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Original article

Enzyme-linked immunosorbent assays for quantification of MMAE-conjugated ADCs and total antibodies in cynomolgus monkey sera

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ABSTRACT

Antibody-drug conjugates (ADCs) are commonly heterogeneous and require extensive assessment of exposure-efficacy and exposure-safety relationships in preclinical and clinical studies. In this study, we report the generation of a monoclonal antibody against monomethyl auristatin E (MMAE) and the development, validation, and application of sensitive and high-throughput enzyme-linked immunosorbent assays (ELISA) to measure the concentrations of MMAE-conjugated ADCs and total antibodies (tAb, antibodies in ADC plus unconjugated antibodies) in cynomolgus monkey sera. These assays were successfully applied to in vitro plasma stability and pharmacokinetic (PK) studies of SMADC001, an MMAE-conjugated ADC against trophoblast cell surface antigen 2 (TROP-2). The plasma stability of SMADC001 was better than that of similar ADCs coupled with PEG4-Val-Cit, Lys (m-dPEG24)-Cit, and Val-Cit linkers. The developed ELISA methods for the calibration standards of ADC and tAb revealed a correlation between serum concentrations and the OD_{450} values, with R^2 at 1.000, and the dynamic range was 0.3–35.0 ng/mL and 0.2–22.0 ng/mL, respectively; the intra- and inter-assay accuracy bias% ranged from −12.2% to −5.2%, precision ranged from −12.4% to −1.4%, and the relative standard deviation (RSD) was less than 6.6% and 8.7%, respectively. The total error was less than 20.4%. The development and validation steps of these two assays met the acceptance criteria for all addressed validation parameters, which suggested that these can be applied to quantify MMAE-conjugated ADCs, as well as in PK studies. Furthermore, these assays can be easily adopted for development of other similar immunoassays.

1. Introduction

Antibody-drug conjugates (ADCs), composed of monoclonal or bispecific antibodies covalently conjugated with cytotoxic payloads via chemical linkers, are designed to selectively attack pathogenic tissues while limiting collateral damage to normal tissues [1–3]. The increasing number of ADCs being developed requires the alignment of bioanalytical methods regarding throughput,

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reproducibility, convenience, and ability to cross host species [4,5]. However, the heterogeneous nature of ADCs presents unique challenges to their quantitative analysis in biological samples [6–9]. At least three different assays are commonly required to detect ADC molecules (carrying at least one payload), total antibodies (tAb, i.e., conjugated plus unconjugated antibodies), and payloads.

The antimitotic agent monomethyl auristatin E (MMAE), which is highly hydrophobic and extremely toxic to use alone, has been successfully adopted as an ADC payload for multiple ADCs [10,11]. The drug product of MMAE-conjugated ADCs is usually a heterologous mixture of molecules having different drug-to-antibody ratio (DAR) values, with the average DAR values typically set between 2 and 4 to retain sufficient efficacy while limiting molecular aggregation caused by the hydrophobicity of MMAE. Owing to the popularity of MMAE-conjugated ADCs being developed across the industry, it is important to develop simple and high-throughput quantification assays to monitor their stability and pharmacokinetic (PK) profiles.

This study generated a specific anti-MMAE monoclonal antibody (mAb) with a high binding affinity for MMAE. Utilizing this antibody, a cost-effective and straightforward enzyme-linked immunosorbent assay (ELISA) was developed and validated for the detection of MMAE-conjugated ADCs. In addition, an ELISA for tAb was developed. Both the assays were successfully applied to in vitro plasma stability and PK studies of SMADC001, an MMAE-conjugated ADC. SMADC001 is one of several ADC molecules under preclinical or clinical development and is composed of trophoblast cell surface antigen 2 (TROP-2) targeting mAb hRS7. TROP-2 is a type I transmembrane glycoprotein overexpressed in several human carcinomas [12,13]. In contrast to other TROP-2-targeting ADCs, SMADC001 adopted a proprietary hydrophilic PEG4-Val-Lys (m-dPEG24) linker, and our results demonstrated that this linker improved the stability and PK profiles of ADC molecules. In addition to measuring SMADC001 and total hRS7 levels, the developed ELISAs can be applied to the stability and PK studies of any MMAE-conjugated ADCs.

2. Materials and methods

2.1. Materials

Triple Quad 5500 Triple Quad LC-MS/MS System (SCIEX, Framingham, MA, USA), Shimadzu LC-30AD high-performance liquid chromatography (HPLC) system (Shimadzu, Tokyo, Japan), MSX electronic balance (Sartorius AG, Göttingen, Germany), and Infinite F50 microplate reader (Tecan Trading AG, Zürich, Switzerland) were used.

