

# Characterization of SGN-CD123A, A Potent CD123-Directed Antibody–Drug Conjugate for Acute Myeloid Leukemia



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## Abstract

Treatment choices for acute myelogenous leukemia (AML) patients resistant to conventional chemotherapies are limited and novel therapeutic agents are needed. IL3 receptor alpha (IL3R $\alpha$ , or CD123) is expressed on the majority of AML blasts, and there is evidence that its expression is increased on leukemic relative to normal hematopoietic stem cells, which makes it an attractive target for antibody-based therapy. Here, we report the generation and preclinical characterization of SGN-CD123A, an antibody–drug conjugate using the pyrrolobenzodiazepine dimer (PBD) linker and a humanized CD123 antibody with engineered cysteines for site-specific conjugation. Mechanistically, SGN-CD123A induces activation of DNA damage response pathways, cell-cycle changes, and apoptosis in AML cells. *In vitro*, SGN-CD123A–mediated potent cytotoxicity

of 11/12 CD123<sup>+</sup> AML cell lines and 20/23 primary samples from AML patients, including those with unfavorable cytogenetic profiles or FLT3 mutations. *In vivo*, SGN-CD123A treatment led to AML eradication in a disseminated disease model, remission in a subcutaneous xenograft model, and significant growth delay in a multidrug resistance xenograft model. Moreover, SGN-CD123A also resulted in durable complete remission of a patient-derived xenograft AML model. When combined with a FLT3 inhibitor quizartinib, SGN-CD123A enhanced the activity of quizartinib against two FLT3-mutated xenograft models. Overall, these data demonstrate that SGN-CD123A is a potent antileukemic agent, supporting an ongoing trial to evaluate its safety and efficacy in AML patients (NCT02848248). *Mol Cancer Ther*; 17(2): 554–64. ©2017 AACR.

## Introduction

Acute myelogenous leukemia (AML) is the most common form of adult leukemia and patients with AML need new therapies. The older patients who are unable to receive intensive chemotherapy have an extremely poor prognosis, with a median overall survival between 5 and 10 months (1, 2). For younger and fit patients, the intensive treatment options include multiagent chemotherapy with or without allogeneic hematopoietic stem cell transplant. However, most AML patients will experience disease recurrence

within 3 years after diagnosis, and will thus require alternative treatment options (2).

One of the approaches is to use monoclonal antibodies that bind to leukemia cells. The alpha chain of interleukin receptor 3, CD123, is an important antigen for AML. Upon binding of IL-3, CD123 forms a heterodimer with the beta subunit of the IL-3 receptor leading to the transduction of intracellular signals associated with cell proliferation, differentiation, and survival (3). Multiple studies have demonstrated that CD123 is expressed on the surface of blasts in the majority of patients with AML (4, 5), with expression levels detected at significantly higher levels compared with those seen in normal CD34<sup>+</sup> hematopoietic progenitors (6, 7). Expression levels of CD123 on AML cells vary depending on genetic and molecular subtypes (5). It has been reported that high CD123 levels on blasts is associated with increased resistance to apoptotic cell death and activation of the signal transducer and activator of transcription 5 pathway (8). In addition, clinical studies showed that increased CD123 expression was associated with higher blast counts at presentation and reduced responses to chemotherapy (9).

In addition to myeloblast cells from patients with AML, CD123 has been found selectively expressed on the leukemic stem cell (LSC) population as measured by both flow cytometry and functional assays (6, 10). The LSC population is of particular interest in AML, because it has been linked to

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chemotherapy resistance and potentially responsible for the persistence of minimal residual disease in patients in hematologic complete remission, which may ultimately lead to relapse (11, 12). Clinical data have demonstrated that patients with elevated LSC populations at baseline (as measured by CD34<sup>+</sup>/CD38<sup>-</sup>/CD123<sup>+</sup> cells) have significantly worse outcomes when compared with patients with minimal LSC populations (13). Importantly, CD123 expression is very low or absent from normal hematopoietic stem cells, suggesting a therapeutic window of opportunity for CD123-directed therapies (14). Preclinical studies with anti-CD123 antibodies have suggested that targeting these cells is associated with a reduction in LSCs and increased survival in xenograft models (10). These studies provide a strong rationale to develop CD123-directed therapy in patients with AML.

In the present study, we report the preclinical development of SGN-CD123A, a pyrrolbenzodiazepine dimer (PBD)-based antibody-drug conjugate targeting CD123 on AML cells. *In vitro*, SGN-CD123A specifically binds to and is internalized into CD123<sup>+</sup> cells. The ADC was found to kill AML cell lines and primary AML samples at concentrations between 0.02 and 2.5 ng/mL. Moreover, neither cytogenetic profiles nor multiple drug resistance (MDR) status affected the activity of SGN-CD123A. In both MDR<sup>+</sup> and MDR<sup>-</sup> xenograft models, treatment with single dose of SGN-CD123A yielded tumor remissions. Furthermore, SGN-CD123A also mediated complete remissions of a patient-derived AML model. Finally, we also demonstrated that SGN-CD123A combines effectively with a FLT3 inhibitor, quizartinib (15), in FLT3-mutated AML models.

## Materials and Methods

### Cell culture

AML cell lines THP-1 (ATCC TIB-20), MV4-11(ATCC CRL-9591), KG-1 (ATCC CCL-246), TF-1 $\alpha$ (ATCC CRL-2451), and HEL92.1.7 (ATCC TIB-180) were obtained from the ATCC. The cell lines HNT-34(ACC-600), MOLM-13(ACC-554), EOL-1 (ACC-386), NOMO-1(ACC-542), SKM-1(ACC-547), GDM-1 (ACC-87), and Kasumi-1(ACC-220) were obtained from German Collection of Microorganisms and Cell Cultures GmbH. All cells were authenticated and confirmed free of *Mycoplasma* using Cell-Check 16 analysis and IMPACT testing (IDEXX BioResearch). RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS (Gibco) was used to grow TF1- $\alpha$ , HEL92.1.7, EOL-1, NOMO-1, THP-1, and HNT-34 cells. RPMI1640 medium supplemented with 20% FBS was used for MOLM-13, SKM-1, GDM-1, and Kasumi-1 cells. IMDM (Thermo Fisher Scientific) supplemented with 20% FBS was used to culture MV-4-11 and KG-1 cells. All cells were maintained in a humidified 37°C incubator with 5% CO<sub>2</sub>.

### Antibody production and conjugation preparation

h7G3ec antibody was produced in Chinese hamster ovary (CHO) cells and purified before conjugation (Seattle Genetics, Inc.). The full amino acid sequence is shown in Supplementary Fig. S4. The conjugation of PBD dimers to antibodies with engineered cysteines (S239C) has been described previously (16). The nonbinding control ADC is composed of a recombinant humanized IgG1 with S239C-engineered cysteine mutations and PBD dimer drug linker.

