Optimization of Desferrioxamine Conjugation to a Cytotoxic ADC for *In Vivo* PET Imaging Studies

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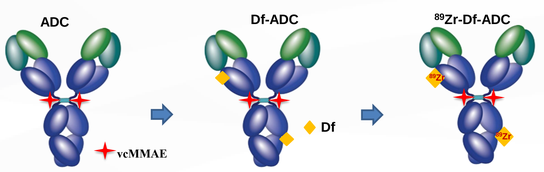
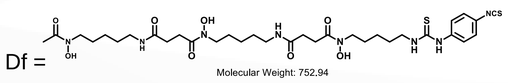
Abstract

Background: Antibody drug conjugates (ADCs) have become an important component of monoclonal antibody based therapies for cancer treatment. Their efficacy, however, is not well understood and may be influenced by poor tumor uptake or by off-target toxicities that prevent attainment of a therapeutic window in patients. The off-target toxicity may be mediated by rapid clearance and target expression on normal tissues (Boswell et al. British Journal of Pharmacology (2013) 168: 445–457). Positron Emission Tomography (PET) imaging with radiolabeled ADCs offers the opportunity to visualize both target expression and biodistribution of the conjugated protein in a patient to predict the efficacy of treatment and reveal sites of target-mediated clearance. Because many ADCs are internalizing, preparation of an imaging reagent requires labeling with a residualizing radiotracer that has a long enough half-life to allow systemic clearance. As such ^{89}Zr ($t_{1/2}$ = 3.3 days) presents a promising strategy for radiolabeling ADCs due to favorable half-life and commercial availability. However, conjugation of an ADC with a readily available complexing agent such desferrioxamine (Df) may present a challenge to the manufacture of a clinical-grade imaging agent owing to (i) it's hydrophobic nature, (ii) propensity of ADCs to aggregate when exposed to the conditions of the reaction and (iii) potential to de-stabilize the cytotoxic drug elements of the ADC.
Methods and Results: An ADC composed of an antibody tethered to the microtubule disrupting drug, vcMMAE, was conjugated to desferoxamine via lysine side chain amino groups. A significant increase in aggregation of the resulting protein prompted us to carefully study the process parameters with respect to reaction time, temperature, per cent co-solvent and Df ratio. We found that gentle reaction conditions with respect to the temperature and pH led to a reduction in aggregation while preserving the binding affinity. An increase in DMSO co-solvent in the reaction was used to counteract the poor solubility of the Df chelator encountered upon lowering the reaction temperature. The resulting conjugates were evaluated and shown to be suitable for imaging on the basis of a variety of analytical methods including: Drug:Antibody Ratio (DAR) by reverse phase HPLC, chelator molar ratio with an Fe(III) chelation assay, purity by SDS PAGE; SE-HPLC and binding affinity by flow cytometry. Selected conjugates were subsequently radiolabeled with ^{89}Zr and shown to retain acceptable immunoreactivity. Finally, imaging studies in tumor bearing mice demonstrated strong and specific uptake of the radioactive ADC tracer. The biodistribution study revealed that a tumor uptake was 2-fold higher for the ADC compared to the radiolabeled parent antibody at 96h, suggesting that (i) this method may add value to understanding product accumulation in low and high antigen copy number models and (ii) biodistribution of an ADC differs from the unconjugated mAb