Tween 20 was purchased from Sinopharm Co., Ltd. (Shanghai, China). Human-TROP-2-ECD-His protein and mouse anti-human IgG-Fc secondary antibody (HRP) were purchased from Sino Biological Inc. (Beijing, China). Cell Counting Kit-8 (CCK8) was purchased from Dojindo Laboratories (Tokyo, Japan). Goat anti-human IgG (H + L) (HRP) was purchased from ABclonal Technology Co., Ltd. (Wuhan, China). Bovine serum albumin (BSA), complete Freund's adjuvant (CFA), Freund's incomplete adjuvant (IFA), diethylenetriaminepentaacetic acid (DTPA), Tris(2-carboxyethyl) phosphine (TCEP), methanol, and acetonitrile were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Formic acid and ammonium acetate were purchased from Aladdin Co., Ltd. (Shanghai, China). MMAE and the innate standard D8-MMAE were purchased from Shanghai Haoyuan Chemexpress Co., Ltd. (Shanghai, China).

Naïve cynomolgus monkey serum/sera (NMS) was obtained from the Center for Drug Safety Evaluation and Research (CDSER) of Shanghai Institute of Materia Medica (SIMM), Chinese Academy of Sciences (CAS). The pooled naïve cynomolgus monkey serum/sera (PNMS) was prepared from samples of ten NMS. The PNMS was used to prepare standards, quality controls, and other test samples.

The humanized TROP-2 antibody hRS7 was expressed using the plasmid constructed by Govindan et al. [14]. It was produced in HEK293F cells (Shanghai Cell Line Bank, Shanghai, China) through transient transfection and purified via protein A affinity chromatography.

The syntheses of hRS7-PEG4-Val-Lys (m-dPEG24)-PAB-MMAE (named SMADC001), hRS7-PEG4-Val-Cit-PAB-MMAE (named PE), hRS7-Val-Cit-PAB-MMAE (named VC), and hRS7-PEG4-Lys (m-dPEG24)-Cit-PAB-MMAE (named LC) have been described previously [15]. Linker drugs were synthesized by Sorrento Therapeutics (San Diego, CA, USA). To prepare ADCs, hRS7 was treated with a 10-fold molar equiv. of TCEP in PBS (pH 7.4) and 1 mM DTPA, for 2.5 h at 37 °C, followed by conjugation to different linker drugs in 75 mM NaAc (pH 6.5) and 1 mM DTPA overnight at 4 °C (20-fold molar equiv.); the buffer was changed to PBS using an Amicon Ultra 30 K centrifugal filter unit (Millipore, Bedford, MA, USA).

2.2. Characterization of SMADC001

2.2.1. Binding to hTROP-2-ECD-His protein

A direct ELISA was used to assess the binding affinity of SMADC001 to the hTROP-2-ECD-His protein. After being coated with 2 μg/mL hTROP-2-ECD-His protein overnight at 4 °C, the 96-well plates were washed with PBS, blocked with 1% casein in PBS (Thermo Fisher Scientific, Waltham, MA, USA), and then incubated with 3-fold serial dilutions of SMADC001 at 26 °C for 1 h. Each sample was assayed in quadruplicate. HRP goat-anti-human IgG (H + L) was added at 1:2000 dilution, and the plate was incubated at 26 °C for 1 h. Then, TMB peroxidase substrate (SeraCare, Milford, MA, USA) was added, and the absorbance was read at 450 nm using a SpectraMax M5e microplate reader (Molecular Devices, LLC, San Jose, CA, USA).

2.2.2. Cytotoxicity assays

The human tumor cell line BXPC-3 purchased from American Type Culture Collection (ATCC) was cultured in RPMI-1640 (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA), and 10,000 cells/well were seeded into 96-well plates. After an overnight incubation, different concentrations of SMADC001 were added, and the plates were incubated in a humidified chamber for 96 h at 37 °C and 5% CO_2. The CCK8 viability assays were performed 4 days after treatment. The absorbance signals at 450 nm were detected using a SpectraMax M5e microplate reader. Growth inhibition was evaluated as the percentage of the growth of treated cells relative to that of the untreated control cells. The IC_{50} values were determined using nonlinear logistic regression.

2.3. Preparation of mAbs against MMAE

2.3.1. Generation of mouse mAbs

BSA was conjugated to the Val-Cit-PAB-MMAE linker-drug to generate the BSA-Val-Cit-MMAE immunogen (Fig. 1). The preparation method was the same as that for ADC synthesis described in Section 2.1. Four- to five-week-old female BALB/c mice (Shanghai Model Organisms Center, Inc., Shanghai, China) were subcutaneously injected with 50 μg of BSA-Val-Cit-MMAE immunogen emulsified in CFA. IFA was used as a booster administered with the same amount of immunogen at 3-week intervals. Seven to ten days after the fourth injection, serum samples were collected and analyzed via ELISA. The mouse with the highest titer was selected and injected intraperitoneally with 50 μg immunogen to boost immunization.

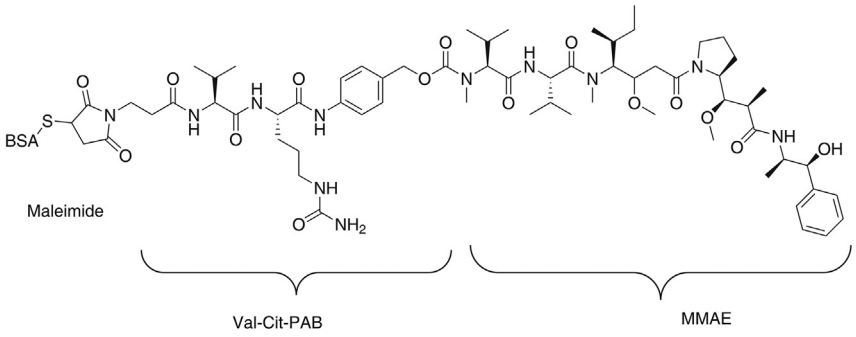

Fig. 1. Chemical structure of monomethyl auristatin E-bovine serum albumin (MMAE-BSA) immunogen.

