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Research Article

Preclinical Characterization of CTL-1, A Biosimilar Anti-EGFR Monoclonal Antibody for Cetuximab

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Abstract

The monoclonal antibody (mAb) market has helped millions of patients across the globe in treating serious and chronic diseases. The expiry of patents for the first generation of approved therapeutic mAbs has led to the development and authorization of biosimilar alternatives for which the market is of great interest due to the demand of affordable treatments. Here we report the preclinical development of a biosimilar mAb to cetuximab (Erbitux®) referred to as CTL-1. Analyses of the physicochemical properties and biological activities of both CTL-1 and cetuximab were performed to allow for comparisons of the primary structure, glycoform heterogeneity, *in vitro* anti-tumor activity, and *in vivo* pharmacokinetics, tissue distribution, and efficacy of the two antibodies. Our results confirm that CTL-1 has the same amino acid sequence and a similar glycosylation profile as the cetuximab reference. *In vitro* bioassays indicate that CTL-1 and cetuximab achieve comparable dose-dependent inhibition of EGFR+ tumor cell proliferation, and maintain similar abilities to induce Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC). Preclinical studies in mice confirm that the proposed biosimilar to cetuximab is comparable with regard to its pharmacokinetic and tissue distribution properties as well as its ability to reduce tumor growth *in vivo*. Based on these comprehensive similarity studies, CTL-1 was confirmed to be highly similar to the reference mAb cetuximab and is suggested as a viable biosimilar alternative to this important therapeutic mAb.

Introduction

Monoclonal antibodies (mAbs), initially introduced for their potential to treat a variety of conditions including cancer, autoimmune diseases, neurological disorders, and infections, now represent one of the fastest growing segments of the biotechnology industry [1]. However, the increasing use of therapeutic mAbs constitutes a major and increasing cost burden for health care systems. Biosimilars are defined as biologically similar, but not identical, alternatives to commercial reference products that are submitted for separate marketing approval following patent expiration [2]. These products have the advantage of improving affordability and accessibility to therapies that otherwise may be restrictive for cost reasons [3]. Regulatory approval is provided on the basis of comparable quality, safety, and efficacy to originator products [4].

Therapeutic mAbs are mainly produced in mammalian cell expression systems, and are thus subject to post-translational modifications (also known as product quality attributes), thereby exhibiting batch-to-batch variability due to potential changes in the manufacturing process [5,6]. Matching biological function and quality attributes is the art of biosimilar development, including demonstration of expected functions without gain of new functions. Thus, thorough comparative analytical characterization, *in vitro* and *in vivo* biological assessment of the attributes between the proposed biosimilar and approved reference product is the first step required in the development of a biosimilar [5]. The proposed CTL-1 antibody is being developed by Cancer Therapeutics Laboratories, Inc. (Los Angeles, CA, USA) as a biosimilar to cetuximab (Erbitux˚; US Patent No. 6,217,866. April 17, 2001), a recombinant mAb that binds to and blocks the extracellular ligand site on the Epidermal Growth Factor Receptor (EGFR) which is important for treating a number of solid cancers [7]. This antibody not only inhibits successive downstream tyrosine kinase activity, but also causes internalization, degradation, and down-regulation of EGFR. The human-murine chimeric IgG1 antibody, cetuximab, binds exclusively to EGFR with a 2-log higher affinity to EGFR than to EGF and TGF-α. Cetuximab also binds, internalizes, and degrades EGFR-vIII, a mutant receptor molecule found in various cancers including head and neck squamous cell carcinoma that has shown resistance to traditional anti-EGFR small molecule therapies. EGFR-bound cetuximab also blocks cellular proliferation via inhibition of progression past the G0/G1 cell-cycle phase by inhibiting the production of vascular endothelial growth factor, IL-8, basic-fibroblast growth factor, and other pro-angiogenic cytokines. It promotes pro-apoptotic protein expression while also decreasing expression of anti-apoptotic proteins. Furthermore, the Fc portion of the antibody can elicit an Antibody-Dependent Cell-Mediated Cytotoxic (ADCC) response from the host immune system. This leads to a wide-spread uptake and destruction of the antibody as well as the antibody-

targeted tumor cell complex. As an IgG1 molecule, cetuximab can induce ADCC responses from a variety of leukocytes including natural killer cells, eosinophils, mast cells, dendritic cells, and B cells [7]. Characteristic of EGFR-blocking antibodies, cetuximab, produces acne-like rashes in about 80% of patients. The severities of these rashes have been correlated to treatment response and survival [8]. It is expected that a biosimilar produced with a glycoprofile matching that of cetuximab will replicate these clinical symptoms to the same extent as the originator antibody [9].

Currently, cetuximab is approved for the treatment of head and neck cancer (particularly those tumors resistant to platinum based therapy) and for patients with EGFR-expressing Metastatic Colorectal Carcinoma (mCRC) without KRAS mutations. Off-label treatment in clinical practice is used for EGFR-expressing Non-Small Cell Lung Carcinoma (NSCLC) [7]. Ongoing clinical and laboratory research has found multiple solid-organ tumors expressing significant amounts of EGFR and exhibiting considerable responses to anti-EGFR antibody treatment. These include: pancreatic carcinoma, gastric cancer, urothelial and renal carcinoma, glioblastoma multiforme, neuroblastoma, and anaplastic thyroid carcinoma [10-15]. This supports the need for an inexpensive, widely available monoclonal antibody targeted against EGFR in order to promote the advancement of wider, more efficient applications for the antibody. At present, the anti-EGFR antibody panitumumab, a recombinant fully human IgG2 monoclonal antibody, is approved as a monotherapy for the treatment of patients with EGFR-expressing metastatic colorectal cancer after disease progression with standard chemotherapy [16]. In addition to cetuximab and panitumumab, a humanized anti-EGFR antibody, matuzumab, is currently in clinical trials for the treatment of stomach, ovarian, and lung cancer [17]. Here we report the pre-clinical characterization of the proposed cetuximab biosimilar CTL-1 using comprehensive analytical techniques to determine its similarity with multiple samples of originator cetuximab reference product. The potential impact of differences in quality attributes was further evaluated in a comprehensive nonclinical development program which comprised comparative studies to show that the effector functions – inhibition of cell proliferation, induction of ADCC activity – are similar *in vitro* between CTL-1 and originator cetuximab. Furthermore, studies comparing biosimilar CTL-1 with the originator product in mice are reported, providing preclinical confirmatory evidence of *in vivo* similarity with regard to pharmacokinetic properties and efficacy, as well as biodistribution in tumor and normal tissues. The overall aim of these comparability studies was to confirm the biosimilarity of CTL-1 and commercially approved cetuximab prior to beginning clinical trials.

