MULTILINK™, A NEW LINKER TECHNOLOGY FOR INCREASING DRUG-TO-ANTIBODY RATIO (DAR)

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SUMMARY

Antibody Drug Conjugates (ADCs) enable the targeted delivery of cytotoxic drugs to tumor cells. Ideally, ADCs should retain the favorable pharmacokinetic and functional properties of antibodies, remain intact and nontoxic in systemic circulation, and become active at the target site with sufficient drug released to kill the target cell. A major challenge in the development of ADCs represents the design of the linker.

Multilink™ is a new linker which is selectively recognized and cleaved by the carboxydipeptidase activity of Cathepsin B[1,2]. This new linker system allows highly efficient and selective drug release. It is also stable in plasma and it enables the preparation of ADCs with high drug-to-antibody ratio (DAR).

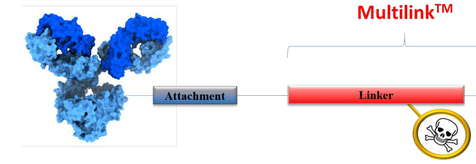
INTRODUCTION

MULTILINK™ IS A PEPTIDIC LINKER

- SPECIFICALLY RECOGNIZED AND CLEAVED BY CARBOXYDIPEPTIDASE ACTIVITY OF CATHEPSIN B
- WELL SUITED FOR MULTIPLE DRUG CONJUGATION

Known technology

Cathepsin B cleavage by endopeptidase activity



Multilink™ technology

Cathepsin B cleavage by carboxydipeptidase activity

METHODS

All in vivo studies were conducted according to the German animal welfare law and the GV-SOLAS guidelines.

ADC Preparation: The linkers were prepared by standard solid-phase peptide synthesis, including on-resin peptide coupling and convergent strategies. The drugs were reacted in solution on the peptides after their cleavage from the resin. The resulting drug-linkers were then conjugated to the monoclonal antibody Trastuzumab using the classic cysteine conjugation resulting from reduction of interchain disulfide bonds.

Release kinetics by Cathepsin B-induced cleavage: The drug release kinetic was evaluated according to the *in vitro* enzymatic cleavage using recombinant human Cathepsin B (BioTechne AG, #953-CY-010). The release kinetics were normalized with cleavage of Cys-MC-Val-Cit-PABC-MMAF (IBIOsource, #S10001) that serves as reference.

Plasma stability: The in vitro plasma stability assay was performed at 37° C in mouse and human plasma over 24h. The integrity of the ADC was controlled by immunoassay using sandwich ELISA (Epitope Diagnostics, #KTR-783).

Binding assay: Binding activities Trastuzumab or ADCs are compared by indirect flow cytometry using a rat anti-human IgG FcmAb (BioLegend, #410706) on SK-BR3 (Her2+) and MDA-MB-231 (Her2 neg) cells.

In vitro cytotoxic activity (performed in different CROs): All assays were performed in triplicate. After overnight resting, serial dilution of drugs were added to the cells. After 72 hours of incubation, either alamarBlue or CellTiterGlo (Promega kit) were added to each well and the plates were incubated for the appropriate amount of time before reading of luminescence. IC50 were calculated using GraphPad Prism.

Mouse xenograft model: (ProQinase) Briefly, 5x10^6 SCOV3 tumor cells in 50% Matrigel were implanted subcutaneously (sc) into the flank of female NMRI nude mice. Animals were randomly assigned to treatment groups when tumors reached approximately 130 mm^3.

Hollow Fiber model: (ProQinase) Briefly, on day 0, Hollow Fibers containing SK-BR3 cells were implanted sc into female NMRI nude mice. Treatment started on day 1. On day 15, animals were euthanized, implanted Hollow Fibers were collected, appropriately processed and a CellTiter Glo® assay for assessing cell viability performed.

RESULTS (1 PAYLOAD PER LINKER)

ADC'S STRUCTURES

mAb	Attachment	Release kinetics ratio (Cathepsin B cleavage)	Payload	DAR	Name
Trastuzumab	Thiol-Maleimide	0.42	Auristatin F	4.2	ADC1
Trastuzumab	Thiol-Maleimide	17.5	DM1	4.4	ADC3
Trastuzumab	Thiol-Maleimide	0.58	DM1	3.5	ADC7

Table 1: Various constructs of ADCs evaluated. The release kinetics ratio indicates the ADCs propensity to be cleaved by Cathepsin B. It corresponds to the ratio between the half life of the ADC over the half life of the reference in the Cathepsin B assay. The DAR was measured by Reversed Phase using an UPLC system.