2.3.2. Production and purification of mAb

Spleen cells from immunized mice were fused with mouse SP2/0 myeloma cells at a ratio of 5:1. Hybridoma supernatants were screened for binding to the MMAE-conjugated hRS7 antibody and BSA-Val-Cit-MMAE. Hybridomas positive for MMAE conjugates were expanded and then assessed for binding to the BSA-Val-Cit-MMAE immunogen via ELISA. The cross-binding positive cell clones were used to obtain the heavy chain and light chain variable region sequences of the candidate anti-MMAE mAb using PCR. After aligning with the sequence of the patented antibody [16], the DNA fragment was cloned into pCAT1.0 and pCAT2.0, and transiently transfected HEK293F cells with polyethyleneimine (PEI, Polysciences, Inc., Warrington, PA, USA). Supernatants were collected after 6 days of culture and purified via protein A affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography (SEC) were used for analyzing antibody purity.

2.3.3. Binding of anti-MMAE antibody to SMADC001

The binding affinity of the mAb against MMAE was determined as follows: after coating with 2 μg/mL of anti-MMAE antibody, a 96-well plate was incubated for 1 h at room temperature with 3-fold serial dilutions of SMADC001 in 1% casein. Each sample was assayed in quadruplicate. The control wells were unconjugated with hRS7 antibodies. The plates were washed with 0.1% PBST thrice and then incubated with HRP goat anti-human IgG (H + L) at 1:2000 dilution and TMB peroxidase substrate. The OD_{450} values were measured using a microplate reader after the reaction was stopped with 2 M H_2SO_4.

2.4. Sandwich ELISA procedure

2.4.1. Assay 1: SMADC001 ADC

The plate was coated with a mouse anti-MMAE antibody (4 μg/mL) and incubated overnight at 4 °C. After washing with 0.1% PBST thrice and blocking with 3% BSA, ADC standard with serial dilutions (0.30, lower anchor point), 0.50 (lower limit of quantification, LLOQ), 0.80, 1.50, 4.00, 10.0, 20.0, 28.0, and 35.0 ng/mL (upper limit of quantification, ULOQ); quality control (QC) samples (0.80, low-quality control, LQC), 10.0 (middle-quality control, MQC), and 26.250 ng/mL (high-quality control, HQC); and "spiked-in" samples with pooled monkey sera were dispensed into different wells and incubated at 37 °C for 1 h. After washing, 100 μL of an HRP-conjugated mouse anti-human IgG-Fc antibody solution (100 ng/mL) was added and the plate was incubated at 37 °C for 1 h. After washing the plate thrice with 0.1% PBST, TMB substrate was added and the reaction was stopped with 2 M H_2SO_4 before detection of the absorbance signal at 450 nm.

2.4.2. Assay 2: SMADC001 tAb

A 96-well plate was coated with hTROP-2-ECD-His (3 μg/mL) protein and incubated overnight at 4 °C. After washing with 0.1% PBST thrice and blocking with 3% BSA, the ADC standard diluted to series of concentrations (0.20 (lower anchor point), 0.60 (LLOQ), 1.00, 3.00, 6.00, 10.0, 15.0, 20.0, and 22.0 ng/mL (ULOQ), QCs (1.50 (LQC), 6.00, (MQC), and 16.5 ng/mL (HQC), and "spiked-in" samples with pooled monkey sera were dispensed into different wells and incubated for 1 h. After washing, 100 μL of HRP-conjugated mouse anti-human IgG-Fc antibody (100 ng/mL) was added to each well, and the plate was incubated at 37 °C for 1 h. After washing thrice, TMB peroxidase substrate was added and the reaction was stopped with 2 M H_2SO_4 before detection of the absorbance signal at 450 nm.

2.4.3. Assay 3: MMAE

Chromatographic separation was performed using an XBridge HILIC column (3.0 mm × 50 mm, i.d. 5 μm; Waters, Milford, MA, USA) with an injection volume of 20 μL. Gradient elution was performed with 95%–50% mobile phase B (0.01% FA and 2 mM NH_4Ac in 98% ACN), whereas mobile phase A consisted of 0.01% formic acid (FA) and 2 mM NH_4Ac in 50% MeOH. The gradient started at 95% B and linearly decreased to 50% B over 2.2 min. It was maintained at 50% for 0.8 min before returning to 95% (over 0.01 min), and reconditioned for 1 min at a flow rate of 0.5 mL/min at 35 °C.

