Preclinical profile of the HER2-targeting ADC SYD983/SYD985; introduction of a new duocarmycin-based linker-drug platform.

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Running title: Preclinical profile of SYD983/SYD985

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ABSTRACT

A linker-drug platform was built based on a cleavable linker-duocarmycin payload for the development of new generation antibody-drug conjugates (ADCs). A leading ADC originating from that platform is SYD983, a HER2-targeting ADC based on trastuzumab. HER2 binding, antibody-dependent cell-mediated cytotoxicity and HER2-mediated internalization is similar for SYD983 as compared to trastuzumab. HER2-expressing cells in vitro are very potently killed by SYD983, but SYD983 is inactive in cells that don’t express HER2. SYD983 dose-dependently reduces tumor-growth in a BT-474 mouse xenograft in vivo. The ADC is stable in human and cynomolgus monkey plasma in vitro but shows relatively poor stability in mouse plasma due to mouse-specific carboxylesterase. SYD983 could be dosed up to 30 mg/kg in cynomolgus monkeys with high exposure, excellent stability in blood and without severe toxic effects. The monkey safety study showed no SYD983-induced thrombocytopenia and no induction of peripheral sensory neuropathy, both commonly observed in trials and studies with ADCs based on tubulin inhibitors. Finally, to improve homogeneity, SYD983 was further purified by hydrophobic interaction chromatography resulting in an ADC (designated SYD985) predominantly containing DAR2 and DAR4 species. SYD985 showed high anti-tumor activity in two patient-derived xenograft models of HER2-positive metastatic breast cancers. In conclusion, the data obtained indicates great potential for this new HER2-targeting ADC to become an effective drug for patients with HER2-positive cancers with a favorable safety profile. More generally, this new generation duocarmycin-based linker drug technology could be used with other mAbs to serve more indications in oncology.
INTRODUCTION

Antibody-drug conjugates (ADCs) are monoclonal antibodies (mAbs) that are chemically linked to cytotoxic agents. Although the concept exists for decades, only recent advances in linker, drug, and antibody technology has turned ADCs into valuable therapeutic agents as illustrated by the recent approvals of brentuximab-vedotin (Adcetris®) and ado-trastuzumab emtansine (T-DM1, marketed as Kadcyla®). In addition to these approved ADCs, over 30 other ADCs are currently in clinical trials of which the majority are based on linker-drugs (LDs) that are similar to those used in brentuximab-vedotin and T-DM1 (1, 2). Like many ADCs in development, both brentuximab-vedotin and T-DM1 carry tubulin-binders (an auristatin and a maytansinoid, respectively) that inhibit tubulin polymerization and cause cell cycle arrest and apoptosis of the targeted cell. Linkers are either cleavable linkers, for example peptide linkers (such as the di-peptide valine-citrulline used in brentuximab-vedotin) or non-cleavable linkers, for example amide or thioether linkers (as used in T-DM1) that depend on complete degradation of the mAb in the lysosome and subsequent release of the cytotoxic agent.

Although the marketed ADCs have improved TIs compared to classical non-targeted chemotherapeutic agents, there is still room for improvement. Brentuximab-vedotin for example reaches its maximum tolerated dose (MTD) in human patients at 2.4 mg/kg every 3 weeks, which is determined by induction of peripheral sensory neuropathy, neutropenia, and thrombocytopenia (3,4,5). Similarly, the MTD of T-DM1 is 3.6 mg/kg every 3 weeks, which is also driven by unacceptable thrombocytopenia and hepato
toxicity at higher dosages, and is accompanied by peripheral sensory neuropathy as well (6,7,8,9).

A new generation platform of LDs has been developed based on chemically synthesized duocarmycins which are DNA-alkylating cytotoxic drugs (10,11) that induce cell death in both dividing and non-dividing cells. The aim is to improve the currently used LDs and increase the TI that can be obtained with ADCs based on these new LDs as compared to that of the earlier generation ADCs. To assess the value of this new LD technology, ADCs were prepared based on the mAb trastuzumab to target HER2 positive tumors. Trastuzumab was chosen for two main reasons. First, the biology of trastuzumab and HER2 is well known (12) and HER2 is a clinically validated target. Second, the safety profile of T-DM1 clearly indicates there is room for a HER2-targeting ADC with an improved TI (6,7,8,9).

