Analytical Challenges of Antibody-Drug Conjugates (ADCs):

"Small Meets Large - ADCs as Example that Size Matters in Bioanalysis“

Introduction to ADC Bioanalysis

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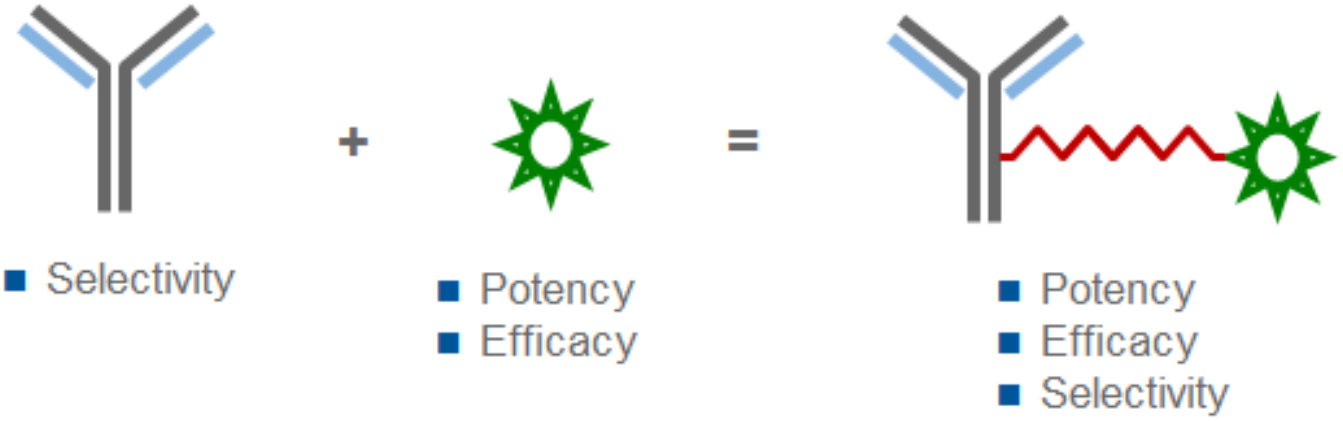
Bernhard Beckermann