A Triple Quad 5500 mass spectrometer equipped with an electrospray ionization (ESI) source operated in the positive ion model was used. The collision energy (CE) for MMAE and the innate standard D8-MMAE were 38 and 37 V, respectively. The declustering potential (DP) for MMAE and D8-MMAE was 180 V. The collision cell exit potential (CXP) was 40 V, dwell time 100 ms, ion source gas 1 (GS1) 45 psi, curtain gas (CUR) 40 psi, collision gas (CAD) 6 psi, entrance potential (EP) 10 V, source temperature 400 °C, and IS 5500. Multiple-reaction monitoring (MRM) was performed to quantify MMAE and the innate standard. The transition *m/z* 718.6/686.5 was detected for MMAE, and *m/z* 726.6/694.6 was used to detect the innate standard.

2.4.4. Statistical analysis

Data were analyzed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), and the results are presented as mean ± standard deviation (SD).

2.5. Validation of method

Detailed validation of the methods was performed according to the US FDA Bioanalytical Method Validation Guidance for Industry (2018).

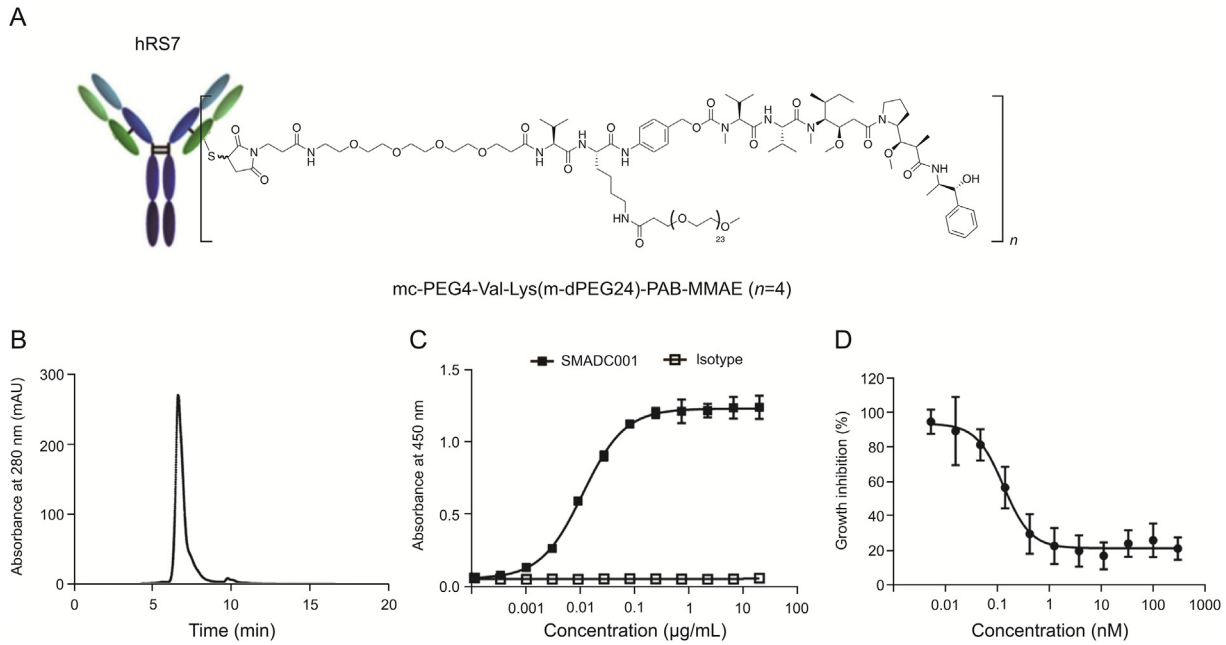

Fig. 2. Structure and characterization of SMADC001. (A) Chemical structure of SMADC001 with linker-payload. (B) SMADC001 appeared to have a single peak with 98.3% purity on the size exclusion chromatography (SEC) column after purification. (C) The EC_{50} value of SMADC001 binding to hTROP-2-ECD-His protein (human trophoblast cell surface antigen 2 extracellular domain, His tag) was 0.0112 μg/mL. Each point represents mean ± SD ($n = 4$). (D) Cytotoxicity assay showed efficacious antitumor activity of SMADC001 in BXPC-3 cells. The IC_{50} value was 0.128 nM. Each point represents mean ± SD ($n = 4$).

2.5.1. Intra- and inter-assay precision and accuracy

Intra- and inter-assay precision and accuracy were determined by analyzing QC samples prepared at five concentration levels (ULOQ, HQC, MQC, LQC, and LLOQ, respectively). Three replicates from each level were measured in one run for the intra-day assay. Two analysts measured three replicates at each level in three different batches to assess inter-day variability. Precision was expressed as the relative standard deviation (RSD, RSD% = standard deviation (SD)/mean × 100%). Accuracy was determined as bias% between the measured and nominal concentrations (bias% = (average measured concentration − nominal concentration)/nominal concentration × 100%). The concentration bias% based on the mean concentration of three sets of samples should be within ±20.0% of the nominal value at each concentration level (±25.0% at the ULOQ, LLOQ). The RSD% concentration based on the concentrations of the three sets of samples should not exceed 20.0% (25.0% at ULOQ, LLOQ). Total error (|Bias%| + RSD%) ≤ 30.0% (ULOQ and LLOQ ≤40.0%).