A set of trastuzumab-based ADCs were prepared by chemical linkage of different LDs to the thiol groups of cysteines generated by random reduction of interchain disulfides on the mAb, using thiol-maleimide chemistry. The profile of lead candidate SYD983 is presented in this paper. We showed that SYD983 combines a high anti-tumor activity with an impressive safety profile indicating its superior preclinical TI compared to existing ADCs.

MATERIALS AND METHODS
Antibodies and ADCs.

Vc-seco-DUBA 1, seco-DUBA 2, and SYD983 were prepared essentially as described previously (13,14) and further detailed in the Supplement. Trastuzumab was expressed using Chinese Hamster Ovary (CHO) cells and purified to homogeneity. IgG1 control antibody infliximab was from MSD, Haarlem, The Netherlands. Non-binding control ADC was vc-seco-DUBA 1 coupled to rituximab (Roche, Basel, Switzerland) using a similar method as for conjugating trastuzumab, resulting in a comparable DAR (see Supplementary Materials and Methods).

Cell lines and reagents.

Human tumor cell lines BT-474, SK-BR-3, SK-OV-3, NCI-H520 and SW-620 were obtained from and characterized by the American Type Culture Collection (Rockville, MD). No further cell-line authentication was conducted. SW-620 and NCI-H520 cells were cultured in RPMI-1640 media (Lonza; Walkersville, MD, USA) supplemented with 10% v/w fetal bovine serum (FBS), Heat-inactivated (HI) (Gibco-Life Technologies; Carlsbad, CA, USA) at 37°C in a humidified incubator containing 5% CO2. SK-BR-3 and SK-OV-3 were maintained in McCoys-5A medium (Lonza) containing 10% v/w FBS HI, and BT-474 cells in RPMI-1640 containing 2mM L-glutamine (Lonza) supplemented with 10% FBS (Lonza). Quantification of cell surface HER2 expression was done using the DAKO Qifikit (DAKO, Glostrup, Denmark), according to the manufacturer’s protocol.

In vitro characterization of SYD983.
Cells in complete growth medium were plated in 96-well plates (90 µl/well) and incubated at 37°C, 5% CO2 at the following cell densities: 6,500 SK-BR-3, 10,000 BT-474, 2,000 SK-OV-3, 4,000 SW-620 and 5,000 NCI-H520 cells per well. After an overnight incubation, 10 µl of mAb, seco-DUBA 2 and/or ADC were added. Cell viability in serial dilutions was assessed after 144 hours (or indicated otherwise) using the CellTiter-Glo™ (CTG) luminescent assay kit from Promega Corporation (Madison, WI, USA) according to the manufacturer's instructions. Metabolic activity of BT-474 cells was measured with an ATP-Lite assay (Perkin-Elmer, Waltham, MA, USA). Cytotoxicity was measured after 96 hours by detection of a protease, which was released by membrane-compromised cells (Cytotox-Glo™ cytotoxicity assay, Promega). Internalization studies are detailed in the Supplement. Antibody-dependent cellular cytotoxicity (ADCC) assays were conducted essentially as described (15) and detailed in the Supplementary Materials and Methods.

**Cell line and patient-derived xenograft studies.**

Studies were approved by the local animal care and use committees according to established guidelines.

The BT474 model was performed at Oncodesign (Dijon, France). Tumors were induced subcutaneously by injecting 2x10^7 BT-474 cells in 200 µl of RPMI-1640 containing matrigel (50:50, v:v, BD Biosciences, France) into the right flank of female Balb/c nu/nu mice. BT-474 tumor cell implantation was performed 48 hours after a whole body irradiation with a γ-source (2 Gy, ^60^Co, BioMep, Dijon, France). Mice were randomized into experimental groups when tumors reached a size between 350-400 mm^3, and
dosing initiated. The mice were dosed i.v. (5-10 ml/kg) in the tail vein with the respective ADCs in vehicle (80 mg/ml trehalose, 10 mM histidine, 0.01% polysorbate-20, pH 6) or with vehicle alone.

Two metastatic HER2 IHC 3+ positive breast cancer patient-derived xenograft (PDX) studies were conducted at Oncotest (Freiburg, Germany). MAXF1322 mammary tumor was a metastasis from a brain tumor of a 49-year old female patient, MAXF1162 mammary tumor was a primary tumor in the breast of a 55-year old female patient. HER2 IHC was performed by using formalin-fixed paraffin-embedded tumor xenograft samples and a polyclonal rabbit anti-human HER2 (DAKO Cat# A0485) antibody and detected by a biotinylated goat anti-rabbit IgG (Jackson Immuno research, Cat# 111-065-04) and a Biozol (Cat # VEC-PK-4000) ABC kit. Staining was evaluated semi quantitatively, using a Zeiss Axiovert 35 microscope. Staining was interpreted as immunoreactivity, based on the proportion of positively-stained cells and on the signal intensity, according to the established guidelines. A known HER2 positive (IHC 3+) and HER2 negative (IHC 0) control tumor slide was included in every HER2 staining procedure (See Supplement Fig S3) to control for specificity.

