

Synthesis and Biological Evaluation of Antibody Drug Conjugates Based on an Antibody Expression System: Conamax

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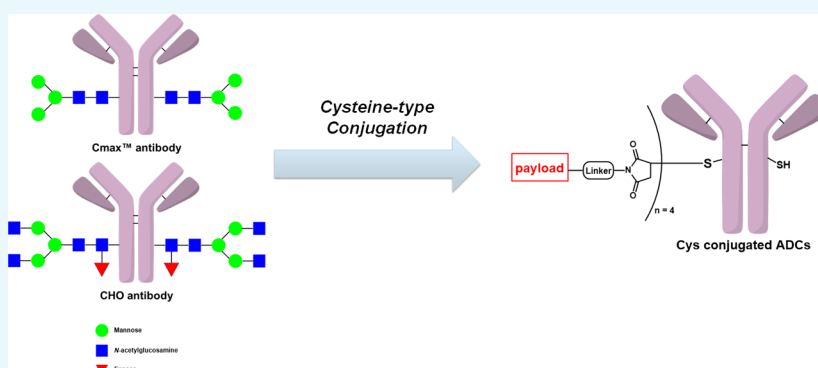
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ABSTRACT: Antibody production for ADCs (or in general) is commonly performed by CHO-based platforms and limited by volumetric productivity, expensive downstream purification, and extended optimization timelines. The Conamax platform is a novel microbial-based protein production and secretion system. A suite of synthetic biology tools have enabled high volumetric productivity (>1 g/L/d) and glycoengineering to produce simple and consistent human-like post-translational modifications. Conamax can be engineered to secrete genuine, functional monoclonal antibodies that have been successfully used to make antibody drug conjugates (ADCs) via cysteine-linked conjugation. Specifically, we evaluated ADCs derived from both a Conamax-produced anti-HER2 antibody and comparable commercially sourced Chinese hamster ovary (CHO)-produced material in an NCI-N87 gastric cancer xenograft model. Conjugation efficiency and resulting analytical data indicated comparable ADC quality and attributes. No statistical difference was observed between Conamax- and CHO-derived test articles thereby indicating similar efficacy and function. These results further demonstrate the potential of Conamax as a useful platform for the discovery and production of therapeutic antibodies and ADCs.

INTRODUCTION

Antibody drug conjugates (ADCs) have become a major class of anticancer therapeutics and remain a common modality for targeted approaches to treating human oncology diseases.¹ Several ADCs are currently on the market, including Adcetris (brentuximab vedotin) from Seattle Genetics, Kadcyla (trastuzumab emtansine) from Genentech,^{2–4} and Mylotarg (gemtuzumab ozogamicin) and Besponsa (inotuzumab ozogamicin) from Pfizer. Additionally, more than 60 ADCs are currently undergoing clinical evaluation.^{1,5}

ADCs consist of a monoclonal antibody covalently linked to a cytotoxic payload. A characteristic of ADCs compared to conventional chemotherapy is their histological selectivity¹ driven by the affinity of antibodies to bind to protein targets and antigens. Generally, approaches to implement ADCs in a human oncological setting involve selecting an antibody against an antigen target, which is expressed by the diseased cells but only lowly (or not) by normal tissues.⁶ In this way,

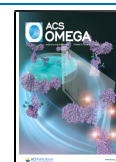
ADCs may afford a widened therapeutic window/index compared to untargeted chemotherapy.⁷

Many commercial manufacturing processes for therapeutic mAbs and ADCs involve the use of the Chinese hamster ovary (CHO) cells.⁸ CHO cells are typically able to express glycoproteins with human-like glycosylation patterns, and over the past few decades, considerable cell line improvements have been made to intensify the production processes to increase volumetric productivity, resulting in an average cost of goods sold for \$71/g in fed batch processes.⁹ Although some perfusion production systems have achieved productivities

