Unraveling the Interaction between Carboxylesterase 1c and the Antibody–Drug Conjugate SYD985: Improved Translational PK/PD by Using Ces1c Knockout Mice

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Abstract

Carboxylesterase 1c (CES1c) is responsible for linker-drug instability and poor pharmacokinetics (PK) of several antibody–drug conjugates (ADC) in mice, but not in monkeys or humans. Preclinical development of these ADCs could be improved if the PK in mice would more closely resemble that of humans and is not affected by an enzyme that is irrelevant for humans. SYD985, a HER2-targeting ADC based on trastuzumab and linker-drug vc-seco-DUBA, is also sensitive to CES1c. In the present studies, we first focused on the interaction between CES1c and SYD985 by size-exclusion chromatography, Western blotting, and LC/MS-MS analysis, using recombinant CES1c and plasma samples. Intriguingly, CES1c activity not only results in release of the active toxin DUBA but also in formation of a covalent bond between CES1c and the linker of vc-seco-DUBA. Mass spectrometric studies enabled identification of the CES1c cleavage site on the linker-drug and the structure of the CES1c adduct. To assess the in vivo impact, CES1c+/− SCID mice were generated that showed stable PK for SYD985, comparable to that in monkeys and humans. Patient-derived xenograft (PDX) studies in these mice showed enhanced efficacy compared with PDX studies in CES1c+/− mice and provided a more accurate prediction of clinical efficacy of SYD985, hence delivering better quality data. It seems reasonable to assume that CES1c+/− SCID mice can increase quality in ADC development much broader for all ADCs that carry linker-drugs susceptible to CES1c, without the need of chemically modifying the linker-drug to specifically increase PK in mice. Mol Cancer Ther; 17(11): 2389–98. ©2018 AACR.

Introduction

Stability of the linker-drug in plasma has been, and still is, a major challenge in the development of antibody–drug conjugates (ADC). On the one hand, linkers should be stable enough to prevent release of the active toxin in plasma, whereas on the other hand, linkers should allow for an efficient release of active toxin once the ADC targets a tumor or tumor cell. The difficulty of developing optimal linker characteristics is illustrated by the limited amount of linker-drug technologies in ADCs that have been studied in the clinic (1, 2), although novel linker-drug strategies are emerging (for recent reviews see 3, 4, 5, 6). For Mylotarg, the first approved ADC based on a pH-labile hydrazine linker, chemical instability of the linker-drug in the blood circulation probably contributed to the temporary withdrawal from the market (7, 8). Besides early cleavage, other possible causes of instability in plasma include maleimide exchange of the cysteine-conjugated linker-drugs to other thiol-containing molecules circulating in plasma, such as serum albumin (9) and enzymatic instability by circulating enzymes in plasma (10, 11), which can cleave functional groups like esters, amides, lactones, lactams, carbamides, sulfonamides, and peptide mimetics (12). SYD985, a HER2-targeting ADC based on trastuzumab and vc-seco-DUBA, a cleavable linker-duocarmycin payload, was found to be stable in human and monkey plasma, but unstable in mouse and rat plasma (13, 14). Despite its instability in mouse plasma, and its subsequent poor exposure in mice, SYD985 was found to be very active in HER2 3+, 2+, and 1+ breast cancer patient-derived xenograft (PDX) models, whereas T-DM1 only showed significant antitumor activity in HER2 3+ models (15). Although good antitumor activity of SYD985 was observed in these mouse models, it was still hypothesized that the poor stability yields an underestimation of the expected anti-tumor activity in humans. Therefore, in parallel to the clinical development of SYD985, subsequent preclinical studies aimed at (i) identifying the cause of instability of SYD985 in mouse and rat plasma, (ii) testing the hypothesis that anti-tumor activity in mice is improved when plasma stability of SYD985 improves, and (iii) delivering a mouse model that more closely resembles the monkey and human pharmacokinetics (PK) to allow for reliable assessment of the minimal effective dose in humans. In addition,
and most ideally, such a mouse model could be used for other ADC programs as well and deliver more relevant pharmacokinetic/pharmacodynamic (PK/PD) data in preclinical studies right away. As reported earlier, the rodent-specific carboxylesterase 1c (CES1c) was identified as the crucial enzyme that cleaves vc-seco-DUBA-based ADCs, leading to the poor PK of SYD985 in mice and rats compared with that observed in human and monkey (14, 15). After these reports, it was published that CES1c is also responsible for cleavage of other valine-citrulline-p-aminoenzoyloxycarbonyl (vc-PABC) containing linker-drugs in mouse plasma and that the extent of CES1c-mediated cleavage depends on the site of conjugation (16). Several additional papers report instability in mouse and rat plasma of vc-PABC-containing linker-drugs (10, 17), indicating CES1c activity might be a more widespread cause of linker-drug instability in these species, hampering the development of ADCs.

