



Cetuximab produced from a goat mammary gland expression system is equally efficacious as innovator cetuximab in animal cancer models

Qian Wang^a, William Gavin^c, Nicholas Masiello^c, Khanh B. Tran^a, Götz Laible^{a,b,d}, Peter R. Shepherd^{a,d,*}

^a School of Medical Sciences, University of Auckland, Auckland, 1023, New Zealand

^b AgResearch, Ruakura Research Centre, Hamilton, New Zealand

^c LFB-USA, Framingham, MA, USA

^d Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand

ARTICLE INFO

Article history:

Received 9 June 2020

Received in revised form 10 September 2020

Accepted 21 September 2020

Keywords:

Recombinant protein production
Humanised monoclonal antibodies
Antibody-drug conjugates
Cetuximab

ABSTRACT

There is increasing demand for improved production and purification systems for biosimilar or biobetter humanised monoclonal antibodies and animal production systems offer one such possible option. Cetuximab, also known as 'Erbix', is a humanised monoclonal antibody widely used in cancer therapy. We have previously reported on a genetically engineered goat system to produce cetuximab (gCetuximab) in milk. Herein we report that gCetuximab has similar bioactivity and pharmacokinetic properties compared with the commercial product produced in mammalian cell culture. In particular both forms have very similar efficacy in a HT29 colorectal cancer xenograft model alone or when conjugated to the toxin MMAE. This also demonstrates that the gCetuximab will be a viable vehicle for antibody drug conjugate based therapies. Taken together, this shows that the goat milk monoclonal antibody production system is an effective way of producing a biosimilar form of cetuximab.

© 2020 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The monoclonal antibody based cancer treatment approach has proven to be one of the most successful cancer therapeutic classes of drugs over the last two decades due to their high target-specificity to tumour cells and low cytotoxicity [1]. Cetuximab, an anti-EGFR monoclonal antibody, is obtained by attaching the Fv variable regions of a murine monoclonal anti-EGFR antibody (C225) to human IgG1 heavy and kappa light chain constant regions [2]. Known by its trade name Erbitux, cetuximab is an epidermal growth factor receptor (EGFR) inhibitor which is used for cancer treatment, especially in advanced colorectal cancer, metastatic non-small cell lung cancer as well as head and neck cancer [3]. Cetuximab is a monoclonal antibody of the immunoglobulin G1 (IgG1) subclass, which binds to EGFR with high affinity, thereby blocking the endogenous EGFR ligands from binding. This results in inhibition of the function of the EGFR receptor [4–6]. Cetuximab is selective and does not bind to other HER family receptors, such as ErbB2, ErbB3 and ErbB4. EGFR is constitutively

expressed in many normal epithelial tissues. Its signaling pathways are involved in the regulation of cell survival, cell cycle progression, angiogenesis, cell migration and cellular metastasis [6–9]. Over-expression of EGFR is frequently detected in many human cancers. Cetuximab was approved by the US Food and Drug Administration (FDA) in February 2004 and by the European Medicines Agency (EMA) in June 2004 as a cancer therapy that used alone or in combination with other medications to treat colon or rectal cancer that has spread to other parts of the body [2].

Clinical grade cetuximab (Erbitux), is produced by expressing it in expensive mammalian cell bioreactors, driving interest in alternative production systems. We have recently reported the development of a system for expressing high levels of cetuximab in the mammary gland of lactating transgenic goats and purifying this version of cetuximab (gCetuximab) from the resulting milk [10]. The high level of production (up to 10 g/L) provides potential for lowering the cost of goods (CoGs) at a commercial level. The gCetuximab produced in goats milk also does not contain α -gal linkages and so significantly reduces potential for inducing immunogenicity that occurs with commercially available cetuximab. This is likely to increase the safety profile of gCetuximab. Furthermore, the gCetuximab has properties consistent with an increased antibody dependent cellular cytotoxicity (ADCC) profile suggesting it may have increased efficacy in humans [10]. This

* Corresponding author at: Department of Molecular Medicine, University of Auckland, Park Road, Auckland, New Zealand.

E-mail address: peter.shepherd@auckland.ac.nz (P.R. Shepherd).

report describes a range of studies that demonstrate that the biological properties of the cetuximab produced in the transgenic milk system are equivalent to those of the commercial cell culture product. Together this provides evidence that the goat produced product could be taken forward as a potential cetuximab biosimilar. Lastly, we also report herein the possible benefits of conjugating this goat derived cetuximab to an anti-cancer molecule thereby significantly increasing its potential efficacy in the current approved cancer indications.

2. Material and methods

2.1. Cetuximab

Cetuximab was expressed in the milk of lactating goats and purified as previously described [10]. Commercially sourced cetuximab was obtained from Onelink (NZ) Ltd. This material was produced by the ImClone Systems Incorporated as previously described [11–13].

2.2. Cell culture

The melanoma cells NZM37 and NZM40 were chosen from a panel of primary melanoma cell lines that were generated from biopsies of metastatic melanoma samples from patients presenting at clinics in Auckland, New Zealand as previously described [14,15]. The cells were maintained in α -modified minimal essential medium (MEM- α) supplemented with antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, and amphotericin B 0.25 μ g/mL; GIBCO Life Technologies), ITS (5 μ g/mL insulin, 5 μ g/mL transferrin and 5 ng/mL sodium selenite; Roche Diagnostics GmbH), and 5 % fetal bovine serum (FBS, HyClone). The colorectal cancer cell line HT29 was obtained from ATCC and maintained in MEM- α supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), and 5 % fetal bovine serum (FBS).

2.3. Western blotting

Protein concentration of total cell lysate was quantified by BCA assay. For western blotting 40 μ g of protein samples were subjected to 10 % in house SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 3% BSA in TBS containing 0.01 % Tween-20 for 1 h at room temperature and then incubated with specific primary antibodies at 4 °C overnight in the blocking buffer. After TBST washing, membranes were incubated with secondary antibody at room temperature for 1 h in the blocking buffer. Detection of specific protein expressions were performed by Clarity Western ECL blotting substrates with Bio-Rad ChemiDoc MP imaging system. Antibodies used for immunoblotting are as follows: total-EGFR (Cell Signaling Technology #2232, 1:1000), phospho-EGFR (Cell Signaling Technology #2234, 1:1000), β -Actin (Sigma #A1978, 1:2000), total-Akt (Cell Signaling #9272, 1:1000), and phospho-Akt (Cell Signaling Technology #9271, 1:1000).

