In vivo detoxification of Amatoxin-based ADCs (ATACs®) by "Click" chemistry

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Introduction

Amatoxin-based antibody-drug conjugates (ATAC®s) are a promising approach for targeted cancer therapy developed by Heidelberg Pharma that uses amanitin as a toxic payload. Amanitin efficiently inhibits cellular transcription by binding to RNA pol II.[1] Despite showing promising results *in vitro* and *in vivo*, liver toxicity, due to unspecific uptake of the ATAC® and/or Amatoxin, remains as a potential dose-limiting factor of this therapy. Off-target toxicity represents in general a significant obstacle in ADCs treatment, and extensive research has demonstrated that target-independent uptake by healthy cells contributes to this issue[2]. In a recent study, a new way to control and modulate post-treatment drug effects was implemented with the use of Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC) reaction directly *in vivo*[3]. In this study, SPAAC was used as a tool to minimize toxin-related adverse effects and increase the therapeutic index (**Figure 1**).

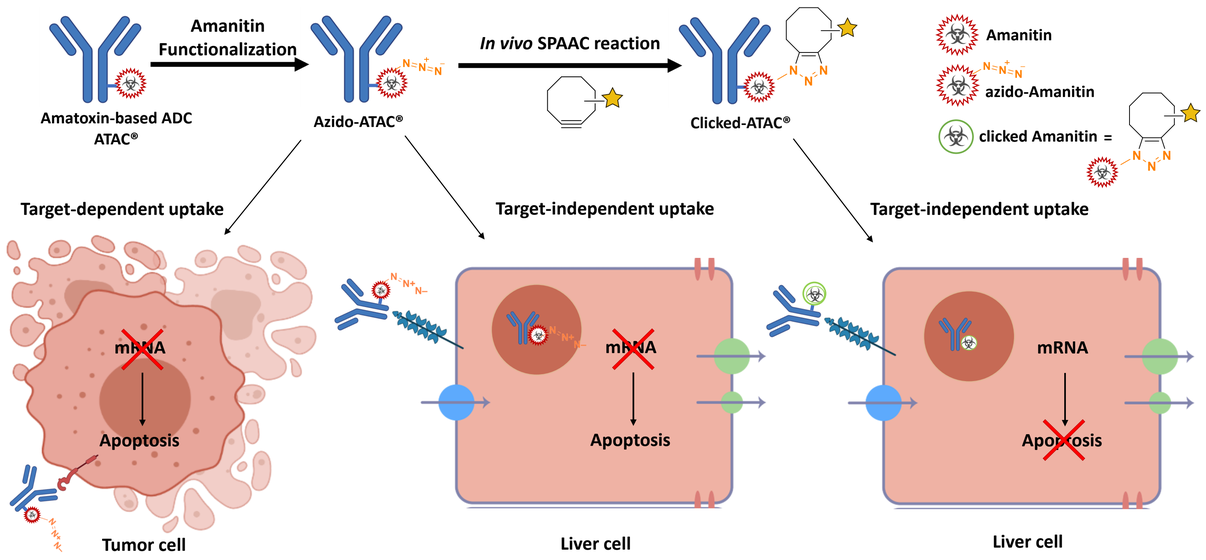

Figure 1. Schematic illustration of the neutralization strategy. Images were created in Biorender.com

Results

Bioactivity of azido-Amanitin and clicked derivatives

The bioactivity of a new azido-Amanitin derivative and its clicked forms with two neutralizing agents containing different alkyne moieties was evaluated *in vitro* (**Figure 2A**). First, the RNA pol II activity and affinity for the amanitin specific organic anion transporter (OATP1B3) was determined. Our findings showed that the bioactivity of the azido-Amanitin **(2)** was comparable to that of natural amanitin **(1).** Compound **2** retained inhibition of RNA pol II activity (**Figure 2B**) and retained affinity for the OATP1B3 transporter, which resulted in cytotoxicity on an OATP1B3+ overexpressing HEK293 cell line (**Figure 2C**). However, when Compound **2** was clicked with either of the two neutralizing agents, a significant retention in RNA pol II activity and HEK293 OATP1B3+ cell viability was observed (**Figure 2A-B**). These results highlight that the click reaction affects the ability of the clicked toxin to bind the RNA pol II enzyme and in turn its cytotoxic activity.