PDX experiments were conducted as described (16). Mice (n = 8-10 per group) were dosed once, i.v. (5-10 ml/kg) in the tail vein with ADCs in vehicle (described above) or with vehicle alone.

For all xenografts, tumors were measured twice a week with calipers and their size (mm$^3$) was calculated as $0.5 \times$ (tumor length) $\times$ (tumor width)$^2$. Individual animals were sacrificed when tumor sizes were 2000 mm$^3$ or at the designated endpoints of the study.
In vitro plasma stability, in vivo pharmacokinetics, and PK ELISAs were done as detailed in the Supplementary Materials and Methods.

Safety.

Groups of 3 naïve female cynomolgus monkeys (macaca fascicularis) received an IV slow bolus injection of 0, 1, 3, 10 or 30 mg/kg SYD983 and were followed for up to 7 weeks until sacrifice. A second dose of 10 or 30 mg/kg was given on day 25 or day 24 in the respective groups. Clinical signs, body weight and clinical pathology were monitored prior to dosing and throughout the study. Upon terminal sacrifice macroscopic and microscopic evaluation of selected tissues was performed.

RESULTS

Design and preparation of SYD983

Duocarmycins and CC-1065 are natural products that were first isolated from Streptomyces bacteria in the late '70s (10). This class of compounds binds to the minor groove in DNA and subsequently alkylates specific adenine residues via ring opening of their cyclopropyl group, which eventually leads to cell death (11). Several synthetic duocarmycin analogs have been taken into development, but they all failed in the clinic or before as a consequence of a limited TI (17,18,19).
We developed a novel class of synthetic duocarmycin derivatives that contain an additional heteroatom in the DNA binding moiety compared to the natural analogs. These duocarmycins were combined with newly designed cleavable linkers in such a way that linker-drugs were obtained in which the duocarmycin drug is inactivated as long as it is bound to the linker. Only after cleavage of the linker, the duocarmycin moiety regains its alkylating properties through a Winstein spirocyclization reaction (Fig1).

Linker-duocarmycins were coupled to trastuzumab after partial reduction of the interchain disulfides to generate on average two free thiol groups per mAb leading to a statistical distribution of HER2-targeting ADCs with an average drug-to-antibody-ratio (DAR) of about two, and low amounts of high molecular weight (HMW) species and residual unconjugated linker-drug.

Evaluation of a large set of ADCs based on different LDs in multiple assays led to selection of SYD983 as our lead candidate for the anti-HER2 program. SYD983 contains a valine-citruline duocarmycine-benzamido-azaindole linker, designated vc-seco-DUBA (Fig1).

**In vitro profile of SYD983.**

Internalization of Alexa488-labeled SYD983 into HER2 positive breast SK-BR-3 cells was compared to that of Alexa488-labeled trastuzumab. Internalization of SYD983 at 37°C is time dependent reaching maximal levels of approximately 60% at 8 hrs, similar to that of the naked Ab (Fig2A). As a control, SYD983 was not significantly internalized.
at 4°C (Fig2A). Binding affinity (K_{\text{D,observed}}) for SYD983 and trastuzumab was 1.1 \times 10^{-9}M and 1.2 \times 10^{-9}M, respectively, indicating that for SYD983 conjugation did not affect HER2 binding affinity. Also the ability of SYD983 to induce antibody-dependent cell-mediated cytotoxicity (ADCC), was not affected by conjugation (Fig2B). It is concluded that conjugation of vc-seco-DUBA 1 to trastuzumab does not alter important characteristics of the mAb, like has been described for T-DM1 (20).