2.4. Construction of CRISPR-mediated EGFR knock out stable cell lines

A pair of guide RNA targeting human EGFR was cloned into pSpCas9(BB)-2A-GFP (p \times 458) plasmid vector (Addgene plasmid #48137) (Addgene, Cambridge, MA) following the depositor's protocol. The sequences are: Forward: 5'-CACCGGGAGCAGC-GATGCGACCCTC-3' and Reverse: 3'-CCCTCGTCGCTACGCTGGGAG-CAAA-5'. Melanoma cell lines NZM37, NZM40 and colorectal cancer cell line HT29 were transfected with pX458-gRNA using

lipofectamine 3000 (Life Technology) according to the manufacturer's instructions. 24 h after transfection, GFP positive cells were sorted by FACSaria II SORP cell sorter and seeded into 96 well plates with single cell per well. Expression of EGFR in expanded colonies was detected by immunoblotting.

2.5. Cell viability assay

Cells were seeded in 96-well plates (5,000 cells/well). After 24 h, cells were incubated with different drugs accordingly with a range of concentrations (from 0.1–100 μ M) for 72 h. Cell viability was determined using the sulforhodamine B (SRB) assay as previously described [16]. Results were plotted as percent of vehicle control from at least two independent experiments conducted in triplicate. Growth curves were analyzed by nonlinear regression using GraphPad Prism V6.0 software (GraphPad Software, San Diego, CA).

2.6. Synthesis and analysis of MMAE and Cy5 anti-EGFR antibody conjugates

Commercial cetuximab (ImClone, 5 mg/mL) and gCetuximab (6.32 mg/mL) were diluted to 2 mg/mL with sterile saline. Diluted cetuximab solutions (2 mg) were treated with 100 μ l Bicine buffer (1 M, pH8.26) and 10 μ l diethylenetriaminepentaacetic acid (DTPA) (100 mM, pH7.0). Then antibodies were reduced by 4 equivalents of tris (carboxyethyl) phosphine (TCEP) at 37 °C for 2 h. After cooling down to room temperature, 4 equivalents of MC-VC-MMAE (MedChem Express, HY-15575) were added and incubated for 30 min. Reaction mixtures were gel-filtered through Sephadex G-25 (Sigma G2580-10 G) and eluted by PBS [17]. Subsequently, the conjugated cetuximab-MMAE mixtures were concentrated by centrifugal concentrator (30 kDa MWCO, Sigma). Before labelling the conjugated cetuximab-MMAE with Cy5-maleimide, 50 mM DTT was added to reduce the remaining disulphide bonds [18]. After 30 min incubation, 2 equivalents of Cy5-maleimide (Abcam ab146489) was added and incubated 30 min at room temperature. Following buffer exchange through Sephadex G-25, the Cy5-cetuximab-MMAE conjugates were concentrated by centrifugal concentrators. Cy5-cetuximab-MMAE conjugates were analysed by hydrophobic interaction chromatography-HPLC using an TSKgel Ether-5PW column (Tosoh Biosciences). Antibody-drug isomers were separated from unconjugated cetuximab by the HPLC method using a linear gradient from 100 % high salt concentration buffer A (0.05 M sodium phosphate buffer, pH7.0; 2 M ammonium sulphate) to 100 % buffer B (80 % v/v 0.05 M sodium phosphate buffer, pH7.0, 20 % v/v 2-propanol) in 45 min [19]. The flow rate was set at 200 μ l/min. The concentrations of antibodies were measured at 280 nm by Nanodrop spectrophotometer.

2.7. Cetuximab half-life assessment

Female CD-1 mice, 5–6 weeks of age, were obtained and maintained in Vernon Janson Unit, University of Auckland. All studies were performed in accordance with University of Auckland animal ethics and animal welfare. Cetuximab was administered by intraperitoneal injection (*ip*) with 12.5 mg/kg dose. Blood samples were collected at 0, 1, 3, 6, 24, 48, 72, 96, and 120 h by cardiac puncture, following a single intraperitoneal injection (*ip*) according to previous publications [20]. The blood samples were centrifuged at 2000xg for 15 min at 4 °C. Plasma aliquots were stored at –80 °C until analysis by an ELISA assay. The ELISA assay was performed according to the manufacturer's instructions (ImmunoGuide IG-AB112). Briefly, the ELISA assay is based on a cetuximab-specific mouse monoclonal antibody pre-coated onto microtiter plates to capture cetuximab in mouse plasma. The

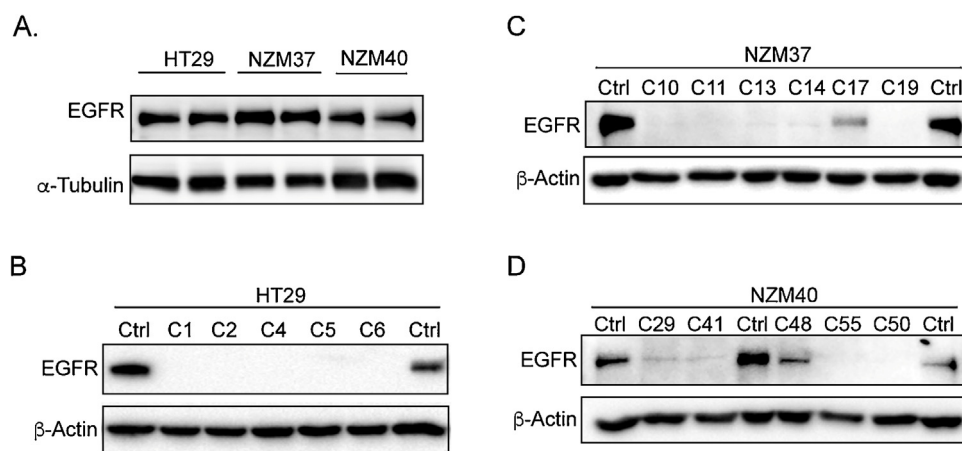


Fig. 1. Evidence for creation of colorectal and melanoma cancer cell lines with EGF receptor deleted. A. Western blots showing EGFR was endogenously expressed in wild type colorectal cell line HT29 and primary melanoma cancer cell lines NZM37 and NZM40. Western blots showing EGFR was successfully knocked out by CRISPR/Cas9 genetic editing strategy in a range of clones of HT29 (B), NZM37 (C), and NZM40 (D) cell lines.