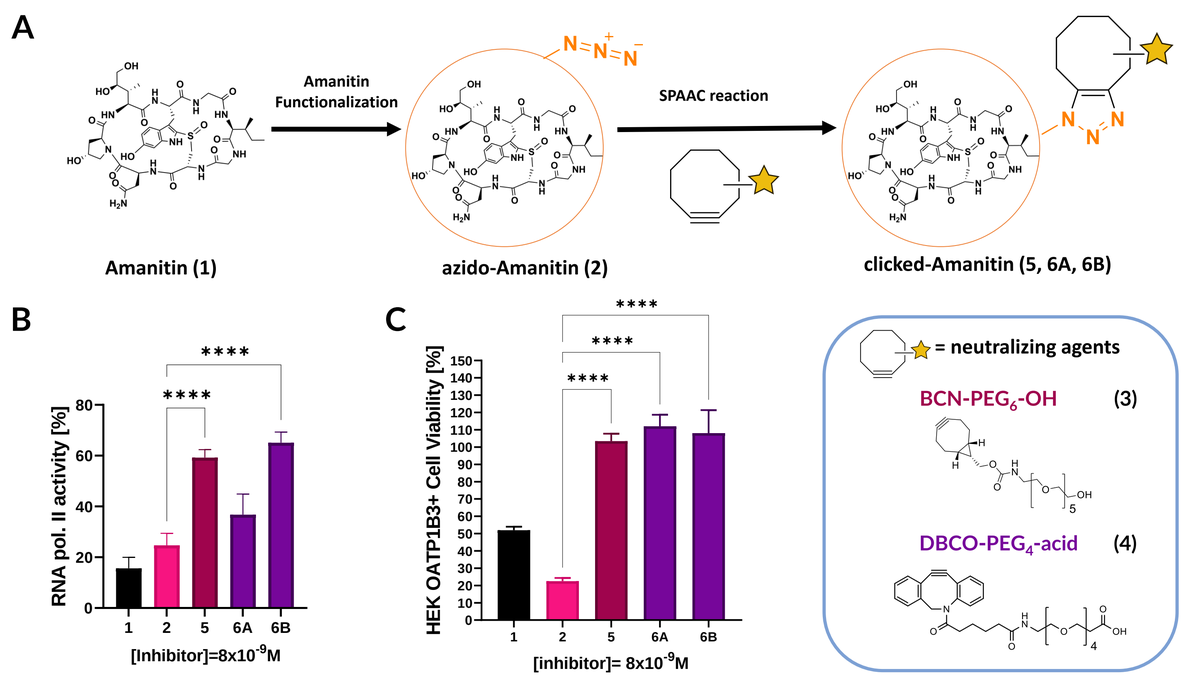

Figure 2. Synthesis and bioactivity of amanitin derivatives (**A**) Amanitin (**1**) was functionalized with an azide moiety to azido-Amanitin (**2**). Reaction of compound **2** with compound **3** in DMF at RT for 4h led to compound **5**. Reaction of compound **2** with **4** in DMF at RT for 4h led to purification of two separated isomers, **6A** and **6B**. Bioactivity of the clicked products was assessed *in vitro* in an RNA Pol II Assay (**B**) and in a cytotoxicity assay on OATP1B3 overexpressing HEK cells (**C**). (**B**) Compounds were incubated in a HelaScribe® Nuclear extract with run-off transcripts template from CMV (Promega, #E3092). Reverse transcription was followed by real time-PCR for the quantification of the mRNA product. RNA detection was performed using a QuantiFast Probe RT-PCR Plus Kit (Qiagen). (**C**) HEK 293 cells that overexpress the OATP1B3 transporter were incubated for 96h with the compounds. Cytotoxicity was detected via BrdU incorporation assay.

