

INTRODUCTION

Antitumor activity of monoclonal antibodies can be dramatically enhanced by conjugation to toxic small molecules. Beside the recent approval of Mylotarg (CD33), Adcetris (CD30), Kadcyla (HER2) and Besponsa (CD20), nearly 60 antibody-drug conjugates (ADCs) have entered clinical trials, promising to strengthen the therapeutic capabilities for cancer treatment in the next decade. Surprisingly most ADCs are based on one of few toxic compounds only and on an even smaller number of toxicity mechanisms: Most antibodies are coupled to the microtubuli-targeting auristatins and maytansines. Toxins that operate through such a mechanism could suffer from limited activity in different cancer indications and in cells expressing resistance mechanisms. 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, so called ATACs (Antibody Targeted Amanitin Conjugates), comprising a new class of ADCs with amanitin as toxic payload (1). Amanitin is the most well-known toxin of the amatoxin family. Amanitin binds to the eukaryotic RNA polymerase II (RNA pol II) and thereby inhibits the cellular transcription at very low concentrations. We accomplished the chemical synthesis of amanitin and were able to synthesize amanitin variants in order to optimize the toxin structure for different tumors and antibodies. To get insight into the relationship between structure and activity, we chemically synthesized six different linker-amanitin derivatives. We characterized these variants with regard to cytotoxicity *in vitro*, efficacy and tolerability *in vivo*.

The amanitin variants were derivatized on the 6-OH-Typtophan or on the sulfoxide group of the Cysteine. Additionally, two linker attachment sites (Asparagine or Tryptophan) were evaluated. In the presented study, six linker-amanitin derivatives were conjugated to different antibodies and tested for *in vitro* toxicity, *in vivo* efficacy against solid and hematologic tumors as well as for tolerability in non-human primates.

METHODS

Cell lines: JIMT-1, SKBR-3, BT474 and NCI-N87 (used for anti-Her2-ATACs); LNCap, 22RV1, MDA-Pca2b and C4.2 (used for anti-PSMA-ATACs); Raji, Raji Luc, Nalm-6 and MEC-2 (used for anti-CD19-ATACs)

Antibodies: Anti-Her-2 (variable domains of trastuzumab; cysteine engineered monoclonal antibody, Heidelberg Pharma); humanized anti-PSMA (Albert Ludwig University Freiburg, medical center; humanization at Lonza Group AG; cysteine engineered monoclonal antibody, Heidelberg Pharma); chimeric anti-CD19 (DKFZ Heidelberg, Germany; cysteine engineered monoclonal antibody, Heidelberg Pharma).

Toxic warhead: Cysteine reactive linker-amanitin constructs were synthesized at Heidelberg Pharma and conjugated site-specifically to the antibodies.

Cell proliferation assay: Quantitative determination of cell viability was performed by CellTiter Glo 2.0 assay (Promega).

Animal models: mouse xenograft tumor models (Her2-, PSMA- and CD19-positive cell lines) were performed in single-dose experiments.

Tolerability was assessed in mice and in non-human primates (NHP).

1. Conjugation of Antibody Targeted Amanitin Conjugate (ATAC)

The RNA polymerase II binding toxin amanitin is conjugated to THIOMABS of the respective antibodies using maleimide chemistry, resulting in homogenous ATACs with a DAR of ~2 toxins per IgG (Figure 1).

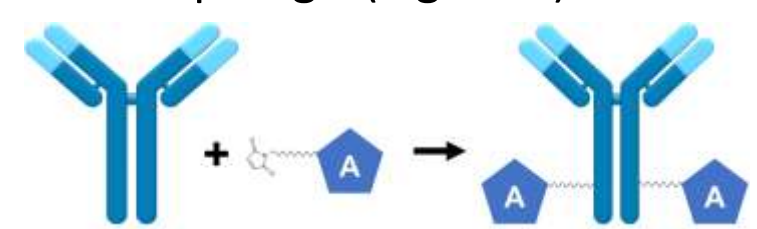


Figure 1: Schematic drawing of conjugation of antibodies with amanitin compounds resulting in an ATAC with DAR of 2.

2. Chemical structure of Amanitin derivatives

In order to improve the efficacy of ATACs, amanitin was derivatized either on the 6-OH-Tryptophan (Figure 2, blue circles) or on the sulfoxide group (Figure 2, red circles). Additionally, previous work showed that there are two possible linker-attachment sites. Either on the Tryptophan (AA4) or on the Asparagine (AA1, green circles). In all cases, a cleavable Valine-Alanine linker was used.