captured cetuximab from plasma was then detected by a horseradish peroxidase (HRP)-conjugated anti-human IgG monoclonal antibody binding to cetuximab Fc fragment. After addition of chromogen-substrate, the colour developed is proportional to the amount of cetuximab in the sample or standard. In order to exclude the affinity differences between commercial and goat-produced cetuximab to mouse anti-cetuximab antibody coating the ELISA plate, a standard curve with goat-produced cetuximab was also included for data analysis.

2.8. Xenograft mouse model

Colorectal cancer cells HT29 (5×10^6) were injected subcutaneously into the right side of the NIH-III immunocompromised nude mice at 5–6 weeks of age. Tumour volume was calculated every 3 days with a caliper using the following formula: $(\pi \times \text{length} \times \text{width}^2) / 6$, where length represents the largest tumour diameter and width represents the perpendicular tumour diameter. When the average tumour volume reached 100 mm^3 , mice

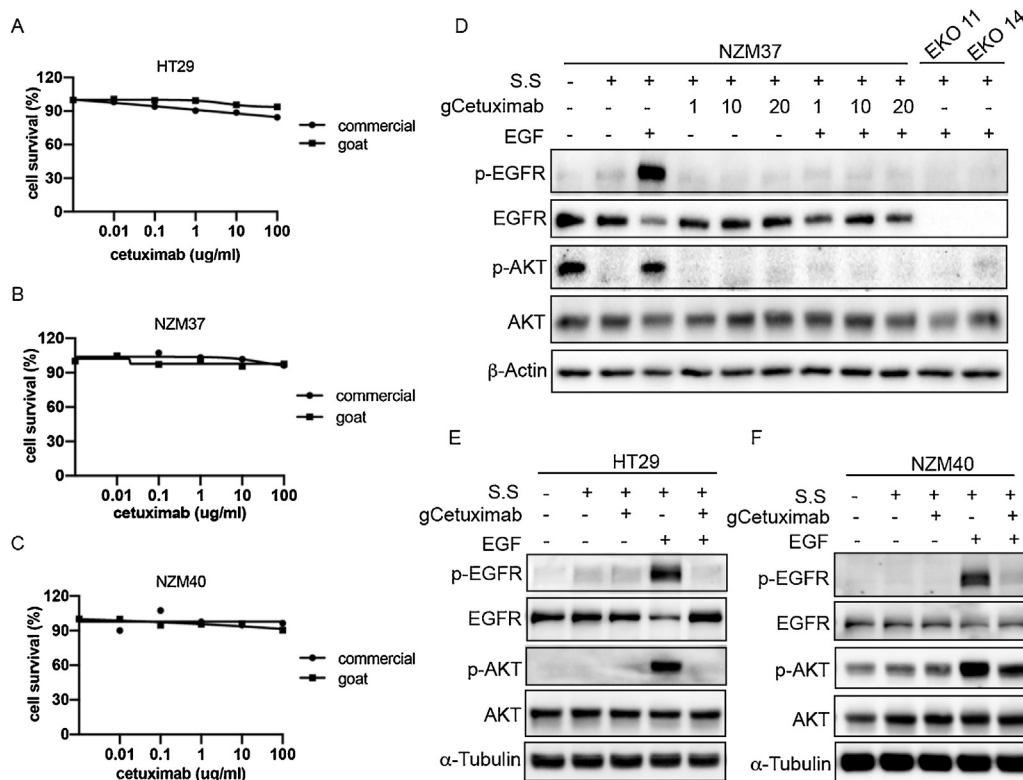


Fig. 2. Evidence that gCetuximab effectively inhibits EGF stimulated EGFR signaling. Effects of both commercial and goat cetuximab on cell growth were tested by SRB growth assay toward HT29 (A), NZM37 (B), and NZM40 (C) cell lines and as expected the antibody did not directly attenuate *in vitro* cell growth. EGF signalling was measured by increased EGFR and Akt1 phosphorylation after 15 min EGF (100 ng/mL) stimulation of serum starved (SS) cells. No stimulation was seen in cells in NZM37 cells in which the EGFR had been deleted (EKO11 and EKO14) (D). Low doses of gCetuximab (100 $\mu\text{g/ml}$ unless otherwise stated) blocked the the EGF stimulation of EGFR and Akt1 phosphorylation in NZM37 cells (D), HT29 cells (E) and NZM40 cells (F).

were dosed every 3 days with commercial cetuximab, goat-produced cetuximab or vehicle control by intraperitoneal injection with the dosage of 10 mg/kg. The data were analysed using a two-way analysis of variance (ANOVA) model.

2.9. Immunohistochemistry

After euthanization of the mice, the tumors were excised and preserved in freshly made 10 % neutral formalin buffer for 48 h. Then paraffin blocks were prepared and sectioned for immunohistochemical (IHC) staining. IHC procedures were performed as follows: paraffin sections were kept in 60 °C for 15 min and then rinsed in fresh xylene twice, 10 min for each

time, following by 100 % ethanol wash (twice, 10 min for each time). Antigen retrieval was applied by incubating sections with 10 mM citric acid buffer at 95 °C for 30 min. After permeabilizing with TBST (0.1 % Triton X-100), the slides were blocked with TBST containing 2 % BSA and 5 % goat serum at room temperature for 1 h. Then slides were incubated with primary antibodies overnight at 4 °C. After washing with TBST the slides were incubated with a secondary antibody for 2 h at room temperature. Slides were sealed with mount media containing DAPI (Invitrogen, ProLong dimand). Antibodies used in immunohistochemical staining were as follows: Ki67 (Abcam, ab8191, 1:100), CD31 (Abcam, ab28364, 1:100).