2.5.2. Matrix effect and selectivity

This method was developed to quantify ADC and tAb concentrations in NMS; therefore, PNMS were selected as matrix blank to prepare calibrator standards and QC samples. Ten individual NMS with different dilutions (1:10, 1:20, 1:50, and 1:100, *V/V*) were compared with the dilution buffer to ensure that the results would not be affected by any endogenous components present in the serum. The minimum required dilution was selected for subsequent sample analysis.

Ten individual NMS were used to evaluate the selectivity of the developed ELISA. SMADC001 was added to make the final

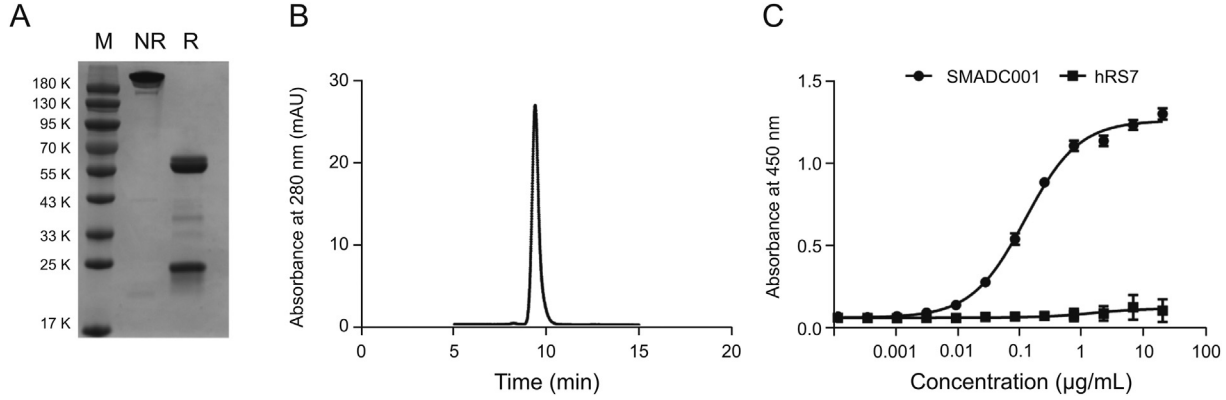

Fig. 3. Biochemical characterization of purified anti-MMAE monoclonal antibody (mAb). (A) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified anti-MMAE monoclonal antibody (mAb). M: standard protein marker; NR: purified mAb under non-reducing conditions; R: purified mAb under reducing conditions. (B) Size exclusion chromatography (SEC) analysis of purified anti-MMAE mAb. No peak (aggregates) of high molecular weight was observed. (C) Binding affinity between anti-MMAE mAb and SMADC001 was analyzed via enzyme-linked immunosorbent assay (ELISA). The EC_{50} value was determined to be 0.120 μg/mL. The unconjugated hRS7 antibody was used as the negative control. Each point represents mean ± SD ($n = 4$).

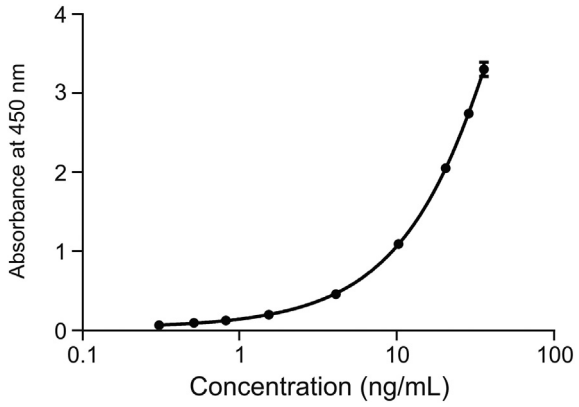

Fig. 4. Typical standard curve for the developed ELISA for measuring SMADC001 in cynomolgus monkey serum with a wide range of concentrations (0.5–35.0 ng/mL; 0.3 ng/mL was set as the lower anchor point). Results were regressed with a 4-parameter logistic model, and each point represents mean ± SD ($n = 3$).

concentrations of the testing samples equal to those of HQC, and the back-calculated concentrations of the individual samples were compared with the nominal concentrations.

2.5.3. Plasma stability

The in vitro plasma stability of the linker between the antibody and the cytotoxic payload was evaluated by spiking MMAE conjugates with different linkers (SMADC001, PE, VC, and LC) into cynomolgus monkey plasma and storing at 37 °C for 0 h, 24 h, 72 h, 5 days, and 7 days. The concentrations of spike-in samples were then assessed.

2.6. PK studies of SMADC001 in cynomolgus monkeys

The PK profiles of SMADC001 were evaluated in cynomolgus monkeys after a single intravenous infusion. Four cynomolgus monkeys (weighing 2.8–6 kg) were divided into two groups: the vehicle group (injected with SMADC001 buffer) and the SMADC001 group (injected with 3 mg/kg SMADC001 via intravenous infusion (hindlimb vein, 30 min/dose/monkey), with one animal/gender/group. The dosing day was defined as day 0. Blood samples were collected at time points of pre-dose (0 h), immediately post-dose (5 min), 1 h, 4 h, 8 h, and 24 h (day 1), 48 h (day 2), 96 h (day 4), 168 h (day 7), 240 h (day 10), 336 h (day 14), 504 h (day 21), and 672 h (day 28) in all groups.