### Flow cytometry

Quantitation of CD123 antigen on cell surface was performed using QIFIKit following the manufacturer's directions (Agilent Technologies). Briefly, cells were washed and stained with primary anti-CD123 antibody (BD Biosciences) or mouse IgG2a (BD Biosciences) on ice for 30 minutes. The cells were then washed and stained with fluorescein isothiocyanate (FITC)-labeled secondary antibody. The calibration beads of the QIFIKit were also stained with FITC conjugate using the same condition. After flow cytometry analysis on FACSCalibur (BD Biosciences), antigen number was determined using the geometric means of the samples stained with CD123 antibody, isotype control, and calibration beads.

To determine the binding affinity on CD123 cells, h7G3ec, SGN-CD123A, and hlgG1 were conjugated with Alexa Fluor-647 (Thermo Fisher Scientific) and incubated with prewashed MV4-11 cells for 60 minutes on ice at various concentrations (between 0 and 996 nmol/L; 1 nmol/L = 151 ng/mL). The stained cells were analyzed on Attune NxT flow cytometer (Thermo Fisher Scientific), and the geometric means of the data were fitted in GraphPad for dissociation constant.

### Western blot analysis

Western blot analyses of phosphorylated H2AX, total H2AX, and phosphorylated-ATM were performed with protein extracts from HNT-34 cells that had been treated with 0, 1, 5, or 10 ng/mL of SGN-CD123A for 48 hours and either lysed using cell lysis buffer (Cell Signaling Technology) or extracted for histone using the EpiSeeker Kit (Abcam). These following primary antibodies were used for Western blot analysis: rabbit anti-H2AX (Abcam), mouse anti-phospho- $\gamma$ H2AX<sub>S139</sub> (Millipore), rabbit anti-cleaved PARP (Cell Signaling Technology), rabbit anti-P-ATM<sub>S1981</sub> (Cell Signaling Technology), rabbit anti-phospho-P53<sub>S15</sub> (Cell Signaling Technology) and mouse anti-beta-actin (Cell Signaling Technology). For detection, membranes were then incubated with secondary antibodies IRDye800CW goat anti-rabbit IgG (LI-COR, Lincoln, NE) or IRDye680RD goat anti-mouse IgG (LI-COR), and imaged on an Odyssey LX imager (Li-COR).

### Internalization assay

Cells were incubated on ice with 1  $\mu$ g/mL SGN-CD123 for 30 minutes before returning to 37°C incubator for 0, 4, or 24 hours. Cells were then washed, fixed, and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) before stained with anti-human IgG Fc antibody (BD Biosciences) and anti-lysosomal-associated membrane protein1 (LAMP-1, BD Biosciences). Nuclei were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Images were acquired with an Olympus IX83 microscope equipped with a Hamamatsu digital camera (C11440).

### *In vitro* cytotoxicity assays

Cells were grown at densities between 5,000 and 10,000 per well in 96-well plates in their respective growth medium, supplemented with 10% heat-inactivated human serum (Gemini Bio-products) to block non-specific uptake of ADCs by Fc $\gamma$ R. Cells were exposed to ADCs for 96 hours at 37°C incubator with 5% CO<sub>2</sub>. Cell viability was determined using Cell-Titer Glo following the manufacturer's instructions. The IC<sub>50</sub> value of each drug was determined using Prism 6 (GraphPad).

The effect of SGN-CD123A on primary patient isolates was determined by incubating samples with increasing concentrations

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of SGN-CD123A for 96 hours before flow cytometric analyses of live/dead cells. Data are expressed as  $IC_{50}$ , the concentration of ADC required to give a 50% reduction in cell viability. CD123 expression on bulk tumor cells and the CD34<sup>+</sup> subset was determined by flow cytometry and expressed as median fluorescence intensity (MFI).

#### Caspase measurement

Caspase activity was measured using Caspas-3/7 Glo kit according to the manufacturer's guide. Briefly, AML cells were cultured in the presence of various concentrations of SGN-CD123A or control ADCs in 96-well plates for 48 hours, before the addition of Caspas-3/7 Glo reagent. The luminescence was then measured on an Envision plate reader (PerkinElmer).

#### Animal studies

All animal experiments were performed according to Institutional Animal Care and Use Committee guidelines. Five million MOLM-13 cells were injected intravenously into severe combined immune deficiency mice (SCID, Harlan Laboratories) to establish disseminated disease. All animals received 10 mg/kg human immune globulin (hIVIg, Grifols Therapeutics Inc.) to block nonspecific uptake from the Fc receptors on blast cells one-day prior ADC administration. ADCs were injected intraperitoneally post hIVIg administration. Animals were monitored for signs of disease, including weight loss and hind limb paralysis as the termination signal. KG-1 cells (ATCC CCL-246) were implanted subcutaneously into SCID mice, and ADCs were administered intraperitoneally when the average tumor volume reached 100 mm<sup>3</sup>. Tumor growth was measured using a digital caliper and the volume was calculated using the formula  $V = 0.5 \times \text{length} \times \text{width}^2$ . The establishment and characterization of the AML patient-derived xenograft model has been described previously (17). Disease burden was determined using flow cytometry of human CD45 in bone marrow. ADCs were given at 300 µg/kg twice intraperitoneally in this model. For combination studies, quizartinib (LC Laboratories) was given orally daily for 21 days, whereas ADCs were given once intraperitoneally at the doses specified in each figure.

#### Statistical analysis

Error bars in xenograft tumor volume measurement represent standard error of means. Two-way ANOVA test was used to compare tumor volume. Survival analysis of xenograft was performed using the time for tumor volume to quadruple for subcutaneous xenograft models. The log-rank test was used to determine statistical significance of survival analysis.

#### Analysis of patient samples

The design of studies using patient samples were reviewed and approved by institutional review boards of either Texas MD Anderson Cancer Center or Fred Hutchinson Cancer Center. Patients were consented for the research use of the samples. CD123 expression on AML cell lines and AML patients was determined using phycoerythrin conjugated mouse anti-CD123 antibody (BD Biosciences). Blasts of AML patients were first characterized by a panel of antibodies (CD45, CD34, CD38, CD3, CD7, CD19, and CD33) that are routinely used for clinical work-up of AML. The blast gating was primarily based on CD45 and side scatter. If the blasts were uniformly positive for CD34, the

CD34 gate was also applied for better delineation of the blast population (Supplementary Fig. S1).

## Results

#### Expression of CD123 on AML patients

It has been reported that CD123 is expressed on the surface of blasts in the majority of patients with AML (4, 5). To confirm the prevalence of CD123 expression, we surveyed a cohort of 52 AML patients using multicolor flow cytometry. Fifteen of these patients had newly diagnosed AML, whereas 37 patients had recurrent or persistent disease. These cases included various morphologic subtypes and AML with high-risk molecular genetic abnormalities. In line with previous studies, CD123 expression was detected on more than 50% leukemic blasts (CD34<sup>+</sup>) among 48 (92%) patients (e.g., Fig. 1A). Furthermore, 45 of these 48 patients (94%) had CD123 expression on that blast that was 10-fold higher than the background (Fig. 1B). These data confirm the potential of CD123 as a target for antibody-based therapies for the treatment of AML.

