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The HB22.7-vcMMAE Antibody-Drug Conjugate Has Efficacy Against Non-Hodgkin Lymphoma Xenografts with Minimal Systemic Toxicity

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Abstract

In this study, the HB22.7 anti-CD22 mAb, was used for specific, targeted delivery of the potent anti-cancer agent, monomethyl auristatin E (MMAE) to non-Hodgkin lymphoma (NHL). MMAE was covalently coupled to HB22.7 through a valine-citrulline peptide linker (vc). Maleimide-functionalized vcMMAE (mal-vcMMAE) was reacted with thiols of the partially reduced mAb. Approximately 4 molecules of MMAE were conjugated to HB22.7 as determined by residual thiol measurement and hydrophobic interaction chromatography-HPLC (HIC-HPLC). HB22.7-vcMMAE antibody drug conjugate (ADC) retained its binding to Ramos NHL cells and also exhibited potent and specific *in vitro* cytotoxicity on a panel of B cell NHL cell lines with IC_{50}s of 20 - 284 ng/ml. HB22.7-vcMMAE also showed potent efficacy *in vivo* against established NHL xenografts using the DoHH2 and Granta 519 cell lines. One dose of the ADC induced complete and persistent response in all DoHH2 xenografts and 90% of Granta xenografts. Minimal toxicity was observed. In summary, HB22.7-vcMMAE is an effective ADC that should be evaluated for clinical translation.

Keywords

HB22.7; lymphoma; antibody-drug conjugate; MMAE; CD22; NHL

Introduction

It is estimated that 70,800 people in the United States alone will be diagnosed with NHL in 2014.[1] The overall 5 and 10-year survival rates are 71% and 63%, respectively.[2] The most common current standard of care for B cell NHL is chemotherapy combined with the anti-CD20 mAb, rituximab. Commonly used regimens include, rituximab (R) cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), bendamustine and rituximab (BR), R-CVP, and fludarabine-based combinations. These regimens have many off-target effects that result in substantial toxicity that limit efficacy. The median age at diagnosis for NHL is 65,

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which highlights the importance of reducing treatment-related toxicity. Monoclonal antibodies (mAb) may be used as targeting molecules with a goal of reducing systemic toxicity while enhancing efficacy by the direct delivery of toxins specifically to cancer cells.[3] Therefore, ADCs may not only potentiate current chemotherapeutics but also permit lower effective doses, thus reducing the toxicity of systemic chemotherapy.

CD22 is a 140-kDa sialo-adhesion protein expressed by normal and malignant B cells and is involved in the regulation of B cell function and survival.[4] CD22 is rapidly internalized upon binding to HB22.7, making it an ideal target for ADC therapy due to efficient intracellular delivery of conjugated payloads.[5]

HB22.7 is an anti-CD22 mAb originally developed to map the CD22 ligand binding domains.[6] Based on the ligand blocking properties, HB22.7 was developed and humanized to aid in the treatment of NHL. We have shown that HB22.7 can be an effective targeting vehicle that mediates cellular uptake of conjugated payloads.[3]

In the present study, we examined the potential of HB22.7 to mediate targeted delivery of a directly conjugated drug, MMAE, (a potent anti-mitotic drug that inhibits cell division and induces apoptosis by binding to microtubules and inhibiting polymerization).[7,8] Several groups have developed mAb-MMAE ADCs to a variety targets, including CD19, 22, 79b, and 30, AGS-5, and guanylyl cyclase C.[9-14] Most have demonstrated significant pre-clinical and clinical efficacy. Moreover, a CD30-targted MMAE ADC was approved by the FDA in 2012 for treatment of relapsed Hodgkin and anaplastic lymphomas and has proven to be an effective and well-tolerated treatment option. Pinatuzmab vedotin (DCDT2980S), an anti-CD22 MMAE conjugate developed by Genentech, is also under clinical investigations for the treatment of relapsed or refractory diffuse large B-cell lymphoma and follicular NHL in combination with rituximab.[10]

The independent lymphomacidal activity of HB22.7 due to its ligand blocking properties (in contrast to other anti-CD22 mAb which do not block ligand binding) rationalizes assessment of the HB22.7-vcMMAE ADC. Herein, we present *in vitro* and *in vivo* data using HB22.7-vcMMAE that demonstrate significant preclinical efficacy.

