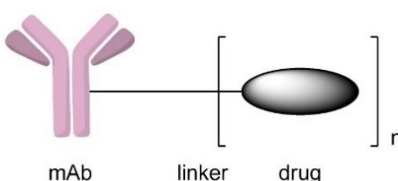
ADC Conjugation by DTT Reduction with Maleimide Drug Linker

Introduction

Maleimide-PEG modified drug can be conjugated to the antibody at the pH 7.5 buffer solution contained 50 mM sodium phosphate, 50 mM sodium chloride and 2 mM EDTA The disulfide bridges in the antibody were then reduced with various molar equivalents of DTT at 30°C for 30 min to afford different numbers of available sulfhydryl groups on the antibody molecule, then the reduced antibody was then conjugated with various molar equivalents of maleimide-PEG modified to afford a range of different DARs.



Condition: a. DTT at 30°C for 30 min; b maleimide-drug, PBS, 7.4, EDTA,

Example protocol

Reduction

To 4.8 mL antibody (10 mg/mL) was added 600 uL of 500 mM sodium borate/500 mM NaCl, pH 8.0, followed by 600 uL of 100 mM dithiothreitol (DTT) in water. After incubation at 37°C for 30 minutes, the buffer was exchanged by elution through Sephadex G-25 resin with PBS containing 1 mM diethylenetriamine pentaacetic acid (DTPA).

The thiol-antibody value was determined from the reduced mAb concentration determined from 280-nm absorbance the thiol concentration was determined by reaction with DTNB (5,5-dithiobis (2- nitrobenzoic acid) and absorbance measured at 412 nm.

Conjugation

PBS containing 1 mM DTPA (PBS/D) was added to the reduced antibody to make the antibody concentration 2.5 mg/mL in the final reaction mixture, and the solution was chilled. The drug-linker solution to be used in the conjugation was prepared by diluting drug-linker from a frozen dimethyl sulfoxide (DMSO) stock solution at a known concentration (approximately 10 mM) in sufficient acetonitrile to make the conjugation reaction mixture 20% organic/80% aqueous, and the solution was chilled on ice. The volume of drug-linker stock solution was calculated to contain 9.5-mol drug-linker/1 mol antibody. The drug-linker solution was added rapidly with mixing to the cold-reduced antibody solution, and the mixture was left on ice for 1 hour.

Separation

A 20-fold excess of cysteine over maleimide was then added from a freshly prepared 100-mM solution in PBS/D to quench the conjugation reaction. While the temperature was maintained at 4°C, the reaction mixture was concentrated by

centrifugal ultrafiltration and buffer- exchanged by elution through Sephadex G25 equilibrated in PBS. The conjugate was then filtered through a 0.2-um filter under sterile conditions and stored at -80°C for analysis and testing.

Analysis

ADCs were analyzed for concentration by UV absorbance, aggregation by size-exclusion chromatography, drug/antibody by measuring unreacted thiols with DTNB, and residual free drug by reverse-phase HPLC.

Note:

1. Reducing the disulfide bonds of a monoclonal antibody should not affect its functions. TCEP and dithiothreitol (DTT) are most often used as reduction reagent.
2. interchain disulfide bonds are easier to be reduced than intrachain disulfide bonds. These allow free thiol groups to be generated under mild reducing conditions while leaving the antibody intact at the same time. Limited reduction with TCEP or DTT predominantly yielded conjugates in which drugs were attached to heavy-light chain disulfides; partial re-oxidation of fully reduced antibodies with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) yielded conjugates that drugs were mainly attached to by heavy-heavy chain disulfides
3. cysteine-maleimide conjugation sites are highly heterogeneous

   In a human IgG1, there are four interchain disulfide bonds that can be used as potential conjugation sites . The four interchain disulfide bonds can be reduced by tris(2-carboxyethyl) phosphine (TCEP) or dithiothreitol (DTT), which results in eight thiol groups that are available for conjugating drug molecules. Through this method, different drug antibody ratio (DAR) conjugates will be obtained when targeting typical DARs of 2–4. In addition, antibody-drug conjugate at each drug antibody ratio has several isomers. Thus, over a hundred different species are present in the antibody-drug conjugate. Although conventional methods that employ cysteine residues as conjugation sites are highly heterogeneous.

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