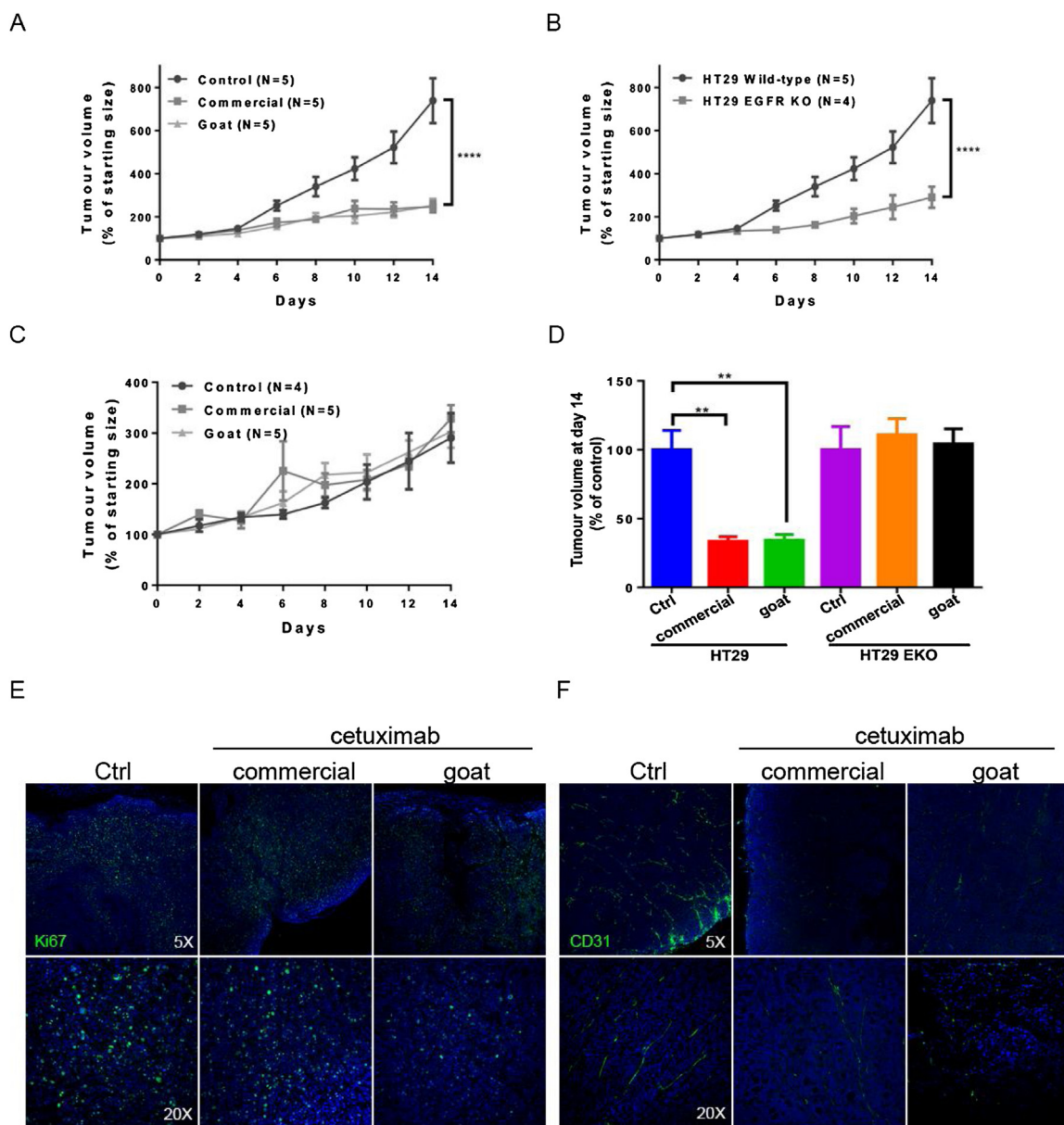


Fig. 3. gCetuximab has equivalent antitumour effect to commercial cetuximab in a mouse tumour xenograft model. Xenografts were established in female NIH-III mice by subcutaneous injection of EGFR knockout or parental HT29 cells. Commercial or gCetuximab (10 mg/kg) was administered by intraperitoneal injection every 3 days. Tumour size was measured every 2 days ($n = 6$). gCetuximab was as effective as commercial cetuximab in slowing growth of xenografts using wild type HT-29 cells (A); Xenografts of EGFR knockout HT29 cells do not grow as fast as parental HT29 cell lines showing that EGF signalling is required for progression of these tumours (B) and cetuximab was no longer effective in blocking growth of the xenografts of EGFR knockout HT29 cells showing effects of the drug were specific to EGF signalling pathways (C); The results of these experiments were quantified and significance assessed using a *t*-test with ** representing significant differences ($p < 0.01$) (D); Immunohistochemical (IHC) analysis of tumour proliferation maker Ki67 and microvessel marker CD31 confirm that the gCetuximab had blocked growth of tumour cells and tumour vascularisation (E,F).

3. Results

3.1. Effects of gCetuximab on EGFR-dependent intracellular signaling

Colorectal cancer cell line HT29 as well as melanoma cancer cell lines NZM37 and NZM40 which have relatively high levels of endogenous EGFR expression were chosen for the following experiments (Fig. 1A). EGFR knock out cell lines were generated using CRISPR-Cas9 and used as negative control. EGFR expression from expanded colonies of each cell line were detected by western blot (Fig. 1B–D). Two knockout clones of each cell line were chosen for the following experiments.