Blood samples were kept at room temperature for at least 1 h before centrifugation (2000 *g*, 10 min, 4 °C) to harvest sera. The sera were divided into five aliquots and stored in a freezer at −80 °C until analysis.

Concentration-time curves were plotted using GraphPad Prism 9. The following parameters were calculated using non-compartmental models: maximum serum concentration (c_{max}), time of the maximum serum concentration (t_{max}), elimination half-life ($t_{1/2}$), area under the serum concentration-time curve (AUC_{0-IFN}), clearance rate (CL), apparent volume of distribution (V_d), and mean retention time (MRT).

All procedures in this study were performed in accordance with the animal welfare policies and the SIMM and CAS guidelines. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of SIMM. The IACUC number is 2016–08-RJ-127.

Table 1
Intra- and inter-assay precision and accuracy of quality control samples (QCs) of antibody-drug conjugate (ADC).

QCs	ULOQ	HQC	MQC	LQC	LLOQ
Nominal concentration (ng/mL)	35.000	26.250	10.000	1.000	0.500
Batch 1	30.570	23.268	8.961	0.901	0.419
Batch 2	32.685	25.150	9.258	0.924	0.442
Batch 3	33.698	25.988	9.792	1.019	0.456
N	3	3	3	3	3
Mean	32.318	24.802	9.337	0.948	0.439
SD	1.596	1.393	0.421	0.063	0.019
RSD (%)	4.9	5.6	4.5	6.6	4.3
Bias (%)	−7.7	−5.5	−6.6	−5.2	−12.2
Total error (%)	12.6	11.1	11.1	11.8	16.5

ULOQ: upper limit of quantification; HQC: high quality control; MQC: middle quality control; LQC: low quality control; LLOQ: lower limit of quantification; SD: standard deviation; RSD: relative standard deviation.

3. Results and discussion

3.1. Biochemical and pharmacological characterization of SMADC001

SMADC001 (Fig. 2A) was generated by conjugating PEG4-Val-Lys (m-dPEG24)-PAB-MMAE to hRS7 antibody (see Section 2). The conjugation reaction yielded a product with a purity of 98.3%, as analyzed via SEC (Fig. 2B). The product was determined, via LC-MS/MS analysis, to be a mixture of ADC molecules with different DAR values, and the average DAR value was 4.3 (Fig. S1). SMADC001 could bind to hTROP-2-ECD-His protein with a high affinity, and the EC_{50} value was 0.0112 μg/mL, as detected with direct ELISA (Fig. 2C). It showed potent antitumor activity against TROP-2-expressing BXPC-3 cells, with an IC_{50} value of 0.128 nM. Our results indicated that SMADC001 was potentially an efficacious antitumor agent (Fig. 2D).

3.2. Generation and characterization of anti-MMAE mAb

The quality, purity, and binding affinity of anti-MMAE mAb were investigated using SDS-PAGE, SEC, and ELISA, respectively. As expected, the light and heavy chains were approximately 25 and 50 kDa, respectively (Fig. 3A), and the purity of the mAb was almost 100% (Fig. 3B). The mAb showed high affinity to SMADC001 (Fig. 3C), with an EC_{50} of 0.120 μg/mL.

3.3. Assay validation

3.3.1. Calibration curves and the accuracy and precision of the QCs

Calibration standards in the range of 0.30–35.0 ng/mL (ADC) and 0.20–22.0 ng/mL (tAb) were prepared with PNMS and diluted to 1:50 (*V/V*; the minimum dilution multiple, MRD) with dilution buffer before analysis. A four-parameter regression model was adopted to convert the OD_{450} values into concentrations. A typical curve is shown in Fig. 4.

Table 2
Intra- and inter-assay accuracy and precision of QCs of total antibody (tAb).

QCs	ULOQ	HQC	MQC	LQC	LLOQ
Nominal concentration (ng/mL)	22.000	16.500	6.000	1.500	0.600
Batch 1	23.163	16.291	5.773	1.596	0.569
Batch 2	20.973	15.612	5.662	1.465	0.523
Batch 3	20.936	15.420	5.416	1.341	0.485
N	3	3	3	3	3
Mean	21.691	15.774	5.617	1.467	0.526
SD	1.275	0.458	0.183	0.128	0.042
RSD (%)	5.9	2.9	3.3	8.7	8.0
Bias (%)	−1.4	−4.4	−6.4	−2.2	−12.4
Total error (%)	7.3	7.3	9.6	10.9	20.4

Table 3
Matrix effects of naïve cynomolgus monkey sera (NMS) (absorbance at 450 nm).

