

Computational and Experimental Design of Precision Therapeutic and Diagnostic Agents

by

Reginald Evans

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Chemical Engineering)
in the University of Michigan
2022

Doctoral Committee:

Associate Professor Greg M. Thurber, Chair
Assistant Professor Sasha-Cai Leshner-Perez
Professor Jennifer J. Linderman
Associate Professor Fei Wen

Reginald C. Evans

regevans@umich.edu

ORCID iD: [0000-0002-0175-9751](https://orcid.org/0000-0002-0175-9751)

© Reginald C. Evans 2022

Dedication

This dissertation is dedicated to all who have made great sacrifices to get me here. My mother, who dedicated her life to get me an education. My sisters, Lauren and Alanah who supported me when I struggled through this process, my mentors for putting me on this path and finally my friends throughout the years who have shaped me into the person I am now.

I would also like to dedicate this PhD. to all black and brown people who are struggling to pursue their dreams – academic or otherwise. The path can be lonely, and often difficult. I hope seeing me reach this milestone, inspires you to continue your path.

Acknowledgements

First, I would like to thank my advisor Prof. Greg Thurber for the opportunity to work in his lab. There has been a lot of ups working in the lab from news of publications and fellowships and downs like my academic probation. Greg has been a strong advocate and great source of support for me. His patience, understanding and eagerness for science motivated me to stay in the program when I grew discouraged. I would also like to thank my committee members Prof. Linderman, Prof. Nagrath and Prof. Tessier for their support and scientific discussions. I would also like to acknowledge the funding from the National Institute of Health, National Science Foundation, Department of Defense, National Institute of Arthritis Musculoskeletal and Skin Diseases, and Rackham Graduate School which made my doctoral studies possible.

Next, I would like to thank the Thurber lab past and present for being an amazing place to work and all the members who made it that way. This includes, Liang Zhang, Cornelius Cilliers, Sumit Bhatnagar, Tejas Navaratna, Lydia Atangcho, Eshita Khera, Bruna Menezes, Ian Nessler, Mukesh Mahajan, Anna Kopp, Marshall Case, Jane Kwon, Celia Dong, Melissa Calopiz, Baron Rubahamya, Tiexin Wang Haolong Huang and Jiakun Guan. All of you have made my time here meaningful through excellent discussions, and most of all teaching me new techniques in the lab. Special shout outs go to Tejas for our deep philosophical discussions in and outside of lab, Eshita for her deep and wide knowledge of the literature, Ian for his unique Iowan humor and Lydia for her deep strength and character. I do not think you know how much I respect you and your journey, and I am unsure if I can find the words to adequately express it.

Outside the lab I appreciate the support and friendship of my cohort, especially Alexander Adams, Heather Fairbairn, Paymon Shirazi, Brian Macdonald, Kathy Macdonald, Joey Valle, Niarra Coleman, Steven Chavez, Alison Banka, Emma Brannon, Carlos Rodriguez and Abdulla Alqubati. You've all kept me grounded, provided me with valuable perspectives, reminded me there is more to life than the lab and made Michigan a fun experience for me.

I appreciate all of my graduate mentors past and present who I could reach out to for my professional pursuits and mental health: Dr. Susan Montgomery, Dr. Lola Eniola, Dr. Laura Hirshfield and Brooke Brantley Gilbert. These women do amazing work and supported me during my lowest points.

I also appreciate my key mentors of my youth. Michael Stigmon, for inspiring me that I could do science, Felipe Galicia, for exposing me to the world of biotechnology and indirectly putting me on this path, Dr. Ian Wheeldon for showing me how engineers can impact biology and medicine, Dr. Phil Christopher for recommending the University of Michigan and finally Dr. Kawai Tam, for being uncompromising and always pushing me to do my best – especially when I did not want to.

Finally, I want to thank my family who has encouraged and supported me every step of the way. My mom for making all the sacrifices in her life, to ensure I would have the best education available, my grandmother for supporting my studies, and my sisters, Lauren and Alanah for being there and making sure I could count on them at any time. Of course there is my niece Brianna who was always a beacon of light and joy even at the darkest times. This PhD is for us and I am blessed to have you all in my life.

Table of Contents

Dedication	ii
Acknowledgements	iii
List of Tables	viii
List of Figures	ix
List of Appendices	xi
Abstract	xii
Chapter 1 Introduction	1
1.1 Motivation for development of Anticancer therapies	1
1.2 Antibody Drug Conjugates.....	4
1.2.1 Antibody	5
1.2.2 Linker	5
1.2.3 Cytotoxic Payload	6
1.2.4 Target.....	7
1.2.5 ADC Resistance.....	8
1.3 Tumor Microenviroment	8
1.4 Computational Models to capture reaction kinetics and drug distribution	9
1.4.1 Kinetic Models	9
1.4.2 Models for Drug Delivery and Distribution	12
1.5 Motivation for development of Diagnostic Imaging Agents	14
1.6 Methods for Diagnosing Rheumatoid Arthritis.....	15

1.7 Route of Administration.....	18
1.8 Depth of Penetration.....	20
1.9 Lipinski's Rule of Five for Oral Delivery	20
1.10 Design of Orally Available Imaging Agents.....	21
1.10.1 High Oral Absorption and High Specific Targeting.....	22
1.10.2 Ideal In vivo Optical Properties.....	22
1.10.3 Target Ligand Selection	23
1.11 Treatment Monitoring of Rheumatoid Arthritis.....	23
1.12 Thesis Summary	25
Chapter 2 Design of High Avidity and Low Affinity Antibodies for In Situ Control of Antibody Drug Conjugate Targeting	26
2.1 Publication Information.....	26
2.2 Abstract	26
2.3 Motivation	27
2.4 Materials and Methods	30
2.4.1 Derivation of Dimensionless Competition Number	39
2.4.2 Derivation of Equilibrium Binding	42
2.4.3 Derivation of Thiele Modulus	45
2.5 Results	45
2.6 Discussion	60
2.7 Conclusion.....	64
Chapter 3 Insights into Design of Bispecific Antibodies Using Computational Modeling.....	65
3.1 Information.....	65
3.2 Abstract	65
3.3 Motivation	66
3.4 Materials and Methods	68

3.5 Results	71
3.6 Discussion	77
3.7 Conclusion.....	80
Chapter 4 Orally Available Imaging Agents for the Early Detection and Treatment Monitoring of Rheumatoid Arthritis	81
4.1 Information.....	81
4.2 Abstract	81
4.3 Motivation	82
4.4 Materials and Methods	85
4.4.1 Synthesis of Imaging Agents.....	85
4.5 Results	88
4.6 Discussion	93
4.7 Conclusion.....	96
Chapter 5 Concluding Remarks and Future Directions	97
5.1 Summary of Research Findings	97
5.2 Future Work and Directions	100
Appendices.....	105
Bibliography	108

List of Tables

Table 1.1 Approved ADCS in the clinic in 2021.....	3
Table 1.2 Flow Table to Develop a Kinetic Model	10
Table 1.3 Comparison of RA Diagnostic Tests.	18
Table 2.1 Constants used in ADC/HALA Models.....	35
Table 2.2 Variables used in ADC/HALA Models	35
Table 3.1 Constants Used in BsAbs Model	71
Table 3.2 Variables Used in BsAbs Model.....	71
Table 4.1 Pharmacokinetic Properties of Proposed Imaging Agents	89
Table A.1 Base case parameters used for Figure 2.2C	105
Table B.1 Purification methods and final product of Imaging agents	106

List of Figures

Figure 1.1 Structure of an ADC and Mechanism of Action	4
Figure 1.2 Diagram of the Krogh Cylinder with four steps of tumor localization.	13
Figure 1.3 Popular Routes of Administration.	19
Figure 1.4 Criterion for an Ideal Near Infrared Imaging Agent	21
Figure 2.1: Improving ADC distribution through coadministration of HALA antibody Administration of an ADC.....	29
Figure 2.2 Competition Kinetics between ADCs and HALA antibodies.	31
Figure 2.3 Validation of Monolayer Simulations. Validation of monolayer simulations of different antibody clones at different cell expression.	46
Figure 2.4 Thermodynamic and Kinetic Control in Simulations.....	47
Figure 2.5 P vs Ψ graphs at varying HALA antibody K_d	48
Figure 2.6: Simulating ADC Distribution in Spheroids.....	50
Figure 2.7. Total Concentration of ADC at center of spheroid vs Ψ and Φ^2	51
Figure 2.8 Simulating distribution of ADC payloads in vivo.	52
Figure 2.9. Distribution of ADC and Payload of co-administered ADC with HALA antibody showing changing concentration and binding affinity in Krogh Cylinder simulations after 48hrs.....	53
Figure 2.10 Krogh Cylinder simulations of a non-bystander payload at 8:1 dose of HALA and ADC with a 3.6mg/kg ADC dose.	54
Figure 2.11 Krogh Cylinder simulations of a bystander payload at 16:1 dose of HALA and ADC with a 1.8mg/kg T-MMAE dose.	55
Figure 2.12 Distribution of ADC and Payload of co-administered ADC with HALA antibody showing changing concentration and binding affinity as a function of Ψ and Φ^2 in Krogh Cylinder simulations after 48 hours.	56
Figure 2.13 Design of optimal HALA with changing antibody dose and affinity after 48hrs.	58

Figure 2.14 Design of Optimal HALA with changing Antibody Dose and Affinity as a function of Ψ and Φ^2 in Krogh Cylinder simulations after 48 hours.	59
Figure 3.1 Competition kinetics between binding arms of a bispecific antibody.....	69
Figure 3.2 Simulated binding profiles of a bispecific antibody.....	72
Figure 3.3 T-cell Activation assay of 3 bispecific antibodies of different binding affinity.....	73
Figure 3.4 Comparison of Crosslinking between invitro and in silico T-cell activation assays of 3 bispecific antibodies of different binding affinity	74
Figure 3.5 Comparison of Crosslinking between invitro and in silico T-cell activation assays of 3 bispecific antibodies of different binding affinity 3 different PD-1 expression levels and different correction factors.	75
Figure 3.6 Comparison of “optimal” Crosslinking in silico T-cell activation assays between 3 sets of reported binding affinity for 3 bispecific antibodies of different binding affinity across 3 different PD-1 expression levels.....	76
Figure 4.1 Structures of Proposed Imaging agents used for early detection and treatment monitoring of Rheumatoid Arthritis	89
Figure 4.2 Subcutaneous and Intravenous Administration of Alendronate 680 RD after 24 hours in healthy mice.....	90
Figure 4.3 Subcutaneous and Intravenous Administration of Folate Cadaverine 680 RD after 24 hours.....	91
Figure 4.4 Subcutaneous Oral and Intravenous Administration of Integrin 800 CW after 48 hours in AIA rat models.....	92
Figure 4.5 Subcutaneous Administration of Integrin 800 CW and Alendronate 680 RD after 48 hours in AIA rat models.....	93
Figure B.1 HPLC Purity Traces of Imaging Agents.....	106
Figure B.2 Maldi Traces of Imaging Agents.	107

List of Appendices

Appendix A Supporting Information for Chapter 2.....	105
Appendix B Supporting Information for Chapter 4.....	106

Abstract

This dissertation investigates the physicochemical and pharmacokinetic factors that affect the distribution and efficacy of antibody therapies and molecular imaging agents. Antibodies are large proteins produced by the immune system that bind and neutralize foreign pathogens but can be engineered to engage disease targets for therapy. Molecular imaging agents are small molecule probes that visualize characterize and quantify biological processes at the molecular, cellular, and anatomical levels in humans and other living systems for purpose of disease detection. In this work, the delivery of both of these agents is described through a multiscale mechanistic approach, examining theoretical and experimental *in vivo* distribution at the molecular, cellular, tissue, organ, and whole-body levels.

First, a computational model was developed to examine the effect of an engineered High Avidity Low Affinity (HALA) antibody carrier dose on the distribution Antibody Drug Conjugates (ADCs) in high and low expressing HER2 cancers. HALA antibodies are designed to compete with the binding of ADCs, forcing them deeper into tumor tissue to improve efficacy in high HER2 expression tumors. However, they do not compete with binding on low expression cancer cells where the ADC needs to bind all receptors for efficacy. A computational kinetic model was developed to simulate the competition, and a dimensionless number that captures the ratio between antibody competition and internalization was developed. The kinetic model was then adapted to include diffusive transport in a spheroid and *in vivo* Krogh cylinder simulations. Using these models, the simulations demonstrated that the HALA carrier dose will automatically adjust competition based on HER2 expression to maintain high efficacy of the ADC. The

dimensionless number can be used to design HALA antibodies with the needed binding kinetics to achieve maximum efficacy independent of target expression level.

Next, the computational model of multivalent binding was applied to simulate the competition and distribution of bispecific antibodies. Briefly, the physiochemical properties of the bispecific antibody and cellular expression levels were varied to capture the amount of antibody crosslinking in an immune synapse. The simulated crosslinking was then validated by comparing results to an *in vitro* T-cell activation assay. Further mechanistic details are needed, but the model can capture *in vitro* results to describe cellular activation.

Finally, guided by computational results indicating low molecular weight probes provide optimal contrast, molecular imaging agents were developed for the early detection and treatment monitoring of Rheumatoid Arthritis (RA). RA is an autoimmune disease that leads to largely irreversible joint damage over time, but effective treatments are available. Thus, there is interest in early detection of RA to prevent further damage, and some studies have even indicated that the disease could be cured if detected early. However, current methods lack the sensitivity to detect RA at an early stage and lack the ability to differentiate RA from other arthritic diseases. Subcutaneous delivery of co-administered Alendronate 680 RD and Integrin 800 CW in an adjuvant induced arthritis rat showed increased uptake in the joints of diseased rats compared to healthy rats. While the imaging agents were unable to detect RA in rats at early (subclinical) time points, the probes were able to detect inflammation of arthritis following oral and subcutaneous delivery.

In summary, computational and experimental approaches to evaluate the pharmacokinetics and efficacy of antibody therapeutics and small molecule imaging diagnostics can be used to identify key considerations and design more effective agents.

Chapter 1 Introduction

This Thesis deals with two distinct research thrusts. The first is using computational modeling to improve distribution and efficacy of anticancer therapeutics (Antibody Drug Conjugates and bispecific antibodies); the second is developing using in vivo approaches to develop imaging agents for early detection of Rheumatoid Arthritis. Due to their distinct differences, each thrust will have an individual introduction and background content.

1.1 Motivation for development of Anticancer therapies

Cancer is the second leading cause of death, contributing to approximately 10 million deaths each year (1). While there are many cancer therapies available in the clinic, including immunotherapy, radiation, laser therapy, and others, chemotherapy followed by surgical removal of the tumor is still the most common intervention (2). Chemotherapy uses potent anticancer molecules in order to kill diseased cancer cells; however, chemotherapy lacks specificity and also kills healthy cells, contributing to harsh side effects. Current chemotherapy advances aim to reduce toxicity and increase the therapeutic index by reducing the minimum effective dose while increasing the maximum tolerable dose (3). However, due to the limited specificity towards cancer cells, more targeted alternatives have been explored (4).

Antibodies are one type of targeted therapy for cancer. Antibodies are large proteins produced by the immune system than target and neutralize foreign pathogens (5). The IgG or immunoglobulin G is the most common antibody in the blood stream. IgGs are 150 kDa “Yshaped” proteins and have two binding arms known as the Fab or fragment antigen region,

allowing IgGs to bind an antigen with high specificity. IgGs also have a crystallizable fragment (Fc) region which allows them to interact with other immune cells.

Antibody therapies are versatile and can combine different mechanisms of action (6). They are engineered to have high binding affinity and specificity towards tumor-related antigens. After binding to the antigen target, some antibodies like trastuzumab and cetuximab can block receptor dimerization, leading to cell apoptosis (6). Additionally, antibody binding can cause internalization, degradation, and downregulation of cell receptors (7). The Fc region of antibodies can direct the immune system to target and kill tumor cells through various mechanism of action (8). Macrophages can phagocytose tumor cells by binding the Fc region through Fc γ receptors (Fc γ R) (9). Antibodies that bind to tumor cells can also bind to C1Q complement protein, which causes a complement cascade and complementary dependent cytotoxicity (CDC), or antibodies bind to Natural killer cells with the Fc γ RIIIa causing antibody dependent cell mediated cytotoxicity (ADCC) through lysis of the targeted tumor cell (8).

Another antibody therapy class is Antibody Drug conjugates (ADCs). ADCs combine the specificity of antibodies and the cell killing potential of small molecule cytotoxic drugs to further improve targeting of cancer tissue (10). Indeed, with 12 Food Drug Administration (FDA) approvals (**Table 1.1**) and hundreds more in clinical or preclinical development, ADCs are one of the most actively researched therapeutic class in oncology today (11, 12).

However, ADC development for solid tumors remain challenging with a 90% failure rate in the clinic (13). These high failure rates are owed in part to the poor ADC distribution, complex Pharmacokinetics (PK) and Pharmacodynamics (PD), and variability in the tumor microenvironment. In the clinic, little ADC reaches the tumor, with less than .01% of injected

dose of ADC reaching 1 gram of tumor (4). Better understanding of the dynamics and mechanism of ADC distribution can reduce the failure rate.

Traditionally, animal models have been used when testing for the efficacy of new drugs and dosing regimens; however, these models are time consuming, expensive, and poorly translate to humans due to differences in species' metabolism (14). Computational models have been developed to guide design of experiments, hasten drug development, and better understand drug and dosing regimens. While simple models are effective in understanding ADC distribution during monotherapy, these models fail to capture more complex phenomena, such as coadministered dosing regimens, alternative antibody constructs, and distribution at various length scales. Thus, more advanced models are required to capture these dynamic systems.

Brand name	Active ingredient	Indication(s)	FDA approval date	Company by BLA submission
Adcetris	brentuximab vedotin	Hodgkin lymphoma; systemic anaplastic large cell lymphoma	2011	Seattle Genetics
Kadcyla	ado-trastuzumab emtansine	HER2-positive, metastatic breast cancer	2013	Genentech (a Roche company)
Besponsa	inotuzumab ozogamicin	relapsed or refractory B-cell precursor acute lymphoblastic leukemia	2017	Wyeth Pharmaceuticals (a Pfizer company)
Mylotarg	gemtuzumab ozogamicin	newly-diagnosed CD33-positive acute myeloid leukemia (AML); relapsed or refractory CD33-positive AML	2017 (initial approval in 2000)	Wyeth Pharmaceuticals (a Pfizer company)
Lumoxiti	moxetumomab pasudotox-tdfk	relapsed or refractory hairy cell leukemia	2018	Innate Pharma/AstraZeneca
Enhertu	fam-trastuzumab deruxtecan-nxki	unresectable or metastatic HER2-positive breast cancer	2019	Daiichi Sankyo/AstraZeneca
Padcev	enfortumab vedotin-ejfv	locally advanced or metastatic urothelial cancer	2019	Astellas Pharma/Seattle Genetics
Polivy	polatuzumab vedotin-piiq	relapsed or refractory diffuse large B-cell lymphoma	2019	Genentech (a Roche company)
Blenrep	belantamab mafodotin-blmf	relapsed or refractory multiple myeloma	2020	GlaxoSmithKline
Trodelyv	sacituzumab govitecan-hziy	unresectable locally advanced or metastatic triple-negative breast cancer; locally advanced or metastatic urothelial cancer	2020	Immunomedics (a Gilead Sciences company)
Tivdak	tisotumab vedotin-tftv	recurrent or metastatic cervical cancer	2021	Seagen
Zynlonta	loncastuximab tesirine-lpyl	relapsed or refractory large B-cell lymphoma	2021	ADC Therapeutics

Table 1.1 Approved ADCs in the clinic in 2021 (15)

1.2 Antibody Drug Conjugates

ADCs consist of three main parts: 1) antibody, 2) payload, and 3) linker (**Figure 1.1 A**). The antibody specifically targets cell receptors expressed on cancer cells. The payload is typically a cytotoxic small molecule drug that kills cells by disrupting cell proliferation via DNA alkylators, DNA crosslinkers or microtubule inhibitors. The linker connects the antibody to the payload and are of two types: cleavable and non-cleavable. Cleavable linkers release the payload once inside the cell's lysosomes (16), while non cleavable linkers will only release the payload once the antibody has been fully internalized and degraded (17). This occurs as a final step in ADC delivery. After intravenous infusion, ADCs extravasate out of the blood vessels into the tumor, diffuse through the interstitium, and bind to the cancer receptor cells. Once they bind, they are internalized, and the antibody degrades in the lysosomes releasing the free payload or payload linker, which binds to the cell target to initiate apoptosis (**Figure 1.1 B**).

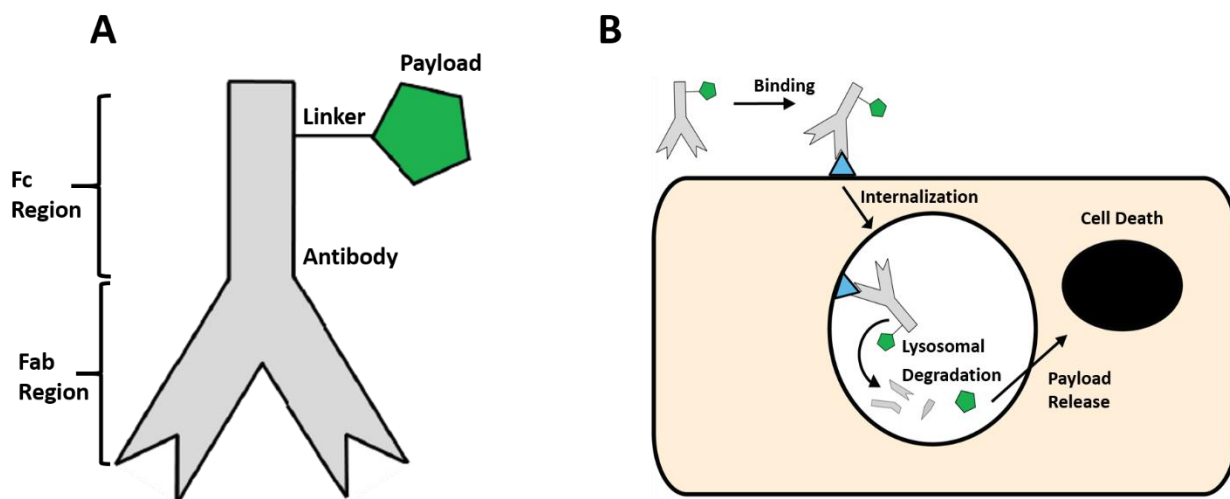


Figure 1.1 Structure of an ADC and Mechanism of Action

(A): The Fab region is the area where the ADC binds to the target and the FC region is where the ADC interacts with immune cells to conduct and immune response. (B): The ADC binds to its target antigen, internalizes, degrades, and releases its payload. Once the payload is released it can exert its cytotoxic effect on the tumor cell.

1.2.1 Antibody

Antibodies are large proteins that are produced by the immune system to combat foreign pathogens in the body. Antibody physiochemical properties can be engineered to bind to select cancer targets with high affinity and specificity. However, changing the physiochemical properties of antibodies can influence the pharmacokinetics of ADCs, affecting the uptake and overall distribution, ultimately altering the efficacy of the ADC. Two key properties affecting ADC efficacy are target binding affinity and size (18,19). Sufficient binding affinity to the target is required for efficacy, as it ensures uptake and internalization of the ADC in the tumor cells. Most approved antibodies and ADCs have effective binding affinities with a nanomolar K_d range ($<10^{-7}$ M). For example, the HER2 targeting antibody trastuzumab, has K_d of 5nM (10^{-9} M) (20). Antibody size is another key property for efficacy of these therapies as size can affect systemic clearance and penetration into the tumor (19,21). Full length antibodies have long half-lives of several days to several weeks compared to small molecules that have half lives in minutes to hours (22). However, due to poor diffusion and low doses, antibodies bind to their targets near blood vessels and do not diffuse deep into the tumor tissue (23,24). Some antibody research is developing various formats to overcome the diffusion limitations such as using antigen binding fragments (50 kDa), single chain, or single domain fragments (25 kDa or 15kDa respectively) instead of the full 150 kDa antibody (25-27). To compensate for the short half-lives of these fragment, various half live extension strategies have been applied (28).

1.2.2 Linker

There are two broad categories of linkers: cleavable and non-cleavable. Cleavable linkers are the preferred choice used in most ADCs; however, they pose greater risk of being cleaved in the plasma instead of inside the tumor cell. Non-cleavable linkers, however, only release the

payload after the ADC has been internalized in the cell and degraded in the lysosomes. A disadvantage to non-cleavable linkers, however, is that payloads are released as an adduct with the linker, which for payloads such as MMAE, is not as effective as its original form. Research has been focused on creating more stable linkers, but less stable linkers have found success, such as sacituzumab govitecan. This ADC targets trophoblastic cell-surface antigen (Trop2) found in many epithelial cancers, and is approved for clinical use (29). While the exact mechanism behind this success is still unclear, researchers are actively looking to understand advantages and disadvantages of each type of linker.

1.2.3 Cytotoxic Payload

Payloads provide the primary mechanism of action for ADC efficacy. In order to achieve efficacy via delivery by cell surface receptors, payloads must be highly potent, which can also result in toxicity. For this reason, FDA approved payloads are too toxic to be administered alone (untargeted) due to their low therapeutic index (30). Indeed, the low potency payloads already used in chemotherapy such as doxorubicin were a main reason for failure in first generation ADCs (31). Second generation ADCs use more potent payloads such as indolinobenzodiazepine DNA-alkylating agents (DGN) or microtubule-inhibiting emtansine (DM1). Some of these payloads proved too potent, and therefore toxic, limiting the dose that could be given (and therefore limiting tumor penetration). Therefore, next generation payloads balance the potency with delivery for an improved therapeutic index.