#### Conjugation and characterization of SGN-CD123A

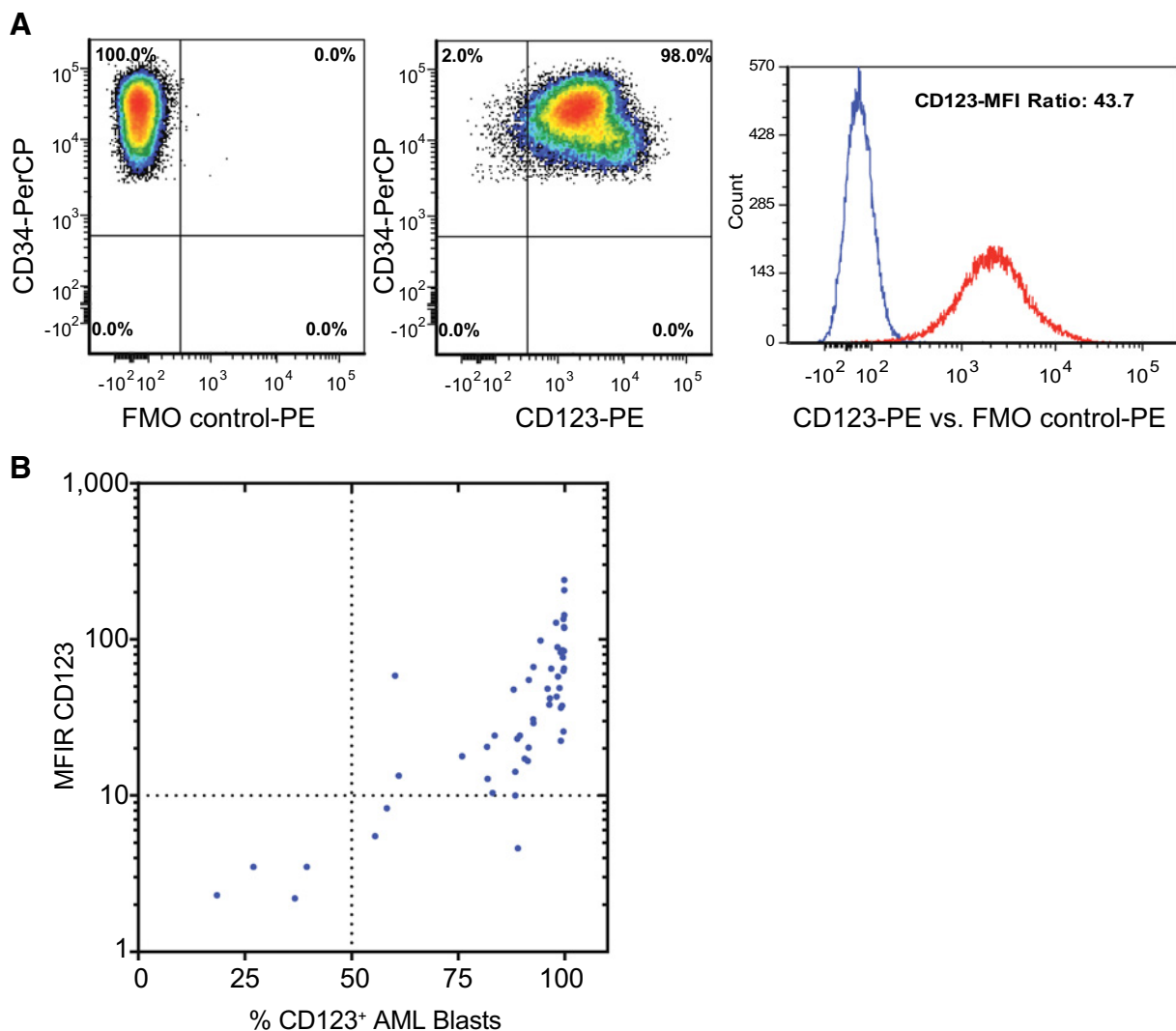
To produce a CD123-targeted ADC, we first humanized a murine anti-CD123 antibody by replacing the complementarity determining regions and select framework residues in a human germline antibody with the corresponding murine sequence to retain binding affinity and maximize homology to the human acceptor sequence. To enable site-specific conjugation, we further introduced an engineered cysteine at amino acid position 239 in each heavy chain, yielding humanized antibody h7G3ec (17, 18). SGN-CD123A is generated by conjugation of SGD-1910, a chemical intermediate that contains both the pyrrolbenzodiazepine dimer (PBD) payload and the valine-alanine dipeptide linker, to the two engineered cysteine residues of h7G3ec (ref. 16; Fig. 2A). SGN-CD123A incorporates approximately two SGD-1910 molecules per antibody molecule.

We next evaluated the binding of h7G3ec and SGN-CD123A to CD123 using a flow cytometry-based cell-binding assay. On CD123<sup>+</sup> MV4-11 cells, both unconjugated antibody and ADC showed high-affinity binding to CD123 (Fig. 2B). The equilibrium dissociation constant values of h7G3ec and SGN-CD123A were 7.6 nmol/L (1.15 µg/mL) and 6 nmol/L (0.92 µg/mL), respectively.

Once bound to surface antigens, ADCs are internalized through endosomes and trafficked to lysosomes for drug release (19). Consistent with this, binding of SGN-CD123A to AML cells resulted in rapid internalization of the ADC-CD123 complex as detected by fluorescence microscopy (Fig. 2C). SGN-CD123A and lysosomes were stained using antibodies recognizing human Fc (red), or the lysosomal marker LAMP-1 (green), respectively. SGN-CD123A was detected on surface and the lysosomes were distinct and punctate at time zero ( $T = 0$ ). Within 4 to 24 hours, SGN-CD123A was readily detected inside cells and co-localized with LAMP-1 (yellow), showing rapid internalization and trafficking to the lysosomes.

#### *In vitro* cytotoxic activity on AML cell lines

We then evaluated the cytotoxic potential of SGN-CD123A in 13 AML cell lines, of which 12 expressed between 2,000 and 26,100 copies of CD123 on the cell surface. SGN-CD123A was



**Figure 1.**

Expression of CD123 in AML patients. **A**, Flow-cytometry staining of CD123 of CD34<sup>+</sup> blasts of one AML patient. FMO: Fluorescence minus one control, which contains all other fluorochromes except CD123. **B**, CD123 expression in 52 AML patients was determined using flow cytometry. MFIR: median fluorescence intensity ratio calculated by the CD123-PE intensity over FMO control.

