

INTRODUCTION

Antitumor activity of monoclonal antibodies can be dramatically enhanced by conjugation to cytotoxic small molecules. By using such antibody-drug conjugates (ADCs) the toxin is delivered to target cells exclusively and thereby kills only those cells. Beside the approval of Kadcyła (T-DM1, 2011) and Adcetris (SGN-35, 2013) nearly 60 ADCs have entered clinical trials, promising to strengthen the therapeutic capabilities for cancer treatment in the next decade. Most ADCs are based on a few cytotoxic compounds only and on an even smaller number of toxicity mechanisms: Most payloads are microtubuli- or DNA-targeting toxins (auristatins and maytansines or duocarmycins and PBDs). Accordingly the use of new drugs that function via alternative toxicity mechanisms could enhance the therapeutic potential of ADCs. Heidelberg Pharma focuses on Amanitin based ADCs or ATACs (antibody targeted Amanitin conjugates) comprising a new class of ADCs with Amanitin as toxic payload. Amanitin is the well-known toxin of the amatoxin family and binds specifically to the eukaryotic RNA pol II thereby inhibiting the cellular transcription process at very low concentrations (1). Heidelberg Pharma also pursues the strategy of site-specific conjugation to limit heterogeneity of drug-antibody species, to improve conjugate stability, and to increase the therapeutic window of ADCs. For this purpose, antibodies are engineered at specific locations to incorporate cysteines resulting in so called Thiomabs. In the current study, *in vitro* and *in vivo* data of Thiomab based ATACs targeting CD19 (also known as B-lymphocyte surface antigen B4, B4, CVID3) are presented. CD19, a class I transmembrane glycoprotein with no significant homology to any known protein, is expressed in B cells and B-cell malignancies like in B-cell acute lymphocytic leukemia (B-ALL) and B cell chronic lymphocytic leukemia (B-CLL) but it is not found on hematopoietic stem cells, and other healthy tissues. Additionally, CD19 has a broad expression profile and high internalization efficacy and is thus an ideal target for Amanitin based ADCs.

METHODS

Cell lines and antibody: The CD19+ cell lines Raji (Burkitt Lymphoma) and Nalm-6 (B-ALL) were obtained from the DSMZ. The chimeric antibody is based on a murine anti-CD19 antibody licensed from the German Cancer Research Center (Heidelberg). Thiomab derivatives thereof were produced by HDP using Expiz93 cells (Life Technologies) and transient transfection methods.

Synthesis of conjugates: Maleimide Amanitin compounds HDP 30.215 and HDP 30.1699, and maleimide MMAE were conjugated to substituted cysteine residues in phosphate buffer, pH 7.4 after reduction with TCEP and re-oxidation of interchain disulfides by dehydroascorbic acid (dhAA). Conjugates were purified by SE-FPLC and dialysis. DAR (drug-antibody ratio) for Amanitin conjugates according to LC-MS analysis was app. 2.0 Amanitins per IgG.

Cell proliferation assay: Quantitative determination of DNA synthesis was performed by WST-1 assay (Roche).

Animal models: Raji intravenous: Six to 8-wk-old female CB-17 SCID mice were obtained from Janvier. Animals were implanted with 2.5×10^6 Raji cells intravenously into their tail vein; treatment was started by a single-dose i.v. application on day 3 post tumor cells inoculation. Clinical signs and survival were registered daily. Animals were sacrificed, when one or more termination criteria arose, such as hind limb paralysis or a weight loss of >20%. Raji subcutaneous: Six to 8-wk-old female CB-17 SCID mice were obtained from Janvier. Raji cells (2.5×10^6) were injected into the flank of each mouse for the development of a tumor. When tumors were well-established (60-80mm³), treatment was started by a single-dose i.v. application. Tumor volume was calculated as $V = a \times b^2 \times 0.5$, where a is the length and b is the width.

Non-human primate tolerability studies were performed at LPT (Germany, Hamburg).

Cytotoxic potency

The CD19+ cell lines Raji and Nalm-6 were used to test the cytotoxic potency of anti-CD19 ADCs. Following ADCs were assessed:

ADC	payload	linker type	DAR (MS)
anti-CD19-Thiomab-30.1699	"natural" Amanitin	cleavable	~2.0
anti-CD19-Thiomab-30.2115	synthetic Amanitin	cleavable	~2.0
anti-CD19-Thiomab-MMAE	MMAE	cleavable	~2.0

In both cell lines, the anti-CD19-Amanitin ADCs showed high cytotoxic activity in a picomolar range and superior activity to the MMAE-conjugate (Figure 1).