Materials and Methods

Cell lines

All cell lines were obtained from the American Type Culture Collection (ATCC) or were gifted to our laboratory. Cell lines were grown in complete medium [RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone, Inc., Logan, UT), non-essential amino acids, penicillin G, and streptomycin (Gemini Bio-Products, West Sacramento, CA)] in humidified, 5% CO_2 incubators maintained at 37°C. The CTL-1 biosimilar anti-EGFR cell line was developed for high secretion of antibody, ability to grow in serum free medium, and near identical physical characteristics. After transfection of the

antibody heavy and light chain DNA, cells were grown in selective medium [Hybridoma SFM without L-glutamine (Gibco/Invitrogen, Carlsbad, CA), 10% dFCS (Hyclone, Inc.), 2% GSEM supplement (Sigma-Aldrich, St. Louis, MO), 1% non-essential amino acids solution (Corning, Inc.), and Penicillin/Streptomycin solution (Corning, Inc.)]. The CTL-1 cell line was then obtained by numerous subcloning procedures by limiting dilution using 50 mL Erlenmeyer flasks with continuous shaking in a 37°C humidified incubator and slow adaptation to serum free medium [(Hybridoma SFM (Gibco/Invitrogen), 250x Cholesterol Lipid Concentrate (Gibco/Invitrogen), 2% GSEM supplement (Sigma-Aldrich), and 1% non-essential amino acids solution (Corning, Inc.)] without antibiotics. For large scale production required for antibody characterization studies, *in vitro* analyses, and *in vivo* efficacy and pharmacokinetic experiments, the cells were grown in 3 liter or 8 liter stir flasks at 37°C with aeration. After confluence, the cells were removed by tandem 5 μm and 0.2 μm filter units (Sartobran P, Sartorius) and subjected to tandem Protein A affinity and ion exchange (SP and Q Sepharose) chromatography using a Pharmacia FPLC apparatus. After elution, the antibody was passed through 0.2 μm syringe filter for sterile vialing. The cell line used to produce CTL-1 is proprietary to the company and in large scale culture, produces 1.5-2g/L.

Antibody sequencing

Prior to mass spectrometry analysis, CTL-1 and cetuximab (Bristol-Myers Squibb, New York, NY) were digested with PNGase F (New England Biolabs, Ipswich, MA) for 18 hours at 37°C to remove N-linked glycans. Antibody heavy chain and light chain fragments were then isolated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (ThermoFisher Scientific, Waltham, MA) as per manufacturer's instructions. Heavy and light chain bands were subsequently excised and sent to the Proteomics Core Facility of the University of Southern California. Three combinations of enzymatic digestions were performed on deglycosylated samples to achieve 100% sequence coverage upon LC-MS analysis: Trypsin + Chymotrypsin, Trypsin + Glu-C, and Trypsin + Asp-N. LC/MS experiments were performed on a Q-Exactive Hybrid quadrupole orbitrap mass spectrometer connected to an Easy-nLC 1000 system. The analytical column was an EASY-Spray column, 25 cm long with 75 μm ID, filled with 2 μm C18 particles with 100 Å pore size (ThermoFisher Scientific, Waltham, MA). The trapping column was a 5 mm guard column with 300 μm ID, filled with 5 μm C18 particles with 100 Å pore size (LC Packings, Amsterdam, Netherlands). The analytical column was operated at 40°C, while the trap column was kept at room temperature. Entire LC/MS connections were connected using nanoViper Fingertight Fittings purchased from ThermoFisher. Tryptic peptide mixture was resolved on a 150-minute gradient. After gradient equilibration for 5 minute at 2% B, gradient increased to 28% B in 100 minute and to 44% B in 40 minutes. The shallow gradient followed with a flushing step at 90% B for 5 minutes. The flow rate maintained at 300 nL/min. Prior to every run, the trap column was equilibrated with 4 μL of buffer A with 0.5 μL/min flow rate, and the analytical column was equilibrated with 12 μL of buffer A with a maximum pressure of 700 bar. The needle and sample loop were washed during LC/MS run three times each of 25 μL of water. Every sample run was followed with a blank run of 5 μL injected buffer A to avoid cross talk among samples. Data-dependent acquisition was performed with a survey scan with a resolution of 70,000 at 200 m/z

with AGC target of 1e6 and maximum injection time of 60 ms with scan range of 375 to 1700 m/z. Top 10 precursor ions were selected for fragmentation with a resolution of 17,500 at AGC target of 5x104 and maximum injection time of 64 ms. The collision energy was set at 27 NCE. The ions with 2 to 6 charges were selected for fragmentation and dynamic exclusion was set at 30 second. Data independent acquisition was performed with 70 consecutive windows with 17,500 at 200 m/z resolution and NCE of 27, ranging from 410 to 900 amu.

NP-HPLC analysis of N-linked glycans

CTL-1 and cetuximab were digested as above with PNGase F (New England Biolabs, Ipswich, MA) for 18 hours at 37°C to remove N-linked glycans. Protein was then precipitated by cold ethanol precipitation for one hour. After centrifugation, supernatant containing the removed glycans was collected and concentrated. Glycans were labeled with 2-aminobenzamide (2-AB) according to manufacturer's protocol (Prozyme, Hayward, CA). Excess dye and other hydrophobic contaminants were removed by use of GlycoProfileTM Glycan Clean-up Cartridges (Sigma-Aldrich). Labeled glycans were then reconstituted in 80% acetonitrile. The normal-phase high performance liquid chromatography (NP-HPLC) method used was adapted from Qian J, et al. [18] with optimization for our HPLC system. A Luna® 5 μm NH₂ 100 Å, LC column (Phenomenex, Torrance, CA) was used to differentiate variants of oligosaccharides. Mobile phase: A: 2% acetic acid and 1% tetrahydrofuran in acetonitrile; B: 5% acetic acid, 1% tetrahydrofuran, 3% triethylamine in water. Pre-run: 20% B for 5 minutes; step 1: 30% B for 2 minutes; Step 3: a linear gradient over a period of 70 minutes from 30%-80% B and a final isocratic elution with 95% B for 5 minutes. Eluted peaks were detected with a fluorescence detector with an excitation of 330 nm and emission 420 nm.

Glycoprofiling by LC-MS

LC-MS glycoprofiling strategies were adapted from Ayoub D, et al. [19]. CTL-1 and cetuximab were cleaved at the hinge region via digestion with IdeS (FabRICATOR, Genovis). Subsequently, Fab and Fc fragments were reduced in 6 M guanidine-HCl with 50 mM TCEP for 30 minutes at room temperature before addition of 10% Trifluoroacetic Acid (TFA) to yield the Fc/2, Fd, and light chain subunits. Mass spectrometry analyses of IdeS digested and TCEP reduced fragments were performed by the Proteomics/Mass Spectrometry Facility located at the Cancer Research Center, University at Albany, supported by the SUNY Research Foundation. LC-MS analyses were performed on an ESI-QSATR XL LC/MS/MS system (ABSCIEX, Framingham, MA, USA). Samples were diluted to 100 μl in 0.1% formic acid. The resulting solutions were injected directly into the RP (R1)-ESI TOF system. The trapping column was at room temperature. The protein mixture was eluted by 75% B (70% ACN and 30% isopropanol + 0.6% FA). BioAnalysis v1.1.5 software was used for deconvolution/transformation of multiple-charged MS spectrum onto a true mass scale. The procedure identified sequences of multiple-charged peaks belonging to a single parent molecule, and transformed the series onto a single peak in the zero-charge spectrum. Cetuximab carbohydrate structures published by Qian J, et al. [18] and Ayoub D, et al. [19] were used as reference to deduce structures of peaks present in LC-MS spectra.

Charge variant analyses

For isoelectric focusing, samples of each antibody were adjusted to a concentration of 0.4 mg/mL before being mixed at a 1:1 ratio with IEF loading buffer pH 3-10 (Life Technologies, Carlsbad, CA). Ten microliters of the mixture (2 μg of antibody) was then run on a Novex Isoelectric Focusing Electrophoresis (IEF) System (Life Technologies). Gels were run at a constant voltage of 100 V for 1 hour, followed by 200 V for 1 hour, and finally 500 V for 30 minutes. Gels were stained with Coomassie Brilliant Blue R-250 (ThermoFisher Scientific) as per manufacturer's instructions. For HPLC analysis, an Antibodix WCX NP5 column (Sepax Technologies, Inc. Delaware, USA) was used to separate charge variants. Mobile phase A: 10mM phosphate buffer, pH 7.5. Mobile phase B: 10mM phosphate buffer, 0.5M NaCl, pH 7.5. The gradient was 0-100% B in 45 minutes with a 15 minute pre-wash. The flow rate was 0.8 mL/min and the UV detector was set to 214 nm.