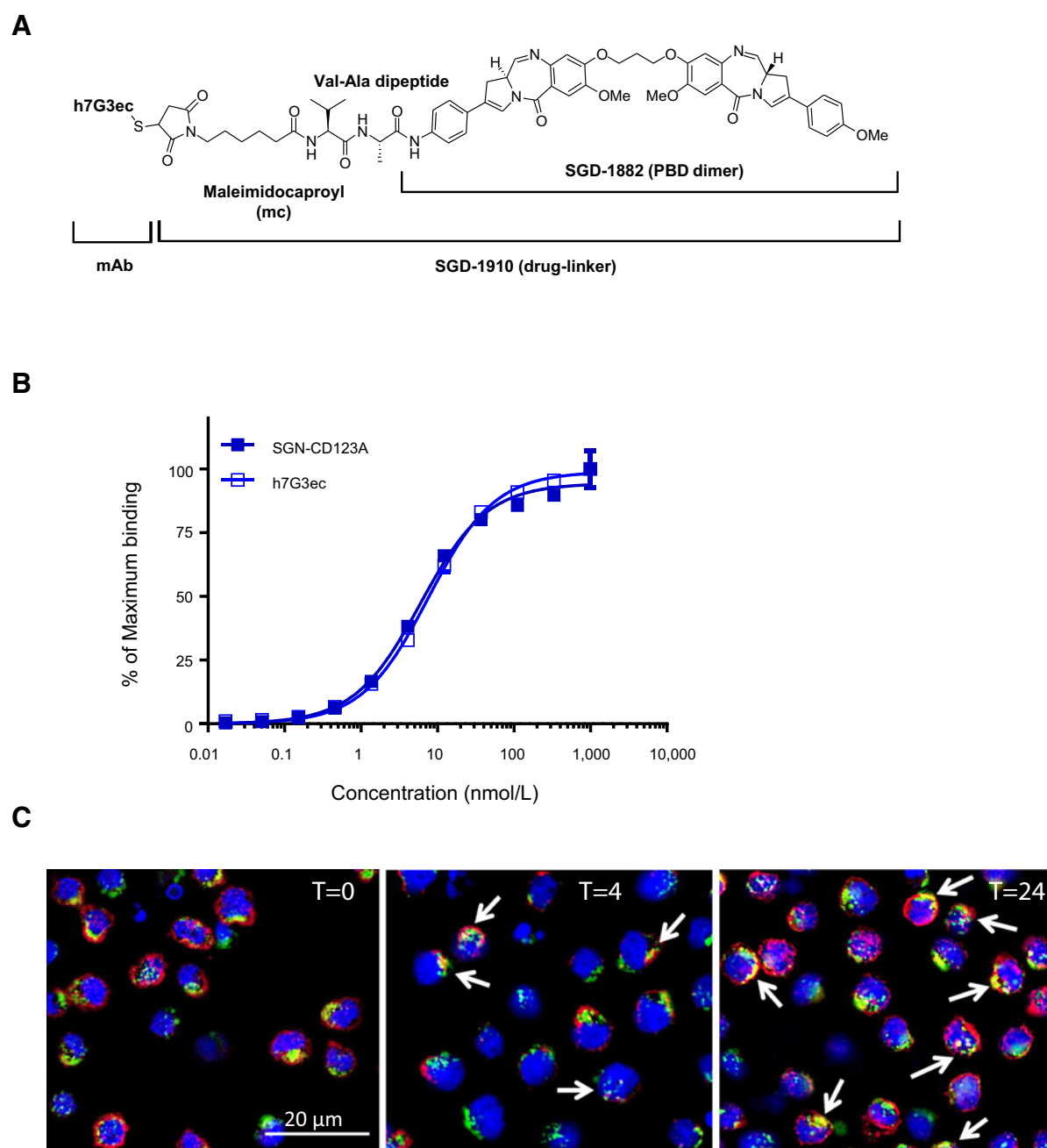
highly active in 11 of 12 CD123<sup>+</sup> AML cell lines tested (mean IC<sub>50</sub>, 6 ng/mL; range of 0.02 to 38 ng/mL). For example, SGN-CD123A killed HNT-34 cells with an IC<sub>50</sub> value of 0.2 ng/mL, whereas neither the control ADC nor unconjugated h7G3ec were cytotoxic (Fig. 3A). The PBD dimer-based payload has been reported to overcome MDR (17). We evaluated the activity of SGN-CD123A in five MDR<sup>+</sup> cell lines (KG-1-ATCC8031 and KG-1-CCL246, GDM-1, Kasumi-1 and TF1- $\alpha$ ). SGN-CD123A was highly active against four of the five cell lines (Table 1). For instance, KG-1 cells are MDR<sup>+</sup>, but SGN-CD123A-mediated cell killing with an IC<sub>50</sub> value of 0.8 ng/mL (Fig. 3B). Importantly, the cytotoxic activity was immunologically specific, as the non-binding control ADC showed minimal activity (Fig. 3A and B). Moreover, SGN-CD123A was not cytotoxic against the CD123-negative HEL92.1.7 AML cell line, further supporting target-specificity (Table 1).

#### ***In vitro* activity of SGN-CD123A in primary AML patient specimens**

We also evaluated the cytotoxic potential of SGN-CD123A on blast samples isolated from 23 AML patients (Table 2). These patients had favorable ( $n = 3$ ), intermediate ( $n = 15$ ), or adverse ( $n = 5$ ) cytogenetic profiles. Fourteen of these patients also had FLT3 internal tandem duplication (FLT3/ITD), a mutation that correlates with poor prognosis in AML (20). Twenty-six percent (6/23) of patients had mutations in nucleophosmin gene (NPM1), which is associated with good prognosis in AML patients (21). Consistent with the cell line data shown in Table 1, the cytogenetic profiles did not affect the cytotoxicity of SGN-CD123A. In fact, 20 of 23 primary samples showed an IC<sub>50</sub> value of 0.8 ng/mL (Table 2 and Supplementary Fig. S2A and S2B). Also in agreement with our cell line data, 14 out of the 15 MDR<sup>+</sup> AML samples had EC<sub>50</sub> values of SGN-CD123A of 0.06 to 2.5 ng/mL,



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**Figure 2.**

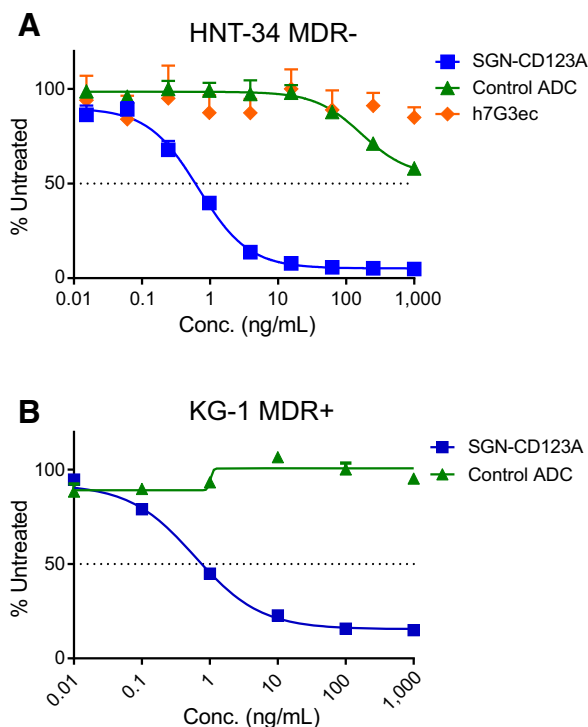
Structure, binding, and internalization of SGN-CD123A. **A**, Chemical structure of SGN-CD123A, comprised of the monoclonal antibody h7G3ec and drug linker (SGD-1910). **B**, Binding kinetics of SGN-CD123A and h7G3ec to CD123<sup>+</sup> MV4-11 cells, as measured by flow cytometry. The highest MFI was set to 100% for normalization. **C**, Immunofluorescence staining of SGN-CD123A (red, staining human IgG Fc kappa chain), lysosome (green, staining LAMP-1), and nucleus (blue, staining 4',6-diamidino-2-phenylindole). Images from 0, 4, and 24 hours post-ADC incubation are represented. When ADC trafficks to the lysosomes and colocalizes with LAMP-1, the resulting signal is projected as yellow in the merged images (arrows).

indicating that SGN-CD123A can also overcome MDR in primary AML samples.

