Metabolic engineering of monoclonal antibody carbohydrates for antibody-drug conjugation

Nicole M. Okeley*, Brian E. Toki, Xinqun Zhang, Scott C. Jeffrey, Patrick J. Burke, Stephen C. Alley, Peter D. Senter

Supplemental information:

EXPERIMENTAL METHODS

mAb production and fucosylation analysis. Evaluation was as described previously[1]. Briefly, mAbs were expressed and purified from CHO-DG44 cells[2] with culture medium containing1 mM fucose analogues during production. Protein A-purified mAbs were reduced (10 mM dithiothreitol, DTT, 37 °C, 15 min) and analyzed by LC-MS (PLRP-S chromatography, electrospray ionization Q-Tof mass spectrometry). Deconvolution of heavy chain data was accomplished with the MaxEnt1 function in MassLynx 4.0. Peak heights of the G0, G0**X** (where **X** = incorporated fucose analogue) and G0F peaks were used for calculation of the incorporation of novel fucose analogues into the mAb.

Conjugation methods. mAbs were conjugated to mc-vc-PAB-MMAE via the interchain disulfide cysteine residues as described previously[3]. In brief, 1-2 eq of tris(2-carboxyethyl)phosphine (TCEP, empirically determined for each mAb) were added to mAb in PBS, pH7.4, 1 mM diethylenetriaminepentaacetic acid for 90 min at 37°C. The resultant reduced antibody was conjugated by the addition of mc-vc-PAB-MMAE in 20%

molar excess for 90 min at 0°C. N-Acetyl cysteine was added to quench the unreacted drug linker (15 min, 0 °C), and the ADC was purified and the buffer exchanged with PBS by use of an Amicon Ultra 30,000 NMWL centrifugal concentration device (Millipore). Thio-sugar conjugated ADCs were generated by full reduction of the antibody interchain disulfides with excess TCEP (19 molar equivalents, 37 °C, 3 hr) followed by removal of excess TCEP using an Amicon Ultra concentration device. Interchain disulfides were re-oxidized by the addition of 50 mM dehydroascorbic acid (dissolved in 100 mM potassium phosphate pH 7.4, 25 molar equivalents) at room temperature (1.5 hr). Excess dehydroascorbic acid was removed by dilution with PBS and concentration in an Amicon Ultra concentration device. The reduced and re-oxidized mAb was conjugated with mc-vc-PAB-MMAE as described above.

Drug loading evaluation and PNGase F treatment. Drug loading was determined using liquid chromatography using a PLRP-S reverse-phase column (Polymer Labs) as described previously[3, 4]. Drug-linker attachment to the carbohydrate was confirmed by reaction of the thio-sugar ADC (20 μg, 0.5 mg/mL) with PNGase F (New England Biolabs, 1 μL) at 37 °C for 1 hr to remove the carbohydrate, followed by DTT reduction (10 mM, 15 min, 37 °C) and liquid chromatography-mass spectrometry using PLRP-S chromatography coupled to a QTOF-API high-resolution mass spectrometer (Waters) to determine the molecular weights of the resultant heavy and light chains.

Cytotoxicity assay. Log phase cultures of cells were collected and plated at 3,000

cells/well in opaque-walled 96-well plates. Serial dilutions of test molecules were added after cell plating and cultures incubated an additional 4 days in a humidified incubator at 37 °C, 5% CO_2. Assessment of cellular growth was determined using Resazurin (Sigma) dye reduction as previously described[5]. Briefly, a freshly prepared 1 mg/mL stock of Resazurin in Hank's balanced salt solution (Life Technologies) was added to each well for a final concentration of 0.1 mg/mL. Cells were incubated an additional 4 hr at 37 °C and dye reduction was measured using a fluorescent plate reader.

Plasma stability. h1F6-**10**-mc-vc-PAB-MMAE or h1F6-mc-vc-MMAE were spiked in citrated Sprague-Dawley rat plasma (Bioreclamation) and incubated at 37 °C for 4 days. Samples were removed for evaluation at 0, 4, 24, 48, 72, or 96 hr followed by immediate freezing and storage at -80 °C. ADCs were then isolated from each sample and MMAE released proteolytically from the isolated ADCs as described previously[6]. The released MMAE was then quantified by LC-MS/MS and normalized to the initial value for each ADC.