In this paper, we will first present the data that led to the identification of CES1c as the enzyme responsible for the instability of SYD985, causing release of active toxin. In order to be complete and set the context right, we needed to recapture a limited amount of data that we published before, mainly as supplementary data. We will subsequently identify the cleavage site of CES1c on the linker-drug vc-seco-DUBA, being different from the cleavage site identified by (16). Furthermore, it was found that CES1c activity not only cleaves the linker-drug, but also leads to the formation of a covalent bond between CES1c and the remaining linker. To circumvent the translational issues caused by the CES1c activity, we developed a mouse model which closely mimics the human PK and is suitable for xenograft studies, by cross-breeding CES1c–/– mice with SCID mice. Using this mouse model we demonstrate that improved PK indeed leads to improved efficacy in PDX studies and improved human dose predictions for SYD985. Potentially, this model would also increase PK/PD relationships of other ADCs that contain linker-drugs that are susceptible to CES1c cleavage.

Materials and Methods

ADCs and related materials
SYD985 was prepared as previously described (15). [3H]-SYD985 was prepared at Pharmaron, Cardiff, UK, using the same procedure and a linker-drug in which two tritium labels were incorporated in the hydroxybenzyl moiety of the toxin. N-acetyl cysteine-quenched linker-drug (NAC-Cys-linker-drug) was prepared by mixing linker-drug and N-acetyl cysteine in a 1:10 molar ratio in an acetonitrile/water mixture for 4 hours followed by purification by preparative HPLC. The head-to-head studies comparing T-DM1 with SYD985 were conducted with two batches of T-DM1 from Roche, EU batch N0001B02 (in nude mice) and N1037B19 (in CES1c–/– SCID mice).

In vitro plasma stability and cleavage by CES1c
The antibody–drug conjugate SYD985 was spiked into pooled female mouse (BALB/c), rat (Sprague Dawley), monkey (Macaca fascicularis), and human K2-EDITA plasma, at a concentration of 100 μg/mL and incubated at 37 °C. After 0, 1, 6, 24, 48, and 96 hours of incubation, plasma samples were snap-frozen and stored at −80 °C until bioanalysis. Recombinant mouse CES1c (rCES1c; Cusabio Biotech, CSB-MP338557MO) was spiked in human K2-EDITA plasma at 0, 10, 100, 200, and 400 μg/mL together with 100 μg/mL SYD985. After 96 hours of incubation at 37 °C, plasma samples were snap-frozen in liquid nitrogen and stored at −80 °C until bioanalysis.

PK studies and bioanalytical assays
Adult female CES1c–/– (B6-Ces1c<sup>±/±</sup>; Charles River) mice, CES1c–/– SCID (B6-Ces1c<sup>±/±</sup> SCID, Rapid); and CES1c–/– SCID mice, (Wistar; Charles River), and monkeys (Macaca fascicularis; Mauritian) were dosed intravenously with 0.3, 1, 3, 8, and/or 10 mg/kg SYD985. Blood samples were taken at multiple time points after dosing, cooled on ice water, and processed to K2-EDITA plasma as soon as possible. Plasma samples were snap-frozen in liquid nitrogen and stored at −80 °C until bioanalysis. SYD985 plasma levels were quantified using ELISA-based methods with anti-idiotype capture and reporting for total antibody, and anti-toxin capture and anti-idiotype reporting for conjugated antibody, as previously described (15). Based on the reported plasma levels, PK parameters were calculated in WinNonlin version 6.3.

Release of DUBA in mouse and rat plasma
Human tumor cell line SW-620 (ATCC number: CCL-227; Lot number: 58483168; P. 86 received on 19SEP2012) was obtained from and characterized by the ATCC. No further cell-line authentication was conducted. Mycoplasma contamination in cell cultures was tested at Minerva Biolabs GmbH using the VenorGeM Prime test. No detectable levels of mycoplasma were found in the working cell bank p86+14 tested on the April 11, 2013. The number of passages between collection and use in the described experiments is 5 and 6. SW-620 cells (90 μL/well, 4,000 cells/well) were plated in 96-wells plates in RPMI1640 medium (Lonza), containing FBS, heat-inactivated (HI; Gibco-Life Technologies) and 80 U/mL Pen/Strep and incubated at 37 °C, 5% CO<sub>2</sub> overnight. After an overnight incubation 10 μL SYD985 or DUBA was added to each well of the 96-wells plate, containing 10% mouse (BALB/c), CES1c<sup>–/–</sup> mouse, rat (Wistar), monkey (Macaca fascicularis), or human plasma. Serial dilutions were made in culture medium with plasma, to reach a final concentration range of 200 nmol/L (10 μg/mL) to 6.3 pmol/L (0.316 ng/mL) for SYD985 and 10 to 0.316 pmol/L for DUBA and 1% plasma. The cell viability was measured after 6 days using the C1G Assay Kit (Promega; G7572).