#### Cell death kinetics induced by SGN-CD123A

The mechanism of SGN-CD123A-mediated cytotoxicity starts with the release of PBD dimers upon internalization. Upon

release, the PBD dimer can crosslink DNA and induce a DNA damage response (17). One of the early markers of DNA damage is phosphorylated histone 2AX (pγH2AX; ref. 22). SGN-CD123A induced pγH2AX in a dose-dependent manner in HNT-34 cells (Fig. 4A). Induction of pγH2AX was also confirmed using flow cytometry, where we found that 5 ng/mL SGN-CD123A induced a

**Figure 3.**

*In vitro* cytotoxicity of SGN-CD123A on leukemia cell lines. **A**, *In vitro* cytotoxicity of SGN-CD123A, a non-binding control ADC, and h7G3ec on HNT-34 cells. ADCs were titrated in growth media and cells were incubated with ADC for 96 hours. Cell viability was normalized to untreated cells. **B**, *In vitro* cytotoxicity of SGN-CD123A and non-binding control ADC on MDR<sup>+</sup> KG-1 cells.

6-fold increase of p $\gamma$ H2AX in HNT-34 cells within 48 hours post ADC treatment (Supplementary Fig. S3A). A similar magnitude of p $\gamma$ H2AX increase was also observed in KG-1 and THP-1 cells after the treatment of SGN-CD123A (Supplementary Fig. S3B and S3C). Accompanying the p $\gamma$ H2AX was the phosphorylation and, hence, the activation of the ataxia telangiectasia–mutated (ATM) kinase (p-ATM, Fig. 4A), which is known to trigger the cascade of events leading to cell-cycle arrest as the cells attempt DNA repair

(23). Sensing DNA damage response, P53 will be phosphorylated and trigger cell-cycle arrest (24). Increased level of phosphorylated P53 protein and cell-cycle arrest were observed in SGN-CD123A–treated cells (Fig. 4B). Indeed, cell-cycle distribution analysis of SGN-CD123A–treated cells confirmed arrest in the G<sub>2</sub>–M phase in a dose-dependent manner (Fig. 4C). Concomitant with the pileup in the G<sub>2</sub>–M phase were marked decreases in the fraction of cells found in the G<sub>1</sub> and S phases.

When DNA repair mechanisms fail, the pathway to apoptosis and cell death is initiated. Activation of cysteinyl-aspartic acid proteases (caspases) is an important early step leading to cleavage of cellular proteins such as PARP and apoptosis (Fig. 4B). Caspase-3 activity was measured by flow cytometry. SGN-CD123A increased caspase-3 activity in HNT-34 cells in the low picomolar range, more potent than the free PBD molecule. In contrast, it took 25  $\mu$ mol/L cisplatin to induce the same level of caspase activity, whereas the non-binding control ADC treatment had no caspase induction (Fig. 4D). SGN-CD123A also induced caspase-3, in a dose-dependent manner, in the GDM-1 cells that express 5500 CD123 on cell surface (Supplementary Fig. S3D).

#### Anti-leukemic activity on xenograft models

Next, we evaluated the activity of SGN-CD123A against a panel of leukemia cell line–based xenograft models. First, in the disseminated disease model established from MOLM-13 cells, a single dose of SGN-CD123A at 10 or 30  $\mu$ g/kg yielded a significant survival advantage over a non-binding control ADC. All eight mice that received the non-binding control died within 32 days. In contrast, none of the SGN-CD123A–treated animals showed any signs of disease by the end of study (Fig. 5A).

In a subcutaneous xenograft model established from HNT-34 cells, a single injection of 25  $\mu$ g/kg of SGN-CD123A led to 50 days of tumor stasis. On the other hand, a single dose of 75  $\mu$ g/kg of SGN-CD123A treatment resulted in remission of tumors in all of the 8 mice. The control ADC (75  $\mu$ g/kg) led to an initial tumor growth delay out to day 40 post-implantation, followed by rapid tumor regrowth. These data indicate potent, antigen-specific activity of SGN-CD123A *in vivo* (Fig. 5B).

To assess the activity of SGN-CD123A in the MDR<sup>+</sup> xenograft models, we implanted KG-1-CCL246 cells subcutaneously in SCID mice. In this model, a single dose of 300  $\mu$ g/kg SGN-

**Table 1.** *In vitro* cytotoxicity of SGN-CD123A on AML cell lines

Cell Line	Mutations <sup>a</sup>			MDR status	CD123 copy number	SGN-CD123A IC <sub>50</sub> , ng/mL
	FLT3	p53	Other			
HNT-34	–	ND	BCR-ABL1	–	24,400	0.2
MOLM-13	+	–	MLL-AF9	–	20,000	0.08
THP-1	–	Mutant	MLL-AF9	–	8,000	24
EOL-1	–	–	MLL-PTD	–	9,000	<0.0001
NOMO-1	–	Mutant	MLL-AF9	–	7,000	38
SKM-1	–	Mutant	–	–	6,600	2.5
MV4-11	+	–	MLL-AF4	–	26,100	0.02
KG1 (ATCC8031)	–	Mutant	NRAS	+	9,400	0.8
KG1 (ATCC246)	–	Mutant	NRAS	+	5,000	0.6
GDM-1	–	Mutant	t(6;7)	+	5,500	2
Kasumi-1	–	Mutant	AML1/ETO	+	2,000	1
TF1- $\alpha$	–	Mutant	Complex	+	2,300	>1,000
HEL 92.1.7	–	Mutant	JAK2V617F	+	0	>1,000

NOTE: *In vitro* cytotoxicity assays were 96-hour assays except for Kasumi and KG1 (ATCC246), which were 7-day assays. CD123 copy number was determined by quantitative flow cytometry using a commercial kit.

Abbreviation: ND, not determined.

<sup>a</sup>Wellcome Trust Sanger Institute (COSMIC) and DSMZ Guide to Leukemia and Lymphoma Cell Lines.