Payloads in ADCs typically impact DNA, microtubulues, or replication machinery that is important for cell proliferation. Thus, disrupting these functions can lead the cells to apoptosis, senescence, or necrosis. (32). The main classes of payloads include microtubules inhibitors (maytansinoids and auristatins), DNA alkylators (pyrrolobenzodiazepine), topoisomerase

inhibitors (SN-38), DNA-intercalators (anthracycline agents), and DNA double strand break inducers (calicheamicin) (33). Beyond toxicity, payloads must meet other requirements for efficacy such as solubility, stability in the plasma, lipophilicity, and immunogenicity risk (34). Once conjugated onto the antibody by a linker, the payload should not change antibody stability that causes premature release or faster clearance of the ADC. A key factor that affects clearance rates in payloads is the Drug to Antibody Ratio (DAR) or the number of payloads per antibody. ADCs with higher DARs often have a faster deconjugation and/or clearance rate than ADCs with a lower DAR (35). The DAR can also have an impact on distribution of ADCs in the tumor. Lower DAR values allow higher ADC doses (greater tolerability) since toxicity is driven by the payload dose, and increased antibody doses have shown improved responses in some ADC treatments (36). Lipophilicity can also impact distribution and uptake, as lipophilic payloads can diffuse across cell membranes, into neighboring cells causing more cell death. This process is called the bystander effect. Payloads with the bystander effect can improve efficacy, compared to hydrophilic payloads that do not easily permeate outside the cell membrane. The bystander effect's role in toxicity is complex, allowing uptake into healthy tissue but also washout of payload from healthy cells.

1.2.4 Target

Target selection is critical for the success and efficacy of ADCs; target expression, the potential for shedding, internalization rates, and trafficking dynamics are key factors to be considered. An optimal target is typically overexpressed on the surface of cancer cells and absent or has reduced expression on healthy cells. Ideally the target is not shed into the interstitial tissues causing off target binding and a decrease in efficacy. Lastly the receptors must be internalized and trafficked to lysosomes for ADC/linker degradation, releasing the payload to kill

cells (37). Human epidermal growth factor receptor 2 (HER2), for example, is over expressed in 25% of cases of breast cancer and is the target of two FDA approved ADCs: trastuzumab emtansine (T-DM1, commercially known as Kadcyla by Genentech, Roche) and trastuzumab deruxtecan (DS-8201 or Enhertu by AstraZeneca/Daiichi Sankyo).

1.2.5 ADC Resistance

ADC resistance in tumors is a continuous challenge for ADC development and can be caused by low receptor expression, defective ADC trafficking, efflux pumps, and other mechanisms (38). Reduction in receptor expression is a common form of ADC resistance. For example, the CD30 receptors in Hodgkin's lymphoma exhibit ADC resistance towards Brentuximab vedotin due to down regulation (39). Receptor expression between cells within the same tumor can be highly variable (40,41). Current research is looking for methods to adapt to ADC resistance through different dosing regimens and ADC mechanisms.

1.3 Tumor Microenvironment

The microenvironment of tumors is composed of many cell types, including tumor stroma, proliferating tumor cells, blood vessels, and infiltrating inflammatory cells, making transport and delivery of large molecules such as ADCs challenging (42- 44). As tumor cells continue to grow, the intratumor pressure increases, reducing convection (45, 46). Molecules therefore penetrate into the tumor tissue through diffusion, and large molecules have low diffusivity coefficients, effectively acting as a kinetic barrier against ADCs. As cells continue to grow and enlarge the tumor, access to oxygen also worsens, causing cells to enter a hypoxic state, leading to reduced drug sensitivity and cell death. Current research is looking into different models to incorporate these dynamics and how they affect drug delivery into simulations.

1.4 Computational Models to capture reaction kinetics and drug distribution

Computational models that capture drug delivery are invaluable to approximate drug reactions, dosing, efficacy, and toxicity (47-49). In the following sections, commonly used models to determine drug binding kinetics and distribution are presented.

1.4.1 Kinetic Models

Kinetic modeling uses mathematical equations to quantitatively illustrate how reactions occur in a biological or chemical process. This is valuable to understand basic biological or chemical phenomena and better predict future experiments in cases such as kinetic binding and competition of different antibodies.

Conventional mechanistic modeling strategies depend on fundamental principles, experimental observation, and empirical knowledge. Conversely, there are purely empirical or unstructured models, also known as “black box” models, that can be used to simulate similar processes such as binding kinetics (50). While black box models are easy to use and readily adapted for data fitting, black box models do not elucidate underlying physical or biochemical mechanisms and can fail to translate beyond the systems where they were initially fitted to data (50).

As modeling research and technology improve, more robust modeling strategies have been developed. However, a fully mechanistic kinetic model that involves thousands of kinetic processes in a biological system would be computationally expensive, even for modern computers. Even so, development of mixed models is an active area of research, which combines the first principles of mechanistic kinetic modeling that can be updated with new information and empirical knowledge of black box kinetic modeling that can handle large volumes of data sets.

Generally, there are three steps in developing kinetic models: mathematical model derivation, data analysis, and model validation (51). Deriving an informative mathematical model is the most crucial part which impacts the accuracy and precision of kinetic modeling.

Step 1 Model Derivation A. Determination of input, output and intermediate variables B. Determination and tabulation of key species, physical properties, and parameters C. Define relationship between variables
Step 2 Data Analysis A. Data collection of physical properties and parameters from estimation or experiments B. Parameters refined to a reasonable range C. Reduction to key equations
Step 3 Model validation A. Validate model with analytical solutions (e.g. limiting behavior at high concentrations, low concentrations, long times) B. Determination of characteristic equations, time, and length scales C. Validate model with experimental results

Table 1.2 Flow Table to Develop a Kinetic Model (51)

1.4.1.1 Types of Kinetic Models

Reactions occurring among a set of reactants define a kinetic reaction network. Traditionally, the set of reactants is described by nonlinear partial differential equations (PDEs) or ordinary differential equations (ODEs). The solutions of these equations provide insight into the dynamics of the studied processes. The size of the reaction network can vary considerably, from the tens to hundreds of thousands, depending on the complexity of the phenomena being described.

Refining a kinetic model requires focus on the most significant events; therefore, only key rate-limiting phenomena are considered. Different kinetic models have been applied to describe binding and/or reaction kinetics of antibodies and binding profiles. A common kinetic model used is the Michaelis-Menten equation. While originally derived to describe enzyme substrate interactions, the approach has been expanded to include other biological events such as antibody-antigen binding and protein-protein interactions (52). Another commonly used model is the Hill equation.

The Michaelis-Menten equation is widely used in many binding kinetic studies due to its ease and convenience for calculations (53). It simplifies the binding reaction network to a single reaction; thus it is most applicable when a single binding event is the limiting condition for the reaction network. However, when multiple reactions becoming limiting (e.g. multivalent, multi-specific, or multi-paratopic binding), the Michaelis-Menten equation is not suitable.

The Hill equation is very similar to the Michaelis Menten equation; however, it includes a “Hill coefficient” which measures binding cooperativity (54). If the Hill coefficient is 1, binding events are completely independent from each other. If the Hill coefficient is greater than 1, there is positive cooperative binding in which binding affinity of the antibody to the receptor increases once bound to an initial receptor. If the Hill coefficient is less than 1, there is negative cooperative binding, in which antibody binding to a receptor goes down after initial binding to a receptor. An advantage of using the Hill equation over the Michalis Menten Equation is that use of the Hill coefficient generally leads to better simulation results. (55). However, the Hill coefficient is largely empirical, so it loses a lot of important mechanistic information, making it suitable for regression and data fitting (56).

As reaction networks grow to be more complex and the understanding of mechanisms expands, more complex kinetic models can be developed. Cell dynamics such as internalization, diffusion, and receptor synthesis rates, can be included in cellular-level kinetic models. Additionally, multiscale modeling, or modeling at different length and/or time scales to study a single system can be employed to expand to larger dimensions (57). These models are linked using analytical or numerical methods, allowing a better understanding of the entire system. In this thesis, a multiscale kinetic model is developed that tracks competition binding kinetics of antibodies and ADCs. These models enable the prediction of antibody distribution and efficacy.

1.4.2 Models for Drug Delivery and Distribution

Kinetic models provide a quantitative understanding of how reactions operate in a biological system; thus, they are useful for simulating ADC binding, specificity, and competition. However, they do not give any information on how the ADC would work as a treatment. Pharmacology handles the topics of pharmacokinetics (PK - what the body does to the drug) and pharmacodynamics (PD - what the drug does to the body). Drugs are Absorbed, Distributed, Metabolized, and Excreted (ADME) and different models can capture these events (58). During these phases, the drug will have an interaction with the biological target which creates a PD response, such as an immune response for antibodies or cell death for ADCs.

Mathematical and computational models can describe PK/PD at a large and dynamic range of scales and sizes and can take many forms including but not limited to: compartmental models, non-compartmental analysis, and physiological or mechanistic models (59-62). Compartmental models divide different regions of interest into compartments, such as parts of the tumor, organs or blood, and track the changes of a substance of interest (e.g. drug concentration) as a function of time. Compartmental models are useful to capture the drug

absorption and clearance in the blood as a function of time (51, 63). In contrast, non-compartmental analysis is independent of the number of compartments and captures “macro” information such as maximum concentration in the blood (C_{max}), total plasma clearance (CL), average drug concentration, and elimination rate constants (64).

Mechanistic models are very common for drug delivery and distribution, capturing details such as drug diffusion, internalization into cells, and intracellular metabolism (65). The Krogh Cylinder model for instance, developed by August Krogh to study oxygen supply in muscle cells, has been expanded to describe ADC distribution from the blood vessel to the tumor tissues (66,67). The model has two cylindrical systems, a large cylinder representing the tumor tissue, and a smaller cylinder within the large cylinder representing the blood vessels. In conjunction with partial and ordinary differential equations, it can describe diffusion, local binding, blood flow, and extravasation among other rates (**Figure 1.2**). Although the Krogh Cylinder has been used to describe ADC distribution of a single species, it has not been used to capture multivalent binding kinetics and competition of different antibody species. In this thesis, a multiscale approach that combines kinetic modeling and mechanistic modeling is used to describe the distribution of ADCs and bispecific antibodies in tumor systems at the cellular, tissue and whole animal/patient length scales.

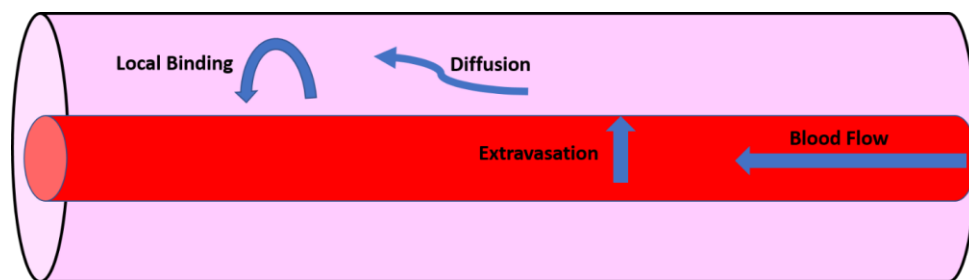


Figure 1.2 Diagram of the Krogh Cylinder with four steps of tumor localization. ADCs are transported through blood vessels, extravasate into the interstitium, diffuse between cells and bind to antigens on the surface of cells.

1.5 Motivation for development of Diagnostic Imaging Agents

Health care is increasingly challenged to meet the demands of improved patient health and outcomes while reducing the costs associated to deliver such innovations. Diagnostic imaging can help meet this demand by providing precise detection of health risks and disease at earlier stages, enabling more rapid treatment and management options, while decreasing overall patient morbidity and costs. Indeed, diagnostics are paramount in health care, directly influencing patient care, health outcomes and costs. Diagnostic imaging consists of no more than 10% and 5% of hospital and Medicare expenditures; however, their results can determine up to 80% of future decision making (68). Thus, the value of diagnostic imaging is not limited to patients and clinicians, but also to governmental and quality assurance agencies that use diagnostics to measure and improve health care.

Diagnostic technology continues to advance as understanding of disease pathology, biological systems, technology, and science improves. Starting from the first x-ray in 1895, medical personnel were able to use ionizing electromagnetic radiation to detect injuries or abnormalities in bone. Today, newly emerging imaging modalities are able to detect diseases with unprecedented resolution, maintaining their accuracy and precision while pushing to further reduce costs. From ultrasound during pregnancy to MRI for Alzheimer's disease, diagnostic imaging is typically the first line of defense for health care. With the push towards personalized medicine, diagnostics are undergoing another revolution, with emerging approaches aimed at individualizing patient care. With the promise to change the paradigm of clinical practice, these advancements are hoping to pair the "right patient with the right treatment at the right time." However, achieve these breakthroughs, approaches must provide more detailed information about disease progression and monitoring treatment response (69).

Molecular imaging can meet these needs. Compared to nonspecific contrast agents, molecular imaging agents specifically interact with a biomarker and are able to provide both spatial and molecular information about the disease. With the continued push of personalized medicine, there will be an increased need for non-invasive approaches to collect molecular information, especially with hard to detect diseases like early-stage Rheumatoid Arthritis (RA).

Rheumatoid arthritis (RA) is a common chronic and extremely disabling autoimmune disorder that affects 1% of the population with an estimated cost of \$19.3 billion annually (70, 71). A characteristic symptom of RA is inflammation of the joints; however long-term symptoms can include irreversible joint damage, increased risk for stroke and heart attack, interstitial lung disease and cancer (72-74). While there are no cures for RA, disease modifying anti-rheumatic drugs (DMARDs) can slow progression and minimize symptoms; therefore early detection is imperative to maintaining the highest possible quality of life and reducing morbidity (75-78). Current research suggests that there is a window of three months of the first symptoms in which DMARDs are more effective at treating RA (79). These studies suggest that early in disease progression, RA is still malleable and has not fully matured, further highlighting the significance of early detection and treatment. Despite significant advancements in RA diagnosis in the past 25 years, detection, quantitative measurement, and monitoring of RA remains challenging.

1.6 Methods for Diagnosing Rheumatoid Arthritis

The features for an optimal imaging modality for the detection of RA would include the ability to provide accurate anatomical and molecular information, a high safety profile, and be noninvasive and inexpensive. While seemingly obvious, accuracy is key for any screening method, because diagnostic error is a significant and under recognized threat to patient safety.

Diagnostic error on average accounts for 10% of patient deaths, therefore, accuracy must always be considered when screening a large population (80).

For RA specifically, diagnostic accuracy is paramount to distinguish RA from other rheumatic conditions within similar symptoms. Safety is also an important consideration when screening healthy populations to avoid adverse effects and improve patient compliance. To improve patient accessibility, the imaging method needs to be inexpensive. As pure anatomical information from medical imaging can lead to false positives, resulting in further testing and increased expenditure, molecular imaging can provide molecular information about the disease – interacting with biomarkers specific to the disease, potentially mitigating many false positives and additional expenditure. Finally, the screening method should be able to have a large depth of imaging in order to provide accurate information when imaging through several layers of human tissue.

With the preceding features in mind, current and proposed modalities for screening of RA were compared with summarized results in **Table 1.3**. The current standard for RA detection is conventional radiography (X-ray) where ionizing radiation is emitted towards skeletal structures, and an image is produced on film (81). Advantages of radiography include the ability to detect structural damage such as osteopenia (loss of mineral density) near the joint, soft tissue swelling, and a smaller than normal joint space. Radiography is also a very fast imaging process, relatively inexpensive, and has high depth of imaging. Limitations of radiography include poor sensitivity, no molecular contrast, and the use of ionizing radiation which can increase cancer risk (82).

MRI is a technique that uses magnetic fields and radio waves to form a detailed image of the entire body. Advantages of MRI over radiography include the ability to scan soft tissues, high spatial resolution, and high sensitivity, which can reveal active inflammation occurring in

the joint capsule or early bone inflammation and destruction prior to permanent damage. Additional advantages include high depth of imaging. MRI, however, takes longer than radiography to complete a scan, is expensive, and can require large doses of imaging agents leading to possible toxicity and low molecular contrast. Ultrasound employs high frequency sound waves to produce a two-dimensional anatomical image based on the how the waves travel through different tissues. Ultrasound can detect joint inflammation and has a spatial resolution of 0.1mm up to 1-2 mm depending on the depth. This technique is inexpensive, can distinguish synovial thickening, detect the presence of fluid in joints, bursae and tendon sheaths, capture basic abnormalities of tendons, ligaments, entheses and small erosions (83), is non-invasive, and uses non-ionizing radiation. This method, however, lacks molecular contrast and has a variable depth of imaging. Blood tests, while not an imaging modality, are an important diagnostic testing method for the detection of RA. The technique withdraws blood from the patient and detects the presence of Rheumatoid Factor, anti-citrullinated protein antibodies, and/or anti-cyclic citrullinated peptide. This technique is inexpensive, does not use ionizing radiation, and provides molecular information; however, it is invasive and has limited sensitivity, as up to 25% of people with RA were considered negative in their tests (84).

Finally, fluorescence imaging has a spatial resolution of 2-3 mm, is relatively inexpensive, uses non-ionizing radiation, and has high sensitivity and molecular contrast. A major limitation of this technique is the low depth of imaging and the fact that it is invasive, as current fluorescent probes are injected intravenously. The ability to provide molecular contrast, low cost and a high safety margin were considered to be most critical for the detection method; thus fluorescence was identified as a potentially ideal method to detect RA. Currently, 3 imaging agents are approved for clinical use: indocyanine green, fluorescein, and aminolevulinic acid

hydrochloride – and all are delivered intravenously. Indocyanine green is used to measure cardiac output, hepatic function, liver blood flow, and ophthalmic blood vessels (angiography) (85,86). Fluorescein, discontinued by the manufacturer, was approved for angiography of the retina and iris vasculature (87). Aminolevulinic acid hydrochloride is a prodrug that is metabolized to form a fluorescent metabolite and has recently been approved as an intraoperative imaging agent for the visualization of malignant gliomas.

	X-Ray	MRI	Ultrasound	Blood Test	Florescence
Non-Ionizing		✓	✓	✓	✓
Inexpensive*	✓		✓	✓	✓
Molecular Contrast				✓	✓
Non-invasive	✓	✓	✓		
Depth of Imaging	✓	✓	✓		

Table 1.3 Comparison of RA Diagnostic Tests MRI diagnosis is estimated to be \$175 making it the most expensive diagnostic technique, versus ultrasound and blood tests, estimated to be \$25. Xray is estimated around \$7 (88). Fluorescence is currently a specialized technique; therefore estimated costs are not listed.

1.7 Route of Administration

Currently, all fluorescent imaging agents are delivered intravenously; however, adverse reactions and toxicity of intravenous injections raise concerns over the benefit for I.V. delivery. Up to 70% of I.V. injections have at least one clinical error, with 25% of those errors causing serious harm to patients (89,90). For example, the risk of anaphylactic shock is much higher in I.V. administration compared to other routes of administration (91-94). Because of this risk, other delivery methods have been considered for imaging agent delivery, primarily oral and

subcutaneous (SC) delivery. Oral delivery is the consumption of a pill which is dissolved in the stomach, absorbed through the intestinal wall, processed through the liver, and then enters the systemic circulation. Oral delivery is completely noninvasive. SC delivery in contrast, is an injection of a dose of an imaging agent or drug in the SC layer between the muscle and the dermis. After SC injection of an imaging agent or drug, the molecule is slowly absorbed into the blood stream. This technique is considered minimally invasive. Studies have also shown that I.V. injection is the least preferred administration method. Patients receiving IV injections would prefer subcutaneous injection due to less pain, irritation, reaction at the injection site, and ability to be self-administered (compared to I.V. delivery, which needs to be administered by a medical professional) (95,96). Similarly, oral delivery is preferred over subcutaneous injection for its completely noninvasive quality (97,98). From a manufacturing perspective, oral delivery is also preferred due to fewer production, formulation and transportation constraints (99). The three delivery systems are visualized in **Figure 1.3**.

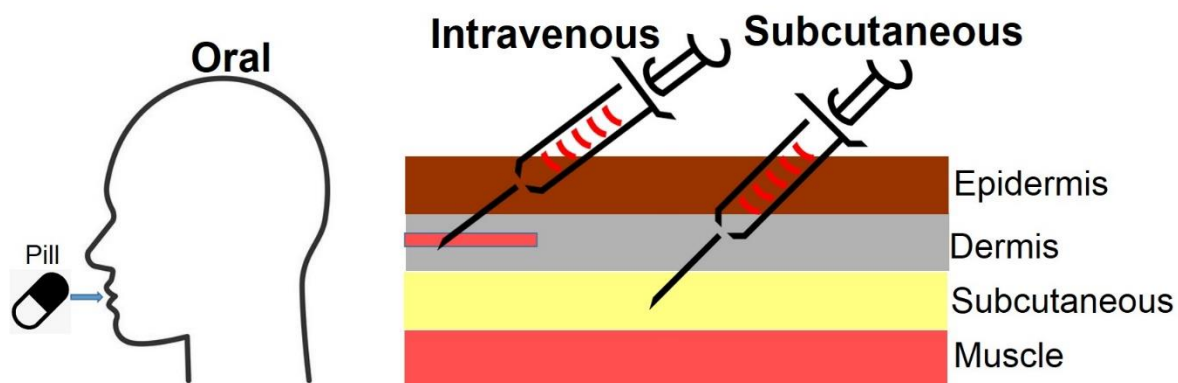


Figure 1.3 Popular Routes of Administration. Oral delivery is consumption of a pill taken by mouth into the stomach. Intravenous Delivery is an injection at the veins of the blood vessels. Subcutaneous delivery is injection of the dose into the subcutaneous layer between the muscle and dermis tissue layers.

1.8 Depth of Penetration

Many molecular imaging agents utilize radioactive probes. The use of radioactive probes has limitations, as ionizing radiation exposure over time can create acute or chronic biological damage, increasing the risk of cancer and inheritable genetic damage (100). Non-ionizing fluorescent probes are recognized as a safer alternative; however, fluorescence imaging is limited by poor depth of penetration and high optical scattering (101). To circumvent this challenge, fluorescent probes with excitation and emission spectra closer to the infrared range can be used. The wavelength range from 650-900 nm is generally regarded as the near infrared fluorescence (NIRF) imaging window (102). NIR light can penetrate several centimeters past the surface of the skin and still be detected (103). NIRF in biological tissues has low absorption, reduced optical scattering, and low auto-fluorescence in tissues, resulting in increased target to background ratios (104,105). FDA-approved ICG is a nonspecific NIRF probe used in the clinic. NIRF of ICG has also shown success in angiograms of the eye, imaging the brain, intraoperative tumor imaging, hepatic output, and colon polyp identifications (106,107).

1.9 Lipinski's Rule of Five for Oral Delivery

Lipinski's rule of five is a widely used metric for oral drug development that evaluates the "drug likeness" or predicted likelihood of a drug lead reaching the market. It states that a drug compound with high oral bioavailability and activity cannot violate more than one guideline; the guidelines include a molecular weight under 500 Daltons, lipophilicity ($\log P$) less than 5], fewer than 5 hydrogen bond donors, and fewer than 10 hydrogen bond acceptors. However, over-reliance of this model can cause missed opportunities to promising drug leads that do not adhere to this model (108). For modern drug development, a significant number of successful oral drugs violate this criterion (109). One of the most limiting guidelines of

Lipinski's rule is the low molecular weight requirement for new drug leads. The specificity of binding can be improved with a larger surface area of interaction, but this conflicts with the molecular weight limit. In fact, one of the major reasons for the specificity of biologics, large drugs that must be delivered parenterally, is their large surface area of contact, enabling both high affinity and specificity. Molecules just outside the limit of Lipinski's Rule but less than the size of biologics, e.g. drugs between 500 and 5,000 Da, provide a novel range for discovery of new agents. For example, the molecular imaging agents of this thesis are within this range.

1.10 Design of Orally Available Imaging Agents

For a near infrared red fluorescent imaging agent to reach successful translation in the clinic, it must exhibit three qualities – high absorption, high specific targeting, and ideal *in vivo* optical properties. Absorption is high dependent on route of administration; therefore, an appropriate delivery method is critical for development of NIRF imaging agents.

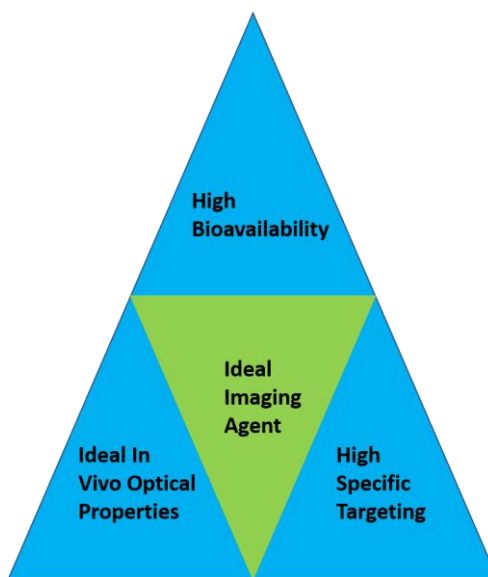


Figure 1.4 Criterion for an Ideal Near Infrared Imaging Agent

1.10.1 High Oral Absorption and High Specific Targeting

The development of orally available NIRF imaging agents is often difficult because of competing constraints between high oral absorption and high specific targeting. Drugs with high oral bioavailability often have low molecular weight and high lipophilicity to increase absorption, while high specific targeting molecules often have high molecular weight and low lipophilicity, to reduce nonspecific sticking and bind with high affinity and specificity. Fortunately, advances in drug development have demonstrated the possibility of high oral bioavailability, good specificity, and > 500 Da size, like Atorvastatin (Lipitor [560 MW]) and Telaprevir (Incivek[680 MW]), showing the potential for imaging agents in this range (110).

1.10.2 Ideal In vivo Optical Properties

While some small molecule binding agents are good for high oral absorption and targeting, they do not intrinsically contain the required optical properties needed for *in vivo* fluorescent imaging. However, they often contain functional groups that can be conjugated to a range of fluorescent dyes using established chemistries. Previous work has shown that the binding affinity, specificity of the targeting ligand to target, the physiochemical properties of the fluorophore (fluorescent dye) such as molecular weight and charge, stability, and lipophilicity must all be considered for *in vivo* diagnostic and therapeutic applications (111). Therefore, conjugation of a parent targeting ligand to a diverse range of fluorophores can create a library of compounds with various physiochemical properties that maintain binding affinity and specificity (112). *In vivo* evaluation of these imaging agents can provide helpful insight towards the design of imaging agents with high contrast (target to background tissue ratios, TBR).