Introduction to ADC Bioanalysis
Overview on Presentation

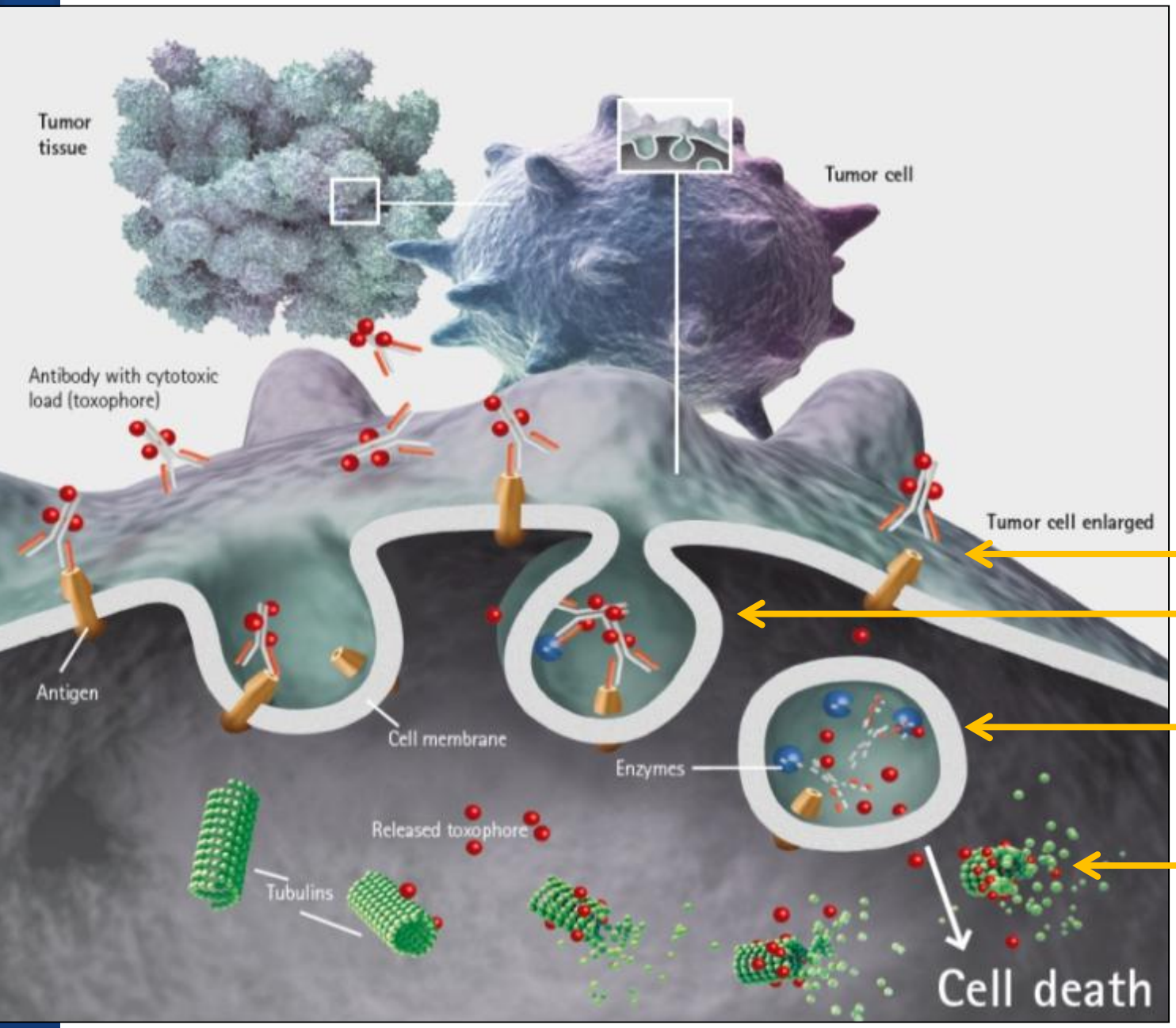
- ADC: Definition, Structure, Mode of Action
- Type of ADCs (Chemistry of Toxophors and Conjug., Biochemistry of Ab)
- Role of Catabolism, Metabolism and PK
- What are the Relevant Analytes for PK
- Criteria for Bioanalytical Strategy
- Assay Types and their Limitations
- Conclusions

How does an ADC work?



ADCs - putting a "warhead" on a "guided missile"

How does an ADC work?



Antigen binding

Internalization

Degradation

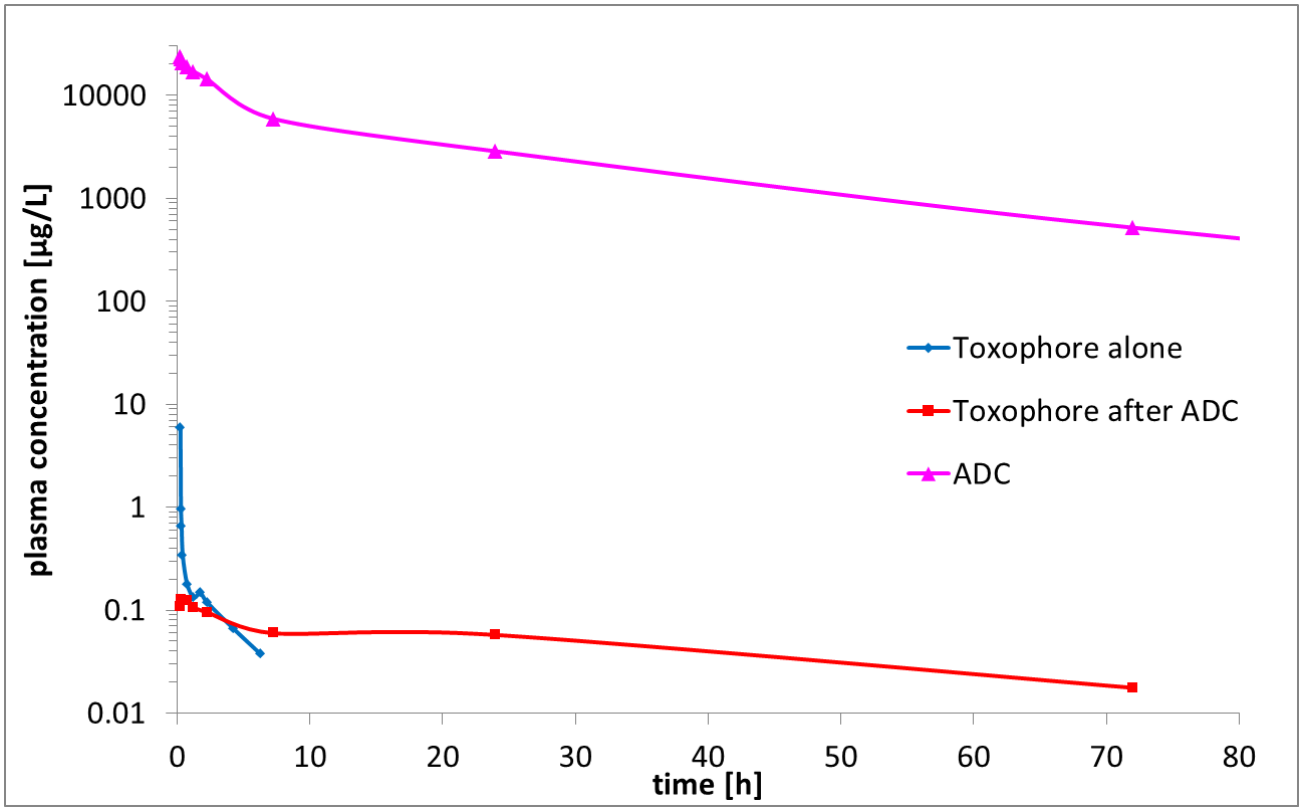
“Toxin action”