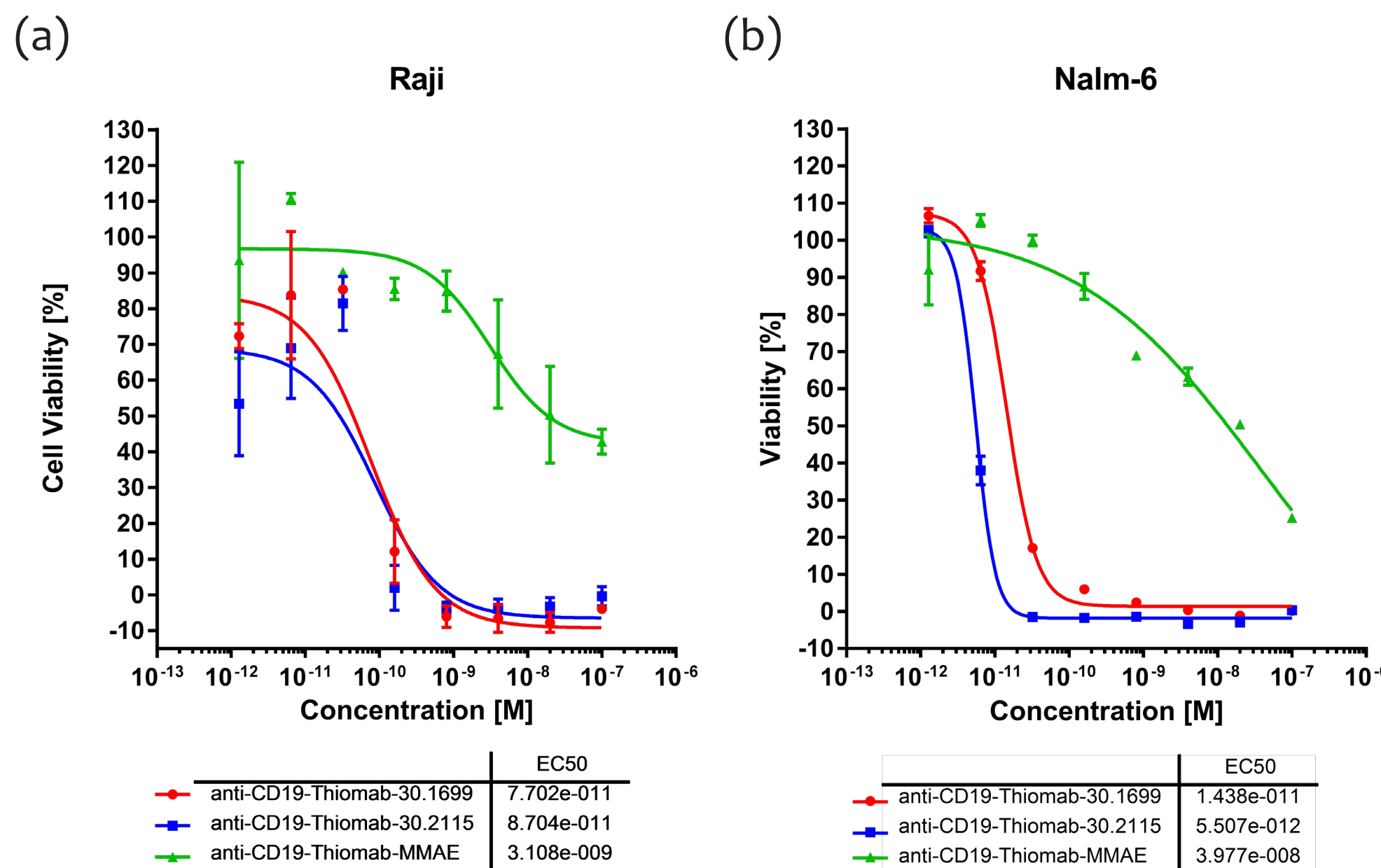


Figure 1: Cytotoxic activity of anti-CD19-Amanitin and -MMAE ADCs on Raji (a) and Nalm-6 (b) cells after incubation for 96h.

Stability of CD19-Amanitin ADCs

To test the stability of anti-CD19-ADCs, compounds were incubated in murine plasma for up to 10 days. After the respective incubation period, the compounds were either analyzed using an anti-Amanitin Western blot or checked for remaining cytotoxic potential in an *in vitro* cytotoxicity assay on Nalm-6 cells. The Western blot analysis showed higher stability of the synthetic Amanitin ADC variant anti-CD19-Thiomab-30.2115 compared to the „natural“ variant anti-CD19-Thiomab-30.1699 (Figure 2a). This finding was confirmed by the decreased cytotoxic efficacy of the plasma incubated ADCs (Figure 2b).

