EUROPEAN JOURNAL OF CANCER 47 (2011) 1736–1746

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

An antibody–cytotoxic conjugate, BIIB015, is a new targeted therapy for Cripto positive tumours

Rebecca K. Kelly, Dian L. Olson, Yaping Sun, Dingyi Wen, Kathleen A. Wortham, Giovanna Antognetti, Anne E. Cheung, Olivia E. Orozco, Lu Yang, Veronique Bailly, Michele Sanicola *

Biogen Idec, Inc., Discovery Oncology, 14 Cambridge Center, Cambridge, MA 02142, USA

ARTICLE INFO

Article history:
Available online 31 March 2011

Keywords:
Cripto
Antibody–cytotoxic conjugate
Targeted therapeutic
Payload

ABSTRACT

BIIB015 is an immunoconjugate created for the treatment of solid tumours and is currently in Phase I of clinical evaluation. BIIB015 consists of a humanised monoclonal antibody against the Cripto protein carrying a payload, via a hindered disulphide linker, of the maytansinoid derivative, DM4. Cripto is a GPI-linked protein required for signal transduction of the TGF-beta ligand, Nodal. Cripto has been previously described as an oncogene and fits the classic pattern of an embryonic gene that is re-expressed in a transformed tumour cell. Cripto expression is highly prevalent on a number of solid tumours, including greater than 75% of breast, lung, and colorectal tumours. Our report documents for the first time that targeting the cell surface Cripto protein with an anti-Cripto antibody–cytotoxic conjugate is an effective means of inhibiting or regressing growth of Cripto positive tumours. BIIB015 which utilises a 'cleavable' linker containing a disulphide bond exhibits superior activity when compared to huB3F6 mAb conjugates with different linker systems, including one with a 'non-cleavable' linker. BIIB015 displays specificity for Cripto in both *in vitro* and *in vivo* experiments. In human xenograft models originating from lung (Calu-6), colon (CT-3), testicular (NCCIT) and breast (MDA-MB-231) tumour samples, BIIB015 shows robust activity with results ranging from >50% tumour inhibition to complete tumour regression. The efficacy seen in the MDA-MB-231 model, a triple negative (-HER2, -ER, and -PR) tumour, is particularly exciting since there is currently no approved therapy for this indication. In addition, BIIB015 can be combined with standard of care chemotherapeutics for enhanced efficacy.

1. Introduction

Cripto is a cell surface GPI-linked protein which is overexpressed on many solid human tumours relative to normal adjacent tissue (for review Bianco et al.[1]). There are five Cripto genes in humans, two of which are expressed (Cr-1 and Cr-3) and three 'pseudo genes' (Cr-2, -4, and -5). The cell surface proteins, Cr-1 and Cr-3, are highly homologous and differ by only three amino acids in the mature protein. Human tumours express Cr-1, Cr-3 or, in some cases, both proteins.[1,2] Functionally, Cripto has been defined as the co-receptor for the TGF-beta growth factor family member, Nodal. The Cripto–Nodal interaction allows Nodal to bind the Alk4-ActRII receptor complex and signal via an SMAD 2,4 transduction pathway.[3,4] Developmental data suggest that the Cripto–Nodal pathway plays a role in determining mesenchymal cell fate.[4–7] A role for Cripto and Nodal during puberty in the developing mammary gland has also been noted.[8] However,

* *Corresponding author:* Tel.: +1 617 679 3307; mobile: +1 617 291 4421.
E-mail addresses: Michele.sanicola@biogenidec.com, Michele.sanicola@gmail.com (M. Sanicola).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.02.023

in normal adult tissues, Cripto is expressed at low levels[8] and a functional role for Cripto and Nodal in the mature adult has not yet been fully defined.

Cripto exhibits the characteristics of a classic 'feto-oncogene', a gene with a key role in embryonic development that is inappropriately re-expressed in transformed cells. *In vitro* Cripto acts as an oncogene in cell transformation assays, promotes cell migration and stimulates growth of mammary gland and colon cells.[4,9] Additional *in vitro* experiments have also shown that Cripto can modulate other signalling pathways such as inhibiting another TGF-beta ligand, Activin B, and stimulating MAPK when it is overexpressed.[1,10–12] *In vivo*, Cripto can transform cells and promote tumour growth as demonstrated by transgenic overexpression of Cripto in the mouse mammary gland driven by a mouse mammary tumour virus promoter (MMTV). 100% of virgin Tg MMTV Cripto mice developed mammary gland hyperplasia, with about one-third of multiparous animals developing mammary tumours as they age.[8,13,14] In humans, Cripto expression is detected on greater than 75% of breast, lung and colon tumours.[1,4] In addition, an increase in Cripto expression has been correlated with human breast, ovarian and cervical cancer progression.[15–17] Furthermore, recent experiments by Hendrix and colleagues suggest re-expression of the Cripto–Nodal embryonic signalling pathway increases metastatic potential of melanomas which is consistent with its developmental role in mesenchymal cell fate and migration.[18,19] Hendrix's group also notes that Cripto transformed cells express the stem cell markers Nanog and Oct 4 and speculates that these cells could represent tumour stem cells.[18–20] In summary, not only is the Cripto protein expressed on many human tumours, biologically it has the oncogenic potential to drive proliferation, migration, cell transformation and tumour formation as well as be a marker of tumour cells with stem cell-like properties. These characteristics make the Cripto cell surface tumour protein an excellent candidate for targeted cancer therapy.

Tumour cell surface proteins, such as Cripto, can be specifically targeted with antibodies and such antibodies can be evaluated for the ability to inhibit growth in human xenograft tumour models. Previously, our group has demonstrated that monoclonal antibodies against human Cripto can inhibit the growth of human xenograft tumours in mice.[10] The activity of the naked antibody was modest but given Cripto's tumour expression profile relative to normal tissue,[1] we decided to examine the therapeutic benefit of targeting a cytotoxic agent directly to Cripto positive tumours. Maytansine is a very potent tubulin polymerisation inhibitor that blocks mitosis, causes death of replicating cells, and was considered as a potential cancer chemotherapeutic agent.[5,21,22] However, clinical development of free maytansine had to be halted due to severe toxic effects.[22] Historically, antibodies have been exploited as vehicles to deliver cytotoxic molecules to tumours and several antibody–cytotoxic conjugates are in development[23–28] and for excellent review see Alley et al.[29] These conjugates offer multiple advantages over free drug, including long half-lives and potential tissue targeting by recognising specific antigens.

Most recently, two antibody drug conjugates in the clinic have demonstrated impressive activity. One is the trastuzumab antibody conjugated to DM1 (T-DM1, Genentech). DM1 is a maytansine derivative that is coupled to trastuzumab and binds to HER2 with an affinity similar to trastuzumab. T-DM1 has demonstrated anti-tumour activity preclinically with both trastuzumab sensitive and resistant lines.[30] Clinically, robust data have been reported in Phase I and Phase II trials.[25,27] Specifically, the activity of T-DM1 was examined in an open-label, single-arm phase II study of 112 patients who had all progressed on HER-2 directed therapy and had received chemotherapy in a metastatic setting. T-DM1 was administered at 3.6 mg/kg intravenously (IV) every three weeks. The objective response rate was 39.3% with a median follow-up of 4.4 months. A subgroup of patients who had a follow-up of >6 months showed an objective response rate of 38.2%. These response rates are impressive in that the study included a population which was heavily pretreated with anti-HER2 therapy and suggests that the targeted delivery of the DM1 via HER2 is very effective.[27] A second immunoconjugate, which uses a completely different payload technology from T-DM1, has also generated impressive clinical data. SGN-35, an anti-CD30 antibody linked to a potent anti-mitotic agent, monomethylauristatin E (MMAE), has been tested in patients with relapsed or refractory CD30 positive haematological cancers, predominantly Hodgkins Lymphoma and anaplastic large cell lymphoma. Bartlett et al.[28] report SGN35 induced durable responses in most patients which at the maximum tolerated dose in this phase I study of 1.8 mg/kg translated into tumour regression for 50% of the patients.