ImmunoPET Tracers

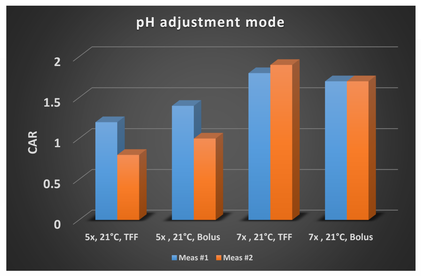




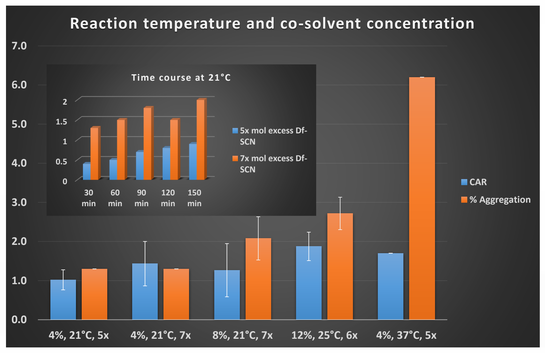
Schematics: Desferrioxamine (Df) isothiocyanate is conjugated on lysine residues of an ADC resulting in a randomly distributed chelator cage with an average CAR of 1.8. Zr-89 positron emitting radionuclide with a $T_{½}$ of 3.1 days was then added resulting in a radio-tracer with radiochemical purity of >97% and specific activity of 5-7 µCi/µL. The Immunoreactivity was assessed using an SEC-based assay to be greater than 75%.

Evaluation of Conjugation Process Conditions

Compatibility of ADC formulation with conditioning regiments



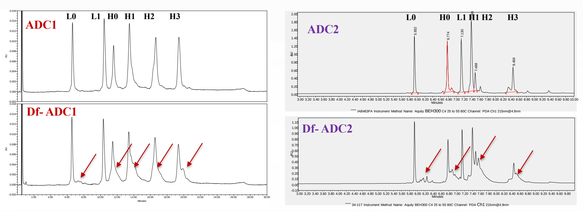
To evaluate whether the formulation buffer components are incompatible with the lysine conjugation chemistry, both bolus pH adjustment and buffer exchange by TFF were carried out. Conjugated ADCs were analyzed for CAR using a UV-based Fe(III) chelation assay.



The reaction time, Df excess, ADC concentration and temperature are important parameters to optimize during process development.

An optimized process results in a consistent Df-to-Antibody ratio. Inset shows the effect of different Df-to-ADC ratios used in the reaction on the resulting conjugated Df.

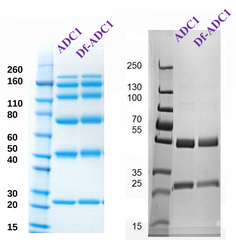
Df Conjugation Did Not Perturb The DAR

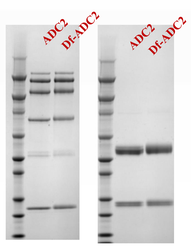


RP-HPLC shows that DAR has not changed.

Additional species and peak tailing are due to the Df conjugation (arrows)

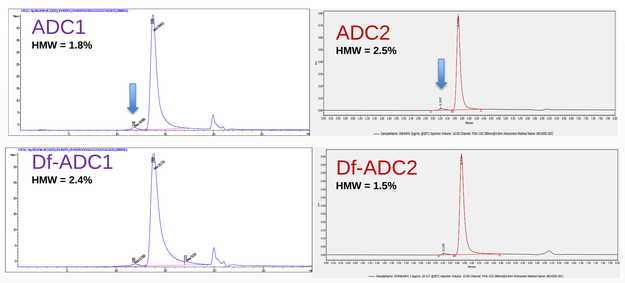
	% Peak Area						
	L0	L1	H0	H1	H2	H3	MR_{MMAE}
ADC1	11.5%	15.1%	12.1%	24.9%	20%	16.5%	4.2
Df-ADC1	10.9%	11.4%	15.9%	25.5%	19.9%	16.6%	4.1
ADC2	14.0%	16.72%	19.1%	27.57	8.7	13.9	3.6
Df-ADC2	11.0%	11.3%	9.4%	17.2	8.0%	7.3%	3.5





Reduced and non-reduced SDS-PAGE demonstrate a similar pattern between the "parent" ADC and Df-ADC.
A slight band retardation is consistent with Df-conjugation

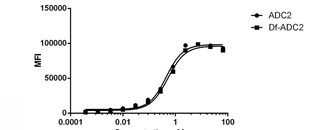
Df Conjugation Did Not Increase Aggregation



SE-HPLC shows that aggregate HWM species (arrow) are controlled under the optimized conditions

