Preclinical evaluation of anti-HER2 Antibody Targeted Amanitin Conjugates (ATACs) on HER2low breast cancer with chromosome 17p deletion

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Focused Cancer Therapies

Introduction

Triple negative breast cancer (TNBC) is the most difficult to treat subtype of breast cancer with limited therapeutic options. At least 50% of TNBC patients have low human epidermal growth factor receptor 2 (HER2; ERBB2) expression with the majority harboring hemizygous loss of POLR2A/chromosome 17p. Anti-tumor activity of monoclonal antibodies can be dramatically enhanced by conjugation to cytotoxic small molecules. By using such antibody-drug conjugates (ADCs) the toxin is exclusively delivered to target cells and thereby kills only those cells. For most of the current ADCs (e.g. anti-HER2 Kadcyla) a medium to high target antigen expression is required. Most ADCs are based on a few cytotoxic compounds only with microtubule- or DNA-targeting toxins as payloads. 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 **ATAC**s (**A**ntibody **T**argeted **A**manitin **C**onjugates), comprising a new class of ADCs with amanitin as toxic payload (1, 2). Amanitin is the well-known toxin of the amatoxin family and binds specifically to the eukaryotic RNA polymerase II thereby inhibiting the cellular transcription process (3, 4). 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 human **HER2low** as well as tolerability studies are presented. For breast cancer patients the treatment with ATACs targeting cancer cells with low expressing HER2 is a new promising approach.

Methods

Cell lines and antibodies: The cancer cell lines JIMT-1 and MDA-MB-453 were obtained from DSMZ, SKBR-3 from ATCC, MDA-MB-468 from CLS and T47D from Sigma-Aldrich. The anti-HER2 wild-type (wt), THIOMAB and the non-targeting (NT) antibodies were produced by Heidelberg Pharma Research GmbH using Expi293 cells (Life Technologies) and transient transfection methods.

Synthesis of conjugates: Maleimide amanitin compound with cleavable linker was conjugated to substituted cysteine residues of anti-HER2 or NT THIOMAB antibody in PBS buffer, pH 7.4 after reduction with TCEP and re-oxidation of interchain disulfides by dehydroascorbic acid (HER2-THIOMAB-ATAC; NT-THIOMAB-ATAC). Amanitin compound with stable linker was covalently conjugated to lysine side chains of anti-HER2 wt antibody by a one-step conjugation reaction in PBS buffer, pH 7.4 (HER2-ATAC). Conjugates were purified by SE-FPLC and DAR (drug-antibody ratio) for amanitin conjugates was analyzed by LC-MS.

Flow cytometry: Binding of HER2-THIOMAB antibody and HER2-THIOMAB-ATAC was analyzed with increasing concentrations. Goat Fab anti-human IgG-AlexaFluor488 secondary antibody (Dianova) was used. Cell binding was measured using FACSCalibur (BD Biosciences).

Cell proliferation assay: Quantitative determination of cell viability was performed by BrdU-ELISA chemiluminescent (Roche) assays.

Animal models: Subcutaneous mouse xenograft tumor models with cell line JIMT-1 were performed in single dose experiment. Six to eight-week-old female NMRI nude mice were obtained from Janvier. 5.0 x 10^6 JIMT-1 cells were injected into the right flank of each mouse for the development of a tumor. When tumors were well-established (ca. 150mm^3), treatment was started by a single dose i.v. application. HER2low heterogeneous TNBC patient derived xenograft (PDX) model studies were performed at XenTech (Evry, France). Non-human primate tolerability study of non-targeting ATAC was performed at LPT (Hamburg, Germany).

Results

1. HER2-THIOMAB antibody and ATAC bind selectively to HER2 on cancer cell lines

For binding analysis, HER2$^+$ SKBR-3, MDA-MB-453, JIMT-1, T47D as well as HER2$^-$ MDA-MB-468 cell lines were incubated with increasing concentrations of the HER2-THIOMAB antibody or HER2-THIOMAB-ATAC. The antibody as well as ATAC showed the same binding to the cancer cell lines expressing HER2 to different levels (Figures 1a+b). The generation of the THIOMAB antibody did not influence the binding towards HER2 (data not shown).

