

Intracellular Activation of SGN-35, a Potent Anti-CD30 Antibody-Drug Conjugate

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Abstract

Purpose: SGN-35 is an antibody-drug conjugate (ADC) containing the potent antimetabolic drug, monomethylauristatin E (MMAE), linked to the anti-CD30 monoclonal antibody, cAC10. As previously shown, SGN-35 treatment regresses and cures established Hodgkin lymphoma and anaplastic large cell lymphoma xenografts. Recently, the ADC has been shown to possess pronounced activity in clinical trials. Here, we investigate the molecular basis for the activities of SGN-35 by determining the extent of targeted intracellular drug release and retention, and bystander activities.

Experimental Design: SGN-35 was prepared with ^{14}C -labeled MMAE. Intracellular ADC activation on CD30⁺ and negative cell lines was determined using a combination of radiometric and liquid chromatography/mass spectrometry-based assays. The bystander activity of SGN-35 was determined using mixed tumor cell cultures consisting of CD30⁺ and CD30⁻ lines.

Results: SGN-35 treatment of CD30⁺ cells leads to efficient intracellular release of chemically unmodified MMAE, with intracellular concentrations of MMAE in the range of 500 nmol/L. This was due to specific ADC binding, uptake, MMAE retention, and receptor recycling or resynthesis. MMAE accounts for the total detectable released drug from CD30⁺ cells, and has a half-life of retention of 15 to 20 h. Cytotoxicity studies with mixtures of CD30⁺ and CD30⁻ cell lines indicated that diffusible released MMAE from CD30⁺ cells was able to kill cocultivated CD30⁻ cells.

Conclusions: MMAE is efficiently released from SGN-35 within CD30⁺ cancer cells and, due to its membrane permeability, is able to exert cytotoxic activity on bystander cells. This provides mechanistic insight into the pronounced preclinical and clinical antitumor activities observed with SGN-35. *Clin Cancer Res*; 16(3); 888–97. ©2010 AACR.

Several monoclonal antibodies (mAb) have established roles in cancer chemotherapy due to their specificities for tumor-associated antigens and their manageable off target toxicities (1, 2). In almost all cases, these agents are used in combination with chemotherapeutic regimens because their activities as single agents are generally suboptimal (3). To extend and enhance this approach, cytotoxic drugs have been linked to mAbs, generating antibody-drug conjugates (ADC) that are capable of selectively delivering drug to target sites. In doing so, it is possible to increase drug activity and at the same time reduce toxic side effects through selective delivery.

Although the ADC concept has long been explored, gemtuzumab ozogamicin (Mylotarg) is the only one that has been approved by the Food and Drug Administration. This

agent is composed of a mAb recognizing the CD33 receptor on acute myelogenous leukemia, modified with the highly potent cytotoxic agent calicheamicin through a hydrolytically unstable linker (4–6). In the years since the approval of gemtuzumab ozogamicin, the progress in developing newer generation ADCs for cancer therapy has been considerable, with increased understanding of the roles that the target antigen, drug potency, linker stability, and conjugation methods play in ADC efficacy and tolerability (7–9). SGN-35 and trastuzumab-DM1 are two such agents that address many of the key parameters, and both have shown activities in phase I and II clinical trials (10–15).

SGN-35 (Fig. 1) is directed against the CD30 antigen, which is highly expressed on such tumors as Hodgkin lymphoma (HL) and anaplastic large cell lymphoma. The cAC10 mAb component of SGN-35 is empowered by the addition of an average of four molecules of monomethylauristatin E (MMAE), a synthetic antimetabolic agent that potently inhibits tubulin polymerization leading to apoptotic cell death (16–20). A dipeptide linker is used to attach MMAE to cAC10 in such a way that upon *in vitro* exposure to proteolytic enzymes such as cathepsin B, free MMAE is released (16, 21). This is expected to occur inside the lysosomes

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Translational Relevance

SGN-35 is one of the few antibody drug conjugates with significant clinical efficacy. The agent consists of a potent cytotoxic drug monomethylauristatin E (MMAE), attached to an anti-CD30 monoclonal antibody through a cleavable dipeptide linker. SGN-35 quantitatively releases and accumulates MMAE inside CD30⁺ cells, and this is accompanied by extended MMAE cellular residence. Sustained contact with the active drug through SGN-35 treatment is clinically relevant because extended exposure may not allow tumor cells a chance to escape the effects of MMAE. We also show that effluxed MMAE can kill CD30⁻ tumor cells cocultured with CD30⁺ cells, suggesting that this targeting technology is applicable to monoclonal antibodies against heterogeneously expressed targets on human tumors. The molecular pharmacology of SGN-35 and the ability of the released drug to exert bystander effects provide a framework for understanding the activities of this agent in treating heterogeneous tumors, such as Hodgkin lymphoma.

of cells after the ADC is internalized through receptor-mediated endocytosis.

The preclinical activities of SGN-35 are pronounced. At the *in vitro* cellular level, the ADC kills CD30-positive cells at low pmole concentrations, several orders of magnitude lower than the amount required for antigen saturation (16, 17). *In vivo* studies have shown that SGN-35 treatment leads to the regression and cure of established HL human tumor xenografts in mice at doses in the range of 1 mg/kg, which is far below the maximum tolerated dose of 120 mg/kg (18). Based on these activities, we initiated a phase I clinical trial of single agent SGN-35 in patients with relapsed, refractory HL, and other CD30-positive hematologic malignancies. Treatment with this ADC has provided multiple objective responses, with a substantial proportion of the patients having complete responses (10, 13, 15).

The identity of the active chemical species released from SGN-35 within cancer cells has not been reported. This issue is of importance to gain further insight into the molecular basis for SGN-35 activity and its application for treating clonal malignant populations that heterogeneously express targeted cell surface antigens, in addition to the fact that mAbs have been shown to distribute within tumors in an uneven manner (22). HL consists of CD30-positive Reed-Sternberg cells surrounded by polyclonal, reactive tumor-associated macrophages, fibroblasts, eosinophils, mast cells, B cells, plasma cells, and T cells that are CD30 negative (23–25). Here, we describe studies that identify MMAE as the released drug within CD30-positive HL cells treated with SGN-35. Intracellular concentrations

of released drug are high over a prolonged time period, yet the amount of effluxed drug is sufficient to exert bystander activity on cocultured antigen-negative cells. These studies provide mechanistic insight into the activities and properties of SGN-35, and a basis for using this agent for the treatment of tumor cell populations that express the CD30 antigen in a heterogeneous manner.