Affinity extraction of SYD985–protein complexes
SYD985 was spiked at 100 μg/mL in mouse (BALB/c) and CES1c<sup>–/–</sup> mouse plasma and incubated up to 48 or 96 hours at 37 °C, followed by storage at −80 °C until extraction. For the T = 0 samples, SYD985 was spiked in plasma and immediately frozen. SYD985 and associated protein complexes were extracted from plasma using an anti-idiotype mini-antibody (Bio-Rad; AbD15916) coupled via amino coupling to NHS-activated protein complexes. 5% ammonium sulfate precipitation step was performed to improve the purity of the extracted samples be selectively precipitating the IgG fraction, including SYD985. Following incubation with the anti-idiotype coupled sepharose, the SYD985 sample was transferred to a disposable column (Thermo Scientific; cat. no. 29920). After removal of the flow-through, sequential washes (1 M NaCl in PBS, 10% acetonitrile in PBS and PBS) were performed to remove nonspecifically bound proteins.
SYD985 and associated protein complexes were eluted with 10 mmol/L glycine-HCl, pH 2.5, followed by immediate neutralization with 0.1 M Tris-HCl, pH 8.5.

**SYD985 analysis by SEC**

Radiolabeled [14C]-SYD985 was incubated at 100 μg/mL in plasma from female mouse (BALB/c), CES1c−/− mouse, rat (Wistar), monkey (Macaca fascicularis), and human for 6 hours at 37°C. The resulting plasma samples were analyzed directly by size exclusion chromatography (SEC) using a Waters BEH200 SEC column (4.6 × 150 mm, 1.7 μm particles) and isotonic elution with 100 mmol/L NaPO₄, pH 6.8 + 0.3 M NaCl. Eluting compounds were detected using a Model 4 β-Ram Radiodetector (LabLogic).

For “cold” (i.e., not radiolabeled) SYD985 that was incubated in plasma of mouse under the conditions mentioned above, antidiotype affinity-extracted material was evaporated to dryness and reconstituted in 100 mmol/L NaPO₄, pH 6.8 + 0.3 M NaCl before analyses by SEC using the same analytical column and solvent as described for radiolabeled SYD985, but using UV absorbance detection at 214 and 330 nm. Eluting compounds, including the high molecular weight (HMW) species, were collected, buffer-exchanged, and concentrated into 50 mmol/L Tris-HCl, pH 8.0 using 10K centrifugal filters (Amicon). 8M urea was added to denature the proteins, followed by reduction and alkylation with dithiothreitol (DTT; 4.5 mmol/L final concentration) and iodoacetamide (IAM, 9 mmol/L final concentration in 50 mmol/L Tris-HCl, pH 8.5, 37°C, 1 hour) and iodoacetamide (IAM, 9 mmol/L final concentration) and iodoacetamide (IAM, 9 mmol/L final concentration in 50 mmol/L Tris-HCl, pH 8.5, 37°C, 1 hour) respectively. Following an overnight (o/n) precipitation in EtOH, the resulting pellets were digested using trypsin (4 hours, 10 mmol/L Tris-HCl, pH 8.5).

**Coomassie blue staining and Western blot analysis**

Samples obtained by affinity extraction were evaporated to dryness, reconstituted in Milli-Q water and electrophoretically separated under nonreducing conditions on a 3% to 8% Tris-acetate gel (Thermo Scientific) for total protein staining, or on a 4% to 12% Bis-Tris gel (Thermo Scientific) for Western blot analysis. Total protein staining was performed with Bio-safe Coomassie stain (Bio-Rad) according to manufacturer’s instructions. For Western blot analysis, proteins were transferred to a PVDF membrane (GE Healthcare) with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The PDVF membrane was blocked with Western blocker solution (Sigma-Aldrich). SYD985 and associated protein complexes were detected with an NAc-Cys-linker-drug complex were per-}

**In-gel protein digestion**

Protein bands of interest were excised from the Coomassie-stained SDS-PAGE and washed with Milli-Q water and acetonitril, to remove Coomassie and SDS before proceeding to the next step. Following in-gel reduction and alkylation with DTT (10 mmol/L final concentration in 10 mmol/L Tris-HCl, pH 8.5, 37°C, 30 minutes) and IAM (20 mmol/L final concentration in 10 mmol/L Tris-HCl, pH 8.5, RT, 45 minutes in the dark), respectively, proteins were in-gel digested using trypsin (o/n, 10 mmol/L Tris-HCl, pH 8.5).

**Incubation of rCES1c with NAc-Cys-linker-drug and SYD985 and subsequent proteolytic digestion**

Recombinant CES1c (rCES1c) was incubated with NAc-Cys-linker-drug or SYD985 in PBS, at a concentration of 100 μg/mL for 96 hours at 37°C under gentle shaking. The resulting sample was acidified to pH 3.5 using glycine-HCl and reduced and alkylated using TCEP (10 mmol/L final concentration, 37°C, 30 minutes) and N-ethyl maleimide (NEM; 20 mmol/L final concentration, RT, 45 minutes in the dark), respectively. Considering that a potential (serine) ester of CES1c with NAc-Cys-linker-drug is probably base-labile, all incubations and sample preparations of the resulting CES1c – NAc-Cys-linker-drug complex were performed at low pH (~3). For protein digestion, pepsin was added and the mixture was incubated at 37°C (pH 3.0, o/n).