ADCs ARE STABLE IN PLASMA AND BINDING AFFINITY IS NOT AFFECTED

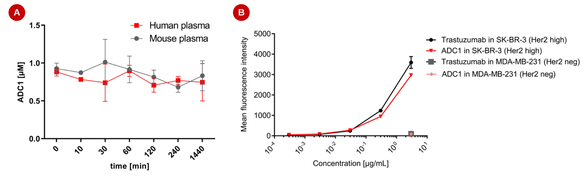


Figure 1: A) Plasma stability of ADC1 tested in mouse and human plasma over 24h. **B) Binding assay** of ADC1 on SK-BR3 cells (breast, Her2 high) and MDA-MB-231 cells (breast, Her2 negative)

ADCs WITH MULTILINK™ ARE ACTIVE IN VITRO

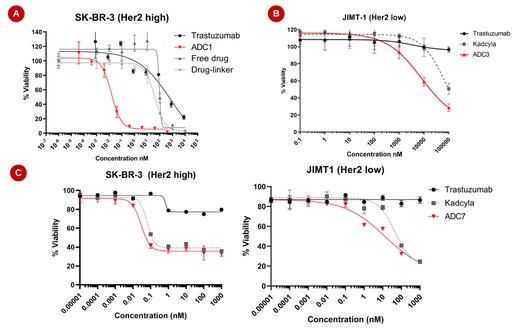


Figure 2: Cytotoxic activity of ADCs in vitro. A) SK-BR3 (breast, Her2 high) cells were incubated with different concentrations of Trastuzumab, ADC1, Auristatin F (free drug) and Auristatin F-Multilink™ (Drug-Linker) respectively. **B)** JIMT1 (breast, Her2 low) cells were incubated with different concentrations of Trastuzumab, Kadcyla and ADC3 respectively. **C)** SK-BR3 and JIMT1 cells were incubated with different concentrations of Trastuzumab, Kadcyla and ADC7 respectively.

ADCs WITH MULTILINK™ ARE WELL TOLERATED AND EFFICIENT IN VIVO

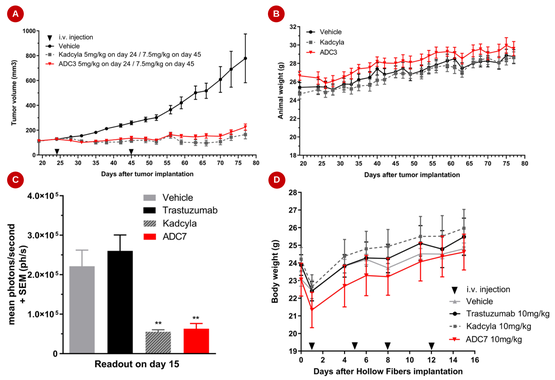
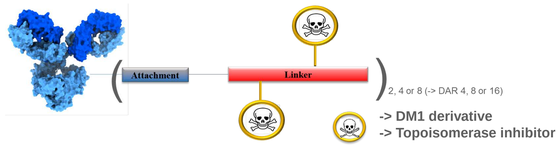


Figure 3: In vivo efficacy models. A) Tumor volumes and **B) Body weight** of SKOV3 xenograft model (breast, Her2 high) were recorded over 78 days. Values shown are mean tumor volumes +/-SEM, N=10 animals per group. On day 24 after tumor implantation, 5mg/kg of Kadcyla or ADC3 were injected i.v., 5ml/kg of PBS for the vehicle group. On day 45, a second i.v. injection of 7.5mg/kg was performed. **C) Mean luminescence** on day 15 and **D) Body weight** over 15 days of Hollow Fiber model with SK-BR3 cells (breast, Her2 high). Values shown are mean tumor volumes +/-SEM, N=5 animals per group. Animals were treated 2x weekly for 2 weeks with 10mg/kg of Trastuzumab, Kadcyla or ADC7. The animals in the vehicle group received 5ml/kg of PBS.

RESULTS (2 PAYLOADS PER LINKER)



DRUG RELEASE KINETICS

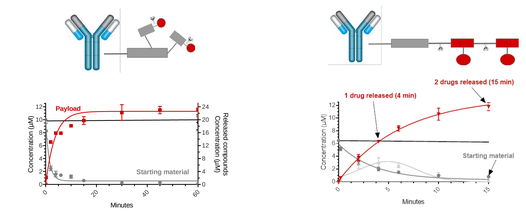


Figure 4: Release kinetics by cathepsin B cleavage on two types of linkers (left: simultaneous cleavage, right: sequential cleavage)

