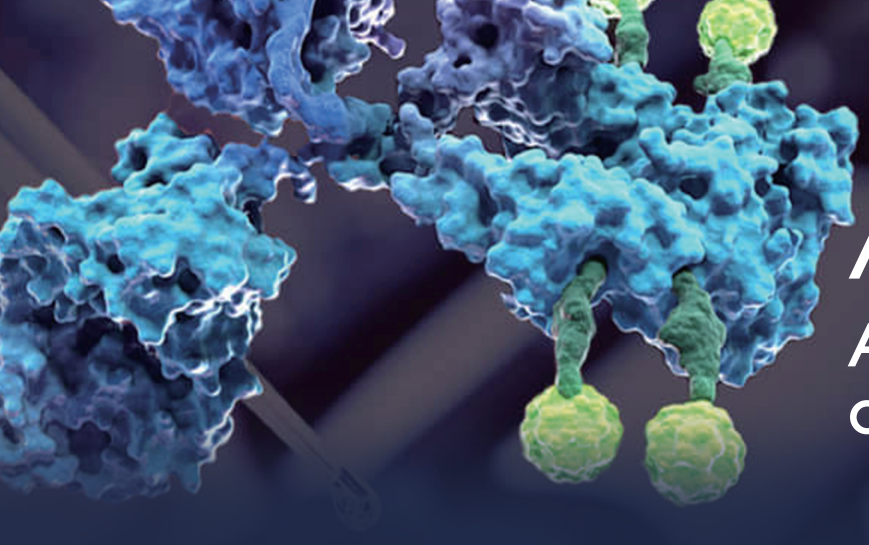


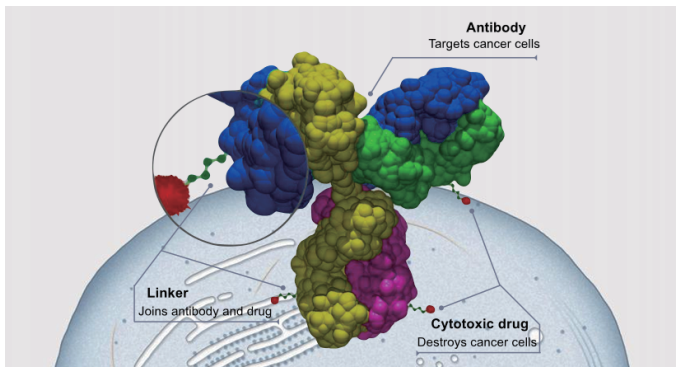
ADC Case Study

Anti-HER2 ADC preparation and potency evaluation against HER2 overexpression human cancer cells

Creative Biolabs
Antibody-drug Conjugate

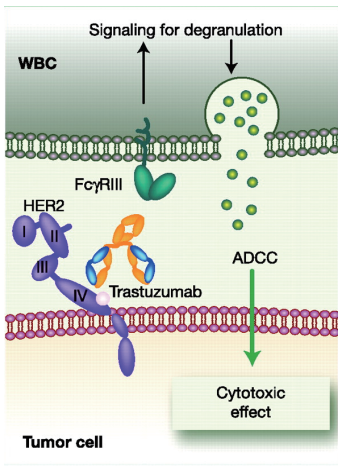
Introduction

ADCs are novel immunotherapeutic agents that are comprised of an antibody, a toxic payload, and a small molecule linker that covalently bridges the other components. With years of experience in providing ADC related products and services, Creative Biolabs has established a comprehensive service portfolio and we are dedicated to offering assistance to clients with various aspects of ADC development projects. Presented here is a case study in which Transtuzumab is formulated into an ADC with monomethyl auristatin E (MMAE). The outcome of ADC formulation and ***in vitro*** efficacy of the resulted ADC is analyzed and compared with un-conjugated Transtuzumab.