Materials and Methods

Radiolabeled drugs and ADCs. ¹⁴C-labeled MMAE and vc-MMAE were prepared through custom synthesis by Perkin-Elmer (¹⁴C-MMAE and ¹⁴C-vc-MMAE). MMAE and vc-MMAE were labeled at the second valine (universally ¹⁴C-labeled, 270 mCi/mmol MMAE and 276 mCi/mmol vc-MMAE; Fig. 1). cAC10-¹⁴C-vc-MMAE was prepared with a method similar to that previously described using partial reduction by DTT (26). The remaining unreacted DTT was removed by PD10 size exclusion chromatography (GE Healthcare) and excess ¹⁴C-vc-MMAE was added to conjugate at 0°C. Any unreacted drug linker was quenched with cysteine and the conjugate was purified from unconjugated small molecule by PD10. The specific activity of the ADC was determined by UV/visible spectroscopy and liquid scintillation counting (LSC). The specific activity of the unconjugated drug linker was used to calculate ADC's drug loading (7.9 μCi/mg, 4.4 drugs/antibody).

Cell culture. The CD30-positive cell line Karpas299 (anaplastic large cell lymphoma) and the CD30-negative line WSU-NHL (non-Hodgkin lymphoma) were obtained from the Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH. The CD30-negative line Ramos was obtained from American Type Culture Collection. The CD30-positive cell line L540cy, a derivative of the HL line L540 adapted to xenograft growth, was provided by Dr. Philip Thorpe (University of Texas Southwestern Medical Center, Dallas, TX). Cells were grown in suspension culture in RPMI 1640 supplemented with either 10% fetal bovine serum (FBS; Invitrogen, Carlsbad; Karpas299, WSU-NHL, and Ramos) or 20% FBS (L540cy) in a humidified environment of 5% CO₂ at 37°C.

Cell volume determination. Cell volumes were determined by estimation of the average viable cell diameter using a Vi-Cell XR2.03 cell viability analyzer (Beckman Coulter) that provides the average cell size in microns. Volume calculation assumed a spherical cell with no correction made for nuclear volume.

ADC catabolism and drug distribution (constant exposure). Cells were seeded at 5 × 10⁵ cells/mL. Cultures were examined in triplicate and typical culture volumes were 30 mL. On each day of the 72-h experiment, cell densities and viabilities were determined by trypan blue exclusion. Radioactive SGN-35 was added to each culture at a final concentration of ~200 ng/mL (6 nmol/L drug equivalent). Flasks were swirled to mix and aliquots were removed from each flask as a reference for the total amount of radioactivity added to the culture. The cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. At the

indicated time points, cultures were mixed and 4 mL aliquots were removed and overlaid on FBS cushions (27) in 15-mL centrifuge tubes. The samples were centrifuged at $390 \times g$ for 5 min at room temperature. From the separated sample, an aliquot of the upper medium phase was removed for further analysis (see below). The remaining supernatant was carefully removed. The pellet was resuspended in 4 mL of ice-cold PBS and 1 mL was removed for LSC quantitation of total cell-associated radioactivity. The remaining 3 mL of cells were pelleted and resuspended in 0.5 mL of proteinase K (5 $\mu\text{g}/\text{mL}$; Promega) in PBS to remove surface-bound ADC. After incubation at 37°C for 10 min, the enzyme was quenched by dilution with 1 mL of FBS-containing medium. The entire sample was overlaid on an FBS cushion (27) and centrifuged at $390 \times g$ for 5 min. The pellet was resuspended in 100 μL of complete medium and was then treated with 900 μL of ice-cold methanol to precipitate protein and permeabilize the cells. An aliquot of this cell precipitation suspension (400 μL) was removed and examined by LSC to quantitate the total intracellular radioactivity. The remaining suspension was stored at -20°C for ≥ 30 min. The sample was then centrifuged at $16,000 \times g$ for 5 min. The clarified supernatant (500 μL) was examined by LSC to quantitate the total intracellular radioactive small molecule.

A sample of the culture medium from each time point was diluted with nine volumes of ice-cold methanol. The resulting suspension was stored at -20°C for ≥ 30 min. The sample was then centrifuged at $16,000 \times g$ for 5 min and the supernatant was counted by LSC for quantitation of the nonprotein-associated radioactivity present in the culture medium. All samples used for LSC were mixed with 4 mL of Ecoscint A scintillation fluid and were vortexed. L540cy cells treated with chloroquine (Sigma) were

preincubated with 100 $\mu\text{mol}/\text{L}$ chloroquine at 37°C for 1 h followed by 5 h of incubation with radiolabeled SGN-35 and subsequent processing as described above.

ADC catabolism and drug distribution (limited exposure). To examine ADC catabolism and drug distribution in cells with limited ADC exposure, cultures were prepared as described above using ice-cold culture medium and were placed on ice before ADC addition. After incubation on ice for ~ 15 min, ^{14}C -SGN-35 was added at ~ 200 ng/mL and the cultures were kept on ice for an additional 30 min. Cultures were then centrifuged at $390 \times g$ for 5 min and were washed with ice-cold PBS followed by resuspension in fresh, warm culture medium. The cultures were placed at 37°C in a humidified 5% CO_2 atmosphere and samples were removed at 24 h and processed as described in the constant exposure method.

Free drug retention. For MMAE retention experiments, 25×10^6 cells were seeded at 5×10^5 cells/mL. Each cell type was treated in triplicate with a concentration of radioactive MMAE determined to provide a similar intracellular concentration for that cell type after 3 h of incubation at 37°C . The cells were washed twice into an equal volume of fresh medium. Each washed culture was split into three 15-mL centrifuge tubes. One-milliliter aliquots were removed immediately from each tube for LSC and a second 1-mL aliquot was layered over 2 mL of FBS in a 15-mL centrifuge tube, centrifuged at $390 \times g$ for 5 min at room temperature. From the separated sample, 0.5 mL of the upper medium phase was removed for LSC. The remaining portion of each sample was frozen in a dry ice bath and the bottom of the tube containing the cell pellet was excised into a scintillation vial containing 0.5 mL of PBS. Samples were vortexed; Ecoscint A scintillation fluid (4 mL; National Diagnostics) was added followed by a