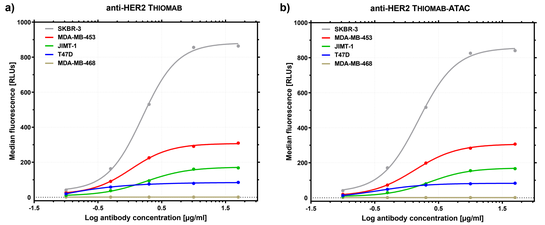

Figure 1: FACS analysis of (a) HER2-THIOMAB-antibody and (b) HER2-THIOMAB-ATAC on SKBR-3, MDA-MB-453, JIMT-1, T47D and MDA-MB-468 cells in increasing concentrations.

2. Cytotoxic potency

The HER2 expressing cancer cell lines SKBR-3, MDA-MB-453, JIMT-1 and T47D as well as HER2 neg. MDA-MB-468 cell line were used to test the cytotoxic potency of HER2-THIOMAB-ATAC with cleavable linker and compared to Kadcyla. While Kadcyla showed only a cytotoxic effect on cell lines with moderate and high HER2 expression, the ATAC showed high cytotoxic activity in a picomolar range on all HER2 high to low expressing cell lines (Figures 2a-d).

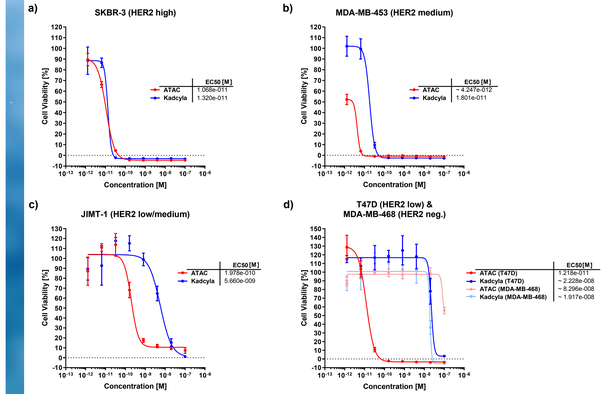

Figure 2: Cytotoxic activity of anti-HER2-THIOMAB-ATAC on (a) HER2 high SKBR-3, (b) HER2 medium MDA-MB-453, (c) HER2 low/medium JIMT-1, (d) HER2 low T47D and HER2 negative MDA-MB-468 cell lines using BrdU ELISA assay after incubation for 96h.

3. Mouse efficacy experiments

The antitumor activity of single dose treatment with anti-HER2 ATACs was determined in JIMT-1 xenograft as well as in patient-derived xenograft (PDX) subcutaneous (s.c.) models *in vivo*.

JIMT-1 s.c. xenograft model

Anti-HER2 ATAC with stable linker (Figure 3a) as well as HER2-THIOMAB-ATAC with cleavable linker (Figure 3b) showed complete remissions after single dose application of 2.9mg/kg and 2.0mg/kg, respectively. In contrast, Trastuzumab conjugated to DM1 (Kadcyla) did not have any effect (Figure 3a).

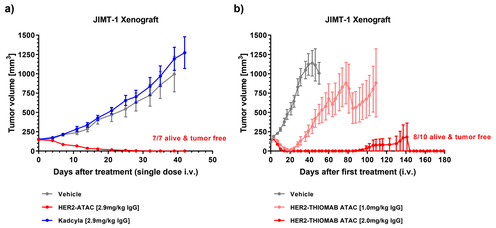

Figure 3: Subcutaneous JIMT-1 xenografts with single dose i.v. application with a) HER2-ATAC with stable linker and Trastuzumab-DM1 as well as b) HER2-THIOMAB-ATAC with cleavable linker. Shown is mean tumor volume ± SEM.

TNBC HER2low Kadcyla-resistant PDX s.c. models

Anti-HER2-THIOMAB-ATAC with cleavable linker showed dose-dependent tumor remission after single dose application of 2.0mg/kg in heterogeneous Kadcyla-resistant HER2low PDX models, which were classified as TNBC. (Figures 4a+b). The efficacy of HER2-THIOMAB-ATACs was more pronounced in PDX models with hemizygous loss of TP53 and POLR2A reflecting a 17p deletion.