1.10.3 Target Ligand Selection

Molecular imaging is contingent on significant accumulation of the imaging agent in diseased tissues compared to healthy tissue via direct interaction of the molecular imaging agent with specific biomarkers. Designing imaging agents with a high likelihood of clinical translation thus requires careful consideration of the pharmacokinetics. Design parameters include an extracellular target for easier access from the blood, high expression of $>10^5$ receptor/cell for high binding to diseased tissue (high target to background ratio), and a significant internalization rate (≤ 15 hr half life) to accumulate large amounts of the probe. To avoid a molecular screening test to identify new binding ligands, known small molecule binders can be used against the molecular target. The binder will have limited heteroatoms to maximize absorption in the intestine. Additionally, the target binder will be selected from a drug candidate that has a good preliminary oral absorption to avoid first pass metabolism in the liver and to avoid additional formulation steps (113).

While oral delivery may provide the least expensive and safest option (with maximum compliance), subcutaneous delivery is another option, possessing many of the benefits of self-administration such as improved compliance and lower risk of reaction at the injection site. Previous synthesized imaging agents have shown 90 -100% SC bioavailability, rapid uptake and clearance, indicating that the imaging agents used for oral delivery also have good properties for SC delivery (114).

1.11 Treatment Monitoring of Rheumatoid Arthritis

Routine monitoring of response to therapy is critical in determining if a current treatment is appropriate or if a dose and/or drug modification is necessary. Due to the inherent variability in patient response to treatment, there are multiple methods used to monitor the efficacy of

treatment. While it is trivial to detect patients who are on either extreme of the response spectrum (patients who are in complete remission or not experiencing any effect), patients who experience moderate efficacy of treatment are those who need more robust and quantitative tools in detection of treatment response to improve their clinical outcomes.

In the United States, use of quantitative clinical scores are somewhat uncommon, instead clinicians typically rely on subjective observations; however, there is significant evidence towards the improvement of patient outcomes if treatment modification is determined through objective means (115, 116). For example, the Tight Control of Rheumatoid arthritis study (TICORA) has suggested that close monitoring of patient's treatment response using quantified measures resulted in better patient outcomes than conventional subjective monitoring of physicians (117). Other clinical studies corroborate these findings, further indicating that incorporation of quantitative assessment tools in treatment management of RA could vastly improve better patient outcomes (118-120).

Current approaches for the treatment of RA involve an iterative process to achieve disease remission due to variability in patient response (95,121). A sensitive and facile measure of drug efficacy would allow physicians to better adjust treatment to match individual patients. While therapeutics focus on molecular disease mechanisms, diagnostic imaging methods are restricted to physiology due to the expense and safety costs of PET molecular imaging and plasma markers that are not indicative of joint response (96). The benefits of early detection and sensitive molecular measurements of treatment response is apparent as it occurs within days (97,122); however, the technology to achieve this goal does not presently exist. The use of a NIRF imaging agent could possibly enable earlier intensification and/or switching therapeutics to obtain a better response in shorter time frame than the current 1-3 months (95).

1.12 Thesis Summary

In this thesis, a multiscale kinetic model is developed to better predict the distribution and efficacy of ADCs and bispecific antibodies. Motivated by modeling results, novel near infrared imaging agents for detection and treatment monitoring of RA are also developed. Chapter 2 describes the development of the model and how it can capture the binding competition kinetics between engineered High Avidity Low Affinity (HALA) antibodies and ADCs. It also demonstrates how this model can be used to predict the efficacy of ADCs in coadministration dose with HALA antibodies at cellular, tissue and in vivo length scales. Chapter 3 extends this model to predict the binding kinetics of bispecific antibodies, where it compares the differences of bispecific antibody distribution using different binding affinities and cellular expression. Chapter 4 outlines the development of novel near infrared imaging agents for early detection and treatment monitoring of RA. Results show that the imaging agents are able to detect RA and with the potential to differentiate RA from similar arthritic diseases. Finally, the last chapter discusses future recommendations of the model for continued development of antibodies and imaging agents.

Chapter 2 Design of High Avidity and Low Affinity Antibodies for In Situ Control of Antibody Drug Conjugate Targeting

2.1 Publication Information

Evans, R., Thurber, G.M. Design of high avidity and low affinity antibodies for in situ control of antibody drug conjugate targeting. *Sci Rep* **12**, 7677 (2022). <https://doi.org/10.1038/s41598-022-11648-0> Modifications have been made to the published document to adapt the content to this dissertation. The goal of this chapter is to develop a new multiscale kinetic model to better understand the efficacy of coadministration between ADCs and engineered High Avidity Low Affinity (HALA) antibodies at different levels of cellular expression.

2.2 Abstract

Antibody-Drug Conjugates (ADCs) have rapidly expanded in the clinic, with 7 new approvals in 3 years. For solid tumors, high doses of ADCs improve tissue penetration and efficacy. These doses are enabled by lower drug-to-antibody ratios and/or co-administration of unconjugated antibody carrier doses to avoid payload toxicity. While effective for highly expressed targets, these strategies may not maintain efficacy with lower target expression. To address this issue, a carrier dose that adjusts binding *in situ* according to cellular expression was designed using computational modeling. Previous studies demonstrated that coadministration of unconjugated antibody with the corresponding ADC at an 8:1 ratio improves ADCs efficacy in high HER2 expressing tumors. By designing a High Avidity, Low Affinity (HALA) carrier antibody, ADC binding is partially blocked in high expression cells, improving tissue

penetration. In contrast, the HALA antibody cannot compete with the ADC in low expressing cells, allowing ADC binding to the majority of receptors. Thus, the amount of competition from the carrier dose automatically adjusts to expression levels, allowing tailored competition between different patients/metastases. The computational model highlights two dimensionless numbers, the Thiele modulus and a newly defined competition number, to design an optimal HALA antibody carrier dose for any target.

2.3 Motivation

Antibody Drug Conjugates (ADCs) are a rapidly growing class of therapeutics for the treatment of cancer with 7 new approvals in the past three years. There are currently 11 approved ADCs and hundreds more in clinical or preclinical development (123). ADCs are composed of an antibody that binds to cancer cells and a cytotoxic payload connected via a linker (4). These therapeutics are advantageous because they combine the high specificity of antibodies with the cell killing potential of chemotherapy. However, these agents also combine the challenges of delivering macromolecules to tumors with the toxicity of small molecules, resulting in many failures over the past two decades (124,125). This leads to challenges in ADC development because of variability in the tumor microenvironment and a generally narrow therapeutic index (126).

There are many strategies to optimize antibodies and ADC chemical properties to expand the therapeutic window and improve efficacy. Antibodies and ADCs distribute heterogeneously within the tumor microenvironment both as a function of target expression and rapid binding within the tissue (binding site barrier effect) (19, 23, 24). While increasing the dose is a well-known technique for more homogenous distribution of antibodies, ADC doses are limited by toxicity due to their payload (127, 128). One method for expanding the therapeutic window is to

decrease the potency of ADCs for high expression targets (129). This enables higher dosing that can increase the tissue penetration of the ADC in the tumor, reaching and killing more cells, while also lowering the target-mediated uptake of ADCs in healthy tissue, reducing toxicity (129). Another strategy is using site specific conjugation, where the payload is conjugated to a specific site of the antibody using a chemical or enzymatic methods(130-132). Site specific conjugation has allowed for more homogenous ADCs which can show better plasma stability, less variability in dose response and less toxicity compared to conventional ADCs for in vivo studies(127, 133-136). A third strategy is modifying the binding affinity of the ADC or utilizing lower molecular weight compounds with faster diffusion in tissue(137-139). The specificity of the ADC to its target cells can also be modified (140-142), such as bispecific agents(143) to control tumor distribution.

One method to reduce potency and increase tissue penetration is coadministration of the unconjugated antibody with the corresponding ADC. For Kadcyla, a ratio of up to 8:1 trastuzumab to Kadcyla dose improves ADC efficacy in xenograft tumors(36, 144-146). Because the unconjugated antibodies are administered at a higher dose, they compete with the ADC and partially block the cell receptors, allowing the ADCs to penetrate deeper into the tumor to improve efficacy. However, many carrier dose studies focused on high expression targets (10^6 receptors/cell). For lower expression level targets, the benefit of a carrier dose is diminished, and at a low enough expression, efficacy is reduced if not enough payload can enter the cell for cell death (e.g. $10^4 - 10^5$ receptors/cell) (147).

In this work, a novel carrier antibody engineered for High Avidity and Low Affinity (HALA) is simulated to determine if it can overcome this limitation by *in situ* adjustment of competitive blocking in the tumor microenvironment. HALA antibodies have a weaker

monovalent binding affinity than ADCs; therefore, the HALA antibodies are hypothesized to compete with the ADCs in tumors with high expression where avidity is strong, pushing the ADCs farther into the tumor to improve efficacy. However, in low expression tumors, where the avidity effect is weaker, the ADC will outcompete the HALA antibody, resulting in high ADC binding and efficacy. Essentially, the HALA antibody is capable of automatically adjusting its competition within different metastases in the same patient, or even cells within the same tumor, to maximize efficacy. A diagram of this model can be seen in **Figure 2.1**.

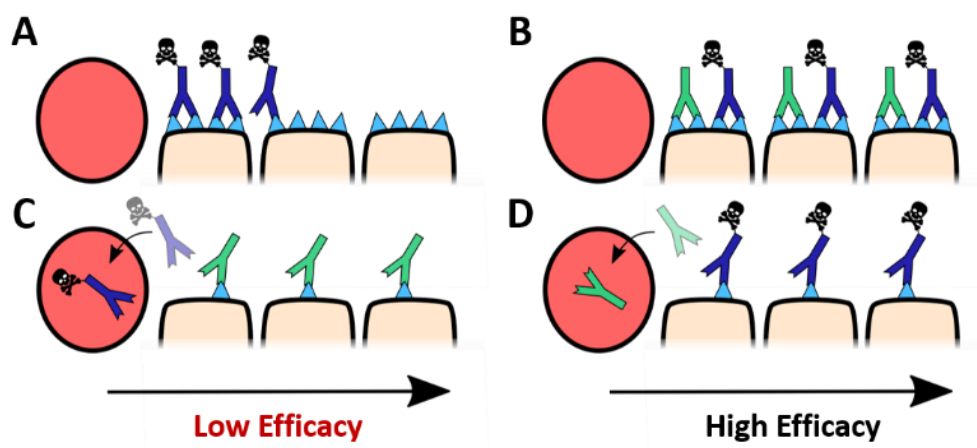


Figure 2.1: Improving ADC distribution through coadministration of HALA antibody
Administration of an ADC (single agent) produces heterogeneous perivascular distribution due to rapid binding relative to transport in the tissue in high expression tumors (e.g. at 3.6mg/kg for Kadcyra, A). Tumor penetration is improved when the ADC is co-administered with a saturating dose of unconjugated antibody (e.g. 8:1 ratio of antibody to ADC, B). The two antibodies compete for binding sites increasing ADC penetration. However, in low expression systems, unconjugated antibodies can outcompete the ADC (C), lowering efficacy. Coadministration of a HALA antibody allows competition in high expression systems (B) while enabling the ADC to outcompete the HALA antibody in low expression systems (D).

To guide the development of HALA antibodies, the HALA competition with an ADC was simulated using validated mechanistic models of bivalent binding in monolayer cells, in tumor spheroids, and *in vivo* using a Krogh Cylinder model. The results provide insight into the dependency of ADC distribution on co-administered HALA antibody binding affinity, receptor expression, and internalization rate. The simulation results can be used to optimize the design of

the co-administered HALA antibody affinity with ADCs to enable maximum efficacy independent of expression heterogeneity.

2.4 Materials and Methods

Three previously validated computational models of increasing complexity (148-150) were used to describe the cellular, tissue, and *in vivo* impact of HALA antibody properties. The first model is a bivalent competition kinetic model used to measure HALA antibody competition versus the ADC on a cell monolayer. The next model incorporated the competition kinetics alongside target internalization and antibody diffusion in the simulation. This model varied HALA antibody binding affinity and concentration in tumor spheroids to determine maximum ADC efficacy as a function of binding affinity. Finally, the competition kinetic model was integrated into a Krogh Cylinder model. The Krogh Cylinder model is a multiscale model that incorporates Pharmacokinetic/Pharmacodynamic (PK/PD) phenomena, such as transport limited by permeability, plasma clearance, payload release, and bystander killing, where the payload is free to diffuse into adjacent cells. This model was used to simulate antibody competition and payload distribution as a function of tumor expression and HALA antibody properties.

Monolayer competition model:

A computational model for competition between two bivalent antibodies was developed by adapting a kinetic model(148, 151) to simulate HALA antibody competition with ADCs on a monolayer. Briefly, the HALA antibody and ADC compete for open binding sites. The monovalently bound HALA antibody or ADC can dissociate or bind bivalently. At the same time, the free binding sites are in competition with new HALA antibodies or ADCs binding to the target. A summary of this phenomenon is depicted in **Figure 2.2A**.

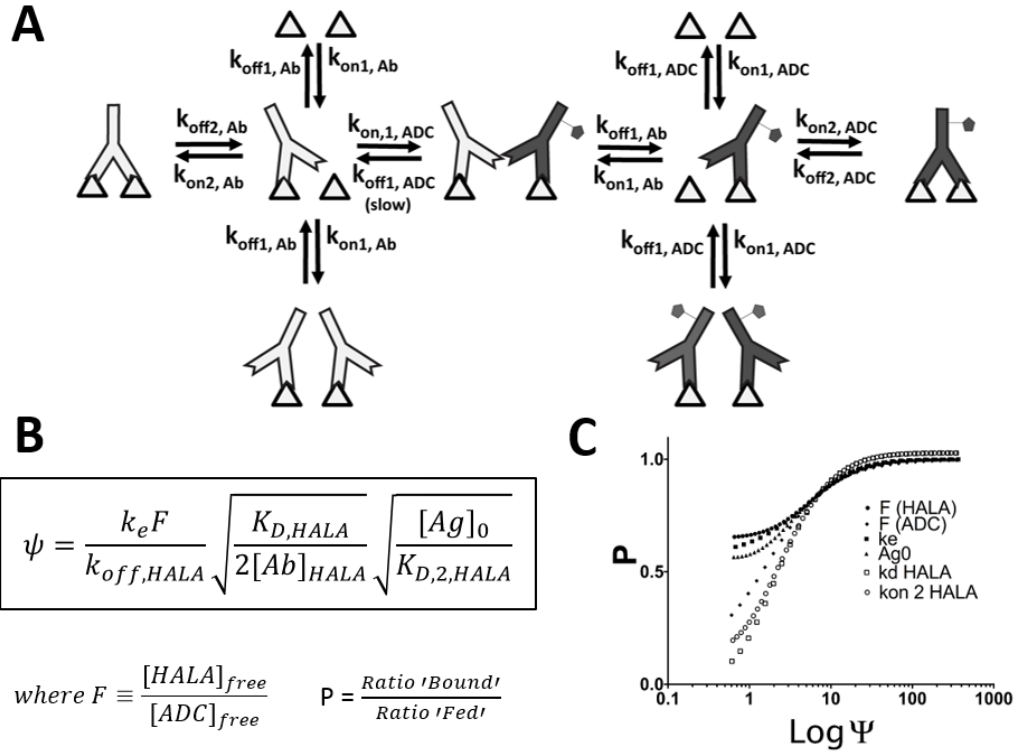


Figure 2.2 Competition Kinetics between ADCs and HALA antibodies. The reaction network of ADC (gray) and HALA antibodies (white) are shown in (A). Two antigens (triangles) are shown to emphasize the bivalent interactions. A dimensionless ‘competition’ number, Ψ , is defined based on assuming irreversible binding of the high affinity ADC (B). The ratio of bound HALA antibody and ADC relative to the ratio of HALA antibody and ADC in the bulk media (P) vs Ψ shows that at Ψ values of 10 or more, the HALA antibody successfully competes for binding with the ADC. The dimensionless number captures the competition rate independent of which parameters are varied (C). For lower values of Ψ , the HALA antibody cannot compete with the ADC, and the bound fraction of ADC is higher than the fraction in solution (low P). Because Ψ is a function of expression, HALA antibodies can be designed to have a large Ψ value for high expressing cells and small Ψ for low expressing cells.

The differential equations were defined from the reaction network, and initial competition parameters were calculated or determined from the literature:

Reaction Network

$$\frac{d[Ab_{ADC}]}{dt} = -2k_{on(1ADC)}[Ag][Ab_{ADC}] + k_{off(1ADC)}[M_{ADC}] \quad 1.$$

$$\frac{d[Ab_{HALA}]}{dt} = -2k_{on(1HALA)}[Ag][Ab_{HALA}] + k_{off(1HALA)}[M_{HALA}] \quad 2.$$

Monovalent Binding event

$$\frac{d[M_{ADC}]}{dt} = 2k_{on(1ADC)}[Ag][Ab_{ADC}] - k_{off(1ADC)}[M_{ADC}] - k_{on(2ADC)}[M_{ADC}][Ag] + 2k_{off(2ADC)}[B_{ADC}] - k_e[M_{ADC}] \quad 3.$$

$$\frac{d[M_{HALA}]}{dt} = 2k_{on(1HALA)}[Ag][Ab_{HALA}] - k_{off(1HALA)}[M_{HALA}] - k_{on(2HALA)}[M_{HALA}][Ag] + 2k_{off(2HALA)}[B_{HALA}] - k_e[M_{HALA}] \quad 4.$$

Bivalent Binding event

$$\frac{d[B_{ADC}]}{dt} = k_{on(2ADC)}[M_{ADC}][Ag] - 2k_{off(2ADC)}[B_{ADC}] - k_e[B_{ADC}] \quad 5.$$

$$\frac{d[B_{HALA}]}{dt} = k_{on(2HALA)}[M_{HALA}][Ag] - 2k_{off(2HALA)}[B_{HALA}] - k_e[B_{ADC}] \quad 6.$$

Antigen Balance

$$\begin{aligned} \frac{d[Ag]}{dt} = & -2k_{on(1ADC)}[Ag][Ab_{ADC}] + k_{off(1ADC)}[M_{ADC}] - 2k_{on(1HALA)}[Ag][Ab_{HALA}] + \\ & k_{off(1HALA)}[M_{HALA}] - k_{on(2ADC)}[M_{ADC}][Ag] - k_{on(2HALA)}[M_{HALA}][Ag] + \\ & 2k_{off(2ADC)}[B_{ADC}] + 2k_{off(2HALA)}[B_{HALA}] + R_s - k_e[Ag] \end{aligned} \quad 7.$$

Receptor Synthesis Rate for Steady State Surface Concentration

$$R_s = k_e[Ag]_0$$

where $[Ab_{ADC/HALA}]$ is the ADC or HALA concentration respectively, $k_{on(1ADC/HALA)}$ is the on rate of the first binding arm of the ADC or HALA antibody respectively, $k_{off(1ADC/HALA)}$ is the off rate of the HALA Antibody or ADC, $[Ag]$ is the concentration of free cell receptors, $[M_{ADC/HALA}]$ is the concentration of the Monovalently bound ADC or HALA complex, $[B_{ADC/HALA}]$ is the concentration of the Bivalently bound ADC or HALA complex, $k_{on(2ADC/HALA)}$ is the on rate of the second binding arm of the ADC or HALA and k_e is the internalization rate.

Full descriptions of the constants and variables used in the model is defined in **Tables 2.1 and 2.2.**

Parameter	Value	Unit	Description	Reference
Parameters used in all 3 Models				
$k_{on,ADC/HALA}$	10^5	$M^{-1}s^{-1}$	ADC or HALA monovalent binding rate	Estimated from (152,153)
$K_{d,ADC}$	5	nM	ADC dissociation constant	(20)
$K_{d,HALA}$	0.1-10,000 ^a 1-10,000 ^{b,c}	nM	HALA dissociation constant	Varies
$k_{off,ADC/HALA}$	= $k_{on,ADC/HALA} * K_{d,ADC/HALA}$	s^{-1}	ADC or HALA dissociation rate	Calculated
$k_{on2,ADC/HALA}$	2.8×10^{-5}	$M^{-1}s^{-1}$	ADC or HALA bivalent binding rate	(51)
$k_{off2,ADC/HALA}$	$k_{off,ADC/HALA}$	s^{-1}	ADC or HALA bivalent dissociation rate	Identical to $k_{off,ADC/HALA}$
Ag_0	$1 \times 10^3 - 1 \times 10^{11}$ ^a 833 ^{b,c}	Receptors $Cell^{-1}$ nM	Initial antigen concentration	(154) ^a Calculated ^{b,c}
k_e	0.01- 1×10^7 ^a 3.3×10^5 ^{b,c}	s^{-1}	ADC/HALA net internalization rate	Varies (150, 22, 155, 156)
$Dose_{ADC}$	0.1-1000 ^a 5 ^{b,c}	nM	Initial Concentration of ADC	Varies Calculated
$Dose_{HALA}$	0.001- 100,000 ^a 1-1000 ^{b,c}	nM	Initial Concentration of HALA	Varies
Parameters used in Spheroid and/or Krogh Cylinder Model				
D_e	$10^{b,c}$	$\mu m^2/s$	Diffusivity of ADC/HALA	(20)
ϵ	0.2	ND	Tumor void Fraction	(157)
$R_{Spheroid}$	200 ^b	μm	Spheroid Radius	(149)
Parameters used in the Krogh Cylinder Model				
R_{Krogh}	75 ^c	μm	Krogh Cylinder (tumor radius)	(158)

$R_{\text{Capillary}}$	8 ^c	μm	Capillary Radius	(159)
A	0.43	ND	Fraction of Alpha Clearance	(20)
k_{α}	0.0866	h^{-1}	Alpha phase clearance rate	(20)
k_{β}	0.0347	h^{-1}	Beta phase clearance rate	(20)
P_{ADC}	3×10^{-9}	ms^{-1}	Vascular Permeability of ADC/HALA	(160)
Q	0.0015	$\text{mLg}^{-1}\text{s}^{-1}$	Tumor Blood Flow Rate	(161)
H	0.45	ND	Hematocrit	(162)
k_{deg}	8×10^{-6}	s^{-1}	ADC lysosomal degradation rate	(163)
DAR	4	ND	Drug to Antibody Ratio	Measured
$k_{\text{in,P}}(\text{DM1})$	5.95×10^{-5}	s^{-1}	Payload influx rate	(150)
$k_{\text{in,P}}(\text{MMAE})$	1.41×10^{-3}	s^{-1}	Payload influx rate	(150)
$k_{\text{out,P}}(\text{DM1})$	3.94×10^{-5}	s^{-1}	Payload efflux rate	(150)
$k_{\text{out,P}}(\text{MMAE})$	6.87×10^{-4}	s^{-1}	Payload efflux rate	(150)
$D_{\text{p}}(\text{DM1})$	9.8	$\mu\text{m}^2\text{s}^{-1}$	Payload diffusion coefficient	(164)
$D_{\text{p}}(\text{MMAE})$	14.8	$\mu\text{m}^2\text{s}^{-1}$	Payload diffusion coefficient	(164,112)
P_{p}	10^{-6}	m s^{-1}	Free payload vascular permeability	(20)
$k_{\text{on,P}}$	8333	$\text{M}^{-1}\text{s}^{-1}$	Payload binding rate	(65)
$k_{\text{off,P}}$	0.003	s^{-1}	Payload dissociation rate	(65)
ε_{p}	0.44	ND	Cell void fraction	(23, 165)

P_{target}	20	μM	Microtubule concentration	(65)
---------------------	----	---------------	---------------------------	------

Table 2.1 Constants used in ADC/HALA Models

A = PSI simulations B= Spheroid simulations C= Krogh Cylinder Simulations

ND = non-dimensional

Variables used in all 3 models		
Variable	Unit	Description
Ab_{ADC}	nM	Free ADC
Ab_{HALA}	nM	Free HALA antibody
Ag	nM	Antigen
M_{HALA}	nM	Monovalently bound HALA antibody
M_{ADC}	nM	Monovalently bound ADC
B_{ADC}	nM	Bivalently bound ADC
B_{HALA}	nM	Bivalently bound HALA
Variables exclusively used in Krogh Cylinder model		
$C_{\text{int,HALA}}$	nM	Internalized Total HALA
$C_{\text{int,MHALA}}$	nM	Internalized monovalently bound HALA
$C_{\text{int,BHALA}}$	nM	Internalized bivalently bound HALA
$C_{\text{int,ADC}}$	nM	Internalized Total ADC
$C_{\text{int,MADC}}$	nM	Internalized Monovalent ADC
$C_{\text{int,BADC}}$	nM	Internalized Bivalent ADC
$C_{\text{ext,P}}$	nM	Extracellular Payload
$C_{\text{int,P}}$	nM	Intracellular Payload
$C_{\text{bound,P}}$	nM	Bound Payload
$C_{\text{lyso,P}}$	nM	Lysosomal Payload

Table 2.2 Variables used in ADC/HALA Models

Spheroid competition model:

The bivalent competition model was adapted to spherical geometry with diffusion using a previous validated model to simulate ADC distribution in spheroids (149). Specifically, partial differential equations describe diffusion of the antibodies along the radius of the spheroid. The simulated spheroids were fixed at a radius of 200 μm in order to capture diffusion gradients

(149). Additionally, the concentration outside the spheroid was fixed to mimic previous studies

(149). Differential equations are shown below.

