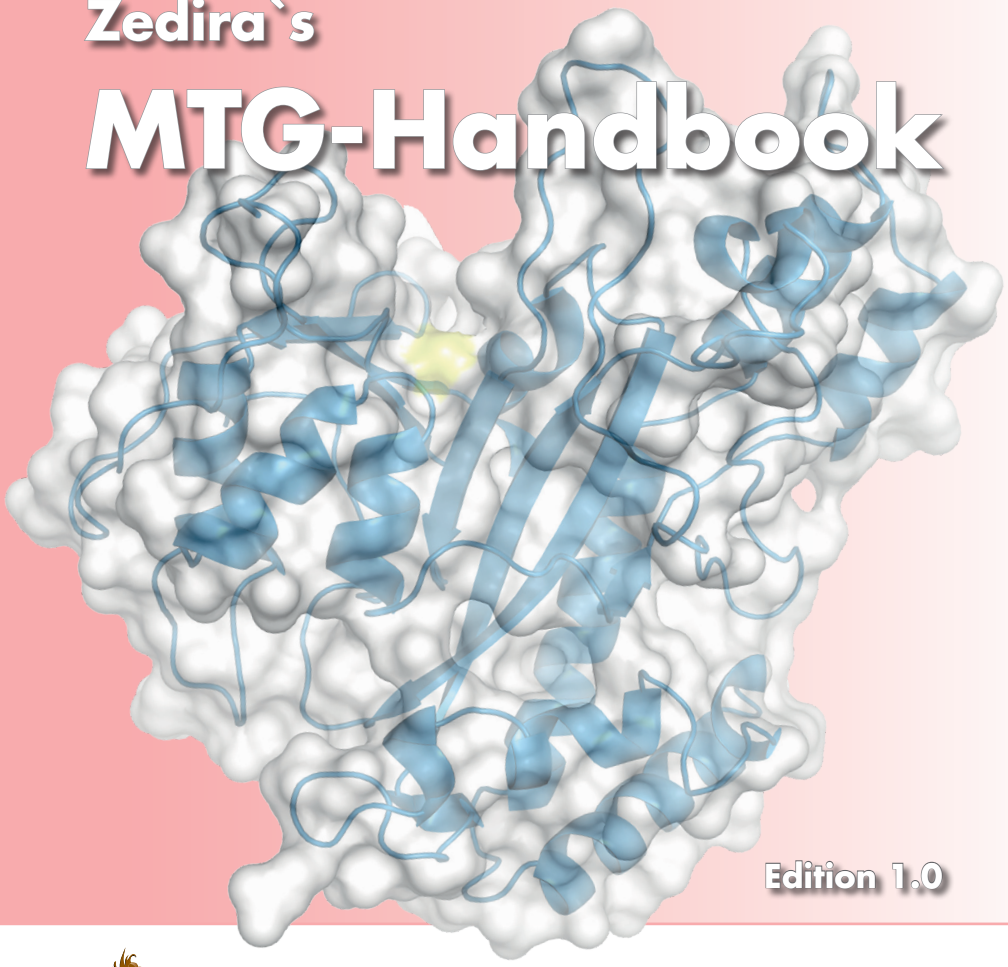
Zedira`s

MTG-Handbook



Edition 1.0

Follow me on a journey on MTG!

Table of content

7. Site Specific Conjugation: ADCs and More

Microbial transglutaminase is a versatile tool for conjugation of proteins or peptides with small molecules, polymers, surfaces, DNA or other proteins (Strop 2014). It yields site specifically labeled, homogenous products. Many applications have been described in scientific literature, including pegylation, labeling with fluorescent dyes and conjugation of enzymes (Kamiya et al. 2009; Pasternack et al. 1998; Sato 2002; Maullu et al. 2009; da Silva Freitas et al. 2013). However, today the manufacturing of ADCs is the main application field for MTG.

Antibody drug conjugate formation by MTG

An emerging field in oncology are antibody drug conjugates (ADCs). In ADC development, the aspects of antibody, payload and linker technology need to be taken into consideration (Figure 13). Each part of the ADC needs to meet high demands in order to provide high efficacy already by low drug doses, which in combination reduces the potential for adverse reactions.

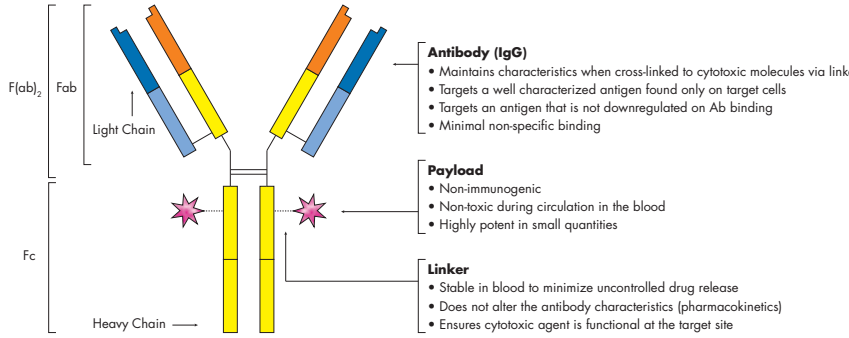


Figure 13: Schematic structure of IgG antibody drug conjugates. Figure adapted from Zolot et al. 2013 and Mulisch 2014.