Our BIIB015 immunoconjugate was generated to harness the potency of maytansine and direct its cell killing activity towards Cripto positive tumours. BIIB015 consists of a humanised monoclonal antibody (huB3F6) capable of binding the Cripto 1 or 3 tumour protein coupled via a hindered disulphide linker (SPDB) to the maytansinoid derivative, DM4. The SPDB linker is highly stable in circulation but unstable once inside the reducing environment of the cytoplasm, where it releases the DM4 payload. Thus, BIIB015 represents a method to target a very potent tubulin inhibitor directly to Cripto positive tumour cells and provide a better therapeutic window than free maytansine. The improved tolerability and safety profile exhibited by maytansinoid conjugates which have been in recent Phase I and Phase II studies as noted above, versus unconjugated maytansine, provide a clinical proof of this concept.[21,25,27,31,32]

In this report we compare the efficacy of BIIB015 to the efficacy of two alternate anti-Cripto monoclonal antibody(mAb)-maytansinoid conjugates which differ in the way the maytansinoid is linked to the antibody. Each of these molecules has been documented to have a different *in vivo* half life. In addition, whilst two of the conjugate linkers utilise a 'cleavable' disulphide linker system (SPP, SPDB); the third uses a 'non-cleavable' linker system (SMCC) containing a thioether bond.[33] In this study, we directly compare the efficacy in xenograft tumour models of these three anti Cripto huB3F6-maytansinoid conjugates to substantiate our selection of BIIB015 as a clinical candidate. In addition, we demonstrate the specificity of BIIB015 *in vivo* and robust tumour growth inhibition in a variety of xenograft tumour models, including regression of large tumours. Lastly, we show enhancement of anti-tumour efficacy when administered in combination with

BIIB015. The data presented here provide experimental evidence that BIIB015 is a potent targeted therapeutic molecule to directly kill Cripto positive tumour cells.

2. Materials and methods

2.1. Antibodies

The murine anti-CR-1 monoclonal antibody muB3F6 was generated as described earlier.[10] A recombinant humanised version of B3F6 was created and expressed in Chinese hamster ovary (CHO) cells.[10] Antibodies were purified using a combination of chromatographic steps on protein A sepharose and ion exchange resins following standard practices.

2.2. Preparation of the antibody–maytansinoid conjugates

Anti-Cripto antibodies were conjugated with maytansinoid derivatives DM1 or DM4 via a stable or reducible linker (Fig. 1A) using proprietary processes by Immunogen, Inc., Cambridge, MA[5,22] to create three antibody–toxin conjugates: huB3F6-SPDB-DM4 (BIIB015), huB3F6-SPP-DM1 and huB3F6-SMCC-DM1. Conjugation was done in two steps. First a bifunctional linker (SPP, SPDB or SMCC) was coupled to the antibody by reaction of its NHS (N-hydroxysuccinimidyl) group with free amino groups (N-termini and lysine residues) of the huB3F6 antibody. The conditions and stoichiometry of this reaction are carefully determined to reproducibly obtain an average of 3 to 4 molecules of a linker attached per antibody. Although some amino groups might react more readily with

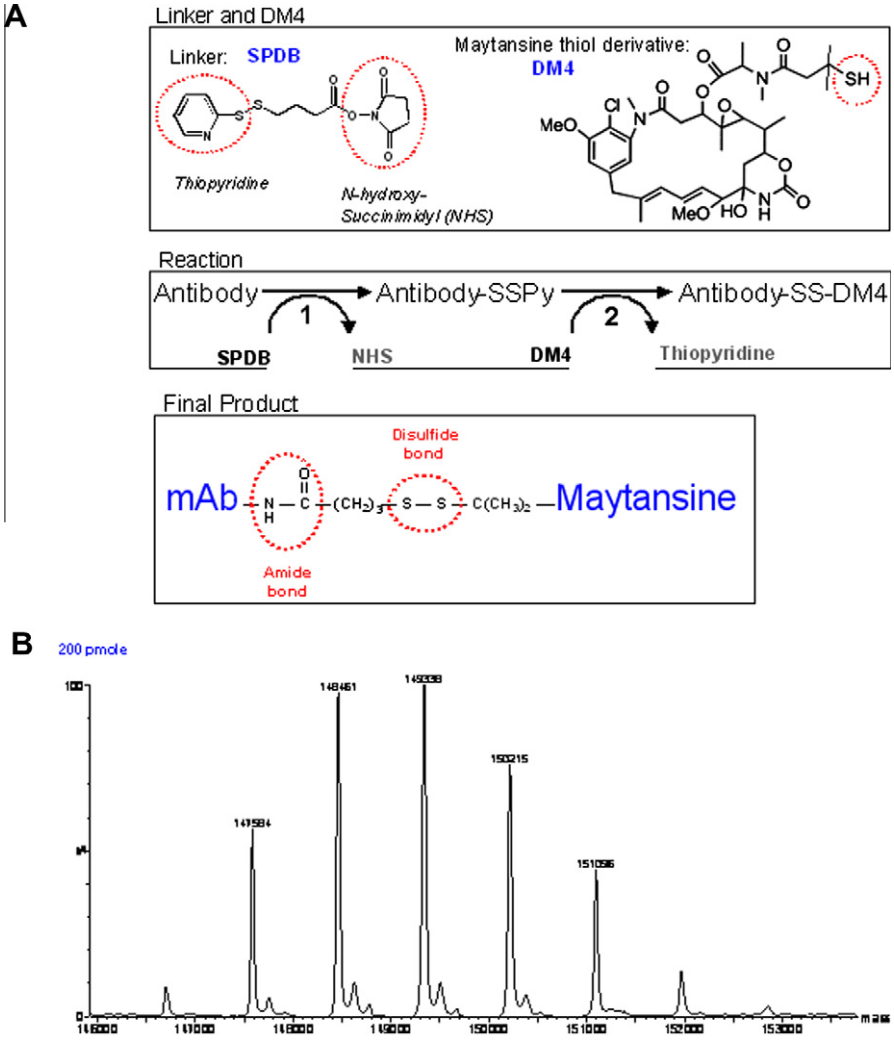

Fig. 1 – Conjugation and characterisation of BIIB015. (A) Schematic representation of the BIIB015 conjugation process. BIIB015 is the humanised IgG1 kappa form of the anti-Cripto monoclonal antibody B3F6 carrying on average 3 to 4 molecules of DM4 via reducible thiobutonyl linker. DM4 is a maytansinoid derivative with a free thiol group $N^{2'}$-deacetyl-N-$^{2'}$(4-methyl-4-mercapto-1-oxopentyl)-maytansine. SPDB 4-(2-pyridyldithio) butanoic acid N-hydroxysuccinimide ester is a common heterobifunctional crosslinker with two reactive groups: an NHS (N-hydroxysuccinimidyl) group for reaction with free amino groups and a pyridine disulphide for reaction with free sulphydryl groups. (B) Panel B shows an example of a deconvoluted mass spectrum for BIIB015.

the linker and be hotspots for conjugation, the large excess of lysine residues over the linker results in an overall randomisation of the conjugation sites. For example, most of the 92 lysine residues of huB3F6 are conjugated but at a frequency of 5% or lower. After gel filtration, the modified antibody is then allowed to react with an excess of DM1 or DM4 to form a covalent bond with defined stability.[5] The conjugated antibodies were purified by gel filtration or chromatography on ceramic hydroxyapatite and formulated by dialysis or diafiltration.

