

# SYD985, a Novel Duocarmycin-Based HER2-Targeting Antibody-Drug Conjugate, Shows Antitumor Activity in Uterine Serous Carcinoma with HER2/Neu Expression

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## Abstract

Uterine serous carcinoma (USC) is an aggressive form of endometrial cancer. Up to 35% of USC may overexpress the HER2/neu oncogene at strong (i.e., 3+) levels by IHC while an additional 40% to 50% express HER2/neu at moderate (2+) or low (1+) levels. We investigated the efficacy of SYD985, (Synthon Biopharmaceuticals), a novel HER2-targeting antibody-drug conjugate (ADC) composed of the mAb trastuzumab linked to a highly potent DNA-alkylating agent (i.e., duocarmycin) in USC. We also compared the antitumor activity of SYD985 in head-to-head experiments to trastuzumab emtansine (T-DM1), a FDA-approved ADC, against multiple primary USC cell lines expressing different levels of HER2/neu in *in vitro* and *in vivo* experiments. Using antibody-dependent

cellular cytotoxicity (ADCC), proliferation, viability, and bystander killing assays as well as propidium iodide-based flow cytometry assays and multiple *in vivo* USC mouse xenograft models, we demonstrate for the first time that SYD985 is a novel ADC with activity against USC with strong (3+) as well as low to moderate (i.e., 1+/2+) HER2/neu expression. SYD985 is 10- to 70-fold more potent than T-DM1 in comparative experiments and, unlike T-DM1, it is active against USC demonstrating moderate/low or heterogeneous HER2/neu expression. Clinical studies with SYD985 in patients harboring chemotherapy-resistant USC with low, moderate, and high HER2 expression are warranted. *Mol Cancer Ther*; 15(8):1900–9. ©2016 AACR.

## Introduction

Endometrial cancer is the most common gynecologic malignancy with approximately 60,050 new cases and 10,470 estimated disease-related deaths in the United States annually (1). Uterine serous carcinoma (USC) is a highly aggressive variant of endometrial cancer. While this histologic subtype represents only 10% of all uterine tumors, it accounts for a disproportionately high number of endometrial cancer-related deaths secondary to its early predilection for deep myometrial invasion, lymph-vascular space invasion, and intra-abdominal as well as

distant spread (2–5). Accordingly, up to two-third of comprehensively staged USC patients are found to have advanced stage disease (i.e., stage III and IV) at the time of initial diagnosis (2–5). Although these patients are treated with "gold standard" chemotherapy regimens (i.e., intravenous carboplatin/paclitaxel), responses are generally nondurable with a median PFS of only 13 months (2–5). The dismal prognosis of USC patients underscores the need to identify novel effective treatment options for patients with advanced or recurrent disease resistant to conventional chemotherapy.

The human HER2/neu (c-erbB2) gene product, like the EGFR, is a transmembrane receptor protein that includes a cysteine rich extracellular ligand-binding domain, a hydrophobic membrane spanning region, and an intracellular tyrosine kinase domain (6–9). With no direct ligand identified to date, HER2/neu functions as a preferred partner for heterodimerization with other members of the EGFR family (namely HER-1 or ErbB1, HER-3 or ErbB3 and HER-4 or ErbB4), and thus plays an important role in coordinating the complex ErbB signaling network that is responsible for regulating cell growth and differentiation (6–9). Recent data from large USC series demonstrated that HER2 overexpression (i.e., HER2/neu 3+ expression by IHC or c-erbB2 gene amplification by FISH) is present in about 35% of the patients with an additional 40% to 50% USC patients harboring tumors with 2+ or 1+ HER2/neu expression

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**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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by IHC (10, 11). Importantly, in these studies, a striking heterogeneity in HER2/neu protein expression was found with 53% of the HER2/neu 3+ positive USC samples demonstrating large areas of disease with low to negligible HER2/neu expression (10). Taken together, these data suggest that while a significant subset of USC patients may potentially benefit from current HER2/neu-targeted therapies such as trastuzumab (Herceptin, Genentech/Roche), a novel HER2/neu-targeting antibody–drug conjugate (ADC) active against USC with heterogeneous or moderate (2+) to low (1+) HER2/neu expression might significantly expand the number of treatment-eligible patients.

Trastuzumab emtansine (T-DM1, Kadcyla, Genentech/Roche) is an ADC with substantial clinical activity in patients with HER2+ breast cancer harboring disease with intrinsic or acquired resistance to trastuzumab. In T-DM1, the antibody and the cytotoxic agent are conjugated by means of a stable (i.e., non-cleavable) linker to combine the HER2-targeting properties of trastuzumab with intracellular delivery of DM1, a highly potent derivative of the anti-microtubule agent maytansine. Once the ADC has been internalized into tumor cells by receptor-mediated endocytosis and processed, DM1 binds to tubulin and inhibits microtubule assembly, ultimately precluding mitosis and causing apoptosis in dividing tumor cells (12–15).

SYD985 (Synthon Biopharmaceuticals BV) is a novel HER2-targeting ADC based on the "cleavable" linker-duocarmycin payload, valine-citrulline-*seco* DUocarmycin hydroxyBenzamide Azaindole (*vc-seco*-DUBA), conjugated to trastuzumab showing impressive preclinical results in breast cancer (16–18) and encouraging clinical activity in phase I clinical trials (data not shown). The toxic payload in SYD985 (i.e., DUBA) alkylates DNA resulting in DNA damage, mitochondrial stress, impaired DNA transcription, apoptosis, and ultimately cell death in both dividing and nondividing cells (16–18). Importantly, cysteine proteases such as cathepsin B present in endosomes and lysosomes are released by human tumors and have been shown to cause both linker cleavage in SYD985 and release of the membrane-permeable active toxin. This property may cause cell killing of not only HER2/neu–positive cells but also of neighboring non-antigen-expressing tumor cells (i.e., bystander killing effect).

The objective of this study was to explore and compare the antitumor activity of SYD985 in head-to-head experiments to T-DM1 against primary USC cell lines with different HER2/neu expression status. We report preclinical data that SYD985 is significantly more potent than T-DM1 in comparative *in vitro* and *in vivo* experiments, especially against USC with 2+ and 1+ HER2/neu expression. Importantly, our results show that SYD985, unlike T-DM1, is able to induce a significant bystander effect against tumor cells with low/negligible HER2/neu expression when admixed with HER2/neu 3+ USC cells, suggesting potential clinical activity against patients harboring USC with heterogeneous HER2/neu expression.