Spheroid competition model

$$\frac{d[Ab_{ADC}]}{dt} = D_{eff} \left(\frac{1}{r^2} \frac{d}{dr} \left(r^2 \frac{d[Ab_{ADC}]}{dr} \right) \right) - 2k_{on(1ADC)}[Ag][Ab_{ADC}] + k_{off(1ADC)}[M_{ADC}] \quad 8.$$

$$\frac{d[Ab_{HALA}]}{dt} = D_{eff} \left(\frac{1}{r^2} \frac{d}{dr} \left(r^2 \frac{d[Ab_{HALA}]}{dr} \right) \right) - 2k_{on(1HALA)}[Ag][Ab_{HALA}] + k_{off(1HALA)}[M_{HALA}] \quad 9.$$

Monovalent Binding event

$$\begin{aligned} \frac{d[M_{ADC}]}{dt} = & 2k_{on(1ADC)}[Ag][Ab_{ADC}] - k_{off(1ADC)}[M_{ADC}] - k_{on(2ADC)}[M_{ADC}][Ag] + \\ & 2k_{off(2ADC)}[B_{ADC}] - k_e[M_{ADC}] \end{aligned} \quad 10.$$

$$\begin{aligned} \frac{d[M_{HALA}]}{dt} = & 2k_{on(1HALA)}[Ag][Ab_{HALA}] - k_{off(1HALA)}[M_{HALA}] - k_{on(2HALA)}[M_{HALA}][Ag] + \\ & 2k_{off(2HALA)}[B_{HALA}] - k_e[M_{HALA}] \end{aligned} \quad 11.$$

Bivalent Binding event

$$\frac{d[B_{ADC}]}{dt} = k_{on(2ADC)}[M_{ADC}][Ag] - 2k_{off(2ADC)}[B_{ADC}] - k_e[B_{ADC}] \quad 12.$$

$$\frac{d[B_{HALA}]}{dt} = k_{on(2HALA)}[M_{HALA}][Ag] - 2k_{off(2HALA)}[B_{HALA}] - k_e[B_{ADC}] \quad 13.$$

Antigen Balance

$$\begin{aligned} \frac{d[Ag]}{dt} = & -2k_{on(1ADC)}[Ag][Ab_{ADC}] + k_{off(1ADC)}[M_{ADC}] - 2k_{on(1HALA)}[Ag][Ab_{HALA}] + \\ & k_{off(1HALA)}[M_{HALA}] - k_{on(2ADC)}[M_{ADC}][Ag] - k_{on(2HALA)}[M_{HALA}][Ag] + \\ & 2k_{off(2ADC)}[B_{ADC}] + 2k_{off(2HALA)}[B_{HALA}] + R_s - k_e[Ag] \end{aligned} \quad 14.$$

Receptor Synthesis Rate for Steady State Surface Concentration

$$R_s = k_e[Ag]_0$$

Krogh Cylinder Model:

The spheroid model does not capture vascular permeability (150), plasma clearance, and payload distribution following release. Therefore, the competition model was adapted to a previously validated Krogh Cylinder geometry to simulate ADC and Payload distribution in vascularized tumors(126,129,143,146, 151, 21). The Krogh Cylinder model assumes radial but not axial distribution because concentration varies little along the capillary length (20). Elevated interstitial pressure results in limited convection and primarily diffusive transport in the tissue (43).

In this model, the antibody and ADC compete for available binding sites as above. Upon binding to a cell receptor, the ADC (or HALA antibody) is internalized into the cell, and the Antibody is degraded, allowing the payload to kill cells. Two types of payloads are included: hydrophilic and lipophilic payloads. Hydrophilic payloads are trapped within the targeted cell, while lipophilic payloads can diffuse outside the cell and into surrounding cells (the bystander effect).

Krogh Cylinder

$$\frac{d[HALA]}{dt} = D_{eff} \left(\frac{1}{r} \frac{d}{dr} \left(r \frac{d[HALA]}{dr} \right) \right) - 2k_{on1(HALA)}[Ag] * \frac{[HALA]}{\epsilon} + k_{off(1HALA)}[M_{HALA}] \quad 15.$$

$$\frac{d[ADC]}{dt} = D_{eff} \left(\frac{1}{r} \frac{d}{dr} \left(r \frac{d[ADC]}{dr} \right) \right) - 2k_{on1(ADC)}[Ag] * \frac{[ADC]}{\epsilon} + k_{off(ADC)}[M_{ADC}] \quad 16.$$

Monovalent Binding event

$$\begin{aligned} \frac{d[M_{HALA}]}{dt} = & 2k_{on(1HALA)}[Ag] * \frac{[HALA]}{\epsilon} - k_{off(1HALA)}[M_{HALA}] - k_{on(2HALA)}[M_{HALA}][Ag] + \\ & 2k_{off(2HALA)}[B_{HALA}] - k_e[M_{HALA}] \end{aligned} \quad 17.$$

$$\begin{aligned} \frac{d[M_{ADC}]}{dt} = & 2k_{on(1ADC)}[Ag] * \frac{[ADC]}{\epsilon} - k_{off(1ADC)}[M_{ADC}] - k_{on(2ADC)}[M_{ADC}][Ag] + \\ & 2k_{off(2ADC)}[B_{ADC}] - k_e[M_{ADC}] \end{aligned} \quad 18.$$

Bivalent Binding event

$$\frac{d[B_{HALA}]}{dt} = k_{on(2HALA)}[M_{HALA}][Ag] - 2k_{off(2HALA)}[B_{HALA}] - k_e[B_{HALA}] \quad 19.$$

$$\frac{d[B_{ADC}]}{dt} = k_{on(2ADC)}[M_{ADC}][Ag] - 2k_{off(2ADC)}[B_{ADC}] - k_e[B_{ADC}] \quad 20.$$

Internalized HALA/ADC

$$\frac{d[C_{int,HALA}]}{dt} = k_e[M_{HALA}] + k_e[B_{HALA}] - k_{deg}[C_{int,MHALA}] - k_{deg}[C_{int,BHALA}] \quad 21.$$

$$\frac{d[C_{int,ADC}]}{dt} = k_e[M_{ADC}] + k_e[B_{ADC}] - k_{deg}[C_{int,MADC}] - k_{deg}[C_{int,BADC}] \quad 22.$$

Extracellular payload

$$\frac{d[C_{ext,P}]}{dt} = D_{eff} \left(\frac{1}{r} \frac{d}{dr} \left(r \frac{d[C_{ext,P}]}{dr} \right) \right) - k_{in(P)} \frac{1-\varepsilon(p)}{\varepsilon(p)} [C_{ext,P}] + k_{out,P} [C_{int,P}] \quad 23.$$

Intracellular Payload

$$\begin{aligned} \frac{d[C_{int,P}]}{dt} = & k_{in(P)} \frac{1-\varepsilon(p)}{\varepsilon(p)} [C_{ext,P}] - k_{out,P} [C_{int,P}] - \frac{k_{on,P}}{(1-\varepsilon(p))R} (P_{target} C_{bound,P}) C_{int,P} + \\ & k_{off,P} C_{bound,P} + k_{in,P} C_{lyso,P} \end{aligned} \quad 24.$$

Bound payload

$$\frac{d[C_{bound,P}]}{dt} = \frac{k_{on,P}}{(1-\varepsilon(p))R} (P_{target} C_{bound,P}) C_{int,P} - k_{off,P} C_{bound,P} \quad 25.$$

Lysosome payload

$$\frac{d[C_{int,P}]}{dt} = DAR * [C_{int,ADC}] - k_{in,P} C_{lyso,P} \quad 26.$$

Antigen Balance

$$\begin{aligned} \frac{d[Ag]}{dt} = & -2k_{on(1HALA)}[Ag][HALA] + k_{off(1HALA)}[M_{HALA}] - 2k_{on(1ADC)}[Ag][ADC] + \\ & k_{off(1b)}[M_{ADC}] - k_{on(2HALA)}[M_{HALA}][Ag] - k_{on(2ADC)}[M_{ADC}][Ag] + 2k_{off(2HALA)}[B_{HALA}] + \\ & 2k_{off(2ADC)}[B_{ADC}] + R_s - k_e[Ag] \end{aligned} \quad 27.$$

Receptor Synthesis Rate for Steady State Surface Concentration

$$R_s = k_e[Ag]_0$$

2.4.1 Derivation of Dimensionless Competition Number

Strategy and Description

To generate a simplified description of the competition between a bivalent HALA antibody and a bivalent ADC (to generate the dimensionless number), several simplifying assumptions were made (which were compared to numerical simulations lacking these assumptions for validation). First, it is assumed that the ADC is high affinity and has effectively irreversible binding. In practice, this means the ADC is internalized before it has time to dissociate. Second, it is assumed that the concentration of the HALA antibody is much greater than the ADC such that the initial fraction bound is much greater for the HALA antibody, and initial bound ADC is ignored.

Following these assumptions, the initial ‘pool’ of antigen available for binding by the HALA and ADC is slowly depleted over time as more ADC irreversibly binds the antigen, depleting the available antigen ‘pool.’ It is assumed that the molecular binding events from the HALA antibody are faster than the binding of the ADC, thereby allowing an equilibrium approximation. Once the equilibrium approximation is made, the rate of ‘depletion’ of the available antigen can be calculated by the binding rate between unoccupied receptor and ADC. This yields the rate of competition by the ADC.

If internalization is not present, the ADC will compete with the HALA antibody indefinitely until it completely occupies all binding sites (due to the irreversible binding assumption). However, the antigen is also being internalized during this time. If the HALA antibody can block binding of the ADC to the target long enough that the target internalizes, the ADC will never be able to bind. Therefore, the overall effective competition of the HALA antibody

is the ratio of the rate of ADC binding to the target (in the presence of the HALA antibody) to the internalization rate. If the affinity, concentration, etc. are sufficient, the HALA antibody will be able to prevent binding of the ADC prior to internalization, and the HALA will be an effective competitor. If the ADC can bind faster than the antigen is internalized, then the HALA antibody will not be able to compete for binding with the ADC.

The mathematical derivation of this strategy is shown below:

M = monovalently bound HALA

B = bivalently bound HALA

Ag = antigen

Ab_{HALA} = HALA antibody concentration

ADC = ADC concentration

F = ratio of HALA to ADC concentration

[Ag]_{PSSH} = The concentration of available antigen for binding by HALA and ADC using the Pseudo-Steady State Hypothesis. This is the sum of Ag, M, and 2B that is depleted by ADC binding.

Equilibrium Constant Definitions:

$$K_{d,1,HALA} = \frac{k_{off,HALA}}{k_{on,HALA}} = \frac{2[Ag][Ab_{HALA}]}{[M_{HALA}]}$$

$$K_{d,2,HALA} = \frac{k_{off,HALA}}{k_{on,2HALA}} = \frac{[M_{HALA}][Ag]}{2[B_{HALA}]}$$

Define the kinetic rate of depletion of the available antigen due to binding:

$$\left. \frac{d[Ag]_{PSSH}}{dt} \right|_{binding} = -2k_{on,ADC} * [ADC][Ag]$$

$$\left. \frac{d[Ag]_{PSSH}}{dt} \right|_{binding} = -2k_{on,ADC} * \frac{[Ab]_{HALA}}{F} [Ag]$$

Assume equilibrium binding of HALA antibody:

$$[Ag]_{o,pssh} = [Ag] + [M_{HALA}] + 2[B_{HALA}]$$

Using Definition of K_{d1HALA} and K_{d2HALA} substitute for $[M_{HALA}]$ and $[B_{HALA}]$

$$\begin{aligned}
[Ag]_{o,pssh} &= [Ag] + \frac{2[Ag][Ab_{HALA}]}{K_{d1_{HALA}}} + \frac{2*[M_{HALA}][Ag]}{2*K_{d1_{HALA}}} \\
&= [Ag] + \frac{2[Ag][Ab_{HALA}]}{K_{d1_{HALA}}} + \frac{[Ab_{HALA}][Ag]^2}{K_{d1_{HALA}}*K_{d2_{HALA}}} \\
[Ag]_{o,pssh} &= [Ag]\left(1 + \frac{2*[Ab_{HALA}]}{K_{d1_{HALA}}}\left(1 + \frac{[Ag]}{K_{d2_{HALA}}}\right)\right)
\end{aligned}$$

If $[Ag]/K_{d2,HALA} \ll 1$, then there isn't any benefit from avidity, so assume $\gg 1$

$$[Ag]_{o,pssh} = [Ag]\left(1 + \frac{2*[Ab_{HALA}]}{K_{d1_{HALA}}}\left(\frac{[Ag]}{K_{d2_{HALA}}}\right)\right)$$

It is also assumed that a large fraction of the Ag is bound (to provide adequate competition), so it is assumed the blue terms are $\gg 1$. Simplifying and solving for Ag:

$$[Ag] = \sqrt{\frac{[Ag]_{o,pssh} * K_{d1_{HALA}} * K_{d2_{HALA}}}{2*[Ab_{HALA}]}}$$

The free antigen concentration is entered into the kinetic equation for total available Ag:

$$\begin{aligned}
\left.\frac{d[Ag]_{Pssh}}{dt}\right|_{binding} &= -2k_{on,ADC} * \frac{[Ab]_{HALA}}{F} [Ag] \\
\left.\frac{d[Ag]_{Pssh}}{dt}\right|_{binding} &= -2k_{on,ADC} * \frac{[Ab]_{HALA}}{F} * \sqrt{\frac{[Ag]_{o,pssh} * K_{d1_{HALA}} * K_{d2_{HALA}}}{2*[Ab_{HALA}]}}
\end{aligned}$$

Simplifying:

$$= -2k_{on,ADC} * \frac{[Ab]_{HALA}}{F} \sqrt{\frac{[Ag]_{o,pssh} * K_{d1_{HALA}} * K_{d2_{HALA}}}{2*[Ab_{HALA}]}} \left(\frac{K_{d1_{HALA}}}{K_{d1_{HALA}}}\right)$$

Rearrange and simplify:

$$= -2k_{on,ADC} * \frac{k_{off,HALA}}{F * k_{on,HALA}} \sqrt{\frac{[Ag]_{o,pssh} * [Ab_{HALA}] * K_{d2_{HALA}}}{2 * K_{d1_{HALA}}}}$$

Assume k_{on} are approximately the same for ADC and HALA and cancel:

$$\left.\frac{d[Ag]_{Pssh}}{dt}\right|_{binding} = -\frac{2k_{off,HALA}}{F} \sqrt{\frac{[Ag]_{o,pssh} * [Ab_{HALA}] * K_{d2_{HALA}}}{2 * K_{d1_{HALA}}}}$$

As the antigen is being bound by the ADC, there is a parallel competing 'reaction' of internalization:

$$\left.\frac{d[Ag]_{Pssh}}{dt}\right|_{internalization} = -k_e * [Ag]_{o,pssh}$$

The ratio of these two competing reactions for $[Ag]_{o,pssh}$, one yielding binding by the ADC and the other yielding effective competition, results in the competition number:

$$\psi \equiv \frac{-ke*[Ag]_{o,pssh}}{\frac{-2k_{off,HALA}}{F} \sqrt{\frac{[Ag]_{o,pssh}*[Ab_{HALA}]^2 K_{d2HALA}}{2*K_{d1HALA}}}}$$

Simplifying:

$$\psi = \frac{ke*F}{2k_{off,HALA}} \sqrt{\frac{2*K_{d1HALA}}{[Ab_{HALA}]}} \sqrt{\frac{[Ag]_{o,pssh}}{K_{d2HALA}}}$$

2.4.2 Derivation of Equilibrium Binding

Derivation of simplified expression for thermodynamic equilibrium with ADC/ HALA binding on a monolayer without internalization

$$K_{d1HALA} = \frac{k_{off,HALA}}{k_{on,HALA}} = \frac{2[Ag][Ab_{HALA}]}{[M_{HALA}]}$$

$$K_{d1ADC} = \frac{k_{off,ADC}}{k_{on,ADC}} = \frac{2[Ag][Ab_{ADC}]}{[M_{ADC}]}$$

$$K_{d2HALA} = \frac{k_{off,HALA}}{k_{on,2HALA}} = \frac{[M_{HALA}][Ag]}{2[B_{HALA}]}$$

$$K_{d2ADC} = \frac{k_{off,ADC}}{k_{on,2ADC}} = \frac{[M_{ADC}][Ag]}{2[B_{ADC}]}$$

Assume Ab_{HALA} and Ab_{ADC} are constant (excess antibody). Use conservation of total antigen, assume equilibrium, and enter values from relationships above:

$$[Ag]_0 = [Ag] + [M_{HALA}] + [M_{ADC}] + 2[B_{HALA}] + 2[B_{ADC}]$$

$$[Ag]_0 = [Ag] + \frac{2[Ag][Ab_{HALA}]}{[K_{d1HALA}]} + \frac{2[Ag][Ab_{ADC}]}{[K_{d1ADC}]} + \frac{2[M_{HALA}][Ag]}{2[K_{d2HALA}]} + \frac{2[M_{ADC}][Ag]}{2[K_{d2ADC}]}$$

Factor out [Ag]:

$$[Ag]_0 = [Ag] * \left[1 + \frac{2[Ab_{HALA}]}{[K_{d1HALA}]} + \frac{2[Ab_{ADC}]}{[K_{d1ADC}]} + \frac{[M_{HALA}]}{[K_{d2HALA}]} + \frac{[M_{ADC}]}{[K_{d2ADC}]} \right]$$

Substitute approximations for M_{HALA} and M_{ADC} again:

$$[Ag]_0 = [Ag] * \left[1 + \frac{2[Ab_{HALA}]}{[K_{d1HALA}]} + \frac{2[Ab_{ADC}]}{[K_{d1ADC}]} + \frac{2[Ag][Ab_{HALA}]}{[K_{d1HALA}][K_{d2HALA}]} + \frac{2[Ag][Ab_{ADC}]}{[K_{d1ADC}][K_{d2ADC}]} \right]$$

Factor out [Ag]

$$[Ag]_0 = [Ag] * \left[1 + \frac{2[Ab_{HALA}]}{[K_{d1HALA}]} + \frac{2[Ab_{ADC}]}{[K_{d1ADC}]} \right] + [Ag]^2 * \left[\frac{2[Ab_{HALA}]}{[K_{d1HALA}][K_{d2HALA}]} + \frac{2[Ab_{ADC}]}{[K_{d1ADC}][K_{d2ADC}]} \right]$$

At equilibrium, the avidity of antibodies results in mostly bivalent binding. Likewise, it is assumed the antibodies are high affinity where the free antigen concentration is low. Therefore, the free antigen and monovalently bound terms are negligible:

$$[Ag]_0 = [Ag]^2 * \left[\frac{2[Ab_{HALA}]}{[K_{d1HALA}][K_{d2HALA}]} + \frac{2[Ab_{ADC}]}{[K_{d1ADC}][K_{d2ADC}]} \right]$$

$$[Ag]^2 = \frac{([Ag]_0/2)}{\frac{[Ab_{HALA}]}{[K_{d1HALA}][K_{d2HALA}]} + \frac{[Ab_{ADC}]}{[K_{d1ADC}][K_{d2ADC}]}}$$

At equilibrium, the avidity of antibodies results in mostly bivalent binding, so the concentration of bivalent antibody at equilibrium is:

$$\frac{[M_{HALA}] + [B_{HALA}]}{[Ag]_0} \approx \frac{[B_{HALA}]}{[Ag]_0}$$

From definitions of equilibrium:

$$[M_{ADC}] = \frac{2[Ag][Ab_{ADC}]}{K_{d1ADC}} \qquad [B_{ADC}] = \frac{[M_{ADC}][Ag]}{2 * K_{d2ADC}}$$

Substitute M_{HALA} into B_{HALA} :

$$[B_{ADC}] = \frac{2[Ag][Ag][Ab_{ADC}]}{2 * K_{d1ADC} * K_{d2ADC}}$$

$$[B_{ADC}] = \frac{[Ag]^2[Ab_{ADC}]}{K_{d1ADC} * K_{d2ADC}}$$

Substitute $[Ag]^2$ into equation:

$$[B_{ADC}] = \frac{[Ab_{ADC}]}{K_{d1ADC} * K_{d2ADC}} * \frac{([Ag]_0/2)}{\frac{[Ab_{ADC}]}{K_{d1ADC} * K_{d2ADC}} + \frac{[Ab_{HALA}]}{K_{d1HALA} * K_{d2HALA}}}$$

Solve for B_{ADC}

$$\frac{[B_{ADC}]}{([Ag]_0/2)} = \frac{\frac{[Ab_{ADC}]}{K_{d1ADC} * K_{d2ADC}}}{\frac{[Ab_{ADC}]}{K_{d1ADC} * K_{d2ADC}} + \frac{[Ab_{HALA}]}{K_{d1HALA} * K_{d2HALA}}}$$

$$\frac{[B_{ADC}]}{([Ag]_0/2)} = \frac{[Ab_{ADC}]}{[Ab_{ADC}] + [Ab_{HALA}] \frac{K_{d1ADC} * K_{d2ADC}}{K_{d1HALA} * K_{d2HALA}}}$$

To simplify the ratio of monovalent and bivalent equilibrium binding, an avidity model is used. In this model, the off-rate is assumed constant, and the effective on-rate is a product of the intrinsic binding rate and local concentration:

$$k_{off,1} = k_{off,2}$$

$$\frac{K_{d_{2ADC}}}{K_{d_{2HALA}}} = \frac{\frac{k_{off,ADC}}{k_{on,2ADC}}}{\frac{k_{off,HALA}}{k_{on,2HALA}}}$$

$$\text{Definition of } k_{on,2} = \frac{3}{8\pi r^2_{cell} r_{IgG} N_a}$$

Reference [51]

Substitute definition into previous equation

$$\frac{K_{d_{2ADC}}}{K_{d_{2HALA}}} = \frac{\frac{k_{off,ADC}}{k_{on,ADC} * \frac{3}{8\pi r^2_{cell} r_{IgG} N_a}}}{\frac{k_{off,HALA}}{k_{on,HALA} * \frac{3}{8\pi r^2_{cell} r_{IgG} N_a}}}$$

Assume r_{cell} and r_{IgG} are equal between ADC and HALA antibody:

$$\frac{K_{d_{1ADC}}}{K_{d_{1HALA}}} = \frac{K_{d_{2ADC}}}{K_{d_{2HALA}}}$$

Plug in definition into B_{ADC} equation:

$$\frac{[B_{ADC}]}{([Ag]_0/2)} = \frac{[Ab_{ADC}]}{[Ab_{ADC}] + [Ab_{HALA}] \left(\frac{K_{d_{1ADC}}}{K_{d_{1HALA}}} \right)^2}$$

Factor out Ab_{ADC}

Assume $K_{d_{ADC}} < K_{d_{HALA}}$

Assume $[Ab_{ADC}] < [Ab_{HALA}]$

$$\frac{[B_{ADC}]}{([Ag]_0/2)} = \frac{1}{1 + \frac{[Ab_{HALA}]}{[Ab_{ADC}]} \left(\frac{K_{d_{1ADC}}}{K_{d_{1HALA}}} \right)^2}$$

$$F = \frac{[Ab_{HALA}]}{[Ab_{ADC}]}$$

$$\frac{[B_{ADC}]}{([Ag]_0/2)} = \frac{1}{1 + F \left(\frac{K_{d1ADC}}{K_{d1HALA}} \right)^2}$$

2.4.3 Derivation of Thiele Modulus

Φ^2 is previously defined as follows from previous literature (22)

$$\Phi^2 = \frac{k_e * [Ag] * (R_{spheroid}^2)}{\varepsilon * D * Ab}$$

R spheroid is the characteristic length of the spheroid which is R/3 thus Φ^2 becomes

$$\Phi^2 = \frac{k_e * [Ag] * (R_{spheroid}^2)}{\varepsilon * D * Ab * 9}$$

In the Krogh Cylinder model Φ^2 is defined as

$$\Phi^2 = \frac{k_e * [Ag] * (R_{Krogh_cylinder}^2)}{2P * R_{Capillary} * Ab}$$

Variable	Description
k_e	Internalization rate
Ag	Total cell receptors
$R_{spheroid}$	Spheroid Radius
$R_{Krogh_Cylinder}$	Krogh Cylinder Radius
D	Diffusion coefficient
Ab	Total Antibody Dose
E	Tumor Void fraction
P	Permeability
$R_{Capillary}$	Capillary Radius

2.5 Results

The model was validated by simulating the amount of bound IgG for affinity variants to cells with different levels of cellular expression (**Figure 2.3**). After validation, an expression “ Ψ ” was derived to describe effective competition of the HALA antibody. It is essentially the ratio between cellular receptor internalization relative to binding competition, **Figure 2.2B**.

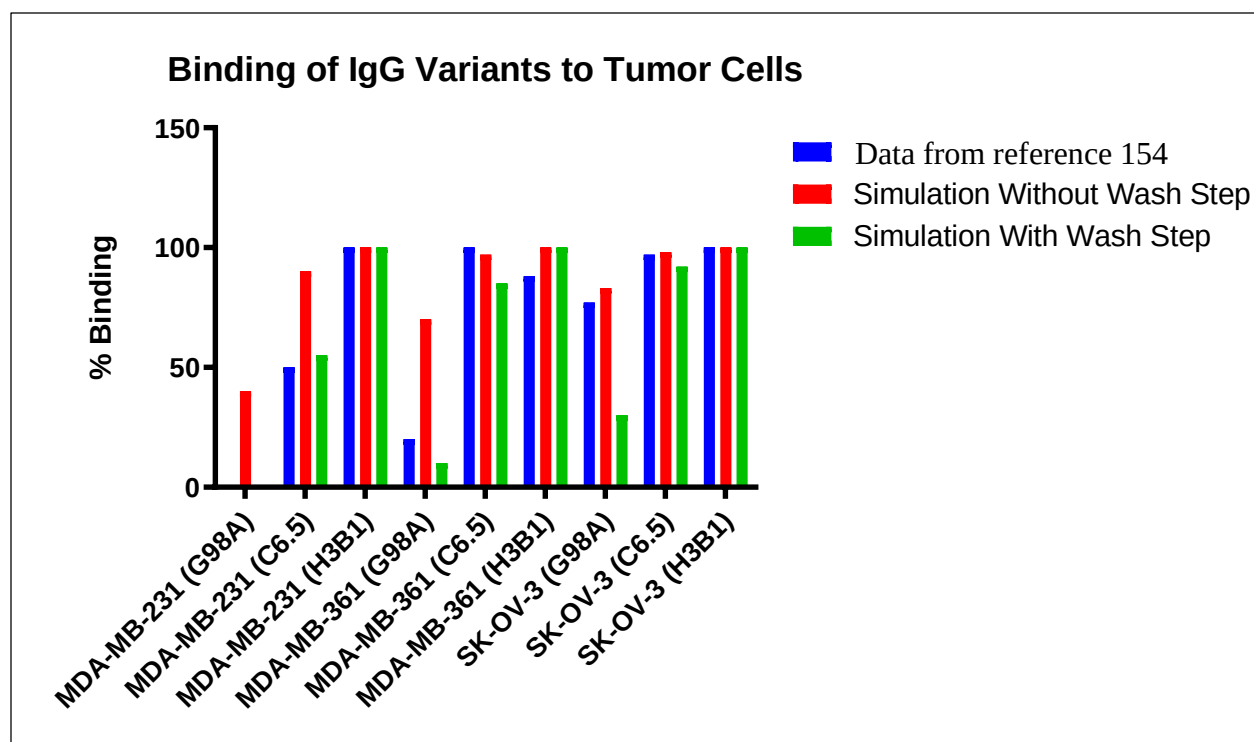


Figure 2.3 Validation of Monolayer Simulations. Validation of monolayer simulations of different antibody clones at different cell expression. Binding assays results were previously published (154). “Unwashed” simulations modeled the association phase of competition binding in the presence of the antibody, which accounts for the higher % of binding. The “washed” simulations include a dissociation phase after the free antibody is removed (simulating a washing step), which results in better congruence with published data.