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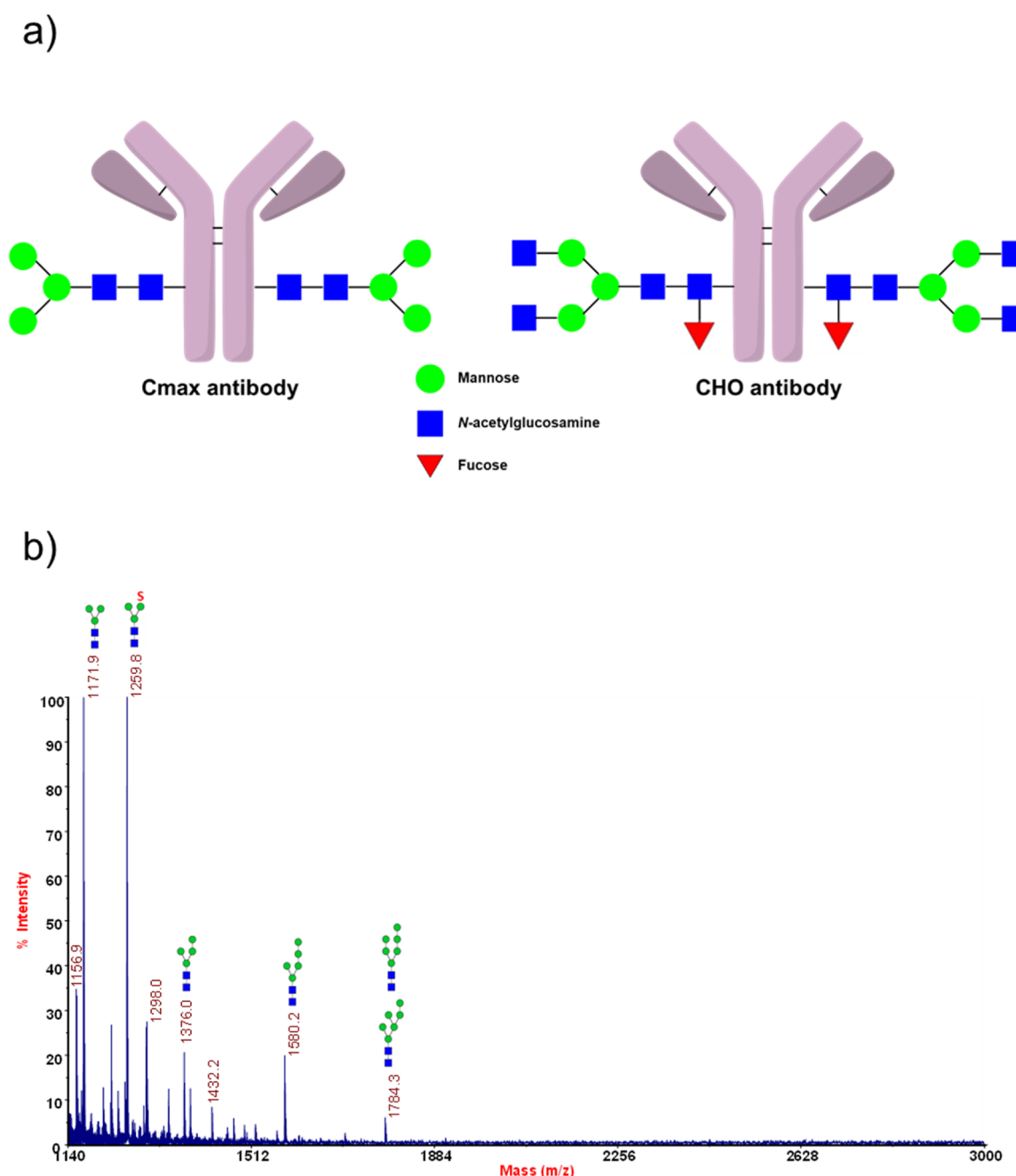


Figure 1. Glycoprofile of Conamax antibody. (a) Predominant glycoform comparison: proposed Conamax antibody structure (left) and known CHO antibody structure (right). (b) Glycoform analysis of an antibody (described here as Conamax anti-ERBB2/HER2) produced by a strain modified to produce primarily paucimannose structures. Reprinted with permission from 13. Copyright 2018.

upward of 2 g/L/d, the productivity gains in CHO have been incremental despite considerable effort. This has led to recent exploration into microbial production platforms, which have numerous production advantages over complex mammalian cells, as alternatives to CHO for the production of complex protein therapeutics like mAbs and ADCs.