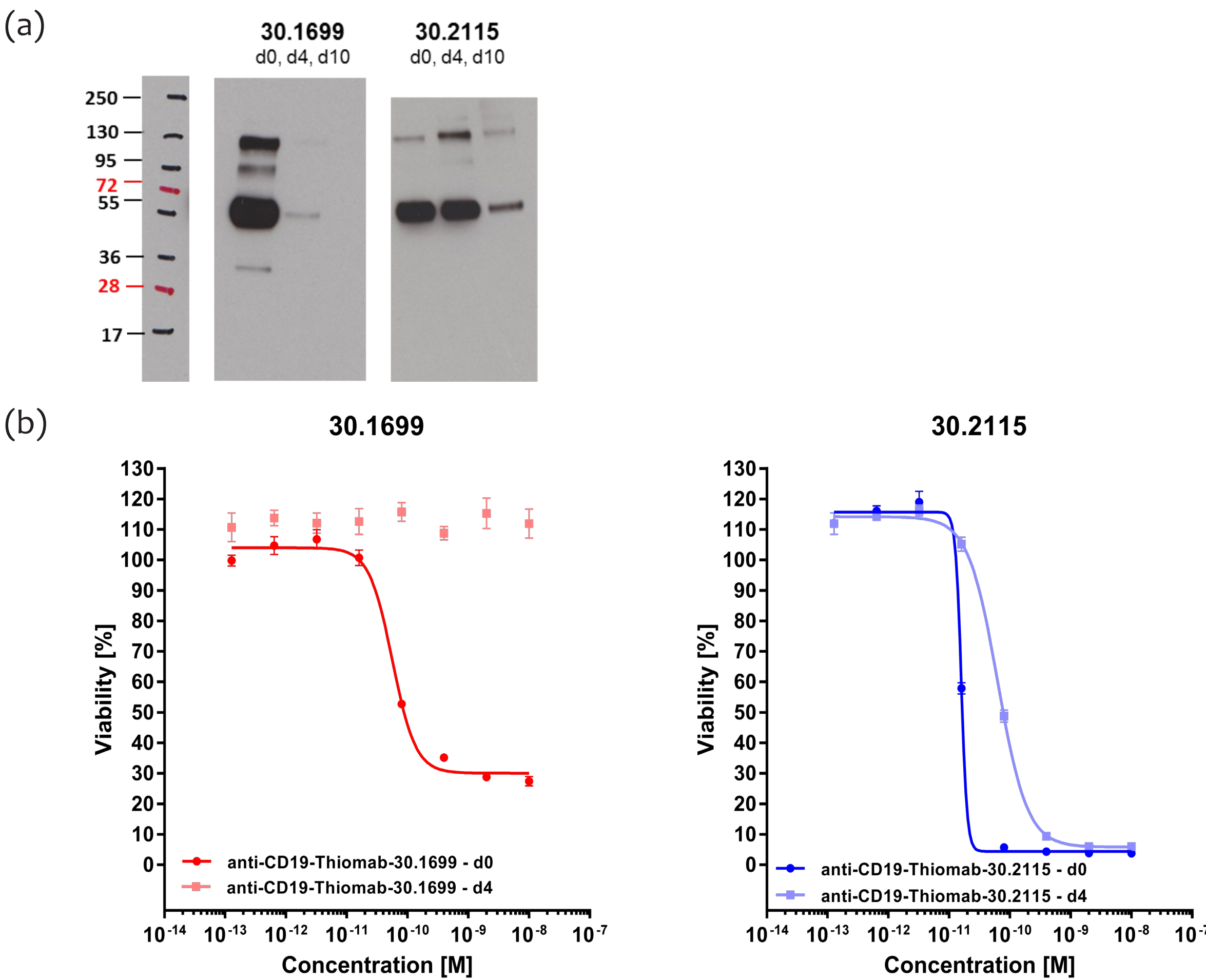


Figure 2: Reducing SDS-PAGE and anti-Amanitin Western blotting of ADCs incubated in murine plasma (a). 30.1699 = anti-CD19-Thiomab-30.1699; 30.2115 = anti-CD19-Thiomab-30.2115. (b): Cytotoxic activity of anti-CD19-Amanitin ADCs in Nalm-6 cells after incubation of ADCs in murine plasma for up to 4 days.

RESULTS

Mouse efficacy and tolerability experiments

Antitumor activities of anti-CD19 ATACs were determined in Raji intravenous (i.v.) and subcutaneous (s.c.) xenograft models *in vivo*.

Raji intravenous model

An increase in survival of mice treated with anti-CD19 ATACs was noticed after single dose application of 2mg/kg and 6mg/kg (Figure 3), which was dose- and Amanitin source-dependent. At the 2mg/kg dose level, the synthetic Amanitin-variant 30.2115 had superior antitumor efficacy compared to the “natural” Amanitin based ADC anti-CD19-Thiomab-30.1699. At the 6mg/kg dose level, a delay in disease onset between control animals and the anti-CD19-Thiomab-30.2115 treated animals, from approximately 20 to more than 60 days was found, with 4 of 10 animals remaining disease free at the end of the experiment on day 155. The “natural” Amanitin based ADC anti-CD19-Thiomab-30.1699 was not tolerated at the 6mg/kg dose level; 8 of 10 animals were found dead after approximately 10 days.