HALA Competition Dimensionless number:

A simplified description of competition kinetics between ADC and HALA antibodies was developed where binding of the ADC was assumed to be irreversible (high affinity). A dimensionless number, Ψ , was derived to capture the ability of the HALA antibody to compete with the higher affinity ADC (**2.4.1**).

Within minutes of adding the antibodies *in silico*, both the ADC and HALA antibody significantly bind the target. Since k_{on} rates are similar for many antibodies (and k_{off} typically distinguishes high versus low affinity antibodies), the amounts of bound ADC and HALA antibody are proportional to their concentrations in solution at early times (**Figure 2.4**). Over the course of hours, the higher affinity ADC starts to outcompete the HALA antibody, increasing the

amount of ADC bound. However, once the receptor is internalized, the ADC can no longer compete for binding. The rate of competition of ADC relative to the rate of internalization is expressed as Ψ , which is dependent on the receptor expression level. At low Ψ values, there is poor HALA antibody binding, and the ADC outcompetes the HALA for binding sites. At high Ψ , the HALA antibody is able to compete with the ADC until the antigen is internalized.

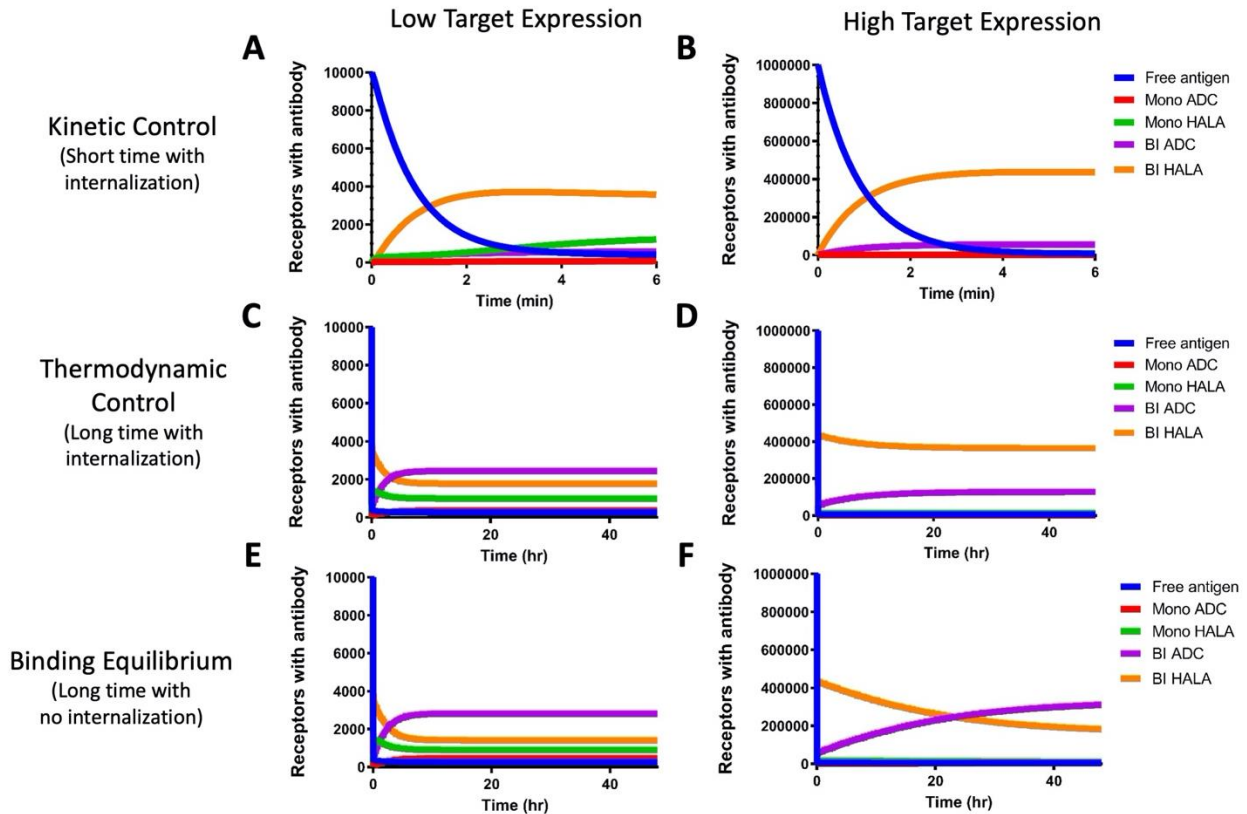


Figure 2.4 Thermodynamic and Kinetic Control in Simulations. During short times (kinetic control) HALA antibody binding outcompetes the ADC because of the higher HALA antibody concentration and similar on-rates for ADC and antibody. Equilibrium is not reached within the 6 minute simulation time for either low expressing cells (A) or high expressing cells (B). ‘Mono’ and ‘Bi’ refer to monovalently and bivalently bound species, respectively. At long times (48 hr simulation time), the ADC begins to compete with the HALA antibody; however, competition is dependent on the expression level. For low expressing systems, the ADC rapidly outcompetes the HALA antibody resulting in the majority of antigen bound by ADC (C). For high expressing systems, the avidity of the HALA antibody results in a slow approach to thermodynamic equilibrium. In fact, internalization removes the bound complex from the cell surface before the ADC can outcompete the HALA antibody, resulting in permanent high HALA antibody binding (D). If there were no internalization, both the low (E) and high (F) expression systems approach thermodynamic equilibrium binding with the ADC dominating the surface binding.

To validate the dimensionless number, numerical simulations were performed, and Ψ was graphed versus P , the amount of HALA antibody bound relative to the initial concentration. When $P = 1$, the amount of HALA binding is proportional to the initial dose, so at an 8:1 HALA antibody to ADC ratio, the HALA antibody binds 8 times more receptors than the ADC. If P is less than 1, the ADC is outcompeting the HALA antibody relative to the initial concentration of each agent, and if P is greater than 1, HALA binding out-competes the ADC (not shown, since this implies a higher affinity HALA antibody). Each parameter of Ψ was varied to validate the relationship between Ψ and P . Regardless of the individual parameter changed, at high values of Ψ , the P value converges to 1. Results are displayed in **Figure 2.2C**. At low values of Ψ , the P value varies depending on the conditions (**Figure 2.5**).

Concentration of ADC Bound as a Function of Psi for Varying HALA Affinities

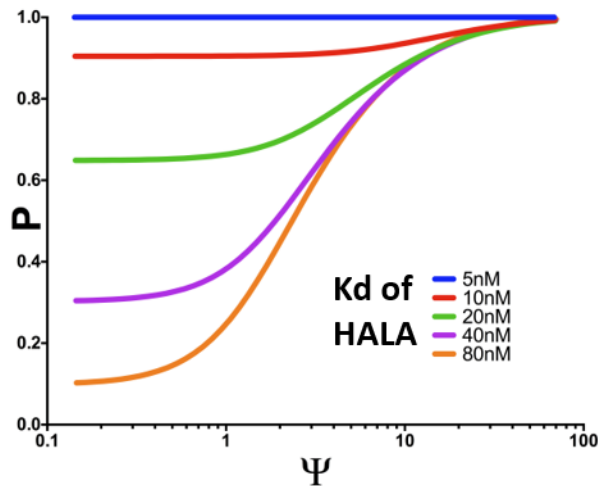


Figure 2.5 P vs Ψ graphs at varying HALA antibody K_d . While higher values of Ψ converge to a P value of 1, individual parameters such as K_d can affect P values at lower Ψ . For example, the changing HALA dose is plotted above at varying affinities (K_d). At low affinities (e.g. 80 nM), the HALA antibody is unable to block uptake faster than the internalization rate, so the ADC outcompetes almost all the HALA antibody. However, as the affinity of the HALA antibody increases (decreasing K_d), the HALA antibody more effectively blocks the ADC at equilibrium. When the HALA affinity equals that of the ADC (5 nM), P always equals 1 since the high ADC affinity assumption fails.

Modeling Spheroids

Previous work demonstrated that a co-administered dose of trastuzumab with T-DM1 at an 8:1 ratio allowed TDM-1 to penetrate farther into a tumor for optimal *in vivo* efficacy²⁷. In this work, the affinity of the competitive antibody was varied to determine the impact on tissue penetration and cellular uptake under different levels of expression. Note that the concentration plotted is for cells at the spheroid center, which is typically lower than peripheral cells. The simulations show that most ADC reaches the center of the spheroid at a binding affinity between 1 and 20 nM and a concentration of initial HALA antibody of 40 nM to 80 nM, with an optimum penetration at 5 nM K_d and HALA antibody concentration of 40 nM (**Figure 2.6A**). This base case condition, using the affinity and dose of trastuzumab shown to work well *in vivo*, is labeled “optimal”. Maintaining the same binding affinity as the “optimal” conditions, but increasing or decreasing the HALA antibody concentration, resulted in decreased ADC penetration, which is labeled “over competition” (too large of a carrier dose) or “concentration limited” (too little carrier dose). Maintaining the optimal HALA antibody concentration but decreasing the HALA antibody binding affinity resulted in low ADC penetration, labeled “affinity limited”. A more generalizable plot of ADC concentration at the spheroid center is shown in **Figure 2.7**, where the value is plotted versus Ψ and the Thiele modulus, ϕ^2 . Dimensional values (i.e. concentration and K_d) are often more intuitive and are therefore used throughout the main figures.

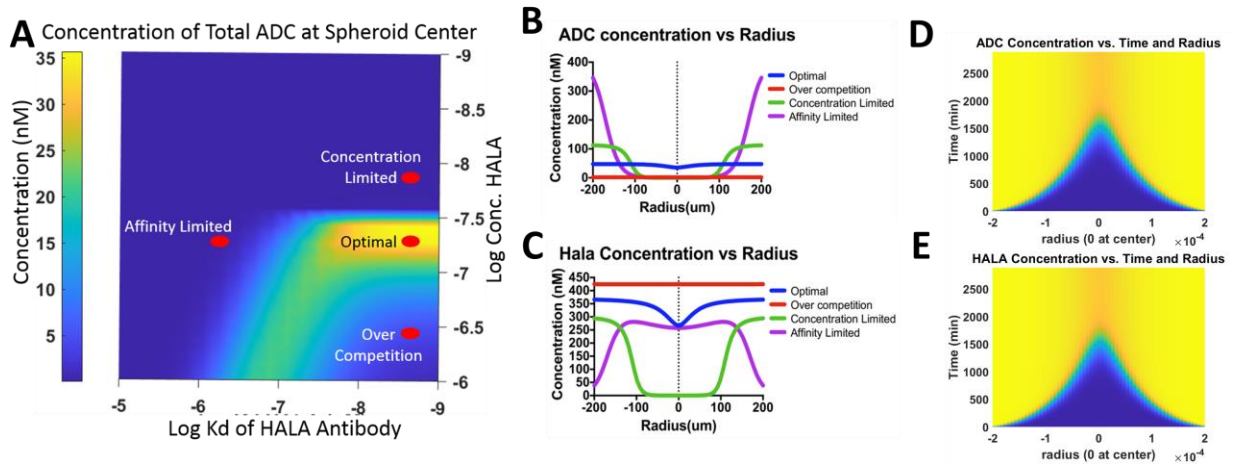


Figure 2.6: Simulating ADC Distribution in Spheroids.(A) Spheroid model simulating the concentration of ADC binding at the center of the spheroid at 48 hrs (after a pseudo-steady state has developed). Both the affinity of the HALA antibody and the (external) concentration of HALA antibody were varied. As the affinity of the HALA antibody is reduced, higher concentrations of HALA antibody are required to maintain competition and drive ADC to the spheroid center. Concentration of the ADC binding (B) or HALA antibody binding (C) over varying distances within the spheroid. Concentration of ADC binding (D) or HALA antibody binding (E) over varying distances within the spheroid and at varying time under “optimal” conditions.

Figure 2.6B and 2.6C show the penetration of the ADC and HALA antibody inside the spheroid as a function of distance/radius. The affinity-limited point shows high ADC concentration at the edge of the spheroid (more than needed for cell death), but the concentration rapidly decreases closer to the center of the spheroid resulting in limited efficacy. Under these conditions, the very low affinity HALA antibody is outcompeted at the edges of the spheroid but reaches the center. In contrast, the model demonstrates homogeneous ADC distribution at the optimal conditions. If the carrier dose is too high, the over-competition point shows a low ADC concentration due to the large dose of HALA antibody. The HALA antibody concentration is large and homogeneous throughout the spheroid. The concentration-limited point shows slightly improved ADC penetration at the edge of the spheroid; however, penetration declines beyond 100 microns. The HALA antibody also lacks tissue penetration. **Figure 2.6D and 2.6E** show the penetration of ADC and HALA as a function of distance and time at “optimal” conditions. Both

the ADC and HALA antibody are able to saturate the center of the spheroid at 1800 minutes (30 hours), indicating the minimum time required to achieve equilibrium.

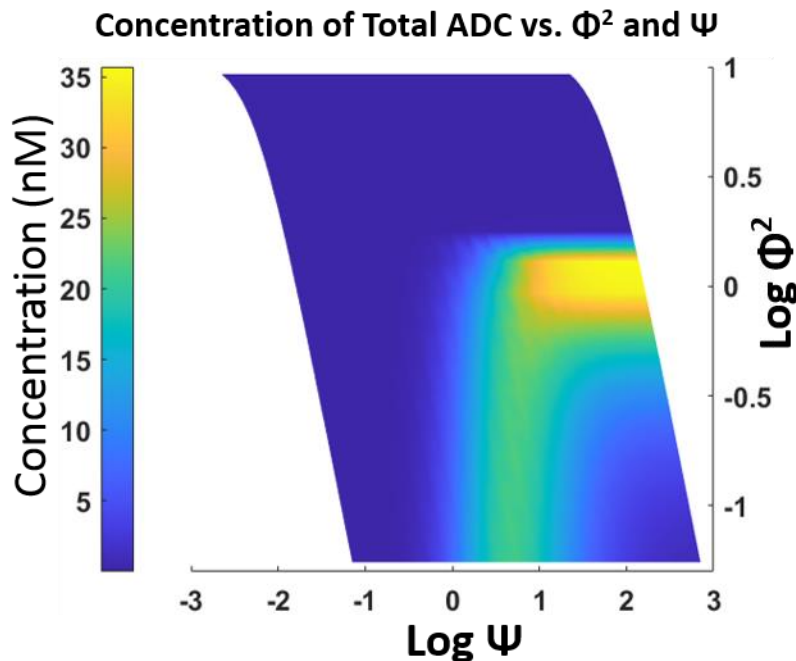


Figure 2.7. Total Concentration of ADC at center of spheroid vs Ψ and Φ^2 . If the HALA antibody concentration is too high (small Φ^2), Ψ is between 1 and 10 (log 0 to log 1) to balance competition. When the optimal amount of HALA is given (to achieve $\Phi^2 \sim 1$), Ψ is > 10 for efficient tissue penetration.

Modeling Krogh Cylinder

After examining the impact of HALA antibody properties on the distribution of ADCs in spheroids, ADC distribution was simulated *in vivo* using a Krogh Cylinder model. This model included vascular permeability, plasma clearance, and bystander payload distribution (**Figure 2.8**).

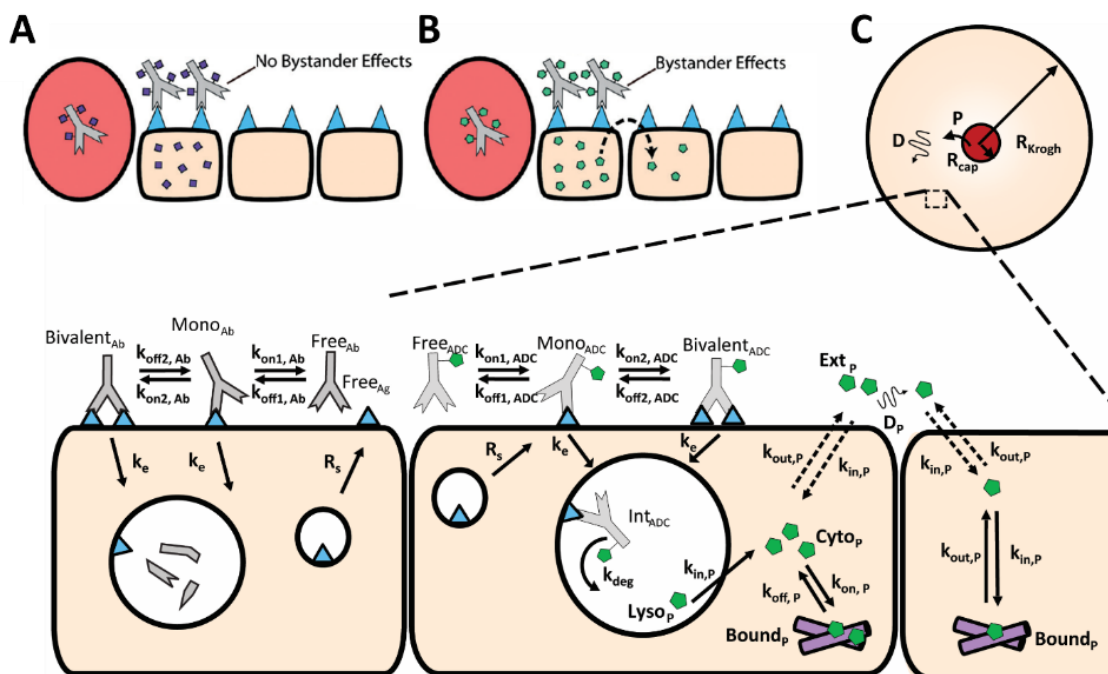


Figure 2.8 Simulating distribution of ADC payloads *in vivo*.(A) Schematic highlighting non-bystander payloads and cell killing with lysine-SMCC-DM1 (maytansinoid with a non-cleavable linker). (B) Schematic highlighting bystander penetration into surrounding cells using MMAE (monomethyl auristatin E). (C) Diagram of competition reactions between ADC and HALA antibody and payload distribution in the Krogh Cylinder Model.

The starting simulations used the Krogh Cylinder model and varied the binding affinity and concentration of the HALA antibody similar to the spheroid model to capture the distribution of the ADC and cytotoxic payload. The model first captures an ADC with the cytotoxic payload released by Kadcyra, lysine-SMCC-DM1 (referred to as DM-1), which is a non-bystander payload. Next, the model simulated an ADC with MMAE, a bystander payload, with both results shown 48 hours after coadministration.

As seen in **Figure 2.9**, the ADC distribution is impacted by both binding affinity and concentration of the HALA antibody. As the initial concentration of the HALA antibody in the plasma increases, the binding affinity must decrease to compensate and maintain good penetration into the tumor. Differences between ADC and Payload distribution after 48 hours can

be attributed to differences in internalization (**Figure 2.10 and 2.11**). **Figure 2.12** demonstrates a more generalizable plot of ADC and Payload concentration as function of Ψ and ϕ^2 .

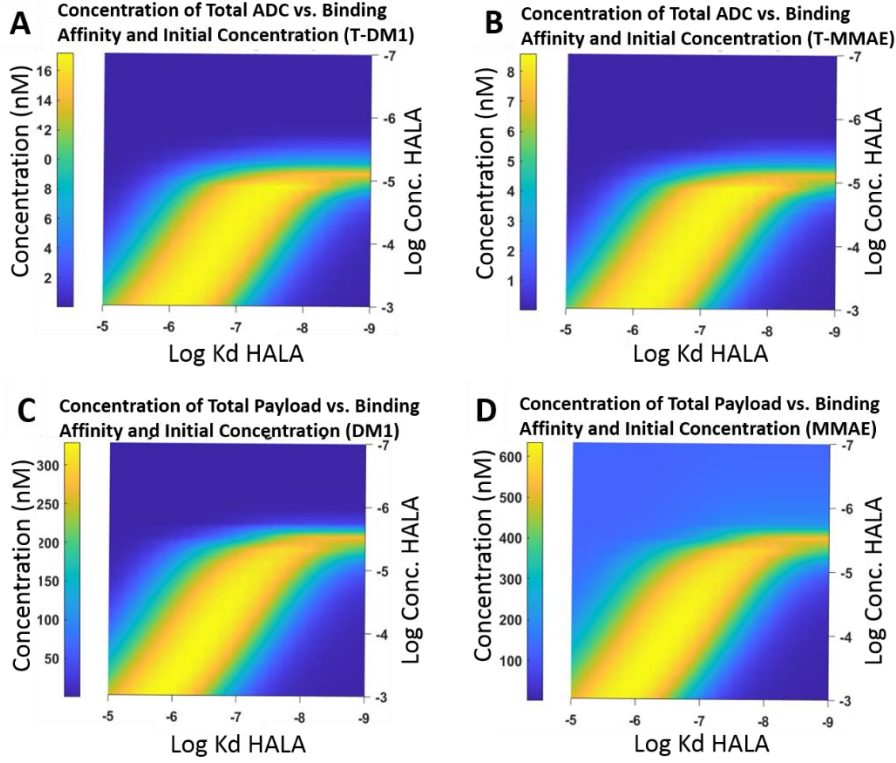


Figure 2.9. Distribution of ADC and Payload of co-administered ADC with HALA antibody showing changing concentration and binding affinity in Krogh Cylinder simulations after 48hrs.(A) Distribution of ADC at a dose of 3.6mg/kg with DM1 payload (non-bystander) (B) Distribution of ADC at a dose of 1.8mg/kg with bystander (MMAE) payload. (C) Payload distribution of ADC with non-bystander payload. (D) Payload distribution of ADC with bystander payload. The lighter blue region above the yellow curve in D has a higher concentration due to MMAE bystander payload distribution. Concentrations are shown at the Krogh cylinder radius, R_{Krogh} , far from tumor blood vessels.

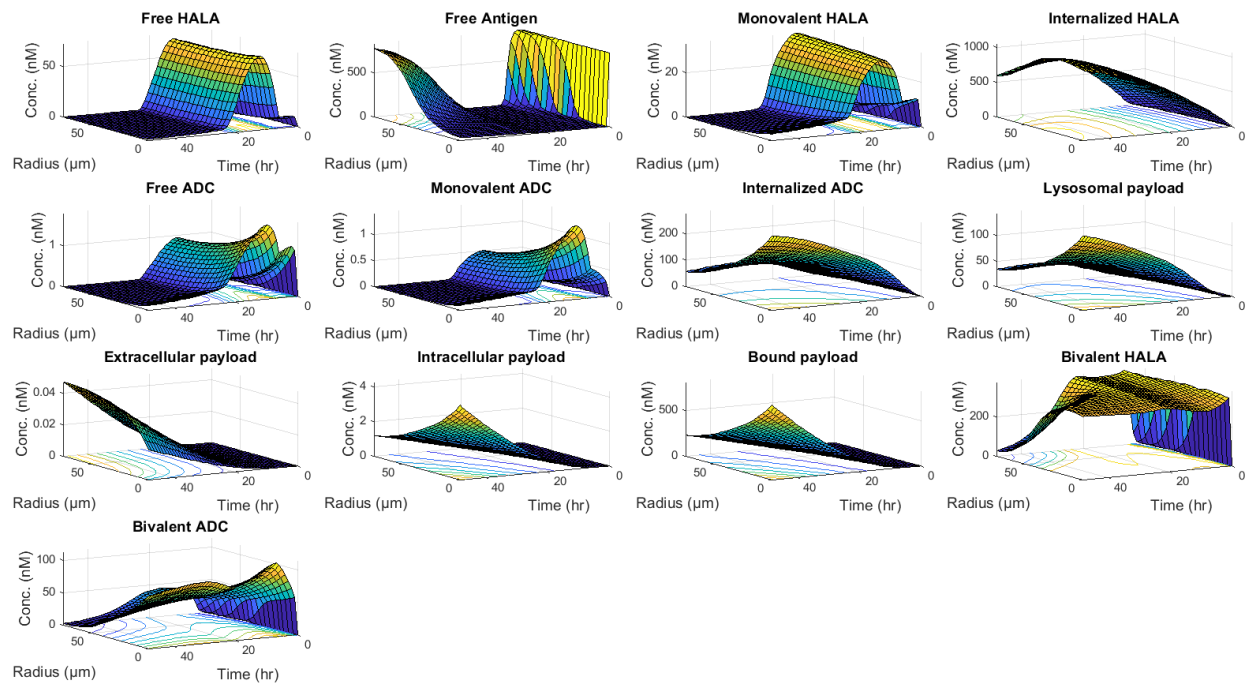


Figure 2.10 Krogh Cylinder simulations of a non-bystander payload at 8:1 dose of HALA and ADC with a 3.6mg/kg ADC dose. Bound Payload is a function of amount of bound ADC and the amount of internalized Payload. Although Bound ADC declines over time, the amount of internalized ADC increases over time, thus the Bound Payload increases.