**LC/MS-MS analysis of proteolytic digests**

Proteolytic peptide mixtures were separated by reversed phase liquid chromatography ( Dionex Ultimate 3000) using a Waters BEH-300 C18 column (1.0 × 150 mm, 1.7 μm particles) and a 40’ 5%–95% linear gradient of acetonitrile + 0.1% formic acid (FA) in Milli-Q + 0.1% FA at a flow rate of 0.1 mL/min and a column temperature of 30°C. Eluting peptides were detected using electrospray ionization mass spectrometry [Thermo Orbitrap Fusion, heated electrospray ionization (HESI) at a spray voltage of 3.8 kV] with MS detection in the Orbitrap at 120K resolution over the scan range m/z 400 to 1,600 and further analyzed using data-dependent tandem mass spectrometry (MS-MS) in the quadrupole, using HCD (30%), on the top-3 most intense precursor ions detected per survey scan to enable protein identification. Fragments were detected in the ion trap. Proteins were identified against the Swissprot database using Mascot search engine.

In addition, MS and MS-MS fragmentation data were interpreted manually using Qualbrowser (Thermo XCalibur 3.0.63) by comparing proteolytic digests of rCES1c with and without NAc-Cys-linker-drug and proteolytic digests of rCES1c and SYD985 individually to that of the incubated mixtures to investigate and characterize cross-linked peptide candidates.

**Patient-derived xenograft studies**

The in vivo antitumor activity of SYD985 versus T-DM1 was tested as single-dose therapy in MAXF1162 breast cancer PDX model (Charles River) in both CES1c−/− NMRI-Foxn1nu mice as well as CES1c−/− SCID mice. Initial tumor volumes at the day of randomization and treatment ranged from 46 to 252 mm³. The HER2 FISH and IHC status of the tumor from the PDX model as determined by the CROs was independently confirmed. Studies were conducted as previously described (15). All in vivo studies and protocols were approved by the local animal care and use committees according to established guidelines.

**Results**

**CES1c expressed in rodent plasma cleaves vc-seco-DUBA**

In vitro plasma stability studies, as well as in vivo PK studies, showed that SYD985 conjugated antibody levels rapidly decrease in mouse and rat plasma, but are stable in monkey
and human plasma (Fig. 1A and B; Supplementary Fig. S1), similar to what was previously found for closely related ADCs (13, 14). A literature study pointed towards mouse and rat-specific CES1c as a potential hydrolyzing enzyme. To assess its role in SYD985 instability, increasing amounts of rCES1c were spiked in human plasma in combination with 100 mg/mL SYD985 and incubated at 37°C up to 96 hours. SYD985 conjugated antibody levels decreased with increasing CES1c concentration (Fig. 1C, previously published as supplementary data; ref. 15). Significant cleavage was observed as of 100 mg/mL CES1c which are physiologically relevant CES1c concentrations, comparable to the in vivo CES1c concentration in mice (80 μg/mL; ref. 16). A PK study with SYD985 in CES1c homozygous (+/+) mice, heterozygous CES1c−/−, and homozygous CES1c−/− wild-type littermates, showed that the PK in homozygous CES1c−/− mice was similar to the PK in monkey and human (Fig. 1D; Supplementary Table S1, previously published as supplementary data in ref. 15).

**Figure 1.**
*In vitro and in vivo kinetics of SYD985.* **A,** Conjugated antibody concentration during a 96-hour incubation of 100 μg/mL SYD985 at 37°C in human, monkey, rat, and mouse plasma (mean ± SEM, n = 3). **B,** Total (TAb) and conjugated antibody (Conj. Ab) concentration (mean ± SEM, n = 2 for rat, three for mouse, and five for monkey) after single-dose IV administration of 10 mg/kg SYD985 to mice, rats (dose normalized from 8 mg/kg), or monkeys. **C,** Conjugated antibody concentrations of 100 μg/mL SYD985 after 96 hours in vitro incubation at 37°C in human plasma with increasing concentrations of mouse rCES1c (mean ± SEM, n = 2). **D,** Conjugated antibody concentrations in plasma in CES1c knock-out (−/−), CES1c heterozygous (+/−), and CES1c wild-type (+/+) mice, after a single IV bolus injection of 5 mg/kg SYD985 (mean ± SEM, n = 3). **E,** Cytotoxic activity of released active toxin on HER2-negative SW-620 cells after 6 days incubation of the toxin DUBA in plasma-free medium (positive control) or SYD985 in the presence of 1% mouse, CES1c−/− mouse, rat, monkey, or human plasma in the culture medium. Data show the percentage of survival ± SD of two experiments performed in duplicate.

