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Liver toxicity of amanitin-based antibody drug conjugates (ATAC®s) is caused by unspecific uptake of the ATAC® into liver cells

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Introduction

Antibody drug conjugates (ADCs) were developed to increase the therapeutic window (TW) of cytotoxins by combining their efficacy with the precision of antibodies. Nevertheless, adverse events (AEs) still limit the use of ADCs in patients. The toxicity profile of an ADC is determined by its on- and off-target effects. On-target toxicity is caused by the specific uptake of the ADC into healthy target-positive cells, while off-target toxicity is caused by target-independent uptake of the ADC, e.g. via the antibody backbone, or by payload release in circulation.

In this study, we unravel the off-target toxicity mechanisms of ADCs that use amatoxins (RNA polymerase II inhibitors) as payload (ATAC®) and use this knowledge to improve the TW of ATAC®s (1). Amatoxins are distinct from other toxins used as payloads of ADC as they are hydrophilic and thus require active transport to pass the cell membrane. The primary transporter of amatoxins in humans is the OATP1B3 transporter, which is exclusively expressed on hepatocytes (2). Thus, unspecific ATAC® toxicity can be caused by free amatoxin, prematurely released in circulation and taken up by liver cells via OATP1B3, or by uptake of the ATAC® via a yet unknown mechanism.

Methods

Synthesis of conjugates: Cysteine-reactive amanitin-linker constructs were synthesized at Heidelberg Pharma and conjugated site-specifically to engineered cysteine residues of monoclonal IgG1 against BCMA (HDP-101), PSMA or digoxigenin (DIG). Drug-Antibody Ratio (DAR) for all conjugates was 2.0 ± 0.1 as determined by LC-MS analysis.

Cell lines: HEKwt and THP-1 cell line were obtained from Cell line service. NCI-H929 and 22Rv1 cell lines were obtained from the American Type Culture Collection and German Collection of Microorganisms and Cell Cultures GmbH, respectively. HEK^{OATP1B3} cell line was generated by Heidelberg Pharma Research GmbH.

Cytotoxicity assay: Quantitative determination of cell viability of adherent cells was performed by BrdU-based chemiluminescent cell proliferation ELISA (Roche). Viability of suspension cells was assessed by CellTiter Glo 2.0 (Promega). FcγR was blocked by adding purified NA/LE human BD Fc Block™ (BD Bioscience) prior the addition of test item dilutions.

Biolayer interferometry (BLI) for Fc/FcγRI affinity measurement: Binding of antibodies to biotinylated human FcγR-I (AcroBiosystem) was measured by using Streptavidin-coated biosensors on the Octet K2 system (Sartorius).

ADC and total antibody detection in serum & tissue: Female CB17-Scid mice or 2-to-4-year-old monkeys were treated with a single dose of ATAC® i.v. and blood samples were taken at different time points. ADC and total antibody levels in serum or tissue were measured by sandwich ELISA.

Determination of amatoxin concentration: Multiple reaction monitoring (MRM)-based targeted metabolomics was used for the quantification of free metabolites in mouse serum or liver tissues after i.v. application of ATAC®.

Intact measurements for DAR species determination: Bead-based affinity capture followed by LC-MS analysis was applied for the measurement of the intact mass of the ATAC® for the determination of DAR in mouse serum or liver tissue after i.v. application of ATAC®.

Tolerability studies: In all *in vivo* tolerability studies, three female or male mice were given a single dose of the test item in PBS on day 0 i.v. The maximal tolerated dose (MTD) was determined by dose escalation of the test item until toxicity was reached.

Immunofluorescence: Tumors were snap frozen and embedded in OCT. 7µm sections were fixed with 4% formalin, permeabilized with 0.1% saponin, blocked with 5% goat serum and 1% BSA and stained with anti-human IgG-AlexaFluor647 and DAPI.

Anti-tumor efficacy studies: 5x10^6 22Rv1 cells were inoculated s.c. in NMRI-Nude mice. When tumor volumes reached a mean of ~150 mm^3, mice were treated with an i.v. dose of a PSMA ATAC®.

Results

Table 1: Similar findings in patients after amanitin intoxication and cynomolgus monkeys treated with ATAC®s

Micro- & macroscopic changes in liver and kidney are similar between patients after amanitin intoxication and cynomolgus monkeys treated with ATAC®s. These findings underline that ATAC®-mediated liver toxicity is eventually driven by amanitin.