## Materials and Methods

### Establishment of USC cell lines

Study approval was obtained from the Institutional Review Board at Yale University, and all patients signed consent prior to tissue collection according to the institutional guidelines. Fifteen primary USC cell lines (cell lines characteristics and tissue source are described in Table 1) were established from chemotherapy-naïve patients at the time of primary staging surgery after sterile

processing of fresh tumor biopsy samples, as described previously, and evaluated in our study (19). Primary cell lines were authenticated by whole exome sequencing (WES) in 2013 at the Yale Center for Genome Analysis (19) and cryopreserved. All revived cells were used within 20 passages, and cultured for less than 6 months. Tumors were staged according to the International Federation of Gynecology and Obstetrics staging system. Patient characteristics are noted in Table 1. Primary USC cell lines with limited passages (i.e., <50) were used in the experiments listed below and corresponding cell blocks were analyzed for HER2 surface expression by IHC and FISH.

### SYD985 and T-DM1

T-DM1 (batch N0001B02; Roche) was purchased by Synthon Biopharmaceuticals BV, Nijmegen, the Netherlands. SYD985 was prepared as described previously (16–18). Briefly, *vc-seco*-DUBA was coupled to a cysteine residue of trastuzumab after partial reduction of the inter-chain disulfides. SYD985 was further purified to deliver a well-defined ADC predominantly consisting of species with a drug to antibody ratio (DAR) of 2 and 4, yielding a mean DAR of 2.8 (16–18).

### Immunostaining of cell blocks of primary USC

Cell blocks were obtained from all fifteen USC cell lines and reviewed by a gynecologic surgical pathologist to confirm the presence of serous carcinoma cells. Briefly, HER2 immunohistochemical staining was performed on paraffin-embedded 5- $\mu$ m sections of cell blocks after deparaffinization and rehydration, using the c-erbB-2 antibody (Thermo Fisher Scientific) at 1:800 dilution. HER2 staining intensity was graded per the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) 2013 breast scoring criteria (20). Appropriate positive and negative controls were used with each case.

### FISH of cell blocks from primary USC

FISH analysis was performed using the PathVysion HER2 DNA FISH Kit (Abbott Molecular Inc.) according to the manufacturer's instructions. Cell block sections of 5  $\mu$ m were deparaffinized and rehydrated, followed by acid pretreatment and proteinase K digestion. A probe mix containing an orange probe directed against the HER2 gene (Vysis, Inc., LSI HER2) and a green probe directed against the pericentromeric region of chromosome 17 (Vysis CEP 17) were added and specimens were denatured for 5 minutes at 73°C. Slides were then incubated overnight in a humidified chamber at 37°C and washed the day after when a fluorescence mounting medium, containing 4, 6-diamidino-2-phenylindole (DAPI), was applied. Fluorescent signals in at least 30 nonoverlapping interphase nuclei with intact morphology were scored using a Zeiss Axioplan 2 microscope (Carl Zeiss Meditec, Inc.) with a 100 $\times$  planar objective, using a triple band-pass filter that permits simultaneous blue, green, and red colors. Tumor cells were scored for the number of orange (HER2) and green (chromosome 17) signals. A case was scored as amplified when the ratio of the number of fluorescent signals of HER2 to chromosome 17 was  $\geq 2$ .

### Polymerase chain amplification

Primers for PCR amplification and sequencing were designed using the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>) and were synthesized by the Yale Keck facility (<http://keck.med.yale.edu/>), based on PIK3CA

sequence obtained from National Center for Biotechnology Information (accession number N M\_006218). Two primer pairs were used to individually amplify exon 9 and exon 20 of PIK3CA from genomic USC primary cell lines DNA. PCR amplification was performed in 20  $\mu$ L reaction volumes that contained 100 ng of DNA, 75 mmol/L Tris-HCl, 1.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 20 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2  $\mu$ mol/L of each primer, 0.2 mmol/L of each deoxyribonucleotide triphosphate, and 1 U of Taq DNA polymerase (BIOTOOLS; B&M Laboratories, SA). The 2 exons were amplified with the following PCR conditions: an initial 5-minute denaturation at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute at 57°C, 1 minute at 72°C, and a final extension of 10 minutes at 72°C.

#### DNA sequencing

PCR products were first purified using the MinElute PCR Purification Kit (Qiagen GmbH) and were bidirectionally sequenced using the original primer pair and Applied Biosystems Cycle Sequencing kit (Applied Biosystem Inc) at the Yale Keck facility (<http://keck.med.yale.edu>). Samples were analyzed on the ABI Prism 3100 Avant instrument (Applied Biosystems), using standard run parameters. The separation matrix used was POP-6 using 1 $\times$  Tris-borate-EDTA buffer with EDTA running buffer (Applied Biosystems).

#### Real-time reverse transcription-PCR

RNA isolation from primary USC cell lines was performed using AllPrep DNA/RNA/Protein Mini Kit (Qiagen), according to the manufacturer's instructions. Quantitative PCR was carried out with a 7500 Real-Time PCR System using the manufacturer's recommended protocol (Applied Biosystems) to evaluate the expression of Cathepsin B (CTSB). The primers and probes were obtained from Applied Biosystems (i.e., CTSB, Assay ID: Hs00947433\_m1). The comparative threshold cycle method was used to determine gene expression in each sample, relative to the value of glyceraldehyde-3-phosphate dehydrogenase (assay ID Hs9999905\_m1) RNA as an internal control.

#### Tests for ADCC

Standard 4-hour chromium (<sup>51</sup>Cr) release assay was performed to measure the cytotoxic reactivity of Ficoll-Hypaque-separated PBLs from several healthy donors in combination with trastuzumab, T-DM1, and SYD985 against primary USC target cell lines at effector to target ratios (E:T) of 20:1 and 40:1. The release of <sup>51</sup>Cr from target cells was measured as evidence of tumor cell lysis after exposure of the tumor cells to 2.0  $\mu$ g/mL of trastuzumab or 2.0  $\mu$ g/mL of T-DM1 and SYD985. Dose-response experiments were performed to determine the optimal antibody dosing for ADCC experiments. The negative control condition was the incubation of target cells alone. As a positive control condition, 0.1% SDS was used to achieve complete lysis of target cells. Chimeric anti-CD20 mAb rituximab 2.0  $\mu$ g/mL was used as the negative control for trastuzumab, T-DM1, and SYD985 in all bioassays. The percentage cytotoxicity of trastuzumab or T-DM1 was calculated by the following formula: % cytotoxicity = 100 X (E-S)/(T-S), where *E* is the experimental release, *S* is the spontaneous release by target cells, *T* is the maximum release by target cells lysed with 0.1% SDS.