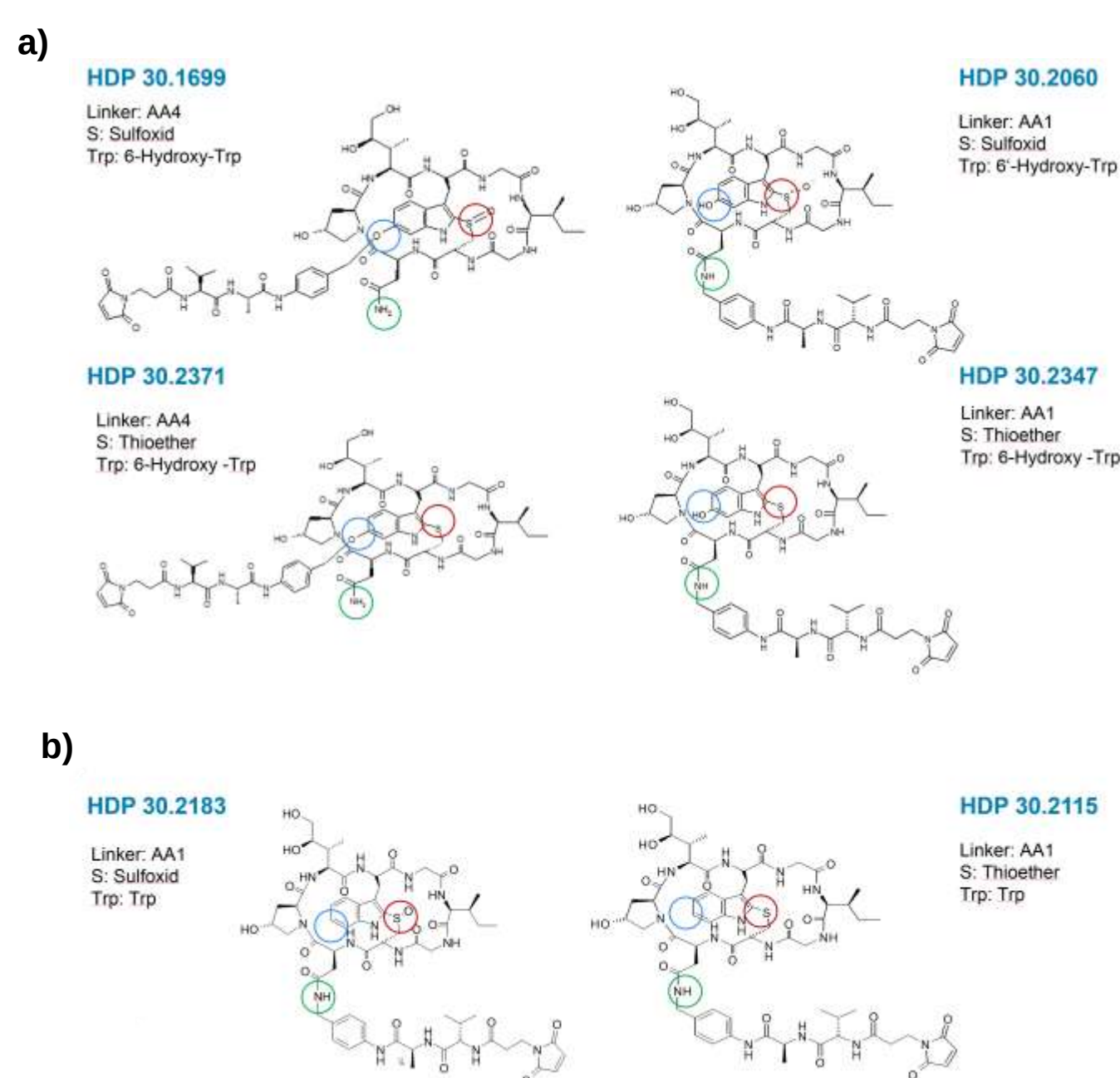


Figure 2: Chemical structures of amanitin derivatives. Colored circles indicate the derivatization sites: Sulfoxide or thioether on the cysteine (red circles), Tryptophan or 6-OH-Tryptophan (blue circles), and the amino group on the Asparagine as possible linker attachment site (green circles). a) Amanitin derivatives based on 6-OH-Tryptophan. b) Amanitin derivatives based on Tryptophan.

3. Cytotoxicity *in vitro*

Three antibodies, directed against Her2, PSMA or CD19, respectively, were conjugated to different linker-amanitin derivatives. The EC50 values of all compounds in target antigen-positive cells were within picomolar range. Slight differences could be detected: Most efficacious compound in the *in vitro* screening was 30.2371, followed by 30.1699, 30.2060 and 30.2347 (data not shown). There was a slightly higher cytotoxic activity for the 6-OH-Tryptophan variants detectable as compared to the Tryptophan derivatives.