Materials and Methods

Materials

Dithiothreitol (DTT) was obtained from Acros Organics. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Thermo Scientific. Diethylenetriaminepentaacetic acid (DTPA) was purchased from Sigma Aldrich. Maleimide-vc-MMAE (mal-vcMMAE) was a gift from Dr. Zhenwei Mao (Concortis Biosystems). HB22.7 was prepared and characterized as previously described [15].

Cell lines

The lymphoma cell lines Ramos, Raji and Granta 519, and the leukemia cell line Jurkat were purchased from the American Type Culture Collection. The lymphoma cell lines SU-DHL-4 and DoHH2 were purchased from the Deutsche Sammlung von Mikroorganismen und

Zellkulturen (DSMZ). All cell lines were maintained with RPMI 1640 supplemented with 10% fetal bovine serum and incubated at 37°C, 5% CO_2 and 90% humidity.

Synthesis of HB22.7-vcMMAE

HB22.7-vcMMAE was prepared using a limited DTT reduction strategy.[16] Briefly, HB22.7 (10-20 mg/mL in PBS) was incubated with 3.25 molar equivalents of DTT for 2 hours at 37°C. Excess DTT was removed from the partially reduced HB22.7 by passing the mixture over a PD-10 column and eluting with PBS. Fractions were collected and assessed at A_{280} to determine the protein-containing fractions. Fractions containing thiolated HB22.7 were pooled and then concentrated with YM-30 Centricon ultrafiltration devices. The final HB22.7 concentration was determined using the A_{280} and a molar extinction coefficient of 1.35. The ratio of thiols per mAb was determined by mixing thiolated HB22.7 with 0.1 mM DTNB (Ellman's reagent) and measuring at A_{412} with a molar extinction coefficient of 13600 M^{-1} (Figure 1). DTPA (1 mM) was added to the reduced HB22.7 to prevent oxidation of thiols.

A stock solution of mal-vcMMAE was prepared in 50% acetonitrile. Partially reduced HB22.7 was mixed with up to 1.5 equivalents of mal-vcMMAE with a final acetonitrile concentration of 5% to ensure solubility. The reaction proceeded for 2 h at 4°C. The degree of drug loading was calculated by quantitation of residual thiols after drug conjugation or by HIC-HPLC.[8,16]

Flow cytometry

To assess CD22 expression and HB22.7-vcMMAE binding, 0.5 x 10^6 Ramos NHL cells per sample were resuspended in 100 uL FACS buffer (PBS + 0.5% FBS) and chilled on ice. HB22.7 (10 ug/mL) was incubated with cells for 30 min on ice, followed by 3 washes with ice-cold FACS buffer. Cells were then incubated with a 1/50 dilution of goat anti-mouse IgG-FITC (Invitrogen) for 30 min on ice. Cells were washed 3 times and 10,000 events were analyzed on a FACScan (BD Biosciences).

MTS assays

In vitro cytotoxicity of HB22.7-vcMMAE was evaluated using an MTS assay. Cells were seeded in 96-well plates at a density of 1 x 10^4 cells/well in 90 μL of media. HB22.7-vcMMAE was serially diluted with media and 10 μL of each dilution was added to the appropriate well and incubated for 72 hours. Cell viability of all treatment groups was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. MTS solution (15 μL) was added to each well and incubated for one hour at 37°C. Cell viability as a percentage of the untreated control was calculated as follows: [(OD_{490} treated – OD_{490} background) / (OD_{490} control – OD_{490} background) x 100]. Data is presented as the mean ± standard deviation (SD) of 3 separate experiments performed in triplicate.