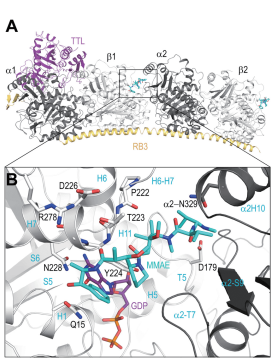


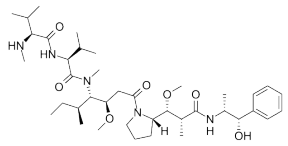
Transtuzumab

Transtuzumab, trade name Herceptin, is a recombinant IgG1 kappa, humanized monoclonal antibody that selectively binds with high affinity in a cell-based assay (Kd = 5 nM) to the extracellular domain of the human epidermal growth factor receptor protein. It is produced in CHO cell culture and induces tumor cell death via effector functions. Currently, it is often used together with pertuzumab as regimen for immunotherapeutics. An ADC, namely Kadcyla, has been developed based on Transtuzumab bearing emtansine as linker-payload.



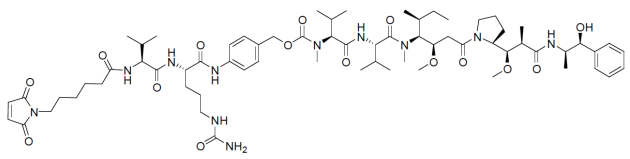
Monomethyl auristatin E (MMAE)



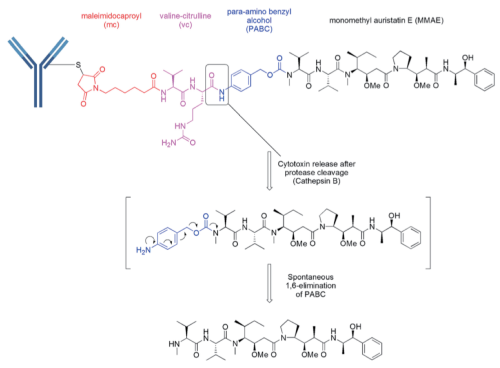


MMAE is an auristatin derivative. It is a microtubule inhibitor that disrupts microtubule function, arrests cell mitosis, and causes cell death via this unique mechanism of actions. MMAE binds to the α as well as β tubulin subunits via interactions with their aromatic amino acid side chains and this strong interaction inhibits the tubulin-dependent GTP hydrolysis, thus stabilizing microtubules. It is the most commonly used payloads for current ADC developments with Adcetris® approved by FDA in 2011. Over 30 ADCs in the clinical trial pipeline is formulated using auristatin derivatives, making these compounds the first line of choice in ADC developments.

Payload-linker



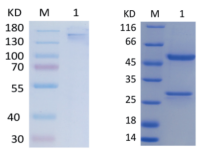
The payload-linker used in this study is Mc-vc-PAB-MMAE (Cat # ADC-S-012). MMAE was firstly coupled with a linker: MC-Val-Cit-PAB-PNP (Cat # ADC-L-008) to form the payload-linker complex. The resulted compound is subsequently conjugated to the Ab via the maleimide portion of the compound. The linker portion, comprised of a di-peptide Val-Cit and a PAB module, undergoes self-elimination to release MMAE in the most native form after the degradation of the "vc" di-peptide by Cathepsin B.



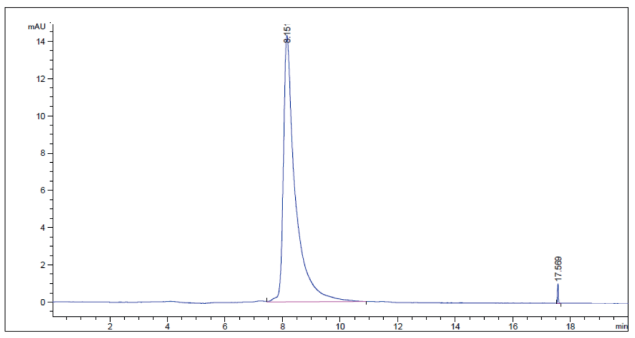
Materials and Methods

Transtuzumab by Creative Biolabs (Cat # TAB-005)

Transtuzumab was expressed using CHO cell line via transient expression.The final protein concentration exceeded 5 mg/ml and exerted high purity (>95%) and small degree of aggregation after protein-A affinity purification, as assayed by SDS-PAGE and SEC chromatography, respectively.