Next some aspects of SYD983 were studied that relate to the duocarmycin payload. A set of 5 human tumor cell lines were selected based on their published HER2-status; breast carcinomas SK-BR-3 and BT-474 (both HER2 3+), ovarian carcinoma SK-OV3 (HER2 2+), and metastatic colon carcinoma SW-620 and lung adenocarcinoma NCI-H520 (both HER2 negative). The HER2 status of these cell lines was confirmed by quantifying HER2 levels using Qifi-kit, indicating 815,000 (SK-BR-3), 910,000 (BT-474), 600,000 (SK-OV3), 150 (NCI-H520), and 1100 (SW-620) HER2 molecules per cell which is in line with previous publications (21,22). First these cell lines were studied for their sensitivity to seco-DUBA 2 (Fig1), which is the active toxin released after intracellular processing of SYD983. All five cell lines were highly sensitive to seco-DUBA 2 with a potency between 0.8 and 4.3 \times 10^{-10}M in all cell lines tested (Fig2C). Incubation with a dose-range of SYD983 showed that potencies and efficacies for SYD983 were in line with HER2 expression; 1.42 \pm 0.06 \times 10^{-10}M (98% cell-killing), 8.07 \pm 0.06 \times 10^{-10}M (98% cell-killing), 7.50 \pm 0.02 \times 10^{-10}M (78% cell-killing) for SK-BR-3, BT-474, and SK-OV3 respectively. An enormous reduction in potency was observed in the cell lines that are HER2-negative (> 5 \times 10^{-8}M) for SW-620 and NCI-H520 (Fig2D). The resulting 1000-fold window of potencies for SYD983 in HER2 positive versus HER2-
negative cell lines indicates that induction of cytotoxicity in HER2-positive cell lines is mediated through HER2. To further study the mechanism of action, the number of viable SK-BR-3 cells was compared after incubation for 4 days with either SYD983, a non-binding control ADC with the same payload and average DAR, or trastuzumab. Trastuzumab lowered the number of viable cells but not to the level at the start of the experiment at day 0 (Fig2E) suggesting that trastuzumab induced partial inhibition of cell proliferation. In contrast, SYD983 reduced the number of viable cells below the initial number at day 0, suggesting induction of cytotoxicity. The non-binding ADC showed no effect on cell viability. Since only SYD983, and neither trastuzumab nor the non-binding control ADC, dose-dependently induced extracellular release of an intracellular protease (Fig2F), we conclude that SYD983, in contrast to trastuzumab, induces cytotoxicity, which is HER2-mediated.

In vivo anti-tumor activity.

The in vivo anti-tumor activity of SYD983 was evaluated in a BT-474 mouse xenograft model. Trastuzumab dosed once at 5 mg/kg showed moderate anti-tumor activity in this model (Fig3A). SYD983 dose dependently reduced tumor growth. At 5 mg/kg the tumor volume was significantly decreased from day 34 onwards (Log transformed data; 2 way ANOVA, multiple comparisons). At 1 mg/kg, SYD983 showed equal anti-tumor activity as 5 mg/kg trastuzumab. This improved anti-tumor activity of SYD983 is due to HER2-targeting of the duocarmycin to the tumor since the combination of 15 mg/kg trastuzumab and the free toxin Seco Drug 2 equimolar to what would be present in a 15
mg/kg SYD983 group, did not improve the anti-tumor activity compared to 15 mg/kg trastuzumab alone. Both treatments were statistically significant from day 33 onwards (Fig3B). Next, it was studied whether anti-tumor activity in the BT-474 xenograft model was dependent on peak levels of SYD983 (Cmax-driven) or on the Area Under the Curve (AUC-driven) of the total exposure to SYD983. To mimic a situation of different Cmax levels, but rather similar AUCs, mice carrying BT-474 tumors were treated either once with 5 mg/kg versus five times with 1 mg/kg SYD983 once every day. Both of these treatments resulted in similar anti-tumor activity, indicating that the AUC drives efficacy (Fig3C). As a control, 1 mg/kg SYD983 dosed once was indeed less effective. In a multiple dose experiment, it was confirmed that the anti-tumor activity of SYD983 in this BT-474 xenograft model was dependent on targeting of the toxin to the target, since a non-binding control ADC with the same payload as SYD983 showed no anti-tumor activity (Fig3D). These data show that SYD983 exhibits potent HER2-mediated anti-tumor activity in vivo.

**Plasma stability and kinetics.**

Vc-seco-DUBA was predicted to be sensitive to certain esterase activity that could hydrolyze the carbamate bond connecting the alkylating moiety of the duocarmycin to the self-elimination module (Fig1) and thereby liberating the toxin. Indeed, the stability of SYD983 in mouse plasma is poor (Fig4A and Supplement Table S1) and solely due to plasma expression of mouse specific carboxylesterase CES1c (23,24), since SYD983 is stable in plasma prepared from CES1c knockout mice (Fig4B). Thus, it
cannot be ruled that some anti-tumor activity of SYD983 in mice is induced by the early release of pay-load in the vicinity of the tumor by mouse-specific CES1c. Stability of SYD983 in plasma from cynomolgus monkeys and humans is good and in line with the absence of carboxylesterase activity in plasma from those species (23).