#### Cell viability assay

USC cell lines were plated at log phase of growth in 6-well tissue culture plates at a density of 20,000–40,000 cells in RPMI1640

media (Life Technologies) supplemented with 10% FBS, 1% penicillin/streptomycin (Mediatech), and 0.3% fungizone (Life Technologies). Cells were incubated at 37°C, 5% CO<sub>2</sub>. After 24 hours of incubation, cells were treated with SYD985, T-DM1, trastuzumab, and isotype control ADC (i.e., rituximab conjugated to *vc-seco*-DUBA: SYD989). SYD985, T-DM1, and SYD989 were used at scalar concentrations of 0.005  $\mu$ g/mL, 0.05  $\mu$ g/mL, 0.5  $\mu$ g/mL, 2  $\mu$ g/mL and 8  $\mu$ g/mL. Trastuzumab was used at concentrations of 1  $\mu$ g/mL, 5  $\mu$ g/mL, 10  $\mu$ g/mL, 40  $\mu$ g/mL, and 100  $\mu$ g/mL. Three and six days after drug treatment, cells were harvested in their entirety, centrifuged, and stained with propidium iodide (2  $\mu$ L of 500  $\mu$ g/mL stock solution in PBS). Analysis was performed using a flow cytometry-based assay to quantify percent viable cells as a mean  $\pm$  SEM relative to untreated cells as 100% viable controls. A minimum of three independent experiments per cell line were performed.

#### Bystander killing

Briefly, a 1:1 ratio of HER2/neu 3+ positive USC cells (i.e., ARK-2) and HER2/neu-negative USC cells (i.e., ARK-4) stably transfected with a GFP plasmid (pCDH-CMV-MCSEF1-copGFP, a kind gift from Dr. Simona Colla, MDACC, Houston, TX), were mixed (20,000 cells/well of each cell type) and plated in 6-well plates (3 mL/well). After an overnight incubation, SYD985, T-DM1, or isotype control ADC at a concentration of 1  $\mu$ g/mL or vehicle were added. After a 72-hour incubation, cells were harvested in their entirety, centrifuged, and stained with propidium iodide (2  $\mu$ L of 500  $\mu$ g/mL stock solution in PBS) to facilitate the identification of dead USC cells. Analysis was performed using flow cytometry-based assay to recognize to quantify percent viable cells as a mean  $\pm$  SEM relative to untreated cells as 100% viable controls. A minimum of three independent experiments were performed.

#### In vivo treatment

The *in vivo* antitumor activity of T-DM1 versus SYD985 was tested in three xenograft models with different HER2/neu expression. Briefly, 6- to 8-week-old CB-17/SCID mice were given a single subcutaneous injection of  $7 \times 10^6$  USC ARK-2 cells (HER2/neu 3+) in approximately 200  $\mu$ L of a 1:1 solution of sterile PBS containing cells and Matrigel (BD Biosciences). A similar procedure was performed for the establishment of ARK-7 (HER2/neu 2+) and ARK-11 (HER2/neu 1+) xenografts. Once the tumor volume was approximately 0.2 cm<sup>3</sup> for each cell line, the mice were randomized into treatment groups (*n* = 5); those treated with SYD985 (3 mg/mg and 10 mg/kg), T-DM1 (10 mg/kg), isotype control ADC (SYD989; 3 mg/mg and 10 mg/kg), and vehicle sterile water. Drug dosages were chosen according to previous studies conducted on different xenograft models (16, 17). All treatment drugs were given as a single intravenous injection based on prior literature (16, 17). Mice were observed for overall survival as the primary outcome measure. Tumor measurements were recorded twice weekly. Mice were sacrificed if tumor volume reached 1.5 cm<sup>3</sup> using the formula (width)<sup>2</sup>  $\times$  height/2. Animal care and euthanasia were carried out according to the rules and regulations as set forth by the Institutional Animal Care and Use Committee (IACUC).

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, Inc.). The differences in ADCC levels

by 4-hour chromium release assays as well as the inhibition of proliferation in the USC cell lines after exposure to SYD985 were evaluated by the two-tailed unpaired Student *t* test. Overall survival data were analyzed and plotted using the Kaplan–Meier method. Survival curves were compared using the log-rank test. Differences in all comparisons were considered statistically significant at *P* values < 0.05.

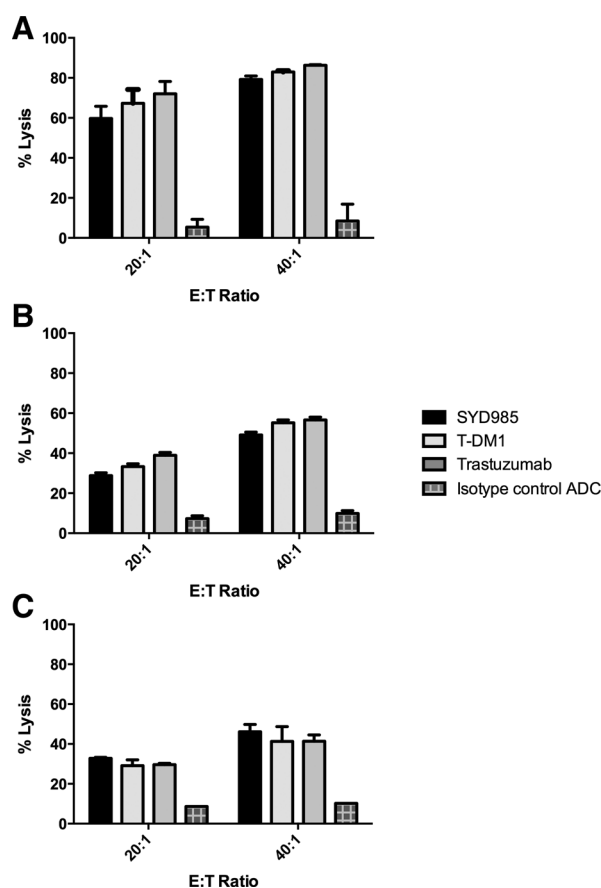
## Results

### HER2/neu and Cathepsin B expression and PIK3CA mutations in primary USC cell lines

We evaluated erbB-2 gene amplification by FISH, HER2/neu protein expression by IHC, and presence of "hot spots" PIK3CA mutations (i.e., exons 9 and 20) by Sanger sequencing in fifteen primary USC culture cell lines (Table 1). We also investigated Cathepsin B expression by qRT-PCR in nine primary USC cell lines. Gene amplification and high levels (3+ staining) of HER2 protein expression by IHC were detected in 33% of the USC cell lines (i.e., 5/15). Six cell lines had low (1+) or negligible (0/1+) HER2 expression on IHC, while the remaining four cell lines had 2+ expression (Table 1). On the basis of the HER2/neu results, we selected a total of 9 primary USC cell lines with similar growth rate and different HER2 expression for the additional *in vitro* and *in vivo* experiments described below. High levels of transcripts encoding for Cathepsin B were detected in all nine primary USC cell lines used for in the *in vitro* experiments with SYD985 (Supplementary Table S1).