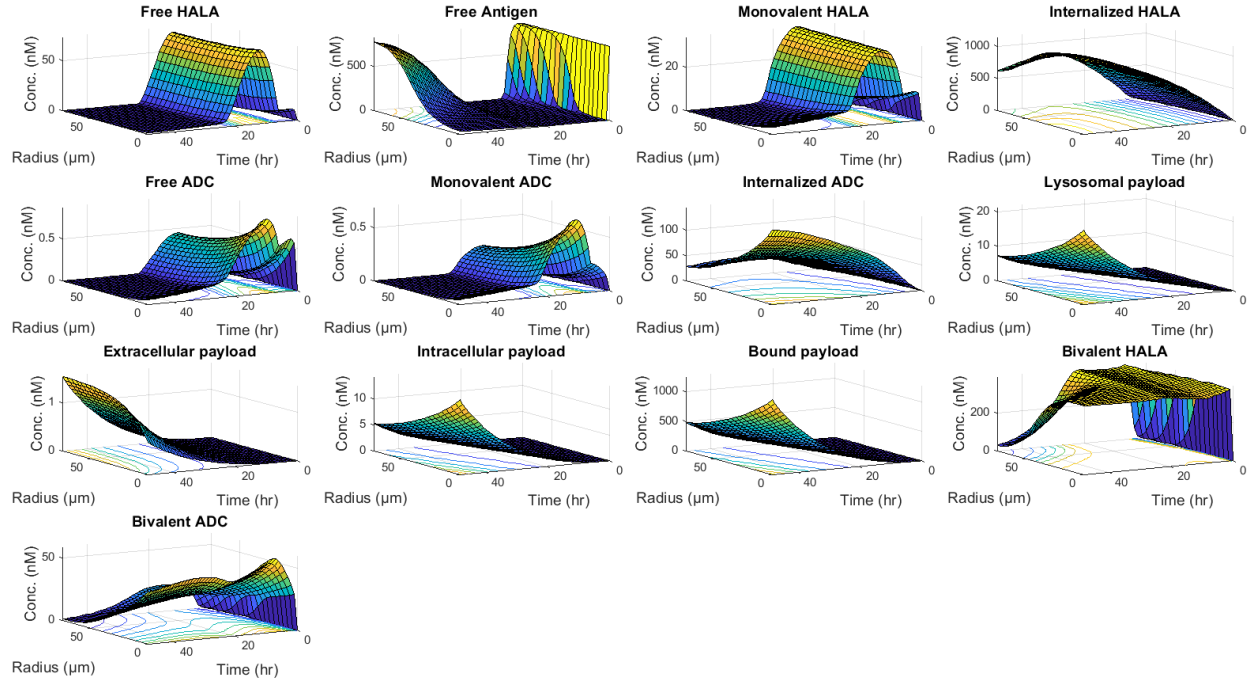


Figure 2.11 Krogh Cylinder simulations of a bystander payload at 16:1 dose of HALA and ADC with a 1.8mg/kg T-MMAE dose. The HALA dose was increased to maintain a total dose that overcomes internalization. Bound Payload is a function of amount of bound ADC and the amount of internalized Payload. Although Bound ADC declines over time, the amount of internalized ADC increases over time, thus the Bound Payload increases. However, due to the bystander effects, the Bound payload amount increases.

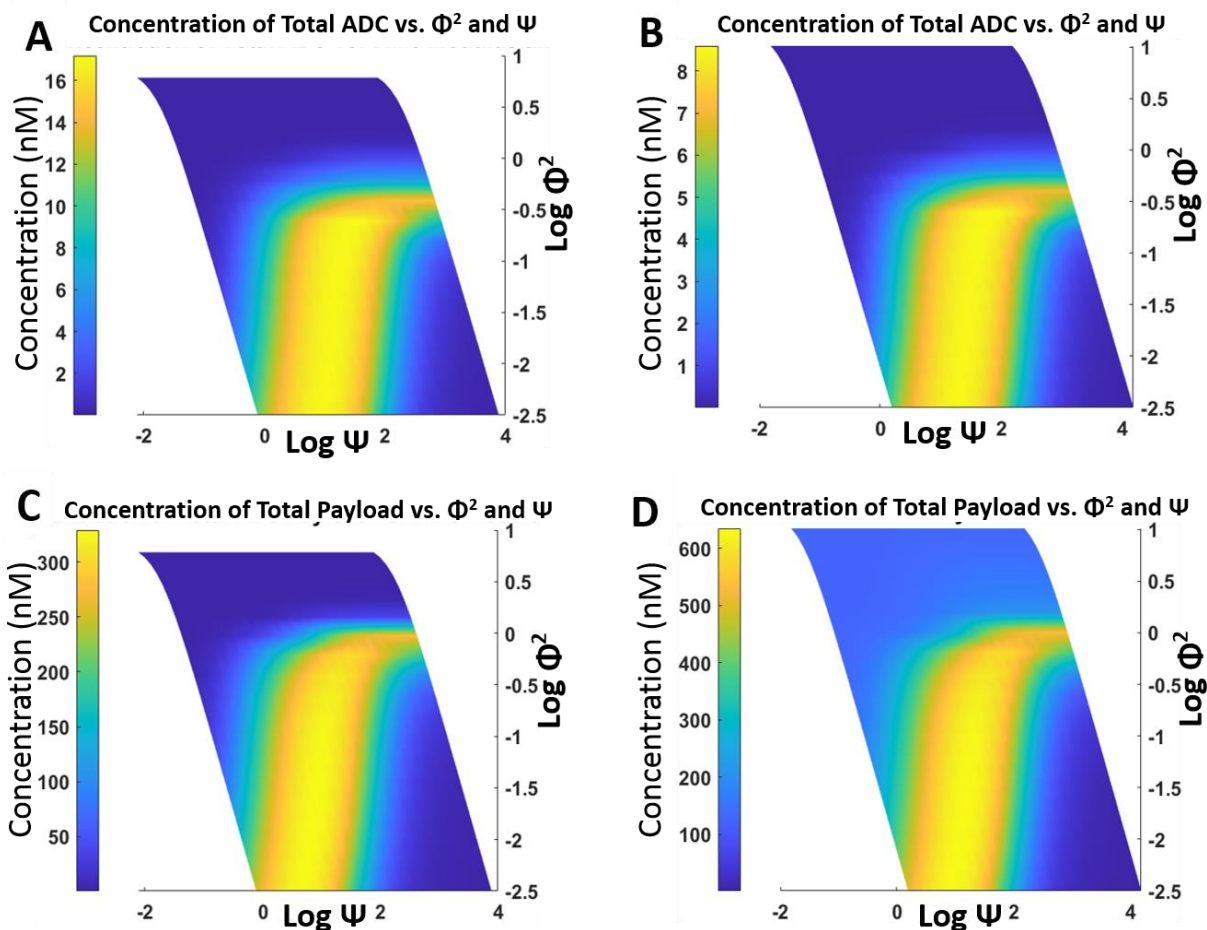


Figure 2.12 Distribution of ADC and Payload of co-administered ADC with HALA antibody showing changing concentration and binding affinity as a function of Ψ and Φ^2 in Krogh Cylinder simulations after 48 hours. (A) Distribution of ADC at dose of 3.6mg/kg with DM1 payload (non-bystander) (B) Distribution of ADC at dose of 1.8mg/kg with bystander (MMAE) payload. (C) Payload of ADC with non-bystander payload. (D) Payload of ADC with bystander payload. The light blue region above the yellow has a higher concentration due to MMAE bystander payload distribution. Due to differences in internalization between the ADC and Payload ADC has a smaller Φ^2 at larger Ψ values, respective to the payload. However, when Φ^2 is close to 1. Ψ is > 1 for good penetration.

Designing Optimal HALA Antibodies

The goal of using HALA antibodies as a carrier dose is to enable efficient penetration in high expression tumors while avoiding competition in cells, tumors, and patients with lower receptor expression. Therefore, from a design perspective, the optimal affinity should be selected based on uptake across a range of receptor expression levels. To accomplish this, the payload

distribution was simulated as a function of affinity and receptor expression to select an optimal affinity. The dose was fixed at an 8:1 HALA to ADC ratio because previous experiments indicated this was an optimal dosing ratio *in vivo* when targeting HER2 using Kadcyla in a high expression tumor (based on tolerability of the ADC). The optimal ratio for other systems will depend on the maximum tolerated dose of the ADC and required antibody dose to saturate the tumor. The binding affinity of the HALA antibody was varied from 1 nM to 10 μ M, and the expression ranged between 10,000 rec/cell to 1,000,000 rec/cell.

Figure 2.13 shows the distribution of the ADC reaching cells far from the tumor vasculature (at the edge of the Krogh cylinder). Stronger binding HALA antibodies increased penetration into high expression tumors, while more weakly bound HALA antibodies were optimal for low expression levels. Around 20 nM, however, the HALA antibody was able to increase uptake in high expression tumors while maintaining payload uptake in moderate expressing tumors.

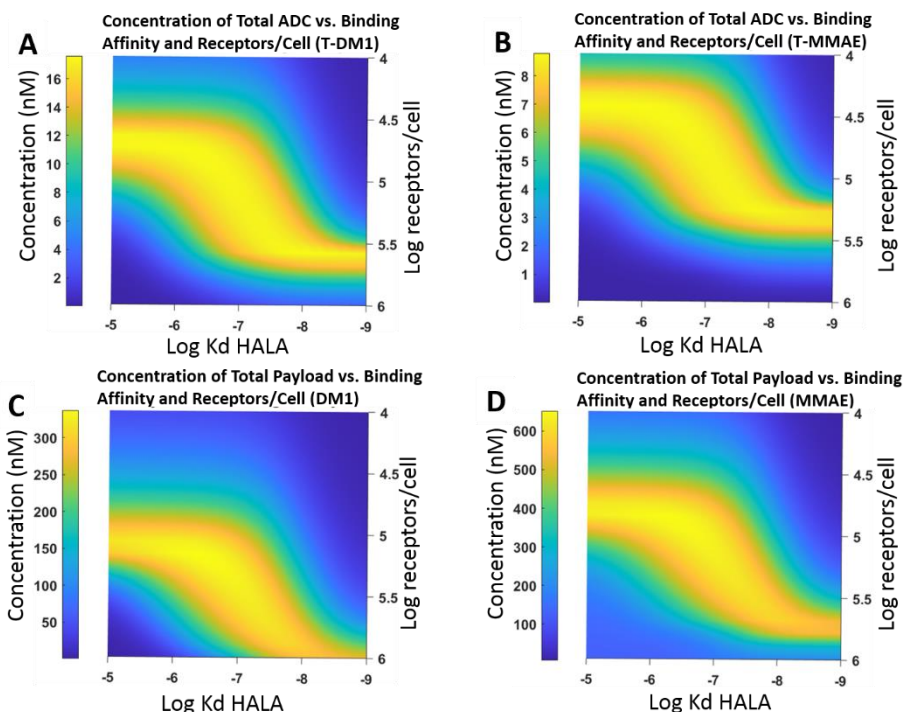


Figure 2.13 Design of optimal HALA with changing antibody dose and affinity after 48hrs.

Distribution of ADC and Payload of coadministered ADC HALA antibody with changing concentration and tumor expression in Krogh Cylinder simulations after 48 hours. (A) Distribution of ADC at a dose of 3.6mg/kg with DM1 payload (non-bystander) (B) Distribution of ADC at a dose of 1.8mg/kg with bystander (MMAE) payload. (C) Payload distribution of ADC with non-bystander payload. (D) Payload distribution of ADC with bystander payload. Difference between ADC and Payload distribution is due to the ADC mostly being cleared at the end of the 48 hours; however, payload is internalized and retained. Concentrations are shown at the Krogh cylinder radius, R_{Krogh} , far from tumor blood vessels.

Figure 2.13 B shows the ADC with MMAE as the payload. It demonstrates similar behavior to the DM1 case; however, the 8:1 ratio is not sufficient to enable full penetration at a 1.8 mg/kg ADC dose, so maximum ADC binding occurs at fewer receptors/cell. Based on these graphs, an optimal HALA antibody affinity can be selected. By appropriately choosing the HALA antibody properties, competition on high expressing cells is efficient, improving tissue penetration several-fold. However, competition on low expressing cells is poor, enabling a majority of receptor to be bound by ADC despite an 8-fold higher concentration of HALA

antibody in solution. **Figure 2.14** demonstrates a more generalizable plot of ADC and Payload concentration as function of Ψ and Φ^2 .

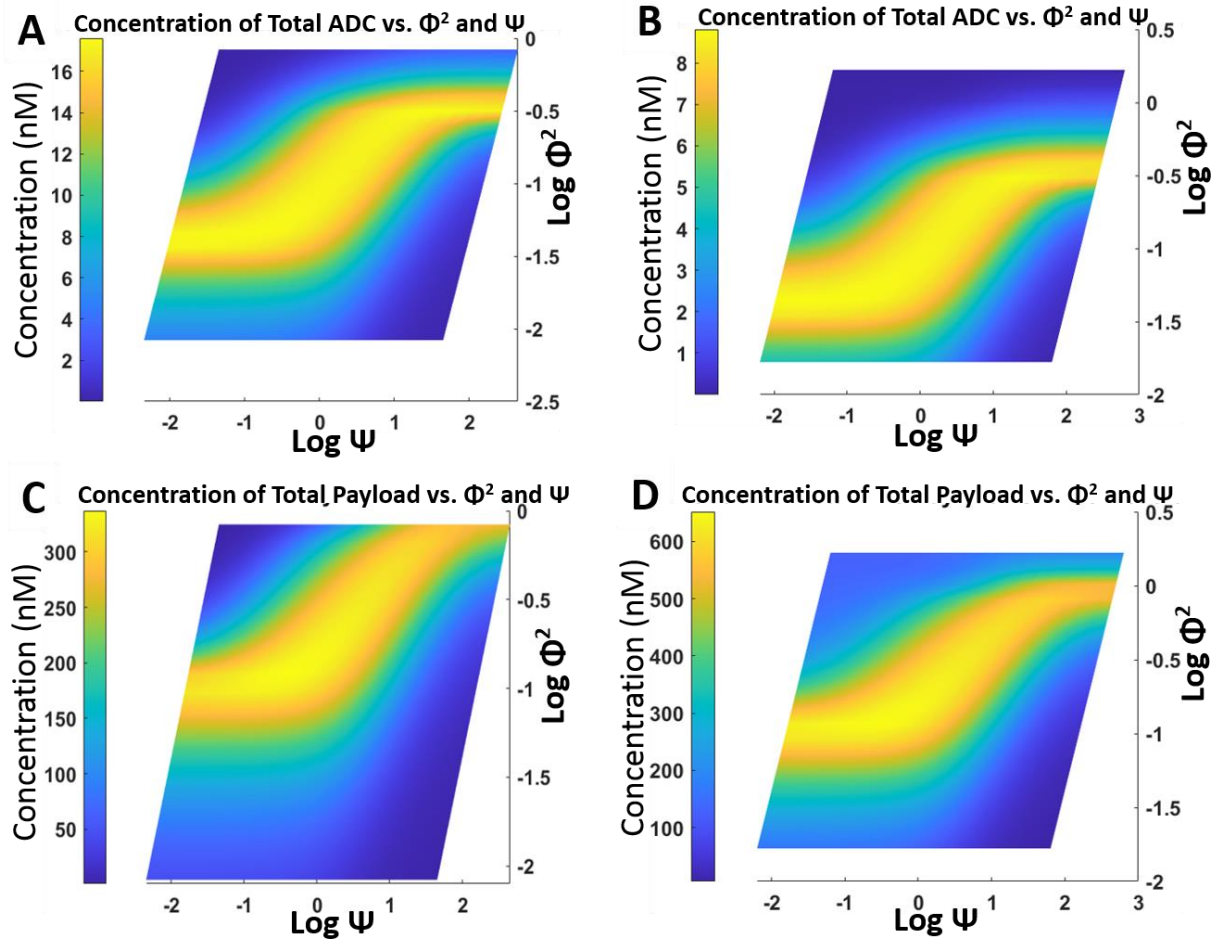


Figure 2.14 Design of Optimal HALA with changing Antibody Dose and Affinity as a function of Ψ and Φ^2 in Krogh Cylinder simulations after 48 hours. (A) Distribution of ADC at dose of 3.6mg/kg with DM1 payload (non-bystander) (B) Distribution of ADC at dose of 1.8mg/kg with bystander (MMAE) payload. (C) Payload of ADC with non-bystander payload. (D) Payload of ADC with bystander payload. The light blue region above the yellow has a higher concentration due to MMAE bystander payload distribution. Due to differences in internalization between the ADC and Payload ADC has a smaller Φ^2 at larger Ψ values, respective to the payload. However, when Φ^2 is close to 1. Ψ is > 1 for good penetration.

2.6 Discussion

Poor tissue penetration of ADCs is a major limitation to their efficacy and has hampered wider clinical success of ADCs(129, 144, 151, 20, 166). Higher antibody doses, whether via lower DAR(166, 167, 168), more tolerable payloads (30), or adding carrier doses(129, 144, 145, 169) can improve the efficacy while lowering target-mediated uptake in healthy tissue to improve the therapeutic window. However, the lower (effective) DAR can also reduce efficacy in lower expression models(126, 170,171). To overcome this limitation, the distribution of a High Avidity, Low Affinity (HALA) carrier antibody was simulated to determine its impact on distribution. The simulations indicate this type of antibody could improve tissue penetration in high expression models while not significantly competing with the ADC in tumors or cells that have lower expression (**Figure 2.1**). This approach could pair synergistically with other strategies such as higher potency, site-specific ADCs to target these lower expression cells.

A simple conceptual picture shows that a HALA antibody binds bivalently (with high avidity) when target expression is high, enabling it to compete with the high affinity ADC. At low expression levels, the HALA antibody binds monovalently (low avidity), resulting in greater competition and binding by the ADC. However, the actual competition is more complex than the diagram, with avidity dependence on valency, affinity, expression density, linker format, etc (172). Antibodies with low (monovalent) affinity can still bind with very high avidity, yielding a low “apparent” K_D on high expressing cells. (“Apparent” K_D and “effective” affinity are commonly used in the field to approximate the avidity of a bivalent antibody by treating it as a monovalent agent when explicitly representing avidity effects is not needed.) For example, clone G98A from Tang et al. has a sub-nanomolar effective affinity on high expressing cells (0.5 nM apparent K_D) despite a low monovalent affinity (270 nM K_D). This also results in negligible

binding to low expressing cells (similar to background) (154). This high effective affinity (greater than the ADC Kadcyla) and negligible binding to low expressing cells may initially appear promising for a HALA antibody. However, in the presence of a high affinity antibody/ADC, this low affinity agent would rapidly be competed off the cell surface due to antibody ‘wobbling’ (173). A more detailed description of binding and competition on the cell surface is needed to identify effective HALA antibodies.

When cells are exposed to a mixture of HALA and ADC antibodies, the on-rate of binding dominates. Since antibodies have similar on-rates, the initial ratio of HALA antibody versus ADC bound is proportional to the concentration (or dose *in vivo*). Analogous to multiple reaction systems, the ADC and HALA antibodies are under kinetic control – the binding is determined by the on-rate, which is proportional to the concentration in solution. As time progresses, the higher affinity ADCs begin to outcompete the lower affinity HALA antibodies to approach the thermodynamic equilibrium. Over long times, the antibodies/ADCs would all reach equilibrium, or thermodynamic control, where the higher affinity ADC dominates binding. However, when targeting live cells, this competition occurs for a limited time – until the target is internalized. If the HALA antibody can outcompete the ADC for this time period, it efficiently competes for binding, allowing deeper tissue penetration. If the shift from kinetic control to thermodynamic control happens faster than the rate of internalization, then the ADC outcompetes the HALA antibody. In other words, if the shift from kinetic control to thermodynamic control happens slower than internalization, the antibodies are ‘trapped’ in their kinetic-controlled state. The amount of competition is dictated by Ψ , the ratio between binding competition and internalization (**Figure 2.2**). The goal of this work is to optimize the kinetics

such that the HALA antibody outcompetes the ADC on high expression cells but not low expression cells. Ψ is therefore large for high expressing cells and small for low expressing cells.

Efficient competition, as determined by Ψ , is not enough to ensure high ADC payload uptake in all cells, however. The total antibody dose (ADC plus HALA antibody) also needs to be sufficient to overcome local metabolism in the tissue. Therefore, there are two requirements to increase penetration to the center of the spheroid. First, the HALA antibody must be able to compete for binding, as determined by Ψ . Second, the total dose of HALA antibody plus ADC must be sufficient to overcome internalization, and this competition is described by the Thiele modulus (20,155,174). These two conditions are required to increase penetration to the center of a spheroid (**Figure. 2.6 and Figure. 2.7**). The dimensionless numbers are more universal for describing uptake, since the explicit values of affinity and dosing are specific to HER2.

However, the dimensional values are more intuitive and therefore shown in **Figure. 2.6**.

When the Thiele modulus is close to one, the dose of HALA antibody and ADC has achieved saturation, which overcomes internalization. If the dose of HALA antibody is too high, then the Thiele modulus will be very low, supersaturating the tumor and lowering ADC uptake. To maintain good penetration, Ψ must also be around 10. If the value is much greater than 10, the ADC will penetrate well for high expression tumors. However, the Ψ value will remain high even in tumors with lower target expression, and the HALA antibody will compete too much with the ADC for these lower expression cells.

The Krogh Cylinder model for *in vivo* simulations adds the additional effects of plasma clearance, blood vessel permeability, and bystander effects (**Figure 2.13**); however, this added complexity does not qualitatively impact how the antibodies distribute within the tissue. The total dose (ADC plus HALA antibody) needs to be sufficient to overcome internalization, and

the affinity must be high enough for efficient competition. For simplicity, plasma clearance is assumed to be the same for both antibodies.

The shape of the curves for both T-MMAE and T-DM1 are similar in the Krogh cylinder model. A sufficient dose and HALA antibody competition are needed to facilitate penetration deeper into the tissue. Overall, the bystander effect from T-MMAE shows better distribution into the cells and is able to maintain a minimum concentration for antitumor activity of 150 nM at a wider range of binding affinity and receptor expression (175). This suggests payloads that exhibit the bystander effect can partially compensate for heterogeneous distribution from either poor ADC tissue penetration or heterogeneous target expression, consistent with clinical experience(176). However, as seen in other systems, higher ADC tissue penetration is still more effective than relying on bystander effects to overcome tissue penetration limitations(177).

To optimally design a HALA antibody for use as a carrier dose, the ADC payload uptake in cells distant from tumor vessels can be graphed as a function of HALA antibody affinity and receptor expression (**Figure 2.13**). By selecting an affinity that is high enough to achieve efficient tissue penetration in high expression tumors but low enough not to compete with the ADC under lower expression conditions, optimal *in situ* competition and maximum efficacy across a range of expression levels can be achieved.

Ultimately, effective ADC treatment requires a positive therapeutic window, where the treatment regimen can achieve significant efficacy relative to toxicity. Therefore, the potential impact of HALA antibodies on toxicity merits discussion. Since HALA antibodies are anticipated to be well-tolerated (similar to typical monoclonal antibodies) they are not expected to increase toxicity by themselves for most targets. Because the toxicity of most ADCs is non-target mediated (178, 179), HALA antibodies are not expected to significantly increase or

decrease toxicity. Rather, they are expected to improve the therapeutic window by having a negligible impact on toxicity while significantly improving efficacy. It should be noted, however, that there are cases where target-mediated toxicity is significant (e.g. TROP-2)(180). In these cases, the HALA antibody is not expected to compete for binding in lower expression tissues. Thus, a higher affinity antibody carrier dose (or lower DAR) may be more appropriate in cases with significant target-mediated toxicity by both lowering uptake in healthy tissue to decrease toxicity and increasing tumor penetration to improve ADC efficacy.

2.7 Conclusion

High Avidity, Low Affinity (HALA) antibodies may serve as optimal carrier doses to increase efficacy of ADCs across a range in cellular expression, even within the same patient or the same tumor. Mechanistic simulations in monolayer, spheroid and Krogh Cylinder models provide insight to the dependency of ADC distribution on coadministered antibody binding affinity, receptor expression, and internalization rate. The data indicate that optimization of the co-administered antibody affinity with ADCs can enable an optimal ratio between competition and internalization to achieve maximum efficacy.

Chapter 3 Insights into Design of Bispecific Antibodies Using Computational Modeling

3.1 Information

The goal of this chapter is to predict the binding and distribution of bispecific antibodies with different affinity on cell types of different expression levels. Notably, the binding between the arms can be binding in ‘trans’ (between two different cell types) rather than in ‘cis’ (binding two antigens on the same cell). This was achieved by updating the models used in Chapter 2 to capture the competition between the cell types in respect to receptor expression and binding affinity of the different binding arms.

3.2 Abstract

Drug resistance occurs when cancer becomes tolerant to an anticancer treatment. This presents a major challenge because 90% of cancer related deaths are attributed to drug resistance. Traditionally drug resistance is overcome through larger doses; however, drugs with cytotoxic payloads (e.g. chemotherapy, ADCs) are limited by their narrow therapeutic index. Bispecific Antibodies (BsAbs) are an emerging class of engineered antibodies that can bind to two different epitopes. BsAbs have many advantages over traditional anti-cancer therapies such as the ability to bind to multiple cell types with different expression levels and induce greater cytotoxicity such as through the activation of T-lymphocytes. However, BsAbs compared to monospecific antibodies have complex binding kinetics. The binding affinity to the epitopes of BsAbs are different than monospecific Abs and cellular expression can be orders of magnitude different.

These contributions have a synergistic effect on bsAbs binding; thus binding kinetics of bsAbs are complex.

Here we use a kinetic model to predict the 'trans' (binding between two different cell types) binding kinetics of three different BsAbs at three different cellular expression levels. The simulations demonstrate that trans binding has significantly different physical constraints than cis (binding between two of same cell type) binding that must be accounted for in the model. Most notably, the avidity effects of a bispecific antibody of trans binding is significantly lower than cis binding. While the model needs further refinement, the data indicates that modeling can capture *in vitro* binding kinetics to describe cellular activation and *in vivo* distribution.

3.3 Motivation

Drug resistance in cancer is a well-documented phenomenon that occurs when cancer becomes tolerant to pharmaceutical treatment. Cancer drug resistance is very challenging because 90% of cancer related deaths are due to drug resistance and ineffectiveness of anticancer therapies (181,182). Drug resistance to anticancer therapies develops by multiple pathways and various mechanisms, such as increased efflux of anticancer drugs that reduces intracellular drug accumulation, acquired mutations that reduces the cellular target expression or affinity, as well as changes in the tumor microenvironment, preventing the drug to fully diffuse into the cells (183, 184). One method to overcome drug resistance is to use higher doses of therapeutic. However, in cases like conventional chemotherapy, where the drug is highly toxic, there are strict limits of how much dose can be increased in order to prevent severe toxicity (185).

Alternative strategies to overcome cancer drug resistance without increasing the dose of therapy is cycling through different types of anticancer monotherapy. One example is using monoclonal antibodies or Antibody Drug Conjugates (ADCs). Antibodies are able to disrupt

downstream signaling pathways and elicit immune cell effector function (e.g. antibody-dependent cellular cytotoxicity), while ADCs have the added utility of releasing a cytotoxic payload when internalized – which all promote cell killing (186). Another example is using hormone therapy. Hormone therapy typically involves the external administration of hormones to inhibit hormone production, which often slow the growth or kill cancer cells (187). Different classes of anticancer therapy are possible because each class uses distinct metabolic pathways. For this very reason, using combination therapy is also a viable strategy to overcome cancer drug resistance. Combination therapy is a strategy that coadministers more than one medication and/or anticancer drug class (188).

