. C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance. Mol Cancer Ther 2002;1:611–6.
33. Minderman H, Suvannasankha A, O'Loughlin KL, et al. Flow cytometric analysis of breast cancer resistance protein expression and function. Cytometry 2002;48:59–65.
34. Hugger ED, Novak BL, Burton PS, Audus KL, Borchardt RT. A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity *in vitro*. J Pharm Sci 2002;91:1991–2002.
35. Lewis Phillips GD, Li G, Dugger DL, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res 2008;68:9280–90.
36. Chari RV. Targeted cancer therapy: conferring specificity to cytotoxic drugs. Acc Chem Res 2008;41:98–107.
37. Homolya L, Hollo Z, Germann UA, Pastan I, Gottesman MM, Sarkadi B. Fluorescent cellular indicators are extruded by the multidrug resistance protein. J Biol Chem 1993;268:21493–6.
38. Raviv Y, Pollard HB, Bruggemann EP, Pastan I, Gottesman MM. Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. J Biol Chem 1990;265: 3975–80.
39. Aller SG, Yu J, Ward A, et al. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 2009;323: 1718–22.