### SYD985, T-DM1, and trastuzumab-mediated ADCC against HER2<sup>+</sup> primary USC

Six representative primary USC cell lines were tested for their sensitivity to PBL-mediated cytotoxicity when challenged with heterologous PBLs collected from several healthy donors in standard 4-hour <sup>51</sup>Cr release assays. USC cell lines were consistently found to be resistant to PBL-mediated cytotoxicity when combined with PBLs and isotype control ADC (2 μg/mL) at E:T ratios of 20:1 and 40:1 (mean ± SEM cytotoxicity of 7.425 ± 1.225% with PBL alone and mean ± SEM cytotoxicity of 8.18 ± 1.99% in the presence of isotype control ADC + PBL, respectively; Fig. 1). We then investigated the sensitivity of



**Figure 1.** A, ADCC results (mean ± SD) of SYD985, T-DM1, trastuzumab and ADC isotype control in a representative HER2 3+ expressing cell line (ARK-2). B, a representative 2+ HER2-expressing cell line (ARK-7). C, a representative 1+ HER2-expressing cell line (ARK-8). No significant differences in ADCC between SYD985, T-DM1, or trastuzumab were detected in USC cell lines with different HER2 expression.

**Table 1.** Patient characteristics, PIK3CA, and HER2/Neu expression in tumors

Patient	Age (years)	Race	FIGO <sup>a</sup> stage	USC histology	FISH	IHC cell block	PIK3CA mutations Exon 9, 20	Establishment of cell lines (year)
<b>USC ARK-1<sup>b</sup></b>	62	AA	IVA	Pure	Amplified	3+	542/1,068	1997
<b>USC ARK-2</b>	63	AA	IVB	Pure	Amplified	3+	Not detected	1998
<b>USC ARK-4</b>	73	C	IVB	Pure	Not amplified	0/1+	Not detected	2004
USC ARK-6	62	C	IB	Mixed	Not amplified	1+	Not detected	2005
<b>USC ARK-7</b>	75	C	IIC	Pure	Not amplified	2+	Not detected	2009
<b>USC ARK-8</b>	88	C	IIIA	Pure	Not amplified	1+	Not detected	2009
USC ARK-9	73	AA	IIIC	Mixed	Amplified	3+	1,044/1,068	2009
<b>USC ARK-11</b>	80	AA	IIIC	Mixed	Not amplified	1+	Not detected	2010
USC ARK-12	64	AA	IVB	Pure	Not amplified	0/1+	Not detected	2010
USC ARK-13	67	C	IVB	Pure	Not amplified	2+	Not detected	2011
USC ARK-14	73	AA	IV	Pure	Not amplified	2+	542/1,068	2011
<b>USC ARK-19</b>	65	C	IA	Pure	Not amplified	2+	Not detected	2012
<b>USC ARK-20</b>	42	C	II	Mixed	Amplified	3+	1,047/1,068	1999
USC ARK-21	70	C	IA	Pure	Not amplified	3+	Not detected	2012
<b>USC ARK-24</b>	54	AA	IIIC	Pure	Not amplified	1+	1,025/1,068	2013

Abbreviations: AA, African-American; C, Caucasian.

<sup>a</sup>FIGO, International Federation of Gynecology and Obstetrics stage 1988.

<sup>b</sup>Primary cell lines used in *in vitro* validation experiments are in listed in bold.

USC cell lines to heterologous PBLs in the presence of trastuzumab, SYD985, and T-DM1 at 2  $\mu\text{g}/\text{mL}$  (Fig. 1). SYD985, T-DM1, and trastuzumab (T) were similarly effective in inducing strong ADCC against primary USC cell lines expressing HER2/neu at high levels (i.e., USC ARK-1, USC ARK-2) with mean cytotoxicity  $\pm$  SEM =  $65.5\% \pm 11.97\%$  for SYD985 vs.  $72.9\% \pm 10.55\%$  for T-DM1 vs.  $76.26\% \pm 10.35\%$  for trastuzumab,  $P = 0.1220$ . Similarly, no significant differences between SYD985, T-DM1, or trastuzumab in the induction of ADCC were detected against USC cell lines with moderate (2+) or low (1+) HER2 expression ( $P = 0.0996$  and  $P = 0.4015$ , respectively, Fig. 1).

#### Cytotoxicity of SYD985 versus T-DM1 *in vitro*

Next, we exposed nine cell lines with different HER2/neu expression (Table 1) to scalar concentrations of ADC for a total of 3 days. As representatively demonstrated in Fig. 2A and Supplementary Fig. S1, SYD985 was more potent in inducing cell death than T-DM1 in all USC cell lines tested regardless of the level of HER2/neu expression (Fig. 2). In the HER2/neu 3+ cell lines, SYD985 exhibited a mean  $\text{IC}_{50}$  of 0.016  $\mu\text{g}/\text{mL}$  while T-DM1 exhibited a mean  $\text{IC}_{50}$  = 0.183  $\mu\text{g}/\text{mL}$  ( $P < 0.0001$ ). In the HER2/neu 2+ cell lines, SYD985 exhibited a mean  $\text{IC}_{50}$  of 0.040  $\mu\text{g}/\text{mL}$  while T-DM1 exhibited a mean  $\text{IC}_{50}$  = 1.374  $\mu\text{g}/\text{mL}$  ( $P < 0.0001$ ). Finally, in HER2/neu 1+ cell lines, SYD985 exhibited a mean  $\text{IC}_{50}$  = 0.058  $\mu\text{g}/\text{mL}$  while T-DM1 a mean  $\text{IC}_{50}$  = 4.391  $\mu\text{g}/\text{mL}$  ( $P < 0.0001$ ). Similar results were obtained exposing representative USC cell lines to a dose of 2.0  $\mu\text{g}/\text{mL}$  (i.e., the dose used in the 4-hour  $^{51}\text{Cr}$  release ADCC assays presented above) for 3 and 6 days, respectively (Fig. 2B) or after the exposure of all nine cell lines to scalar concentrations of ADC for a total of 6 days (Supplementary Fig. S2). In the HER2/neu 3+ cell lines, both SYD985 and T-DM1 induced significant cell death leaving less than 10% of the cells viable. In the HER2/neu 2+ and 1+ cell lines, SYD985 consistently performed better than T-DM1 in inducing cell death (Fig. 2B). Isotype control ADC (based on rituximab) was the least potent in inducing cell death in HER2/neu 3+ cell lines, while in HER2/neu 2+ and 1+ cell lines performed better than T-DM1. No significant differences in SYD985-induced cytotoxicity were noted when cell lines harboring PIK3CA driver mutations (i.e., ARK-1 and ARK-20) were compared to USC cell lines harboring wild-type PIK3CA genes (i.e., ARK2; data not shown).