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**Table 2.** *In vitro* cytotoxicity of SGN-CD123A on primary AML patient samples

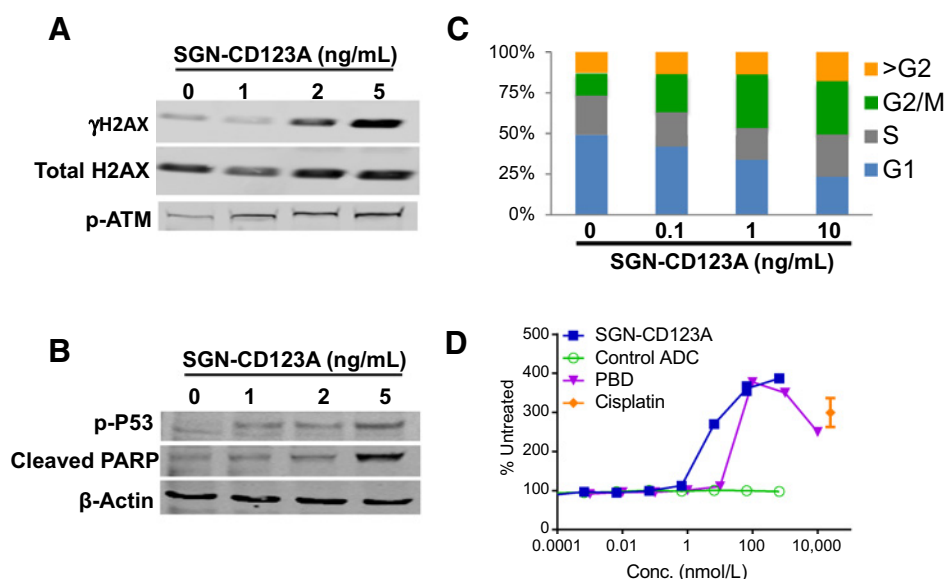
Sample	Cytogenetics	Mutations FLT3/ITD	Mutations NPM-1	MDR status	CD123 (MFI)	SGN-CD123A IC <sub>50</sub> , ng/mL
SG037	Intermediate	+	+	+	480	0.68
SG016	Intermediate	ND	ND	+	595	1.4
SG048	Intermediate	+	-	+	1,070	1.9
SG025	Intermediate	+	-	+	1,190	>2.5
SG034	Intermediate	+	+	+	1,200	0.22
SG045	Intermediate	+	-	+	2,030	1.2
SG040	Intermediate	+	ND	+	2,400	0.37
SG039	Intermediate	+	+	+	2,670	0.075
SG038	Intermediate	-	-	+	2,950	0.18
SG018	Intermediate	ND	ND	+	3,140	0.12
SG036	Intermediate	+	+	+	3,260	0.16
SG026	Intermediate	+	-	+	4,600	0.22
SG047	Intermediate	+	+	-	790	>2.5
SG023	Intermediate	+	+	-	2,280	0.06
SG046	Intermediate	+	ND	-	2,530	0.17
SG028	Favorable	-	-	-	830	1.9
SG019	Favorable	-	-	-	1,480	>2.5
SG022	Favorable	-	-	-	1,485	0.56
SG044	Adverse	ND	ND	+	1,140	2.5
SG043	Adverse	ND	ND	+	1,370	2.5
SG029	Adverse	-	-	+	1,820	0.46
SG042	Adverse	+	-	-	270	0.11
SG021	Adverse	+	-	-	1,560	1.6

NOTE: Primary AML samples were incubated with increasing concentrations of SGN-CD123A for 96 hours before flow-cytometry analyses of live/dead cells. Data are expressed as the IC<sub>50</sub> value, the concentration of ADC required to give a 50% reduction in cell viability. CD123 expression on bulk tumor cells and the CD34<sup>+</sup> subset was determined by flow cytometry and expressed as MFI.

Abbreviations: MDR status, fluorescence in the presence of MDR pump inhibitors more than 2-fold above background; ND, not determined.

CD123A significantly decreased tumor growth, including four durable remissions ( $P = 0.008$  using log-rank test). This is in contrast with treatment with a non-binding control ADC, which did not slow the growth rate (Fig. 5C).

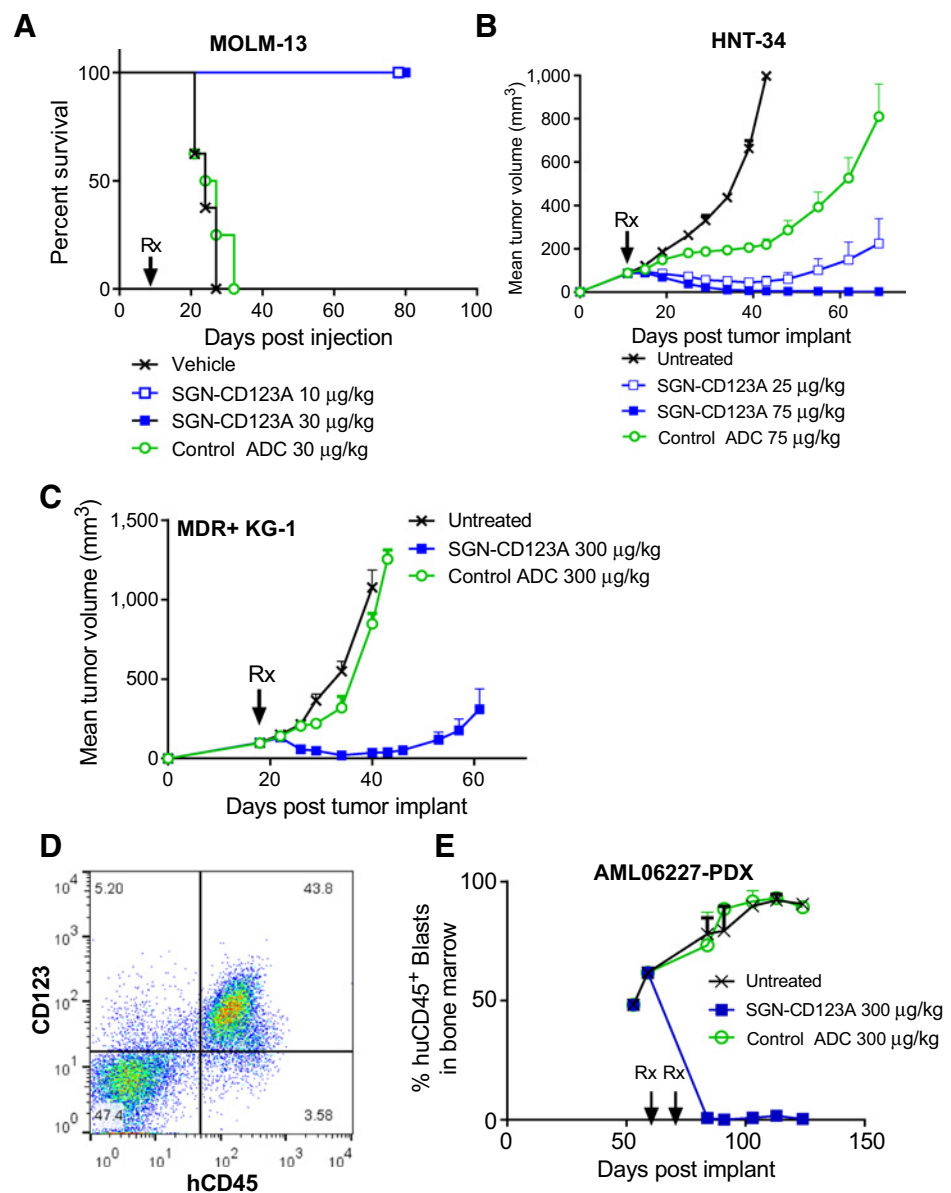
Patient-derived xenograft models are thought to better mimic the physiology of AML patients, and thus better predict the clinical performance of drugs (25). The anti-leukemic activity of SGN-CD123A was also evaluated in a primary AML