RESULTS

4. Tolerability in mice

All ATACs were checked for tolerability (Table 1) and efficacy (Figure 3) in mouse models. The ATACs tested were tolerable in mice at ≥ 4 mg/kg. 30.2115 was most tolerable as CD19-ATAC with a maximal tolerated dose (MTD) of > 15 mg/kg. There seems to be a slightly better tolerability for derivatives which use AA1 as linker attachment site.

Derivative	Code*	MTD [mg/kg]		
		Her2-ATAC	PSMA-ATAC	CD19-ATAC
30.1699	SO 6-OH-Trp AA4	4	4	4
30.2060	SO 6-OH-Trp AA1	>10	>10	>10
30.2371	S 6-OH-Trp AA4	4	4	4
30.2347	S 6-OH-Trp AA1	4	4	6
30.2183	SO Trp AA1	>10	>10	>10
30.2115	S Trp AA1	4	10	>15

Table 1: Tolerability of different ATACs in mice. * the code indicates the derivatization site. S or SO: Cysteine as thioether or sulfoxide; 6-OH-Trp or Trp: Tryptophan as 6-OH variant or without 6-OH group; AA1 or AA4: linker attachment site on amino acid 1 or amino acid 4.

5. Efficacy in mouse xenograft models

For assessment of efficacy of the different ATACs, subcutaneous and intravenous xenograft models were performed. Animals were allocated to groups at a mean tumor volume of $\sim 120\text{mm}^3$. Treatment: single dose i.v. In subcutaneous models 6-OH-Tryptophan-based derivatives show superior efficacy as compared to the Tryptophan-based variants. With regard to the oxidation state at the sulfur atom, no clear influence could be observed. The linker attachment site does not seem to have an impact on the efficacy. In the disseminating model, efficacies of the Tryptophan and the 6-OH-Tryptophan derivatives were comparable. This is in agreement with data from other ATACs targeting hematologic tumors, were Tryptophan derivatives were even superior to 6-OH-Tryptophan variants (data not shown).