Proliferation assay

Five thousand Difi human colorectal carcinoma cells were plated in each well of a 96-well plate in 100 μL of complete medium. Cells were allowed to attach overnight. The following day, original medium was removed from each well and antibodies diluted in complete medium were added in a 2-fold series dilution at 100 μL per well. Concentrations ranged from 1.28 μg/mL to 5 ng/mL as well as no antibody control wells with complete medium only. Cells were cultured with or without antibody for 84 hours. Twenty microliters of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) was then added to wells and plates were incubated at 37°C for 2 hours before absorbance was read on a Synergy HT microplate reader (Bio-Tek, Winooski, VT). Data shown are averages of the absorbance values of four wells relative to no antibody controls.

ADCC assay

A431 human epidermoid carcinoma cells were labeled for 30 minutes at 37°C with 10 μM Calcein AM Viability Dye (eBioscience, San Diego, CA) in RPMI-1640 medium. Cells were plated in complete medium in 96-well tissue culture plates at 1x104 cells per well and incubated overnight at 37°C. The following day, PBMCs were isolated from the blood of a healthy adult donor via differential density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden). Appropriate numbers of effector PBMCs were added to each well of the 96-well plate to achieve effector:target cell ratios of 0:1, 5:1, 10:1, and 20:1. Cetuximab (Bristol-Myers Squibb, New York, NY) or CTL-1 was added to the plates at various concentrations in duplicate wells. Quadruplicate wells were included for target cell spontaneous release (target cells alone in complete medium) and target cell maximum release (target cells alone in complete medium supplemented with 2% Triton X-100). For all wells, total volume was 150 μl. Plates were incubated for 4 hours at 37°C. After incubation, 50 μl of supernatant was removed from each well and transferred to 96-well black polysterene plates. Fluorescence (excitation 485/20, emission 528/20) was measured on a Synergy HT microplate reader (Bio-Tek, Winooski, VT) with gain set to 50. Percent cytotoxicity was calculated as follows: [(Test release – spontaneous release)/(maximum release – spontaneous release)] X 100.

FcRn binding assay

FcRn binding was evaluated by Enzyme-Linked Immunosorbent Assay (ELISA). Human FcRn (gifted to the lab by Dr. Pamela Bjorkman at the California Institute of Technology) was biotinylated with EZ-LinkTM Sulfo-NHS-LC-Biotin (ThermoFisher Scientific) and subsequently bound to the Pierce™ NeutrAvidin™ coated high capacity plates (ThermoFisher Scientific). Serial dilutions of cetuximab or CTL-1 were added to the plate and allowed to bind hFcRn in a low pH buffer. Bound antibody was detected with peroxidase-conjugated goat F(ab′)2 anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with 3,3′,5,5′-tetramethylbenzidine substrate (Biolegend, San Diego, CA) before absorbance was measured at 450 nm using a Synergy HT microplate reader (Bio-Tek).

Mice

Six-week-old female BALB/c and athymic nude BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). All studies were planned and conducted according to institutional guidelines for *in vivo* animal experiments and with approval from the University of Southern California's Institutional Animal Care and Use Committee (IACUC).

Radiochemistry

Antibodies were labeled with Iodine-125 using PierceTM Iodination Tubes (ThermoFisher Scientific). One millicurie of Iodine-125 (PerkinElmer, Waltham, MA) was diluted in PBS and added to a PierceTM Iodination Tube for five minutes. Oxidized Iodine-125 was subsequently transferred to a separate tube containing the antibody to be labeled. The labeling reaction was incubated for one minute before being transferred to an illustra NAP5™ column (GE Healthcare Bio-Sciences, Pittsburgh, PA). The average yield of recovered radiolabeled product was approximately 75%–85%. The radiolabeled antibody preparations were diluted in PBS for injection, stored at 4°C, and administered within 24 hours of labeling. The radiochemical purity of the radiolabeled antibodies was determined via an instant Thin Layer Chromatography (ITLC) system consisting of silica gel impregnated glass fiber (Gelman Sciences, Ann Arbor, MI). Strips (2 x 20 cm) were activated by heating at 110°C for 15 minutes before use, spotted with 1 μL of sample, developed with 80% methanol, and analyzed for protein-bound and free radioiodine. Radioactivity was measured with a 2480 Wizard2 automatic gamma counter (PerkinElmer, Waltham, MA).

Blood pharmacokinetics

Six-week-old female BALB/c mice were provided drinking water supplemented with potassium iodide beginning two days before the administration of radioiodinated antibodies to minimize thyroid sequestration of I-125. Mice were randomized into two groups (n=8) and administered a single i.v. injection of 5 μCi ^{125}I-cetuximab or ^{125}I-CTL-1 spiked into either unlabeled cetuximab or unlabeled CTL-1, respectively. The total antibody doses administered were 0.1 mg/kg (2μg/mouse). Blood was collected and massed from the tail veins of four mice from each group at 3, 6, 24, 48, 72, 96, 120, and 168 hours post-injection. Each mouse was bled no more than four total times and no more than twice in 24 hours. After the final collection, the radioactivity of each tissue sample was measured using a 2480

Wizard2 automatic gamma counter (PerkinElmer). For each mouse, data were analyzed and expressed as percent of injected dose per gram of blood (%ID/g). Statistical analyses were carried out using GraphPad Prism 6 software (La Jolla, CA). Beta phase elimination half-life – $t_{1/2}(\beta)$ – was calculated by nonlinear regression utilizing a one-phase exponential decay model. Regression was based on data collected at least 24 hours post-injection in order to eliminate the influence of alpha phase distribution.

Tissue distribution

Tumors were implanted by subcutaneous injection of 5x10^6 HT-29 human colorectal adenocarcinoma cells in a 0.2 mL inoculum into the left flank of six-week-old female athymic nude mice. As stated above, potassium iodide supplemented water was provided to mice prior to administration of radioiodinated antibodies to minimize thyroid sequestration. When average tumor sizes reached ~0.5 cm in diameter, all mice were randomized into treatment groups (n=4). Each group received a single i.p. injection of 5 μCi ^{125}I-cetuximab or ^{125}I-CTL-1 spiked into either unlabeled cetuximab or unlabeled CTL-1, respectively. The total antibody doses administered were 0.1 mg/kg (2μg/mouse). Animals were euthanized 1, 2, 3, 5, and 7 days post-injection and tissues (blood, various organs, and tumor) were harvested and massed. Perfusion of residual blood from tissues was not performed. Eight days after initial injection, once all tissues had been collected, the radioactivity of each tissue sample was measured using a 2480 Wizard2 automatic gamma counter (PerkinElmer). For each mouse, data were analyzed and expressed as percent of injected dose per gram of tissue (%ID/g) and tumor-to-organ ratio. Statistical analyses were carried out using GraphPad Prism 6 software (La Jolla, CA). The %ID/g and tumor-to-organ ratios were analyzed by two-way ANOVA with time and organ as the independent variables.