#### Bystander killing *in vitro*

Next, we evaluated the ability of SYD985 and T-DM1 to induce bystander cytotoxicity of USC cells with low/negligible HER2 expression (i.e., GFP-ARK-4 cells) when admixed with ARK-2 (HER2/neu 3+) cells for 96 hours. As shown in the Fig. 3A for SYD985, while no increase in ARK-2 killing was noted when ARK-2 cells were cocultured with ARK-4 ( $P = 0.9570$ ), ARK-2/ARK-4 cocultures yielded a significant increase (i.e., 42%,  $P = 0.0011$ ) in the amount of bystander killing of HER2 low/negligible ARK-4 cells when compared with controls. In contrast, minimal bystander cytotoxicity was detected when ARK-2/ARK-4 cocultures were challenged with T-DM1 (i.e., 13%,  $P = 0.29$ ) or isotype control ADC for 96 hours (Fig. 3B and C, respectively).

#### *In vivo* antitumor activity of SYD985 versus T-DM1

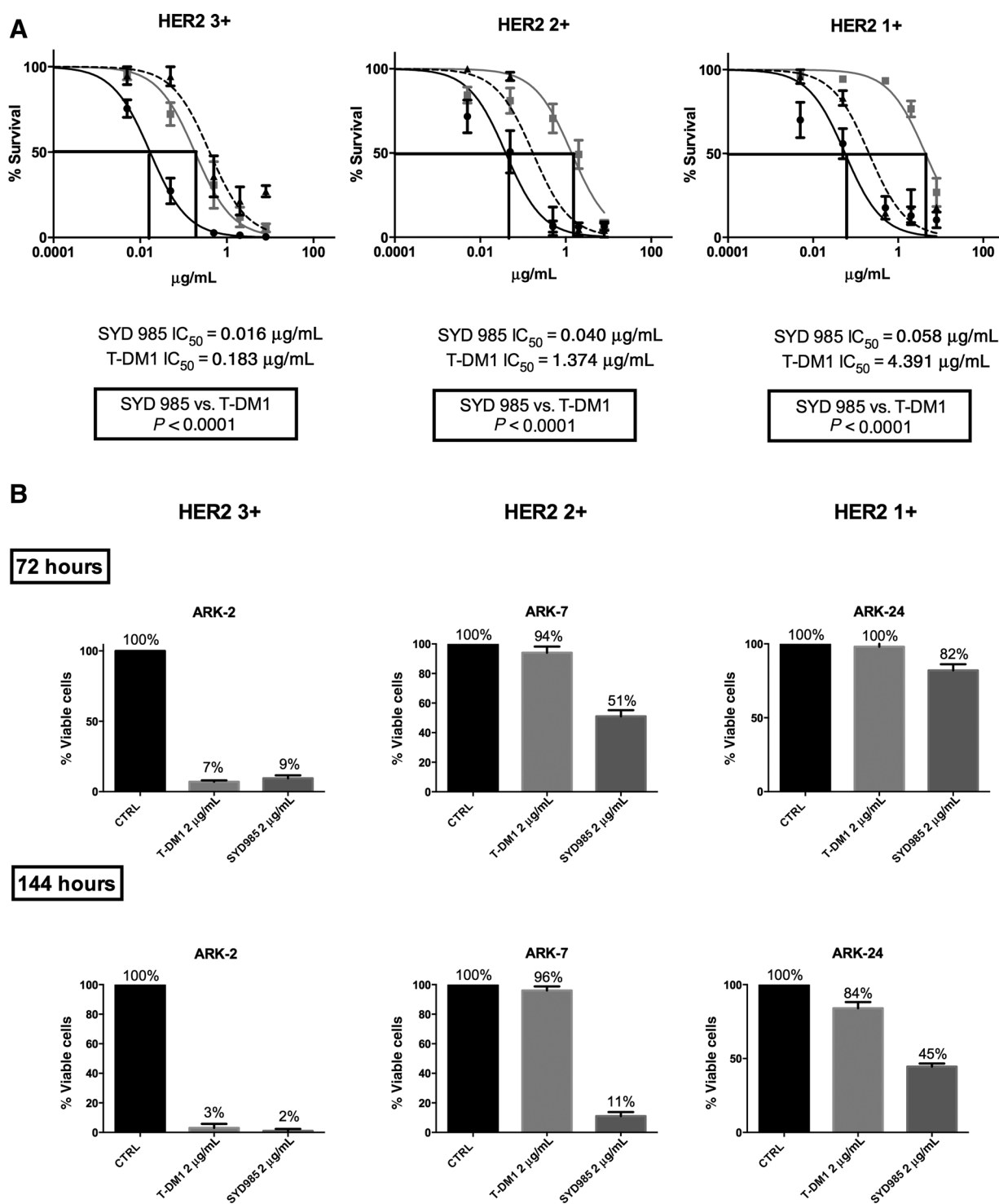
The *in vivo* effects of SYD985 and T-DM1 were determined by establishing multiple xenografts of primary USC cell lines with different HER2/neu expression [i.e., ARK-2 (HER2/neu 3+), ARK-