ADC: Definition, Scope, Mode of Action

- ADCs are pro-drugs with drugs covalently bound to MoAb‘s
- The antibody serves to deliver the drug to the intended site of action by binding to antigens at the site of action (e.g. tumor, targeting approach)
- The binding of the antibody has to be very specific (also off-target binding)
- The drug is slowly to be released in the target cells (also in off-target cells)
- If the drug is a cytotoxic drug for cancer treatment, it‘s called toxophor (the total drug load is often called „Payload“)
- The total amount of conjugated drug (payload) is limited (<2% of Ab)
- Therefore, the toxophor has to show high potency (=> ng/L)
- **D**rug to **A**ntibody **R**atio („DAR“) > 6 critical for antibody stability

Example: An ADC is a Slow Release Prodrug of the Toxophore

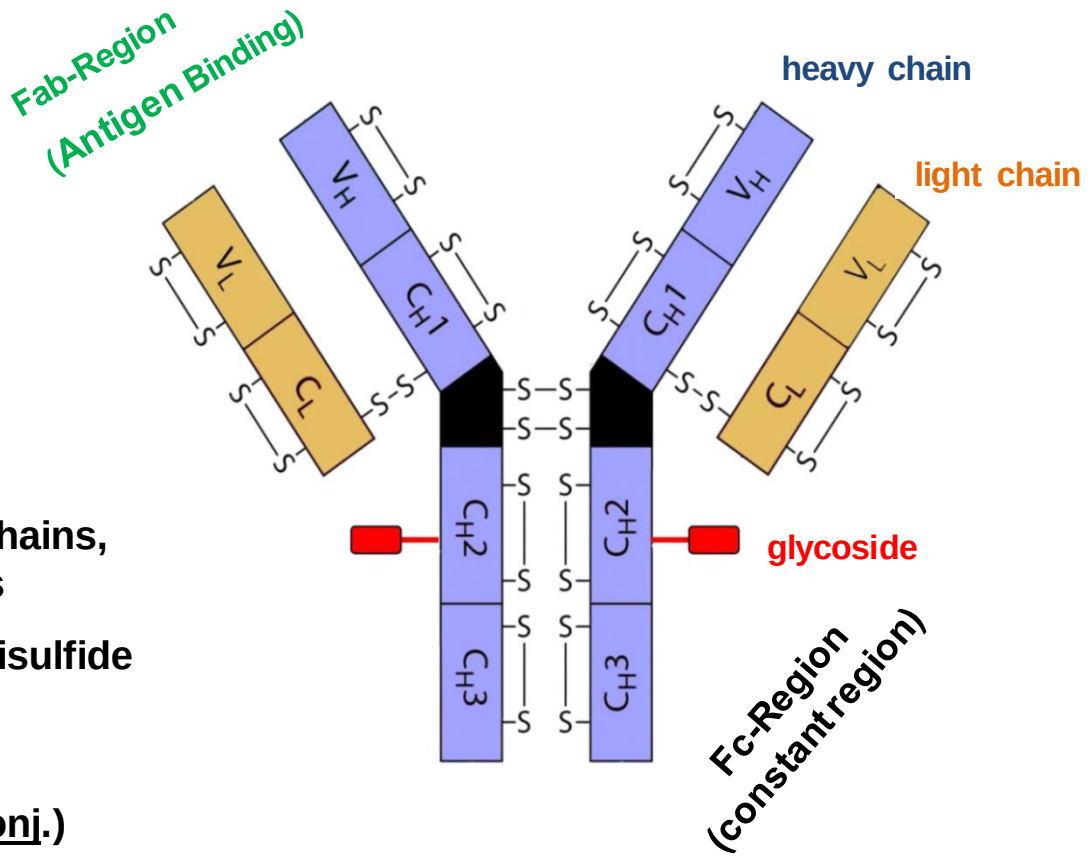
Toxophor given alone (blue curve): very short half-life