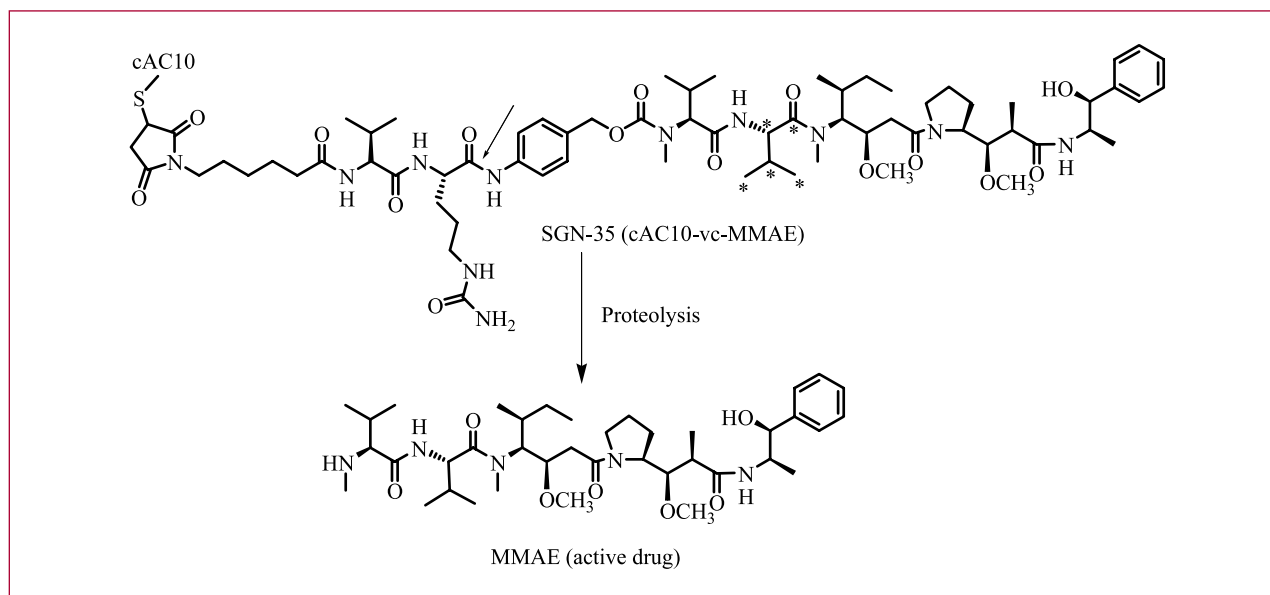


Fig. 1. Structure of SGN-35. *, the location of ^{14}C in the radiolabeled drug linker.

second vortex; and the samples were counted by LSC (Beckman LS6000IC, Beckman Coulter). Further aliquots (1 mL) were processed over FBS, as described, at the indicated time points. For the calculation of intracellular drug concentrations, cell densities were redetermined by trypan exclusion after washing into nondrug-containing medium and again after 24 h.

Radioactivity calculations. Calculations were made after subtracting the background from all the disintegration per second values. The triplicate background-corrected disintegration per second readings were averaged and the SD for those values was calculated using the STDEVPA function in Microsoft Excel. Average disintegration per second values were converted to μCi and subsequently to pmole of the drug using the specific activity of the radioactive drug or drug linker used in the experiment. For each mathematical manipulation, a propagation of error calculation was done using standard propagation of error formulas for propagation of SD values. The intracellular drug concentrations in nmole were calculated using the estimated volume of 1×10^6 cells.

Mass spectrometric drug quantitation. Triplicate samples of cells were washed into fresh medium at $\sim 5 \times 10^5$ cells/mL. ADC was added (200 ng/mL) and the cultures were incubated at 37°C in 5% CO_2 . For intracellular drug quantitation, at 24 h, the cells were enumerated by trypan blue exclusion and a known volume of cells was harvested by centrifugation ($360 \times g$ rpm, 5 min, 4°C). The cells were washed with an equal volume of ice-cold PBS ($360 \times g$, 5 min), repelleted, and resuspended in complete culture medium to provide final volumes of 150 μL . Two volumes of ice-cold methanol were added and the samples were stored at -20°C for ≥ 30 min. For released drug quantitation in the culture medium, cells were prepared in the same manner; however, aliquots were removed over 3 d. Culture medium was recovered by simple centrifugation of the samples and removal of the supernatant, taking care not to disturb the pellet. The culture medium samples (150 μL) were mixed with 150 μL of a 50-ng/mL internal standard in the same culture medium. These were precipitated with two volumes of ice-cold acetonitrile. Samples of medium and cell pellets not treated with ADC were used for standard curves and were prepared following the same precipitation procedures. Eight-point standard curves were made using varying amounts of MMAE plus constant internal standard in untreated matrix. All samples were centrifuged at high speed to remove protein, and the supernatants were removed and dried in a centrifugal evaporator.

Samples were reconstituted in 33% acetonitrile and were examined by LC/MS using online solid phase extraction. To derive an equation for the quantitation of released drug in the experimental unknown samples, the peak area for each drug standard was divided by the peak area obtained for the internal standard. The resultant peak area ratios were plotted as a function of the standard concentrations and the data points were fitted to a curve using linear regression. The peak area ratios obtained for the released drug to internal standard in the experimental sam-

ples were converted to drug concentration using the derived equation.

ADC-treated culture conditioned medium bioassay. Samples of spent culture medium from SGN-35-treated cells were added to CD30-negative Ramos cell cultures in six different dilutions. In parallel, Ramos cells were incubated with eight MMAE concentrations as cytotoxicity standards. After a 96-h incubation at 37°C , the Ramos cultures were developed using resazurin (Sigma; relative fluorescence, excitation = 530-560 nmol/L, emission = 590 nmol/L). Using the average relative fluorescence unit (RFU) measurement for Ramos cells incubated with the MMAE standards, the percentage of viable cells relative to untreated Ramos cells (% untreated) was plotted as a function of MMAE concentration (nmol/L). A four-parameter curve fit was used to generate an equation for the quantitation of released MMAE in the spent culture samples. Cell viability measurements of the spent culture dilutions were transformed into MMAE concentrations (assuming cytotoxicity is attributed solely to MMAE) using this equation. Only cell viability measurements falling between 15% and 85% of the untreated Ramos cells were used to calculate the concentration of MMAE.