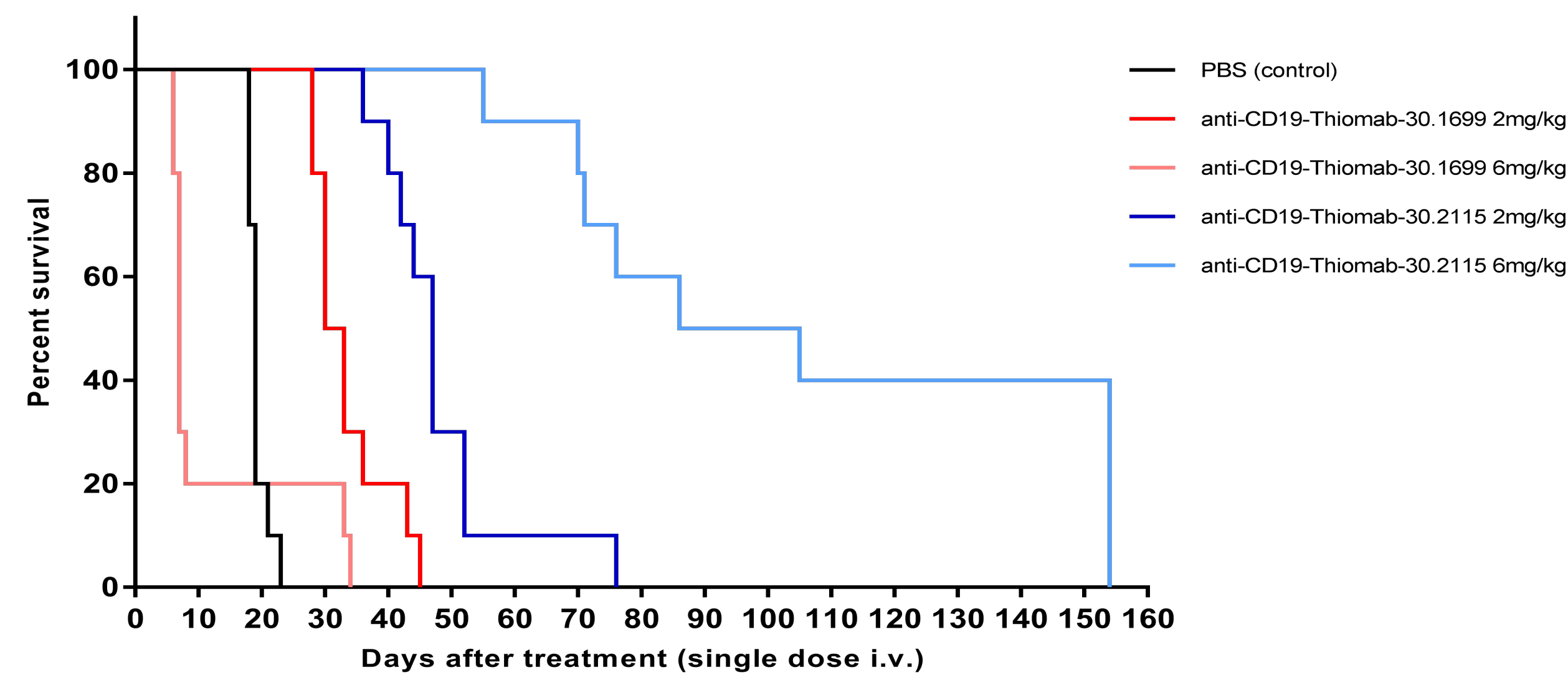


Figure 3: Survival of mice implanted with Raji cells via tail vein injections. Single dose i.v. treatment on day 3 after tumor implantation.

Raji subcutaneous model

In Raji s.c. model the synthetic Amanitin-variant 30.2115 again had superior antitumor efficacy compared to the “natural” Amanitin based ADC anti-CD19-Thiomab-30.1699 after single dose application between 2mg/kg and 6mg/kg (Figure 4). In the Raji i.v. model, the “natural” Amanitin based ADC anti-CD19-Thiomab-30.1699 was not tolerated at the 6mg/kg dose level (Figure 3). The highest tolerable dose for that ATAC was found with 3mg/kg. At this dose level the anti-CD19-Thiomab-30.1699 ATAC lead to a delay in tumor growth which was comparable to the effect of the synthetic Amanitin based ADC anti-CD19-Thiomab-30.2115 at the 2mg/kg dose level when tested against Raji s.c. tumors. As already observed in the Raji i.v. model, the synthetic Amanitin based ADC anti-CD19-Thiomab-30.2115 was well tolerated at the 6mg/kg dose level. In Raji s.c. model, significant inhibition of tumor growth was observed at the 6 mg/kg dose level.