- Toxophor: very short intrinsic terminal half-life (blue curve)
- Antigen conjugation: prolongation of toxophor half-life **(red curve)**
- Cave: Toxophor LLOQ: < 1 ng/L versus Ab: 1000 µg/L

Biochemistry of an Antibody (IgG1 Type)

Structures are important for understanding of ADC chemistries and bioanaytical approaches



- **> 650 AA**
- **arranged in four polypeptide chains, two light and two heavy chains**
- **cross-linked by 4 inter-chain disulfide bridges (=> max 8 Cys-SH for conjugation).**
- **(=> about 30 Lysine-NH2 for conj.)**

ADCs: highly diverse mixture of several different molecular entities

Selection Criteria for ADC Design

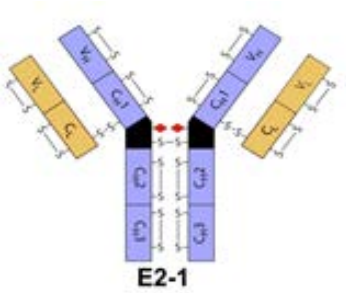
- Selection of the antibody: based on the intended tumor target antigen
- Selection of the toxophore: based on potency and intended mechanism of action (e.g. tubulin)
- Selection of linker (between Ab and Txp): based on intended cleavage
- Selection of the conjugation site: based on available amino acids for conjugation
- Conjugation limited to AA-SH of Cysteins and AA-NH_2 of Lysines
- Final ADC molecule elements:

  Antibody <- > Ab-Amino Acid + Maleimid-Linker + Spacer + Toxophor

- ADC: MW ~ 150 KDa; Drug: MW ~ 0.3 to 1 KDa !

Conjugation Strategies
How is the Drug Linked-In® ?

- Maleimid-activated linker-toxophors most often used
- Conjugation with interchain Cystein-SH
  - after reduction of interchain disulfid bridges (n=4 per antibody)
  - => max. 8 positions, max. 12 different biochemical entities
- Conjugation with Lysine-NH2
  - n= about 30 per antibody, 10 on light chain plus 20 on heavy chain
  - Several 100 different biochemical entities resulting (DAR ~ 4)
- Conjugation with an engineered add. Ab-Cystein-SH
  - „Site specific conjugation“ (THIOMAB)
  - ☹ Technically less feasable
  - ☺ Lower no. of biochemical entities (still instability issues, deconjugation)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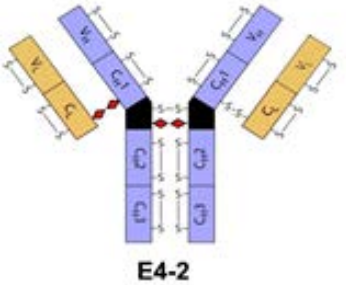
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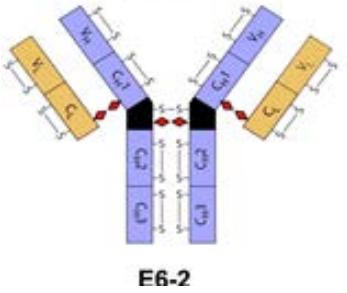
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Chemistry of the ADCs
Current Approaches

Diversity of ADC and challenges for bioanalysis due to combination of

- Different toxophor lead structures
- Different linker-strategy for toxophor cleavage
- Different antibody amino acids used for conjugation