In vivo plasma kinetics was studied in healthy Balb/c mice and in tumor-bearing nu/nu mice. Total antibody (TAb) levels in healthy Balb/c mice are in line with previously reported stability for trastuzumab in mice (25), with a terminal half life of 309 hours and a clearance of 0.34 mL/h/kg at 1 mg/kg SYD983 (Fig4C and Supplement Table S2). This indicates that the conjugation has no clear effect on TAb PK. In tumor-bearing mice the clearance was 6 fold higher (2.13 mL/h/kg), in line with target-mediated drug disposition (Fig4D and Supplement Table S3). In contrast, ADC exposure rapidly declines resulting in much higher clearance values, being similar for healthy (14.3 mL/h/kg) and tumor-bearing mice (13.8 mL/h/kg) at 1 mg/kg. This rapid decline is in line with the plasma stability data and probably caused by CES1c esterase activity and much more pronounced than the target-mediated clearance in tumor-bearing mice.

In monkey, TAb PK showed slightly higher clearance (e.g. 0.58 ±0.03 mL/h/kg at 3 mg/kg) compared to previously reported data for trastuzumab (25,26,27), but also showed non-linear PK at the lower dosages (Fig4E). Distinct from the mouse data, ADC concentrations hardly decline resulting in clearance values (e.g. 0.64 ±0.03 mL/h/kg) that are only slightly higher than TAb clearance values (Supplement Tables S4 and S5).

**Safety and therapeutic index.**
The tissue-specific expression of HER2 in cynomolgus monkey closely resembles that of humans (28), and trastuzumab does bind to cynomolgus HER2 with a comparable affinity to human HER2. The plasma exposure of SYD983 in cynomolgus monkeys is high and plasma stability of SYD983 in cynomolgus and human plasma is similar. Although we cannot rule out that processing of the linker-drug might be different in monkey versus human, we do consider cynomolgus monkeys to be the most relevant species for SYD983 nonclinical safety studies.

SYD983 was very well-tolerated in cynomolgus monkeys up to 2 dosages of 30 mg/kg 3.5 weeks apart. Exposure of SYD983 was high and in line with the dosing regimen (Fig4E). Effects observed were all mild and transient (Table 1). The highest non-severely toxic dose was estimated to be ≥ 30 mg/kg. Most interestingly, no hepatotoxicity, thrombocytopenia and no signs of peripheral sensory neuropathy were observed which was confirmed by pathology assessment. The latter two observations are of special interest since they may be important potential differentiators to other LD technologies in general and to T-DM1 in particular.

The AUC at the HNSTD (30 mg/kg/cycle) in cynomolgus monkeys is 100051 ug.h/ml, which is a factor of 300 higher than the exposure of 333 ug.h/ml in mice at the effective dose of 5 mg/kg. These data indicates a large TI of SYD983 in the preclinical setting, suggesting sufficient window to reach anti-tumor activity at a dose with an acceptable safety profile in humans. Since the TI was obtained in two different species, one of
which is the mouse where SYD983 has a poor PK, translatability of these data to human will only be proven in clinical trials.

**HIC purification of SYD983; preparation of SYD985.**

SYD983 is a heterogeneous mixture of non-conjugated Abs and conjugated Abs with a DAR of 2, 4, 6, and 8 (Supplementary Fig S1A). For further (pre-)clinical development, we preferred an ADC that is more homogeneous and preferably does not contain naked Ab, to increase the DAR and reduce competition of naked Ab on the tumor antigen and thereby increase its potential anti-tumor activity. Hydrophobic Interaction Chromatography (HIC)-based purification was used to deliver a well-defined ADC consisting predominantly of DAR2 and DAR4 species (Supplementary Fig S1B). This more homogeneous ADC was designated SYD985. SYD985 has an average DAR of 2.7 versus 2.0 for SYD983. As can be expected based on the increased DAR in SYD985, its potency to kill HER2-expressing SK-BR-3 cells *in vitro* is indeed increased (two-fold) compared to SYD983 (Supplementary Fig S2).