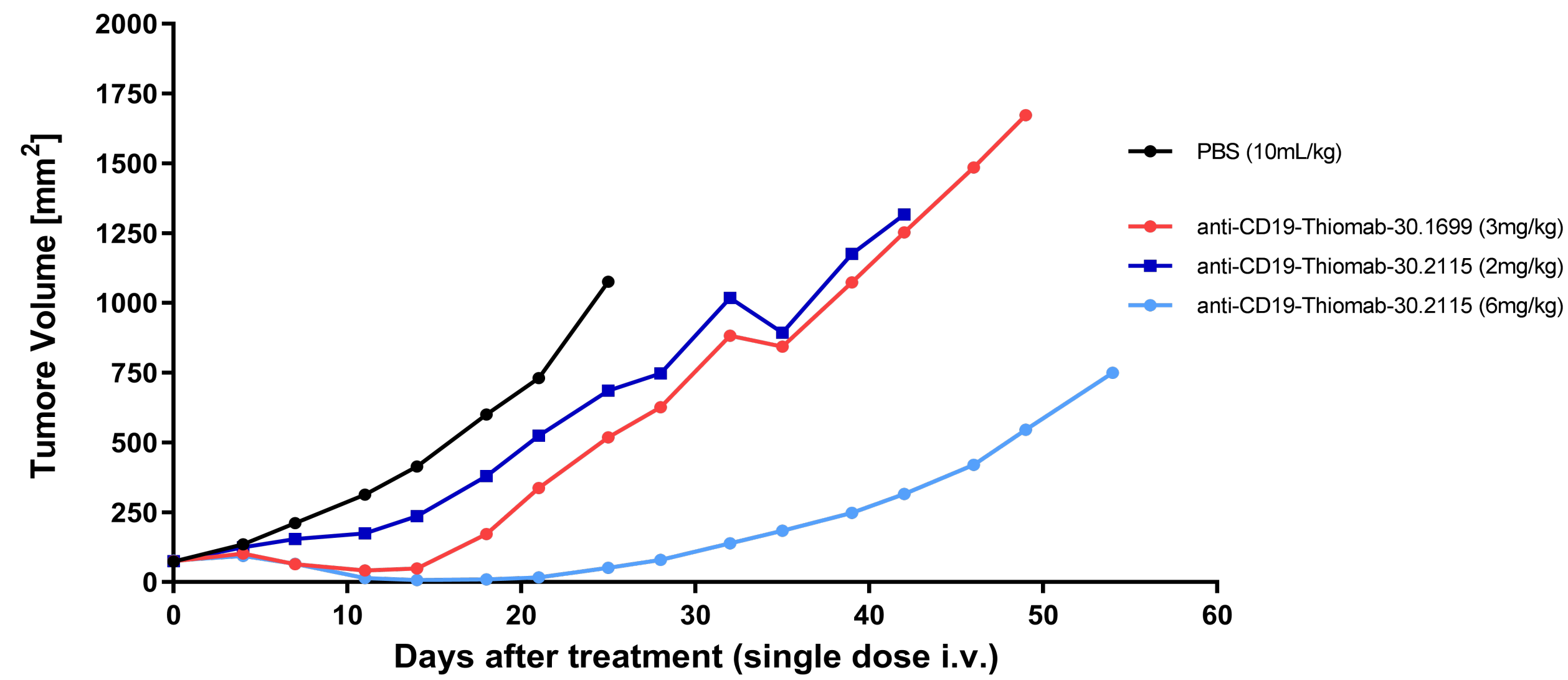


Figure 4: Subcutaneous xenograft model based on Raji cells. Single dose i.v. application.

Non-human primate tolerability study

Anti-CD19-Thiomab-30.2115 was assessed for a dose-escalating tolerability study in cynomolgus monkeys. The ATAC was applied sequentially at doses of 0.3mg/kg, 1.0mg/kg, 3.0mg/kg and 5.0mg/kg to the same animals. Before and after application biochemical and hematological blood parameters were evaluated extensively (selected parameter: Figure 5). Histopathological examination is ongoing.