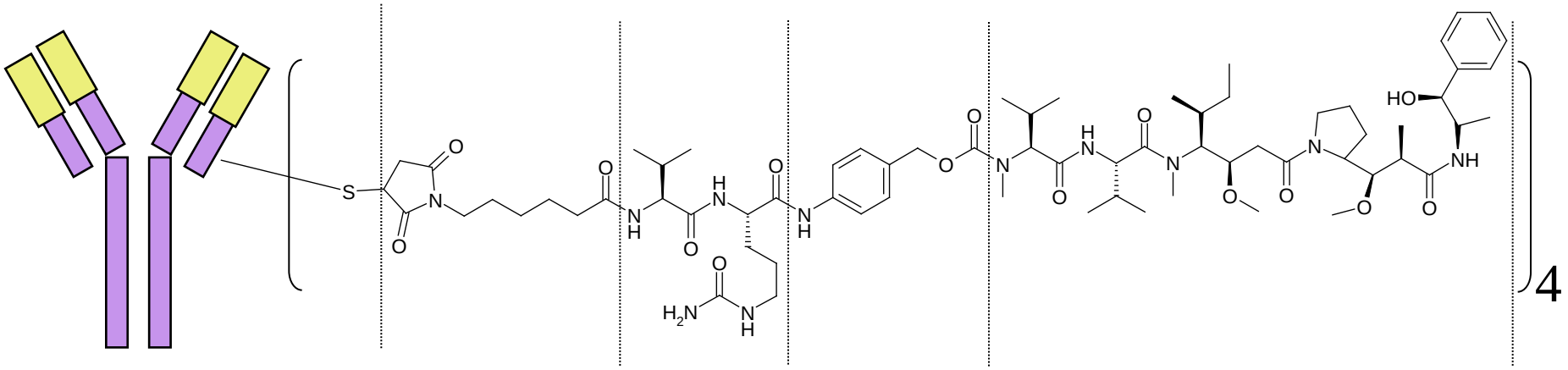
Toxophor-Classes

- 2 major classes
  - Auristatin-based
  - Maytansinoid-based

Linker Strategies = > analytes:

- Cleavable linker: unconjugated („free“) toxophor = active drug (Analyte)
- Stable linker: Amino-Acid + Linker + toxophor construct = active drug (Analyte)

Example 1: Mono-Methyl-Auristatin (MMAE) based ADC



Antibody Against Tumor Target Antigen

N-Maleimid Linker to Ab

Val-Cit (substrate for Cathepsin induced cleavage)

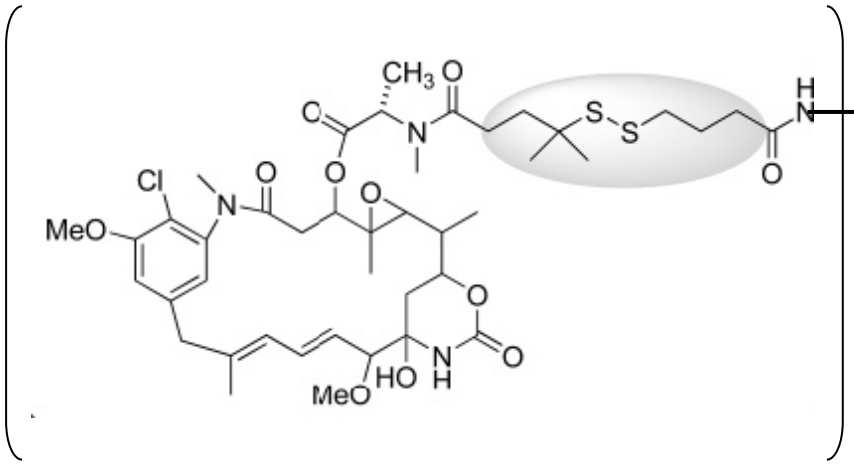
PABC (spacer, self-immolative)

MMAE (Toxophore, active drug)

Drug to Antibody Ratio DAR = 4

- ADC **binds** to tumor-specific antigen at tumor cells via FAB
- ADC is **internalized** into tumor cells (-> Lysosomes)
- Toxophor is **released** from the antibody (lysosomal peptid cleavage)
- Toxophor **kills** cells through inhibition of tubulin polymerization