NHL xenograft Experiments

Female (6-8 weeks old) ICR-SCID mice were obtained from Charles River and maintained in micro-isolation cages under pathogen-free conditions. All animal experiments were

performed in compliance with institutional guidelines and according to the Animal Use and Care Administrative Advisory Committee at the University of California, Davis. Mice were allowed to acclimatize for at least 4 days prior to the start of any experiment. To enhance tumor engraftment, three days prior to tumor cell implantation, mice received whole body radiation (400 rads). Granta 519 or DoHH2 cells (10^7) were re-suspended in PBS and injected subcutaneously into the flank of each mouse. Tumors were allowed to grow to a volume of 100-200 mm^3 and this was then designated as day 0. Mice were then randomly separated into two treatment groups (8-10 per group) consisting of PBS or HB22.7-vcMMAE (7.5 mg/kg). Treatments were administered on day 0 by intraperitoneal (IP) injection. Tumor volume was measured using digital calipers and calculated by the formula $(LxW^2)/2$, where L is the longest and W is the shortest in tumor diameter (mm).

Body weight and signs of toxicity were monitored twice a week. Mice were sacrificed when tumor volume exceeded 1500 mm^3 or 20 mm in diameter. Blood was drawn from the lateral saphenous vein (n=3) and collected into EDTA-lined tubes for complete blood counts (CBC).

Results

Development of HB22.7-vcMMAE

As stated above, HB22.7-vcMMAE was developed as previously described with some modifications necessary to optimize reaction conditions for HB22.7. The ADC was produced in house, and the drug to antibody ratio (DAR) of 4.6 was calculated by a third party (Concortis) via HIC-HPLC (data not shown).

Flow cytometric analysis of HB22.7-vcMMAE binding

To ensure that MMAE conjugation to HB22.7 did not affect CD22 binding, the CD22+ NHL cell line, Ramos, was used to compare the binding of the parent (HB22.7) to that of HB22.7-vcMMAE. MMAE conjugation to HB22.7 had no effect on its binding to Ramos cells (Figure 1).

HB22.7-vcMMAE and free-MMAE in vitro cytotoxicity

We used a panel of CD22+ NHL cell lines representing NHL sub-types to assess the *in vitro* cytotoxicity of HB22.7-vcMMAE. As shown in Figure 2A, all cell lines tested were sensitive to free-MMAE with IC_{50}s ranging from 0.099 to 1.348 nM. HB22.7-vcMMAE was effective in all CD22+ NHL cell lines tested, Figure 2B. IC_{50}s for the cell lines ranged from 0.017 to 0.241 nM and while all cell lines expressed CD22, the degree of cytotoxicity did not correlate with CD22 expression levels (data not shown). DoHH2 cells were the most sensitive with an IC_{50} of 0.017 nM. Although the CD22- cell line Jurkat was very sensitive to free-MMAE with an IC_{50} of 0.099 nM it was, as expected, resistant to HB22.7-vcMMAE with an IC_{50} over 2.114 nM, Figure 2 & Table 1. The lack of killing of Jurkat cells by the conjugate, while unconjugated MMAE was toxic, suggests that the conjugate is CD22 specific, was stable and did not release free MMAE into the culture media.

In vivo efficacy of HB22.7-vcMMAE

The pre-clinical efficacy of HB22.7-vcMMAE was evaluated using cell lines representing human transformed follicular (DoHH2) or mantle cell lymphoma (Granta 519). NHL cells were implanted into the flanks of ICR-SCID mice, and tumors were allowed to develop until the average tumor volume reached approximately 100 mm^3 and 170 mm^3 for DoHH2 and Grant 519, respectively. In untreated mice, tumors reached 1500 mm^3 within 15 to 20 days. In comparison, treatment with one dose of HB22.7-vcMMAE (7.5 mg/kg) induced complete, durable tumor remission in all mice bearing DoHH2 xenografts (Figure 3A) and in 9 out of 10 mice bearing Granta xenografts (Figure 3B). Body weight and CBCs were used to assess treatment-induced toxicity. Average percent of starting body weights in the treated groups were not significantly different from controls (Figure 4A & 4B). Mice treated with HB22.7-vcMMAE had blood counts within the normal range and they did not deteriorate over time (Figure 4C - 4E).