Tumor treatment studies

Tumors were implanted by subcutaneous injection of 5x10^6 HT-29 or A431 human epidermoid carcinoma cells in a 0.2 mL inoculum into the left flank of six-week-old female athymic nude mice. When average tumor sizes reached ~100 mm^3, all mice were randomized into treatment groups (n=7). Groups of mice were treated with cetuximab, CTL-1, or received no treatment (PBS vehicle control only). Treatment groups were given 250 μg of antibody diluted in PBS by intraperitoneal (i.p.) injection every three days for a total of five treatments. Tumor volumes were determined by length, width, and height measurements using a caliper (volume = length × width × height). For survival analysis, an event was considered to have occurred if a mouse was deceased, or when euthanasia of a mouse was required due to conditions previously specified in IACUC protocols.

Results and Discussion

Antibody sequencing and carbohydrate analysis

Full coverage sequence analysis of cetuximab and CTL-1 was achieved in three rounds of enzymatic digestions coupled with LC-MS/MS (data not shown). The analysis confirmed CTL-1 to share an identical primary sequence with cetuximab based on previously published sequence data [20]. However, antibody function is not solely dependent on primary sequence. Post-translational modifications, especially N-linked glycosylations, that occur to the antibody during production have important implications in altered antibody function.

In development of biosimilar mAbs, the importance of glycoform similarity is often overlooked. Therefore, extensive physiochemical studies were performed in our analyses to confirm that CTL-1 and cetuximab share similar glycoprofiles.

As described in the literature, cetuximab contains two well-characterized and biologically significant sites for N-linked glycosylation [18,21]. In this study, initial characterization of glycoprofiles at these sites was achieved by normal phase HPLC analyses of 2-AB-labeled N-linked oligosaccharides released from these sites by PNGase F digestion. As shown in Figure 1, the virtually indistinguishable chromatograms for cetuximab and CTL-1 produced in this analysis indicate that a similar array of glycans exists on each antibody in remarkably similar ratios. These data provide an important global view of the heterogeneity of carbohydrates present on the surface of each antibody. However, this analysis does not identify specific carbohydrate structures or differentiate between the two glycosylation sites on the antibodies. Therefore, to elaborate on this finding in a site-specific, structure-elucidative manner, antibody subunits were separated prior to analysis by mass spectrometry. This protocol was adapted from Ayoub D et al. [19] in order to characterize in an accurate manner the N-linked oligosaccharide profiles at each site on the two antibodies. To do this, the antibodies were cleaved at the hinge region via IdeS digestion before being completely reduced with TCEP. This procedure separated the three major antibody subunits (light chain, Fd, and Fc/2) and allowed for analyses of the N299 glycosylation site (on the Fc/2 subunit) and the N88 glycosylation site (on the Fd subunit) independent of one another (Supplemental Figure S1). It should be noted that obtaining an exact glycoprofile with similar amplitudes of spikes is a very difficult task. In addition, our results were obtained in 3L and 8L reactors. When manufacturing is scaled up to 300L or higher, it is expected that changes in the amplitude of spikes and perhaps minor changes in the glycoprofile will be seen. The important point, however, is that despite the minor differences seen, we did not detect any changes between CTL-1 and commercial Erbitux in ADCC, *in vivo* efficacy, biodistribution, and FcRn binding which are the critical parameters for performance of the molecule in patients. Mass spectrometric analysis was also performed on each subunit after

enzymatic separation in order to identify the molecular weights of the various glycans present at each site (Supplemental Figures S1 and S3). Carbohydrate structures were then extrapolated from m/z values based on published structures from previous studies on cetuximab glycoprofiles [18,21]. The relative abundance of various glycans at each glycosylation site on the antibodies is illustrated in Figure 2. It can be seen in this figure that these two antibodies share exceptional similarity in their glycoprofiles. While most glycans are present in relatively similar ratios, it is of note that commercial cetuximab contains a higher proportion of mannose-5 (Man5) glycans at its N299 site. High-levels of Fc Man5 glycans have been correlated with increased Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), enhanced binding to FcγRIIIA, and more rapid clearance *in vivo* [21]. Although the levels of Man5 are only modestly higher in cetuximab as compared to CTL-1, this is an important difference to note. However, apart from the slightly altered abundance of this specific glycan, the overall oligosaccharide profiles of these two antibodies remain extraordinarily similar. This extensive structural analysis provides a unique and important insight into the biosimilarity of our produced antibody that is often not afforded in characterizations of mAb biosimilars. While glycoform analysis confirmed similar post-
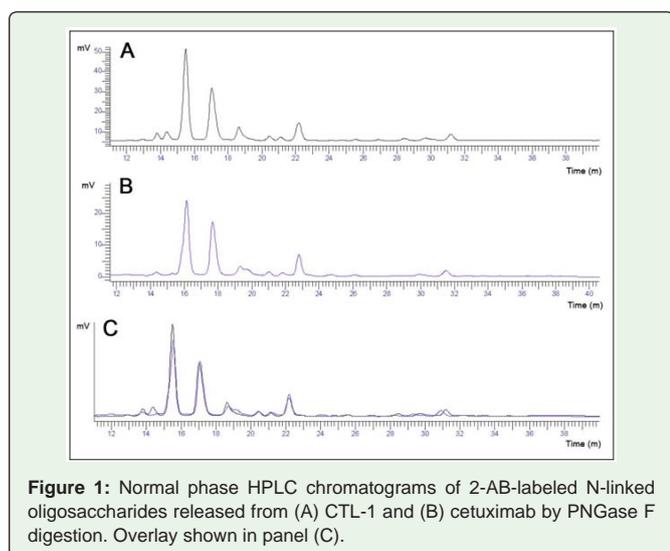
Figure 2: Relative abundance of glycoforms at each glycosylation site on cetuximab and CTL-1. Antibodies were IdeS cleaved and TCEP reduced to separate the major antibody subunits. Fc/2 and Fd regions were isolated and run by LC-MS to analyze glycoform heterogeneity at each glycosylation site. Peaks in the LC-MS chromatograms were correlated to their respective glycan structures based on previously published work on cetuximab glyco-profiling [18,21]. Sugar residues are N-acetylglucosamine (□), mannose (○), galactose (◇), N-glycolyl neuraminic acid (○), and fucose (△). Glycan compositions are given in terms of hexose (H), N-acetylhexosamine (N), deoxyhexose (F), and N-glycolyl neuraminic acid (NGNA).