A recent collaboration among Amyris Inc. and Biogen that was funded by the Bill and Melinda Gates Foundation assessed the attributes of eight eukaryotic microbes for potential development platforms to produce mAbs.¹⁰ Some key characteristics that were outlined by this group and others include rapid growth, dedicated organelles and functions for protein processing and secretion, genetic amenability, robust-

ness with respect to glycoengineering, high specific productivities, a minimal secretome with no co-secreted proteases, low-cost media, and process tolerance toward shear, pH, and temperature.^{10,11}

Conamax (formerly Cmax), a proprietary microbial protein production and secretion system, was developed by Synthetic Genomics Inc. and later acquired by Conagen, Inc. for use as an alternative to CHO for the manufacture of complex protein therapeutics.^{12,13} The Conamax system is based on a marine protist in the genus *Aurantiochytrium* of the class Labyrinthulomycetes, which is emerging as an attractive group of hosts for production of pharmaceutically useful compounds. Like industrial yeasts, the Conamax organism is robustly ferment-

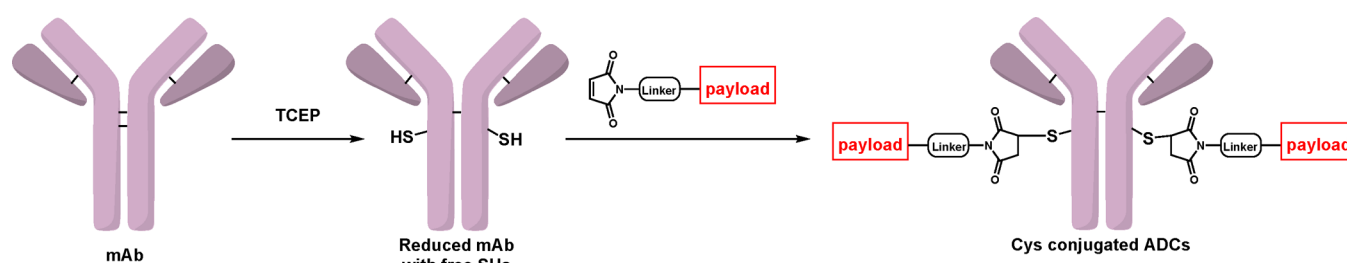


Figure 2. Conventional cysteine-type conjugation utilizing reduction of interchain disulfide bonds and conjugation to a maleimide linker payload.

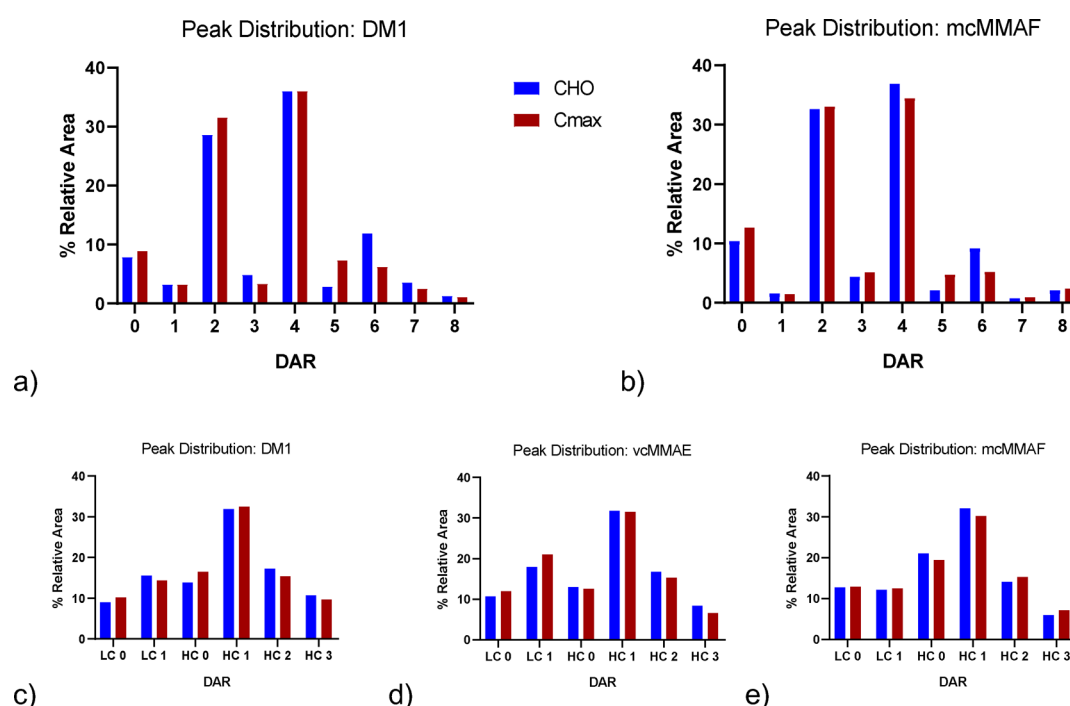


Figure 3. Peak distribution profiles from HIC-HPLC for Conamax anti-ERBB2/HER2 and commercially sourced CHO trastuzumab conjugated to (a) DM-1 and (b) mcMMAF. Additionally, reduced RP-HPLC distribution profiles for conjugated light chain (LC) and heavy chain (HC) are shown for both antibodies conjugated to (c) DM-1, (d) vcMMAE, and (e) mcMMAF.