In order to compare the bioactivities of gCetuximab with the commercial cetuximab, we firstly investigated the *in vitro* effects of cetuximab on the growth of EGFR expressing melanoma and colorectal cell lines using the SRB cell viability assay. After 72 h exposure, both forms of cetuximab elicited minimal growth inhibition effect on both colorectal and melanoma cells (Fig. 2A–C). This was consistent with previous findings that cetuximab showed limited growth inhibition on cancer cell lines growing in

serum *in vitro* as the immune cells required to induce ADCC were not present [21]. However, it suggested gCetuximab has minimum non-specific cytotoxicity to cells and could be safely used in animal studies. We then investigated the effects of gCetuximab on EGFR signaling that was induced by EGF ligand stimulation of the cells. Cells were serum starved for 24 h and then stimulated with EGF (100 ng/mL) for 15 min with or without cetuximab preincubation and the phosphorylation of EGFR and Akt were monitored as read outs of signalling through the EGFR signalling pathway. Immunoblotting results show the complete inhibition by low doses of both commercial cetuximab and gCetuximab of EGFR and Akt1 phosphorylation in all three cell lines tested (Fig. 2D–F). These results indicate that the gCetuximab and commercial cetuximab have equivalent effects in blocking EGF stimulated EGFR signaling.

3.2. Anti-tumor effects of gCetuximab on a colorectal cancer xenografted model

We next investigated the anti-tumour effect of gCetuximab *in vivo* using a tumour xenograft model. Firstly, neither gCetuximab

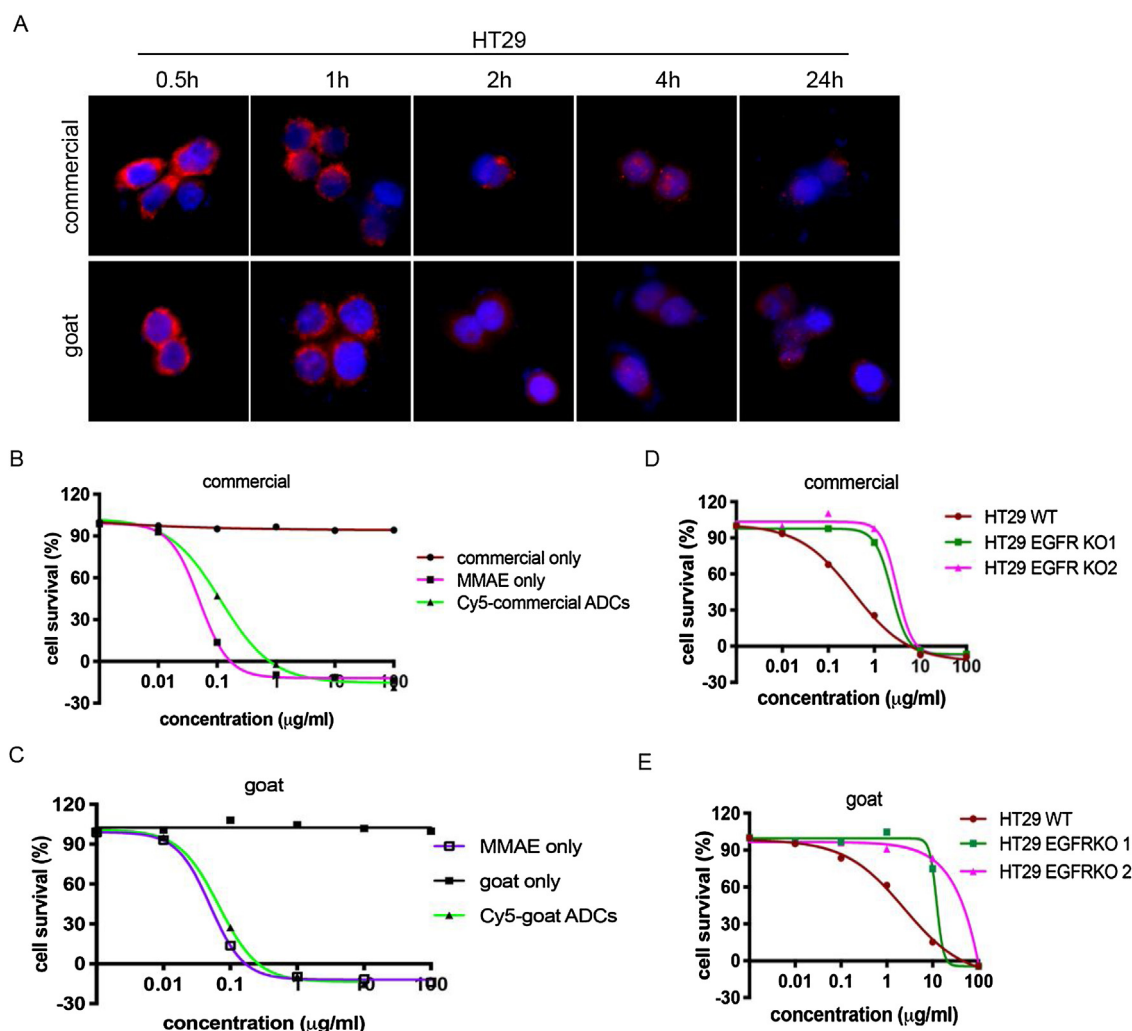


Fig. 4. gCetuximab can be successfully conjugated to toxin MMAE. A. To test uptake of antibodies colorectal cancer cells HT29 were incubated for 0.5 h with either gCetuximab or commercial cetuximab that had been conjugated with MMAE and fluorescently labelled with Cy5. Fresh culture media was added and uptake of fluorescent label into cells monitored for 1, 2, 4, and 24 h. This shows the Cy5 labelled cetuximab-MMAE conjugates could bind to the cell surface and then gradually be internalised (A). To test efficacy of the MMAE labelled cetuximabs (cy5-ADCs) HT29 cells were incubated with cetuximab only, MMAE only or cy5-ADCs for 72 h. SRB cell growth assay was performed to determine cell growth inhibition by different treatments. These show that both commercial cetuximab-MMAE conjugate (B) and gCetuximab-MMAE conjugate (C) were equally effective as the free MMAE drug alone whereas unconjugated cetuximab was ineffective. These effects were lost in EGFR knockout HT29 cells whereas the free MMAE drug alone remained effective showing the effects of the cy5-ADCs were specifically mediated by the presence of EGFR on the cells (D commercial cetuximab; E, gCetuximab).

nor commercial cetuximab caused any overall weight loss in the mice indicating they were not having direct toxicity in the animals (Data not shown). However, both drugs similarly inhibited the rate of growth in the volume of the HT29 xenograft tumours (Fig. 3A) and this can be seen as significant differences in final tumour size at day14 of the experiment (Fig. 3D).