Figure 1: Normal phase HPLC chromatograms of 2-AB-labeled N-linked oligosaccharides released from (A) CTL-1 and (B) cetuximab by PNGase F digestion. Overlay shown in panel (C).
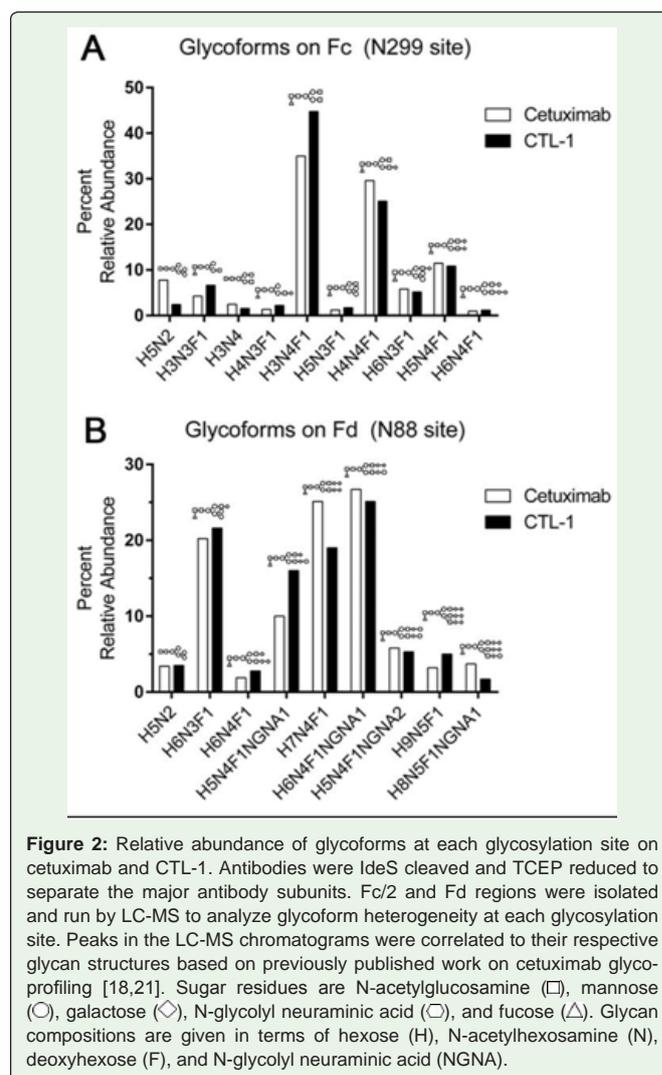
translational glycosylation patterns, analysis of antibody charge variants was performed to analyze the effects of other modifications known to alter protein isoelectric point. These analyses included ion-exchange HPLC and Isoelectric Focusing (IEF). For HPLC, batches of each antibody were examined using a Sepax Antibodix™ WCX-NP5 weak-cation exchange column. Ion chromatograms from this analysis are shown in Figure 3 and demonstrate the near identical similarity observed in the ratios of charge variants present in each lot of the two antibodies. The retention properties of cetuximab and CTL-1, and their charge variants were evaluated by calculating the Relative Retention Time (RRT). The acidic species eluting earlier than the main peak have a RRT of 4.37 and 4.31 for cetuximab and CTL-1, respectively. The basic peaks eluting later than the main peak have a RRT of 2.81 and 2.77. These data are directly supported by the results of IEF analyses which produced completely indistinguishable patterns of bands between the two antibodies when run on pH 3-10 gels (Figure 3). Combined, these data illustrate the striking similarity in charge variants present in batches of each antibody, further confirming the similarity in post-translational modifications that occurs to these therapeutic mAbs. This level of similarity in charge variants was only achieved after numerous rounds of subcloning in which one of several selection criteria was based upon IEF analyses as well NP-HPLC analysis to confirm initial similarity in glycoprofiles.

In vitro anti-tumor activity

To confirm similar *in vitro* biological activity between antibodies, anti-proliferation assays and ADCC assays were performed. The two antibodies exhibited an equal ability to inhibit tumor cell proliferation in assays with the EGFR+ colorectal carcinoma cell line Difi. As shown in Figure 4, curves illustrating the effect of increasing concentrations of antibody treatment on tumor cell proliferation are virtually indistinguishable between the two antibodies. From these curves, nearly identical IC50 values of 48.3 ng/mL and 51.1 ng/mL were derived for cetuximab and CTL-1, respectively. ADCC assays were performed with effector PBMCs isolated from a healthy adult donor directed against the EGFR+ human epidermoid carcinoma cell line A431. As seen in Figure 4, similar percent cell cytotoxicity was observed in response treatment with each antibody at various effector:target cell ratios and with varying concentrations of antibody. Relative to controls without antibody treatment, PBMC tumor cell co-cultures incubated with either antibody resulted in significant and similar increases in percent cell cytotoxicity. Although largely similar, cetuximab did induce a greater degree of tumor cell cytotoxicity as

compared to CTL-1 in some conditions (Figure 4). These observed differences in ADCC may be attributed to the slightly greater levels of Fc Man5 glycans in cetuximab (Figure 2). As stated above, higher levels of Fc Man5 glycans have been demonstrated to enhance ADCC activity, presumably due to the concomitant increase in binding affinity to FcγRIIIA [21]. While ADCC activity *in vitro* may differ as a result of increased abundance of Man5 glycans, this does not always translate to significant changes to *in vivo* efficacy due to the associated increased clearance of antibodies with high abundance of Man5 glycan [22].

FcRn-binding and blood pharmacokinetics

In vitro FcRn-binding assays and *in vivo* blood pharmacokinetic studies suggest similar pharmacokinetic profiles for each antibody. FcRn-binding curves produced by ELISA indicate similar binding affinities for both cetuximab and CTL-1 (Figure 5). Similar FcRn binding profiles are important to note, as antibody binding to FcRn is a suggested explanation for the long half-life of IgG antibodies in the blood as compared to other proteins. Similar FcRn binding profiles suggest similar degrees of antibody recycling in the blood and thus increased similarity in pharmacokinetic and plasma retention profiles [23]. *In vivo* blood pharmacokinetics of I-125 labeled cetuximab and CTL-1 in non-tumor bearing BALB/c mice confirmed the similarity of blood clearance profiles of these antibodies (Figure 5). Data from 24 hours onward were used to calculate the elimination half-lives of each antibody to avoid contributions of distribution in the α-phase. As depicted in Figure 5, CTL-1 and cetuximab were eliminated nearly
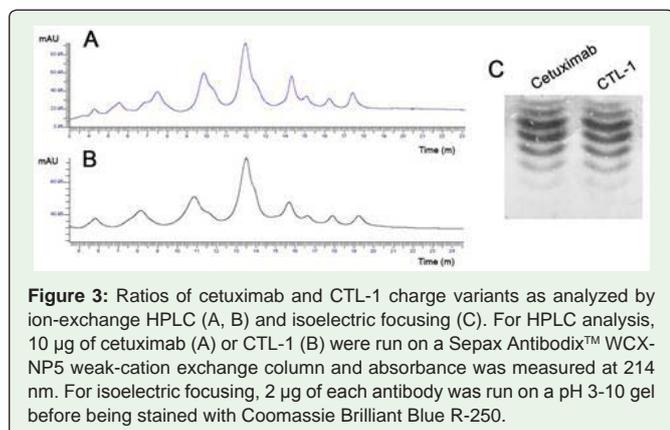

Figure 4: Cell growth inhibition (A) and antibody-dependent cell-mediated cytotoxicity (ADCC). (B) assays demonstrating the *in vitro* efficacy of cetuximab and CTL-1 against two carcinoma cell lines. *A*, Difi colorectal carcinoma cells were incubated for 3 days with varying concentrations of cetuximab or CTL-1. Percent proliferation was then calculated relative to untreated controls. IC50 values for cetuximab and CTL-1 were calculated to be 48.3 and 51.1 ng/mL, respectively. *B*, A431 epidermoid carcinoma cells were labeled with Calcein AM Viability Dye (eBioscience) and plated with varying numbers of PBMCs isolated from a healthy donor. Co-cultures were either left untreated or treated with varying concentrations of cetuximab or CTL-1 for four hours. Fluorescence was then measured as an indicator of cytotoxicity.