Coculture experiments. Karpas299, L540cy, or Ramos cells in single culture were seeded at 2.5×10^5 cells/mL in culture volumes of 1.5 mL, whereas cocultures of CD30-positive and CD30-negative pairs consisted of 1.25×10^5 cells/mL of each cell type in 1.5 mL of culture medium (1:1 mixture of cells, RPMI 1640 + 10-15% FBS). The culture medium used in the coculture experiments adequately supports the growth of all three cell types. Cultures were treated with vehicle control, 1 $\mu\text{g}/\text{mL}$ SGN-35, or IgG-vc-MMAE nonbinding control. After a 72-h incubation, cultures were fed with 60% medium containing the requisite treatment type. Cultures were examined for cell count and viability (Vi-Cell XR2.03 cell viability analyzer, Beckman Coulter) after 120 h and the surviving cells were stained with anti-CD30-Phycoerythrin (BD Biosciences) and anti-CD19-FITC (BD Biosciences) antibodies to determine the distribution of each cell type in the surviving cultures. Staining was accomplished by harvesting the cells by centrifugation at 1,200 rpm for 5 min, plating $\sim 5 \times 10^5$ cells per well in a 96-well plate in 20 μL of fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FBS), and adding the labeled antibodies to the desired wells without dilution (5 $\mu\text{L}/\text{well}$). The plate was incubated on ice for 30 min before centrifugation at 1,500 rpm for 5 min and before the removal of the supernatant by tapping the plate. The cells were washed thrice with PBS (200 μL) before resuspending in 250 μL FACS buffer and before storage at 4°C for subsequent analysis by flow cytometry on a BD FACScan instrument.

Results

Drug release from SGN-35. The kinetics of SGN-35 internalization, drug release, and the extent of intracellular, released drug retention were determined with a radiolabeled

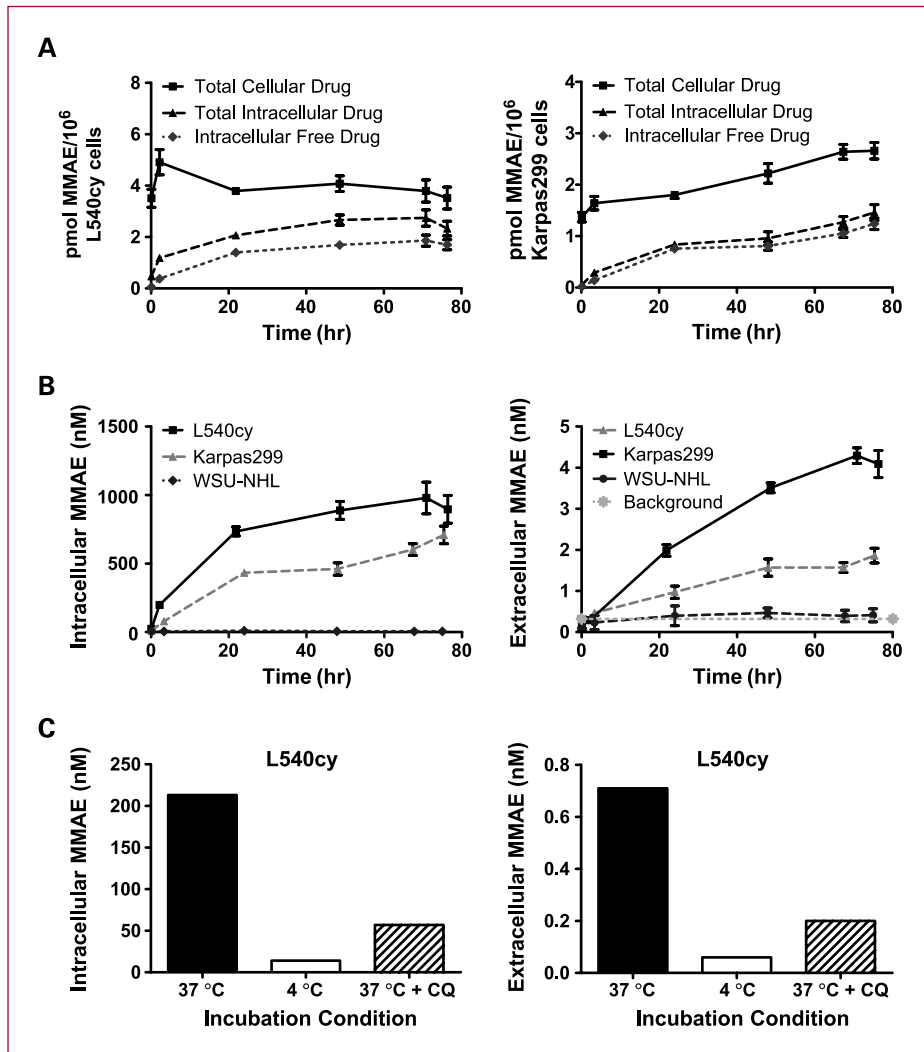


Fig. 2. A, generation of MMAE in cells treated with MMAE-containing ADCs. Radiolabeled MMAE detected in L540cy and Karpas299 cells treated with 200 ng/mL ³H-SGN-35. B, intracellular and extracellular concentrations of small-molecule radioactivity detected in antigen-positive (Karpas299 and L540cy) and antigen-negative (WSU-NHL) cell culture. C, intracellular and extracellular small-molecule radioactivity detected in L540cy culture after 5 h of ¹⁴C-SGN-35 treatment (200 ng/mL) at 4°C and 37°C, with and without 100 μmol/L chloroquine (CQ).

version of the ADC prepared using ¹⁴C-MMAE conjugated to cAC10 using the mc-valine-citrulline-PABC linker shown in Fig. 1. The resulting ADC contained an average of 4.4 MMAE molecules attached to interchain disulfides as previously described (18, 26). The fate of SGN-35 on cultured cells was examined by the incubation of cells with radiolabeled SGN-35 and the determination of the fraction of ADC in the medium versus associated with cells. ADC bound to the cell surface was removed by proteinase K to distinguish it from intracellular ADC. For the media and intracellular fractions, conjugated MMAE was distinguished from released MMAE by precipitation in organic solvent.

CD30-positive L540cy HL and Karpas299 anaplastic large cell lymphoma cells were treated with a constant exposure of ¹⁴C-labeled SGN-35 at ~200 times the IC₅₀ concentration. The total amount of cell-associated drug and intracellular drug are shown in Fig. 2A. Initially, most of the drug was associated with the membrane, but intracellular bound and released drug built up over the course of the assay. Beyond the earliest time point, the vast majority

of intracellular drug was free, suggesting that upon internalization, the release of drug from SGN-35 is quite facile.