7 (HER2/neu 2+), and ARK-11 (HER2/neu 1+)]. After the tumors had reached the goal volume (i.e., 0.2  $\text{cm}^3$ ), animals were randomized into treatment groups and treated as described in the Materials and Methods section. Tumors were assessed twice weekly and mice were sacrificed if tumors became necrotic, reached a volume of 1.5  $\text{cm}^3$ , or mice appeared to be in poor health. Treatment with a single injection of SYD985 inhibited tumor growth across all levels of HER2/neu expression (Fig. 4). In ARK-2 (HER2/neu 3+) xenografts, we detected a significant difference in growth inhibition between animals treated with SYD985 3 mg/kg ( $P = 0.045$  at 17 days) and 10 mg/kg ( $P = 0.0007$  at 9 days) when compared with T-DM1 at 10 mg/kg. In ARK-7 (HER2/neu 2+) xenografts, a significant difference in growth inhibition between animals treated with SYD985 3 mg/kg ( $P = 0.004$  at 11 days) and 10 mg/kg ( $P < 0.0001$  at 7 days) when compared with T-DM1 10 mg/kg was also noted (Fig. 4). Finally, in ARK-11 (HER2/neu 1+) xenografts, a significant difference in growth inhibition between animals treated with SYD985 3 mg/kg ( $P = 0.001$  at 7 days) and 10 mg/kg ( $P = 0.0006$  at 7 days) after single injection when compared with T-DM1 10 mg/kg was demonstrated (Fig. 4).

A significant survival advantage was also seen across all levels of HER2/neu amplification in SYD985 treated xenografts (Fig. 5). Specifically, in the ARK-2 (HER2/neu 3+) xenograft model, when comparing SYD985 3 mg/kg single injection to T-DM1 10 mg/kg single injection, there was a significant difference in mean overall survival of 42 days versus 24 days, respectively ( $P = 0.0045$ ). A similar survival benefit was seen in SYD985 10 mg/kg single injection when compared with T-DM1 10 mg/kg single injection ( $P < 0.0001$ ). Notably, 2 of 5 (40%) of the mice were alive and disease free at 180 days after a single injection of SYD985 at a dose of 10 mg/kg. In the ARK-7 (HER2/neu 2+) xenograft model, when comparing SYD985 3 mg/kg single injection to T-DM1 10 mg/kg single injection, there was a significant difference in overall survival of 25 days versus 14 days, respectively ( $P = 0.008$ ). This same survival benefit was seen in SYD985 10 mg/kg single injection when compared with T-DM1 10 mg/kg single injection, with an overall survival of 39 days versus 14 days, respectively ( $P = 0.023$ ). Finally, in the ARK-11 (HER2/neu 1+) xenograft model, when comparing SYD985 3 mg/kg single injection to T-DM1 10 mg/kg single injection, there was a significant difference in overall survival of 28 days versus 11 days, respectively ( $P = 0.0018$ ). This same survival benefit was seen in SYD985 10 mg/kg single injection when compared with T-DM1 10 mg/kg single injection, with an overall survival of 42 days versus 11 days, respectively ( $P = 0.0018$ ). In agreement with our *in vitro* results, isotype control ADC rituximab provided a superior survival benefit when compared with T-DM1 in the HER2/neu 2+ and 1+ xenograft models. Survival benefit in mice treated with isotype control ADC was however consistently inferior to SYD985 across all xenograft models with different levels of HER2/neu expression (Fig. 5), indicating that SYD985-induced antitumor activity in these models is at least partly mediated through HER2.

## Discussion

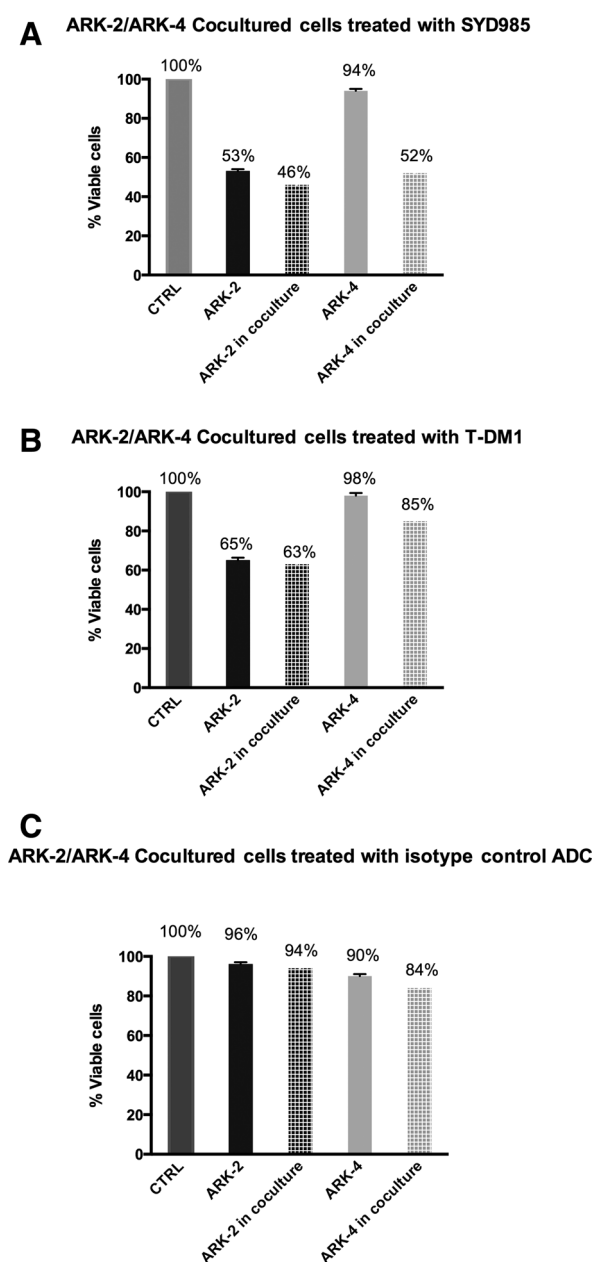
Our group has recently used next-generation sequencing (NGS) to analyze the genetic landscape of a large number of USC (19). In this comprehensive report, we found somatic copy number



**Figure 2.** A,  $IC_{50}$  dose-response curves of SYD985, T-DM1, and ADC isotype control in all USC cell lines tested *in vitro* (i.e., HER2 3+ cell lines,  $P = <0.0001$ ; HER2 2+ cell lines,  $P = <0.0001$ ; and HER2 1+ cell lines,  $P = <0.0001$ ) at 3 days. B, cytotoxicity in representative HER2 3+, 2+, and 1+ USC cell lines after 3 and 6 days exposure to 2.0  $\mu\text{g/mL}$  of ADC SYD985 versus T-DM1.