Figure 3: Ratios of cetuximab and CTL-1 charge variants as analyzed by ion-exchange HPLC (A, B) and isoelectric focusing (C). For HPLC analysis, 10 μg of cetuximab (A) or CTL-1 (B) were run on a Sepax Antibodix™ WCX-NP5 weak-cation exchange column and absorbance was measured at 214 nm. For isoelectric focusing, 2 μg of each antibody was run on a pH 3-10 gel before being stained with Coomassie Brilliant Blue R-250.
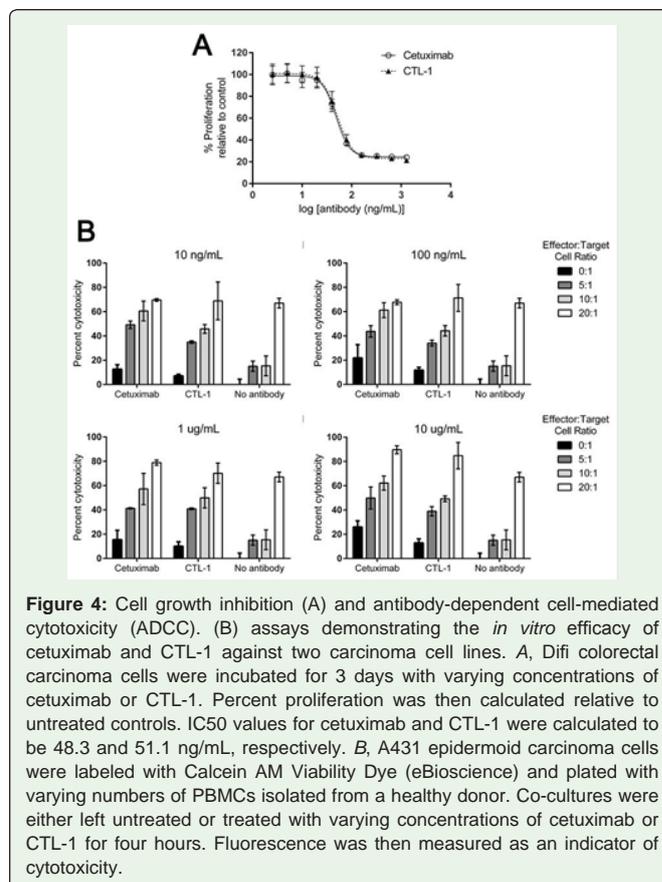
identically with β-phase half-lives of 186.0 hours and 186.4 hours, respectively. In addition, the elimination rate constants (Ke) for each antibody were determined to be statistically indistinguishable from one another by an extra sum-of-squares F test (p=0.9915). Given the equal terminal clearance rates and the noted similarity in FcRn binding, it is clear that these antibodies experience identical biological processes of antibody recycling and elimination that provides for the equivalent plasma half-lives of these therapeutic mAbs. It should be noted that blood PK for cetuximab and CTL-1 in healthy BALB/c mice confirmed the similarity of blood clearance profiles of both CTL-1 and cetuximab (Figure 5). Immunogenicity would have altered the half-life of the antibody so we are confident that this difference in various glycans (e.g. Man5) at each glycosylation site on the antibodies (Figure 2 and Supplemental Figures S1-S3) will not be significant for these parameters.
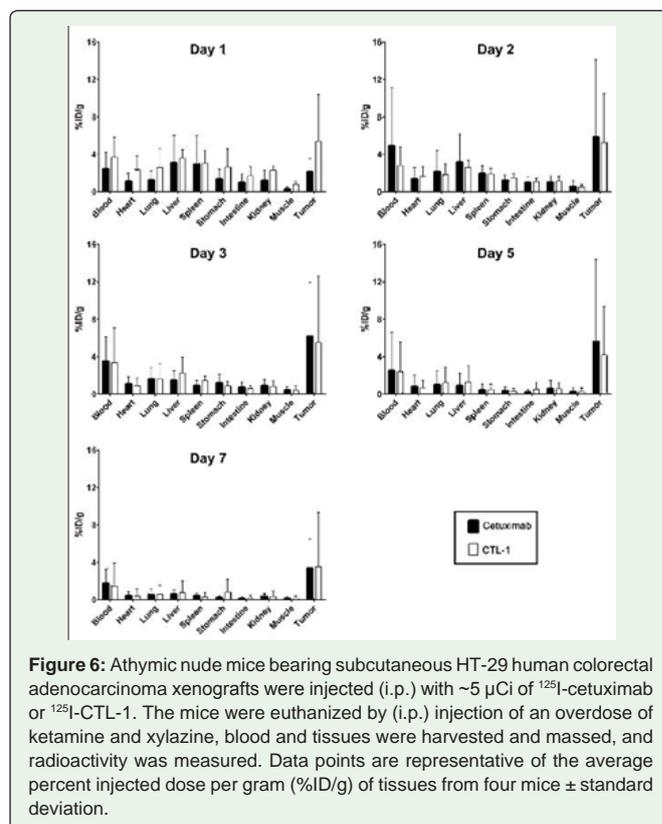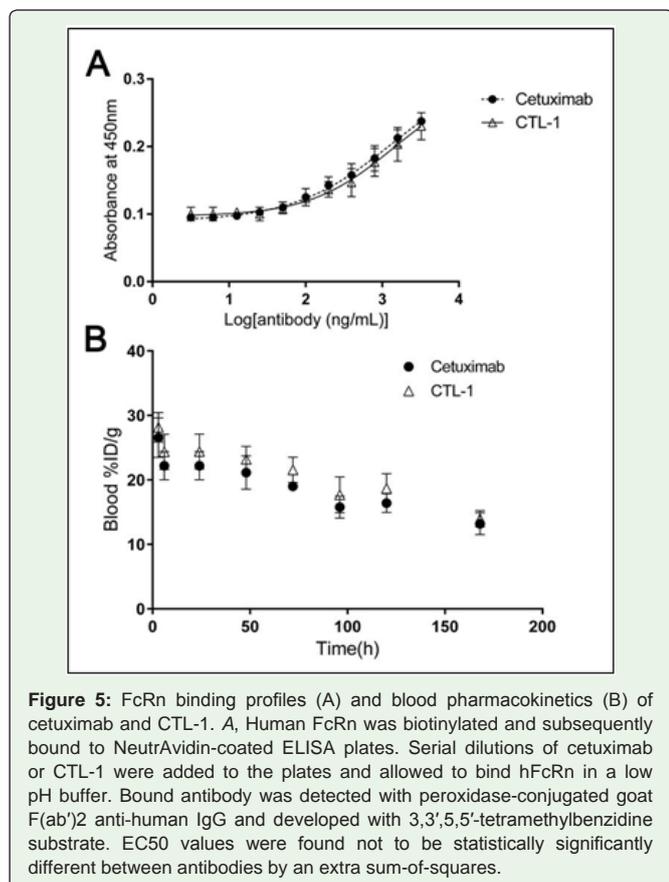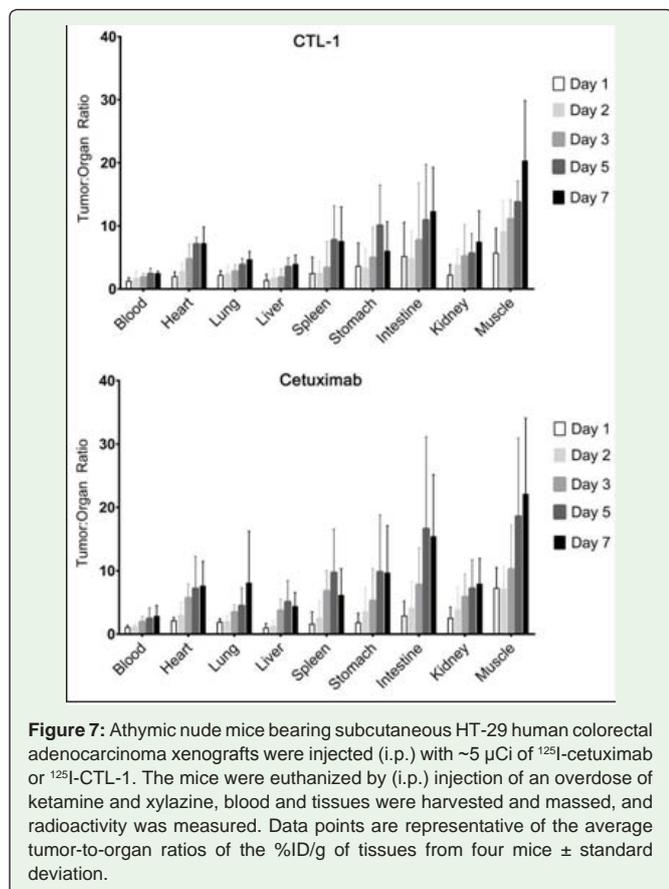
Tissue distribution