**Anti-tumor activity in a breast cancer patient-derived xenograft.**

Finally, efficacy of SYD985 was studied in two breast cancer patient derived xenograft models (PDX) named MAXF1322 and MAXF1162. Both of these tumors are HER2 3+ (Fig5A-B). It was shown that these PDX models do not respond to a high dose of trastuzumab, dosed 4-times at a 4-day interval (Fig5C-D). In both tumor models,
SYD985 dose-dependently reduced tumor growth after a single compound administration. This effect was dependent on HER2 expression on the tumors since the anti-tumor activity of SYD985 was significantly better than that of the non-binding isotype control ADC (Fig5E-F). Thus, SYD985 induces strong HER2-mediated anti-tumor activity in these HER2 positive-breast cancer PDX models, illustrating its potential clinical benefit in this patient group.

DISCUSSION

Many of the ADCs currently in clinical and preclinical development are based on LDs that are present in either brentuximab-vedotin (auristatins) or T-DM1 (maytansinoids) (1,2). The TI of the current ADCs on the market is limited and does indicate the need for new generation LDs with greater benefit/safety ratios. We have developed a LD technology based on the highly potent cytotoxic duocarmycin class of compounds that is bound in an inactive form to a cleavable linker that is highly stable in human plasma but sensitive to proteases in the tumor. We have evaluated this technology by profiling a HER2-targeting ADC, SYD983, based on the mAb trastuzumab and vc-seco-DUBA, one of our new generation LDs.

SYD983 was selected as the best performing ADC from a series of related ADCs profiled in a Lead Optimization program. Since conjugation occurs randomly on cysteine residues, SYD983 has a theoretical DAR distribution encompassing DAR0, 2,4,6, and 8 species. Higher DAR species (DAR6 and DAR8) are generally less stable, clear more rapidly, more easily exchange LD to thiol-reactive constituents like albumin in plasma, and therefore have a relative large contribution to toxicity (29). The DAR0 species,
which is trastuzumab in the case of SYD983, does not contribute to toxin-mediated anti-tumor activity and even will compete for antigen binding sites, reducing the potential anti-tumor activity of the ADC. Therefore, an ADC with a more defined DAR distribution containing DAR2 and DAR4 species only is preferred. We have used HIC to purify SYD983 and thereby remove DAR0, DAR6, and DAR8 species. The resulting SYD985 predominantly contains DAR2 and DAR4 species with a mean DAR of 2.7. SYD985 behaves as a potent anti-tumorigenic ADC in relevant patient-derived xenografts.

In line with what has been observed for other ADCs and especially T-DM1 (30), our in vitro studies support a mechanism of action in which SYD983 binds to HER2, internalizes, and releases its cytotoxic payload. We showed that the potent in vitro cell cytotoxicity does translate into effective anti-tumor activity in vivo in xenografts, including in PDX models that represent the current target cancer population for T-DM1 treatment, e.g. HER2 positive metastatic breast cancer patients.

SYD983 has a poor stability in mouse plasma which induces poor exposure of intact ADC in mice. In contrast, stability of SYD983 in cynomolgus monkey plasma is very high resulting in very stable PK in cynomolgus monkeys after dosing in vivo. Thus for vc-seco-DUBA, in vitro plasma stability does predict for stability and PK in vivo. It is therefore expected that the high stability of SYD983 in human plasma will lead to high exposure of intact ADC in patients. The poor stability of SYD983 in mouse plasma is solely due to CES1c activity, since SYD983 is very stable in plasma from CES1c knockout mice. The poor exposure in terms of AUC of SYD983 in mice is very likely affecting its anti-tumor activity in mice. As for T-DM1 (31), also anti-tumor activity of SYD983 is driven by the AUC rather than Cmax. Thus, the anti-tumor activity of
SYD983 and SYD985 will most likely increase once the AUC of intact ADC will increase. It cannot be ruled out though that some anti-tumor activity is induced by the release of the pay-load in the vicinity of the tumor by mouse-specific CES1c. Clinical studies with SYD985 will demonstrate whether indeed this is the case. In addition, efficacy studies in CES1c knockout mice might importantly contribute to better understanding the PK/PD relationship.