The concentration of intracellular and extracellular released drug in cell lines treated with constant SGN-35 exposure is shown in Fig. 2B. WSU-NHL cells are CD30 negative and did not release detectable levels of drug through the entire 3-day course of the assay. In contrast, both CD30-positive cell lines generated released drug, with high intracellular concentrations (>400 nmol/L) reached within 24 hours of treatment. This indicates not only that SGN-35 was processed in CD30-positive cells, but that the released drug accumulated and was retained within the cells at concentrations much higher than the initial treatment ADC concentration of 6 nmol/L. Appearance of free drug inside and outside the L540cy cells was greatly reduced at 4°C or when cells were treated with chloroquine before ADC exposure (Fig. 2C). Chloroquine raises lysosomal pH and reduces the activity of lysosomal proteases having optimal activities under acidic conditions (28). Taken together, these results are consistent with

intracellular drug generation by CD30-mediated internalization of SGN-35, and accumulation of extracellular drug in the culture medium through escape due to the inherent membrane permeability of MMAE (29, 30).

The combined intracellular and extracellular released drug over the 72-hour constant exposure incubation provides a basis for estimating the total number of SGN-35 molecules that were internalized by the cell and catabolized. This number can be compared with the number of CD30 receptors present in the cell culture at 72 hours and hence provide an estimate of the turnover of CD30 in each cell line (Table 1). The calculated number of ADCs internalized and catabolized is 2.5 and 3.4 ADCs per receptor for Karpas299 and L540cy cells, respectively, indicating that over the course of the 72-hour SGN-35 incubation, there was either recycling of CD30 to the cell surface or synthesis of new receptor. This is supported by the amount of ADC that is turned over when cells are treated with a limited exposure to the ADC, in which ice-cold cells are initially treated with 200 times the IC₅₀ concentration of ¹⁴C-SGN-35 as in the constant exposure experiment, but subsequently are washed to remove unbound ADC, providing a treatment condition beginning with only CD30-bound ¹⁴C-SGN-35. Under these conditions, approximately one ADC is present per receptor. At 24 hours, 0.5 and 0.3 ADCs were turned over per receptor in L540cy and Karpas299 cells, respectively, compared with 1.2 and 1.1 ADCs per receptor in the same cells under constant exposure conditions (Table 1). Synthesis or recycling of the receptor would be required to obtain an increased number of ADCs catabolized after 24 hours in the constant versus limited exposure conditions. This finding shows the potential for

receptor recycling or resynthesis in a tumor mass during the course of treatment, thus providing new binding sites for the remaining SGN-35 present in circulation. Synthesis of new receptor or recycling helps explain the sustained concentration of MMAE observed in the CD30-positive cell culture in light of the fact that MMAE is observed to be accumulating in the culture medium (Fig. 2B).

Identification of released drug. To determine if the identity of the released drug was exclusively MMAE, bioassay and mass spectrometry studies were undertaken on the soluble small molecules generated from CD30-positive cells treated with SGN-35. The data were compared with the total methanol-soluble radioactivity derived from the cells as shown in Fig. 3A and B. In the bioassay, antigen-negative Ramos cells were treated with the spent culture medium from SGN-35-treated antigen-positive cells. Using the cytotoxicity of Ramos cells to authentic MMAE standards, the effective MMAE concentrations in the spent culture samples were calculated. Over time, there was an increasing antigen-independent cytotoxin concentration that overlapped with the presumed MMAE concentration from LSC (Fig. 3A). Furthermore, quantitative mass spectrometric analysis of released drug in the cell culture supernatants, as well as inside cells (Fig. 3A and B), indicated that a species indistinguishable from MMAE by mass and retention time was present in concentrations that also overlapped with the presumed MMAE concentration from LSC.

Cellular efflux of MMAE. Because the escape of released drug from antigen-positive cells may affect therapeutic efficacy, we further established that this type of behavior can be observed with cells loaded with free MMAE by measuring

Table 1. Catabolism of SGN-35 by CD30-positive cells

	L540cy cells (HL)			Karpas299 cells (ALCL)		
	CD30*: 587,511/cell			CD30*: 290,676/cell		
	Limited, [†] 24 h	Continuous, [†] 24 h	Continuous, [†] 72 h	Limited, [†] 24 h	Continuous, [†] 24 h	Continuous, [†] 72 h
1 × 10 ⁶ cells/mL	0.53	0.52 ± 0.01	0.33 ± 0.04	0.49	0.65 ± 0.002	0.45 ± 0.02
Intracellular released MMAE (pmol/mL)	0.31	0.72 ± 0.04	0.62 ± 0.02	0.13	0.49 ± 0.02	0.57 ± 0.03
Extracellular released MMAE (pmol/mL)	0.93	2.0 ± 0.1	4.3 ± 0.2	0.24	1.0 ± 0.2	1.9 ± 0.2
Total released MMAE (pmol/mL)	1.2	2.7 ± 0.6	4.9 ± 0.2	0.37	1.5 ± 0.1	2.4 ± 0.2
¹⁴ C-SGN-35 catabolized per receptor [‡]	0.54	1.2 ± 0.1	3.44 ± 0.02	0.35	1.1 ± 0.1	2.55 ± 0.01

Abbreviation: ALCL, anaplastic large cell lymphoma.

*CD30 levels expressed as the number of mAb molecules bound per cell.

[†]Limited exposure refers to cells treated initially at 0°C with 200 ng/mL ¹⁴C-SGN-35 and subsequently washed to remove unbound ADC before incubation at 37°C. Continuous exposure refers to treatment of cells at 37°C with 200 ng/mL ¹⁴C-SGN-35.

[‡]Calculated using a drug loading of 4.4 drugs/mAb and the average number of cells remaining per milliliter at the time of sample collection.

the loss of intracellular drug over 24 hours, during which time the cells maintain their viability. To accomplish this, L540cy and Karpas299 cells were treated with sufficient ^{14}C -MMAE to achieve intracellular concentrations of approximately 3 to 4 $\mu\text{mol/L}$ and the rate of drug loss was measured, as shown in Fig. 3C. For both cell lines, 50% of the initial intracellular MMAE was retained for approximately 16 to 22 hours, and the remaining material was found in the culture supernatant. L540cy displays limited efflux of rhodamine dye whereas Karpas299 does not (Supplementary Fig. S1), indicating that the former but not the

latter expresses an unidentified multidrug resistance protein. Despite this difference, the efflux of MMAE from the two cell lines is very similar (Fig. 3C), which suggests that MMAE can diffuse from viable cells regardless of whether a multidrug resistance protein is present. Because MMAE is only observed in the cell culture medium of SGN-35-treated CD30-positive cells and its appearance is inhibited by the inhibition of lysosomal proteases, the observation that MMAE can slowly diffuse out of cells bolsters the hypothesis that the extracellular-free drug found in SGN-35-treated culture medium originated from intracellular processing of the ADC, followed by subsequent passive or active drug efflux.

