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Targeted Delivery of TLR-Agonists Using ADC Technology

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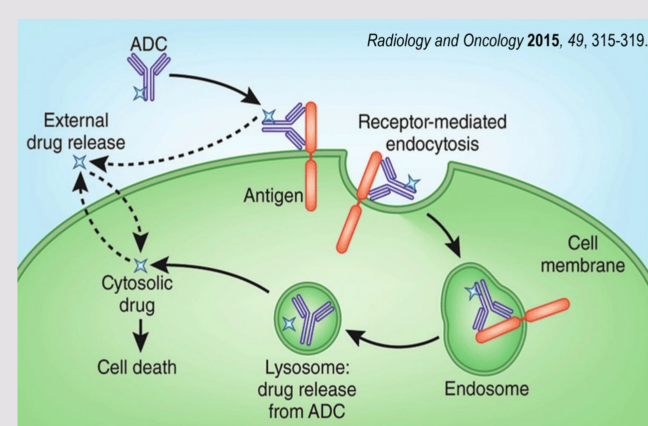
Abstract

Antibody-drug conjugates (ADCs) are a method of targeted drug delivery that transport a payload to a desired cell type. By directly targeting the cell type of choice, off-target effects experienced by non-diseased cells can be mitigated. Currently, the majority of clinical-stage ADCs are directed to tumor cells and contain a cytotoxic payload. In contrast, this work focuses on the design and evaluation of ADCs that deliver Toll-Like Receptor (TLR)-7/8 agonists to B-cells. TLR-7/8 agonists activate an endosomal receptor that leads to the activation of NFκB, which results in the production of proinflammatory cytokines. This forms a link between the innate and adaptive immune system by promoting T cell activity. We will present work that focuses on 3 aspects of these TLR-activating ADCs: 1) Evaluation of payload and linker-payload efficacy in B-cells; 2) Understanding ADC stability and catabolism; and 3) Evaluating the specificity and activity of the TLR-activating ADCs.

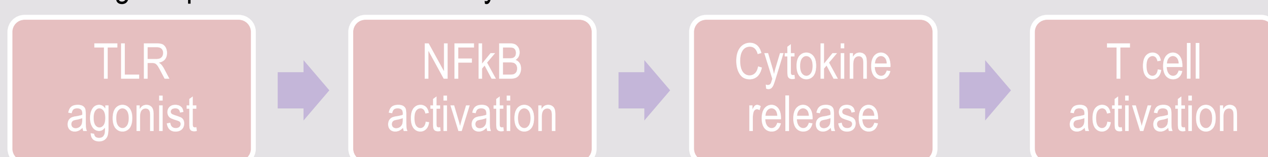
Nine payloads were synthesized and evaluated and found to exhibit sub-μM potency: Resiquimod, E66, and E104. Of these three, E104 had the highest potency followed by Resiquimod. Each payload was evaluated using three different linkers: mc, mc_ValCit, and mc_ValCitPABC. Mc has been shown to be non-cleavable linker via catabolism studies, while mc_ValCitPABC has been shown to be cleavable. Quanti-blue assays in Ramos Blue cells demonstrated that mc_E104 is the most potent linker-payload when attached to a B-cell targeting antibody. This non-cleavable ADC was significantly more potent than the corresponding cleavable (ValCitPABC) ADC. We will present catabolism and permeability data that may explain this unexpected finding.

Introduction

Antibody drug conjugates (ADCs) are targeted agents that deliver a drug only to cells that express antigens recognized by the cognate antibody. ADCs enter the cell via receptor-mediated endocytosis and are then trafficked to the lysosome, where they are degraded to release the attached payload. This type of delivery system is conventionally used for the delivery of cytotoxic drugs since it helps to alleviate off-target and side effects.



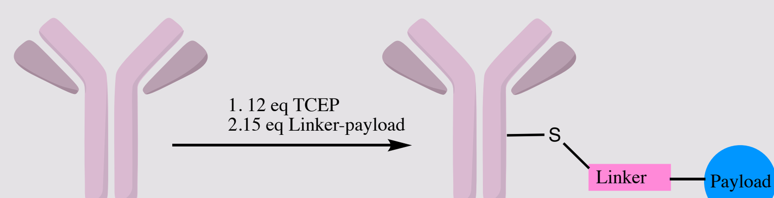
However, this work focuses on the delivery of Toll-Like Receptor (TLR)-7/8 agonists to B lymphocytes. The TLR-7/8 receptor is within the endosome, so upon degradation of the ADC, there is activation of the receptor. As opposed to cytotoxic drugs, TLR-7/8 agonists lead to the activation of a signal cascade that has a downstream affect of activating the transcription factor NFκB, which results in the production of proinflammatory cytokines. This forms a link between the innate and adaptive immune system by promoting T cell activity and ideally reprogramming the immune system to destroy the tumor using the patient's own immune system