**Mechanism of action of CES1c-mediated vc-seco-DUBA cleavage**

To study if CES1c activity results in release of the toxin DUBA, SYD985 was incubated with HER2-negative cells in the presence of 1% plasma. Compared with the medium control, presence of 1% plasma caused a clear shift in the IC50 for rat (8.08 nmol/L) and especially mouse (0.82 nmol/L) plasma, but not with monkey, human, or CES1c−/− mouse plasma, all showing an IC50 > 100 nmol/L.

As part of a metabolism study, radiolabeled SYD985 was analyzed by size exclusion chromatography (SEC) after incubation in mouse, rat, CES1c−/− mouse, monkey, and human plasma. In SEC profiles of mouse and rat plasma a new peak appeared (Fig. 2B and D), eluting at an earlier retention time (7.15 minutes) compared with the SYD985 peak (RT = 7.8–8.02 minutes). This new peak was hardly observed in samples taken from CES1c−/− mouse and monkey, human plasma (Fig. 2A, C, and E), suggesting that a SYD985-containing compound with a higher molecular weight is

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weight than SYD985 alone was formed in rat and mouse plasma. To identify the compound(s) eluting in this mouse and rat-specific peak, mouse plasma was incubated with SYD985 up to 48 hours and SYD985 and SYD985-associated material was subsequently extracted from plasma using anti-idiotype extraction. SEC profiles of the affinity-extracted material confirmed the appearance of a SYD985-containing peak, at an earlier retention time as compared with SYD985 itself (Fig. 2F). The extract was

Figure 2. A–E, SEC-HPLC radiochromatograms of [3H]-SYD985 after 6 hours incubation in plasma of indicated species at 100 µg/mL and 37°C. F–I, Analysis of HMW species in affinity-extracted material obtained from BALB/c mouse plasma incubated with SYD985. F, Confirmatory SEC-HPLC UV chromatogram of non-radiolabeled SYD985 and associated material affinity-extracted after 48 hours of incubation in wild-type mouse plasma at 37°C. G, Coomassie staining of affinity-extracted samples from BALB/c mouse plasma using non-reducing SDS-PAGE revealed an increase in the formation of HMW species over time. H, A Western blot analysis with the anti-idiotype antibody directed against SYD985 showing the presence of SYD985 in the HMW species. I, A Western blot analysis with an anti-CES1c antibody showing the presence of CES1c in the HMW species.
also analyzed using nonreducing SDS-PAGE and Coomassie staining, which revealed a unique band pattern around 200 to 250 kDa (Fig. 2G). Although SYD985 has a molecular weight of ~150 kDa, bands were also observed at lower molecular weight in the extracted samples. This is due to the fact that as a result of conjugation on interchain cysteines, used to manufacture SYD985, its structural integrity (partially) relies on noncovalent interactions that are disrupted during SDS-PAGE analysis. The 200 to 250 kDa bands were cut out of the gel, trypsin-digested and analyzed by LC/MS-MS. Next to trastuzumab, CES1c (Uniprot entry P23953) was confidently identified based on 6 unique peptides (9% sequence coverage, Mascot score 337) in the lower migrating band (around 200 kDa), whereas CES1c was also identified in the higher migrating band (around 250 kDa) based on 12 unique peptides (25% sequence coverage, Mascot score 745). CES1c was also observed by LC/MS/MS analysis of a direct (i.e., in solution) digest of affinity-extracted material from wild-type mouse plasma following incubation of SYD985 at 37°C for 96 hours. Western blot studies showed the presence of both SYD985 and CES1c in the 200 to 250 kDa bands, because it stained positive for SYD985 using an anti-idiotypic antibody, and for CES1c using an anti-CES1c antibody (Fig. 2H and I), respectively.

Next, it was attempted to identify the CES1c cleavage site(s) on the linker-drug in SYD985 and the position of the covalent bond between CES1c and SYD985. Covalent interactions between carbamates and esterases have been described for human CES1 and CES2 (19). Based on that work, it was hypothesized that the interaction, potentially occurring via the active site serine (Ser 221) in CES1c and one of the carbamates present in the linker-drug (Fig. 3A). Due to the structural orientation of the carbamates, the covalent bond formation with the ADC would most likely proceed via the carbamate connecting the linker to the DNA-alkylating moiety of the toxin, resulting in concomitant release of the toxin.

To investigate this hypothesis, rCES1c was first incubated with N-acetylcysteine-quenched linker-drug (NAC-Cys-linker-drug), for 72 hours at 37°C in PBS. Following reduction, alkylation and proteolytic digestion with pepsin (all at pH ~3), LC/MS/MS analysis of the generated peptide mixture resulted in the identification of five covalently modified CES1c peptides (Fig. 3B–E; Table 1), all including the active site serine Ser 221. The proposed covalent interaction was confirmed by the accurate masses of these modified peptides and the concomitant mass increase of 968.1 Da with respect to the corresponding original (unmodified) peptide mass as a result of the binding of NAC-Cys-linker-drug (minus the released toxin DUBA). Furthermore, the characteristic fragment ions observed in the corresponding MS/MS data, such as the neutral loss of 779.3 Da, corresponding to the majority of the NAC-Cys-linker-drug modification; the loss of the complete NAC-Cys-linker-drug modification (985.4 Da) resulting in the formation of a dehydroalanine moiety from the serine residue, and several NAC-Cys-linker-drug specific fragment ions (Fig. 3E) supported the hypothesized structure of the CES1c-linker-drug cross-link. In addition, free sec-DUBA, the pro-toxin that is released upon linker-drug cleavage and is stable at acidic pH, was also identified in the incubated digest at pH <3, confirming the release of the toxin.