Discussion

In previous studies, our group used HB22.7 as a vehicle for targeted delivery of saporin (SAP) to CD22+ NHL. SAP was chosen as the cytotoxic payload because of its potency and ease of chemical conjugation to mAb. Although the HB22.7-saporin conjugate was efficient at targeting CD22+ malignancies, it did not induce complete regression in human NHL xenograft tumor models. Moreover, clinical development of a mAb-SAP conjugate may be challenging due to hepatotoxicity and the development of neutralizing anti-SAP antibodies.[17] Several groups have conjugated SAP to mAbs for targeted delivery to tumor cells and demonstrated significant preclinical anti-tumor activity.[18,19] However, toxins produced by plants, fungi, and bacteria are easily recognized as foreign molecules by the immune system leading to the development of neutralizing antibodies. This leads to rapid clearance of mAb-SAP conjugates and a reduction in the level of ADC available for uptake by target cells.

As an alternative to SAP, potent small molecule drugs such as those derived from the auristatin, monomethyl auristatin E (MMAE), and maytansine (DM1) families have shown promising results as the cytotoxic component of ADCs. These drugs have similar or higher potency compared to SAP but have the distinct advantage of not producing the above-mentioned side effects. Several groups have successfully conjugated MMAE onto mAb and showed promising pre-clinical and clinical efficacy. Recently, Genentech has developed anti-CD22 antibody and MMAE conjugate (DCDT2980S) which has shown promising efficacy against NHL xenograft models. DCDT2980S, pinatuzmab vedotin (PiV), is currently undergoing Phase II clinical trials in combination with rituximab in patients with relapsed or refractory B-cell NHL.[10,20]

The independent lymphomacidal activity and successful utilization of HB22.7 to target CD22 positive B-cell malignancies led us to further investigate the use of a more potent and non-immunogenic drug such as MMAE[3] in conjunction with an anti-CD22 mAb that has ligand blocking activity. HB22.7-vcMMAE consists of the anti-CD22 mAb, HB22.7, chemically conjugated to MMAE, a stable biodegradable peptide linker composed of valine and citrulline. MMAE is a synthetic anti-mitotic agent that inhibits cell division by blocking the polymerization of tubulin.[21] The linker between HB22.7 and MMAE is stable in

extracellular fluid, but is cleaved by cathepsin once the conjugate has entered tumor cells, thus activating the anti-mitotic mechanism of MMAE intracellularly.[22]

All the cell lines tested in this study were sensitive to free MMAE, with IC_{50} values less than 0.3 nM. Our *in vitro* results show that, when conjugated to HB22.7, MMAE selectively kills CD22+ NHL cell lines but not the CD22 negative cell line, Jurkat. While the toxicity of this ADC is dependent on CD22 expression, and suggests that the delivery of the drug is indeed dependent on antibody targeting, the degree of cytotoxicity did not correlate with CD22 expression levels. The lack of correlation between CD22 expression levels and cytotoxicity has been observed with other MMAE constructs[8] and may suggest that the high potency of MMAE allows for lower target thresholds. To study the *in vivo* efficacy of HB22.7-vcMMAE, we treated two xenograft models that represent particularly resistant subtypes of human NHL: transformed follicular and mantle cell lymphoma. In both models a single dose of the HB22.7-vcMMAE ADC induced durable complete response in nearly all mice (Figure 3). These results suggest that HB22.7-vcMMAE has potent anti-tumor activity *in vivo* with no observable toxicity (Figure 4).