This work utilizes an engineered B lymphocyte line that enables the analysis of NFκB activation which allows for the evaluation of various payloads, linker-payloads, and ADC efficacy. Additionally, mass spectrometry was utilized to characterize the stability and catabolism of these ADCs.

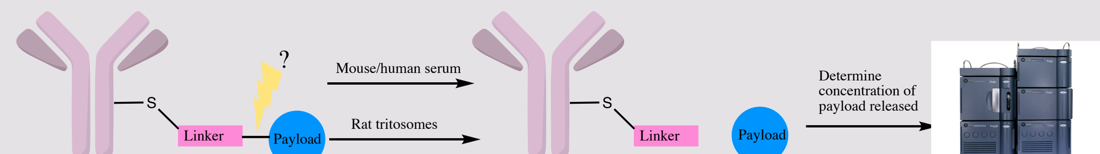
Methodology

ADC Synthesis



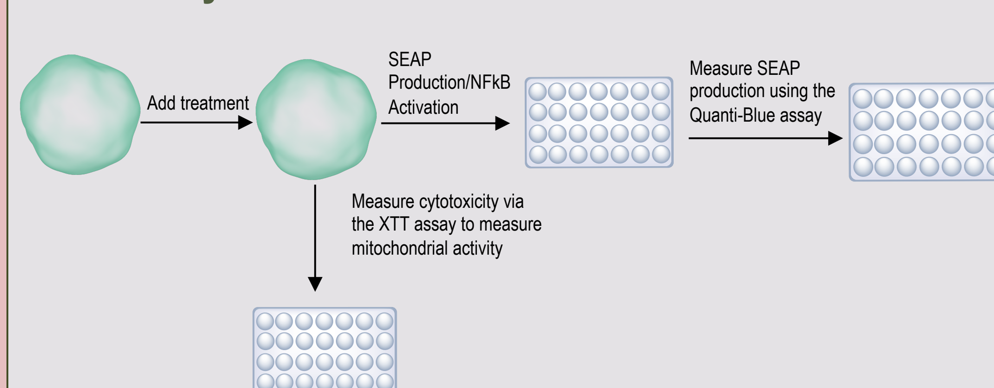
ADCs were synthesized using the conventional method of native cysteine conjugations. Drug-to-Antibody Ratio (DAR) between 6 to 8 was determined by Mass Spectrometry. Aggregation was checked by SEC.

Stability and Catabolism Studies



Stability studies were performed in both mouse and human serum over the course of 7 days. All time points were analyzed using a Tandem-Quad Mass spectrometer against a standard curve of the desired compound to determine the amount of payload release over that time period.
Lysosomal catabolism studies at pH 4.7 were performed over the course of 4 hours. All time points were analyzed using a Tandem-Quad Mass spectrometer against a standard curve of the desired compound to determine the amount of payload release over that time period.

Cell Assays:



The Ramos Blue cell line (a B-cell lymphoma) contains a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of NFkB. TLR7 agonists (or ADCs) were incubated with this cell line for 2-4 days to induce NFkB activation, and thereby promote SEAP expression. Cell media samples were incubated with the Quanti-Blue SEAP substrate to assess NFkB activation spectrophotometrically. Compounds or ADCs were generally incubated with the Ramos blue cells for 2-4 days. Cell viability was assessed using an XTT mitochondrial function assay.

Payload Potency:

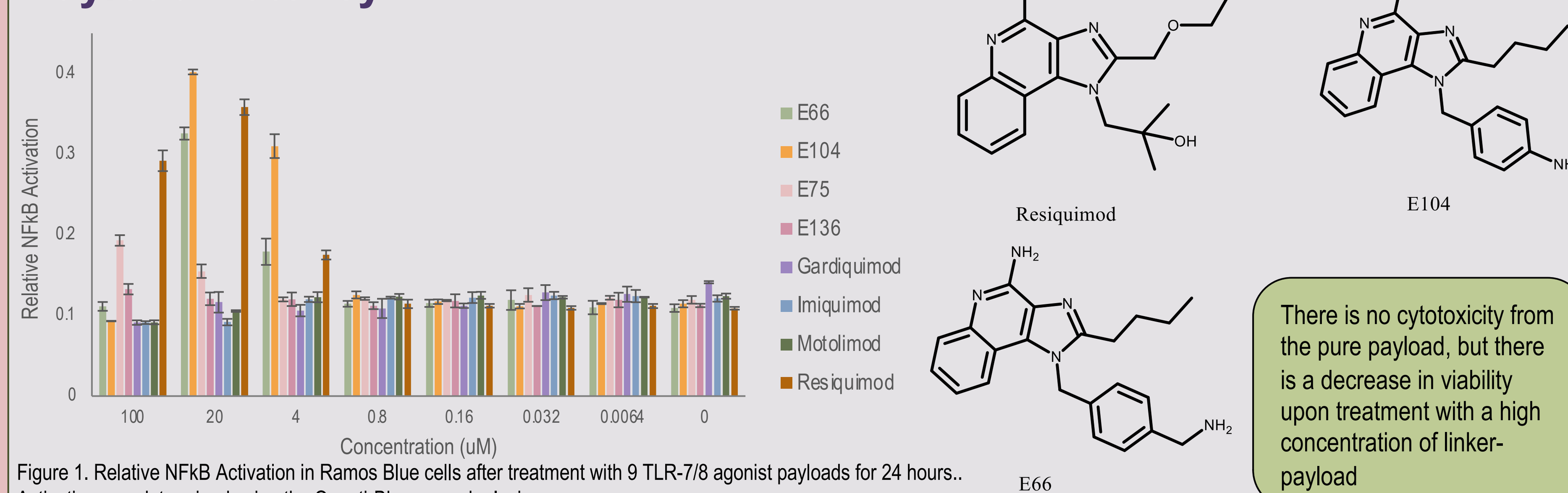


Figure 1. Relative NFkB Activation in Ramos Blue cells after treatment with 9 TLR-7/8 agonist payloads for 24 hours.. Activation was determined using the Quanti-Blue assay by Invivogen

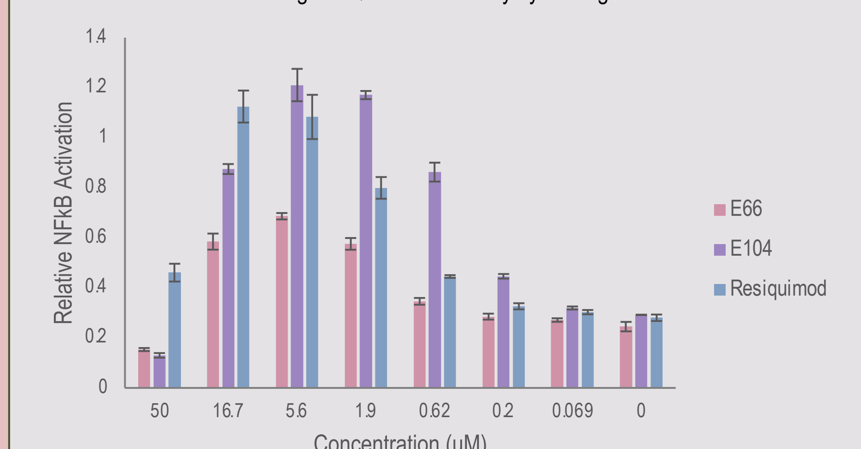


Figure 2. Relative NFkB activation of Ramos Blue Cells after treatment with the 3 most active TLR-7/8 agonist payloads for 72 hours.

There is no cytotoxicity from the pure payload, but there is a decrease in viability upon treatment with a high concentration of linker-payload

Figure 3. Percent viability of Ramos Blue cells after a 3-day incubation with 3 TLR-7/8 agonist payloads

Linker-payload activity:

Table 1. Relative activation of NFkB in Ramos Blue cells upon treatment with linker-payload at a concentration of 5 uM

Linker-payload	Relative Activation	STD
Mc_E104	704	10.8
Mc_ValCit_E104	237.56	17.83
Mc_ValCitPABC_E104	12.73	18.4
Mc_E66	118.6	22.26
Mc_ValCit_E66	124.81	22.41
Mc_ValCitPABC_E66	7.15	13.37
Mc_Resiquimod	17.58	22.47
Mc_ValCit_Resiquimod	11.41	12.92
Mc_ValCitPABC_Resiquimod	18.62	20.23