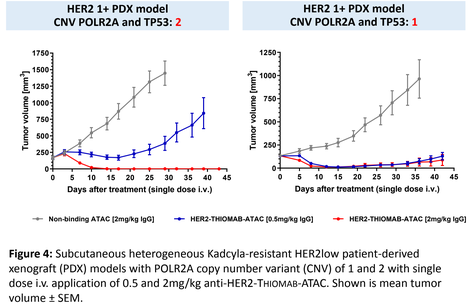

Figure 4: Subcutaneous heterogeneous Kadcyla-resistant HER2low patient-derived xenograft (PDX) models with POLR2A copy number variant (CNV) of 1 and 2 with single dose i.v. application of 0.5 and 2mg/kg anti-HER2-THIOMAB-ATAC. Shown is mean tumor volume ± SEM.

4. Non-human primate tolerability study (off-target tox)

Non-targeting THIOMAB-ATAC 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 and repeated dosing of 3.0mg/kg to the same animals. Biochemical and hematological blood parameters were evaluated extensively during the study (selected parameter: Figure 5).

The NT-THIOMAB-ATAC was well tolerated even with repeated dosing of 3mg/kg dose levels:

- Transient and mild increase in liver-relevant biochemical parameters after application of 3mg/kg.
- No signs of systemic intolerance
- No sign of kidney damage by serum parameters.
- Food consumption and body weight remained unaffected at doses 0.3 and 1mg/kg and was slightly lowered at dose 3mg/kg

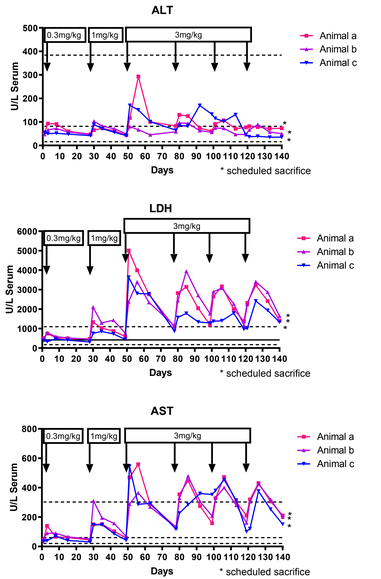

Figure 5: Selected biochemical serum parameters in cynomolgus monkeys treated with escalating doses of non-targeting (NT)-THIOMAB-ATAC. Dashed lines reflect the mean, min. and max. values of untreated animals (predose values).

Conclusion

Breast cancer is the most commonly occurring cancer in women world-wide. It is classified into several subtypes. HER2 is amplified in a variety of breast cancer patients and even other breast cancer subtypes (e.g. TNBC) express low levels of HER2.

In the current study, the mode of action of the payload amanitin led to an efficient anti-tumor potential *in vitro* and *in vivo*. Targeted cytotoxic drug delivery to low, medium and high HER2 expressing cell lines was achieved by using anti-HER2 ATACs. In mouse xenografts anti-HER2 ATACs showed clear anti-tumor activity in Trastuzumab-resistant JIMT-1 model. Heterogeneous Kadcyla-ineffective TNBC PDX models with low levels of HER2 were sensitive to ATAC treatment. Loss of POLR2A/chromosome 17p increased susceptibility to anti-HER2 ATAC making 17p del TNBC a suitable indication for optimized anti-HER2 ATACs.
Safety profiling in cynomolgus monkey revealed a good tolerability and low off-target tox for non-targeting ATAC.

In summary, amanitin as a toxic warhead for ADCs targeting HER2low seems to be a suitable therapeutic option because of the unique mode of action and the molecular characteristics of the toxin. Amanitin is also active in drug resistant cells, independent of the expression of multi-drug resistance transporters. This is a potential advantage for amatoxin payloads in slow proliferating or dormant cancer cells. With POLR2A we identified a possible predictive biomarker for ATAC therapies, which might be used to stratify patients.

References

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