In summary, our results indicate that the HB22.7-vcMMAE ADC has potent pre-clinical efficacy against two very difficult-to-treat NHL, mantle cell and transformed follicular lymphoma. Nearly all tumors completely responded to only one dose of HB22.7-vcMMAE. The results from this study are very comparable to Genentech's ADC, PiV, and other MMAE ADCs that have been translated to clinical studies. In addition, HB22.7's unique ligand blocking properties may contribute additional cytotoxicity based on its ability to block B-cell survival signals. This makes HB22.7-vcMMAE an attractive candidate for clinical translation of a more efficacious and less toxic NHL therapeutic.

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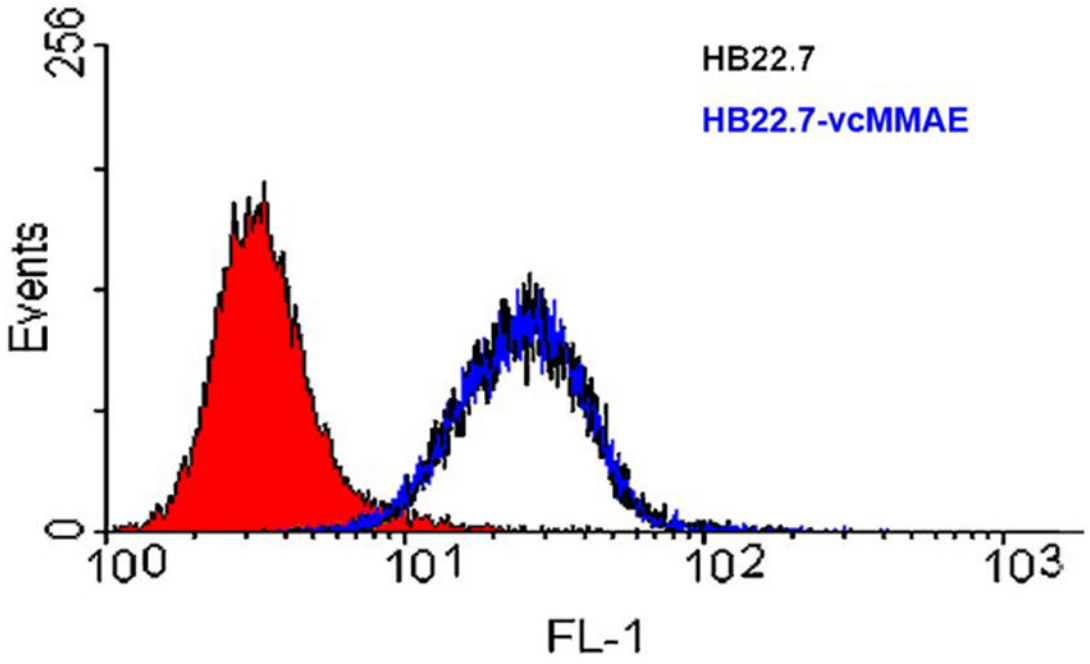


Figure 1: Flow cytometric analysis of HB22.7-vcMMAE binding.

The binding affinity of HB22.7 after conjugation with MMAE was verified by flow cytometric analysis. Ramos were used to compare the binding of unmodified HB22.7 to that of HB22.7-vcMMAE. MMAE conjugation to HB22.7 had no effect on its binding to Ramos cells.