Next, SYD985 was incubated with rCES1c in PBS during 72 hours at 37°C. Following the same, low pH, sample work up as described for the NAC-Cys-linker-drug incubated sample, the generated peptide mixture was analyzed using LC/MS/MS. This dataset revealed several candidate cross-linked peptide molecules. These were confirmed using a combination of their accurate mass and the distinctive fragment ions that were also observed when rCES1c was incubated with NAc-Cys-linker-drug, corresponding to the rCES1c part of a cross-linked peptide. Subtracting the mass of the rCES1c sequence (with part of the linker-drug on it), as well as the remainder of the linker-drug part that was conjugated to the cysteine residue originating from SYD985, from the precursor mass, revealed one of the cross-linked peptide candidates to contain the trastuzumab sequence KSFRNRCG (Supplementary Fig. S2), originating from a conjugated trastuzumab light chain. This confirmed the presence of a SYD985-rCES1c cross-link through the linker-drug side chain via the proposed mechanism.

Finally, this conclusion was underscored by the identification of a cross-link between a rCES1c peptide and the SYD985 hinge region-containing heavy chain peptide THTC(NEM)PCEA-PELGGLGPSVF (Supplementary Fig. S2), that was originally conjugated to one linker-drug. The other cysteine in the sequence was found to be alkylated with N-ethylmaleimide as a result of the sample preparation. Overall, four different rCES1c sequences (all containing the active site serine, Ser 221) were found to be cross-linked to three different peptides originating from the light chain, and to one peptide originating from the hinge region of the heavy chain (Table 2).

### Efficacy studies in CES1c knockout mice

Since in vivo CES1c activity could not be completely blocked by cysteine esterase inhibitors (Supplementary S3), efficacy studies were performed in CES1c −/− SCID mice. Before efficacy studies were initiated, it was first verified whether the SYD985 PK in CES1c −/− SCID mice was identical to that in the original CES1c+/+ strain. As shown in Fig. 4A, the PK profiles indeed overlapped. Pilot efficacy studies with CES1c−/− SCID mice using different PDX models (15), showed tumor take and growth rates similar to those observed in the nude mice. In these models, efficacy was indeed improved at 3 mg/kg SYD985 (Supplementary Fig. S4). To confirm the data obtained at a single dose level of 3 mg/kg, and to get an accurate estimate of the fold change in efficacy, an experiment comprising of different dose levels was performed in the HER2 3+ MAXF1162 PDX model. The efficacy of different SYD985 dosages was compared, head-to-head against T-DM1, similar to a previous study performed in nude mice (15), to control for potential strain differences between the nude and SCID background. A non-binding isotype control ADC, bearing the same linker-drug and with the same drug-antibody ratio, was included to study the effect of improved PK on efficacy for a non-target-binding ADC. The efficacy of SYD985 was improved almost threefold in CES1c−/− SCID mice showing tumor-regression at 3 mg/kg SYD985 in these animals whereas 10 mg/kg SYD985 induced tumor-stasis in CES1c+/+ nude mice. The isotype control also showed efficacy at 3 mg/kg, in contrast to the lack of efficacy in nude mice. This difference is most likely also caused by an increased exposure of intact isotype control ADC. Extracellular cleavage or pinocytosis of the isotype control could explain the efficacy, which is not uncommon for the isotype control (15). As expected, since T-DM1 is not susceptible to CES1c cleavage, T-DM1 showed similar efficacy in nude mice versus CES1c−/− SCID mice, with prolonged tumor stasis at 10 mg/kg.

Recently, phase 1 clinical data were published describing the efficacy of SYD985 at the recommended phase II dose of...
Figure 3.