able. It and a number of other related species are routinely grown at scales over 100 kL in fermentations for food or feed applications. Genera related to *Aurantiochytrium* were first commercialized for their highly efficient lipid metabolism, which appears to also translate to efficient exosome production. This feature in particular has previously been exploited for production of a vaccine, which was found to be very effective at preventing influenza in a murine challenge model.¹⁴ Unlike Ascomycete yeasts, the Conamax organism does not natively produce hyper-mannosylated glycoproteins. Instead, this organism's glycoproteins are generally decorated with smaller, high-mannose glycans (fewer than nine mannose molecules branching from the di-*N*-acetylglucosamine stem, GlcNAc₂). Furthermore, this host has been manipulated to produce a paucimannose proteoglycan (GlcNAc₂Man₃), which comprises a minority portion of the glycoform profile of CHO cells and human glycoproteins (Figure 1). The occupancy rate of the paucimannose glycan was also enriched, yet despite all of these manipulations, the physiology and robustness in fermentation of Conamax were preserved.¹³ These and other intrinsic features of the platform have led to the development of a suite of synthetic biology tools tailored for biologics production, which has enabled high-productivity (>1 g/L/d)

mAb production with high purity. This platform has intrinsic advantages for biologics production over mammalian-based and cell-free systems.

The distinct advantages of Conamax in terms of cost, speed, and increased productivity prompt us to conduct further investigation of Conamax-produced antibodies for use in ADCs. We report here preliminary studies showing comparable conjugation efficiency and drug distribution profiles of Conamax-produced anti-ERBB2/HER2 conjugated to maytansinoid and auristatin payloads via cysteine-linked conjugation.^{15,16} Trastuzumab is a humanized IgG1 monoclonal antibody that binds to the human ERBB2/Her2 antigen, which is highly expressed on breast, ovarian, and gastric cancers.¹⁷ We evaluated ADCs derived from both Conamax and commercially sourced CHO-produced trastuzumab in an NCI-N87 gastric cancer xenograft model. The evaluation of CHO and Conamax expression systems for use in antibody drug conjugates (ADCs) utilizes the clinically validated and commercially available trastuzumab antibody and Conamax-produced anti-ERBB2/Her2 antibody. No statistical difference was observed between Conamax- and CHO-derived test articles thereby indicating similar efficacy and function. Our experiments and *in vivo* efficacy results serve as the initial proof

of concept for the use of the microbial Conamx-derived antibodies in ADC technology as a challenger to traditional CHO technology.

RESULTS AND DISCUSSION

The antibody-drug conjugates (ADCs) were synthesized by conventional stochastic cysteine-maleimide conjugation resulting in a heterogeneous distribution of drugs per antibody (Figure 2).¹⁵

Preliminary evaluation of drug incorporation and conjugation feasibility of the two antibodies was conducted utilizing two linker payloads, DM1 and mcMMAF. Equivalent reaction conditions were utilized to produce four ADCs: Conamx-DM1, CHO-DM1, Conamx-MMAF, and CHO-MMAF. The ADCs were characterized by HIC-HPLC for the drug to antibody ratio (DAR); additionally, an orthogonal comparison was conducted by RP-HPLC with the addition of two ADCs, Conamx-MMAE, and CHO-MMAE.¹⁶ The evaluation results were comparable as shown in Figure 3 (detailed method and procedures are described in the Experimental Section).

HIC-HPLC results of the four ADCs showed similar trends and drug distribution profiles (Table 1), and the orthogonal

evaluation by reduced RP-HPLC also confirmed the trends observed by HIC. However, a little discrepancy of DAR distributions between CHO trastuzumab conjugates and Conamx trastuzumab conjugates was observed in the HIC chromatogram (Figure 3a,b).^{18–20} HIC analysis is known to provide a relatively lower peak resolution, which has a potential risk to cause overlapping with each peak;²¹ therefore, we selected RP-HPLC for further comparison study.

The initial conjugation study indicated that Conamx anti-ERBB2 and commercially sourced CHO trastuzumab have similar conjugation efficiency (maximum of 0.3 DAR unit variability).²²

In addition to DAR comparison, an initial stability study utilizing size exclusion chromatography (SEC) was conducted to compare aggregation levels both before and after conjugation (Figure 4). The conjugation and purification steps involve exposure to organic solvent and several buffer exchange steps. This comparison was useful and served as an initial stability assessment. These stability study results indicated that both trastuzumab-CHO- and trastuzumab-Conamx-derived ADCs have no significant difference in relative monomer amounts before and after conjugation (less than 1%).