2.3. Immunohistochemistry

Human breast, colon, lung and ovarian formalin-fixed paraffin-embedded tumour tissue arrays (Imgenex, San Diego, CA, Folio Biosciences, Columbus, OH and Zymed, San Francisco, CA) were stained as described previously.[10] NCCIT and CT-3 formalin-fixed paraffin-embedded tumour xenograft tissues were stained using the DAKO Envision system (DAKO, Carpinteria, CA). Slides were deparaffinised and hydrated, then treated with peroxidase blocking solution. Slides were incubated with muB3F6 antibody at 2 μg/ml and then anti-mouse HRP labelled polymer (DAKO) and developed using DAB chromogen. Images were captured using a Zeiss Axioplan 2 microscope with a 40×/0.75 Plan-Neofluor lens.

2.4. Caspase assay

CHO cells were transfected with Cripto as described earlier.[34] CHO–CR and CHO cells were harvested with 20 mM EDTA, washed 1× and re-suspended in their respective normal growth media (10% FCS alpha minus MEM for CHO-CR, 10% FCS alpha plus MEM for CHO) to obtain a cell concentration of 4.0×10^5 cells/ml. The cells were then added to a 96-well white microtitre plate with a plating density of 20,000 cells per well (50 μl/well). A dose response curve was generated with BIIB015 (lot 2000-112) at 2× final assay concentration in the same media and added to the plate at 50 μl /well. The assay plate was then incubated for 48 hours at 37 °C with 5% CO_2. Caspase 3 and 7 were measured with the Caspase-Glo® 3/7 Assay Kit (Promega, cat# G8091). After a 10 minute dark adaptation period, the luminescence readout was detected with the PerkinElmer Topcount Microplate Counter and reported as counts per second. The data were plotted versus the conjugate concentration and fitted with a 4-parameter curve fit to determine an EC50.

2.5. Transfection of 293 cells with Cripto-1 or Cripto-3

293 cells transformed human Kidney cells (American Tissue Culture Collection (ATCC), Manassas, VA) were seeded 48 hours before transfection at 2.0×10^6 cells per 100 mm plate. Transfections were performed according to manufacturer's recommendations using Roche Diagnostics Corporation's Fugene-6 Transfection Reagent (Indianapolis, IN). Briefly 15 μl of Fugene was combined with 5 μg of an expression vector containing either Cripto-1 or Cripto-3. A third group was mock transfected with no DNA to confirm selection efficiency. At 48 hours post transfection the cells were selected for and maintained in media supplemented with 250 μg/ml G418.

2.6. Sorting based on Cripto expression levels

293 cells expressing Cripto-1 or Cripto-3 surface protein were harvested using 5 mM EDTA in PBS and re-suspended at 3×10^7 cells/ml in DMEM containing 10% Fetal Calf Serum (FCS). The cells were combined with huB3F6 at 10 μg/ml for 30 minutes at 4 °C, washed twice with complete DMEM/10%FCS and then combined with R-Phycoerythrin labelled Goat Anti-Human IgG H&L (Jackson Labs, West Grove, PA) for 30 minutes at 4 °C. Cells were then washed twice with complete DMEM/10%FCS. To identify dead cells, 1 μg/ml Propidium Iodide (Becton Dickenson, San Diego, CA) was added and cells were sorted using an Influx Sorter from Cytopeia (Seattle, WA). The sorted cell populations were then maintained separately in culture.

2.7. Quantification of Cripto surface antigen number and effect on cell viability

293 cells expressing Cripto-1 or Cripto-3, as well as Cripto null 293 cells were harvested with 5 mM EDTA in PBS, washed once with PBS and resuspended in FACS buffer (PBS, 1% BSA 0.1%, Na Azide). The cells were then combined with Bangs Laboratories micro spheres (Fishers, IN), which express known numbers of surface receptors, and were treated with 10 μg/ml of huB3F6 for 30 minutes at 4 °C. The cells were then washed twice with FACS buffer, combined with R-Phycoerythrin labelled Goat Anti-Human IgG H&L (Jackson Labs, West Grove, PA) for 30 minutes at 4 °C, washed 2× with FACS buffer and fixed in 2% Para-formaldehyde in PBS. Fixed cells were acquired on a FACS Caliber (Becton Dickinson, San Jose, CA) and analysed using FlowJo software (Treestar Inc., Ashland, OR). The mean fluorescence intensity (MFI) of the individual bead populations was determined and entered into a linear regression model supplied by Bangs Labs. The relative number of Cripto surface molecules on transfected 293 cell populations was then determined from their respective MFI's using the same linear regression model.

2.8. Cell killing assay

293 cells were plated at 1.5×10^4 per well into white clear bottom 96 well microtitre plates (Costar, Corning, NY). A dose curve starting with a concentration of 10 μg/ml of BIIB015 was added to the wells. After incubation for 72 hours at 37 °C in a humidified incubator, cell viability was determined by using the ATP-lite kit supplied by Perkin-Elmer (Boston, MA) according to manufacturer's instructions. The plates were read in a Packard Bio Science Top Count NXT (Boston, MA) micro plate scintillation & luminescence counter. The means of triplicate samples were calculated and plotted. The concentration at which 50% of the cells were no longer viable was determined.

2.9. Human xenograft tumour models

All *in vivo* procedures were performed in accordance with Biogen Idec Institutional Animal Care and Use Committee guidelines. NCCIT, a mediastinal mixed germ cell human testicular carcinoma cells (ATCC, Manassas, VA) and MDA-MB-231,

human breast carcinoma cells (ATCC), were cultured and inoculated into mice as previously described.[10] NCCIT cells were inoculated into 8-week old male athymic nude mice (Hsd:Athymic Nude-*Foxn1^nu*, Harlan Sprague Dawley Inc., Indianapolis, IN). MDA-MB-231 cells were inoculated into 7-week old female CB17 SCID mice (CB17-*Prkdc*^{scid}/NCrCrl, Charles River Laboratories, Inc., Wilmington, MA). Calu-6, human non-small cell lung cancer cells (ATCC) were maintained in MEM Earles's BSS/NEAA/10%FBS media without antibiotics and inoculated subcutaneously (SC) (5×10^6/mouse in 200 μl media) into the right flank of 9-week old female athymic nude mice (Hsd:Athymic Nude-*Foxn1^nu*, Harlan Sprague Dawley Inc., Indianapolis, In). For the CT-3 tumour model, primary human colon tumour tissue (Sera Care, Inc., Oceanside, CA) was serially transplanted *in vivo* to establish a SC xenograft model. Cryopreserved tumour fragments were thawed and serially passaged SC in female SCID beige mice at 8–10 weeks old (C.B-17/IcrHsd-*Prkcd*^{scid}*Lyst*^{bg}, Harlan Sprague Dawley Inc., Indianapolis, IN) for two to five generations prior to implantation for studies.

Tumours were measured at least twice a week using digital calipers. Tumour volume was calculated using the formula: $L \times W^2/2 = mm^3$. When the majority of tumours reached 100–200 mm^3 mice were assigned to treatment and control groups and initial treatments were administered. Tumours were size-matched across groups. BIIB015, the corresponding vehicle buffer, and free DM-4 were administered via tail vein injection. Paclitaxel (clinical-grade Bedford Laboratories (Bedford, OH) was administered via intraperitoneal injection every four days for a total of three doses (q4dx3). 5-fluorouracil (clinical grade Adrucil, Sicor Pharmaceuticals, Irvine, CA) was administered via intraperitoneal injection every two days for a total of six doses (q2dx6). Student's *t*-test (one-way, two-tail) was used to determine statistical significance of differences in growth of test groups compared to control groups.