A, Structure of NAc-quenched linker-drug with carbamate moieties indicated by circles. The blue-circled group was hypothesized to be the one resulting in the covalent interaction upon binding of CES1c. B, Proposed structure of the linker-drug–modified CES1c active site-containing peptide GESSGGISV. C and D, Proposed structures of the corresponding predominant fragment ions formed from the linker-drug–modified CES1c active site-containing peptide GESSGGISV upon collision-induced dissociation: one that loses part of the linker moiety (C) and one that loses the complete side chain modification, resulting in the formation of a dehydroalanine (dhA) residue (D). Please refer to the middle spectrum in E for the MS-MS data of this modified peptide. E, Ion trap MS-MS fragmentation spectra (HCD @30 eV) of the five linker-drug–modified CES1c peptides that were found after incubation of the carboxylesterase with the NAc-Cys-linker-drug. The predominant fragment resulting from the loss of most of the conjugated NAc-Cys-linker-drug moiety (780.3 Da) from the precursor ion is clearly visible (note: all precursors are doubly-charged), just like the presence of the dehydroalanine-containing peptide fragments (at m/z “precursor ion”: 987.3 Da) and linker-specific fragments, such as those at m/z 163.1, 207.2, 240.3, and 349.1.
1.2 mg/kg in HER2 positive metastatic breast cancer patients (20, 21). The 1.2 mg/kg dose was shown to be efficacious with an overall response rate (ORR) of 33% (16 of 48 patients) and a progression-free survival (PFS) of 9.4 months (95% CI, 4.3–12.4). The human PK of SYD985 at 1.2 mg/kg (AUCinf = 1,725 h.µg/mL) almost overlaps with the PK observed in MAXF1162 tumor bearing CES1c−/− SCID mice at the 1 mg/kg dose that shows tumor stasis (AUCinf = 1,332 h.µg/mL; Fig. 4D; Supplementary Table S3).

Discussion

Until recently, all xenograft studies with vc-seco-DUBA-based ADCs, including SYD985, were performed in immune-compromised mice expressing CES1c. Despite the low exposure levels for conjugated antibody in these mice, SYD985 showed good efficacy, which was superior to that of T-DM1 in numerous xenograft models with different levels of HER2 target expression (15). However, because the SYD985 PK profiles in the mouse were dramatically different from those in monkey and the anticipated human profile in human based on in vitro plasma stability data, an accurate estimation of an effective human dose based on efficacy in PDX models was not possible. Based on extrapolation of available PK data for SYD985 in CES1c−/− mice (either nude or SCID) AUCs of conjugated antibody at tumor static dosages are estimated to be between 150 and 260 h.µg/mL. This is based on data generated in the MAXF1162 PDX model and the BT474 model (15) and in normal (non-immune-deficient) mice. This AUC is more than tenfold below the observed AUC in the clinic at the recommended phase II dose of 1.2 mg/kg that showed efficacy (21). Thus, using the AUC at tumor static dose in CES1c−/− mice to predict the effective human dose would lead to a significant under-prediction of this dose. It was anticipated that the translational value of PDX models would improve if the PK profile in mice was more in line with the anticipated human PK profile. As will be discussed later, the AUC of conjugated SYD985 at a tumor static dose of 1 mg/kg in CES1c−/− mice in the MAXF1162 PDX model very nicely correlated with that in humans at the recommended phase II dose at 1.2 mg/kg which turned out to be efficacious.

Based on the in vitro PK data it was reasoned that the instability of SYD985 is most likely caused by cleavage or release of the linker-drug, rather than clearance of the entire ADC, because total antibody levels were similar in all tested species. It was hypothesized that the instability is the result of hydrolysis of one of the carbamate moieties in the linker-drug because these are known substrates for esterase-like activity (22, 23). Preliminary studies with human esterases suggested that neither human CES1 and CES2 nor the plasma esterase BCHE seem to be able to cleave the linker-drug in SYD985 (data not shown). Based on a literature study focused on species differences in esterase activity in plasma, the mouse- and rat-specific CES1c was identified as a candidate hydrolyzing enzyme (18, 24, 25). Incubation of SYD985 in human plasma spiked with rCES1c indeed revealed that CES1c caused instability of SYD985. The final evidence was provided by a PK study in CES1c−/− mice, showing a PK profile similar to the PK in monkey and human. The identification of CES1c triggered additional studies to reveal its mode of action. The increase in potency observed after in vitro incubation of SYD985 with HER-2 negative cells and 1% mouse or rat plasma, compared with medium, human, monkey and CES1c−/− plasma suggested active toxin DUBA is released by CES1c activity. LC/MS-MS analysis confirmed the presence of significant amounts of DUBA in plasma samples from mice and rats dosed with SYD985.

Most surprisingly, CES1c activity not only results in release of DUBA: here it is shown that it also results in the formation of HMW species, as identified by SEC. Using SDS-PAGE, Western blotting and LC-MS/MS, these HMW species were found to contain both trastuzumab and CES1c, which suggests that at least one CES1c molecule is capable of covalently binding to SYD985. Next, attempts to identify the site of interaction between CES1c and the linker-drug disclosed it to be the carbamate group connecting the linker and toxin. Other carbamates in the linker-drug seem to remain unaffected. CES1c activity results in release of

### Table 2. Characteristics of the peptides containing the CES1c active site serine (Ser221) that were found to be conjugated to NAc-Cys-linker-drug