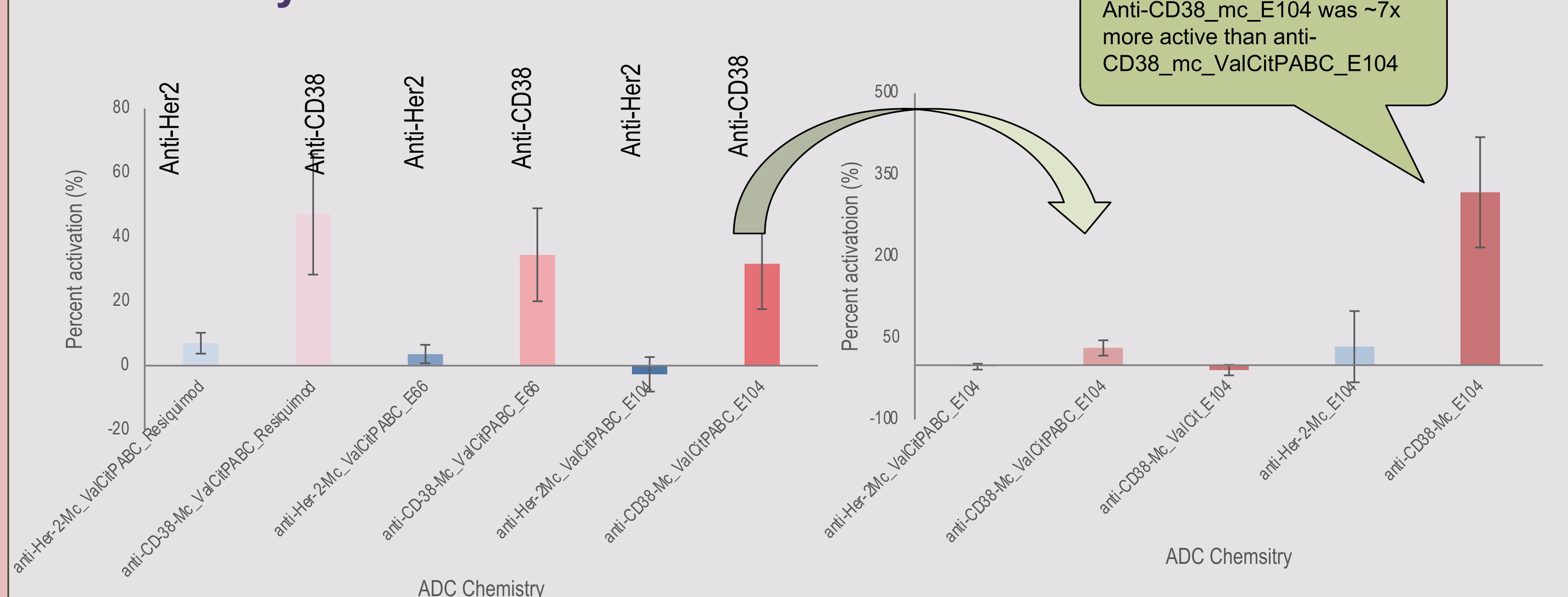
Figure 4. Percent viability of Ramos Blue cells after a 3 day incubation with select linker-payloads

Antibody selection:

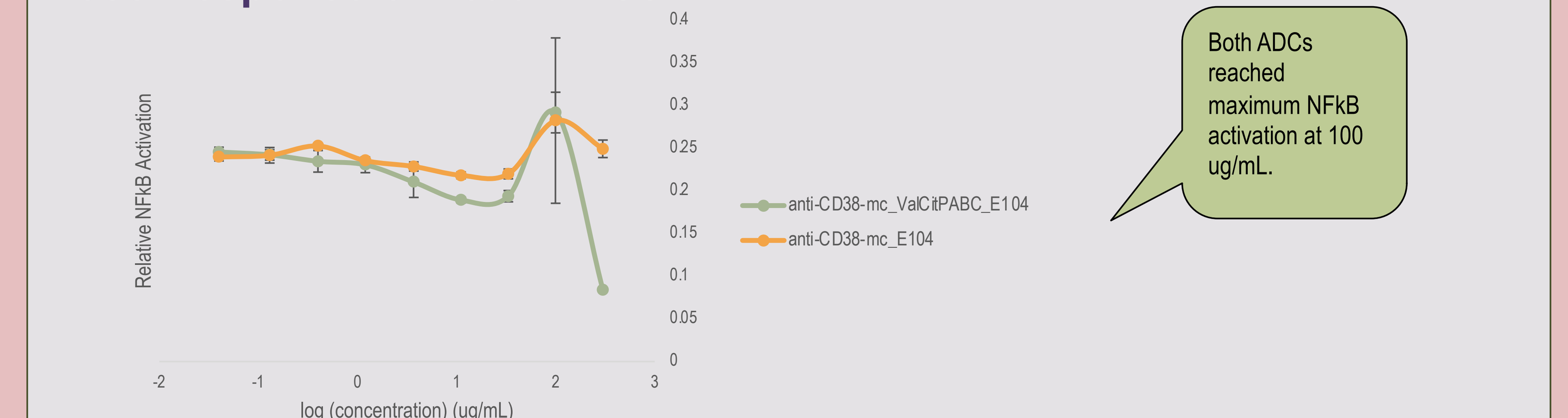
Target	MFI fold enhancement	IC_{50} of MMAE ADC against Ramos cells
Her2	1.46x	**10.5** (6.6-17.7)
VEGFR2	1.48x	**1.74** (0.39-18.8)
IL6-R	1.61x	**1.18** (0.41-5.2)
CD-38	6.7x	**0.12** (0.085-0.174)
CD-20	5.0x	**0.76** (0.45-1.3)
CD79b	3.2x	**0.42** (0.31-0.56)
TNFα	TBD	**2.22** (0.48-14.5)
CD11a	TBD	**1.56** (0.48-6.03)

Anti-CD-38 was selected as the targeted antibody since the naked antibody did not result in any activation of the NFkB pathway, but the ADCs did.

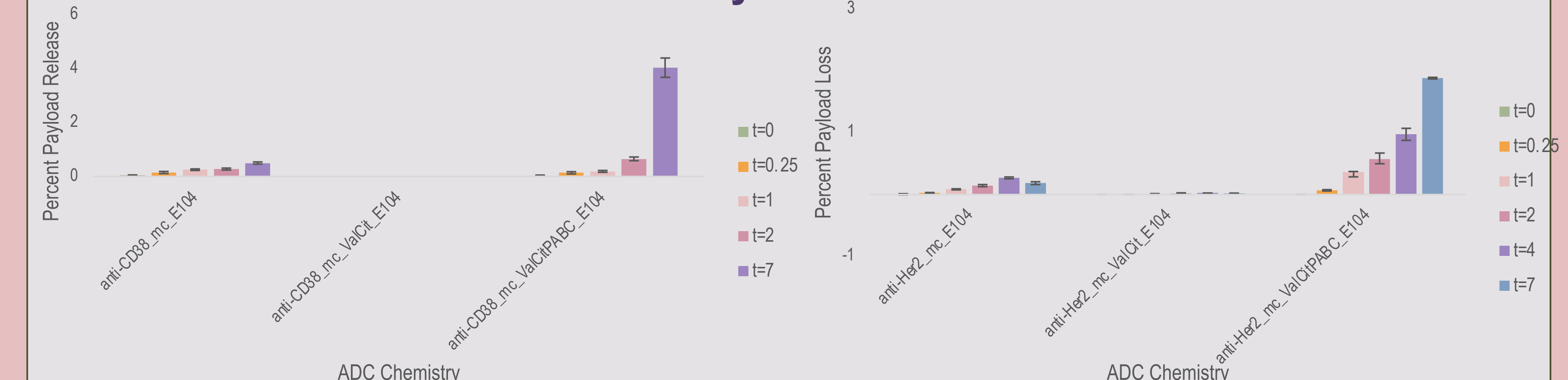
ADC efficacy:



Dose Response of Lead ADCs

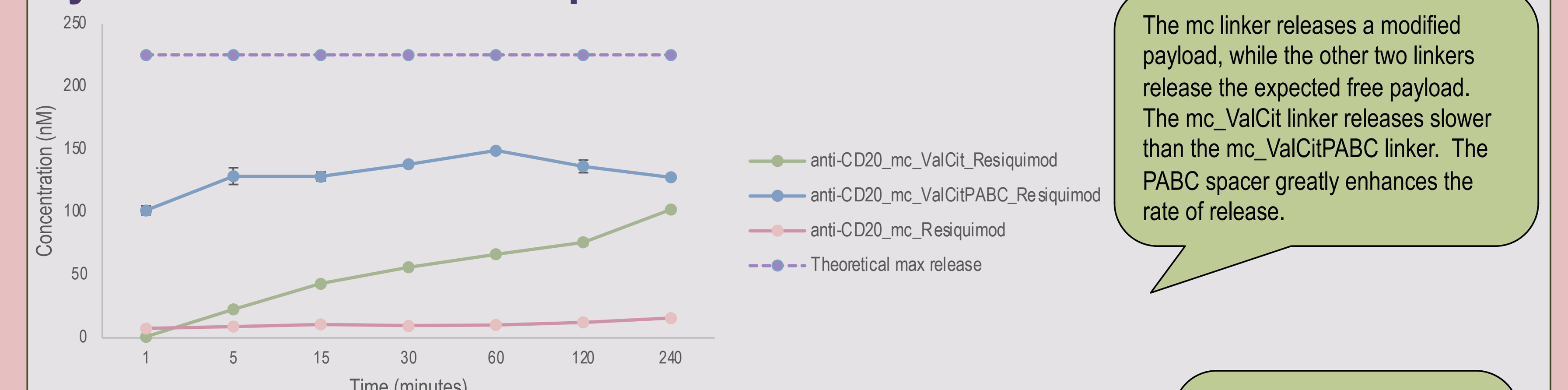


Mouse and Human Serum Stability

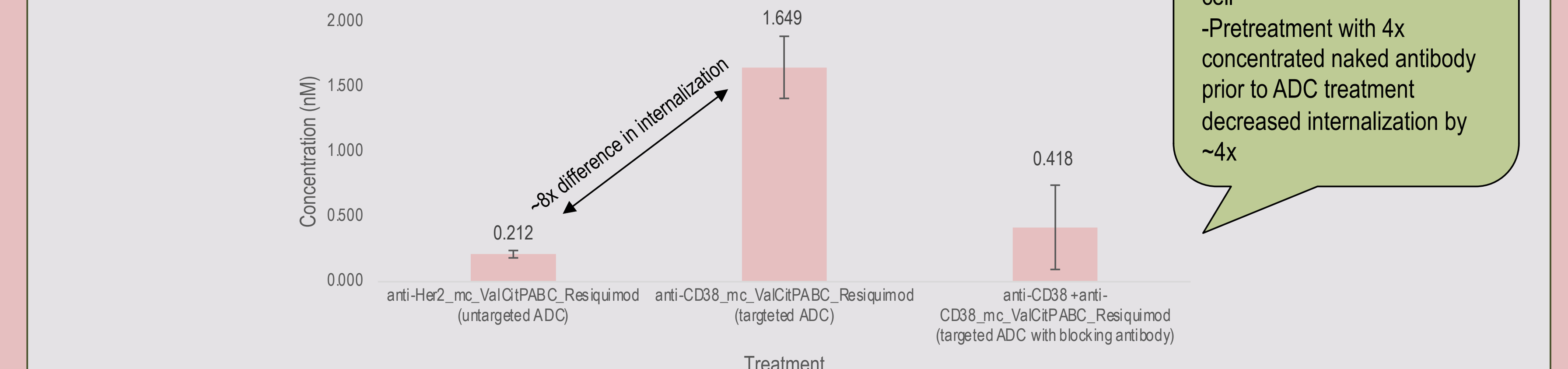


ADCs exhibited very little release of payload over the course of 7 days when incubated at 37°C in mouse and human serum. Incubation in mouse serum results in <10% release and incubation in human serum had a negligible amount of release (not shown).

Lysosomal catabolism at pH 4.7



Cellular catabolism



Conclusions

3 TLR-7/8 agonists were identified as active in Ramos Blue cells at a range of concentrations. Studies to determine the ideal targeted antibody for Ramos Blue cells concluded anti-CD38 to be the targeted antibody, with the non-targeted control being anti-Her-2. Further tests identified the mc_E104 linker payload to be the most potent as both a linker-payload and ADC. Cleavable linkers also demonstrated activity within the assay. Both the targeted and non-targeted ADCs are stable in human and mouse serum over the span of 7 days.
Cellular catabolism results reiterated the selectivity by a nontargeted antibody to have ~8x less payload within the cell. A decrease in payload in the cell can also be shown when to pretreate the cells with naked antibody shows. This shows the process of internalization is via receptor-mediated endocytosis.
Future work includes determining amount of cytokine induction from ADCs and indirect delivery of ADCs to activate T cells.

Acknowledgements

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