Example 2: Maytansinoid based ADC

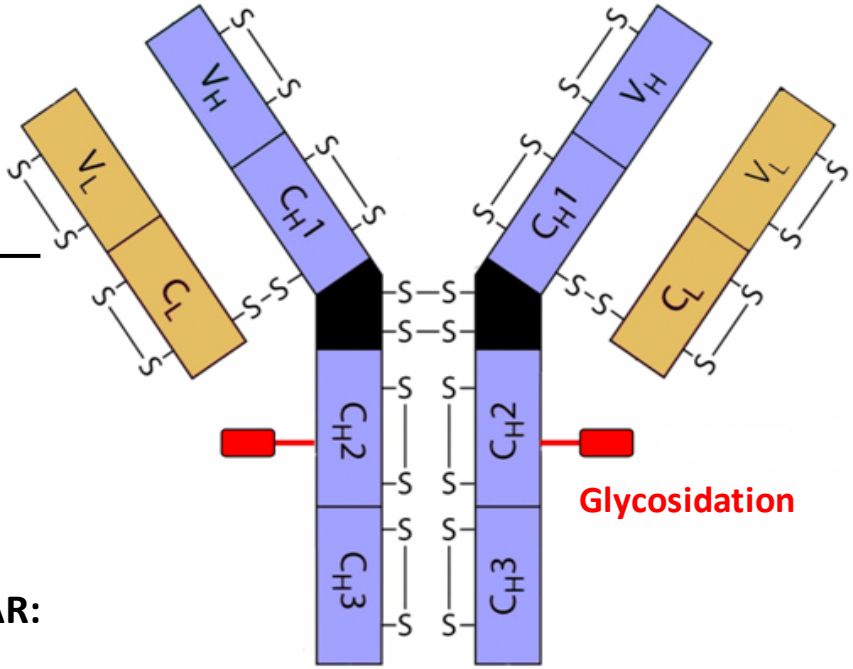


Maytansinoid-derivative DM4

SPDB (disulfide linker)

DAR:

n = ~3-4



Antibody

Active Drug(s) (Analytes)

- Disulfide cleavage to free thiol DM4
- Acive metabolite of toxophor via S-Methylation to S-Methyl-DM4

ADC PK - Role of Distribution and Catabolism
Main processes with bioanalytical relevance:

1. Antibody-related (mainly intracellular catabolism)

- Like antibodies, ADCs are specifically taken up into cells via target antigens **plus** unspecifically via Fc and FcRn receptors ("on-target/off-target").
- Besides tumor targets cells, also non-tumor cells can take up ADCs: either
  * on-target due to tissue cross-reactivity or
  * off-target (low affinity / high capacity binding)
- Intracellular clearance of antibodies in tissues (e.g. liver, tumor) and macrophages via proteolysis and cleavage to amino acids or toxophor-containing amino acids, resp. (digestion)
- In cases of non-cleavable linkers and stable toxophors digestion is the most relevant process for releasing the active drug

ADCs: Role of Metabolism and Clearance (2)

2. Toxophor related: mainly metabolism + systemic clearance

- both intracellular and intravasal (plasma) metabolism
- reductive cleavage of disulfide bridges (e.g. some Maytansinoids)
  - can result in instable (but still active) metabolites
  - (oxidation, S-methylation and „disulfide-shuffling“)
- peptid-cleavage to unconjugated MMAE (e.g. some Auristatins)
- linker cleavage (e.g. ring opening of maleimid, M+18 in MassSpec)
- Renal and/or hepato-biliar clearance

Slow intracellular release and high plasma CL => ng/L

Relevance of ADC Bioanalysis for Drug Development

PK of the ACD as well as the active drug has to be determined for evaluation of efficacy, toxicity and exposure / response (PK/PD):

- Selection of drug candidate with appropriate half-life in Research
- PK/PD support for selection of appropriate dose and dosing schedules in tumor models (e.g. AUC at effective dose, effective dosing interval)
- Preclinical Safety Studies
  - Establish AUC and Cmax at NOAEL or MTD (Maximum Tolerated Dose)
  - Ensure multiples of intended human exposure in animals (MoE)
- Clinical Development Studies (from 1st in Man to Submission)
  - Rational dose regimen for FiM and further studies
  - PK PD, PoP PK etc

Requests for Bioanalysis in ADC Programs
(most relevant analytes coloured red)

Analytes

- DAR (drug/antibody ratio) in vivo (change over time after adminstration)
- **Sum of all conjugated antibodies (drug loaded antibodies)**
- Total antibody (sum of conjugated and unconjugated antibodies)
- Unconjugated antibodies (optimization of clearance)
- **Unconjugated (free) toxophor**
- Unkonjugated toxophor-metabolite (if still active)
- **Anti-Drug Antibodies (ADA)**
  - binding ADAs and neutralizing ADAs (=> lack of efficacy)
- Sum of ADA-bound ADC
  - (Is safety relevant due to toxophor payload)

Bioanalytical ADC Assays
Strategy to be defined early on!

