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Amanitin-based ADCs with an improved therapeutic index

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INTRODUCTION

Antitumor activity of monoclonal antibodies can be dramatically enhanced by conjugation to toxic small molecules. Beside the recent approval of Kadcyla (T-DM1) and Adcetris (SGN-35) more than 30 antibody-drug conjugates (ADC) have entered clinical trials, promising to strengthen the therapeutic capabilities for cancer treatment in the next decade. Surprisingly most ADCs are based on a few toxic compounds only and on an even smaller number of toxicity mechanisms: Most antibodies are coupled to the microtubuli-targeting auristatins and maytansines (1). 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.In the present study we evaluated the antitumor potency of monoclonal antibodies conjugated to small molecules from the amatoxin family. Amanitin, the most well-known toxin of the amatoxin family, binds to the eukaryotic RNA pol II and thereby inhibits the cellular transcription at very low concentrations (2). In our experiments, we tested a site-specific strategy to covalently conjugate amanitin to HER2-targeting trastuzumab and generated conjugates with low aggregation and high affinity for the target antigen. For site-specific conjugation, trastuzumab was engineered at different locations to incorporate cysteines. Trastuzumabs with engineered cysteines at different positions were compared *in vitro* and *in vivo* to identify the best localization for drug attachment. Safety profiling of a site-specific amanitin ADC in cynomolgous monkeys revealed good tolerability and a favorable therapeutic index.

METHODS

Cell lines and antibody: The human breast cancer cell line JIMT-1 was obtained from the DSMZ, the ovarian cancer cell line SK-OV-3 and monocytic THP-1 cells were obtained from the ATCC and grown according to the suppliers instructions. Antibodies were produced by HDP using Expi293 cells (Life Technologies) and transient transfection methods.

Synthesis of amanitin-ADCs: Maleimide amanitin compounds HDP 30.0880 and HDP 30.1699 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 SEC-HPLC and dialysis. DAR (drug-antibody ratio) according to LC-MS analysis was ca. 1.90 to 2.05 amanitins per IgG.

Cell proliferation assay: Quantitative determination of DNA synthesis was performed by a chemoluminescent BrdU incorporation assay (Roche).

Animal models: Six to 8-wk-old athymic female NMRI nude mice (nu/nu) were obtained from Janvier. JIMT-1 or SK-OV-3 cells (5.0 x 10^6), respectively, were injected into the flank of each mouse for the development of a tumor. When tumors were well-established (150-250 mm^3), 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. NHP studies were performed at LPT (Germany, Hamburg).

RESULTS

Cysteine substitutions in trastuzumab heavy chains

HER2 targeting trastuzumab antibody was modified by substitution of endogenous amino acids to cysteine at eight different positions (HC1 to HC8) in the constant regions of heavy chains (Fig. 1). The RNA polymerase II binding toxin amanitin was conjugated by stable linker (HDP 30.0880) or cleavable linker (HDP 30.1699) technology and maleimide chemistry, resulting in homogenous ADCs with a DAR of ca. 2 toxins per IgG. Two of the drug positions (HC4 and HC6) were selected for interfering with binding of the antibodies Fc domains to Fcγ receptors, thus reducing the cytotoxic activity on Fcγ receptor expressing cells, e.g. macrophages.

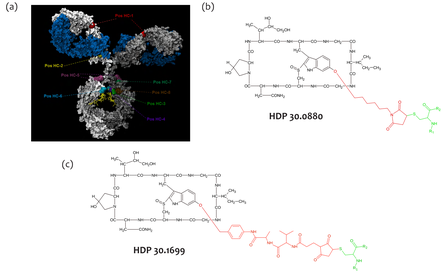

Figure 1: (a) Positions of cysteine substitutions in trastuzumab heavy chains. (b, c) Chemical structures of cysteine reactive amanitin-linker compounds.

Site-specific coupling of amanitin to heavy chains

Reducing SDS-PAGE and Western Blotting detection with an anti-amanitin antibody demonstrated site-specific payload conjugation to the heavy chains of trastuzumab (Fig. 2a). In parallel a heavy and light chain LC-MS analysis documented a mass shift to a higher molecular weight only for heavy chains, reflecting the selective reaction of amanitin-linker compounds to the substituted cysteines (Fig. 2b). All ADCs showed low aggregation (<2%) and high homogeneity by analytical SEC chromatography.

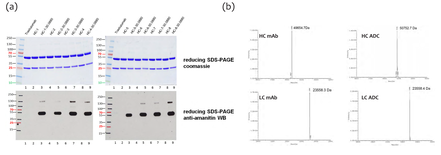

Figure 2: (a) Reducing SDS-PAGE and anti-amanitin Western Blotting of antibodies and ADCs. (b) LC-MS analysis of heavy chains and light chains of Trastuzumab-HC4 and Trastuzumab-HC4-30.0880.

Cytotoxic potency in Fcγ receptor- and HER2-positive cells

The monocytic THP-1 cell line was used as a model to test the cytotoxic potency of the different amanitin ADCs on Fcγ receptor expressing cells. ADCs based on the trastuzumab variants HC4 and HC6 showed clearly lower cytotoxicity on THP-1 cells compared to the other site-specific ADCs and the heterogeneous Her-30.0643 ADC (Fig 3). This reflects the low binding affinity of HC4 and HC6 variants to Fcγ receptors, resulting in reduced cellular uptake and intracellular release of toxic payload.