Purity assay: non-reduced (left) and reduced (right) SDS-PAGE

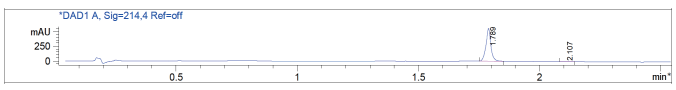


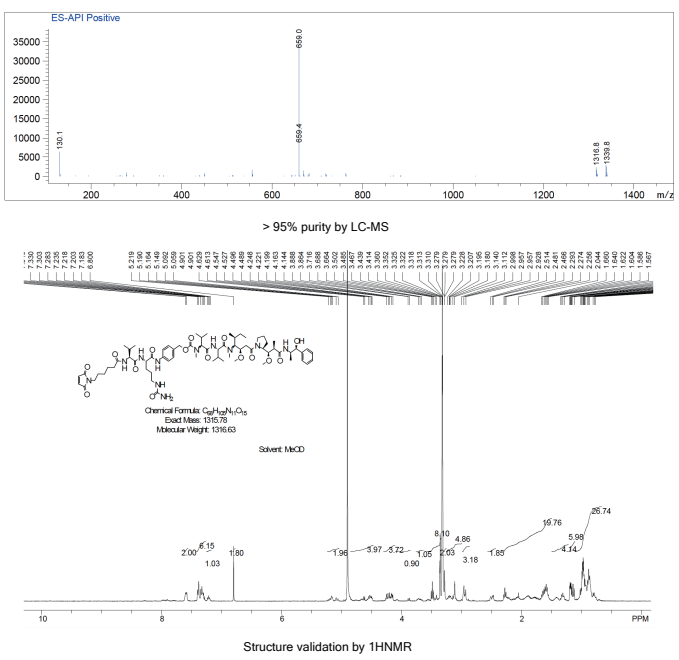

```
Signal 1: VWD1 A, Wavelength=280 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]      [min]   [mAU*s]    [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   8.151 VB    0.3997  408.76608   14.29320  99.4546
   2  17.569 VB    0.0339    2.24155    1.05228   0.5454
```

Transtuzumab purification: protein A affinity chromatogra-

Mc-vc-PAB-MMAE from Creative Biolabs (Cat # ADC-S-012)

Mc-vc-PAB-MMAE was synthesized by coupling MMAE with Mc-vc-PAB-PNP linker. The resulted payload-linker showed >95% purity and structure validated by NMR.





> 95% purity by LC-MS

Structure validation by 1HNMR

Transtuzumab was first treated with DTT for partial reduction and then conjugated with Mc-vc-PAB-MMAE to form the ADC. Optimization rounds were performed to establish the reaction conditions for both reduction time and ratio for Ab: payload-linker. Final conjugation was carried out by the following protocol:

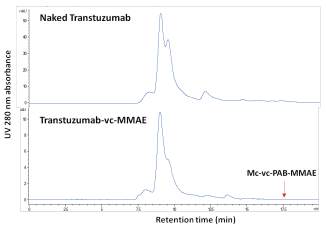
1. Antibody was treated with DTT, which was subsequently removed by ultrafiltration to obtain the reduced Transtuzumab.
2. Determine the thiol concentration of the reduced Transtuzumab by A280 and DTNB test to calculate the molar ratio of thiol: Ab.
3. Dissolve payload-linker in DMSO to form a stock solution, which was added to the antibody solution to initiate the conjugation reaction.
4. The conjugation was allowed for 3 hr at RT. The free drug and other small-molecule species were removed via constant-volume UF/DF to obtain the final conjugate: Trastuzumab-VC-MMAE.
5. The DAR (drug to antibody ratio) of the ADC was determined using the drug/mAb ratio, as measured by the absorbance at 252 nm and at 280 nm. HIC chromatogram was also used to confirm the DAR and assay for residual free drug.
6. Naked Transtuzumab underwent partial reduction was used as control for chromatography assays.