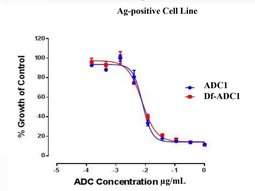
In Vitro and Vivo Results

Binding Affinity: ADC2



	ADC2	Df-ADC2
EC50	0.4471	0.5174

Potency of ADC1:

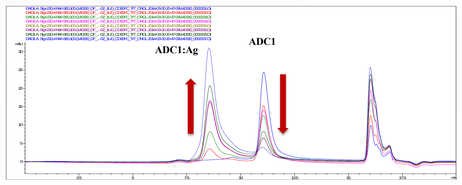


Antigen binding and cytotoxicity are not impacted following DFO conjugation
- ADC2 and Df-ADC2 showed concentration-dependent binding with EC_{50} of 0.44 nM and 0.51 nM, respectively
- Cell killing potency remained the same (Cell-Titer Glo assay)

Radiolabeling and Immunoreactivity:

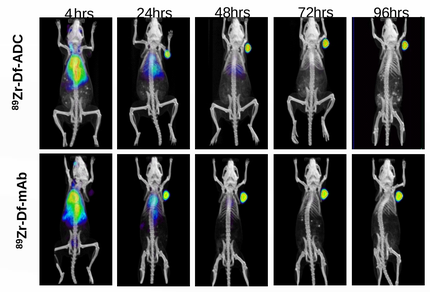
Radiolabeling w/ ^{89}Zr	Results
Total radioactivity	335.0 µCi
Total protein	50 µg
Labeling efficiency (ITLC, n=2)	99.4%
Specific activity	6.6 µCi/µg
Purification	
Radioactivity recovered	221.0 µCi
% of total radioactivity recovered	65.9 %
Purity (ITLC)	99.32%

rhAgFc: ADC1 Ratio Optimization		
	ADC1 peak (r.t.=11.1 min)	
Ratio	Df-ADC1 AUC	%-Remaining Df-ADC1
1:0	730	100%
1:0.7	457	63%
1:1	389	53%
1:1.6	56	8%
1:2	N/A	0%



ADC: PET/CT Imaging of Pancreatic Tumors:

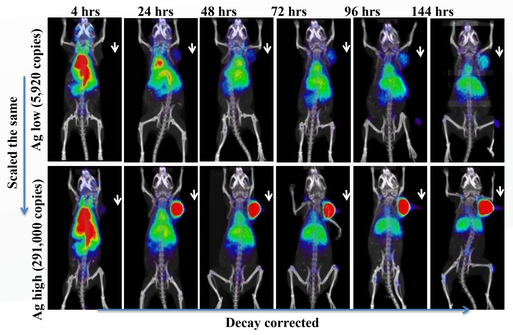
Sequential PET images from a single mouse scanned at the indicated time. Top panels show the mouse that was injected with ^{89}Zr-Df-ADC and the bottom panels shows the mouse that was injected with radiolabeled parent mAb, ^{89}Zr-Df-mAb. Images are scaled the same.



Imaging/biodistribution studies revealed 2-fold higher uptake of ADC in tumors compared to the unconjugated mAb

ADC1: PET/CT Imaging of High and Low Antigen Expressing NHL Tumor Models

Sequential PET images from a single mouse scanned at the indicated time. Representative Df-ADC1 exhibited excellent targeting in both low and high antigen expressing tumor models *in vivo* showing that low antigen expressing targets can be visualized with PET



Acknowledgements:

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Conclusions

- Process conditions were optimized to allow efficient conjugation of multiple ADCs .The desired chelator was consistently achieved and ADC aggregation was minimized
- The DAR, potency and binding affinity were not perturbed as shown with a panel of assays including RP-HPLC, SE-HPLC, binding by FACS and cell cytotoxicity prior to radiolabeling and by retention of immunoreactivity post-radiolabeling
- Representative Df-ADC showed excellent targeting of the HPAF-II tumors but the tumor accumulation was 2-fold higher (215% vs. 125% ID/g) than in the "parent" unconjugated mAb. Representative Df-ADC1 showed that both
- PET imaging using radiometal labeled ADCs is a promising approach to determine tumor targeting and tissue biodistribution in real time and for use as a patient selection tool