Tolerability of azido-ATAC in combination with the neutralizing agents

Azido-Amanitin (**2**) was functionalized with a cleavable linker and linked via site-specific bromo-acetamide conjugation to engineered cysteine residues of a Trastuzumab-based antibody (THIOMAB ® approach†). Following conjugation, the maximum tolerated dose (MTD) was determined in NMRI nude mice to be 20 mg/kg. In a next step, the ability of the neutralizing agents to increase the *in vivo* tolerability of the azido-ATAC was evaluated. Mice were first treated with a lethal dose of Azido-ATAC (30 mg/kg = 1.5-fold MTD) and 12h later with a single dose of the neutralizing agents (**Figure 4**). Remarkably, the groups that were treated with both the ATAC and the neutralizing agents survived up to 14 days (termination date) whereas mice treated with ATAC only (vehicle control) died after 3 days. This demonstrated the ability of the neutralizing agents to improve tolerability by reducing the treatment-related toxicity.

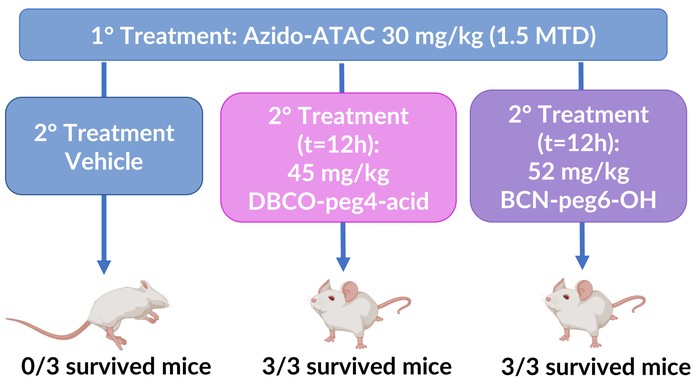

Figure 4. Illustration of the tolerability study conducted in NMRI nude female mice (n=3 per group). Compounds were administered i.v. Mice were monitored for 14 days after treatment (planned termination date). Animals that were found in poor conditions were sacrificed prior the planned termination date. Images were created in Biorender.

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Results

Efficiency of click reaction *in vivo*

To compare the efficiency of the click reaction of the two neutralizing agents *in vivo*, the plasma concentration of azido-ATAC and total ATAC (azido-ATAC + clicked ATAC) at various timepoints was monitored. Two different ELISAs were developed, one of which detecting only ADCs with an unclicked azide moiety (azido-ATAC) and the other detecting ADCs with both, clicked and unclicked azide moiety (total ATAC). Mice were treated with azido-ATAC followed by one of the neutralizing agents after one hour. At 15 minutes, the group treated with BCN-PEG_6-OH showed a two-fold decrease of unclicked Azido-ATAC concentration compared to the control group, while an eight-fold decrease was seen in the group treated with DBCO-PEG_4-acid (**Figure 5A**). On the other hand, the concentration of total ATAC remained constant for each group at all timepoints, indicating the presence of both clicked ATAC and unclicked ATAC in circulation (**Figure 5B**). These results suggested that the click reaction with DBCO-PEG_4-acid was more efficient with 90% of the azido-ATAC being already clicked after 15 minutes. In contrast, with BCN-PEG_6-OH, the percentage of clicked ATAC remained around 40-60% even 60 minutes after administration of the neutralizing agent (**Figure 5C**).