After preliminary conjugation assessment, Conamx-derived ADCs and CHO-derived ADCs and the two corresponding parent antibodies were evaluated in an HER2-positive, NCI-N87 gastric cancer xenograft model. Xenografts were grown to an average tumor volume of 120 mm³ and placed into 12 treatment groups. Treatments occurred twice a week for 2 weeks. Tumor volume and body weight were measured twice a week during the treatment period.

The average tumor volume for each arm of the study is separated by dosage in Figure 5a (full *in vivo* plot in Figure S1). There were four treatment groups (trastuzumab-CHO-MMAE at 2.5 and 5 mg/kg and trastuzumab-Conamx-MMAE at 2.5 and 5 mg/kg) where the tumors shrunk to a point where they could no longer be measured accurately (Figure 5f). It should be noted that, in the measurements, when only scar tissue was visible, a measurement of 2 × 2 mm was recorded. In most instances, a 2 × 2 mm measurement meant no discernable

Table 1. Comprehensive Comparison of Drug Distribution Profiles by RP-HPLC^a

production system	drug linker					
	DM1		mcMMAF		vc-MMAE	
	CHO	Conamx	CHO	Conamx	CHO	Conamx
LC 0	9.0	10.2	11.6	12.9	10.7	12.0
LC 1	15.6	14.4	14.0	12.5	17.9	21.0
HC 0	13.9	16.5	15.8	19.4	13.0	12.6
HC 1	31.9	32.5	31.6	30.2	31.8	31.5
HC 2	17.2	15.4	16.9	15.3	16.8	15.3
HC 3	10.7	9.6	8.7	7.1	8.4	6.6
DAR by RP-HPLC	3.9	3.7	3.6	3.3	3.8	3.8

^a% Relative area from Figure 3c–e.

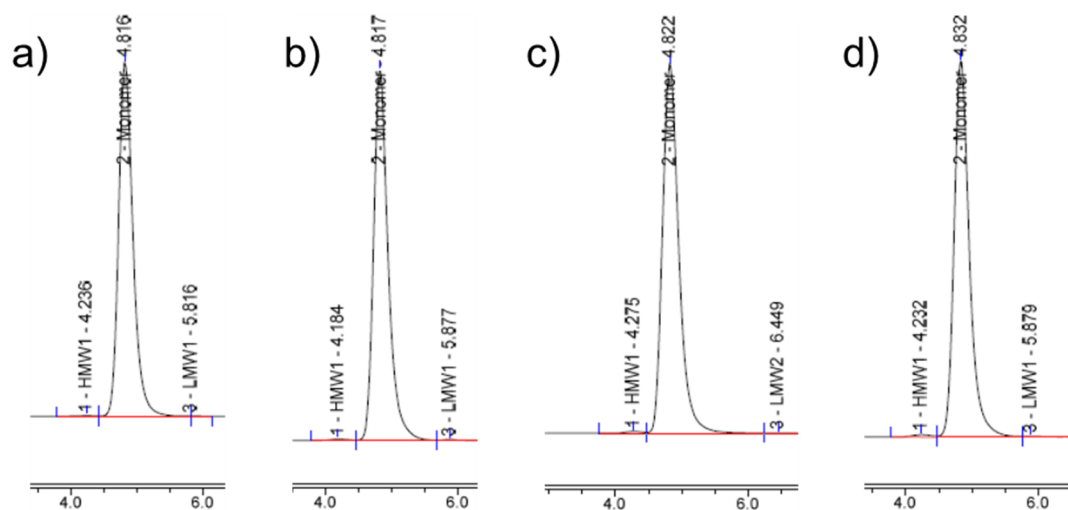


Figure 4. Initial stability study by SEC: (a) trastuzumab-CHO (99.8% monomer), (b) trastuzumab-Conamx (99.1% monomer), (c) trastuzumab-CHO-MMAE (99.4% monomer), and (d) trastuzumab-Conamx-MMAE (98.8% monomer).