In order to confirm the tumor growth inhibition effect was due to the specificity of gCetuximab binding to EGFR, xenograft studies were performed using the EGFR knock out HT29 cell line. When transplanted into mice, EGFR knock out HT29 cells showed delayed tumour growth compared to HT29 parental tumours which is consistent with EGF signalling playing an important role in the ability of HT29 cells to form tumours (Fig. 3B). When these tumors were treated with either gCetuximab or commercial cetuximab there was no effect on the growth of EGFR knock out HT29 xenograft tumours (Fig. 3C). Together this shows that the drugs were specifically targeting EGFR on HT29 cancer cells in these xenograft models and not having non-specific effects on the tumours.

Immunohistochemical (IHC) analysis was performed to investigate the impacts of the drugs on different cell types in the tumours. This shows both gCetuximab and commercial cetuximab had a direct effect in reducing the mitotic index of cetuximab treated HT29 wild type tumours as shown by a reduction in the proliferation marker Ki67 (Fig. 3E). In addition, microvessel density of cetuximab-treated xenograft tumours was also assessed using CD31 as a marker and both gCetuximab and commercial cetuximab decreased levels of vasculature in the tumours (Fig. 3F). For both Ki67 and CD31 staining there was no significant difference between commercial and gCetuximab treatments. Taken together, these data again suggested that gCetuximab could effectively inhibit tumor growth *via* EGFR signaling *in vivo*.

3.3. Effects of toxin conjugated gCetuximab and commercial cetuximab on colorectal cancer cells

Antibody-drug conjugates (ADCs) for cancer therapy has been attracting significant attention over the past few years due to its

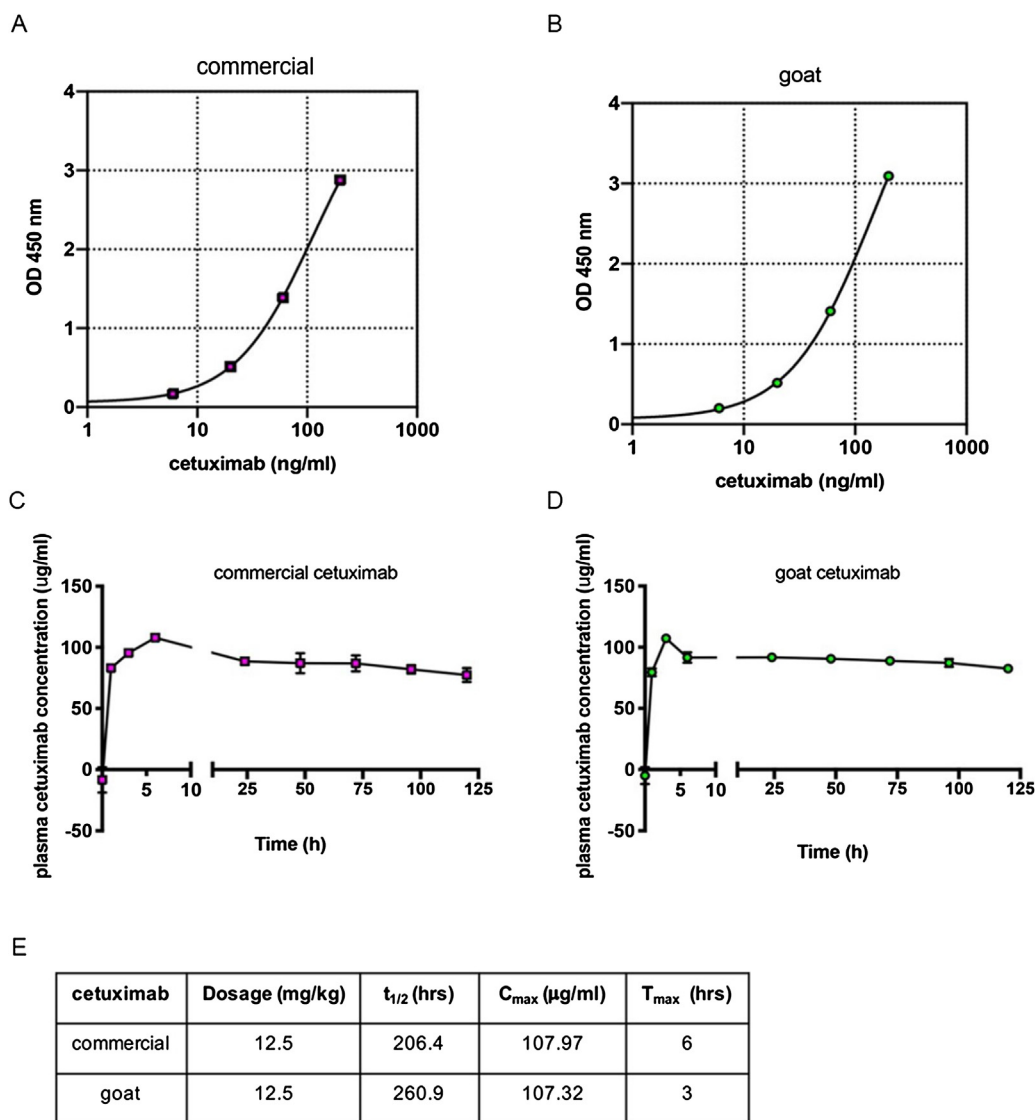


Fig. 5. Pharmacokinetics of commercial cetuximab and gCetuximab are similar. Both commercial cetuximab (A) and gCetuximab (B) were both found to respond equally to an ELISA assay that was subsequently used for pharmacokinetic assays. To test pharmacokinetic properties CD1 mice were administered with a single dose of either commercial (C) or gCetuximab (D) (12.5 mg/kg). Plasma were sampled at 0, 1, 3, 6, 24, 48, 72, 96 and 120 h ($n = 3$). Cetuximab concentration in plasma samples were determined by ELISA assay. Pharmacokinetic parameters were found to be similar (E).

precision in cancer target therapy. Monomethyl auristatin E (MMAE), a potent antimitotic drug, cannot be used as a drug itself due to its toxicity. Instead, it can be linked to monoclonal antibodies to form ADCs for targeted cancer therapy that will specifically take the drug to the tumour cells without potentially affecting other cells in the body. In this study, we tested the ability of gCetuximab to be linked with MMAE and investigated the effectiveness of gCetuximab-MMAE conjugates at targeting toxins to cells that contain the EGFR.