Results

ADC preparation: Transtuzumab-vc-MMAE

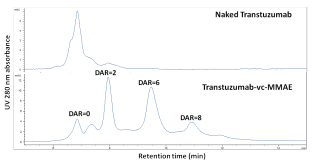
SEC chromatography analysis of ADC:

1. Partial reduction caused small aggregation of Transtuzumab and in the resulted ADC.
2. The addition of the payload-linker did not cause a significant shift in MW of the ADC, resulting in a purity at ~90%.
3. Free payload-linker was not observed in the SEC chromatograph, indicating the thorough removal of free drug.



HIC chromatography analysis of ADC:

1. Several conjugation species bearing DAR from 2~8 were observed by HIC.
2. The ratio of unconjugated Ab (DAR=0) was assessed at ~10%.
3. Final DAR of the ADC was calculated at ~4.5 based on HIC results.
4. The DAR measured by UV/VIS was 4.3, in agreement with HIC results.



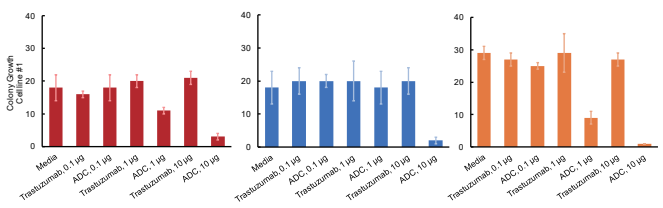
DAR calculation by UV/VIS

$$DAR = (\varepsilon^{p}_{252nm} - \varepsilon^{p}_{280nm} R)/(\varepsilon^{d}_{280nm} R - \varepsilon^{d}_{252nm})$$

Summary of ADC preparation

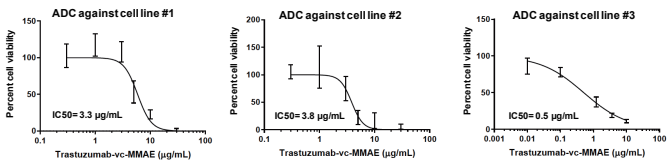
1. High purity Transtuzumab and payload-linker were produced by recombinant expression and synthesis, respectively.
2. 10 mg Transtuzumab at 5 mg/ml was used for ADC preparation, which resulted in ~ 5 mg ADC. SEC analysis of the AD C indicated a purity at ~90% with no trace of free drug
3. HIC analysis of the ADC revealed the major conjugated species bearing DAR of 2 or 6 and the
4. DAR of the ADC was calculated to be 4.5 by HIC and 4.3 by UV/VIS.

Transtuzumab-vc-MMAE potency assessment: Colony growth inhibition



Three human cell lines over-expressing HER2 were chosen to assess the potency of the Transtuzumab-vc-MMAE ADC. In the first assay, tumor growth inhibition was assayed using different dosages of the ADC and unconjugated Transtuzumab as control. The results indicated a strong inhibitory potency of the ADC at 1 μg and 10 μg doses. Near complete growth inhibition was observed in all cell lines at relatively high ADC dosage.

Transtuzumab-vc-MMAE potency assessment: IC50 determination



IC50 values of the Transtuzumab-vc-MMAE ADC were determined against the three selected cell lines. Comparing to the IC50 of naked Transtuzumab against these cell lines, which were all >30 μg/ml, the cytotoxicity of he ADC is significantly improved (~10-fold improvement) with the attached payload-linker.

Summary of Transtuzumab-vc-MMAE potency assessment

1. Transtuzumab-vc-MMAE ADC showed good ***in vitro*** cytotoxicity against all three HER2-over expression cell lines.
2. Higher dosage of the Transtuzumab-vc-MMAE ADC exerted excellent inhibitory effect of tumor growth in all three cell lines.
3. With IC50 at ~3 μg/ml, the Transtuzumab-vc-MMAE ADC was shown to be over 10-fold more potent that unconjugated Transtuzumab.

References

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