Bystander effects. Having shown that the released drug from SGN-35 is MMAE, that the drug is able to diffuse slowly out of the cell from which it was derived (Figs. 2B and 3C), and that SGN-35 is stable under these culture conditions with no generation of MMAE from antigen-negative cell lines (Fig. 2B), we explored whether the ADC elicits bystander activity in cocultures with CD30-positive and CD30-negative cell lines. We have previously shown (17) that Ramos Burkitt's lymphoma cells are CD30 negative and are as sensitive to free MMAE (IC_{50} , 0.04 nmol/L) as Karpas299 (IC_{50} , 0.07 nmol/L) and L540cy (IC_{50} , 0.21 nmol/L) cells, with IC_{50} values that are representative of the sensitivity of cancer cells to MMAE. Being CD30 negative, Ramos cells were relatively insensitive to SGN-35 (IC_{50} , 3,300 ng/mL with eight drugs per mAb) compared with Karpas299 (IC_{50} , 1.3 ng/mL with eight drugs per mAb) and L540cy (IC_{50} , 9.9 ng/mL with eight drugs per mAb) cells (17). FACS analysis of cocultures of L540cy and Ramos cells showed that treatment with 1 $\mu\text{g/mL}$ SGN-35 eliminated both populations of cells equally well, whereas a similarly treated mixed cell population with a nonbinding control ADC were unaffected (Fig. 4A and B). Similar treatment of the Karpas299/Ramos cell mixture with 1 $\mu\text{g/mL}$ SGN-35 produced the same outcome (Fig. 4C and D). These results show an antigen-independent cytotoxic effect on the CD30-negative cells that is likely caused by the released MMAE from the cocultured CD30-positive cells. A 5-fold lower ADC dose was also examined under the same conditions and displayed a less potent reduction of the CD30-negative cells (data not shown), suggesting that the amount of small-molecule cytotoxic agent generated is dependent on the amount of SGN-35 added to the culture. Thus, SGN-35 has strong bystander activity on neighboring antigen-negative cells in culture.

Discussion

Recent advances in ADC research have led to the development of SGN-35, an agent that has shown considerable activity, both in preclinical models and in early clinical trials (7, 10, 13–16, 18). This molecule incorporates a highly potent payload, linker technology that is considerably more stable than earlier generation hydrazone and disulfide linkers (7, 8, 31, 32), and conjugation

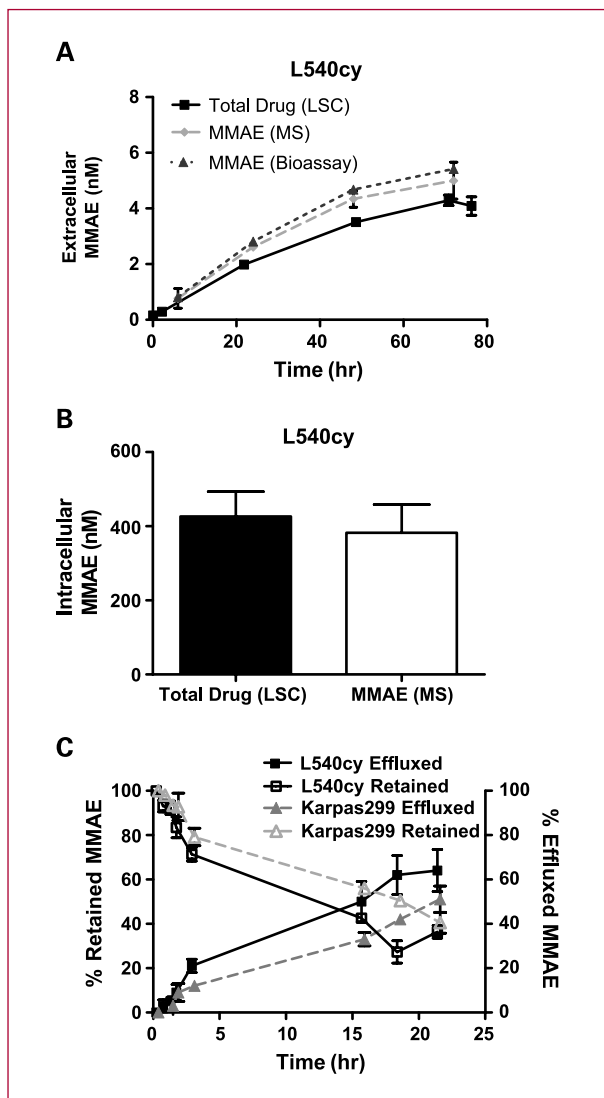
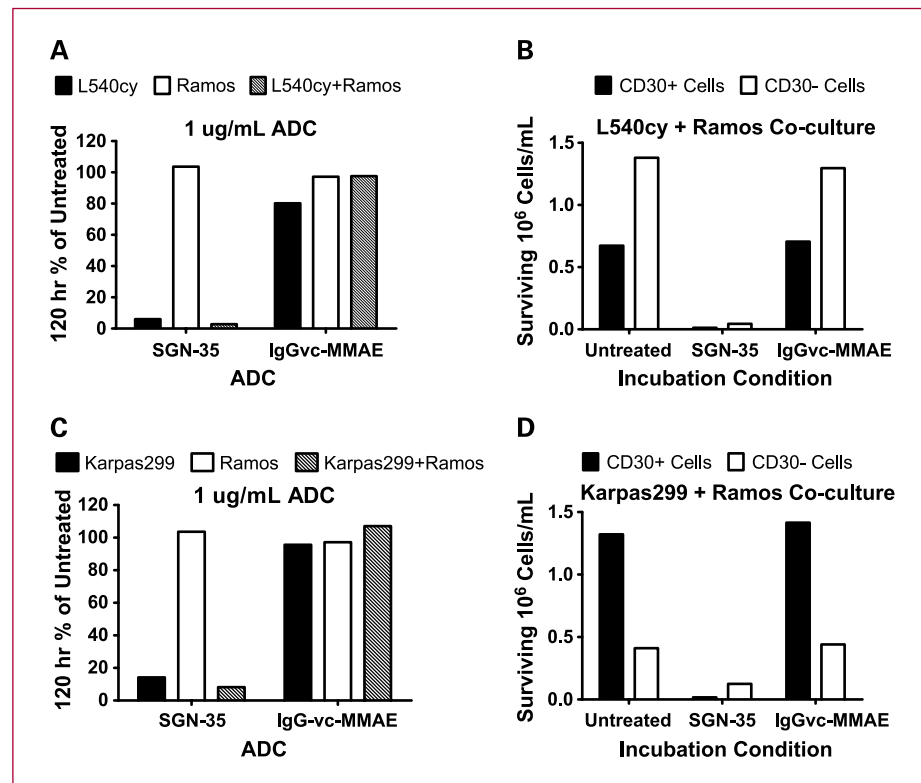