Data from safety studies in cynomolgus monkey were used to compare the SYD983 profile to T-DM1 (28) as the main comparator competitor drug. In clinical practice, the main adverse effects of T-DM1 are induction of thrombocytopenia and of peripheral neuropathy, which is a major, possibly irreversible, disabling adverse effect for patients (6,7,8,9). Platelet counts did decrease approximately 30% in cynomolgus monkeys treated with T-DM1 at 30 mg/kg/cycle (28). Thrombocytopenia does limit the maximal human dose of T-DM1 to 3.6 mg/kg every 3 weeks. In cynomolgus monkey, SYD983 did not show any signs of hepatotoxicity or peripheral neuropathy, by visual examination of the animals and pathological examination of the spinal cord. In addition, no effects on thrombocytes were noted. Clinical studies should prove whether these potential differentiators to T-DM1 will lead to benefits in terms of absence of neuropathy and/or in terms of the ability to dose SYD983 higher leading to potentially better clinical outcome. For both T-DM1 and brentixumab-vedotin, dose-limiting-toxicity in cynomolgus monkeys is driven by toxic effects in the hematopoietic system and thought to be mediated through plasma levels of the free toxins (28, 32), which are DM1 and MMAE respectively. The first signs of toxicity of SYD983 in cynomolgus monkeys are hyperpigmentation in the skin which is thought to be mediated through HER2-positive
melanocytes (33). Thus, in contrast to T-DM1 and brentuximab-vedotin, the first signs of toxicity for SYD983 seem target-related rather than related to free-toxin levels. Future vc-seco-DUBA-based ADC programs directed towards other antigens will show whether this potential benefit for the vc-seco-DUBA LD technology sustains and does translate into humans.

In addition to the proposed mechanism of action where the ADC is internalized and toxin is released within the lysozome by proteases like cathepsins, vc-seco-DUBA may be cleaved in the tumor by extracellularly available proteases, thereby releasing the cytotoxic payload outside the target cells and inducing a bystander effect. Proteases such as cathepsins, especially cathepsin B and L, are highly expressed in a wide range of tumors, including breast cancer tumors, where they have been implicated in tumorigeneisis (34) and associated with a poor clinical outcome (34,35,36,37). It is tempting to speculate that the cleavable linker in SYD985 will indeed allow a significant bystander effect and perhaps enable successful treatment of tumors in which not all tumor cells express high levels of HER2. Further experiments are ongoing to test that hypothesis.

The duocarmycin class of compounds has been explored by several others for application in ADCs (38,39,40). The anti-CD70 ADC MDX-1203 is the only duocarmycin-based ADC, however, that has been taken into the clinic. The main difference between the linker-duocarmycin used in MDX-1203 versus SYD983 is that in MDX-1203 the linker is connected to the DNA binder moiety, which necessitates the use of an additional cleavable protecting group on the hydroxyl group of the DNA alkylator to stabilize the LD, whereas the linker in SYD983 is directly coupled to the hydroxyl group.
of the DNA alkylator, thereby eliminating the need for a second promoiety. The double prodrug approach as applied in MDX-120-3 therefore requires two cleavage steps in the tumor cell to release the active toxin, which potentially increases the risk of insufficient activation.

We conclude that SYD983 combines a high anti-tumor activity with an impressive safety profile. The superior preclinical TI compared to established LD technologies formed the basis for the decision to progress our HER2-targeting ADC towards clinical studies. This will be done with SYD985, a HIC-purified form of SYD983 that is more homogeneous and predominantly contains DAR2 and DAR4 species. SYD985 is the most progressed ADC based on a new duocarmycin-based LD technology platform. When taken more generally, the data presented in this paper indicate that vc-seco-DUBA, a new generation LD as used in SYD983/SYD985, might be used on many other mAbs to create effective and safe ADCs for many more indications in oncology.

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Table 1. Summary of findings in dose-range-finding toxicity and PK study in cynomolgus monkey.

<table>
<thead>
<tr>
<th>Parameter/organ system</th>
<th>Effect</th>
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<td>Body weight</td>
<td>Maximum transient body weight loss: 8.7% (2nd cycle)</td>
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<tr>
<td>White Blood Cell populations</td>
<td>Mild transient decreases at ≥ 10 mg/kg/cycle</td>
</tr>
<tr>
<td>Red Blood Cell parameters</td>
<td>Transient decrease followed by rebound in reticulocytes at ≥ 10 mg/kg/cycle</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>No reduction below 95% confidence interval of population predose values (NOEL: ≥ 30 mg/kg/cycle)</td>
</tr>
<tr>
<td>Liver</td>
<td>No effect on transaminases, albumin, bilirubin and histopathology (NOEL: ≥ 30 mg/kg/cycle)</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>No effects on behavior and histopathology (NOEL: ≥ 30 mg/kg/cycle)</td>
</tr>
<tr>
<td>Ovaries</td>
<td>Mild effects in line with target expression</td>
</tr>
<tr>
<td>Skin</td>
<td>Hyperpigmentation at dosages ≥ 10 mg/kg/cycle. Reversible facial swelling in 1 animal at 10 mg/kg/cycle.</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