Due to the antibody's high selectivity, the minimal effective dose can be delivered to a target (e.g. cancer cell) leading to low unspecific binding though providing the maximal tolerated dose (Schumacher et al. 2016).

The mode of action of ADCs is based on their degradation in cell's lysosomes and subsequent payload release. To achieve that, the antibody first needs to bind the antigen at the tumor cells surface. Receptor mediated endocytosis in early endosomes imports the ADC into the cell. If the ADC's Fc-domain binds to the FcRn-receptor of the endosome, it is re-exported out of the cell. Thus, antibodies to be used as ADC should exhibit low or no binding to FcRn.
Late endosomes finally fuse with lysosomes leading to proteolytic degradation of the ADC. Depending on the selected linker technology, the payload is released either by low pH or by proteolysis. The cytotoxic payload, usually DNA-binding or microtubule polymerization inhibiting substances, leaves the lysosome and can access DNA or microtubules, leading to immediate cell death or apoptosis initiation (Peters and Brown 2015).
Drawback of this ADC mode of action can be the so called "Bystander Effect", where the released drug diffuses through the cell membrane and affects neighboring healthy cells (Bouchard et al. 2014).

ADCs can be generated using chemical, physical or enzymatic conjugation. For all methods mild reaction conditions are required, in order to maintain the antibody's native structure and functionality. Homogenous conjugates guarantee batch independent drug efficacy, required for pre-clinical and clinical development and assessment of adverse effects (Kline et al. 2015).
MTG can label native antibodies, especially IgGs and can therefore be used for the generation of ADCs.
IgGs heavy chains from various species exhibit a conserved sequence of Q295[F/Y]N (Figure 14). Q295 is recognized by MTG as substrate. However, the Asp297 in proximity is glycosylated leading to sterical hindrance of MTG binding and catalysis (Figure 15).
Deglycosylation of Asp297 by the enzyme PNGase F, rendering Gln295 accessible for microbial transglutaminase, resulting in 2 labels pers antibody (1 per heavy chain, Jeger et al. 2010; Dennler et al. 2014).

```
                                                295
                                                ↓
Mouse IgG1      SKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRV
Rabbit IgG      SQDDPEVQFTWYINNEQVRTARPPLREQQFNSTIRVVSTLPIAHQDWLRGKEFKCKV
Rat IgG         SHEDPQVKFNWYVDGVQVHNAKTKPREQQYNSTYRVVSVLTVLHQNWLDGKEYKCKV
Human IgG4      SQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKV
Human IgG3      SHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTFRVVSVLTVLHQDWLNGKEYKCKV
Human IgG2      SAEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKV
Human IgG1      SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
                . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

Figure 14: Sequence alignment of IgG Fc-region of various species. Red: Conserved sequence Q[F/Y]N. Sequences taken from RCSB Protein Data Base. From top to bottom: 3HKF/2VUO/1FRT/5HVW/6D58/4L-4J/5D4Q. Sequence-alignment using Clustal Ω.

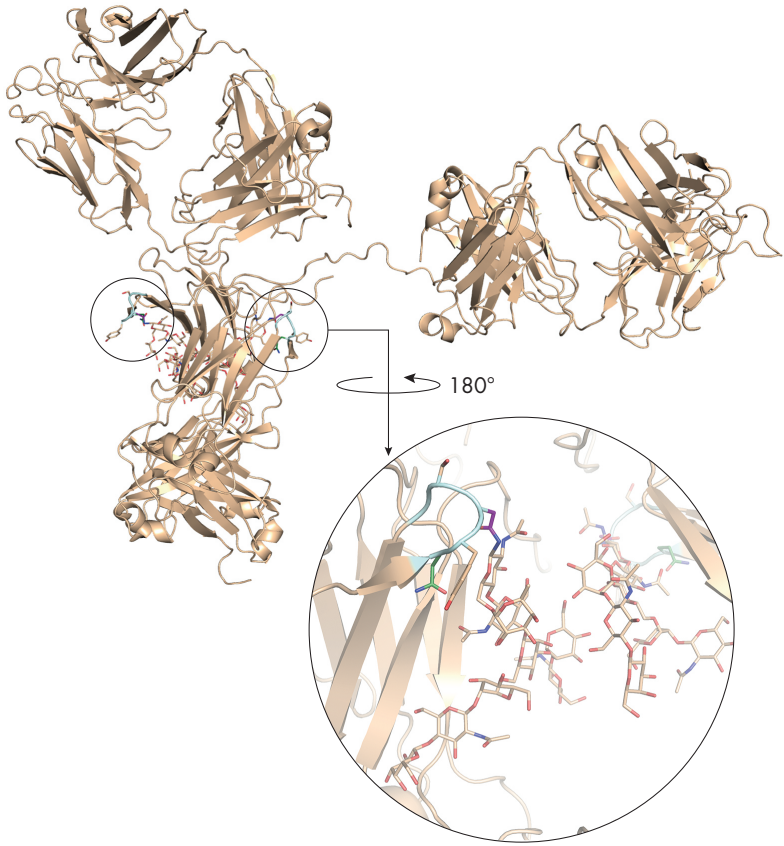


Figure 15: Crystal structure of human IgG. Enlarged: Gln295 and glycosylated Asp297 (black circle). Green: Glutamin 295; Purple: Asparagine 297; Blue: backbone of C/E-Loop QYNST. Crsytal structure from Saphire et al., 2001 (PDB-ID: 1HZH). Programm: Pymol. (Bitsch 2016).