Typical **Assays Formats** for ADC Analytes

- DAR in vivo: **(MS of intact antibodies, high resolution, e.g. qTOF-MS)**
- Sum of all conjugated antibodies („ADC assay“):

  Elisa with Toxophor capture and Fab Detection, range: mg/l)

  Elisa with anti-human capture- and detection- Ab (non-human species, research phase)
- Total antibody (sum of conjugated and unconjugated antibodies) **(„total Ab Assay“):**
  - **Elisa with Fc capture (animals) or Fab capture (human) plus Detection via Fab**
- Unconjugated antibodies: (difference **„total Ab Assay“ minus „ADC assay“**)
- Unconjugated toxophor: **(LC-MSMS , e.g. Triple Quad)**; range: ng/L (100.000 fold lower)
- Anti-Drug Antibodies (ADA): **(Ligand Binding Assay Formats**)
- Sum of ADA-bound and free ADC: (**no state of the art assays available**)

  (Top down approach with tryptic Ab digestion and **LC-MCSMS ?**)

Bioanalytical Issues with ADCs (1)

Elisa for PK samples:

- Availabilities of well characterized reference ADCs
- Availabilities of anti-toxophor detection Abs and antigens for Ab capturing

ADA (anti-drug antibodies):

- Availability of reference Abs and establishment of cell based assays

DAR (Drug Antibody Ratio):

- Availability of HRMS and reference ADCs (DARs)
- LLQQ / Sensitivity for ex vivo plasma samples (at late time points)

Bioanalytical Issues with ADCs (2)

Toxophor:

- Availability of Stable Isotope Labeled Internal Standard
- Instability of ADC and toxophor in matrix / critical sample handling
  - release of toxophor from ADC in vitro
  - instability of the toxophor itself
- Toxophor-impurity in reference ADC
- Inaccuracy of toxophor analysis in presence of excess ADC (cleavable linker)
  - In vivo: Cleaved toxophor distributes extravasal => low plasma concentrations
  - ex vivo after sampling (artefacts!):
    - toxophor cleaved from ADC after sampling can not distribute in to Vss
    - Amount may significantly contribute to overestimation of concentration result

Summary and Conclusion (1)

ADC Bioanalysis is very challenging and requires:

State of the art HRMS-, 3qMS-, LBA- technologies for detection (LLOQ; selectivity)

Antibody LC (HILIC) and know-how on immuno-affinity extraction (hyphenated techniques) for separation (selectivity, precision)

Early access to LBA reagents (ELISA) and stable isotope label IS (LC-MSMS) time critical for method development and validation

Early access to certified reference compounds essential for accuracy, proof of selectivity and stability investigation

Summary and Conclusion (2)

Small meets large: size and site (of conjugation) matters !

Integrated approach:

- Combined expertise in Biochemistry and Chemistry
- Cutting edge LBA and MS technologies
- Well interfacing into CMC (use of available analytical know for drug substance (API)!)

Thank you for your attention !

Acknowledgements

to my Bayer colleges:

Manuela Braun (LBA of Ab PK and ADA)

Mark Gnoth (LC-MSMS Toxophor analysis)

Suggested further readings

S. Kour: Bioanalytical strategies for the development of antibody-drug conjugate biotherapeutics. Bioanalysis (2013) 5(2), 201-266

K. Xu: Characterization of intact antibody-drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography MS. Anal. Biochem. (2011)

B. Gorovits: Bioanalysis of ADCs: AAPS ADC-Working Group Position Paper. Bioanalysis (2013)

EBF Topic Team (to come …)