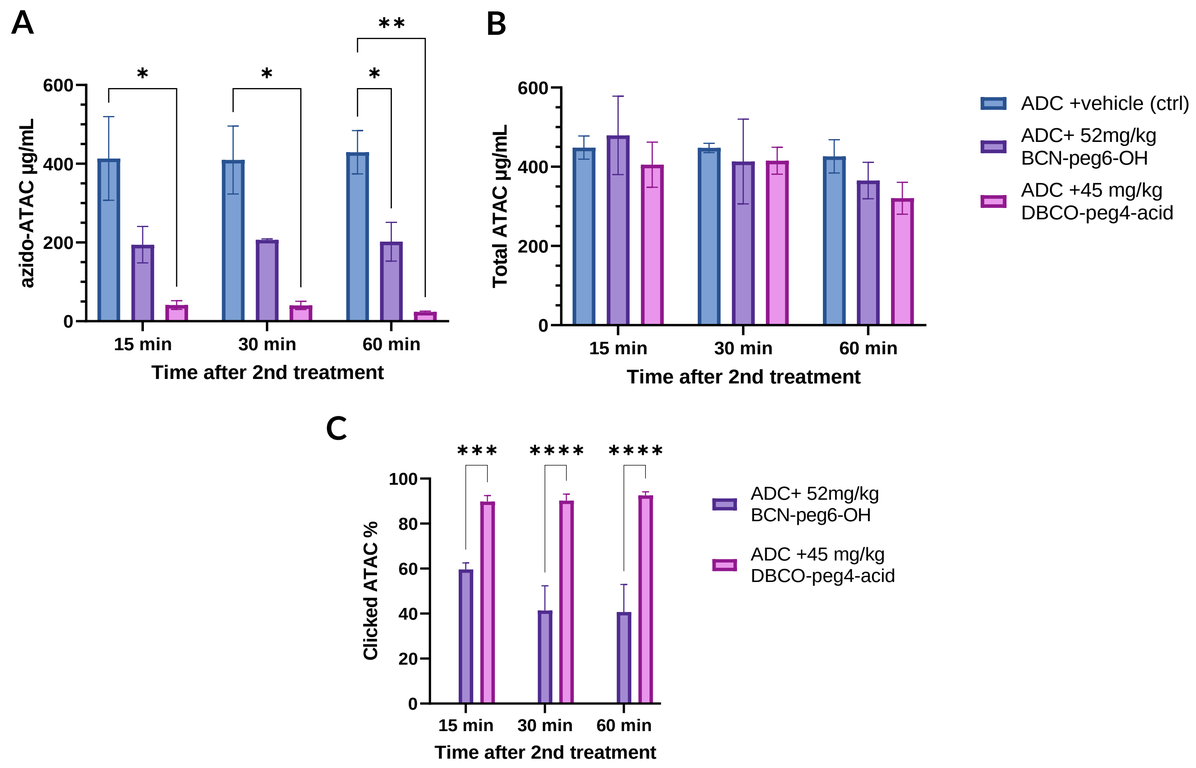

Figure 5. Click reaction efficiency study in mice. NMRI nude female mice (n=3 per group) were treated i.v. with 30 mg/kg azido-ATAC (1.5x MTD) and 1h later with either 52 mg/kg BCN-PEG_6-OH or 45 mg/kg DBCO-PEG_4-acid. Plasma was collected 15, 30 and 60 minutes after treatment with the neutralizing agents. Samples were analysed via sandwich ELISA: (**A**) Unclicked azido-ATAC was detected using streptavidin coated plates incubated with DBCO-peg4-biotin. (**B**) Total ATAC was detected using plates coated with an anti-amanitin polyclonal antibody. (**C**) Concentration of Azido-ATAC was divided by concentration of Total ATAC and values were normalized.

Efficacy of azido-ATAC in a JIMT-1 xenograft model

The efficacy of the Azido-ATAC was tested *in vivo* in a JIMT-1 xenograft mouse model. All groups treated with a single dose of Azido-ATAC at three different doses showed complete and persistent tumor remission, with mice remaining tumor-free for up to 95 days post-treatment.(**Figure 3A**). Differences in body weight among groups were in correlation with the increase in ATAC dosage (**Figure 3B**). Survival of Azido-ATAC treated animals greatly outperformed the PBS control group (**Figure 3C**). These results confirmed the activity of the Azido-ATAC with azido-Amanitin (**2**) as payload.