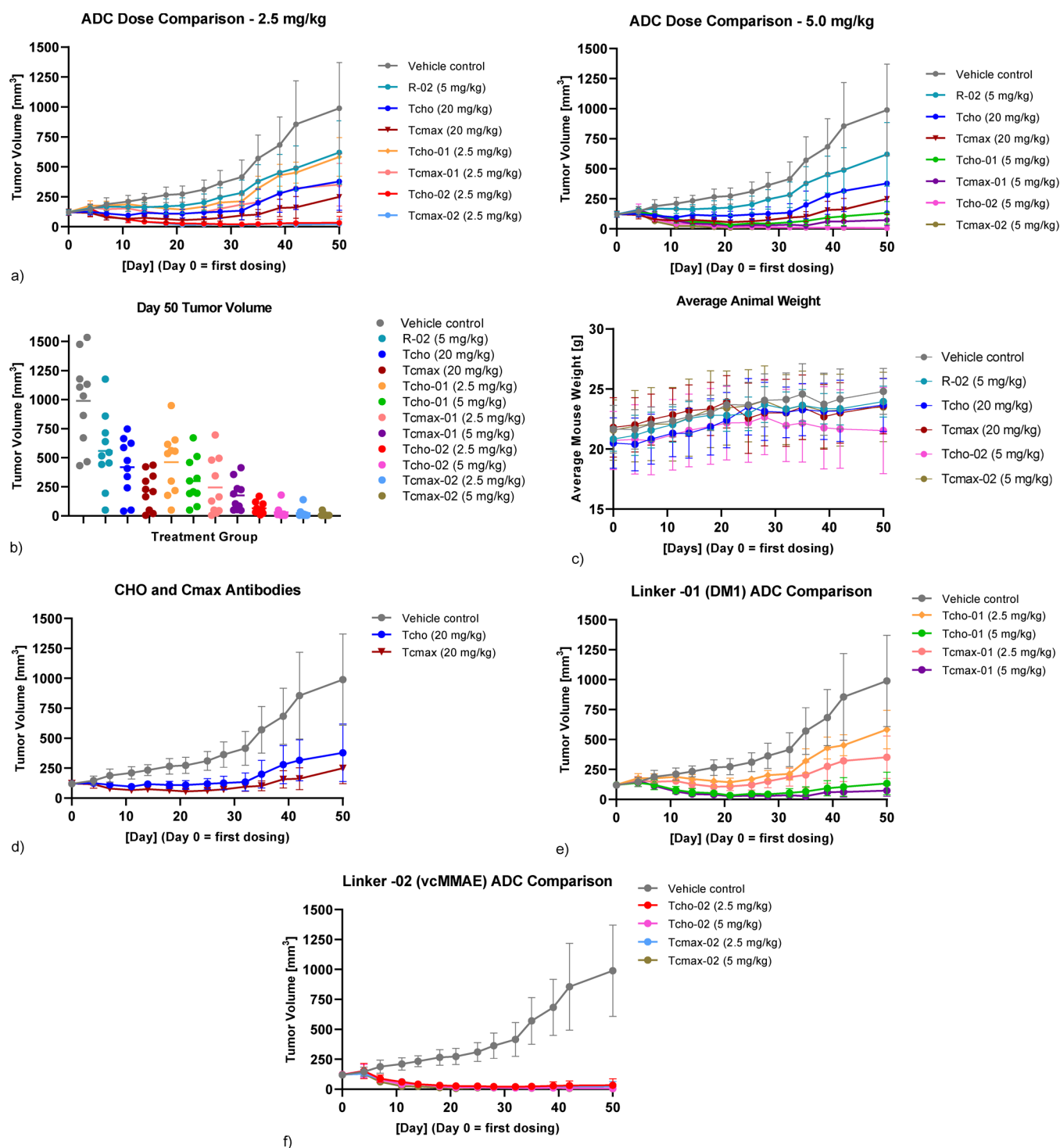


Figure 5. Antitumor activity of anti-HER2 ADCs in the NCI-N87 xenograft tumor models. Each arm of the study is shown in the (a) ADC dose comparison plots at 2.5 and 5.0 mg/kg doses. Treatment was administered to mice when the mean tumor volume reached ~ 120 mm³. Error bars represent s.d. (see Experimental Section for full *in vivo* data). (b) Individual tumor volumes on day 50 in each cohort with mean and standard errors. (c) Average animal weight from the highest-dose group and most toxic drug linker, along with Tcho and Tcmax, for the duration of the study. (d) parent antibody comparison, Conamax-anti ERBB2 and CHO-trastuzumab, (e) linker-01 (DM1) ADC comparison, and (f) linker-02 (vcMMAE) ADC comparison.

tumor. Figure 5b includes the individual tumor volumes on day 50 in each cohort with mean and standard errors.