Fig. 3. Fate of MMAE in cells treated with SGN-35. A, overlay of the extracellular MMAE and small-molecule radioactivity detected by LC/MS, bioassay, and LSC. B, intracellular concentration of MMAE in L540cy cells treated with SGN-35 for 24 h detected by LC/MS compared with the value of the total small-molecule radioactivity obtained by LSC. C, cellular retention of free MMAE over 24 h.

Fig. 4. Bystander activities of MMAE containing ADCs. Coculture experiments included L540cy and Karpas299 (CD30+, CD19-) and Ramos (CD30-, CD19+) cells. A, cell count of L540cy/Ramos cocultures treated with 1 μ g/mL SGN-35 and a corresponding nonbinding control ADC (IgG-vc-MMAE). B, L540cy/Ramos cocultures were stained with anti-CD30-PE and anti-CD19-FITC following treatment, allowing the determination of the number of surviving CD30-positive and CD30-negative cells. C, cell count of Karpas299/Ramos cocultures treated with 1 μ g/mL SGN-35 and a corresponding nonbinding control ADC (IgG-vc-MMAE). D, Karpas299/Ramos cocultures were stained with anti-CD30-PE and anti-CD19-FITC following treatment, allowing the determination of the number of surviving CD30-positive and CD30-negative cells.



methodology that provides high yields of well-defined ADCs with drugs attached at specific mAb sites that are distal to the variable region (18, 26). The results with SGN-35 have provided the basis for extending the findings to include such antigens as LeY (16), CD20 (33), CD22 (30), CD70 (19), CD79b (30), MUC16 (34), EphA2 (35), PSMA (36), and many others. In addition, *in vivo* model studies have shown that SGN-35 added to standard chemotherapeutic regimens leads to improved activity over either treatment group alone (19). Given the broad applicability of ADCs using this technology, it is of importance to have a molecular understanding of how this ADC functions at a molecular level, the subject of investigations reported here.

It has previously been shown that the nature of drug release from ADCs can have a profound influence on activity. For example, maytansinoid ADCs release lysine adducts if they are linked to mAb lysines through thioether linkers, but yield S-methylated derivatives if linked to lysines through disulfide linkers (37). These agents have distinct *in vitro* and *in vivo* activity profiles, and the charged lysine adduct was shown to be much less active than the thioether derivative as a free drug. It was therefore not surprising that the thioether adduct elicited a strong bystander effect, whereas the lysine adduct did not (38). We have also observed that the nature of the linker can influence the structure of the released drug, based on studies with thioether-linked auristatins that released cysteine adducts through mAb degradation (29).

In the work described here, we show that CD30-positive cell lines process SGN-35 by releasing MMAE in a chemically unmodified form. The results are consistent with the proteolytic cleavage at the citrulline-PABC amide bond, which leads to the release of MMAE after spontaneous fragmentation of the PABC spacer (Fig. 1). This is supported by inhibition studies with chloroquine (Fig. 2C), a lysosomotropic agent that reduces lysosomal protease activity through pH modulation. Upon release, the MMAE diffuses through cell membranes and accumulates in culture media, albeit at concentrations that were \sim 250 times lower than that found inside the cells, presumably due to dilution into the relatively larger volume of medium (Fig. 2B). Despite the great differential, extracellular drug was able to kill CD30-negative cells cocultured with CD30-positive cells. Thus, SGN-35 has the potential to act on cells within a heterogeneous tumor cell population that do not bind sufficiently high amounts of ADC for effective direct cytotoxic activity. In general, this is of importance because mAbs have been shown to distribute within tumors in an uneven manner and many tumors are heterogeneous with respect to antigen expression (22, 39, 40). In targeting HL with antibodies directed against CD30 in particular, the ability to eradicate antigen-negative cells within the tumor mass may be useful because only a small percentage of the cells are CD30 positive and thought to be a part of the clonal malignancy (25). Because this bystander cytotoxic effect would be localized to the tumor

microenvironment, with cells near the CD30-positive cells exposed to a concentration gradient of MMAE that decreases with increasing distance, it is likely to minimize toxic systemic exposure of diffusible tumor-released MMAE. In summary, targeted *in vitro* delivery of MMAE to CD30-expressing cells with SGN-35 leads to high and sustained intracellular MMAE levels, and successfully ablates both CD30-expressing malignant cells and neighboring malignant cells that do not express the target antigen. This may be of significance in treating tumors that are heterogeneous with respect to both antigen presentation and ADC distribution (9, 41). The results reported here provide insight into the pronounced activities associated with this promising ADC.