3. Results

3.1. Cripto protein expression is prevalent in many different solid tumour types

In the literature, several anti-Cripto antibodies have been used to document Cripto expression by immunohistochemistry (IHC) on different tissues.[1,15–17] Cripto expression can be detected on greater than 50% of most solid human tumours.[1] We examined the immunoreactivity of the anti-Cripto muB3F6 mAb on a panel of primary human tumours and the results are summarised and compared to the data reported by Bianco et al.[1] in Table 1. The prevalence of Cripto expression in human solid tumours is high and can be detected in more than 75% of the breast, lung and colon tumour samples. The number of different solid tumour types that express Cripto suggests that BIIB015 could target multiple tumour types and be therapeutically beneficial across multiple cancer indications.

3.2. Characterisation of the antibody–maytansinoid conjugates

Basic characterisation of the conjugated antibodies (BIIB015, huB3F6-SPP-DM1, huB3F6-SMCC-DM1) included the measure of the antibody and DM4 concentrations by UV absorbance and the measure of the percentage of aggregates by size exclusion chromatography.[33] Mass spectrometry was done after removal of N-glycans to evaluate semi-quantitatively the overall relative distribution of the antibody molecules with the defined number of linker-maytansinoid adducts. An example of a deconvoluted mass spectrum for deglycosylated BIIB015 is presented in Fig. 1B and illustrates a typical Poisson distribution of masses for a population of conjugates with 0 to 7 or more linker-DM4 per antibody molecule.

3.3. Comparison of huB3F6-SPDB-DM4 (BIIB015) with alternate huB3F6-maytansinoid conjugates

Linker selection is a critical component of an antibody–cytotoxic conjugate generation. For maximal efficacy with minimal toxicity, the ideal linkage should be stable whilst in circulation and yet allow for the liberation of an active cytotoxic moiety upon binding to and internalisation into a target cell. We evaluated three different huB3F6-maytansinoid conjugates that differ in the structure of the linkage used between the antibody and the maytansinoid moiety (huB3F6-SPP-DM1, huB3F6-SMCC-DM1 and huB3F6-SPDB-DM4)[22,33,35,36] for their activity in human tumour xenograft models in mice. The BIIB015 conjugate (huB3F6-SPDB-DM4)

Table 1 – Immunoreactivity of anti-Cripto antibodies on primary human tumours.

Tissue of origin	Tumour type	
	% Anti-Cripto muB3F6 immunopositive (%)	% Anti-Cripto rabbit polyclonal Ab immunopositive* (%)
Breast	82% (31/38 samples)	78
Colon	78% (136/174 samples)	73
Lung	78% (51/65 samples)	91
Ovary	59% (38/65 samples)	50
Bladder	No data	60
Cervix	No data	39
Testis	No data	57
Stomach	No data	42
Endometrium	No data	58

* From Bianco et al.[1]

contains a cleavable hindered disulphide bond (Fig. 1) similar to that found in IMGN242 (huC242-DM4) which has a terminal half-life of about 5 days in circulation.[5] huB3F6-SPP-DM1 also contains a cleavable disulphide bond. The reduced hindrance of the disulphide bond in huB3F6-SPP-DM1 compared to huB3F6-SPDB-DM4 would be expected to result in a reduced half-life in circulation. An important feature of both disulphide linkages is that they have been shown by Erickson et al.[33,37] to be capable of generating maytansinoid metabolites after lysosomal degradation inside the cell that can diffuse out of the cell membrane allowing for 'nearest neighbour' killing, an effect that could be an additional benefit in a heterogeneous tumour. In contrast, huB3F6-SMCC-DM1 contains a thioether bond which is not susceptible to reduction or disulphide exchange with an estimated half-life similar to that of the antibody itself. Intracellular processing of this type of conjugate results in the release of an active metabolite without the bystander activity observed with the disulphide containing conjugates. Fig. 2 compares the efficacy of BIIB015 (huB3F6-SPDB-DM4) with huB3F6-SMCC-DM1 in the NCCIT xenograft model. The NCCIT xenograft tumour model shows strong tumour epithelial Cripto expression (Fig. 2A). Both conjugates inhibited tumour growth with the SPDB conjugate exhibiting higher activity (tumour growth inhibition 80–90%) than the SMCC conjugate (tumour growth inhibition 50%) (Fig. 2B). We also compared the efficacy of these two conjugates and huB3F6–SPP-DM1 in the CT-3 colon model, whose tumours typically have low overall Cripto expression with some regions of patchy heterogeneous expression (Fig. 2A). Treatment with huB3F6-SMCC-DM1 did not inhibit tumour growth in this model, however, BIIB015 and huB3F6-SPP-DM1 inhibited tumour growth by about 90% and 80%, respectively (Fig. 2C).

3.4. *Specificity of BIIB015 anti-tumour activity*

In order to assess the specificity of the *in vitro* cytotoxicity of BIIB015, CHO cells transfected to express Cripto-1 and parental Cripto-negative CHO cells were incubated with BIIB015 for 48 hrs and then assayed for the activation of the cell death markers Caspase 3 and Caspase 7. BIIB015 selectively induced activation of cell death markers in Cripto-1-positive CHO cells with an EC50 of 0.982 μg/ml, compared to an EC50 of 12.8 μg/ml in the Cripto-negative CHO cells (Fig. 3A). Similar *in vitro* studies have demonstrated the ability of BIIB015 to kill the Cripto-positive tumour cell lines NCCIT (testicular carcinoma) and GEO (colon carcinoma) (data not shown). These results support that BIIB015 will bind to cell surface Cripto and then kill the cell after internalisation and release of the DM4.

The *in vivo* specificity of BIIB015 was confirmed by the ability of an excess of unconjugated huB3F6 to inhibit the anti-tumour activity of BIIB015 in a tumour xenograft study (Fig. 3B). In this experiment, mice bearing subcutaneous CT-3 colon tumour xenografts were treated with 20 mg/kg BIIB015 with or