No significant weight change was observed for the groups from the first day of treatment through day 50 (Figure S2). The highest-dose group and most toxic drug linker along with trastuzumab-CHO and trastuzumab-Conamax for the duration

of the study are shown in Figure 5c. All groups gained weight, which would be expected for young mice. The significantly lower average weight loss of the trastuzumab-CHO-MMAE (5 mg/kg) group on day 50 was primarily due to just two outliers. It is not known if this was related to toxicity of the test agent or some other external factor. All animals in groups 1–12 made it

through the study without outward manifestations of morbidity or health complications (no mice died while on study).

The parent antibodies, trastuzumab-CHO (Tcho) and trastuzumab-Conamax (Tcmax), dosed at 20 mg/kg were comparable during the course of the study. Initially, the tumor volume was stabilized, but after day 35, growth was observed (Figure 5d). These results indicated that a 20 mg/kg dose of both parent antibodies did not qualify as a minimum effective dose (MED); however, the curve of the tumor volume treated by Tcho matched with the results of Tcmax. Both Tcho and Tcmax unconjugated antibodies showed some tumor growth inhibition in this model, and this has been seen by our research group before in a previous unrelated study.²³

Tcho-01 (trastuzumab-CHO-DM1) and Tcho-02 (trastuzumab-CHO-MMAE) are more efficacious at the higher dose of 5 mg/kg (Figure 5e,f). The MED of Tcho-01 was determined to be 5 mg/kg, which was identical with that of Tcmax-01 (trastuzumab-Conamax-DM1). In the case of MMAE-type ADCs, the MED of Tcho-02 was decreased to 2.5 mg/kg, which was identical with that of Tcmax-02 (trastuzumab-Conamax-MMAE). The negative control ADC (Rituximab-MMAE) did not show tumor inhibition.

These three comparative results (naked antibody, DM1-type ADC, and MMAE-type ADC) indicate that antibodies produced with Conamax display the same efficacy and tumor selectivity as CHO-produced antibodies.

Additionally, we conducted a statistical analysis using ANOVA with posthoc analysis (DUNNETT) for all of the groups. The results showed a statistical difference at day 50 compared to the vehicle-treated mice (Table S2).

The number of drugs per antibody (DAR) was targeted to be equivalent for the test article samples. We report herein comparable conjugation efficiency, drug distribution profiles and *in vivo* xenograft efficacy results using ADCs based on antibodies produced from both CHO and Conamax platforms. The principal structural difference between conventional trastuzumab produced by CHO cells and Conamax anti-ERBB2/HER2 is in their respective glycostructures. Glycoform analysis of trastuzumab glycans as produced by the Conamax system is shown in Figure 1. Glycoforms of conventional trastuzumab were reported elsewhere but chiefly comprise three or four fucosylated forms with either terminal GlcNAc or Gal residues.^{24,25} Minor glycan species with terminal mannose residues, similar to those produced by the Conamax system, were also reported in this work. It is unlikely that these differences in glycan profiles would contribute to functional differences using the immunocompromised nude mouse model of the present study as the effector cells that are sensitive to Fc glycosylation are depleted. The dominant mechanism of action for ADCs is broadly understood to be receptor-mediated delivery of the payload to the tumor site (or vicinity). However, the potential contribution of other activities related to Fc-receptor binding should not be ignored. Future investigations will address these structure–function questions and may also seek to optimize Conamax glycans for cell-mediated cytotoxic effects. With that said, even in immunocompetent subjects, there is some debate as to whether antibody glycan structures have any impact on the effectiveness of ADCs. The results from this work indicate that the differing glycostructures of Conamax and CHO antibody–drug conjugates did not confer differing effects. Considered together, these results indicate that the Conamax antibody

production technology platform is a viable challenger to the commonly used CHO production platforms.

In summary, Conamax anti-ERBB2/HER2 and commercially sourced CHO trastuzumab were conjugated utilizing the cysteine-linked conjugation platform and achieved similar DARs for vcMMAE, mcMMAF, and DM1. The corresponding ADCs displayed similar drug distribution profiles as reported by HIC-HPLC and RP-HPLC. An *in vivo* study was initiated to compare the antitumor effects of ADCs derived from the Conamax production platform to those from traditional CHO-derived ADCs using the HER2+NCI-N87 gastric cancer xenograft model: no statistical difference was observed in tumor volume reduction. The experiments included in this paper serve as the initial proof of concept for the microbial Conamax-derived antibodies in ADC technology as a challenger to traditional CHO technology. Future studies of Conamax technology as the next leading platform for production of therapeutic antibodies and ADCs are in progress.