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Supplemental Figure 1

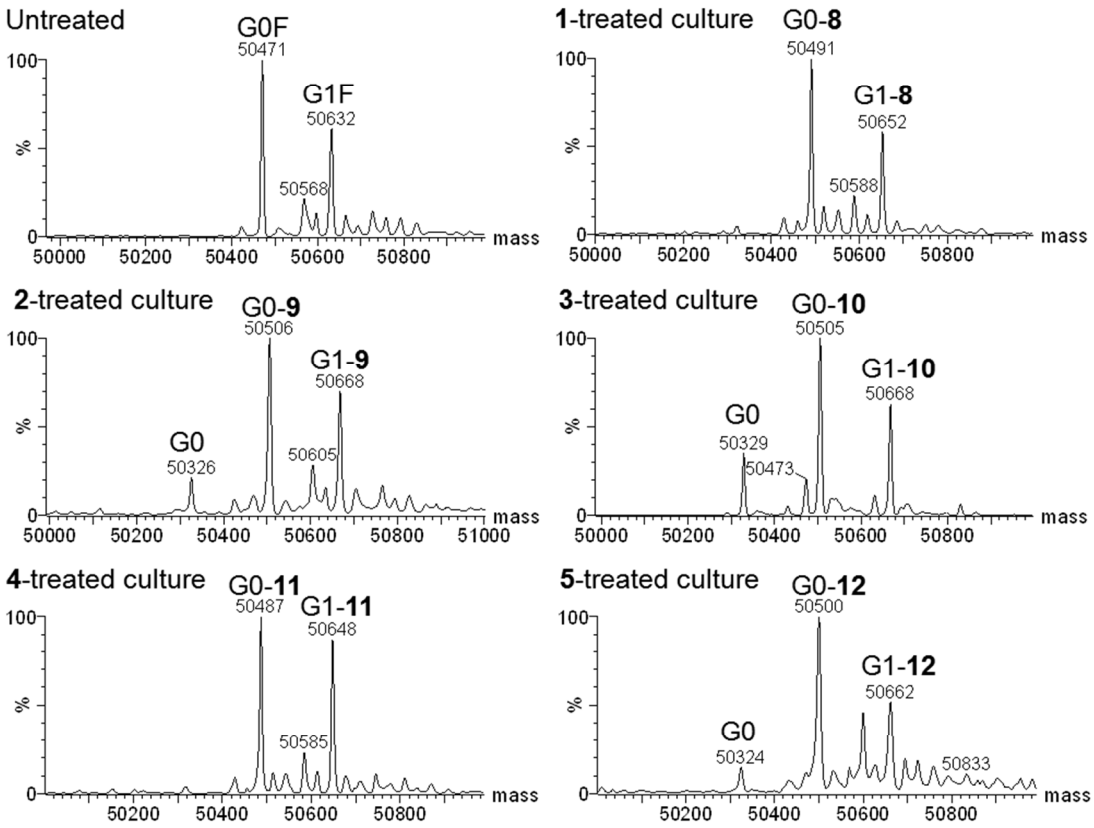

Figure S1. Mass spectral analysis of glycosylated heavy chains of antibodies generated by CHO cells treated with 1 mM **1**, **2**, **3**, **4**, or **5**. Expected mass of G0F = 50474, G0-**8** = 50492,G0-**9** = 50508, G0-**10** = 50506, G0-**11** =50488, G0-**12** =50500.

Supplemental Figure 2

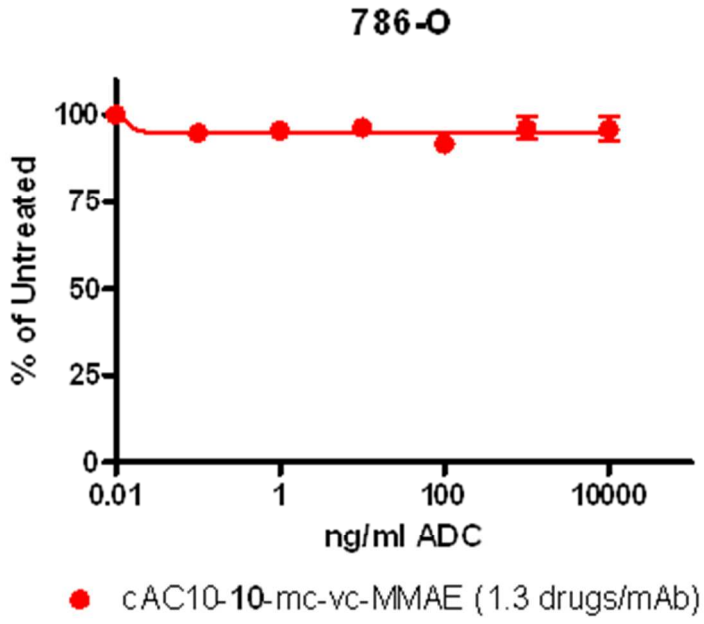

Figure S2. Cytotoxicity evaluation of the anti-CD30 thio-sugar ADC made with mc-vc-PAB-MMAE attached through the antibody carbohydrate-incorporated **10** (cAC10, anti-CD30 mAb) on an antigen negative cell line (786-O, CD30 negative). No cytotoxic activity is observed.