variations (CNV) to play a major role in the pathogenesis of USC (19). Consistent with this view, CNV analyses identified amplification of *HER2* gene in 44% of the whole exome-sequenced

USC tested with an additional 16% and 52% of the USC samples, respectively, demonstrating mutations or CNV gain in the *PI3K* gene (19). *PI3K* gene is located downstream to *HER2/neu* in the



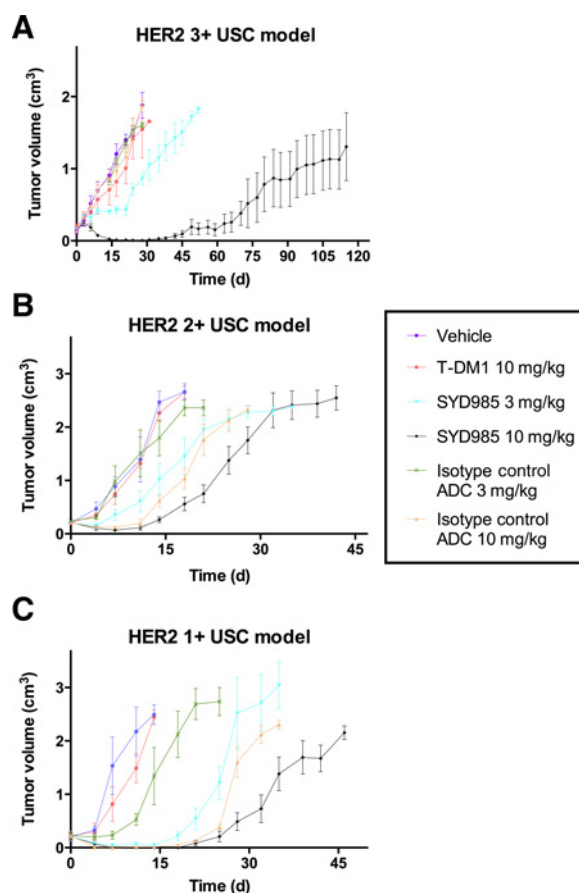
**Figure 3.**

**A**, cytotoxicity induced on HER2 3+ USC (ARK-2), HER2 0/1+ USC (ARK-4), and ARK-2/ARK-4 cocultures with 1  $\mu$ g/mL of SYD985, T-DM1, and ADC isotype control. A significant increase in the amount of killing of HER2 0/1+ USC cells was detected when compared with control ( $P = 0.001$ ) after treatment with SYD985.

**B**, cytotoxicity induced on HER2 3+ USC (ARK-2), HER2 0/1+ USC (ARK-4), and ARK-2/ARK-4 cocultures with 1  $\mu$ g/mL of SYD985 (T-DM1). No significant increase in the amount of killing of HER2 0/1+ USC cells was detected when compared with control ( $P = 0.29$ ) after treatment with T-DM1. **C**, cytotoxicity induced on HER2 3+ USC (ARK-2), HER2 0/1+ USC (ARK-4), and ARK-2/ARK-4 cocultures with 1  $\mu$ g/mL of SYD985 (isotype control ADC). No significant increase in the amount of killing of HER2 0/1+ USC cells was detected when compared with control ( $P = 0.51$ ) after treatment with isotype control ADC.

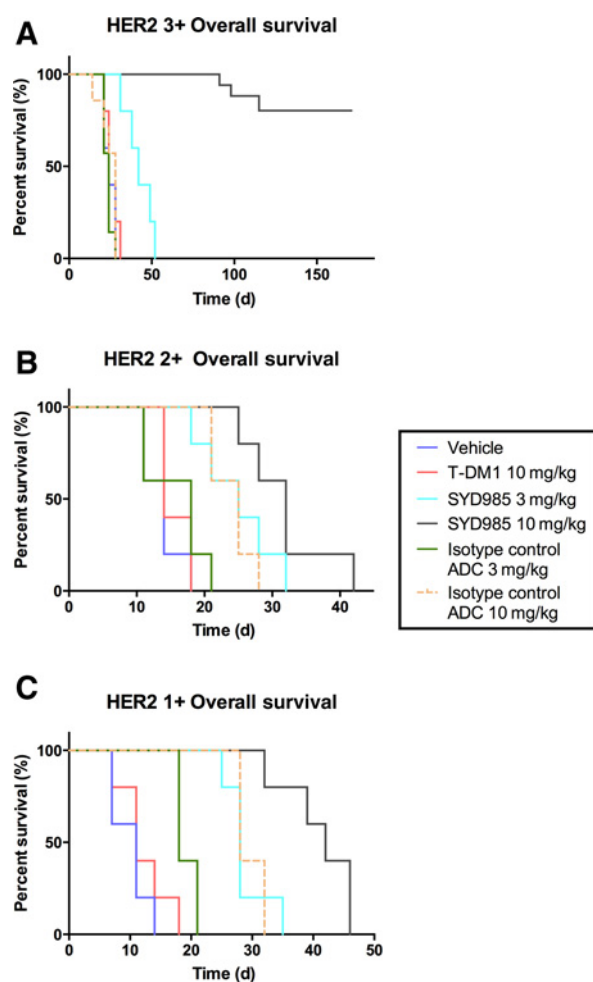
HER2/PIK3CA/AKT/mTOR pathway and is known to play a major role in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and impor-

tantly, resistance to chemotherapy (6–9, 21, 22). Along these lines, we recently hypothesized that the high prevalence of PI3K mutations in HER2/neu amplified USC may constitute a major mechanism of resistance to trastuzumab treatment (23). Accordingly, we recently demonstrated that: (i) about two-third of the USC cell lines with HER2/neu-amplified by FISH harbor oncogenic PIK3CA-mutations, (ii) HER2/neu-amplified/PIK3CA-mutated USC are highly resistant to trastuzumab when compared with HER2/neu-amplified/wild-type-PIK3CA cell lines, and (iii) HER2/neu-amplified/PIK3CA-wild-type cell lines transfected with oncogenic PIK3CA-mutations significantly increase their resistance to trastuzumab (23). Taken together, these data suggested that PIK3CA mutations may represent a biomarker to identify patients unlikely to respond to single-agent trastuzumab-based therapy (23, 24). More importantly, these results strongly support the view that novel HER2/neu-targeted agents effective in the treatment of PI3K-mutated/trastuzumab-resistant USC (i.e., T-DM1 and/or afatinib; refs. 25–27) or SYD985 (this article), might represent more effective treatment strategies against this subset of biologically aggressive endometrial cancers.