Combination therapy is an attractive method to overcome cancer drug resistance because the therapy can target cancer pathways in an additive or synergistic manner. Additionally, combination therapy is often deemed safer than monotherapy because the dosage required for each individual drug may be lower than its monotherapy counterpart. (189, 190). Together, combination therapy can produce more effective treatment responses in fewer cycles, reducing the likelihood of acquired drug resistance. (191,192).

While combination therapy is effective in overcoming drug resistance by engaging multiple pathways simultaneously, combination therapy can be expensive and complicated because, with each additional drug administered, the factors pertaining to the combination drug becomes more complex (e.g. ratio of drugs used, absolute doses, timing, drug-drug interactions); thus a single drug is preferred (190,193).

An emerging class of therapeutics includes bispecific antibodies (BsAbs) that combine the advantages of combination therapy with antibody monotherapy. BsAbs are engineered antibodies that can bind to two different epitopes or targets with different specificities (194).

BsAbs have advantages over single antibodies, which include activation of T-lymphocytes (by cross-linking cancer and immune cells to induce killing), greater cytotoxicity, and the ability to bind to target cells with poor expression (195-197). Additionally, due to their ability to target multiple epitopes, BsAbs can block different targets in a pathway, which helps further drive efficacy, circumventing drug resistance (198).

Despite the significant advantages BsAbs have over monoclonal Abs, they also have a plethora of challenges as well. One challenge is complex binding kinetics. Unlike monospecific antibodies, the binding affinity of the binding arms for BsAbs are different, and the expression levels of the epitopes can be different. Because of this, the binding kinetics of BsAbs are synergistic and highly dependent on local conditions.

In this work, we use a kinetic model to predict bispecific binding *in silico*. Our simulations are able to capture the dependence of bispecific antibodies on binding affinity and cellular expression, in order to better design BsAbs and guide experiments

3.4 Materials and Methods

A previously validated computational model was used to describe the cellular impact of bispecific antibodies. The model used a bispecific competition kinetic model to measure the crosslinking of Abs with the different binding affinity of the binding arms and validated using *in vitro* T-cell activation data. Different BsAb clones were generated by Crescendo Biologics, and Anna Kopp of the Thurber Lab conducted the *in vitro* T-Cell activation assays.

Monolayer competition model:

A computational model describing the binding of two antibody domains of a single bispecific antibody was developed by adapting a previously validated bivalent kinetic model to simulate the crosslinking of the bispecific antibody (51,148). Briefly, the two arms of the

bispecific antibody engage open binding sites. The binding arms can bind to free receptor CD-137 or PD-1 and then can bind to the opposite cell receptor (on a different cell) to become crosslinked or bivalently bound. A summary of this phenomenon is depicted in **Figure 3.1**

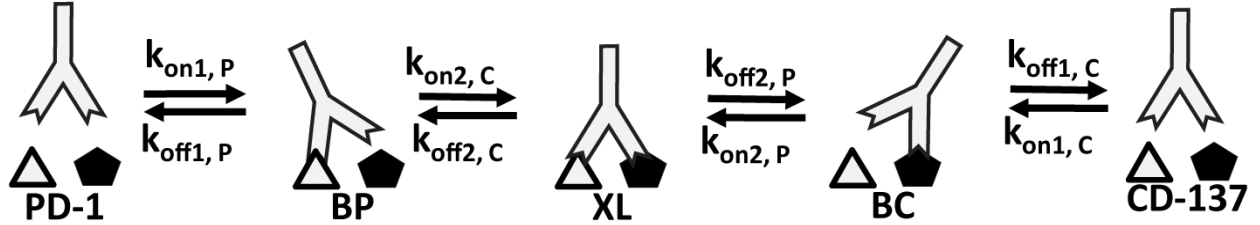


Figure 3.1 Competition kinetics between binding arms of a bispecific antibody. The reaction network with PD-1 receptors (white triangles) and CD-137 receptors (black pentagons) are shown in. Antibodies can bind monovalently bound to PD-1 (BP) or CD-137 (BC) or crosslinked – bivalently bound to both cell receptors (XL).

The differential equations were defined from the reaction network, and initial competition parameters were calculated or determined from the literature:

Free Receptors

$$\frac{d[PD]}{dt} = -k_{on(1P)}[Ab][PD] + k_{off(1P)}[BP] - k_{on(2P)}[BC][PD] + k_{off(1P)}[XL] \quad 1.$$

$$\frac{d[CD]}{dt} = -k_{on(1C)}[Ab][CD] + k_{off(1C)}[BC] - k_{on(2C)}[BP][CD] + k_{off(1C)}[XL] \quad 2.$$

Single Binding event

$$\frac{d[BP]}{dt} = k_{on(1P)}[Ab][PD] - k_{off(1P)}[BP] - k_{on(2C)}[BP][CD] + k_{off(1C)}[XL] \quad 3.$$

$$\frac{d[BC]}{dt} = k_{on(1C)}[Ab][CD] - k_{off(1C)}[BC] - k_{on(2P)}[BC][PD] + k_{off(1P)}[XL] \quad 4.$$

Crosslinking event

$$\frac{d[XL]}{dt} = k_{on(2C)}[BP][CD] + k_{on(2P)}[BC][PD] - (k_{off(1C)} + k_{off(1P)})[XL] \quad 5.$$

where $[Ab]$ is the antibody concentration, $k_{on(1P/1C)}$ is the on rate of the first binding arm to PD-1 or CD-137 cell receptor respectively, $k_{off(1P/1C)}$ is the off rate of the first binding arm to PD-1 or CD-137 cell receptor respectively, $[PD]$ is the concentration of free PD-1 receptors, $[CD]$ is the

concentration of free CD-137 receptors, [BP] is the concentration of the monovalent complex bound to PD-1, [BC] is the concentration of the monovalent complex bound to CD-137, [XL] is the concentration of the bivalently bound complex (Crosslinked), $k_{on(2Pp/2C)}$ is the on rate of the second binding arm to PD-1 or CD-137 cell receptor respectively.

Full descriptions of the constants and variables used in the model is defined in **Table 3.1** and **Table 3.2**.

Parameter	Value	Unit	Description	Reference
$k_{on,1P/1C}$	10^5	$M^{-1}s^{-1}$	On rate of first PD-1 or CD-137 binding arm	Estimated from (152,153)
$K_{d,1P}$	$5.31^a 53^b$ $20.5^a 8^b$ $80^a 90^b$	nM	PD-1 monovalent dissociation constant	TPP 1010 TPP 1246 TPP 983 a= flow cytometry b= fitted
$K_{d,1C}$	$9.39^a 94^b$ $2.48^a 10^b$ $.3^a 6^b$	nM	CD-137 monovalent dissociation constant	TPP 1010 TPP 1246 TPP 983 a= flow cytometry b= fitted
$k_{off,1P/1C}$	$= k_{on,1P/1C} * K_{d,1P/1C}$	s^{-1}	PD-1 or CD-137 dissociation rate	Calculated
$K_{d,2P}$	$= 3.5 \times 10^9 * CF * K_{d,1P}$	Cell	ADC dissociation constant	(51)
$K_{d,2C}$	$= (K_{d,2P} * K_{d,1P}) / K_{d,1C}$	Cell	ADC dissociation constant	Equilibrium approximation
$k_{on,2Pp}$	$= k_{off,1P} / K_{d,1P}$	$M^{-1}s^{-1}$	Crosslinking on rate PD-1 arm	Calculated
$k_{on,2C}$	$= k_{off,1C} / K_{d,1C}$	s^{-1}	Crosslinking on rate CD-137 arm	Identical to $k_{off,ADC/HALA}$
$k_{off2,P/C}$	$= k_{off,1P/1C}$	s^{-1}	PD-1 or CD-137	Identical to $k_{off,1P/1C}$

			dissociation rate (2 nd arm)	
PD-1 ₀	$1 \times 10^4 - 4 \times 10^6$	Receptors Cell ⁻¹	Initial PD-1 concentration	Varies
CD-137 ₀	66,000	Receptors Cell ⁻¹	Initial CD-137 concentration	Calculated

Table 3.1 Constants Used in BsAbs Model

Variable	Unit	Description
Ab	nM	Free Antibody
PD	Receptors Cell ⁻¹	Free PD-1 Receptors
CD	Receptors Cell ⁻¹	Free CD-137 Receptors
BP	Receptors Cell ⁻¹	Ab monovalently bound to PD-1 Receptor
BC	Receptors Cell ⁻¹	Ab monovalently bound to CD-137 Receptor
XL	Receptors Cell ⁻¹	Crosslinked Ab

Table 3.2 Variables Used in BsAbs Model

3.5 Results

Validating the Model

The model was validated by reproducing the binding profiles of a bispecific antibody that binds to CD4 and CD70 on the same cell type (binding in ‘cis’) in a previously validated model with varying Ab concentrations. (51, 199). Results can be seen in **Figure 3.2**

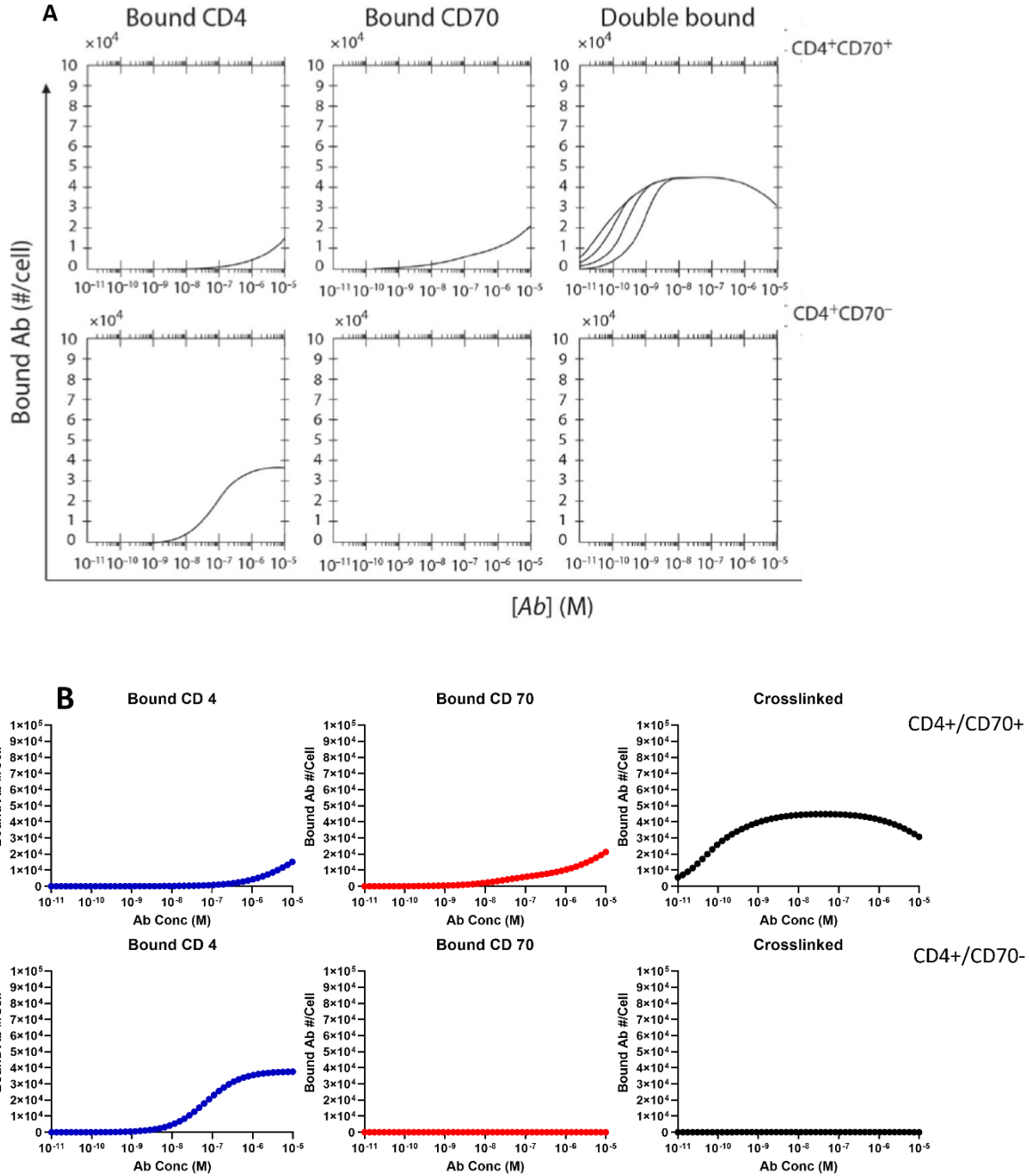


Figure 3.2 Simulated binding profiles of a bispecific antibody. (A) Original binding profiles after 0.3, 1, 3, and 10 hours for a bispecific antibody with 25 nM affinity for CD70 and 70 nM affinity for CD4 on cells expressing both targets (top) or predominantly CD4 (bottom). Each of the three modes of antibody binding is shown (B) Reproduced binding profile after 10 hours for the bispecific antibody using bispecific binding kinetic model.

Fitting a binding affinity

Once the model was validated for binding in the ‘cis’ conformation, we moved to adapt the model for binding in ‘trans’ or binding across two different cell receptors using *in vitro* data obtained from a T cell activation assay. This assay measured the amount of fold change in signal in 3 different bispecific antibody clones with different binding affinities to PD-1 and CD-137 – TPP 983, TPP 1010 and TPP 1246, at various cellular expression levels of PD-1 and a fixed concentration of CD-137 over a range of Ab concentrations. The results of the T activation assay are shown in **Figure 3.3**

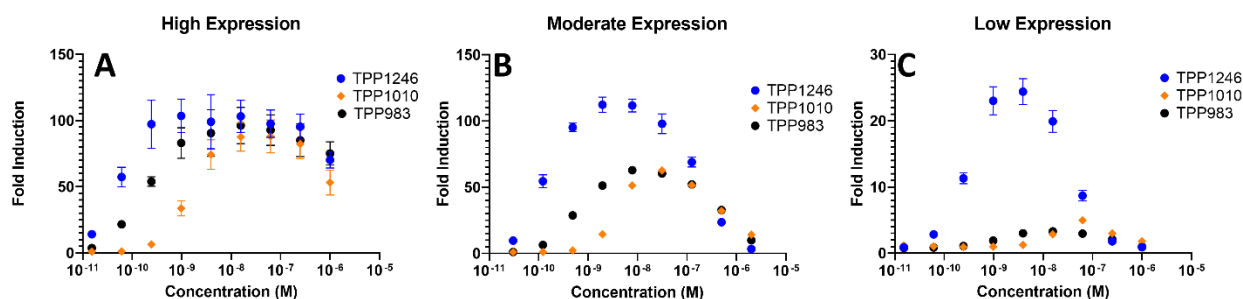


Figure 3.3 T-cell Activation assay of 3 bispecific antibodies of different binding affinity - TPP 1246 (high PD-1 affinity, high CD-137 affinity), TPP 1010 (low PD-1, affinity low CD-137 affinity), TPP 983 (low PD-1 affinity, high CD-137 affinity) at 3 different PD-1 expression levels. Fold induction is change between the original measurement and subsequent measurement. Data was collected by Anna Kopp of the Thurber Lab.

The fold change data was normalized and converted to a fraction of the lowest expressed target (CD-137) as a proxy for the amount of crosslinking. The binding affinities reported from a flow cytometry assay were used in the model, as measured by collaborators at Crescendo. The unmodified model over-predicted the amount of crosslinking in the simulation compared to *in vitro* data without making any adjustments for binding in ‘trans’ versus ‘cis’. With this over-prediction, a data fitted model was used to estimate the apparent binding affinities of the different bispecific antibody clones by fitting the *in vitro* data. The binding affinities of the fitted model were determined to be weaker than the binding affinities measured by flow cytometry.

The comparison between *in vitro* determined and fitted binding affinity data is shown in **Figure 3.4**.

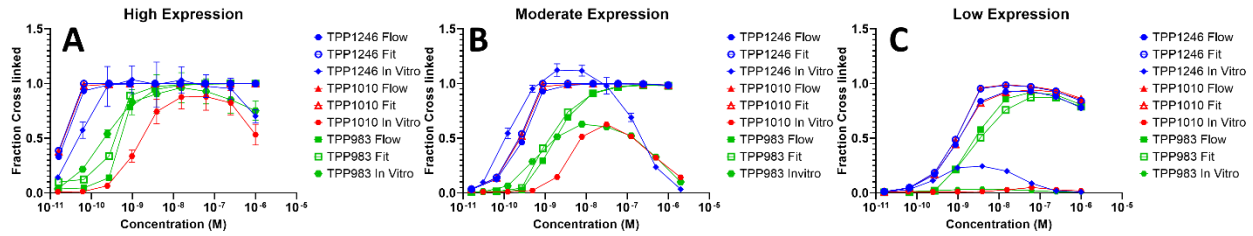


Figure 3.4 Comparison of Crosslinking between *in vitro* and *in silico* T-cell activation assays of 3 bispecific antibodies of different binding affinity -TPP 1246 (blue), TPP 1010 (red), TPP 983 (green) at 3 different PD-1 expression levels. Simulations overestimate the amount of crosslinking due to overestimation of avidity effects

The changing of the binding affinity alone did not generate good agreement of the simulations compared to the *in vitro* data, so we modified the binding affinity of the 2nd binding arm, effectively investigating the impact of binding avidity in the simulations.

Investigating Avidity Effects

The over-prediction of binding in the model compared to the T-cell activation data is hypothesized to be due to the model having a faster ‘second arm’ binding rate (the effective on rate) or stronger avidity binding compared to the *in vitro* data. The original model uses assumptions about the local concentration of an antigen on the same cell surface as the bound antibody (“cis” binding) versus the current binding event which occurs on a separate cell (“trans” binding). To compensate for a slower 2nd binding on rate, a correction factor term was introduced when calculating the dissociation constant K_d of the second binding arm. Physically, the correction factor is accounting for the structural changes that BsAbs experience during “trans” binding which weakens binding avidity at the immune synapse (e.g. stretching to bind far spaced receptors and/or contortion to bind to a non-uniform distribution of cell receptors) Different correction factors were tested across multiple expression levels for each of the antibody clones

and compared to the *in vitro* data, where an “optimal” correction factor was chosen which provided modest reproduction of the *in vitro* data at all expression levels. To determine if the reported binding affinities were valid, an equivalent correction factor was calculated by taking the ratio of the 2nd dissociation constant of the PD-1 binding arm using the optimal correction factor. Results are presented in **Figures 3.5 and 3.6**

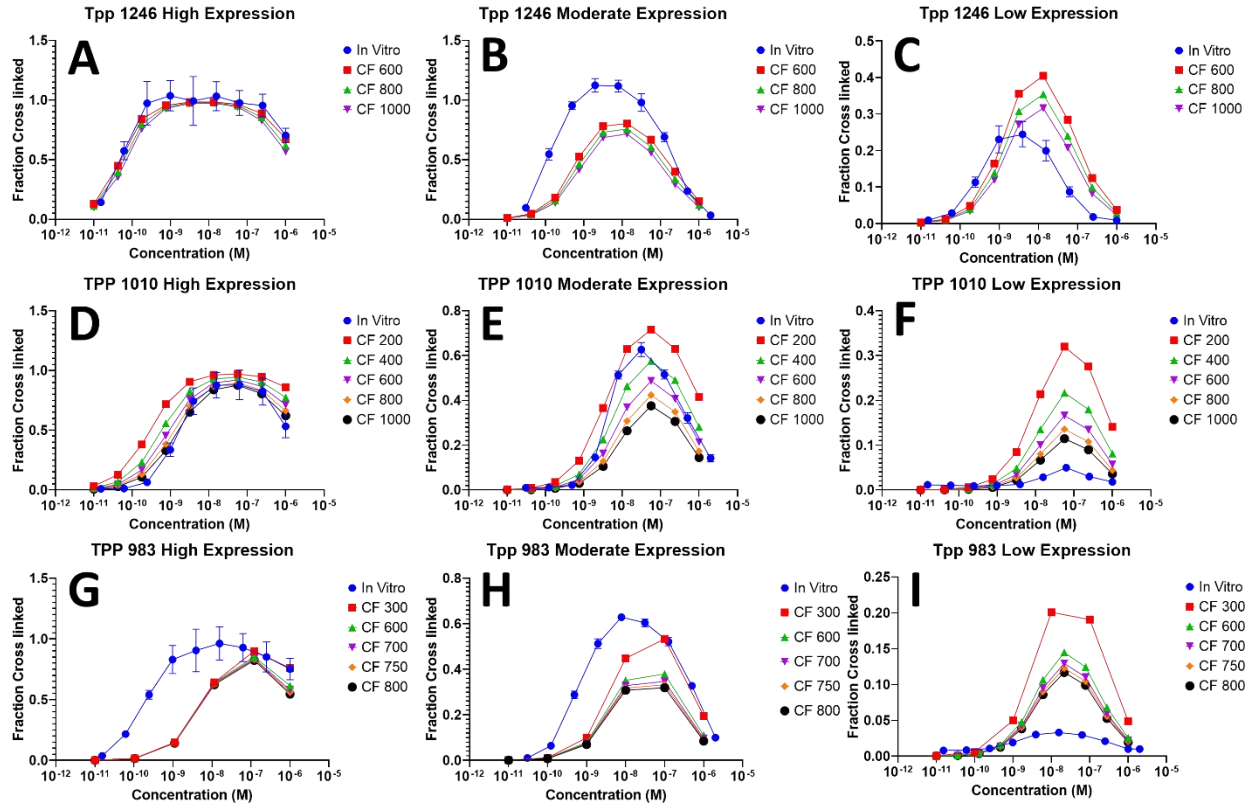


Figure 3.5 Comparison of Crosslinking between invitro and in silico T-cell activation assays of 3 bispecific antibodies of different binding affinity 3 different PD-1 expression levels and different correction factors.

Figure 3.5 compares the amount of cross linking between different bispecific antibodies within the simulation and compares it with *in vitro* data. The high expression cell line simulations show a good agreement with *in vitro* results and a general insensitivity to the correction factor. For clone TPP 983 in the high expression cell line (**Figure 3.5 G**), simulations demonstrate an IC₅₀ shift towards the right indicating slower binding kinetics. In contrast, the moderate expression and low expression cell line simulations demonstrate a strong sensitivity to

the correction factor. Generally, a smaller correction factor results in a higher amount of crosslinking. However, there are trade-offs when choosing a correction factor. A smaller correction factor can yield better agreement with the *in vitro* results, but the low expression cell line simulation with the corresponding correction factor overestimates the amount of crosslinking. Therefore, a correction factor of 1000, 600, and 600 were selected for TPP 1246, TPP 1010 and TPP 983 respectively as a balanced correction factor which yielded a modest agreements for all cell lines.

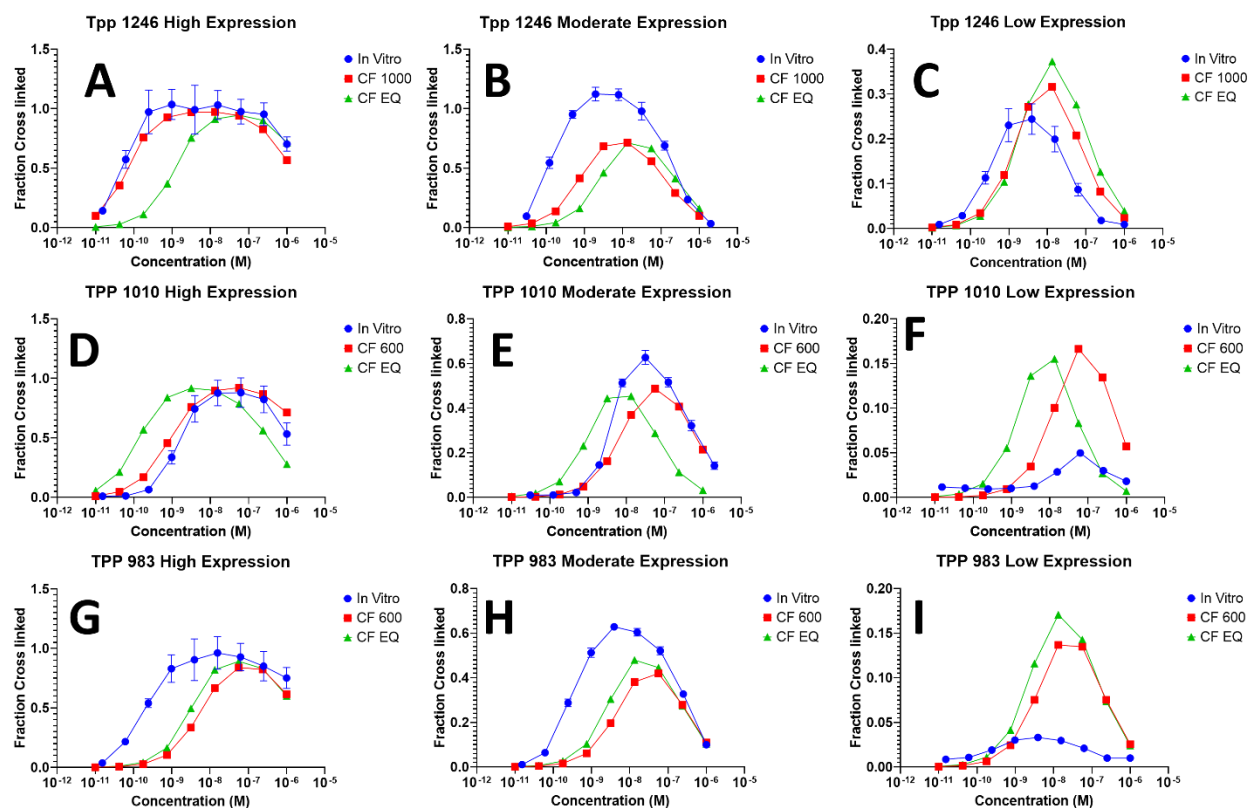


Figure 3.6 Comparison of “optimal” Crosslinking in silico T-cell activation assays between 3 sets of reported binding affinity for 3 bispecific antibodies of different binding affinity across 3 different PD-1 expression levels.