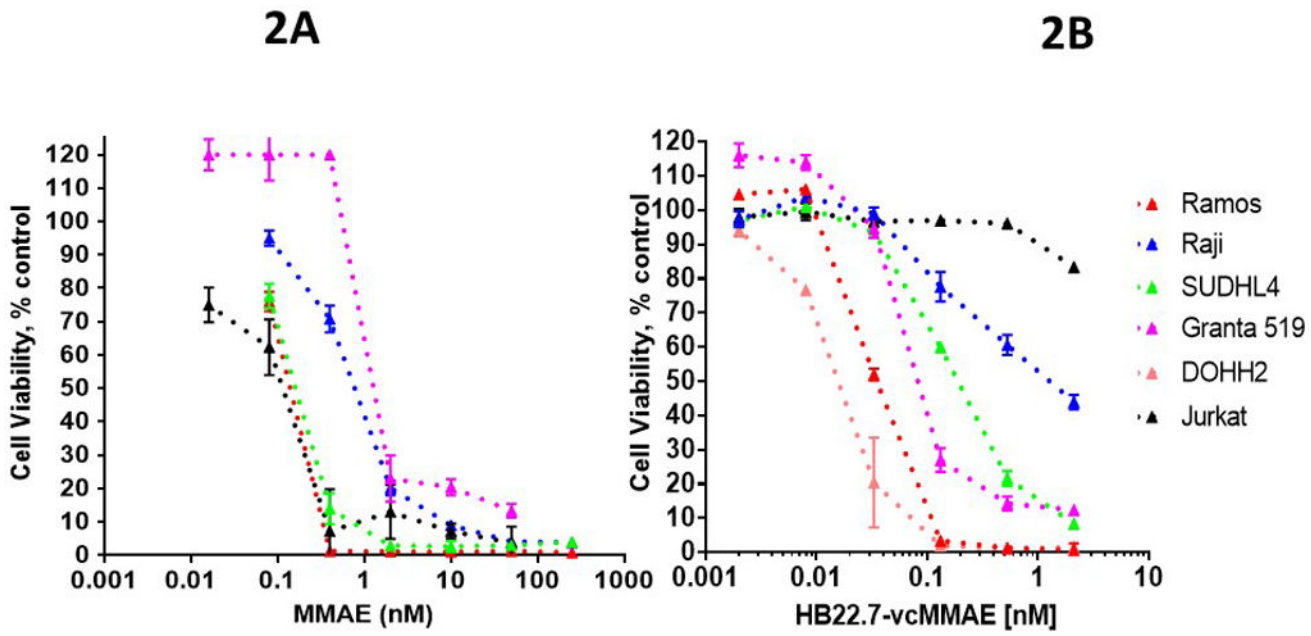


Figure 2: *In vitro* cytotoxicity and selectivity of HB22.7-vcMMAE.

All cell lines tested were sensitive to free-MMAE (A). Indicated NHL cells were also treated with escalating doses of HB22.7-vcMMAE; however, only CD22+ cells were selectively killed by the ADC (B). Cells were assessed for cytotoxicity by MTS after 72 hours of continuous exposure. The percent viability, relative to untreated control, was plotted versus the ADC concentration. Results for each study are the average of quadruplicate determinations. Error bars indicate ± SD.