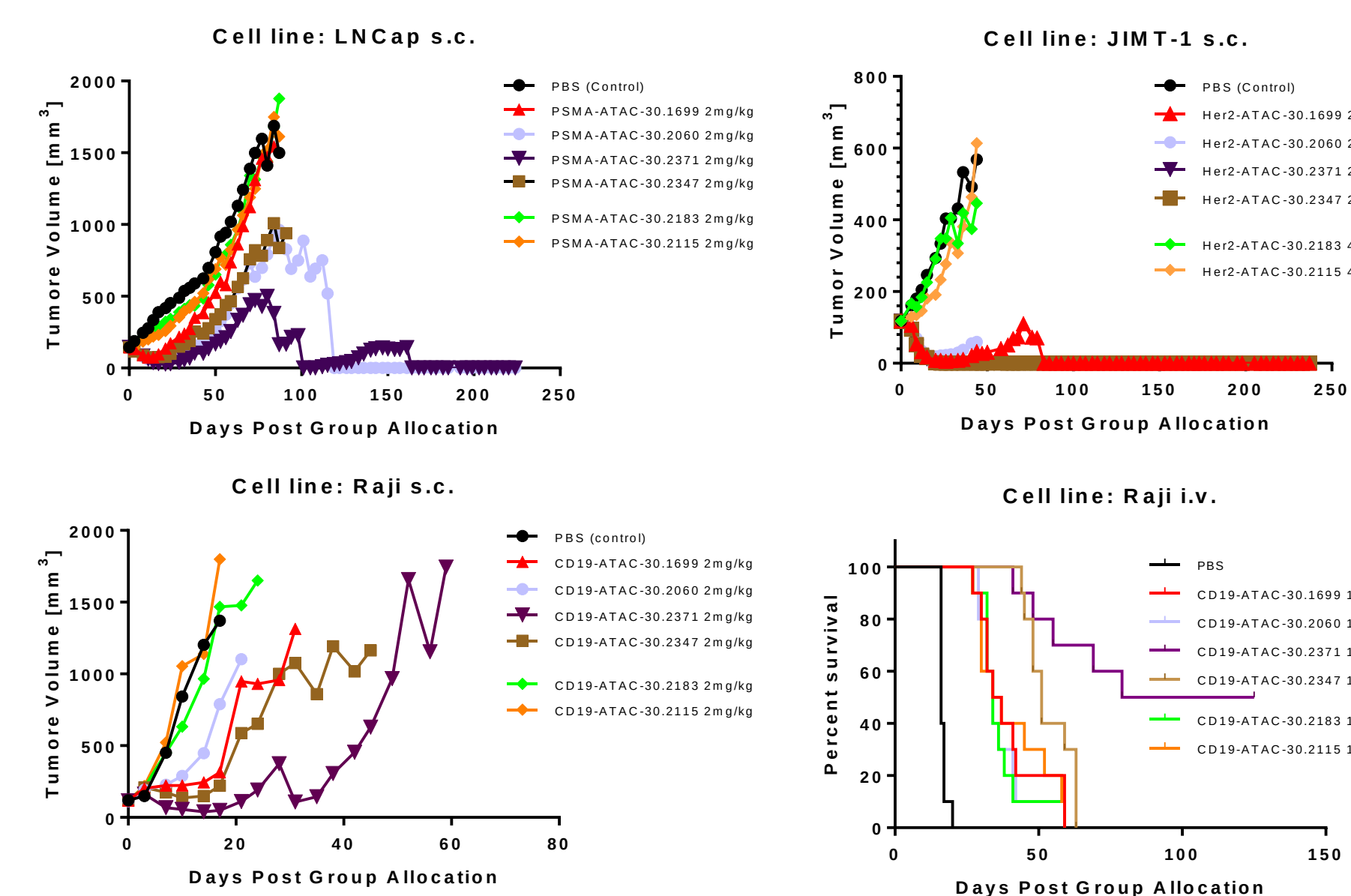


Figure 3: PSMA-, Her2-, and CD19-ATACs in LNCap, JIMT-1 and Raji xenograft models. Animals were allocated to groups at tumor volumes of $\sim 125\text{mm}^3$ or 1 week post i.v. inoculation (Raji i.v.).

6. Non-human primate tolerability study

To assess off-target tolerability in non-human primates, Amanitin derivatives were conjugated to a THIOMAB of a non-binding anti-Digoxigenin-antibody using maleimide chemistry, resulting in homogenous ATACs with a DAR of ~ 2 toxins per IgG. The resulting ATACs were assessed in a dose-escalating tolerability study in cynomolgus monkeys. Escalating doses of individual ATACs were sequentially given to the same animals. Before and after application biochemical and hematological blood parameters were evaluated extensively. Figure 4 shows the AST and LDH data. Most tolerable compounds were DIG-30.2115 and DIG-30.2060, followed by DIG-30.1699, DIG-30.2347 and DIG-30.2371. No correlation of structural variants and toxicity could be detected.