HIGH DAR ADCs CHARACTERIZATION

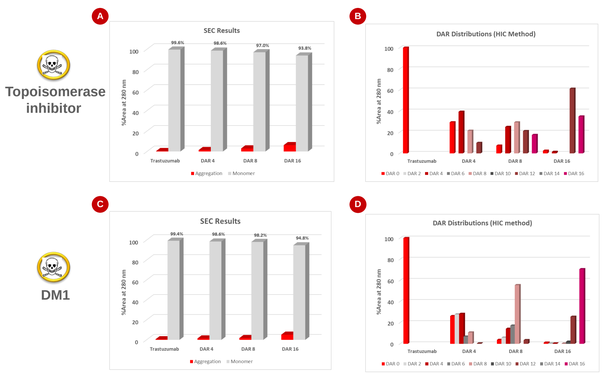


Figure 5: A) and **C) Size Exclusion Chromatography** of Trastuzumab and ADCs-Multilink™-2 payloads with respectively theoretical DAR 4, 8 and 16. **B)** and **D) DAR distribution** determined by Hydrophobic Interaction Chromatography for Trastuzumab and ADCs-Multilink™-2 payloads with respectively theoretical DAR 4, 8 and 16. Top graphs: ADCs with Multilink™-2 topoisomerase inhibitor. Bottom Graphs: ADCs with Multilink™-2 DM1.

HIGH DAR ADCs WITH MULTILINK™ ARE ACTIVE IN VITRO

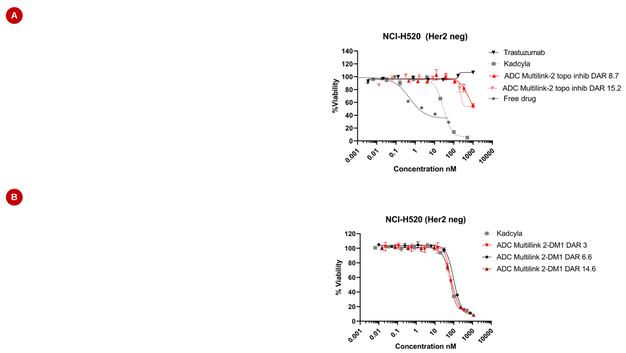
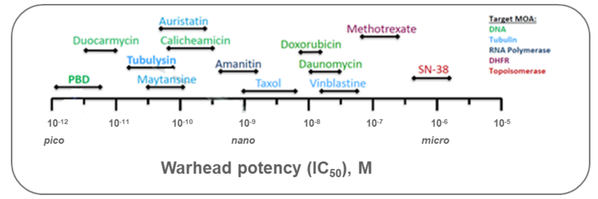


Figure 6: Cytotoxic activity of High DAR Multilink™ ADCs. A) ADC Multilink™ containing topoisomerase inhibitor as payload (topo inhib) with respective DAR of 8.7 and 15.2, Trastuzumab, Kadcyla and the free drug were tested in SK-BR3 (breast, Her2 high), JIMT-1 (breast, Her2 low) and NCI-H520 (lung, Her2 neg) cells at different concentrations. **B)** ADC Multilink™ containing DM1 as payload with respective DAR 3, 6.6 and 14.6, and Kadcyla were tested in SK-BR3, JIMT-1 and NCI-H520 cells at different concentrations.

CONCLUSION

The Multilink™ system allows the attachment of several payloads per linker. It thus enables the preparation of ADCs with high drug-to-antibody ratio (DAR) which, in the end increases the amount of drug that can be delivered to cancer cells.

The Multilink™ technology has a potential for well tolerated payloads of medium cytotoxicity that need to be conjugated with a high DAR.



Multilink™ for increasing DAR

Site-specific technologies for decreasing DAR

NEXT STEPS

- Evaluate the Multilink™-ADCs with 2 payloads per linker *in vivo*
- Evaluate other conjugation technologies
- Evaluate ADCs with combined payloads

Figure 7 : Release kinetics by cathepsin B cleavage on linkers with combined payloads

REFERENCES

(1) M. Renko et al., FEBS J., 277 (2010) 4338-4345

(2) V. Turk et al. Biochim. Biophys. Acta (2012) 1824, 68-88.

ACKNOWLEDGEMENTS

We thank all collaborators from ABZENA that are involved in this project for their helpful discussions and their work.

FEATURES OF MULTILINK™

Multilink™ is a new linker-drug-conjugate which is selectively recognized and cleaved by the carboxypeptidase activity of Cathepsin B. This technology provides significant advantages for next ADCs development.

1. **Highly efficient and selective drug release**
2. **Tunable drug release kinetics**
3. **Formation of high DAR ADCs**
4. **Suitable for combination of different payloads**
5. **Compatible with various mAbs and payloads**
6. **Compatible with various conjugation technologies**

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