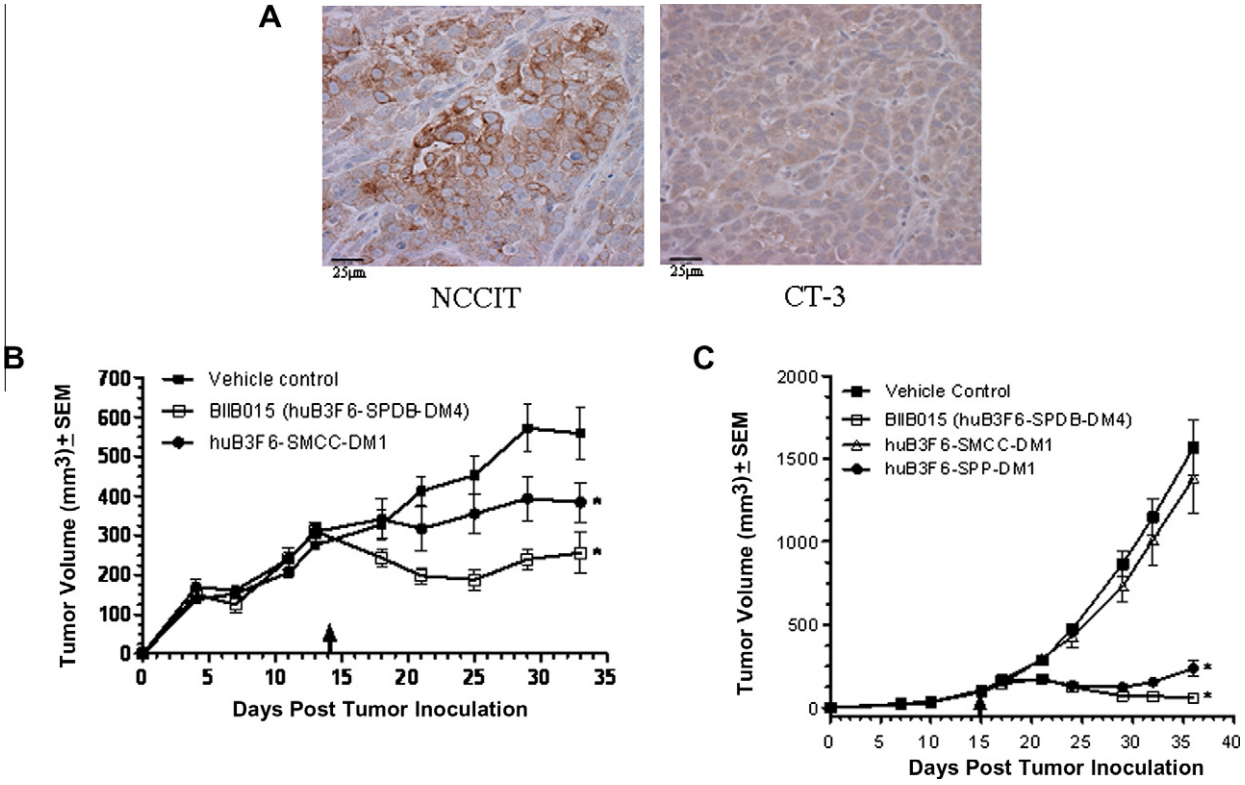

Fig. 2 – SPBD linker conjugate shows superior efficacy. (A) Cripto expression by IHC staining in NCCIT (left) and CT-3 (right) xenograft tumour sections. (B) Response of NCCIT xenograft tumours to treatment with BIIB015 (15 mg/kg; ~45 mg/m^2), B3F6-SMCC-DM1 (15 mg/kg; ~45 mg/m^2) or vehicle control. (C) Response of CT-3 xenograft tumours to treatment with BIIB015, B3F6-SMCC-DM1, B3F6-SPP-DM1, or vehicle control. All treatments single dose, administered i.v. on day indicated by arrow. *Points*, mean; *bars*, SE. *$P < 0.05$ versus vehicle control.

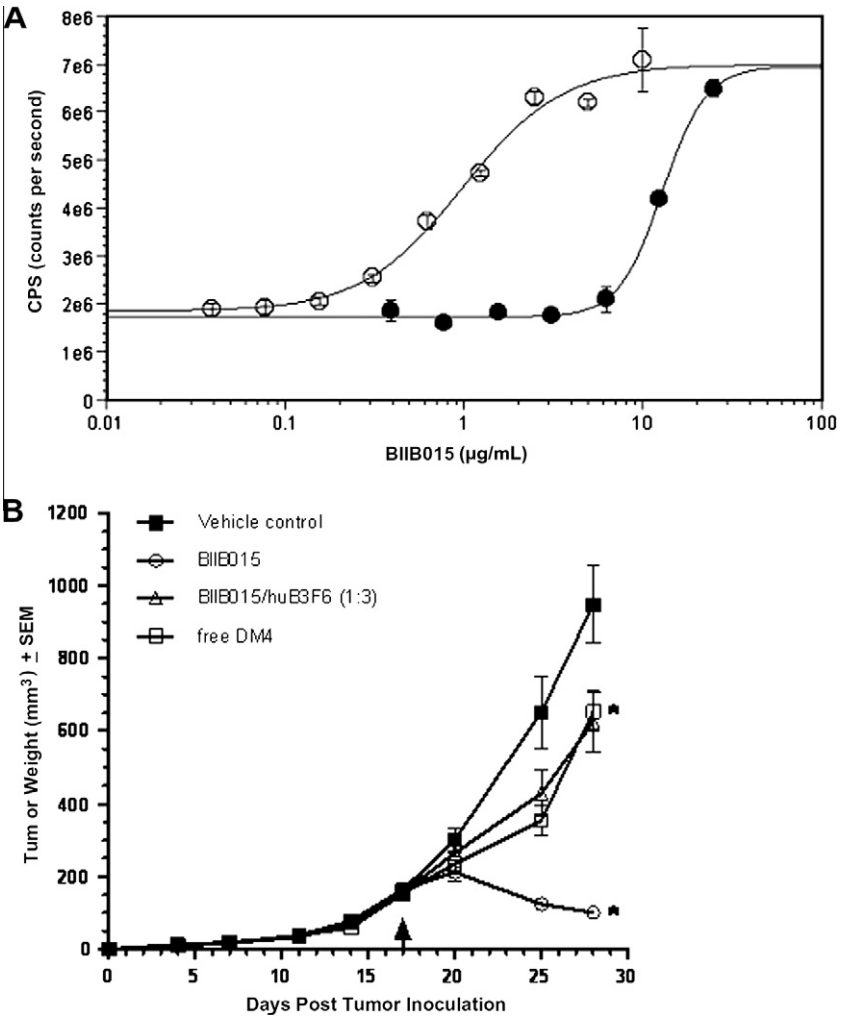

Fig. 3 – BIIB015 specifically kills Cripto positive cells, *in vitro* and *in vivo*. (A) Dose response curves for caspase 3/7 activation with CHO-Cripto (open circles) and CHO cells (solid circles). (B) Cripto specific antitumour activity of BIIB015. Mice bearing CT-3 human colon tumour xenografts were treated with BIIB015 (20 mg/kg; ~60 mg/m^2), BIIB015 (20 mg/kg; ~60 mg/m^2) in the presence of a 3-fold excess of naked huB3F6 anti-Cripto antibody (60 mg/kg), an equivalent dose of unconjugated DM4 (0.4 mg/kg) or with vehicle (10 ml/kg). All treatments single dose, administered i.v. on day indicated by arrow. *Points*, mean; *bars*, SE. *P < 0.05 versus vehicle control.

without a three-fold excess of naked huB3F6 (60 mg/kg). Treatment with BIIB015 alone inhibited tumour growth by 75–80%. In contrast, tumour inhibition by BIIB015 was reduced to approximately 25% in the presence of excess huB3F6, suggesting that a majority of the observed anti-tumour activity of BIIB015 is through the specific targeting of Cripto.

3.5. *Correlation of Cripto expression levels with in vitro cell killing activity*

In vitro studies assessed the effect of Cripto cell surface receptor levels on the ability of BIIB015 to kill Cripto expressing cells. Populations of 293 cells expressing various levels of either Cripto-1 or Cripto-3 were cultured in the presence of BIIB015 and cell viability was determined. Table 2 summarises the Cripto expression level of each of the cell populations as determined by FACS analysis (mean fluorescence intensity; MFI), the corresponding number of Cripto molecules on the cell surface per cell and the *in vitro* IC50 for BIIB015. The results show that the *in vitro* cell killing activity of BIIB015 correlated with the relative receptor expression level of the cells. As the average Cripto-1 or Cripto-3 expression increased, the IC50 for BIIB015 decreased. Significant Cripto-dependent activity of BIIB015 was observed against Cripto-expressing cells even if Cripto levels were very low (3,500–5,900 copies/cell) as compared to the Cripto-negative 293 parent cells.

3.6. *BIIB015 is efficacious on solid tumours*

BIIB015 can inhibit tumour growth or even induce tumour regressions in a number of xenograft models in mice, including breast, colon, lung and testicular tumour models. Initial

Table 2 – Summary of BIIB015 killing in sorted 293 cell populations expressing various levels of Cripto receptors (Cr-1 or Cr-3).