Findings in patients after intoxication with amanitin:	Observed after ATAC® treatment in cynomolgus monkeys?
Liver	
Yellow; of creamy consistency	
Hemorrhagic foci	✓
Edematous hepatic stroma	✓
Centrilobular hepatic necrosis	✓
Vacuolar degeneration of hepatocytes	✓
Kidney	
Multiple hemorrhages	✓
Necrosis of convoluted tubules	✓
Hyaline tubular casts	✓

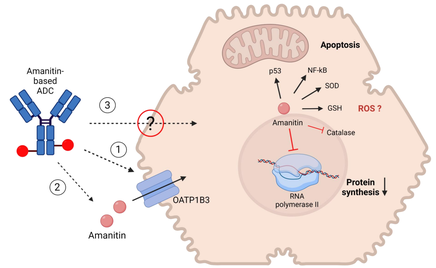
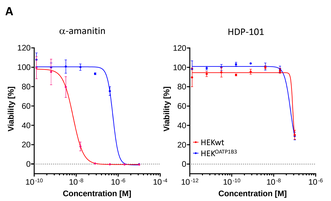


Fig. 1: Potential uptake mechanism of ATAC®s

ATAC®-mediated liver toxicity is driven by amanitin. The inhibition of RNA polymerase II by Amatoxins causes reduced protein synthesis as well as elevated ROS levels leading to enhanced apoptosis of hepatocytes. However, it is unclear whether free amatoxin or intact ATAC® enters hepatocytes. Potential uptake mechanism of ATAC®s include the uptake of ATAC®s via OATP1B3 transporter **(1)**, the release of free payload in circulation **(2)** or the unspecific uptake of ATAC®s via yet unknown mechanisms **(3)**.

1. Off-target toxicity is not mediated by uptake of the ATAC® via the OATP1B3 transporter.



B

Test item	MTD [mg/kg]	
	In wild type mice	In *Oatp1a/b* KO mice
α-amanitin	0.3	0.5
active metabolite	1.25	≥2.5
HDP-101	6	6

Fig. 2: ATAC®s are not a substrate of OATP1B3 transporter

Cytotoxicity of α-amanitin (left) and HDP-101 (right) on HEKwt (red) or OATP1B3 overexpressing cells (blue) was assessed **(A)**. While α-amanitin is more cytotoxic on OATP1B3 overexpressing cells, HDP-101 shows no difference in cytotoxicity on both cell lines. Maximal tolerate dose (MTD) of α-amanitin, HDP-101 and the active metabolite of HDP-101 was determined in FVB wildtype or *Oatp1a/b* knockout (KO) mice after a single i.v. dose of the test items **(B)**. Mice were observed for up to 14 days after dosing. The MTD of α-amanitin and the active metabolite are increased in *Oatp1a/b KO* mice as compared to wild type mice. In contrast, the MTD of HDP-101 remained unchanged in mice lacking the *Oatp1a/b* gene cluster as compared to wild type mice.

2. Off-target toxicity is not mediated by the release of amatoxins in circulation

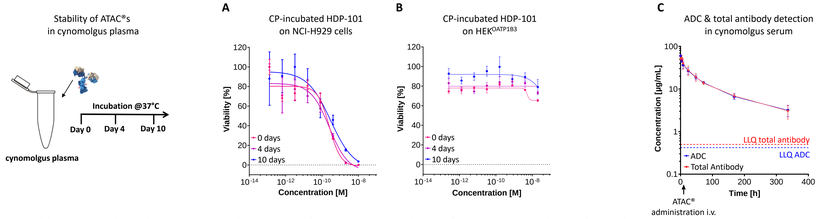


Fig. 3: ATAC®s are stable in cynomolgus plasma *in vitro* and no release of amatoxins in circulation of NHPs treated with ATAC® was observed

HDP-101 was incubated in cynomolgus-plasma (CP) at 37°C for 0 to 10 days and cytotoxicity was assessed on target positive NCI-H929 **(A)** or OATP1B3 expressing HEK^{OATP1B3} cells **(B)**. HDP-101 retained its cytotoxicity on target positive cells while no toxicity was observed on HEK^{OATP1B3} cells.
Cynomolgus monkeys were treated i.v. with 1 mg/kg HDP-101 and total antibody and ADC levels in serum were analyzed by sandwich ELISA **(C)**. Half-life of ADC and total antibody in cynomolgus monkey serum up to 21 days after dosing with HDP-101 was similar. Means and SD of 10 animals/time point are shown.

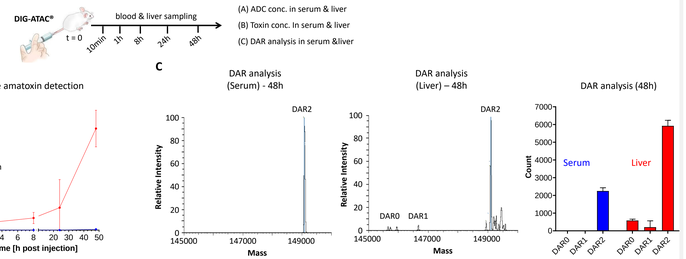


Fig. 4: ATAC®s are stable in murine serum and amanitin is released in liver cells only