By using a reducing reagent, MMAE was successfully linked to both the gCetuximab and commercial cell culture produced cetuximab. To test if these two ADCs can deliver MMAE specifically to tumour cells, the ADCs were labelled with the fluorescent dye Cy5 for tracking. The EGFR expressing colorectal cancer cell line HT29 was treated with either the goat derived or the commercial Cetuximab-MMAE-Cy5 conjugated antibody and analyzed for Cy5 fluorescence to understand where the antibody was. After 30 min, either the goat or commercial Cetuximab-MMAE-Cy5 conjugated antibodies were specifically localized at the cell surface and were gradually internalised and degraded within 24 h concurrent with EGF receptor internalization (Fig. 4A). This would have also released the MMAE into those cells. We then investigated the cell killing effect of both the goat or commercial Cetuximab-MMAE-Cy5 conjugated antibodies using *in vitro* SRB cell growth assay. Both the goat or commercial Cetuximab-MMAE-Cy5 conjugated antibody were more effective than cetuximab alone at killing HT29 colorectal cancer cells, with both being as effective as the MMAE drug added alone (Fig. 4B, C). In contrast, the goat or commercial Cetuximab-MMAE-Cy5 conjugated antibodies were far less effective in killing HT29 EGFR knockout cells than the MMAE alone showing that the conjugated antibodies were indeed selectively targeting cells expressing EGFR (Fig. 4D, E).

3.4. *In vivo* half-life of gCetuximab compared to commercial cetuximab

To investigate cetuximab half-life, blood samples were collected by cardiac puncture from CD1 mice following a single dose 12.5 mg/kg of either form of cetuximab. Plasma concentration of cetuximab was quantified by ELISA assay. Cetuximab exhibited a one phase exponential decay. In order to exclude the difference of binding affinity between commercial and gCetuximab to precoated ELISA plates, we performed two sets of standard curves: gCetuximab and commercial cetuximab standard curves. There was no significant difference between these two sets of standard curves (Fig. 5A, B). gCetuximab and commercial cetuximab plasma concentrations were calculated according to the standard curve, respectively. The maximum plasma concentration of gCetuximab was 107 µg/mL which was peaked at 3 h post-administration; while for commercial cetuximab a similar maximum plasma concentration, 108 µg/mL, was peaked at 6 h after administration (Fig. 5C, D). The half-life ($t_{1/2}$) of gCetuximab was calculated as being 260 h comparing to that of commercial cetuximab at 206 h (Fig. 5E). There were no significant statistical differences in maximum plasma concentration and $t_{1/2}$ between commercial and gCetuximab. It suggests similar pharmacokinetics of gCetuximab and commercial cetuximab in mice.

4. Discussion and conclusion

High costs of monoclonal anti-cancer antibody therapies restrict their overall routine use in the clinic. We have recently described a transgenic animal production system for the monoclonal antibody cetuximab (gCetuximab) which produces high levels (up to 10 g/L) of this drug by expressing it in the mammary gland of lactating goats [10]. The transgenic produced gCetuximab also has other potential

advantages in having reduced potential for inducing immunogenic reactions against the drug as the production in goats does not produce α -gal linkages as is seen with the innovator and mammalian cell culture derived cetuximab product [10]. Additionally, the gCetuximab has potential for increased efficacy based on its increased antibody dependant cellular toxicity (ADCC) profile [10].

In this study, we investigated antitumour effects of goat-produced cetuximab by a range of biological assays both *in vitro* and *in vivo*. Our results provide evidence that the cetuximab produced in and purified from goat milk is as equally effective in targeting and inhibiting the EGF receptor as is the cetuximab produced by traditional cell culture based methods. Despite the differences in the production systems, the goat-produced cetuximab showed no obvious difference in its toxicity profile in mice as assessed by weight loss. We also report that, compared to the commercially available cetuximab, the goat derived cetuximab is well suited as an antibody for attaching a toxin to create an ADC form of cetuximab. We show that it can selectively target the toxin MMAE to HT29 cells as the gCetuximab-MMAE conjugate required the presence of EGFR to have potent cell killing effects on the cells. This shows the gCetuximab is a good vehicle for treatment regimes based on antibody drug conjugates.

Taken together, this work confirms that the gCetuximab produced in mammary glands of transgenic goats is as effective in blocking EGF signalling and in attenuating EGF dependent growth of tumour models as the current commercial product and therefore suitable as a candidate for clinical biosimilar development. Further, this provides continuing support for the goat milk production system as a commercial and proven (multiple agency approvals worldwide) platform for cost effective human recombinant protein therapeutic production and now to be applied to potential biosimilars going forward.

CRediT authorship contribution statement

Qian Wang: Investigation, Formal analysis, Writing - review & editing. **William Gavin:** Conceptualization, Funding acquisition, Resources, Writing - review & editing. **Nicholas Masiello:** Investigation, Resources. **Khanh B. Tran:** Investigation, Methodology. **Götz Laible:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing. **Peter R. Shepherd:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

WG and NM are employees of LFB-USA and GL is an employee of AgResearch Ltd and PS is an employee of the University of Auckland and all these organisations have a commercial interests or potential commercial interests in the production of gCetuximab.