The ATAC anti-CD19-Thiomab-30.2115 was well tolerated up to the 5mg/kg dose level:

- transient increase in liver-relevant biochemical parameters after application of 3mg/kg and 5mg/kg.
- no sign of kidney damage by serum parameters.
- food consumption and body weight remained unaffected at doses applied

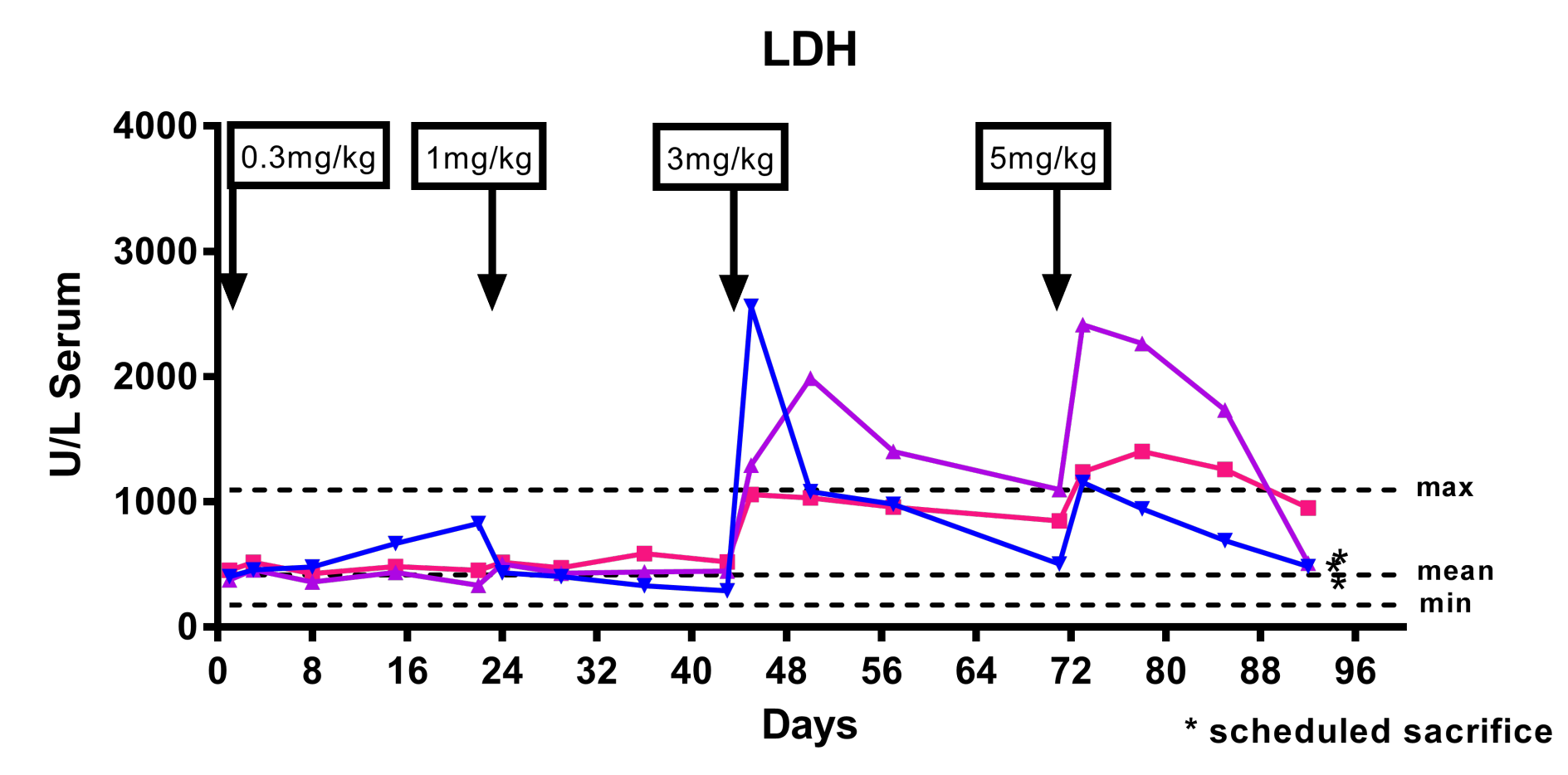
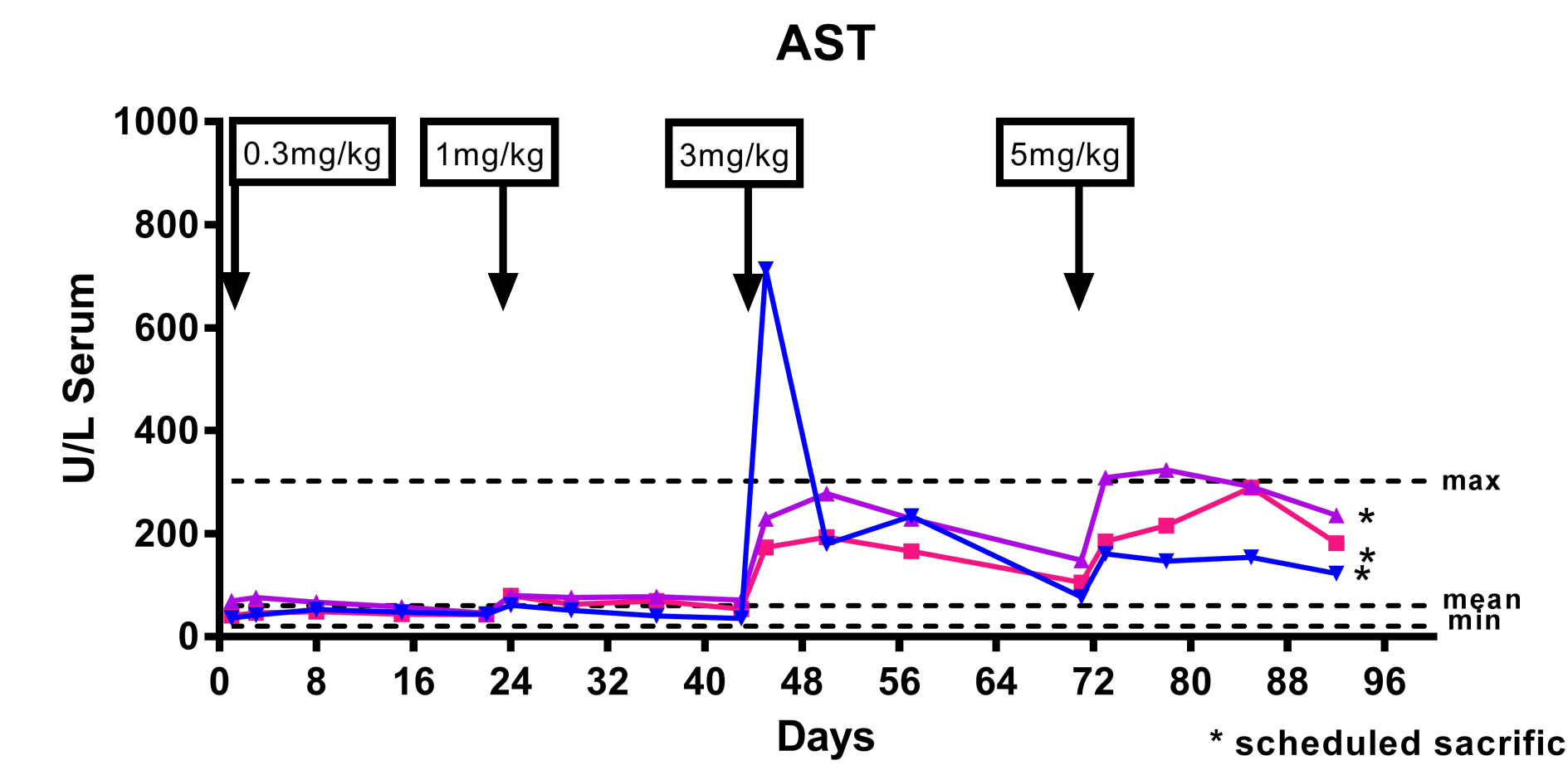
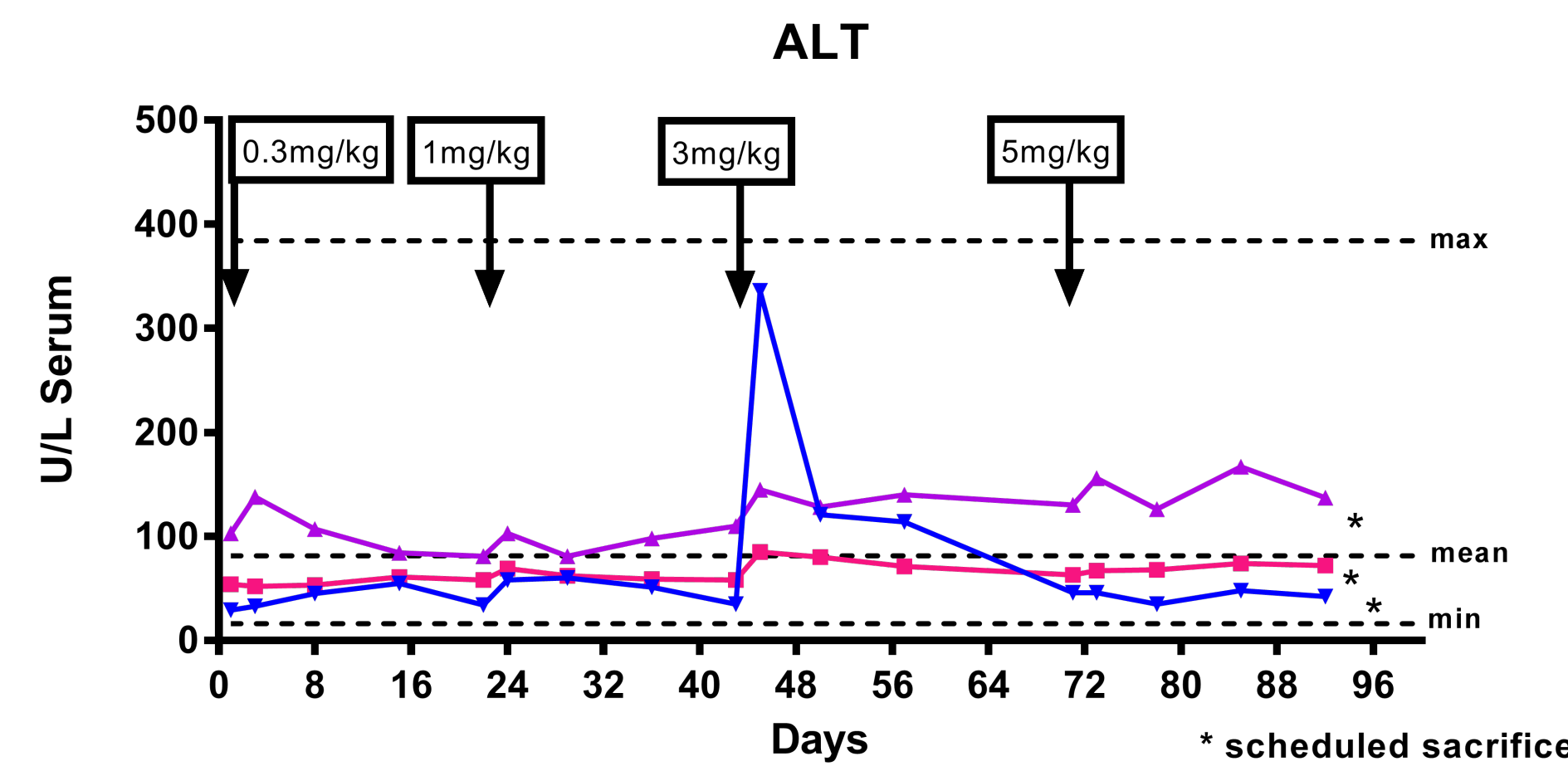