	Mean fluorescence intensity	Cr-3 receptor copy number	50% viability (μg/mL)
Hu 293 cells – no added Cripto	15.55	<500	1.00
Hu 293–Cr-3 Population A	149.12	5,900	0.30
Hu 293–Cr-3 Population B	264.28	10,349	0.15
Hu 293–Cr-3 Population C	509.35	19,704	0.09
Hu 293–Cr-3 Population D	733.19	28,543	0.06
Hu 293 cells – no Added Cripto	6.89	<500	1.40
Hu 293–Cr-1 Population A–L	51.60	3,500	0.22
Hu 293–Cr-Population B	225.91	14,806	0.10
Hu 293–Cr-1 Population D–R	519.67	33,644	0.08

studies exploring different dosing regimens for BIIB015 in mice indicated that frequent dosing of BIIB015 was not required to achieve significant anti-tumour activity with dosing regimens of q4D, q2W, q3W or single dose being effective (data not shown). Significant tumour growth inhibition was observed by BIIB015 against established (100–300 mg tumours at the start of treatment) NCCIT (Fig. 4Ai), Calu-6 (Fig. 4Aii) and CT-3 (Fig. 4B) tumour xenografts at doses that were well tolerated (data not shown). Comparison of single dosing with repeated dosing in the CT-3 model demonstrated an increased duration of response with repeated dosing (Fig. 4B). BIIB015 also demonstrated significant activity in mice bearing very large tumour xenografts (from 550–775 mg). Mice with large CT-3 xenograft colon tumours were dosed with either a vehicle control, 15 mg/kg of BIIB015 or 25 mg/kg of BIIB015 on Day 30 post tumour implantation. As shown in Fig. 4C, BIIB015 at 15 mg/kg and 25 mg/kg inhibited tumour growth approximately 50% compared to control ($P < 0.05$) and 70% compared to control ($P < 0.01$), respectively, with partial regressions of tumours observed in three mice at 25 mg/kg (data not shown).

Additional studies in xenograft models show that BIIB015 can enhance efficacy when dosed in combination with standard of care chemotherapeutic agents. Mice bearing CT-3 colon xenograft tumours were treated with BIIB015 and 5-flourouracil (5-FU), one of the agents used as part of the standard of care for colon cancer patients in the clinic. Treatment with a single dose of BIIB015 at 15 mg/kg or treatment with 5-FU at its maximum tolerated dose (MTD) in mice (30 mg/kg) q2dx6 resulted in approximately the same percentage of tumour growth inhibition (~30%). However, treatment with BIIB015 and 5-FU in combination resulted in tumour growth inhibition of 80%, more than double the response of either agent alone (Fig. 4Di). In another study, using the MDA-MB-231 breast xenograft model, mice received BIIB015 at 20 mg/kg alone or in combination with 25 mg/kg, the maximum tolerated dose, of paclitaxel (a standard of care agent for breast cancer). Tumour growth inhibition was augmented by treatment with the combination of drugs (~90% inhibition) compared to treatment with either of the agents alone (Fig. 4Dii). Paclitaxel animals dosed at the MTD exhibited a recoverable weight loss of approximately 5% body weight. The combination treatment group exhibited a very similar recoverable weight loss, suggesting no additional toxicities of the combined treatment (data not shown).

4. Discussion

A means to combine the precise tumour targeting power of a monoclonal antibody with the potency of a chemotherapeutic is the main idea driving the development of antibody–drug conjugates. Whilst many conjugates have entered the clinic (for reviews, see Wu and Senter[24] and Alley et al.[29]), early antibody drug conjugates often failed because linker systems were not stable enough in serum. The lack of stability resulted in unacceptable toxicities and/or lack of a therapeutic window. In other cases, the inherent potency of the chemotherapeutic used or low antigen density prevented the accumulation of a lethal payload inside the tumour cell. These problems have been addressed by the development of more stable linker systems which also now allow a greater number of cytotoxic molecules to be linked to the antibody. In addition, newer conjugates take advantage of more potent cytotoxic agents, such as calicheamican, monomethyl auristatin E and maytansinoids thus increasing the likelihood that a moderately overexpressed tumour antigen can be targeted and a lethal amount of cytotoxic agent delivered. The only cytotoxic conjugate that is approved for use in the clinic is mylotarg.[23] In addition, conjugates using peptide linker approaches with auristatins[23] as well as conjugates using disulphide or thioether containing linkers with maytansinoids are in clinical trials and reports of *in vivo* activity are being noted.[22,31,32,36] These developments suggest that immunoconjugate strategies may finally begin to be brought to fruition.

Here we report the generation of an anti-Cripto conjugate that carries multiple maytansinoid (DM4) molecules utilising an effective linker chemistry that balances the requirements for stability and efficacy. We have developed a reproducible method for generating an anti-Cripto conjugate antibody with approximately 3 DM4 molecules per antibody. We have evaluated the difference in efficacy of three different disulphide based Immunogen Inc., proprietary linker systems (SPP, SPDB, SMCC) which differ in stability and have been evaluated to select the best linker system for our anti-Cripto antibody. We compared the three linkers in single dose efficacy experiments (Fig. 2B and C) are found whilst all three conjugates exhibited activity, the SPDB linker system had superior efficacy overall (Fig. 2). The SPDB conjugate is reported to have a longer *in vivo* half-life than the SPP linker system[33,37] and this claim is supported by the clinical data with