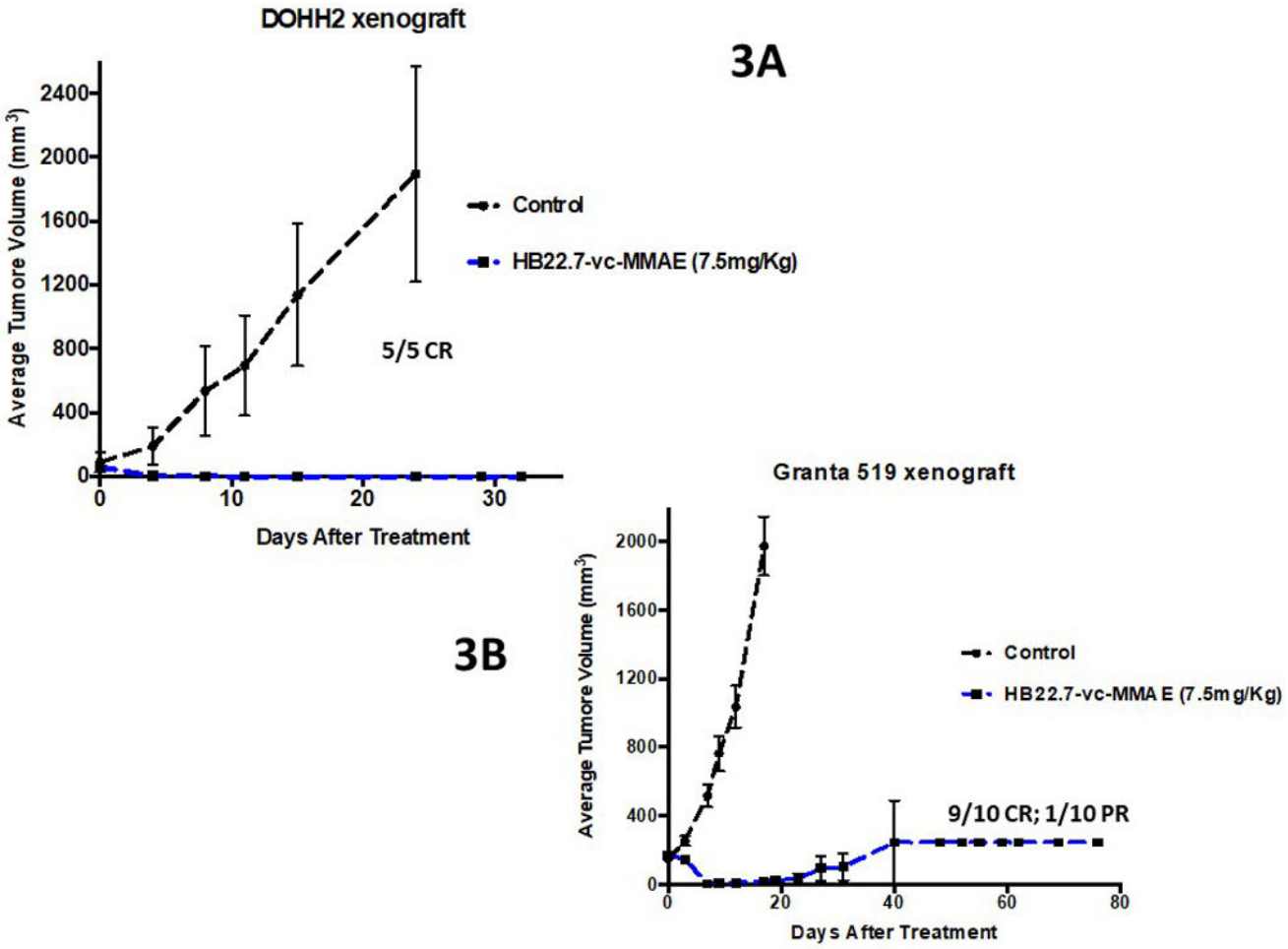
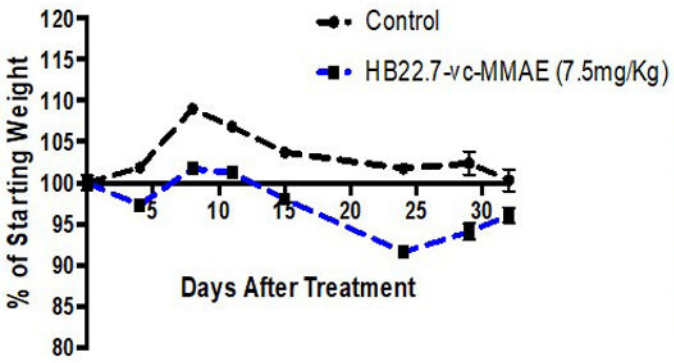


Figure 3: Efficacy of HB22.7-vcMMAE in transformed follicular and mantle cell lymphoma xenograft models.

Antitumor activity of HB22.7-vcMMAE on subcutaneous DoHH2 and Granta tumors in ICR-SCID mice. Mice were implanted with 10^7 DoHH2 cells (A) or Granta cells (B). Mice (5 – 10/group) were treated with a single dose of HB22.7-vcMMAE at 7.5 mg/kg (black) when the average tumor size reached100 mm^3 (DoHH2) and 170 mm^3 (Granta 519). Control mice (5/group) were monitored without treatment (blue) until the tumor size reached 1500 mm^3 at which point they were euthanized.