Matrix No.	100% serum	10% serum	5% serum	2% serum	1% serum	Dilution buffer
1	0.373	0.083	0.058	0.052	0.042	0.036
2	0.224	0.082	0.052	0.043	0.042	0.041
3	0.213	0.087	0.059	0.045	0.040	0.038
4	0.126	0.071	0.051	0.044	0.038	0.035
5	0.183	0.125	0.089	0.052	0.048	0.039
6	0.314	0.075	0.050	0.044	0.047	0.040
7	0.291	0.083	0.060	0.045	0.049	0.037
8	0.179	0.096	0.064	0.044	0.039	0.038
9	0.550	0.119	0.068	0.047	0.049	0.037
10	0.688	0.070	0.054	0.043	0.045	0.036

Table 4
The Selectivity for the SMADC001 and tAb. Each point represents mean ± SD ($n = 3$).

Matrix No.	Bias (%)[a]	
	ADC	tAb
1	−27.7	−14.8
2	2.4	18.0
3	17.1	17.8
4	11.2	16.5
5	9.9	3.3
6	−18.0	−9.0
7	−15.4	−4.9
8	−3.6	6.3
9	19.0	6.0
10	6.8	27.9

[a] From the nominal concentration of HQC ADC: antibody-drug conjugate; tAb: total antibodies. ADC: antibody-drug conjugate; tAb: total antibodies.

3.3.2. Accuracy and precision of the assay

The results of intra- and inter-assay accuracy and precision of the QCs of ADC and tAb are summarized in Tables 1 and 2. The intra- and inter-assay bias ranged from −12.2% to −5.2% and from −12.4% to −1.4%, respectively, and the RSD% was less than 6.6% and 8.7%, respectively. The total error was below 20.4% for all the calibration standards.

3.3.3. Matrix effects and selectivity

A summary of the matrix effects of NMS is provided in Table 3. The OD_{450} values at each concentration measured in different diluted NMS were not significantly different from those of the dilution buffer, and samples were diluted 1:20 or below. The instrument signals from 10 NMS samples diluted 50 times or more approached those of the dilution buffer to set an MRD of 50 to reach a concentration within the dynamic range, without altering the assay precision or accuracy (Tables S1 and S2).

To further evaluate the interference from matrix components, HQC level selectivity test samples were prepared from ten individual sera. The bias% of nine out of ten test samples was within 20.0%, indicating that the measurements of SMADC001 or tAb were not affected by the matrix (Table 4).

3.3.4. Plasma stability

The in vitro plasma stability assay is a commonly applied method that demonstrates ADC stability and is a helpful tool to evaluate stability across nonclinical animal species applied in toxicity and efficacy studies, including in humans [9]. Given that the properties of linkers strongly influence the PK profiles of ADCs, four MMAE-conjugated ADCs with different linkers were prepared, and their in vitro plasma stability was assessed. Table 5 shows that the HQC and LQC of SMADC001 maintained at 37 °C were stable for up to 5

Table 5
Plasma stability of SMADC001 and ADCs with different linkers after different storage time.

Sample	Concentration (ng/mL)	Measured concentration (ng/mL)					Bias of the 0 h[a] (%)			
		0 h	24 h	72 h	5 days	7 days	24 h	72 h	5 days	7 days
SMADC001-HQC	26.250	25.462	24.495	23.699	20.849	19.733	−3.8	−6.9	−18.1	−22.5
SMADC001-LQC	1.000	1.073	0.952	0.954	0.885	1.306	−11.3	−11.1	−17.5	21.7
PE-HQC	26.250	14.738	10.250	9.334	8.436	8.017	−30.5	−36.7	−42.8	−45.6
LC-HQC	26.250	10.906	8.179	8.290	7.978	7.466	−25.0	−24.0	−26.8	−31.5
VC-HQC	26.250	14.168	6.803	5.785	5.129	5.667	−52.0	−59.2	−63.8	−60.0

[a] Measured concentration bias (%) at different storage times compared to 0 h. PE: hRS7-PEG4-Val-Cit-PAB-MMAE; LC: hRS7-PEG4-Lys (m-dPEG24)-Cit-PAB-MMAE; VC: hRS7-Val-Cit-PAB-MMAE.

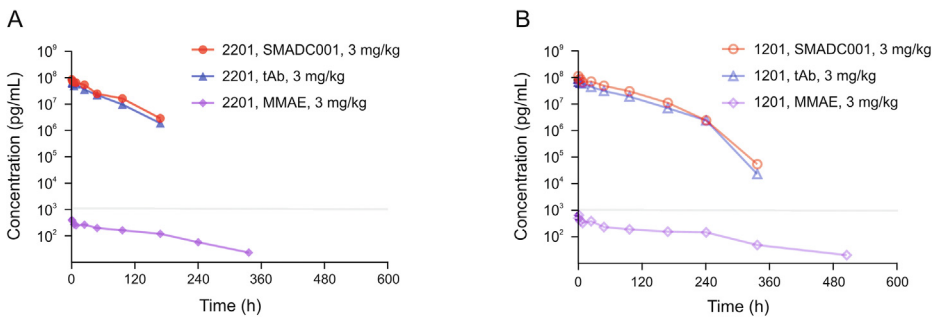

Fig. 5. Pharmacokinetic profiles of SMADC001, tAb, and free MMAE in cynomolgus monkeys following a single intravenous infusion (3 mg/kg) of SMADC001. Serum concentrations of SMADC001, tAb, and free MMAE were measured via ELISA and LC-MS/MS. (A) Female cynomolgus monkeys. (B) Male cynomolgus monkeys. Each point represents mean ± SD ($n = 2$).