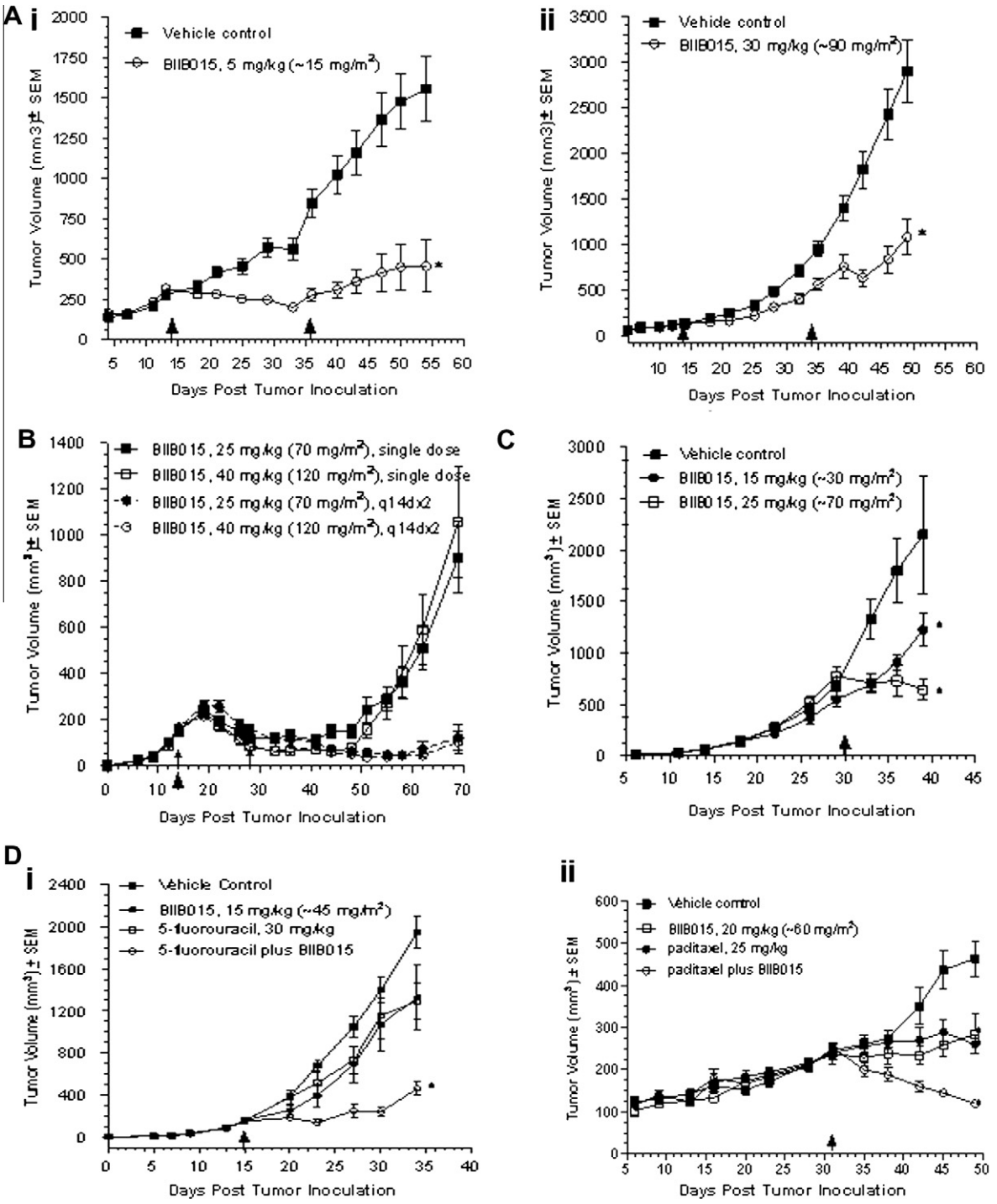

Fig. 4 – *In vivo* efficacy of BIIB015. (Ai) Response of NCCIT xenograft tumours to BIIB015 or vehicle control. (Aii) Response of Calu-6 xenograft tumours to BIIB015 or vehicle control. (B) Tumour growth in mice bearing CT-3 xenograft tumours after treatment with BIIB015 once (arrowhead) or on an every-14-day schedule (arrows). The second dose of BIIB015 extends efficacy. (C) Mice bearing large (550–775 mm³) CT-3 tumours were dosed with BIIB015 once on Day 30 (arrow). (Di) Mice bearing CT-3 xenograft tumours were treated with vehicle control, BIIB015, 5-FU (q2dx6, IP) or both BIIB015 and 5-FU (q2dx6, IP). (Dii) Mice bearing MDA-MB-231 xenograft tumours were treated with vehicle control, BIIB015, paclitaxel (q4dx3, IP) or both BIIB015 and paclitaxel. All doses administered i.v. on day indicated by arrow, unless otherwise specified. *Points*, mean; *bars*, SE. *$P < 0.05$ versus vehicle control.

C242-SPP-DM1 versus C242-SPDB-DM4.[36] We believe increased stability leads to the superior efficacy of huB3F6-SPDB-DM4 (BIIB015) 90% tumour inhibition, versus huB3F6-SPP-DM1, 80% tumour inhibition in our experiments. Furthermore, we observed a significant difference in activity of the SMCC–DM1 B3F6mAb conjugate depending on the xenograft

model tested. For example, in the NCCIT model which has xenograft tumours with higher, more homogeneous Cripto expression compared to the CT3 xenograft tumours with lower, more variable Cripto expression there was a dramatic difference in activity noted. In the NCCIT model, the SMCC conjugate resulted in 50% tumour inhibition compared to negligible tumour inhibition in the CT3 model. In contrast, there was no significant difference in activity noted in BIIB015's ability to inhibit tumour growth in these two models. As discussed above, Erickson et al.[33,37] suggest that the SMCC linker system is metabolised within an antigen positive tumour cell to yield a maytansine derivative that can not diffuse out of the cell and kill the nearest neighbour tumour cell. This model is consistent with our data that the anti-Cripto SMCC conjugate is active in homogeneous and/or high level expressing Cripto xenograft models (NCCIT, Fig. 2B), but is inactive in a model with lower Cripto expression and/or heterogeneous antigen expressing xenograft tumour (CT-3, Fig. 2C.) Since most human tumours are heterogeneous in nature, we believe the 'nearest neighbour' killing potential of the SPP and SPDB conjugates could make the difference in efficacy, as noted in our studies. Okeley et al.[38] also report data with the clinically active SGN-35 that indicates an important contribution to efficacy of 'nearest neighbour' killing in heterogenous tumour cell populations. However, it is prudent to note that a potential downside of 'nearest neighbour' killing could be toxicity to normal tissue. Maytansine and its derivatives like DM4 are only active on dividing cells, so non-mitotic normal cells would likely be spared. For any chemotherapeutic regimen, antibody drug conjugates included, the therapeutic window will determine the success or failure of the drug candidate. Animal studies with BIIB015 to date do not suggest normal tissue toxicity, but clinical data from an ongoing Phase I trial will likely best answer this question. Finally, the longer stability of the SPDB linker versus the stability of the SPP linker led us to ultimately choose the huB3F6-SPDB-DM4 conjugate (BIIB015) as our clinical candidate to target not only high expressing Cripto tumours, but also tumours with lower and/or heterogeneous Cripto expression.

Antigen density is believed to play a role in the ability of a conjugate antibody to effectively target and kill a tumour cell. Our *in vitro* cytotoxicity experiments clearly indicate that a lower EC50 of BIIB015 is needed with tumour cells containing a greater number of copies of cell surface Cripto (Table 2). However, *in vivo*, exact quantification of the receptor expression level on tumours is much more difficult. By FACS, NCCIT cells show Cripto expression in the range of 10,000–30,000 receptors per cell. In contrast, Calu-6 cells show levels in the 5,000–10,000 range (data not shown). Although the changes that occur in receptor levels once the cells are grown in mice are an unknown, we have noted that NCCIT testicular tumour xenografts show higher levels of Cripto expression by immunohistochemistry than Calu-6 lung tumour xenografts. In xenograft experiments, 30 mg/kg (~85–90 mg/m^2 of DM4) of BIIB105 is needed to inhibit the growth of Calu-6 tumours whilst a dose as low as 5 mg/kg (12–15 mg/m^2 of DM4) leads to regression of NCCIT tumours (Fig. 4Ai and ii). Both tumours can be effectively growth inhibited with an appropriate dose, however, this difference in sensitivity to BIIB015 suggests a possible correlation of receptor expression level with efficacy *in vivo* as well as *in vitro*. A retrospective correlation of clinical efficacy versus Cripto expression by immunohistochemical analysis should answer this question in the future.

In summary, our therapeutic hypothesis is that BIIB015 will bind to Cripto positive tumours, will be internalised and release DM4 which will kill the tumour cell upon division. Since the B3F6 antibody portion of BIIB015 targets the molecule to the tumour cells, we expect this therapy to be less toxic than free DM4. For this hypothesis to work, Cripto must be expressed on tumours and B3F6 must be specific for Cripto. We have demonstrated Cripto expression in human tumours, the specificity of BIIB015 *in vitro* and *in vivo*, and selected the SPDB linker conjugate for the most efficient killing of tumour cells. We have also shown that BIIB015, at non-severely toxic doses, inhibited growth and/or regressed tumours in all Cripto positive human tumour xenograft models tested. Efficacy was achieved with many different dosing paradigms, including once every three week dosing and good activity was observed even when BIIB015 was used to treat very large tumours (greater than 500 mm^3). BIIB015's inhibition of growth of the MDA-MB-231 triple negative (-HER2, -ER, -PR) breast tumour is particularly exciting since there is currently no approved therapy for the patients with tumours of this type. In addition, experimental results indicate that BIIB015 efficacy can be additive with chemotherapeutics (5-FU, paclitaxel). Interestingly, BIIB015 was additive even with another microtubular inhibitor, paclitaxel, when paclitaxel was dosed at its maximum tolerated dose (25 mg/kg). This result provides further evidence of the tumour specificity of BIIB015, since administration of an additional microtubular inhibitor (BIIB015) increased tumour growth inhibition without any additional toxicity compared to paclitaxel alone. The evidence of additivity with standard of care chemotherapeutics in xenograft models may signify a greater chance of success for BIIB015 in the clinic. Furthermore, data from the Hendrix lab[18] support the hypothesis of Cripto and Nodal as tumour stem cell factors which, intriguingly, suggest that targeting Cripto will result in a very potent therapeutic effect by killing stem cell populations within the tumour. Taken together, the experimental results presented here validate the cell surface Cripto protein as a target for a toxin conjugated therapeutic antibody approach. BIIB015 has recently entered the clinic and Phase I studies have been initiated in patients with Cripto positive tumours where the safety and tumour inhibition activity of BIIB015 will be evaluated for therapeutic benefit in man.