In contrast, all site-specific ADCs showed comparable cytotoxicity on HER2 expressing tumor cells, e.g. SK-OV-3 ovarian cancer cells. There was only a minor difference in cytotoxic potency between the different ADCs with respect to payload positioning. Because of the release of an intracellular amanitin metabolite and not an intracellular amanitin-linker metabolite, cleavable linker conjugates based on HDP 30.1699 showed ca. 10 times higher activity compared to stable linker conjugates based on HDP 30.0880.

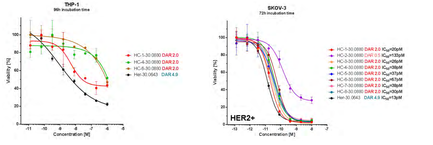

Figure 3: Cytotoxic activity of amanitin conjugates in THP-1 and SK-OV-3 cells after incubation for 120h or 72h, respectively.

Mouse efficacy and tolerability experiments

Antitumor activities of ADCs were determined in JIMT-1 and SK-OV-3 subcutaneous xenograft models *in vivo*. All ADCs showed dose-dependent tumor regression and complete remissions after single dose application between 2mg/kg and 10mg/kg (Fig. 4). There were only slight differences between anitumor activities with respect to drug positioning, but significant difference between linker structures. Cleavable linker structure were ca. 3-times more potent compared to stable linker derivatives. Tolerability was checked for the site-specific stable linker ADCs in mice. In a dose escalation experiment was a clear difference of ADCs in tolerability, with MTDs between ca. 15mg/kg and more than 37.5mg/kg.

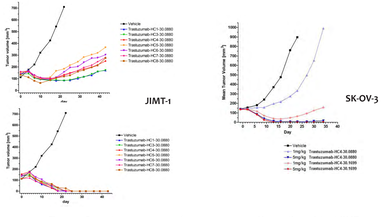

Figure 4: Subcutaneous xenograft models based on Herceptin-resistant JIMT-1 cells and Herceptin-sensitive SK-OV-3 cells. Single dose i.v. application.

Non-human primate tolerability study

The site-specific ADC based on trastuzumab HC4 showing the highest tolerability in rodents was used for a dose-escalating study in cynomolgus monkeys. Amanitin ADC was applied sequentially at doses of 0.3mg/kg, 1.0mg/kg, 3.0mg/kg and 10.0mg/kg. Before and after application biochemical and hematological blood parameters were evaluated extensively (Fig. 5).

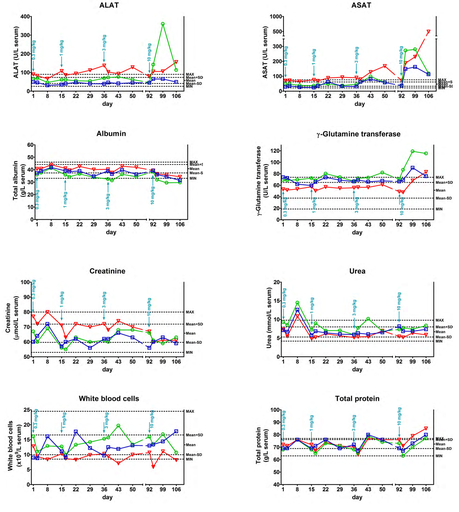

Figure 5: Biochemical serum parameters and hematology in cynomolgus monkeys treated with escalating doses of Trastuzumab-HC4-30.0880.

Trastuzumab-HC4-30.0880 ADC was well tolerated after dosing at 0.3mg/kg, 1.0mg/kg and 3.0mg/kg with no significant increase in liver-relevant biochemical parameters There was a slight increase of ALAT and γ-glutamine transferase and a moderate increase of ASAT after an additional adminstration of 10mg/kg ADC. There was no sign of kidney damage by serum parameters.

Food consumption and body weight remained unaffected at doses up to 10mg/kg. One animal showed occular disturbances at highest doses, potentially a result of HER2 on-target toxicity caused by the ADC.

CONCLUSIONS

Breast cancer is one of the most common malignancies and is the leading cause of cancer death among women in developed countries. While significant therapeutic advances have been made, therapeutic options remain unsatisfactory. Amanitin-based ADCs using an anti-Her2 antibody showed high antitumor activity in a series of models of preclinical oncology. Amanitin as a toxic warhead for ADCs in breast cancer 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 resistant transporters because of its hydrophilic structure. By inhibition of RNA polymerase II amanitin-binding leads not only to apoptosis of dividing cells, but also of slowly growing cells, as observed in dormant cells and cancer-initiating cells.

HDP has constructed more than 70 different linkers allowing the conjugation of amanitin to lysine-residues, cysteine-residues and non-natural amino acids of antibodies. In the presented work we characterized a site-specific strategy to covalently conjugate amanitin to HER2-targeting trastuzumab. Trastuzumabs with engineered cysteines at different positions were compared *in vitro* and *in vivo* to identify the best position for drug attachment. The most promising ADC was assessed for its safety profile in a dose escalating study in cynomolgous monkeys and revealed very good tolerability and a favorable therapeutic index.

These positive findings of these initial experiments encourage Heidelberg Pharma to further proceed with site-specific amanitin-based ADCs towards clinics.

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