A tissue biodistribution study was performed in HT-29 human colorectal adenocarcinoma tumor-bearing nude mice to demonstrate equivalent uptake of each antibody in various tissues, as well as similar tumor-targeting capabilities *in vivo* (Figure 6 and 7). Percentage of injected dose per gram of tissue (%ID/g) was calculated as a dose-normalized unit of uptake to demonstrate the specificity of distribution, which can be used to quantify and compare different tissue concentrations. Analyses of tissue uptake one to seven days post-injection of ^{125}I-labeled antibody demonstrate that both antibodies preferentially localize to tumor tissue over all other

analyzed tissues, including blood. Both antibodies are shown to clear from blood and other tissues more rapidly than tumor tissue, indicating active binding and sustained localization at the tumor site up to 7 days post-injection. The level of both antibodies is similar in all tissues analyzed at every time point studied, establishing similar tissue distribution profiles for each antibody (Figure 6). These similar profiles are notable due to the distinct and direct localization of each antibody to the tumor microenvironment in this EGFR+ tumor model. The distribution data were further analyzed to calculate the tumor-to-organ antibody uptake ratios. These ratios were computed to normalize for the effect of different circulating levels of antibody in the blood and other normal tissues. In this analysis, remarkably similar distribution profiles were observed. The increasing tumor-to-organ uptake ratios observed over time confirm the presence of antigen specific accumulation at the tumor site for both cetuximab and CTL-1 (Figure 7). Overall, the tumor tissue exhibited sustained antibody uptake 7 days post-injection as compared to control tissues, resulting in proportional increases in the tumor-to-organ uptake ratios over time for all tissues, including blood. These data illustrate the identical *in vivo* behavior of these antibodies to specifically target and accumulate at an EGFR+ human tumor microenvironment while being cleared from the rest of the body.

Efficacy Studies

In vivo efficacy studies of cetuximab and CTL-1 were performed in six-week-old female nude mice using the EGFR-expressing A431 human epidermoid carcinoma and HT-29 human colorectal adenocarcinoma cell lines. Both cetuximab and CTL-1 produced nearly identical tumor growth inhibition curves compared to vehicle

Figure 5: FcRn binding profiles (A) and blood pharmacokinetics (B) of cetuximab and CTL-1. *A*, Human FcRn was biotinylated and subsequently bound to NeutrAvidin-coated ELISA plates. Serial dilutions of cetuximab or CTL-1 were added to the plates and allowed to bind hFcRn in a low pH buffer. Bound antibody was detected with peroxidase-conjugated goat F(ab')2 anti-human IgG and developed with 3,3',5,5'-tetramethylbenzidine substrate. EC50 values were found not to be statistically significantly different between antibodies by an extra sum-of-squares.

Figure 6: Athymic nude mice bearing subcutaneous HT-29 human colorectal adenocarcinoma xenografts were injected (i.p.) with ~5 μCi of ^{125}I-cetuximab or ^{125}I-CTL-1. The mice were euthanized by (i.p.) injection of an overdose of ketamine and xylazine, blood and tissues were harvested and massed, and radioactivity was measured. Data points are representative of the average percent injected dose per gram (%ID/g) of tissues from four mice ± standard deviation.

Figure 7: Athymic nude mice bearing subcutaneous HT-29 human colorectal adenocarcinoma xenografts were injected (i.p.) with ~5 µCi of ^{125}I-cetuximab or ^{125}I-CTL-1. The mice were euthanized by (i.p.) injection of an overdose of ketamine and xylazine, blood and tissues were harvested and massed, and radioactivity was measured. Data points are representative of the average tumor-to-organ ratios of the %ID/g of tissues from four mice ± standard deviation.

Figure 8: Athymic nude mice were injected (s.c.) with 5 x 10^6 HT-29 human colorectal adenocarcinoma cells (*A,C*) or A431 human epidermoid carcinoma cells (*B,D*). Seven days after injection, when average tumor volumes reached ~100mm³, groups of seven mice were treated with PBS, cetuximab, or CTL-1 every three days for a total of five doses. *A* and *B*, average tumor volumes of mice (n=7) ± standard deviation. *C* and *D*, percentage survival of mice over time.

control mice. These important data demonstrate that comparable therapeutic results were obtained for both reagents against high-expressing EGFR human tumor xenografts. As shown in Figure 8, mice treated with cetuximab or CTL-1 exhibited reductions in A431 tumor growth by 67.0% or 64.3%, respectively, compared to PBS-treated mice fourteen days after treatment commencement. By reducing tumor growth, both CTL-1 and cetuximab significantly improved survival of A431 tumor bearing mice compared to PBS-treated control animals. While all mice from the PBS treatment group (n=7) had died or been euthanized due to tumor progression by day 31, only one to two of the seven mice treated with cetuximab or CTL-1 had died or been euthanized by day 45. As shown in the Kaplan Meier Plot presented in Figure 8, the survival curves demonstrate a similar and significant increase in the survival of mice treated with either cetuximab or CTL-1.In our studies, the HT-29 colon carcinoma model was much less sensitive to anti-EGFR antibody treatment. Cetuximab and CTL-1 exhibited near identical efficacy against tumor growth in this model, though the reduction in growth was more modest - 25.8% and 33.9% tumor reduction in response to cetuximab and CTL-1 treatment, respectively – as opposed to the more than 60% reduction in growth observed in the A431 model. Despite a similar and significant reduction in tumor growth, neither antibody increased overall survival to a significant degree up to 25 days after the initiation of treatment. It should be noted that the response differences seen are between cell lines not between the test and commercial products for a given cell line. The reason for this is that the HT-29 and A431 human tumor cell lines have different sensitivities to Erbitux treatment. Though both antibodies proved less efficacious in the colorectal carcinoma model, these studies validate that our generic CTL-1 antibody has an equivalent ability to the commercial cetuximab in inhibiting human tumor growth *in vivo*.
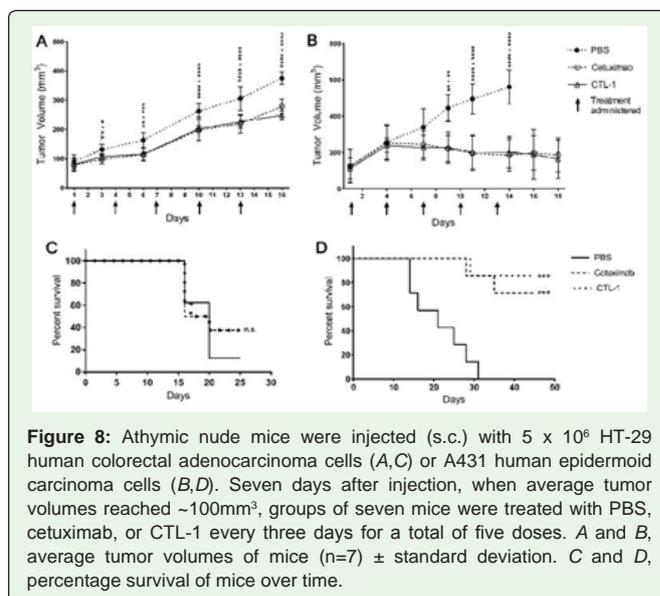
Conclusion