**Figure 4.**

DNA damage responses and cell-cycle changes induced by SGN-CD123A. **A**, Western blot of phosphorylated H2AX, total H2AX, and phosphorylated-ATM in protein extracts from HNT-34 cells treated with 0, 1, 2, or 5 ng/mL of SGN-CD123A for 48 hours. **B**, Western blot staining of phosphorylated P53, cleaved PARP, and beta-actin of HNT-34 cells treated with SGN-CD123A at indicated concentrations for 48 hours. **C**, Cell-cycle analysis of HNT-34 cells after treatment of SGN-CD123A at indicated concentrations. The cell-cycle phase was determined using propidium iodide staining. **D**, Flow-cytometry analysis of cleaved caspase-3 of HNT-34 cells after the treatment with SGN-CD123A, control ADC, free PBD drug, or cisplatin. The x-axis represents the molar concentrations of each reagents and y-axis represents the fold difference of mean fluorescence intensity between treated cells and untreated cells. Each data point is the average of two replicates and the experiment has been repeated twice.

**Figure 5.**

*In vivo* anti-leukemic activity of SGN-CD123A. **A**, Survival plot of disseminated leukemia model MOLM-13 after SGN-CD123A treatment. MOLM-13 cells were intravenously implanted in SCID mice and animals received a dose of SGN-CD123A (10 or 30  $\mu\text{g}/\text{kg}$ ) or control ADC (30  $\mu\text{g}/\text{kg}$ ). All animals who received SGN-CD123A remained alive until the end of study (day 80).  $N = 8$  mice per group. **B**, Tumor volume measurement of HNT-34 xenografts. HNT-34 tumors were subcutaneously implanted on SCID mice and ADCs were given once when average tumor volume reached 100  $\text{mm}^3$  ( $n = 8$  per group,  $P < 0.0001$  using two-way ANOVA test). **C**, Tumor volume measurement of MDR<sup>+</sup> KG-1 xenograft implanted subcutaneously in SCID mice. Animals were dosed once with either SGN-CD123A or control ADC at 300  $\mu\text{g}/\text{kg}$  ( $n = 8$  each group,  $P = 0.0003$  using two-way ANOVA test). **D**, Flow cytometry of staining for CD123 and human CD45 of leukemic blasts from patient-derived xenograft model AML06227 grown in NSG mice. **E**, Measurement of CD45<sup>+</sup> blasts in bone marrow of AML06227 PDX mice that were treated with SGN-CD123A or control ADC. Mice were randomized and treated when the average blast in the bone marrow cells reached 50%. Two mice were sacrificed and analyzed for blast percentage at each time point.



model, AML06-227 (17). Flow-cytometric analysis showed that that approximately 47% of the bone marrow consisted of CD45<sup>+</sup> human blasts at 53 days post implant. Moreover, 93% of the blasts expressed CD123 (Fig. 5D). Remarkably, two injections of SGN-CD123A eliminated the blasts cells in the bone marrow until the end of study (124 days post implant). In contrast, the non-binding control ADC had no impact on the disease burden during the course of study (Fig. 5E).

#### Combination of SGN-CD123A and quizartinib

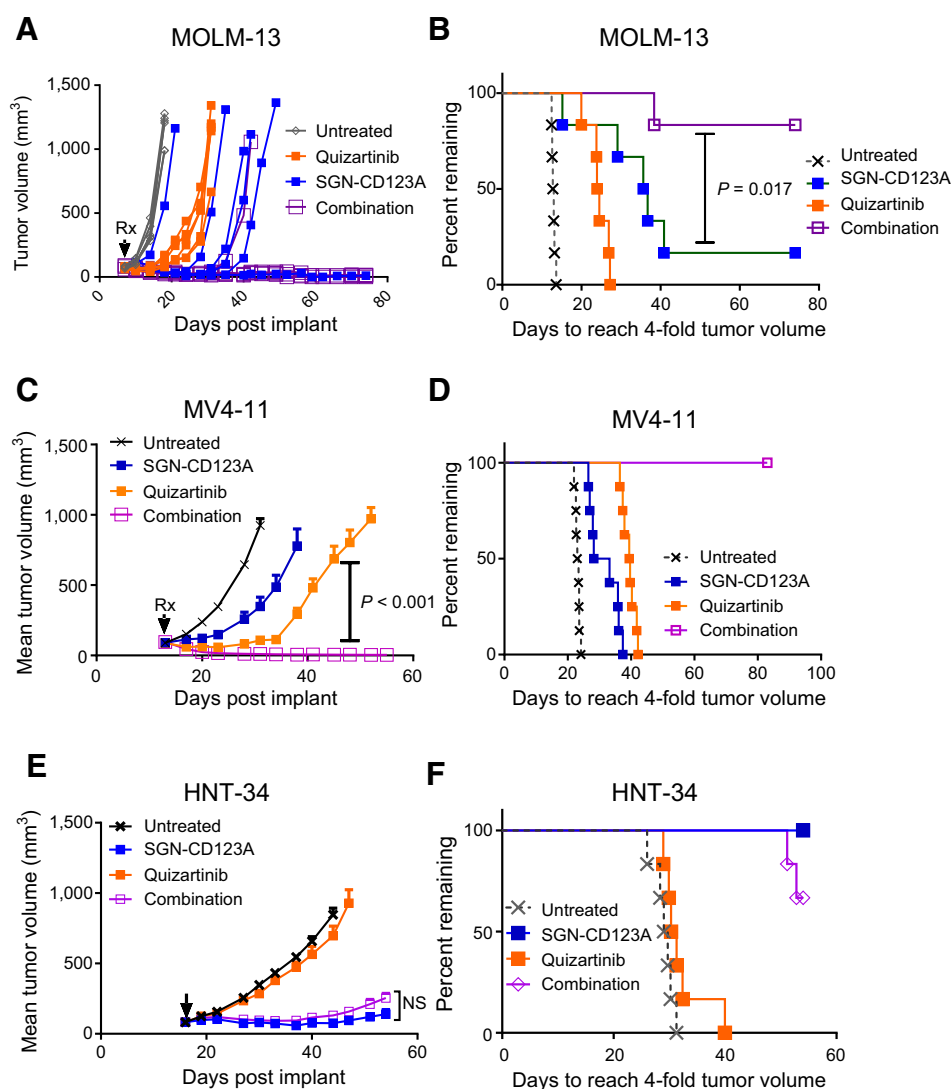
Approximately 23% of AML patients carry a mutation in the FLT3 gene (20, 26). Coincidentally, AML patients carrying FLT3 mutations have higher level of CD123 expression (27, 28). Because it is possible that FLT3 mutated AML patients will receive both a FLT3 inhibitor and SGN-CD123A during clinical studies, we evaluated whether the combination of quizartinib, a FLT3-ITD

specific inhibitor, and SGN-CD123A provides additional efficacy *in vivo*.