<table>
<thead>
<tr>
<th>Precursor (1−)</th>
<th>CES1c fragmenta</th>
<th>Linker mass</th>
<th>Residual mass</th>
<th>SYD985 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light chain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>828.04 (3−)</td>
<td>2482.13</td>
<td>1140.6</td>
<td>618.28</td>
<td>723.25</td>
</tr>
<tr>
<td>773.74 (3−)</td>
<td>2319.23</td>
<td>979.5</td>
<td>618.28</td>
<td>723.30</td>
</tr>
<tr>
<td>741.32 (3−)</td>
<td>2221.98</td>
<td>880.4</td>
<td>618.28</td>
<td>723.30</td>
</tr>
<tr>
<td>813.03 (3−)</td>
<td>2437.11</td>
<td>880.4</td>
<td>618.28</td>
<td>938.43</td>
</tr>
<tr>
<td>684.32 (4−)</td>
<td>2734.28</td>
<td>880.4</td>
<td>618.28</td>
<td>1235.60</td>
</tr>
<tr>
<td>Heavy chain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>836.39 (4−)</td>
<td>3342.55</td>
<td>680.3</td>
<td>618.28</td>
<td>2045.99</td>
</tr>
<tr>
<td>899.18 (4−)</td>
<td>3561.70</td>
<td>680.4</td>
<td>618.28</td>
<td>2064.02</td>
</tr>
<tr>
<td>1181.89 (3−)</td>
<td>3543.69</td>
<td>880.4</td>
<td>618.28</td>
<td>2046.01</td>
</tr>
</tbody>
</table>

aCES1c peptide fragments (uncharged): 680.3 = GESSGG; 880.4 = GESSGGIS; 979.5 = GESSGGISV; 1140.6 = IFGESSGGIS; “+H2O” refers to hydrolysis (ring opening) of the maleimide moiety in the linker drug.
DUBA and formation of a covalent bond between SYD985 and CES1c, most likely via the serine (Ser221) in its active site, although it cannot be excluded that the modification occurs on serine-222, adjacent to the active site serine. CES1c-SYD985 cross-links were observed both in the hinge region of SYD985 heavy chain and SYD985 light chain. All together, these interaction studies clarified the structure of the covalent bond between CES1c and SYD985 that is -unexpectedly- formed upon CES1c cleavage of the vc-seco-DUBA linker-drug at a specific carbamate.

Clearly, CES1c activity is a more general concern in the development of ADCs. In a recent publication, Dorywalska and colleagues (15) showed that CES1c also cleaves other linker-drugs. Their vc-PABC containing linker-drug was postulated to be cleaved at the carbamate C-terminal to the citrulline moiety. Cleavage by, or covalent interaction with, CES1c would not result in a complex between the ADC and the esterase due to the structural orientation of the carbamate in the linker-drug (19). Even though this linker-drug and vc-seco-DUBA share the vc-PABC structure, we found no evidence for CES1c-mediated cleavage C-terminal to citrulline, or for hydrolysis at the other carbamate positions in vc-seco-DUBA, underlining the complexity of the structure–activity relationship of CES1c. Several other publications describe carbamate-containing linker-drugs that are unstable in studies with mice and rat and are therefore potentially sensitive to cleavage by CES1c (17, 26). So far, modifying the structure of the linker-drug is the general mitigation to enhance ADC stability in mouse plasma, even though the linker-drug is perfectly stable in human plasma and from that perspective does not require further optimization. In order to circumvent linker-drug optimization specifically for the mouse, it was attempted to block mouse-specific CES1c activity in vivo. Initial experiments with esterase inhibitors failed to completely block CES1c activity. Thus, immune-compromised CES1c−/− mice were needed to evaluate the full impact of the absence of CES1c activity on PK and in vivo efficacy in PDX models. Cross-breeding of CES1c−/− mice with SCID and nude mice was setup in parallel. The cross-breeding of CES1c−/− mice with nude mice showed poor reproductive performance, whereas the SCID cross-breeding was successful. Pilot PDX studies in CES1c−/− SCID mice suggested improved efficacy of SYD985, compared with previous studies in CES1c+/+ nude mice. The full PKPD study in the MAXF1162 PDX model with T-DM1 to control for potential strain differences confirmed improved efficacy of SYD985 in CES1c−/− SCID mice showing tumor stasis at 1 mg/kg. At this dose, the PK profile and AUC are very similar to those at the recommended phase II dose in human. This shows that use of CES1c−/− mice is a good and for us the preferred alternative to unnecessary structural optimization of the linker-drug that might even compromise on human-relevant parameters.

Conclusions

Even though it is known that the rodent-specific esterase CES1c is able to cleave linker-drugs on ADCs at distinct sites, the impact of CES1c activity on the predictive value of PK, efficacy and safety studies with ADCs in mice and rats should not be underestimated. Here it is shown that cleavage of vc-seco-DUBA results in the formation of a covalent bond between CES1c and the ADC, which...
indicates that the structure-activity relationship of CES1c is more complex than originally thought. It not only depends on the structure of the linker-drug, but also on the site of conjugation in the ADC. Consequently, developing linker-drugs that are stable in both mice and humans, remains challenging. The use of CES1c in mice widens the spectrum of linker-drugs suitable for the development of ADCs, thereby increasing the chance that ADCs are successfully developed.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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