Mice were injected i.v. with a single dose of an ATAC® and bioanalytical analysis were performed at different time points up to 48h. ADC concentration was analyzed by ELISA **(A)** while free amatoxin **(B)** was measured by MRM-based targeted metabolomics in serum (blue) or perfused livers (red). ATAC® concentration in liver tissue increased at the same time point as the concentration in serum dropped, indicating uptake of the ATAC® into liver cells. Almost no amatoxin was detectable in serum at any time point and it accumulated in the liver (red) with a constant increase rate. Means of three animals/group/time point are shown. DAR analysis of serum and liver tissue (perfused) of mice was performed by intact LC-MS measurement **(C)**. At any time point, solely DAR2 species were detected in serum. In contrast, DAR0 and DAR1 species were detected in liver tissue 48h post injection, indicating payload release inside of liver cells.

3. Liver toxicity of ATAC®s is at least partially caused by FcγR-mediated uptake into liver cells

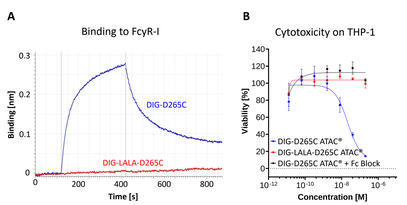


Fig. 5: ATAC®s interact with FcγR

The binding affinity of an anti-DIG antibody to FcγR-I was analyzed by BLI **(A)**. While DIG-D265C antibody binds to FcγR-I with a KD of 30mM, its binding was abolished by silencing mutations (LALA) in the Fc backbone. Cytotoxicity based on FcγR-mediated endocytosis was assessed for DIG ATAC®s on FcγR-expressing THP-1 cells **(B)**. The DIG-D265C ATAC® leads to a significant cytotoxicity on THP-1 cells, which was not observed with DIG-LALA-D265C ATAC®. Furthermore, addition of a FcγR blocking agent eliminates the cytotoxicity of the DIG-D265C ATAC®, demonstrating that the cytotoxicity is caused by FcγR-mediated uptake of the ATAC®.

A

species	MTD [mg/kg]	
	D265C	LALA-D265C
mouse	40	70
cyno	3	7.5

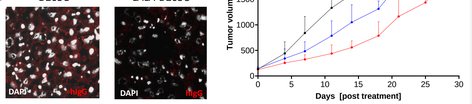


Fig. 6: Fc silencing of ATAC® improves tolerability while maintaining efficacy

MTD of a PSMA-LALA-D265C ATAC® (silenced) or PSMA-D265C ATAC® (non-silenced) was tested in mice and cynomolgus monkey **(A)**. The PSMA-LALA-D265C ATAC® was better tolerated than the PSMA-D265C ATAC® in both species. IF liver image of mice injected i.v. either with an PSMA-D265C ATAC® with or without LALA mutation **(B)**. While a strong staining was detected in livers of mice treated with the PSMA-D265C ATAC®, the signal was significantly reduced in livers of mice treated with the anti-PSMA-LALA-D265C ATAC®. Efficacy of a PSMA-D265C ATAC® with or without LALA mutations was investigated in a hard-to-treat s.c. 22Rv1 prostate cancer CDX model **(C)**. PSMA-D265C and PSMA-LALA-D265C ATAC® showed a similar anti-tumor efficacy. Mean tumor volume and SD is shown (n= 8 or 9).

Conclusion

Our data show how the off-target liver toxicity of ATAC®s is driven by the unspecific uptake of the intact ADC into liver cells and causes liver damage independent of the free payload.
Although the toxicity profiles of free amatoxins and ATAC®s are highly similar and their toxic effects are paradoxically identical, we demonstrated that the uptake via the OATP1B3 transporter into hepatocytes, as described for amatoxins as free toxins (2), does not play a role in the liver toxicity of ATAC®s. Further, upon the high stability of ATAC®s in circulation, no premature release of amatoxins was observed.
Instead, we could show that intact ATAC®s are taken up into liver cells via FcγR upon target-independent binding to the Fc-part of the antibody. The elimination of FcγR binding by insertion of the LALA mutation into the Fc backbone of the antibody increased the tolerability of ATAC®s, while the anti-tumor efficacy remained unchanged eventually leading to a truly broadened TW. Since additional uptake mechanisms known to cause liver toxicity of other drugs (3-4) could also play a role for ATAC® mediated liver toxicity, further work is needed to elucidate the impact of uptake e.g. via macro- and micropinocytosis or via other receptors such as C-type lectin receptors (e.g. Mannose receptor).

Key findings

- ATAC®-mediated off-target toxicity is driven by amatoxins
- ATAC®s are stable in circulation and free amatoxin is released only in liver cells
- Liver toxicity of ATAC®s is determined by the amount of ATAC® that is taken up by liver cells
- ATAC®s interact with FcγR leading to receptor-mediated endocytosis
- Fc silencing of ATAC®s improves tolerability while maintaining efficacy

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