As outlined in the studies above, the produced EGFR-targeting monoclonal antibody CTL-1 is chemically, structurally, and biologically biosimilar to the commercial antibody cetuximab. Mass spectroscopy studies confirmed identical amino acid sequences as well as similar glycosylation profiles on each antibody. Isoelectric focusing and ion-exchange chromatography demonstrated similar charge variants in lots of each antibody. As predicted by the comparable glycosylation profiles of each antibody, the two antibodies have similar *in vitro* binding affinities to human FcRn as well as identical blood clearance profiles in BALB/c mice. Each antibody was able to inhibit proliferation of EGFR+ tumor cell growth as well as induce an equivalent ADCC response against these cells *in vitro*. Tissue distribution studies demonstrated tumor-specific uptake of both antibodies over a seven day period. The two antibodies demonstrated indistinguishable tissue distribution profiles, tumor uptake, and blood clearance rates. Both antibodies were shown to inhibit human tumor growth and improve overall survival to identical degrees in two different mouse xenograft models. Given these extensive pre-clinical data, we propose that CTL-1 is a viable candidate as a generic antibody alternative to cetuximab for treatment of EGFR+ tumors.

Acknowledgments

Authors Contributions

Leslie A. Khawli and Ryan Graff were responsible for the overall

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Page 8/9

design and execution of the analyses of *in vitro* and *in vivo* biological activity. Long Zheng was responsible for antibody engineering and clonal development as well as physiochemical characterization of produced constructs. Peisheng Hu is the subject expert in the field of antibody engineering and was responsible for the design and cloning procedures, and comparative biochemical and structure analysis. Alan L. Epstein is the lead investigator and the subject expert in the field of cell culture processes for monoclonal antibody production. All authors participated in the writing, reviewing and technical editing of the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute.

References

1. Reichert JM. Marketed therapeutic antibodies compendium. MAbs. 2012; 4: 413-415.

2. Schellekens H. Biosimilar therapeutics—what do we need to consider? NDT Plus. 2009; 2: 27-36.

3. Cornes P. The economic pressures for biosimilar drug use in cancer medicine. Targ Oncol. 2012; 7: 57-67.

4. McCamish M, Woollett G. The state of the art in the development of biosimilars. Clin Pharmacol Ther. 2012; 91: 405-417.

5. Da Silva A, Kronthaler U, Koppenburg V, Fink M, Meyer I, Papandrikopoulou A, et al. Target-directed development and preclinical characterization of the proposed biosimilar rituximab GP2013. Leuk Lymphoma. 2014; 55: 1609-1617

6. Schiestl M, Stangler T, Torella C, Čepeljnik T, Toll H, Grau R. Acceptable changes in quality attributes of glycosylated biopharmaceuticals. Nat Biotechnol. 2011; 29: 310-312.

7. Martinelli E, De Palma R, Orditura M, De Vita F, Ciardiello F. Anti-epidermal growth factor receptor monoclonal antibodies in cancer therapy. Clin Exp Immunol. 2009; 158: 1-9.

8. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med. 2004; 351: 337-345.

9. Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. Nat Rev Drug Discov. 2009; 8: 226-234.

10. Morgan MA, Parsels LA, Kollar LE, Normolle DP, Maybaum J, Lawrence TS. The combination of epidermal growth factor receptor inhibitors with gemcitabine and radiation in pancreatic cancer. Clin Cancer Res. 2008; 14: 5142-5149.

11. Michaelis M, Bliss J, Arnold SC, Hinsch N, Rothweiler F, Deubzer HE, et al. Cisplatin-resistant neuroblastoma cells express enhanced levels of epidermal growth factor receptor (EGFR) and are sensitive to treatment with EGFR-specific toxins. Clin Cancer Res. 2008; 14: 6531-6537.

12. Shimura T, Kataoka H, Ogasawara N, Kubota E, Sasaki M, Tanida S, et al. Suppression of proHB-EGF carboxy-terminal fragment nuclear translocation: a new molecular target therapy for gastric cancer. Clin Cancer Res. 2008; 14: 3956-3965.

13. Patra CR, Bhattacharya R, Wang E, Katarya A, Lau JS, Dutta S, et al. Targeted delivery of gemcitabine to pancreatic adenocarcinoma using cetuximab as a targeting agent. Cancer Res. 2008; 68: 1970-1978.

14. Black PC, Brown GA, Inamoto T, Shrader M, Arora A, Siefker-Radtke AO, et al. Sensitivity to epidermal growth factor receptor inhibitor requires E-cadherin expression in urothelial carcinoma cells. Clin Cancer Res. 2008; 14: 1478-1486.

15. Kim S, Prichard CN, Younes MN, Yazici YD, Jasser SA, Bekele BN, Myers JN. Cetuximab and irinotecan interact synergistically to inhibit the growth of orthotopic anaplastic thyroid carcinoma xenografts in nude mice. Clin Cancer Res. 2006; 12: 600-607.

16. Hecht JR, Patnaik A, Berlin J, Venook A, Malik I, Tchekmedyian S, et al. Panitumumab monotherapy in patients with previously treated metastatic colorectal cancer. Cancer. 2007; 110: 980-988.

17. Seiden MV, Burris HA, Matulonis U, Hall JB, Armstrong DK, Speyer J, et al. A phase II trial of EMD72000 (matuzumab), a humanized anti-EGFR monoclonal antibody, in patients with platinum-resistant ovarian and primary peritoneal malignancies. Gynecol Oncol. 2007; 104: 727-731.

18. Qian J, Liu T, Yang L, Daus A, Crowley R, Zhou Q. Structural characterization of N-linked oligosaccharides on monoclonal antibody cetuximab by the combination of orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole-quadrupole time-of-flight tandem mass spectrometry and sequential enzymatic digestion. Anal Biochem. 2007; 364: 8-18.

19. Ayoub D, Jabs W, Resemann A, Evers W, Evans C, Main L, et al. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. MAbs. 2013; 5: 699-710.

20. Dubois M, Fenaille F, Clement G, Lechmann M, Tabet JC, Ezan E, and Becher F. Immunopurification and mass spectrometric quantification of the active form of a chimeric therapeutic antibody in human serum. Anal Chem. 2008; 80: 1737-1745.

21. Yu M, Brown D, Reed C, Chung S, Lutman J, Stefanich E, et al. Production, characterization and pharmacokinetic properties of antibodies with N-linked mannose-5 glycans. MAbs. 2012; 4: 475-487.

22. Reusch D, Tejada ML. Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology. 2015; 25: 1325-34.

23. Datta-Mannan A, Wroblewski VJ. Application of FcRn binding assays to guide mAb development. Drug Metab Dispos. 2014; 42: 1867-1872.

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Page 9/9