In the FLT3/ITD, subcutaneous MOLM-13 xenograft model, 2 mg/kg daily dosing of quizartinib led to a modest growth delay. Injection of 25  $\mu\text{g}/\text{kg}$  SGN-CD123A led to growth delay in five animals and remission in another animal. Strikingly, combination of SGN-CD123A and quizartinib led to durable remissions in five out the six tumor-bearing animals (Fig. 6A). Using tumor-quadrupling time as a measurement of survival, the combination group showed significant extension, with a median survival longer than 70 days. In contrast, the median survival for quizartinib and SGN-CD123A was 24 and 36 days, respectively (Fig. 6B). In addition, we observed a similar combination benefit between SGN-CD123A and quizartinib in another FLT3/ITD xenograft model MV4-11. Although SGN-CD123A and quizartinib led to growth delays, the combination resulted in remissions lasting longer than 80 days (Fig. 6C and D). Furthermore, the quizartinib



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**Figure 6.**

Combination of SGN-CD123A and quizartinib. **A**, Tumor volume measurement of MOLM-13 xenografts after the treatment of quizartinib (2 mg/kg, qdx21 po), SGN-CD123A (25  $\mu$ g/kg, once i.p.), or the two agents given in combination. Each line represents the tumor volume of an individual animal ( $n = 6$ /group). The experiment was repeated in an independent study. **B**, Survival plot of the MOLM-13 tumor-bearing animals using tumor volume quadrupling time as readout. The median survival time in untreated, quizartinib, SGN-CD123A, or combination group was 13 days, 24 days, 36 days, and  $\geq 74$  days, respectively.  $P = 0.017$  using log rank test comparing SGN-CD123A with combination treatment. **C**, Tumor volume measurement of MV4-11 xenografts after the treatment of quizartinib (0.4 mg/kg, qdx21 p.o.), SGN-CD123A (10  $\mu$ g/kg, once i.p.), or in combination ( $n = 8$  in each group).  $P < 0.001$  by two-way ANOVA test. **D**, Survival analysis of MV4-11 model using tumor volume quadrupling as terminal read. The median survival time for animals in untreated, quizartinib, SGN-CD123A, or combination group was 23, 40, 30, and  $\geq 80$  days, respectively.  $P < 0.0001$  using log rank test comparing SGN-CD123A with combination. **E**, Tumor volume of HNT-34 xenografts after the treatment of quizartinib (2 mg/kg, qdx21 p.o.), SGN-CD123A (20  $\mu$ g/kg, once i.p.), or combination ( $n = 6$  in each group).  $P = 0.05$  using two-way ANOVA test comparing the SGN-CD123A and combination. **F**, Survival plot of the HNT-34 study using tumor volume quadrupling as termination criteria. The median survival time for animals in untreated, quizartinib, SGN-CD123A, or combination group was 29 days, 30 days, unreachd, and unreachd, respectively.

and SGN-CD123A were specific to FLT3-mutant tumor models, as in FLT3 wild type HNT-34 tumor model, quizartinib has no activity on its own and it did not influence the activity of SGN-CD123A (Fig. 6E and F).

## Discussion

Every year about 38,000 patients are diagnosed with AML in the United States and European Union (29). Although induction

chemotherapy can be effective in some patients, between 10% and 40% of newly diagnosed AML patients are refractory or resistant to the regimen. Even among patients who achieve remission, the majority will relapse between several months and several years after initial treatment (29). These refractory and relapsed AML patients are in need of novel therapeutic approaches.

One hurdle in these patients is MDR, which confers resistance to gemtuzumab ozogamicin, as calicheamicin is a substrate of

MDR proteins (30). PBD dimers-based ADCs, have demonstrated antitumor activity in MDR<sup>+</sup> xenograft models and show activity in AML patients (16, 31). Moreover, other PBD-based ADCs, including rovalpituzumab tesirine and ADC-301, have demonstrated clinical activities in small cell lung cancer as well as non-Hodgkin's lymphoma (32, 33). Our preclinical results demonstrate that PBD dimers conjugated to a CD123-binding antibody are active in AML cell lines, patient isolates, and xenograft models. Together with the clinical trial results of other PBD-based ADCs, these data support further evaluation of these ADCs for cancer treatment.

Another challenge is the persistence of leukemic stem cells. Multiple studies have demonstrated that there is a hierarchical organization of AML (34), which is thought to be responsible for the relapse of AML. Importantly, CD123 was discovered as a marker of LSC and high CD123 expression on the CD34<sup>+</sup>CD38<sup>-</sup> population is associated with poor prognosis (6, 9). CSL362, a CD123 antibody, was shown to reduce the LSC population in xenograft studies (10). In line with this hypothesis, we also observed long-term remission of patient-derived xenografts after SGN-CD123A treatment, suggesting that the drug eradicated the LSC in this model. On the other hand, CD123 was also detected on low percentage of hematopoietic progenitor cells, which may lead to on-target but off-disease side effects of CD123-targeted therapies (7). Because the h7G3ec antibody does not bind to murine CD123, we could not study the potential impact on hematopoietic cells in this study.

A third challenge for AML drug development is the resistance to targeted small-molecule inhibitors (2). For example, FLT3 inhibitors represent a major class of the drugs in development for FLT3-mutated AML patients (26). However, although patients can respond to treatments with FLT3 inhibitors, they almost invariably develop resistance (26). The rationale to combine FLT3 inhibitors with CD123 targeted drugs include: (i) FLT3-mutated patients, especially those carrying the FLT3/ITD mutations, often have higher CD123 than those without FLT3 mutations (5); and (ii) FLT3 mutation and CD123 expression may overlap on the leukemic stem cells (35). Remarkably, our study shows that a low dose of SGN-CD123A significantly improved the activity of quizartinib in two xenograft models. These data may support clinical evaluation of SGN-CD123A in combination with a FLT3 inhibitor.

The overexpression of CD123 on putative LSCs raised major interest in developing CD123-targeting therapeutics as AML

therapies. Examples include unconjugated monoclonal antibodies, T-cell engaging bispecific antibodies, immunotoxins, and chimeric antigen receptor (CAR)-modified T cells (36–41). Although each of the modality has its own advantages, our study shows that SGN-CD123A is highly active against AML models regardless of cytogenetic profiles and MDR status, and can be effectively combined with quizartinib. These data support the ongoing clinical trial to evaluate its utility in AML treatment.

### Disclosure of Potential Conflicts of Interest

D. Sussman and K. Klussman have ownership interest (including patents) in Seattle Genetics, W. Zeng is a senior research associate at Seattle Genetics, T.S. Lewis is an associate director—Cell Biology and has ownership interest (including patents) in Seattle Genetics, Inc., E.J. Feldman is a medical director, reports receiving a commercial research grant, and has ownership interest (including patents) in Seattle Genetics, D.R. Benjamin has ownership interest (including patents) in Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M.K. Sutherland, R.B. Walter, L. Westendorf, J. Valliere-Douglass, A. Cronkite, D. Sussman, K. Klussman, M. Ulrich, M.E. Anderson, W. Zeng, T.S. Lewis, M. Goswami, S.A. Wang

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** F. Li, M.K. Sutherland, J. Valliere-Douglass, L. Pan, A. Cronkite, D. Sussman, K. Klussman, M. Jonas, T.S. Lewis, S.A. Wang, C.-L. Law, E.J. Feldman

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