MTG mediated antibody conjugation is depicted in Figure 16. An in-depth description of MTG technology is given by Schibli and Spycher in the next section.

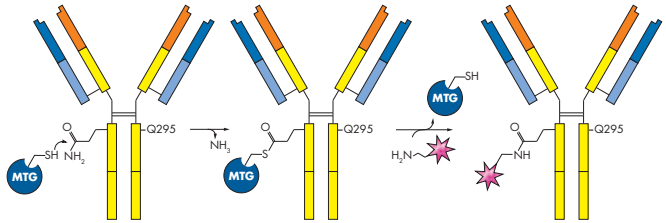


Figure 16: MTG reaction pathway. Antibody heavy chains are conjugated with a drug (linked to a primary amine) by MTG on position Q295, resulting in an ADC with two site specifically conjugated drug molecules. Here, the conjugation of only one Q295 is shown.

An alternative to deglycosylation was discovered by Spycher et al. Using lysine-containing peptides as primary amine substrates which contain an additional positively charged amino acid results in efficient labeling without deglycosylation (Spycher et al. 2019). The technology is now available at the swiss company Araris.

The Rinat-Pfizer group engineered the transglutaminase recognition sequence (Q-tag) LLQA to several positions of the heavy and light chain of IgG and successfully conjugated fluorophores and auristatin derivates resulting in drug to antibody ratio (DAR) 1.2 - 2 (Strop et al. 2013). However, the MTG mediated conjugation lead to unspecific reactivity at Q295, which could be avoided by Q295N mutation of IgG's heavy chain (Farias et al. 2014).

The Darmstadt, Germany, based academic groups of Harald Kolmar and Hans-Lothar Fuchsbauer commonly designed Q-tag sequences based on microbial transglutaminase's natural substrates DAIP and SPI_p (Siegmund et al. 2015; Ebenig 2019), showing improved reaction kinetics. Here, to avoid intermolecular cross-linking of IgG the terminal K447 had to be removed.

A further Q-tag sequence named CovIsoLink™ was developed by the French Company Covalab (El Alaoui and Thomas 2016). They showed that sequence environment, conformation of the antibody, and type of spacer can influence the conjugation. Conjugation was improved when the Q-tag was fused to the heavy chain's C-terminus in comparison to the light chain's C-terminus (Martin et al. 2018).

An alternative to conjugation at glutamine residues was developed by Morphotek/Eisei (Spidel and Albone 2019; Spidel et al. 2017) using antibody's lysine-residues and glutamin containing peptides as substrates. In analogy to the Q-labeling approach, either native or engineered lysines can be addressed for conjugation.

Targeting
Transglutaminase
for research and medicine!

Microbial transglutaminase (MTG) is an enzyme originally produced by *Streptomyces mobaraensis*. MTG catalyzes cross-linking of proteins in the absence of calcium. Current application field of the enzyme is processesing of meat, sausage, dairy, bakery, and pasta products to improve food properties.

However, MTG cannot only cross-link proteins, it can also incorporate primary amines to proteins in a covalent, irreversible manner. This feature allows linking of a whole bundle of labels to proteins, including biotin, fluorescent dyes, click chemistry substrates and cytotoxins.

MTG's unique properties can be utilized to generate site specific and homogenous antibody drug conjugates (ADCs). Today, ADCs manufactured with MTG reached clinical trials.

Zedira's MTG-Handbook shall provide an overview on this fascinating enzyme. While this first edition is just the beginning – future editions will follow to include latest developments and to cover novel applications.

zedira®

Zedira GmbH
Roesslerstrasse 83
64293 Darmstadt
Germany

Phone: +49 61 51 / 32 51 00
Fax: +49 61 51 / 32 51 19
E-mail: contact@zedira.com
Web: www.zedira.com