■ EXPERIMENTAL SECTION

Materials. CHO-produced trastuzumab (Herceptin) of 150 mg was purchased from Roche Pharmaceutical Company (Switzerland). Cmax-produced anti-ERBB2/HER2 was provided from Synthetics Genomics, Inc. (U.S.A.). SMCC-DM-1, MC-VC-MMAE, and MC-MMAF were purchased from Abzena (U.S.A.). All other chemical reagents were purchased from Sigma-Aldrich (U.S.A.).

General ADC Conjugation Process. The antibodies were initially buffer-exchanged into the appropriate reaction buffer, pH 7.5, 50 mM PBS, and 10 mM EDTA, to prepare for the conjugation process. The reduction reaction began with the addition of a defined molar ratio of tris-(2-carboxyethyl) phosphine hydrochloride (TCEP HCl) to the antibody and proceeded with incubation for 1.5 h at 20 °C. Conjugation began with the initial addition of cosolvent DMA (8% v/v) to ensure solubility, and then 7 equiv of the drug linker was added and mixed for 2 h at 20 °C. The unreacted drug linker was quenched with the addition of 25 equiv of *N*-acetyl-L-cysteine (NAC) and mixed for 1 h at 20 °C. The final mixture was purified using illustra G-25 desalting columns and eluted with pH 5.2 20 mM histidine, 5.5% trehalose.

DAR Determination by HIC-HPLC. The average drug to antibody ratio (DAR) was determined by HIC on a MabPac HIC Butyl, 4.6 × 100 mm, 5 μm (Thermo Fisher Scientific), connected to an Agilent 1260 HPLC system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and diode array detector. The system ran at 0.8 mL/min at 30 °C using 1.5 M (NH₄)₂SO₄, 100 mM NaHPO₄/NaH₂PO₄, pH 7.0 (mobile phase A, MPA) and 100 mM NaHPO₄/NaH₂PO₄, pH 7.0 (mobile phase B, MPB), and the absorbance was monitored at 280 nm (reference wavelength at 450 nm). All samples (1 mg/mL, 20 μL) were injected into the system sequentially at 0.8 mL/min and eluted with a 25 min method consisting of a 2 min wash at 100% MPA, 12 min linear gradient from 100% MPA to 100% MPB, 2 min wash using 100% MPB, 1 min linear gradient from 100% MPB to 100% MPA, and 8 min re-equilibration at 100% MPA.

DAR Determination by RP-HPLC. An orthogonal method for DAR determination was conducted under denaturing and reducing conditions using a reversed-phase HPLC column. Reduced samples were prepared as follows: 1.0 mg/mL ADCs in 20 mM histidine, 5.5% trehalose was diluted to 0.64 mg/mL

with the addition of 500 mM tris, pH 7.5, and 8 M guanidine-HCl and reduced with addition of 1 M DTT. The mixture was incubated at 80 °C for 10 min. RP-HPLC analysis was performed on an Advance Bio RP-mAb Diphenyl, 2.1 × 100 mm, 3.5 μm (Agilent, PN: 795775-944), connected to an Agilent 1260 HPLC system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and diode array detector. The system ran at 0.3 mL/min at 70 °C using 0.1% trifluoroacetic acid (TFA), 2% ACN in water (mobile phase A, MPA) and 0.1% TFA in acetonitrile (ACN) (mobile phase B, MPB), and the absorbance was monitored at 280 nm (reference wavelength at 450 nm). All samples (0.64 mg/mL, 20 μL) were injected into the system sequentially and eluted with a 33 min method consisting of a 2 min isocratic hold at 32% MPB, 25 min linear gradient from 32% to 48% MPB, 4 min wash using 95% MPB, and 3 min re-equilibration at 32% MPB.

Initial Stability Study by SEC. Monomer amounts of the ADCs were determined by SEC using an AdvanceBio SEC 300 Å, 4.6 × 150 mm, 2.7 μm column (Agilent) connected to an Agilent 1260 HPLC system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and diode array detector. The system was run at 0.25 mL/min at 30 °C using 100 mM NaHPO₄/NaH₂PO₄, 250 mM NaCl, and 10% v/v isopropanol, pH 6.8 (mobile phase, MP), and the absorbance was monitored at 280 nm (reference wavelength at 450 nm). All samples (1 mg/mL, 40 μL) were injected into the system sequentially and eluted over 15 min using 100% MP.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.9b03628>.

In vivo xenograft results as described in the text (PDF)

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Notes

The authors declare no competing financial interest.

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