Acknowledgements

Funding was provided by the Ministry of Business Innovation and Employment in New Zealand (contract ID is C10 × 1504), AgResearch and from the Maurice Wilkins Centre for Molecular Biodiscovery. The authors wish to thank Lynn Peters for the purification work and Dan Pollock, Harry Meade and Li How Chen for reviewing the draft and also Christina Buchanan for assistance with HPLC analysis. We thank Dr Moana Tercel for advice on developing ADCs.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00533>.

References

- [1] A.M. Scott, J.P. Allison, J.D. Wolchok, Monoclonal antibodies in cancer therapy, *Cancer Immun.* 12 (2012) 14.
- [2] B. Vincenzi, A. Zoccoli, F. Pantano, O. Venditti, S. Galluzzo, Cetuximab: from bench to bedside, *Curr. Cancer Drug Targets* 10 (1) (2010) 80–95.
- [3] J. Graham, M. Muhsin, P. Kirkpatrick, Cetuximab, *Nat. Rev. Drug Discov.* 3 (7) (2004) 549–550.
- [4] S. Li, K.R. Schmitz, P.D. Jeffrey, J.J. Wiltzius, P. Kussie, K.M. Ferguson, Structural basis for inhibition of the epidermal growth factor receptor by cetuximab, *Cancer Cell* 7 (4) (2005) 301–311.
- [5] E.S. Kim, F.R. Khuri, R.S. Herbst, Epidermal growth factor receptor biology (IMC-C225), *Curr. Opin. Oncol.* 13 (6) (2001) 506–513.
- [6] E. Martinelli, R. De Palma, M. Orditura, F. De Vita, F. Ciardiello, Anti-epidermal growth factor receptor monoclonal antibodies in cancer therapy, *Clin. Exp. Immunol.* 158 (1) (2009) 1–9.
- [7] A. Ahsan, S.M. Hiniker, M.A. Davis, T.S. Lawrence, M.K. Nyati, Role of cell cycle in epidermal growth factor receptor inhibitor-mediated radiosensitization, *Cancer Res.* 69 (12) (2009) 5108–5114.
- [8] L.M. Ellis, Epidermal growth factor receptor in tumor angiogenesis, *Hematol. Oncol. Clin. North Am.* 18 (5) (2004) 1007–1021 viii.
- [9] A. Holsken, M. Gebhardt, M. Buchfelder, R. Fahlbusch, I. Blumcke, R. Buslei, EGFR signaling regulates tumor cell migration in craniopharyngiomas, *Clin. Cancer Res.* 17 (13) (2011) 4367–4377.
- [10] G. Laible, S. Cole, B. Brophy, P. Maclean, L.H. Chen, D.P. Pollock, et al., Transgenic goats producing an improved version of cetuximab in milk, *FASEB BioAdv.* (2020), doi:<http://dx.doi.org/10.1096/fba.2020.00059>.
- [11] D. Patel, A. Lahiji, S. Patel, M. Franklin, X. Jimenez, D.J. Hicklin, et al., Monoclonal antibody cetuximab binds to and down-regulates constitutively activated epidermal growth factor receptor vIII on the cell surface, *Anticancer Res.* 27 (5A) (2007) 3355–3366.
- [12] S. Kim, C.N. Prichard, M.N. Younes, Y.D. Yazici, S.A. Jasser, B.N. Bekele, et al., Cetuximab and irinotecan interact synergistically to inhibit the growth of orthotopic anaplastic thyroid carcinoma xenografts in nude mice, *Clin. Cancer Res.* 12 (2) (2006) 600–607.
- [13] J.F. Doody, Y. Wang, S.N. Patel, C. Joynes, S.P. Lee, J. Gerlak, et al., Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non small cell lung cancers, *Mol. Cancer Ther.* 6 (10) (2007) 2642–2651.
- [14] K.B. Tran, G. Gimenez, P. Tsai, S. Kolekar, E.J. Rodger, A. Chatterjee, et al., Genomic and signalling pathway characterisation of the NZM panel of melanoma cell lines: a valuable model for studying the impact of genetic diversity in melanoma, *Pigment Cell Melanoma Res.* (2020), doi:<http://dx.doi.org/10.1111/pcmr.12908>.
- [15] M. Sweetlove, E. Wrightson, S. Kolekar, G.W. Rewcastle, B.C. Baguley, P.R. Shepherd, et al., Inhibitors of pan-PI3K signaling synergize with BRAF or MEK inhibitors to prevent BRAF-Mutant melanoma cell growth, *Front. Oncol.* 5 (2015) 135.
- [16] V. Vichai, K. Kirtikara, Sulforhodamine B colorimetric assay for cytotoxicity screening, *Nat. Protoc.* 1 (3) (2006) 1112–1116.
- [17] S.R. Adams, H.C. Yang, E.N. Savariar, J. Aguilera, J.L. Crisp, K.A. Jones, et al., Anti-tubulin drugs conjugated to anti-ErbB antibodies selectively radiosensitize, *Nat. Commun.* 7 (2016) 13019.
- [18] M.M. Sun, K.S. Beam, C.G. Cerveny, K.J. Hamblett, R.S. Blackmore, M.Y. Torgov, et al., Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides, *Bioconjug. Chem.* 16 (5) (2005) 1282–1290.
- [19] K.J. Hamblett, P.D. Senter, D.F. Chace, M.M. Sun, J. Lenox, C.G. Cerveny, et al., Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate, *Clin. Cancer Res.* 10 (20) (2004) 7063–7070.
- [20] F.R. Luo, Z. Yang, H. Dong, A. Camuso, K. McGlinchey, K. Fager, et al., Correlation of pharmacokinetics with the antitumor activity of Cetuximab in nude mice bearing the GEO human colon carcinoma xenograft, *Cancer Chemother. Pharmacol.* 56 (5) (2005) 455–464.
- [21] T. Matsuo, S.S. Nishizuka, K. Ishida, T. Iwaya, M. Ikeda, G. Wakabayashi, Analysis of the anti-tumor effect of cetuximab using protein kinetics and mouse xenograft models, *BMC Res. Notes* 4 (2011) 140.