Conflict of interest statement

All of the authors are employed at the biotech company, Biogen Idec.

Acknowledgements

The authors would like to thank the following scientists for constructive critique and comment throughout the course of this work: Mitchell Reff, Laurie Phillips, Jeremy Barton, David Salomon, Ingrid Joseph, Alan Wahl, Robert Lutz, Godfrey Amplett, Deborah Meshulam, John Lambert, Fang Liu, Yong

Wang, Rajesh Krishnamurthy, Lintao Wang, Amy Leung, Paul Weisbach, Michael Fleming, Cheryl Dionne, Nathan Fishkin, Kate Lai and Rajeeva Singh. We would also like to thank Cindy Bottiglio and Keli Perron for technical assistance.

REFERENCES

1. Bianco C, Strizzi L, Normanno N, et al. Cripto-1: an oncofetal gene with many faces. *Curr Top Dev Biol* 2005;**67**:85–133.
2. Sun C, Orozco O, Olson DL, et al. CRIPTO3, a presumed pseudogene, is expressed in cancer. *Biochem Biophys Res Commun* 2008;**377**:215–20.
3. Yeo CY, Whitman M. Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell* 2001;**7**:949–57.
4. Strizzi L, Bianco C, Normanno N, et al. Cripto-1: A multifunctional modulator during embryogenesis and oncogenesis. *Oncogene* 2005;**24**:5731–41.
5. Chari RV. Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc Chem Res* 2008;**41**:98–107.
6. Brennan J, Lu CC, Norris DP, et al. Nodal signalling in the epiblast patterns the early mouse embryo. *Nature* 2001;**411**:965–9.
7. Lu CC, Robertson EJ. Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior–posterior patterning. *Dev Biol* 2004;**273**:149–59.
8. Kenney NJ, Adkins HB, Sanicola M. Nodal and Cripto-1: Embryonic pattern formation genes involved in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia* 2004;**9**:133–44 (Review).
9. Ciardiello F, Dono R, Kim N, Persico MG, Salomon DS. Expression of Cripto, a novel gene of the epidermal growth factor gene family, leads to *in vitro* transformation of a normal mouse mammary epithelial cell line. *Cancer Res* 1991;**51**:1051–4.
10. Adkins HB, Bianco C, Schiffer SG, et al. Antibody blockade of the Cripto CFC domain suppresses tumor cell growth *in vivo*. *J Clin Invest* 2003;**112**:575–87.
11. Gray PC, Harrison CA, Vale W. Cripto forms a complex with activin and type II activin receptors and can block activin signalling. *Proc Natl Acad Sci USA* 2003;**100**:5193–8.
12. Kelber JA, Shani G, Booker EC, Vale WW, Gray PC. Cripto is a noncompetitive activin antagonist that forms analogous signaling complexes with activin and nodal. *J Biol Chem* 2008;**283**:4490–500.
13. Wechselberger C, Strizzi L, Kenney N, et al. Human Cripto-1 overexpression in the mouse mammary gland results in the development of hyperplasia and adenocarcinoma. *Oncogene* 2005;**24**:4094–105.
14. Wechselberger C, Ebert AD, Bianco C, et al. Cripto-1 enhances migration and branching morphogenesis of mouse mammary epithelial cells. *Exp Cell Res* 2001;**266**:95–105.
15. Gong YP, Yarrow PM, Carmalt HL, et al. Overexpression of Cripto and its prognostic significance in breast cancer: A study with long-term survival. *Eur J Surg Oncol* 2007;**33**:438–43.
16. D'Antonio A, Losito S, Pignata S, et al. Transforming growth factor alpha, amphiregulin and Cripto-1 are frequently expressed in advanced human ovarian carcinomas. *Int J Oncol* 2002;**21**:941–8.
17. Ertoy D, Ayhan A, Sarac E, et al. Clinicopathological implication of Cripto expression in early stage invasive cervical carcinomas. *Eur J Cancer* 2000;**36**:1002–7.
18. Topczewska JM, Postovit LM, Margaryan NV, et al. Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. *Nat Med* 2006;**12**:925–32.
19. Postovit LM, Margaryan NV, Seftor EA, et al. Human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of aggressive cancer cells. *Proc Natl Acad Sci USA* 2008;**105**:4329–34.
20. Strizzi L, Abbott DE, Salomon DS, et al. Potential for Cripto-1 in defining stem cell-like characteristics in human malignant melanoma. *Cell Cycle* 2008;**7**:1931–5.
21. Issell BF, Crooke ST. Maytansine. *Cancer Treat Rev* 1978;**5**:199–207.
22. Liu C, Tadayoni BM, Bourret LA, et al. Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. *Proc Natl Acad Sci USA* 1996;**93**:8618–23.
23. Carter PJ, Senter PD. Antibody–drug conjugates for cancer therapy. *Cancer J* 2008;**14**:154–69 (Review).
24. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol.* 2005;**23**:1137–46 (Review).
25. Krop IE, Beeram M, Modi S, et al. Phase I study of trastuzumab-DM1, an HER2 antibody–drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *Clin Oncol* 2010;**28**:2698–704.
26. Niculescu-Duvaz I. Trastuzumab emtansine, an antibody–drug conjugate for the treatment of HER2+ metastatic breast cancer. *Curr Opin Mol Ther* 2010;**12**:350–60.
27. Burris III HA, Rugo HS, Vukelja SJ, et al. Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2) – positive breast cancer after prior HER2-directed therapy. *JCO* 2011:398–405.
28. Younes A, Bartlett NL, Leonard JP, et al. Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. *N Engl J Med* 2010;**363**:1812–21.
29. Alley SC, Okeley NM, Senter PD. Antibody–drug conjugates: targeted drug delivery for cancer. *Curr Opin Chem Biol* 2010;**14**:529–37.
30. Lewis Phillips GD, Li G, Dugger DL, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody–cytotoxic drug conjugate. *Cancer Res* 2008;**68**:9280–90.
31. Tolcher AW, Ochoa L, Hammond LA, et al. Cantuzumab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I, pharmacokinetic, and biologic correlative study. *J Clin Oncol* 2003;**21**:211–22.
32. Eagan RT, Ingle JN, Rubin J, Frytak S, Moertel CG. Early clinical study of an intermittent schedule for maytansine (NSC-153858): brief communication. *J Natl Cancer Inst* 1978;**60**:93–6.
33. Erickson HK, Park PU, Widdison WC, et al. Antibody–Maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. *Cancer Res* 2006;**66**:4426–33.
34. Schiffer SG, Foley SKaffashan A, et al. Fucosylation of Cripto is required for its ability to facilitate nodal signaling. *J Biol Chem* 2001;**276**:37769–78.
35. Alley SC, Benjamin DR, Jeffrey SC, et al. Contribution of linker stability to the activities of anticancer immunoconjugates. *Bioconjug Chem* 2008;**19**:759–65.
36. Rodon J, Garrison M, Hammond LA, et al. Cantuzumab mertansine in a three-times a week schedule: a phase I and pharmacokinetic study. *Cancer Chemother Pharmacol* 2008;**62**:911–9.
37. Erickson HK, Widdison WC, Mayo MF, et al. Tumor delivery and *in vivo* processing of disulfide-linked and thioether-linked antibody–maytansinoid conjugates. *Bioconjug Chem* 2010;**21**:84–92.
38. Okeley NM, Miyamoto JB, Zhang X, et al. Intracellular activation of SGN-35, a potent anti-CD30 antibody–drug conjugate. *Clin Cancer Res* 2010;**16**:888–97.