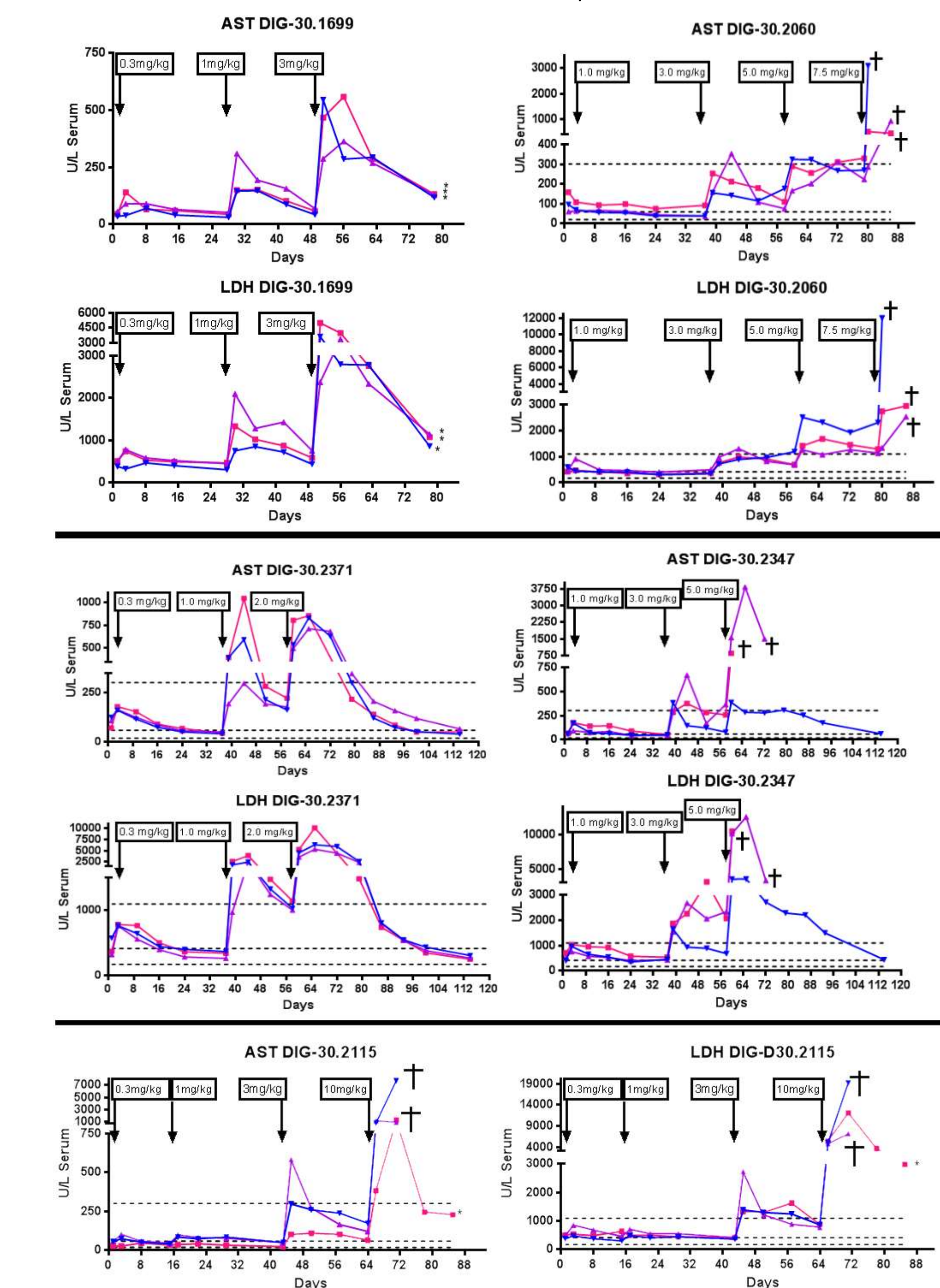


Figure 4: Selected biochemical serum parameters in cynomolgus monkeys (3 animals) treated with escalating doses of anti-DIG ATACs. Dashed lines reflect the mean, min. and max. values of untreated animals.

CONCLUSION

To get insight into the relationship between structure and activity, we chemically synthesized six different amanitin-linker-derivatives. These amanitin variants were derivatized on the 6-OH-Typtophan or on the sulfoxide group of the Cysteine. Additionally, two linker attachment sites (Asparagine or Tryptophan) were evaluated.

All ATACs showed *in vitro* cytotoxicity on target positive cell lines in the picomolar range. Mouse tolerability studies of ATACs showed a MTD of at least 4 mg/kg. However, 3 derivatives had superior tolerability: the 6-OH-Tryptophan variant 30.2060 and the Tryptophan variants 30.2183 and 30.2115. In mouse xenograft models, ATACs with amanitin based on 6-OH-Tryptophan caused dose-dependent tumor regression and complete remission after a single i.v. dose of 2.0 mg/kg in s.c. xenografts irrespective of the antibody and target used. In contrast the other two linker-amanitin derivatives (Tryptophan-based) were only poorly effective *in vivo* while showing comparable activities *in vitro*. When comparing subcutaneous and intravenous xenograft models using the same cancer cell lines, the differences in efficacy were much more pronounced in subcutaneous than in intravenous xenografts. This is in agreement with data from other ATACs targeting hematologic tumors, were Tryptophan derivatives were at least equivalent, sometimes even superior to 6-OH-Tryptophan variants (data not shown).

Tolerability studies in non-human primates using ATACs with a non-binding anti-Digoxigenin-antibody showed overall good tolerability at ≥ 3 mg/kg. Superior tolerability of > 5 mg/kg was observed for 30.2060 (6-OH-Trp, SO, AA1) and 30.2115 (Trp, S, AA1).

In conclusion, different efficacy of linker-amanitin derivatives with regard to mouse xenograft models was detected: Amanitin derivatives based on 6-OH-Tryptophan were superior compared to the Tryptophan in solid tumors. For hematologic indications, both variants are suitable. The oxidation state of the sulfur in the cysteine and the attachment site of the linker do not have significant impact on efficacy or tolerability.

REFERENCES

(1) Anderl J, Echner H, Faulstich H. Chemical modification allows phallotoxins and amatoxins to be used as tools in cell biology. Beilstein J Org Chem. 2012; 8:2072-84

CONTACT

Dr. Michael Kulke

Heidelberg Pharma Research GmbH
Schriesheimer Str. 101
68526 Ladenburg
Germany

Phone: +49-6203-1009 0

Email: Michael.Kulke@hdpharma.com
<https://www.heidelberg-pharma.com>