Table 6
Pharmacokinetic (PK) parameters in cynomolgus monkeys after a single intravenous infusion of SMADC001.

PK parameter	ADC (3 mg/kg)	tAb (3 mg/kg)
$t_{1/2}$ (h)	25.2 ± 4.95	30.8 ± 4.65
t_{max} (h)	0.083 ± 0	0.083 ± 0
c_{max} (μg/mL)	101 ± 19.8	77.7 ± 17.5
$AUC_{0\text{-}INF}$ (h × μg/mL)	5810 ± 2150	4060 ± 1280
V_d (mL/kg)	20.9 ± 11.4	33.7 ± 5.71
CL (mL/h/kg)	0.554 ± 0.205	0.778 ± 0.246
$MRT_{0\text{-}INF}$ (h)	57.7 ± 13.4	55.8 ± 15.5

$t_{1/2}$: the elimination half-life; t_{max}: time of the maximum serum concentration; C_{max}: the maximum serum concentration; $AUC_{0\text{-}INF}$: the area under the serum concentration-time curve; V_d: the apparent volume of distribution; CL: the clearance rate; $MRT_{0\text{-}INF}$: the mean retention time.

days, and the concentration in the plasma was beyond the acceptance criteria by 7 days. As the measured LQCs of ADCs with PE, LC, and VC linkers were below the LLOQ of the assays, only the stability of their HQC samples was compared. The measured concentrations of PE, LC, and VCs at 0 h differed from the theoretical value of 26.250 ng/mL (approximately 1/2 of the theoretical value), which may be attributed to the standard curve prepared with SMADC001, as there might be some detection errors. The bias% of PE, LC, and VCs was greater than 20.0% at 24 h, indicating that most of the reduction in concentration and instability occurred during the first 24 h, with a more gradual decrease during 7 days. SMADC001 appeared stable during the first 24 h, with a bias of −3.8%, and decreased gradually over time. In addition, we observed that the deconjugation rates of SMADC001, PE, LC, and VCs were identical during 24 h–7 days (80%, 78%, 91%, and 83%, respectively), which might be due to nonspecific protease activity occurring during incubation in monkey plasma at 37 °C. In conclusion, the in vitro plasma stability of SMADC001 was better than that of ADCs with PE, LC, and VC linkers. The ELISA assay utilizing a MMAE antibody can be applied to the plasma stability study of MMAE-ADCs similar to that of another mAb, SG3.33, described by Shen et al. [17]. Both our antibody and SG3.33 could recognize conjugated MMAE. Sanderson et al. [18] also described an mAb (SG3.218) to recognize and quantify free MMAE. However, it is not clear whether our antibody or SG3.33 can recognize free MMAE.

3.4. PK studies

The PK profiles of SMADC001, tAb, and MMAE in cynomolgus monkeys following a single intravenous infusion (3 mg/kg) of SMADC001 are shown in Fig. 5. Serum concentrations of SMADC001, tAb, and free MMAE were measured as described in Section 2. The corresponding PK parameters are listed in Table 6. The serum concentration of SMADC001 was similar to that of tAb throughout the time course, indicating a slow release of the drug by SMADC001 in the circulation, which is consistent with our result of free MMAE measurement. Although the overall AUC of SMADC001 appeared to be slightly higher than that of tAb, they were essentially the same, considering the margins of errors of the two different assays. This indicates that the developed ELISAs are specific and sensitive enough to quantify MMAE-conjugated ADCs and tAbs for pharmacokinetic studies in cynomolgus monkeys.

4. Conclusions

This study generated a specific antibody with a high affinity for MMAE using hybridoma technology. The generated antibody was used to develop a direct ELISA for MMAE-conjugated antibody detection with a calibration range of 0.5–35.0 ng/mL. We also developed and evaluated an ELISA suitable for measuring the serum concentrations of total antibodies in cynomolgus monkeys, with a calibration range of 0.6–22.0 ng/mL. Compared to LC-MS/MS-based assays to detect conjugated antibodies and total antibodies, ELISA methods facilitate testing of samples without further pre-treatment, such as pre-affinity capture or proteolytic digestion, which saves the analysis time and ensures sensitivity, reproducibility, and high-throughput capacity. The developed assays were successfully applied to the plasma stability study and PK studies of SMADC001, an ADC composed of humanized TROP-2 antibody hRS7, MMAE payload, and optimized linker PEG4-Val-Lys (m-dPEG24), and demonstrated that the ADC was stable in monkey plasma, both in vitro and in vivo. These assays can be applied to quantify any MMAE-conjugated ADCs, and the development and validation steps described in this study can be easily adopted for development of other similar immunoassays.

CRediT author statement

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Declaration of competing interest

Chunhe Wang is an employee of Dartsbio Pharmaceuticals, China. The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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