**Figure 4.**

Antitumor activity of SYD985 compared with T-DM1 and ADC isotype control in USC xenograft tumor models including ARK-2 (HER2/neu 3+; **A**), ARK-7 (HER2/neu 2+; **B**), and ARK-11 (HER2/neu 1+; **C**). Mice were treated with a single dose administered intravenously as described in Materials and Methods. A significant difference in growth inhibition was detected in SYD985-treated groups at the dose of 3 mg/kg and 10 mg/kg when compared with the other treatment groups.



**Figure 5.** Overall survival in mice inoculated with USC xenografts including ARK-2 (HER2/neu 3+; **A**), ARK-7 (HER2/neu 2+; **B**), and ARK-11 (HER2/neu 1+; **C**) after treatment with vehicle, single injection SYD985 (3 mg/kg and 10 mg/kg), and single injection T-DM1 (10 mg/kg) and ADC isotype control. Significantly prolonged overall survival across SYD985-treated groups was detected when compared with the other treatment groups.

The population of cancer patients who may benefit from HER2/neu-targeted therapy could be dramatically increased by the use of more effective HER2/neu targeting ADCs which can trigger killing of target cells with moderate and low HER2/neu expression as well as of neighboring non-HER2/neu positive bystander tumor cells growing in their proximity. Consistent with this view, in this study, we evaluated for the first time the preclinical activity of SYD985, a novel HER2-targeting ADC composed of trastuzumab linked to the highly potent DNA-alkylating agent *seco*-DUBA with a cleavable linker against multiple USC primary cell lines with different HER2/neu expression (i.e., 1+, 2+, and 3+). Our experiments demonstrate that SYD985 when compared with T-DM1 is endowed with consistently stronger cytotoxicity against HER2/neu-positive USC cell lines both *in vitro* and *in vivo* in SCID mice harboring USC xenografts with different HER2/neu expression. In this regard, while SYD985 and T-DM1 evoked similar levels of ADCC against HER2/neu-expressing USC cell lines in the

presence of effector PBLs, SYD985 was significantly more cytotoxic against primary USC cell lines with HER2/neu expression of 1+, 2+, and 3+ in absence of PBLs. Specifically, in HER2/neu 3+ cell lines, we found SYD985 to be more than 10-fold more potent than T-DM1 ( $P = 0.0468$ ) in inducing cell death. In HER2/neu 2+ cell lines, SYD985 was 34 folds more potent than T-DM1 ( $P = 0.0498$ ), while in HER2/neu 1+ cell lines, SYD985 was 75 folds more potent than T-DM1. Importantly, similar to our recently reported studies with T-DM1 in HER2/neu 3+ USC models (5, 7), we found that the cytotoxic activity of SYD985 was not affected by the presence of PI3K mutations or resistance to trastuzumab treatment as demonstrated by the high sensitivity of both PIK3CA-mutated (USC ARK-1) and PIK3CA-wild type (USC ARK-2) cell lines to the cytotoxic activity of SYD985.

Despite the over-expression of HER2 in a significant subset of USC (10, 11) and the publication of encouraging case reports using trastuzumab in combination with chemotherapy on a limited number of patients with recurrent disease (28–30), single-agent trastuzumab 4 mg/kg in week 1 then 2 mg/kg weekly until disease progression in stage III/IV or recurrent endometrial cancers failed to demonstrate significant clinical activity at the phase II level (GOG-181B) (24). While the negative results of the Gynecologic Oncology Group study have been challenged due to the many shortcomings in the design of the GOG181B trial (31), these negative clinical findings do suggest that a significant number of endometrial cancer patients may potentially harbor disease endowed with primary resistance to trastuzumab secondary to PIK3CA mutations and/or caused by a highly heterogeneous HER2/neu expression (10, 11). Importantly, our preclinical data with SYD985 clearly demonstrate that PI3K-mutated/T-resistant USC primary cell lines may be highly sensitive to this novel ADC and that SYD985 may be significantly more effective than T-DM1 in inducing bystander killing of low/negative HER2/neu-expressing USC cells admixed with HER2/neu-positive tumors. In this regard, proteases like cathepsin B are expressed in a wide variety of human tumors including USC and are released by malignant cells (32). Cleavage of duocarmycine from its linker may therefore take place in USC not only within HER2/neu overexpressing tumor cells after antibody internalization (16, 17) but also extracellularly within the tumor microenvironment, inducing a potent bystander effect. *In vivo* data in multiple animal models harboring USC xenografts with differential HER2/neu expression were confirmatory of the *in vitro* results demonstrating high efficacy of SYD985 against USC 3+, 2+, and 1+. Importantly, previous pharmacokinetic results demonstrated a poor SYD985 stability in mouse plasma secondary to the presence of a mouse-specific carboxylesterase, CES1c, which is not expressed in human or cynomolgus monkey (16–18). Thus, efficacy studies with SYD985 *in vivo* in mice most likely lead to an underestimation of antitumor activity in species that will have higher exposure to SYD985, such as humans. A possible limitation in the use of SYD985 in the clinical setting is its potential toxicity. It is therefore worth noting that no evidence of *in vivo* acute or chronic toxicity was detected in animals treated with SYD985 with doses up to 15 mg/kg/weekly.

Taken together, these results strongly suggest that SYD985 may represent a significantly more effective therapeutic tool when compared with T-DM1 or trastuzumab in HER2/neu-expressing USC. Moreover, SYD985 may be effective against USC with low to moderate HER2/neu expression and may significantly increase the number of USC patients potentially benefitting from this



targeted therapeutic approach. Consistent with this view, encouraging clinical results have recently been reported in breast cancer with the administration of SYD985 in phase I clinical trials (data not shown).

In conclusion, we have demonstrated that SYD985 is a novel ADC with activity against USC with strong (3+) as well as low to moderate (i.e., 1+/2+) HER2/neu expression. SYD985 is significantly more potent than T-DM1 in comparative experiments and unlike T-DM1, it may be active against USC demonstrating heterogeneous HER2/neu expression. Clinical studies with SYD985 in USC patients harboring disease resistant to standard salvage chemotherapy are warranted.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J. Black, G. Menderes, F. Ferrari, F. Predolini, C. De Haydu, N. Buza, P. Hui, S. Lopez, M. Azodi, A.D. Santin

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