References

- Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. Monoclonal antibody successes in the clinic. *Nat Biotechnol* 2005;23:1073–8.
- Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. *Nat Rev Drug Discov* 2007;6:349–56.
- Clavio M, Vignolo L, Albarello A, et al. Adding low-dose gemtuzumab ozogamicin to fludarabine, Ara-C and idarubicin (MY-FLAI) may improve disease-free and overall survival in elderly patients with non-M3 acute myeloid leukaemia: results of a prospective, pilot, multi-centre trial and comparison with a historical cohort of patients. *Br J Haematol* 2007;138:186–95.
- Bross PF, Beitz J, Chen G, et al. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin Cancer Res* 2001;7:1490–6.
- Damle NK. Tumour-targeted chemotherapy with immunoconjugates of calicheamicin. *Expert Opin Biol Ther* 2004;4:1445–52.
- Linenberger ML. CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. *Leukemia* 2005;19:176–82.
- Carter PJ, Senter PD. Antibody-drug conjugates for cancer therapy. *Cancer J* 2008;14:154–69.
- Chari RV. Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc Chem Res* 2008;41:98–107.
- Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol* 2005;23:1137–46.
- Bartlett N, Forero-Torres A, Rosenblatt J, et al. Complete remissions with weekly dosing of SGN-35, a novel antibody-drug conjugate (ADC) targeting CD30, in a phase I dose-escalation study in patients with relapsed or refractory Hodgkin lymphoma (HL) or systemic anaplastic large cell lymphoma (sALCL). *J Clin Oncol (Meeting Abstracts)* 2009;27:8500.
- Beeram M, Krop I, Modi S, et al. A phase I study of trastuzumab-MCC-DM1 (T-DM1), a first-in-class HER2 antibody-drug conjugate (ADC), in patients (pts) with HER2+ metastatic breast cancer (BC). *J Clin Oncol (Meeting Abstracts)* 2007;25:1042.
- Krop I, Beeram M, Modi S, et al. A phase I study of trastuzumab-DM1, a first-in-class HER2 antibody-drug conjugate, in patients with advanced HER2+ breast cancer [abstract 310]. 30th Annual San Antonio Breast Cancer Symposium; 2007.
- Younes A, Forero-Torres A, Bartlett NL, et al. Multiple complete responses in a phase 1 dose-escalation study of the antibody-drug conjugate SGN-35 in patients with relapsed or refractory CD30-positive lymphomas. *ASH Annual Meeting Abstracts* 2008;112:1006.
- Younes A, Forero-Torres A, Bartlett N, et al. A novel antibody-drug conjugate, SGN-35 (anti-CD30-auristatin), induces objective responses in patients with relapsed or refractory Hodgkin lymphoma, preliminary results of a phase I tolerability study [abstract PO99bis]. 7th International Symposium on Hodgkin Lymphoma; 2007.
- Younes A, Forero-Torres A, Bartlett NL, Leonard JP, Kennedy DA, Sievers EL. Robust Antitumor Activity of the Antibody-Drug Conjugate SGN-35 when Administered Every 3 Weeks to Patients with Relapsed or Refractory CD30 Positive Hematologic Malignancies in a Phase 1 Study [abstract 0503]. European Hematology Association 14th Congress; 2009.
- Doronina SO, Toki BE, Torgov MY, et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat Biotechnol* 2003;21:778–84.
- Francisco JA, Cerveny CG, Meyer DL, et al. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood* 2003;102:1458–65.
- Hamblett KJ, Senter PD, Chace DF, et al. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin Cancer Res* 2004;10:7063–70.
- Oflazoglu E, Kissler K, Sievers E, Grewal I, Gerber H. Combination of the anti-CD30-auristatin-E antibody-drug conjugate (SGN-35) with chemotherapy improves antitumor activity in Hodgkin lymphoma. *Br J Haematol* 2008;142:69–73.
- Sanderson RJ, Hering MA, James SF, et al. *In vivo* drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. *Clin Cancer Res* 2005;11:843–52.
- Sutherland MSK, Sanderson RJ, Gordon KA, et al. Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by peptide-linked anti-CD30-auristatin conjugates. *J Biol Chem* 2006;281:10540–7.
- Jain RK, Baxter LT. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. *Cancer Res* 1988;48:7022–32.
- Kuppers R. The biology of Hodgkin's lymphoma. *Nat Rev Cancer* 2009;9:15–27.
- Schmitz R, Stanelle J, Hansmann ML, Kuppers R. Pathogenesis of classical and lymphocyte-predominant Hodgkin lymphoma. *Annu Rev Pathol* 2009;4:151–74.
- Kasamon YL, Ambinder RF. Immunotherapies for Hodgkin's lymphoma. *Crit Rev Oncol Hematol* 2008;66:135–44.
- Sun MM, Beam KS, Cerveny CG, et al. Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides. *Bioconjug Chem* 2005;16:1282–90.
- Kassis AI, Adelstein SJ. A rapid and reproducible method for the separation of cells from radioactive media. *J Nucl Med* 1980;21:88–90.
- Tu C, Ortega-Cava CF, Chen G, et al. Lysosomal cathepsin B participates in the podosome-mediated extracellular matrix degradation and invasion via secreted lysosomes in v-Src fibroblasts. *Cancer Res* 2008;68:9147–56.
- Doronina SO, Mendelsohn BA, Bovee TD, et al. Enhanced activity of monomethylauristatin F through monoclonal antibody delivery:

Disclosure of Potential Conflicts of Interest

All authors are employed by and have ownership interest in Seattle Genetics.

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- effects of linker technology on efficacy and toxicity. *Bioconjug Chem* 2006;17:114–24.
30. Polson AG, Calemine-Fenau J, Chan P, et al. Antibody-drug conjugates for the treatment of non-Hodgkin's lymphoma: target and linker-drug selection. *Cancer Res* 2009;69:2358–64.
 31. Saleh MN, Sugarman S, Murray J, et al. Phase I trial of the anti-Lewis Y drug immunoconjugate BR96-doxorubicin in patients with Lewis Y-expressing epithelial tumors. *J Clin Oncol* 2000;18:2282–92.
 32. Tolcher AW, Ochoa L, Hammond LA, et al. Cantuzumab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I, pharmacokinetic, and biologic correlative study. *J Clin Oncol* 2003;21:211–22.
 33. Law CL, Cervený CG, Gordon KA, et al. Efficient elimination of B-lineage lymphomas by anti-CD20-auristatin conjugates. *Clin Cancer Res* 2004;10:7842–51.
 34. Junutula JR, Raab H, Clark S, et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat Biotechnol* 2008;26:925–32.
 35. Jackson D, Gooya J, Mao S, et al. A human antibody-drug conjugate targeting EphA2 inhibits tumor growth *in vivo*. *Cancer Res* 2008;68:9367–74.
 36. Ma D, Hopf CE, Malewicz AD, et al. Potent antitumor activity of an auristatin-conjugated, fully human monoclonal antibody to prostate-specific membrane antigen. *Clin Cancer Res* 2006;12:2591–6.
 37. 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.
 38. 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.
 39. Fargion S, Carney D, Mulshine J, et al. Heterogeneity of cell surface antigen expression of human small cell lung cancer detected by monoclonal antibodies. *Cancer Res* 1986;46:2633–8.
 40. Grainger JL, von Brunn A, Winkler MM. Transient synthesis of a specific set of proteins during the rapid cleavage phase of sea urchin development. *Dev Biol* 1986;114:403–15.
 41. Jain RK. Tumor physiology and antibody delivery. *Front Radiat Ther Oncol* 1990;24:32–46.