Figure 5: Selected biochemical serum parameters in cynomolgus monkeys treated with escalating doses of anti-CD19-Thiomab-30.2115. Dashed lines reflect the mean, min. and max. values of untreated animals (predose values).

CONCLUSION

Leukemia in general ranks as the 6th most common type of cancer and Non-Hodgkin-Lymphoma as the 10th most common type of cancer in the United States. CD19 (also known as B-lymphocyte surface antigen B4, B4, CVID3) is expressed on cells of the B cell lineage, ranging from the pre-B cells until the terminal differentiation to plasma cells and, therefore, it is expressed in most acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemias (CLL) and B cell lymphomas (2). Thus, CD19 antigen is an ideal target for immunotherapy of B-cell malignancies. In the current study, *in vitro* and *in vivo* data of Amanitin-ADCs targeting CD19 are presented. Amanitin as a toxic warhead for ADCs targeting CD19 seems to be a suitable therapeutic option because of the unique mode of action and the molecular characteristics of the toxin. Amanitin is highly active in drug resistant cells, independent of the status of expression of multi-drug resistance transporters because of its hydrophilic structure. By inhibition of RNA pol II Amanitin-binding leads not only to apoptosis of dividing cells, but also of slowly growing cells and dormant cells. This is a potential advantage for amatoxin payloads in certain leukemias, e.g. for slow proliferating CLL cells. HDP has constructed more than 70 different linkers allowing the conjugation of Amanitin to lysine-residues, cysteine-residues (both, genetically engineered and interchain cysteines) as well as non-natural amino acids of antibodies. In the presented work we characterized ADCs directed against CD19 with synthetic and “natural” Amanitin payloads linked via a cleavable linker to genetically engineered cysteine residues. *In vitro* data of anti-CD19-ATACs showed cytotoxicity on CD19 positive cell lines in a picomolar range and superior activity to the MMAE comparison conjugate. Further the synthetic Amanitin ADC variant anti-CD19-Thiomab-30.2115 showed a superior stability compared to the “natural” variant anti-CD19-Thiomab-30.1699 in murine plasma. In mouse xenograft models, especially the anti-CD19-Thiomab-30.2115 conjugate showed clear anti-tumor activity. Safety profiling in Cynomolgus monkey revealed a good tolerability and therapeutic index for anti-CD19-Thiomab-30.2115. The positive findings of these experiments warrant the clinical development of anti-CD19-ATACs.

REFERENCES

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