**Figure 1.** Mechanism of activation of SYD983/SYD985 inside the tumor cell to release the toxic duocarmycin drug. SYD983 consists of trastuzumab linked to vc-seco-DUBA via reduced interchain disulfides. When SYD983 has been taken up into the tumor cell by endocytosis and the linker is cleaved in the lysozome by proteases, such as cathepsin B. Subsequently, two self-elimination reactions occur to generate the seco-DUBA, which then spontaneously rearranges to form the activated duocarmycin drug, which can then bind and alkylate DNA.

**Figure 2.** *In vitro* profile of SYD983. **A,** Percentage of internalization in time for SYD983 versus trastuzumab in SKBR3 cells. **B,** Induction of ADCC by SYD983 and trastuzumab. **C and D,** Cytotoxicity induced by seco-DUBA (C) or SYD983 (D) in a series of cell lines. **E and F,** The effect of SYD983, trastuzumab, and non-binding isotype control ADC on cell viability (E) and release of intracellular protease in the medium (F) of SK-BR3 cells.

**Figure 3.** Antitumor activity in BT-474 xenograft tumor model. Mice were treated intravenously as indicated by the arrow on the X-axis (A,B,C,D). **A,** The effect of SYD983 in a dose range compared to trastuzumab. **B,** Effect of adding free toxin to trastuzumab on anti-tumor activity. The free toxin seco-DUBA was added to 15 mg/kg trastuzumab at an equi-molar concentration compared to that present in SYD983. **C** Anti-tumor activity of SYD983 in the BT-474 xenograft model is AUC-driven. **D,** The effect of SYD983 compared to a non-binding isotype control ADC.
**Figure 4.** *In vitro* and *in vivo* kinetics of SYD983 in different species. **A, B,** ADC concentration after 96 hrs incubation of 100ug/mL SYD983 at 37°C in **A**, human, monkey and mouse plasma or **B,** plasma from mouse carboxylesterase 1c (CES1c) knockout mice versus wild-type mice. **C, D, and E,** Time concentration curves of total antibody (TAb) or ADC concentration (n = 3, ± SEM) after single dose intravenous administration of 0.2, 1 or 5 mg/kg SYD983 to healthy Balb/c mice (C), 1 or 5 mg/kg SYD983 to tumor bearing Balb/c nu/nu mice (D), or 1,3,10 or 30 mg/kg SYD983 in cynomolgus monkeys (E). The concentration of intact ADC or TAb was quantified by sandwich ELISA.

**Figure 5.** The effect of SYD985 in HER2 IHC 3+ breast cancer patient-derived xenografts. **A and B,** HER2 expression in MAXF1322 and MAXF1162 tumor xenografts stained with HER2 and counterstained with hematoxylin. The insert shows staining of a duplicate slide with hematoxylin only. **C-F,** antitumor activity in MAXF1322 and MAXF1162 PDX models *in vivo*. Mice were IV treated with trastuzumab (C and D) or SYD985 and non-binding isotype control ADC (E and F) as indicated by the arrow on the X-axis. **C and D,** Mice were treated with either vehicle or trastuzumab, which was dosed once at 60 mg/kg (loading dose) and three times at 30 mg/kg. **E and F,** Mice were treated (single-dose) with a dose range of either SYD985 or non-binding isotype control ADC.
Figure 1.
Figure 2.

A. SYD983 - 4C

B. SYD983

C. seco-DUBA 2

D. SYD983

E. SYD983

F. SYD983

Legend:
- SYD983
- trastuzumab
- no antibody
- cell viability on day 0
- isotype control ADC
Figure 3.

A. [Graph showing tumor volume over time for different treatments.]

B. [Graph showing tumor volume over time for different treatments.]

C. [Graph showing tumor volume over time for different treatments.]

D. [Graph showing tumor volume over time for different treatments.]
Figure 4.
Figure 5.

A. MAXF 1322

B. MAXF 1162

C. MAXF 1322

D. MAXF 1162

E. MAXF 1322

F. MAXF 1162

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Preclinical profile of the HER2-targeting ADC SYD983/SYD985; introduction of a new duocarmycin-based linker-drug platform.

Wim H Dokter, Ruud Ubink, Miranda van der Lee, et al.

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