4A



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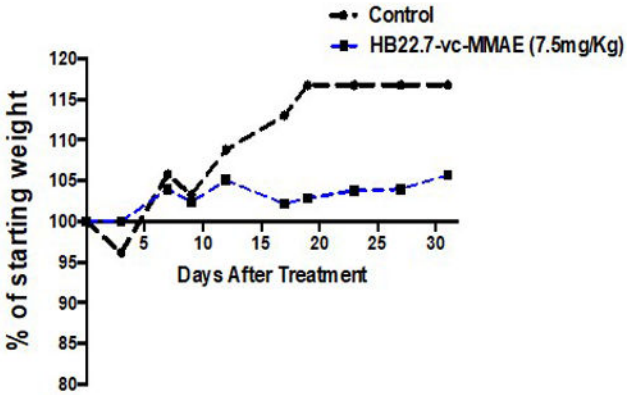

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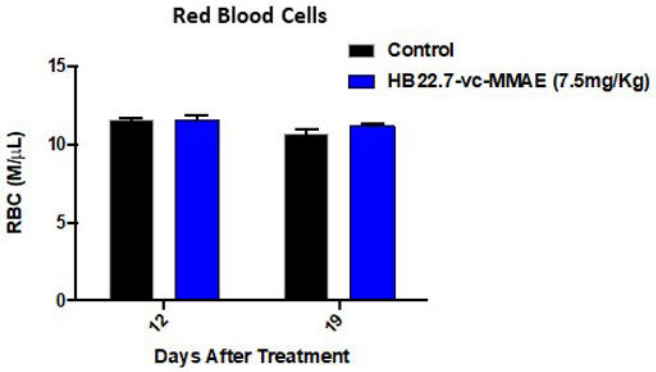

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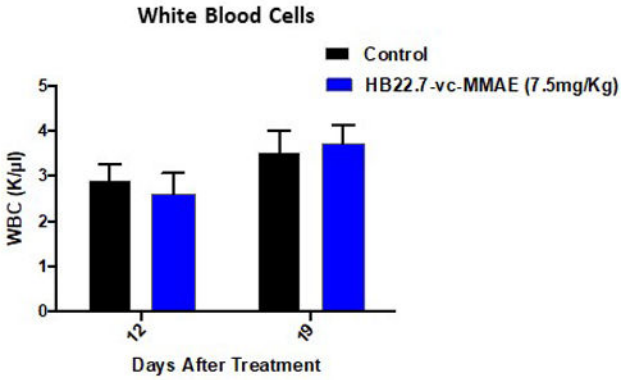

4E

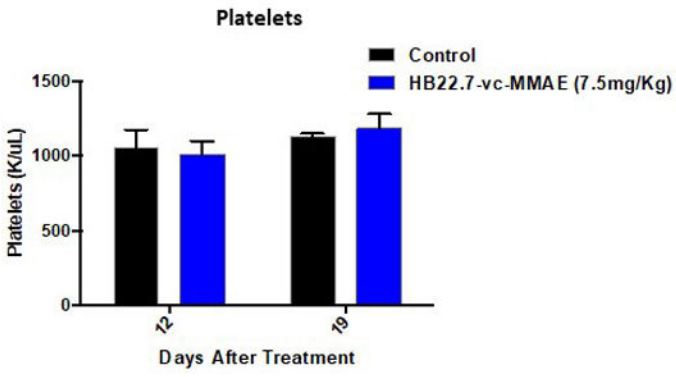

Figure 4: No signs of toxicity were observed in mice treated with HB22.7-vcMMAE compared to untreated control.

Toxicity was assessed by body weight (A & B) and complete blood count (C – E). Body weight was measured twice a week and the percent of starting weight was calculated.

Table 1:

IC_{50} values for free MMAE and HB22.7 on NHL cell lines.

Cell lines	IC50	
	MMAE(nM)	HB22.7-vcMMAE (nM)
Ramos	0.104	0.032
Raji	0.705	0.241
SUDHL4	0.292	0.163
Granta 519	1.348	0.060
DOHH2	—	0.017
Jurkat	0.099	>2.114