Topic team 43 - Antibody Drug Conjugates

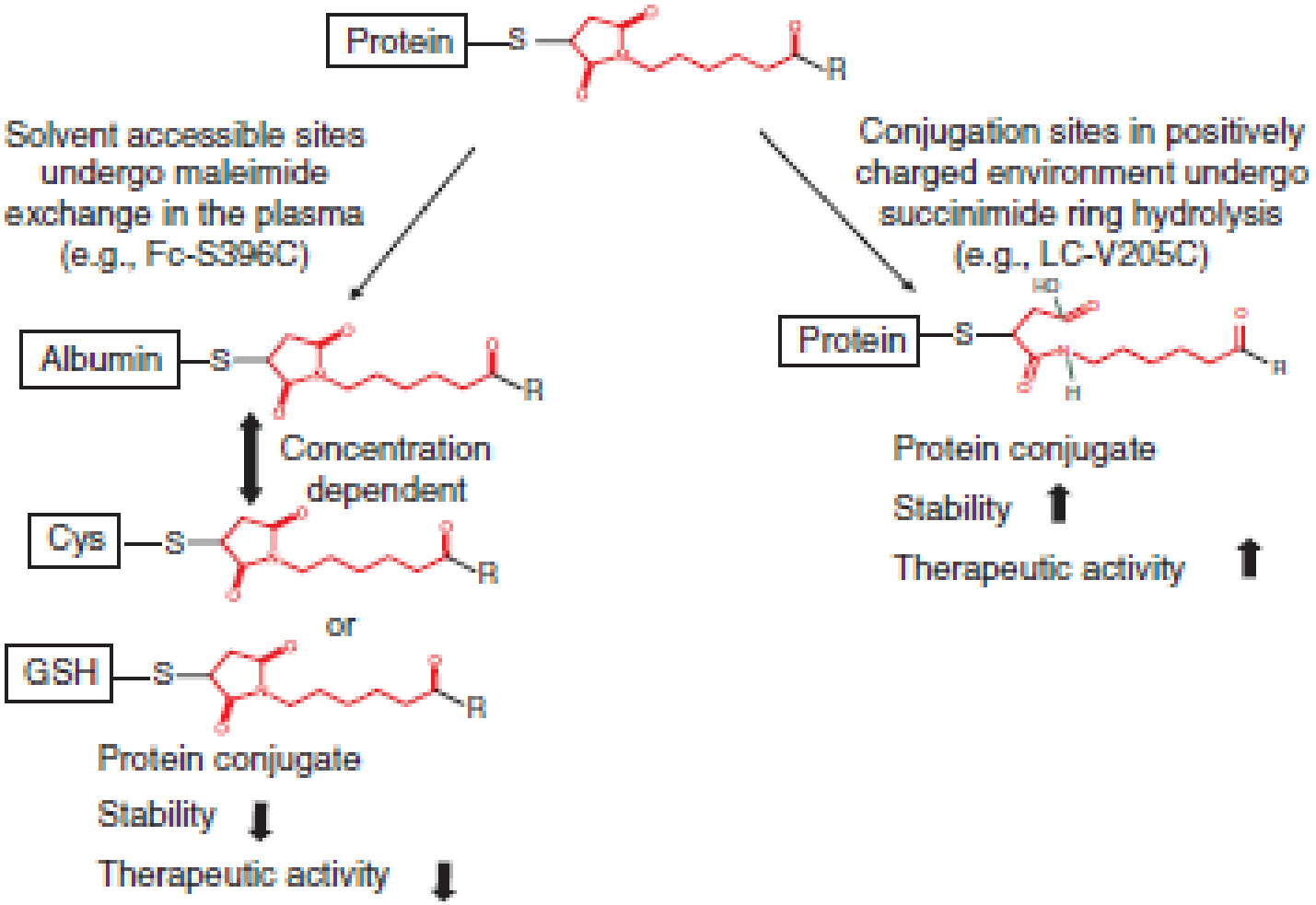
The Team

- Matt Barfield, GSK (Lead)
- Bernhard Beckermann, Bayer
- Margarete Brudny-Kloeppel, Bayer (Sponsor)
- Stephanie Fischmann, Abbvie
- Kirsty Jackson-Addie, Astrazeneca
- Martin Nemansky , PRA
- Monique Putman, QPS
- Andrew Roberts, Quotient
- Melody Sauerborn, TNO

Team goals

Share best practice, information gathering and disseminate across the community

Cystein-Linked ADC: Example for S-Maleimid based de-conjugation followed by "Disulfid-Shuffling" versus ring-opening



Source: B. Chen, Nature Biotechnology, 2012