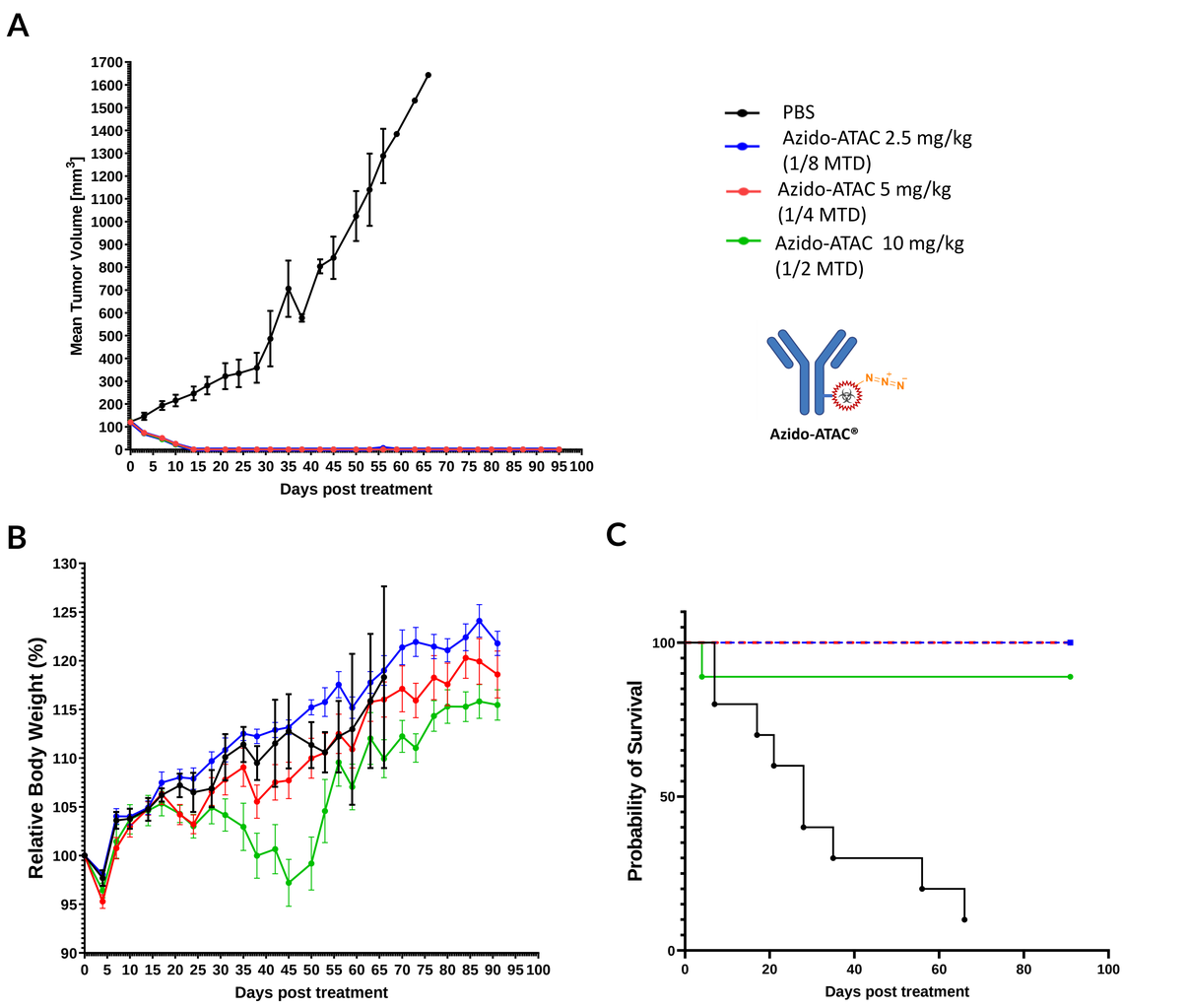

Figure 3. Efficacy study in NMRI nude female mice xenografted with JIMT-1 tumors. Mice were allocated to groups at tumor volume of 80-150 mm^3 and treated with different single *i.v.* doses of Azido-ATAC (**A**) Mean tumor volume (mm^3) . (**B**) Relative body weight of animals (%). (**C**) Probability of survival.

Conclusions

Although antibody-drug conjugates (ADCs) offer high selectivity and specificity for delivering toxic payloads to tumor cells, the risk of on- and off-target toxicity remains a major concern. Through the selective attachment of neutralizing agents containing an alkyne moiety to the Amanitin payload of an ATAC via click chemistry, tolerability was significantly increased and ATAC anti-tumor efficacy was retained. By using this de-toxification strategy, the risk of toxic effects due to the payload of an ADC can be greatly reduced. This facilitates higher and more frequent dosing broadening the therapeutic window. Overall, our study highlights the potential of click chemistry as a versatile tool for modulating the toxicity of ADCs, providing new opportunities for optimized cancer treatments and improved patient outcomes.

References

Pahl A, et al. *Drug Discov Today: Technol* **2018; 2.** Mahalingaiah et al. *Pharmacol. Ther.* **2019**, 86:41-55**; 3.** Ursuegui S, et al. *Nat Commun* **2017,** 8:15242**; 4.**Porte K, et al. *ChemBioChem* **2021**, *22*, 100-113