Figure 3.6 compares the amount of crosslinking between the correction factor when using the fitted binding affinities and the same correction factor with the binding affinity reported by flow cytometry (CF EQ). For the TPP 1246 and TPP 983 clones, the CF EQ has generally good agreement between the *in vitro* results and previous simulation. However, there is

a shift of the IC_{50} towards the right. However, for the TPP 1010 clone, the CF EQ simulations have a similar magnitude as the CF 600 simulations but an IC_{50} shifted to the left, with less crosslinking at higher concentrations. In general, higher binding affinity to the targets shifts the curves left while an increase in the correction factor (a reduction in avidity) lowers the amount of crosslinking, particularly for low expression systems.

3.6 Discussion

Drug resistance of anticancer therapies is a significant challenge that causes the majority of cancer related deaths (181,182). Cycling through different drugs of the same class can improve efficacy using different metabolic pathways, and combination therapy via hormone therapy, immunotherapy, targeted molecular therapy, etc. can circumvent drug resistance using drugs that utilize different mechanisms of action (200-202). However, finding the right combination therapy can be challenging given the complexity of identifying an optimal dose ratio, varying pharmacokinetics, and the potential for increased toxicity (203).

To overcome these limitations, the use of bispecific antibodies has been proposed for their improved cytotoxic effects, ability to bind to low expression targets, and ability to induce novel mechanisms of actions (194,204). However, the binding kinetics of BsAbs are complex; therefore, a mechanistic kinetic model was developed to determine the distribution and binding kinetics of BsAbs to better guide the design of BsAb structure and experiments.

The reaction network and model (**Figure 3.1**) show that the BsAbs bivalent binding to a second cell type (trans binding) is fast after it has bound to the first cell receptor. However, the actual competition is more complex than the reaction network, with avidity effects that the model does not completely capture (205,206). These include the effects of linker length and flexibility, epitope location, and cofactors in the synapse among other factors. When compared to *in vitro*

results of the T-cell activation assay, it was determined that the initial binding avidity is too fast since simulations overestimated the amount of crosslinking. One hypothesis for this discrepancy is the effective binding affinity. Binding avidity of BsAbs in an immune synapse cannot be directly measured. Challenges of predicting crosslinking and avidity effects include estimating the levels of target engagement for both targets, which are affected by absolute levels of target expression for both cell types, target mobility in the synapse, and the potential for steric hindrance in the simultaneous binding of the two targets (207). Therefore, the affinity of each individual binding arm was fit independently since experimental methods can also impact the reported results by several orders of magnitude (208-211). Without a direct measurement of affinity under identical conditions as the T-cell activation assay, it is difficult to directly compare the computational and experimental results. For the T-cell activation assay, while the target expression is fixed, it is unknown if the distribution is uniform. Uniformity of expression and the distance between the cells can impact avidity effects, which the model does not account for. This could explain why the model estimates higher binding and crosslinking, requiring the need for a correction factor and new fitted binding affinity parameters to account for these differences (205).

After using fitted binding affinity data and correction factors to account for the trans binding avidity effect, the simulations provide reasonable agreement with the *in vitro* T-cell activation assay. At high PD-1 expression levels, there was an overall insensitivity to the correction factor for all three clones. This insensitivity is likely due to the high density of PD-1 receptors. At higher receptor densities, the probability of binding increases significantly, reducing the dependency of binding via an avidity effect.

As PD-1 expression levels decrease, the dependence on binding avidity increases with lower CF values simulated for high levels of binding. As the CF values decrease, the binding avidity increases (lower K_d = stronger binding) resulting in more crosslinking. In contrast, at low PD-1 expression, the binding avidity is apparently too strong, overestimating the amount of crosslinking in simulations compared to the *in vitro* results.

Despite the agreement between *in vitro* results and numerical simulations, there are some discrepancies with the results. While the fold induction was assumed to be a proxy for crosslinking, the relationship between the amount of T-cell activation and the amount of crosslinking is currently unknown. The current model assumes a linear relationship (proportionality constant). The relationship is significant because currently, the minimum and maximum threshold for T-cell activation is unknown. T-cell activation likely requires a minimum number of CD-137 receptors to be activated before the signal is transduced and could have a maximum limit where more activated CD-137 receptors do not cause a higher signal – effectively creating a minimum and maximum threshold (212-216). This is significant because for moderate PD-1 expression, while it appears that the model underestimates the amount of crosslinking compared to *in vitro* T-cell activation results, it is possible that the amount of crosslinking reaches the maximum threshold of T-cell activation, despite less crosslinking. Similarly for low PD-1 expression, more BsAbs are crosslinked; however, the lower value may not be enough to reach the minimum threshold for T-cell activation.

The differences in binding avidity and affinity between *in vitro* results and simulations may also account for the shifting of the IC_{50} . The simulations are shifted to the right, suggesting that the fitted binding affinities for the simulations are weaker than *in vitro* binding affinity, resulting in less potent clones. (217, 218)

Interestingly, TPP 983 demonstrated different trends compared to the TPP 1010 and TPP 1246. TPP 983 simulations demonstrated a discrepancy where the IC_{50} was shifted to the right (weaker activation) relative to experimental data; however, the magnitude of the discrepancy is reduced when comparing signaling with lower PD-1 expression. This is because of the binding affinity of TPP 983 has low binding affinity to PD-1 and high affinity to CD-137. Because of the relative lower abundance of CD-137 receptors compared to PD-1, the effect of the potency from the high affinity CD-137 arm is outweighed by the lower affinity of the PD-1 arm, reducing the amount of crosslinking in the simulation. When the expression of PD-1 is reduced, where it is closer in abundance to CD-137 expression, the simulated crosslinking is in closer agreement with the experimental data. In other words, at very high expression levels of PD-1, the expression helps compensate for the lower PD-1 affinity more than the simulation captures.

3.7 Conclusion

Bispecific antibodies may serve as next generation therapeutics to increase efficacy and avoid drug resistance of anticancer drugs. Mechanistic kinetic binding simulations in the immune synapse provide insight on the dependency of bispecific antibody distribution to the antibody binding affinity and avidity of each arm, and receptor expression of both cell types. Significantly, the avidity of a bispecific antibody binding in ‘trans’ is significantly less than a bispecific antibody binding in ‘cis,’ which must be included in kinetic models. While the model needs further refinement, the data indicates that the model can capture trends with *in vitro* binding kinetics and can be improved to describe cellular and *in vivo* distribution and activation.

Chapter 4 Orally Available Imaging Agents for the Early Detection and Treatment Monitoring of Rheumatoid Arthritis

4.1 Information

The goal of this chapter is to develop orally available Near Infrared imaging agents for the early and specific detection of Rheumatoid Arthritis for early treatment. This was achieved by synthesizing targeting ligands that bind to different cellular targets and conjugating them to near infrared fluorophores (molecular dyes) of non-overlapping wavelengths (one in the 700 nm channel and another in the 800 nm channel). Co-administration of multiple imaging agents that bind to different targets can provide increased molecular information for more specific and accurate disease diagnosis.

4.2 Abstract

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that causes irreversible damage of the joints. However, drugs exist that can stop or reverse disease progression; thus there is intense interest in early detection and treatment monitoring to improve patient outcomes. Imaging has the potential for early detection, but current methods are time-consuming, expensive and/or lack the sensitivity to differentiate RA from similar arthritic diseases. In this study, we examined the self-administration of molecular imaging agents through subcutaneous (SC) and oral administration of an integrin and hydroxyapatite binding near-infrared (NIR) fluorescent imaging agent for regular screening of RA. Using NIR fluorescent imaging, in comparison to current imaging methods, does not use ionizing radiation, is low cost,

minimally invasive, and provides both spatial and molecular information. The study demonstrates that for SC, oral, and intravenous administration, there is an increase in uptake of Integrin-800CW and Alendronate-680RD in the inflamed joints of an adjuvant induced arthritis (AIA) rat model compared to healthy rat joints. While the signal to noise was not sufficient for the imaging agents to detect RA at subclinical time points, coadministration and targeting of the imaging agents could help differentiate different arthritic conditions.

4.3 Motivation

Rheumatoid arthritis (RA) is a common chronic and extremely disabling autoimmune disorder that affects 1% of the population with an estimated cost of \$19.3 billion annually (70, 71). A characteristic symptom of RA includes inflammation of the joints; however long-term symptoms can include irreversible joint damage, increased risk for stroke and heart attack, interstitial lung disease, and cancer (72-74). While there are no cures for RA, disease modifying anti-rheumatic drugs (DMARDs) can slow progression and minimize symptoms. Therefore, early detection is imperative to maintaining the highest possible quality of life and reducing morbidity (75-78). Current research suggests that there is a window of three months after first symptoms in which DMARDs are more effective at treating RA (79). These studies suggest that early into disease progression, RA is still malleable and has not fully matured, further highlighting the significance of early detection and treatment. Despite significant advancements in RA diagnosis in the past 25 years, detection, quantitative measurement, and monitoring of RA remains challenging.

Common techniques for detection of RA include blood tests, radiography, MRI, and ultrasound imaging. Blood testing is inexpensive, but achieving high specificity and sensitivity for accurate diagnosis is a challenge, nor does it provide any information on the condition of the

joint (84, 219). Conventional radiography is a standard choice for RA imaging; due to its ability to consistently detect structural damage, however, this technique lacks the sensitivity of detecting early inflammation and has some risk due to use of ionizing radiation(220, 221). Magnetic Resonance Imaging (MRI) overcomes the sensitivity and safety limitations of radiography; however, it is restricted to high operational and technical costs (222). Ultrasound is another common diagnosis technique for its low cost; however, it is the most operator dependent (223). Quality of sonographic images is highly variable depending on the skill of sonographers, and inexperienced sonographers may not take adequate scans and/or misinterpret the results (224). Thus, an inexpensive, accurate, safe, and early diagnosis to detect RA would create a dramatic improvement in patient outcomes.

Optical molecular imaging is a developing modality for early screening of RA, potentially able to detect the molecular, cellular, and physiological changes that precede anatomical damage, without the use of ionizing radiation (225). There are previous reports relating fluorescent intensity and joint inflammation in molecular probes (226 - 228). Indocyanine green (ICG), a non-specific dye that binds to plasma proteins, has been studied in a few reports since it is the only clinically approved near-infrared (NIR) dye (229). Although ICG has been reported to differentiate RA joints from healthy joints during later stages of the disease (230), detection at the earliest stages, ideally subclinical inflammation for screening purposes, remains challenging and unrealized (227, 231, 232).

For feasibility of an optical molecular imaging agent, a safe, inexpensive, and convenient (to improve patient compliance) delivery method is needed. Self-administration routes like oral delivery (PO) or subcutaneous delivery (SC) are ideal for their ability to screen populations, patient preference, time, and cost savings (233, 234). Intravenous (I.V) delivery, while the most

common delivery route, has a higher chance of anaphylaxis (235, 236). Additionally, even the most quickly cleared imaging agents require imaging to happen several hours post-delivery to allow the agent to clear from the body, resulting in multiple or extended visits to the clinics. These visits can increase financial and personnel burdens for both patient and health care staff. Self-administration of imaging agents would allow a single visit to the clinic, mitigating screening costs.

Previously, an experimental integrin ($\alpha_v\beta_3$) binder conjugated to increasingly negatively charged NIR fluorophores was developed and tested in mouse studies for detection of RA (114). The integrin binder conjugated with IRDye800CW with an overall charge of -4 was determined to have a maximum oral absorption of 2% and provided sufficient accumulation in inflamed mouse joints for imaging. However, RA is a complex disease with multiple biomarker associations such as metalloproteinases, the folate receptor, and somatostatin receptor 2, which are not specific solely to RA (237-239). For example, integrin $\alpha_v\beta_3$ is highly expressed on macrophages that are present at the sites of inflammation in diseases such as cancer, osteoporosis, and RA(114, 240, 241). Thus, the integrin imaging agent alone lacks specificity and cannot individually distinguish between RA and other inflammatory arthritic conditions. Therefore, additional molecular information must be obtained before making a positive confirmation.

To guide development of imaging agents for specific detection of RA, 3 additional imaging agents were developed using integrin, alendronate, and folate. Their bioavailability, and optical properties were then tested in mice models. The lead agents were coadministered in order to determine if a dual channel approach is possible for early, specific detection, treatment

monitoring of RA, and effective across multiple animal models (rat and mouse models). The results can be used to guide the development of dual imaging agent technology.

4.4 Materials and Methods

4.4.1 Synthesis of Imaging Agents

Integrin Agents

The integrin agent was synthesized similarly to previous reports (240). Briefly, the targeting ligand (ChemPartner, Waltham, MA) was commercially synthesized as an ester and resuspended in DMSO in the lab at a concentration of 300 mg/ml. Half of the ester group on the integrin binder was hydrolyzed to a carboxylic acid by mixing it with 150 μ L of ethanol and 7 μ L of 1M NaOH per mg of drug with continuous stirring overnight. This mixture was then neutralized with 1M HCL and the solvents were evaporated. IRDye 800CW was obtained from LI-COR (Lincoln, NE)). The hydrolyzed integrin binder and unhydrolyzed “prodrug” variant were reacted with the fluorescent dye at a 2:1 molar ratio in the presence of 2 μ L of triethylamine per mg of drug in separate reaction mixtures overnight.

Alendronate Agent

The drug alendronate (Sigma Aldrich, St Louis, MO) was used as a targeting ligand by resuspending in DI H₂O to react with IRDye 680 RD (obtained from LI-COR, Lincoln, NE). The solution was reacted with fluorescent dye at a 5:1 molar ratio in the presence of 10% v/v NaHCO₃ for 1 hour.

Folate Agent

Folate (Sigma Aldrich, St Louis, MO) was used to target the folate receptor by first resuspending the agent in DMSO. EDC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride) and NHS (*N*-hydroxysuccinimide) were added at a 2:2:1 ratio of EDC:NHS: folate and mixed

for 16 hours. Afterwards a 5 eq molar of N-boc cadaverine to folate was added along with 2 μ L of triethylamine per mg of folate and incubated overnight. The mixture was purified using a preparative scale Luna C18(2) column (Phenomenex; Torrance, 62 CA) on a Shimadzu reverse phase HPLC. The purified linker product was confirmed by electrospray ionization mass spectrometry.

After purification and cleavage of the Boc protecting group, the product was resuspended in DMSO. The solution was reacted with IR Dye 680 RD fluorescent dye (LI-COR, Lincoln, NE) at a 5:1 molar ratio in the presence of 10% v/v NaHCO_3 for 1 hour.

Imaging Agent Purification

The imaging agents were purified using a preparative scale Luna C18(2) column (Phenomenex; Torrance, 62 CA) on a Shimadzu reverse phase HPLC. Full purification methods are provided in the **Appendix Table B.1**. The purified products were confirmed using MALDI-TOF MS (High affinity IRDye800CW agent: m/z calculated 1393, found 1396; Prodrug 800 CW agent: m/z 1421, found 1423.63, Alendronate 680 RD agent m/z 1093, found 1094.88, Folate-Cadaverine 680 RD agent m/z 1367, found 1368.17. The purities of these agents (254 nm) were measured on HPLC (High affinity IRDye800CW agent: 95% (99.9% fluorescence purity); Prodrug agent: 97.4% (99.9% fluorescence purity) Alendronate agent: 93% (95% fluorescence purity); Folate Cadaverine agent: 91% (92% fluorescence purity).

In vivo experiments

To determine the absorption following PO and SC administration, 1.5 nmoles of the imaging agent (~0.1 mg/kg) were administered in separate 8-10 week old female black-6 mice via dorsal SC injection or tail vein intravenous (IV) injection or 75 nmoles were administered (5mg/kg) via oral gavage (PO delivery). In rat models 7.5 nmoles of the imaging agent (~0.1

mg/kg) were administered in separate 8-10 week old female Lewis Rats via SC and intravenous (IV) injection or 375 nmoles were administered (5mg/kg) via oral gavage. Retro-orbital blood sampling was conducted at 5, 15, 30, 60 120, 240, 360, 1440, and 2880 minutes post-administration for SC and PO delivery and at 1, 3, 5, 15, 30, 60, 180, 360, 1440 and 2880 minutes post-administration for IV delivery. 10 μ l of blood was mixed with 20 μ l PBS-EDTA and spun down at 2000Xg for 1 minute. The plasma (supernatant) was then pipetted into a 384 well plate and the fluorescence of the sample was measured on an Odyssey CLx. The signal was converted into a plasma concentration of the imaging agent using a calibration curve in mouse plasma. Area under the curves (AUC) for the mice were calculated using the trapezoidal rule and % absorbed was determined by the ratio of the AUC of SC to IV. For oral delivery, the percent absorbed was measured as previously reported in Bhatnagar et al (240).

The Adjuvant Induced Arthritis (AIA) model was used for imaging (Chondrex; Redmond, WA). Lewis Rats of 8-10 weeks of age were used for this study. 1 mg/ml of Complete Freund's Adjuvant (CFA) was administered by SC injection into rats at the base of the tail (Jackson Laboratories; Bar Harbor, ME). The extent of paw and ankle inflammation was measured using calipers and the rats were administered doses of the imaging agents after 3, 5, 7, 10, and 14 days.

Imaging agents were administered by either SC injection (dorsal side between the shoulder blades) or PO administration to the AIA model mice with inflamed joints or to control mice with no inflammation. For SC delivery, the animals were anesthetized using 2.5% isoflurane and the imaging agents were either co-administered at a dose of 7.5 nmole. For oral delivery, a 18-gauge needle (Fine Science Tools; Forster City, CA) was used to administer a 375 nmole dose. Post injection, the rats were imaged on an IVIS Spectrum (Perkin Elmer; Waltham,

MA) at 48 hours. After the 48-hour image, the rats were euthanized and all the organs were resected for biodistribution measurement. The biodistribution protocol is adapted from previous studies (242). Briefly the tissue was minced with a razor blade, weighed, and digested in Eppendorf tubes using a collagenase (Worthington Biochemical; Lakewood, NJ; Cat. No. CLS-4) solution (5 mg/ ml) in RIPA buffer (Boston BioProducts; Ashland, MA) at 37°C for 20 minutes. The organs were sonicated, digested for 20 minutes at 37°C using a 50:50 trypsin and RIPA buffer solution and sonicated again. A dilution series of the resulting mixture was scanned in a black walled 96-well plate using an Odyssey CLx. Absolute quantification was obtained by comparison with a calibration curve. The uptake values were normalized to the average amount of probe that reached the systemic circulation (absorbed dose) per gram of tissue.

4.5 Results

NIRF imaging agents and their properties

Previous studies have shown that a negative charge on imaging agents can facilitate sufficient targeting following oral delivery (240) by increasing the target specificity, extending circulation in the body long enough to be taken up by antigen positive cells, retaining the agent in cells after receptor mediated internalization (due to their residualizing nature), and increasing oral absorption. In this study, the pharmacokinetic properties of Integrin 800 CW, Prodrug Integrin 800 CW, Alendronate 680 RD, and Folate Cadaverine 680 RD were tested in rat and/or mouse models. The structures of the tested imaging agents are shown in **Figure 4.1** and the key pharmacokinetic data are shown in **Table 4.1**

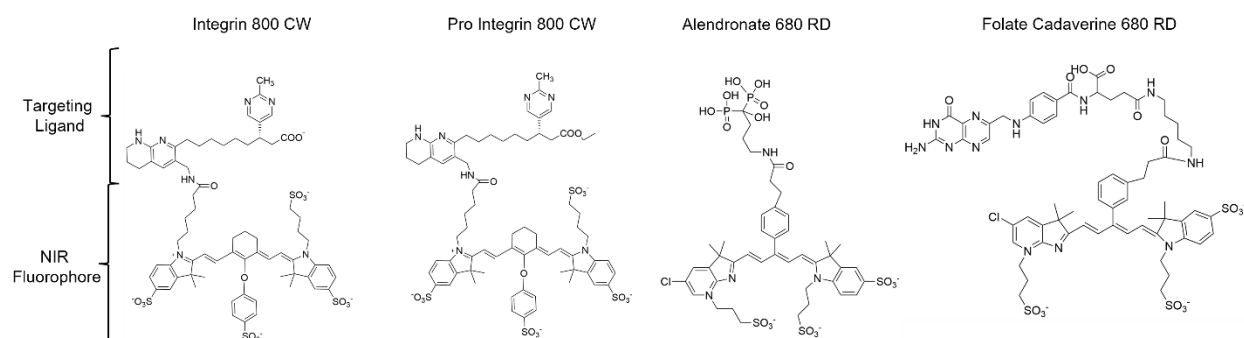


Figure 4.1 Structures of Proposed Imaging agents used for early detection and treatment monitoring of Rheumatoid Arthritis

Compound	MW	Animal	% Bioavailability
Int-800 CW	1393	Rat	67 ± 8 (SC) 2 ± 1.1 (PO)
Prodrug Int-800 CW	1421	Mouse	0.2 ± .8(PO)
AA-680 RD	1093	Mouse	46±(SC) .05±.87 (PO)
Fol-Cad-680 RD	1367	Mouse	67±3.4 (SC) 0.4 ± .21(PO)

Table 4.1 Pharmacokinetic Properties of Proposed Imaging Agents

Detection of disease

To study the effect of the physicochemical properties of the agents on absorption and targeting, candidates with successful PK properties (AA680 RD, Fol Cad 680) were tested by administering SC or IV delivery in C57BL/6 (B6) or CAIA mouse models and imaged after 24 hours. Images show that AA 680 RD provides significant contrast in the joints in healthy B6 mice, compared to uninjected control mice for both IV and SC delivery. Signal at the joints overall is higher for IV administration. Results are shown in **Figure 4.2**.

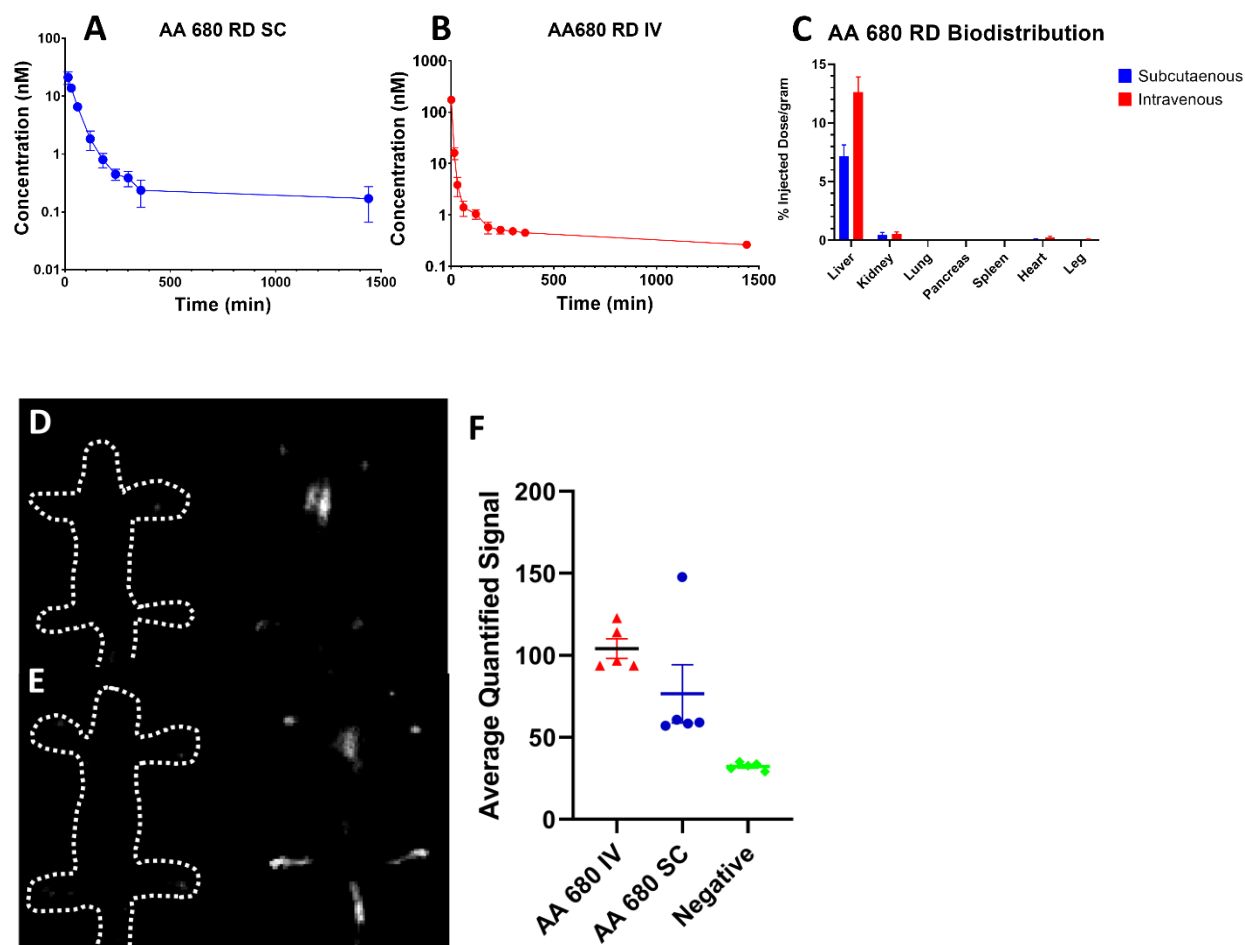


Figure 4.2 Subcutaneous and Intravenous Administration of Alendronate 680 RD after 24 hours in healthy mice (A). Plasma clearance of Alendronate 680 RD administered SC. (B). Plasma clearance of Alendronate 680 RD administered IV. (C) Biodistribution and average signal (F) were compared between both routes of administration. (D,E) IVIS images taken at 24 hours post administration for both administration routes. Mouse outlines show uninjected mice (autofluorescence).

Healthy mice administered with Fol Cad 680 RD did not have a significant increase in signal compared to the uninjected mice indicating a low level of specific uptake of the probe. CAIA mice were then dosed IV with Fol Cad 680 RD. Signal of the CAIA mice was not significantly higher than healthy mice despite over expression of folate receptor (243). Results are shown **Figure 4.3**.

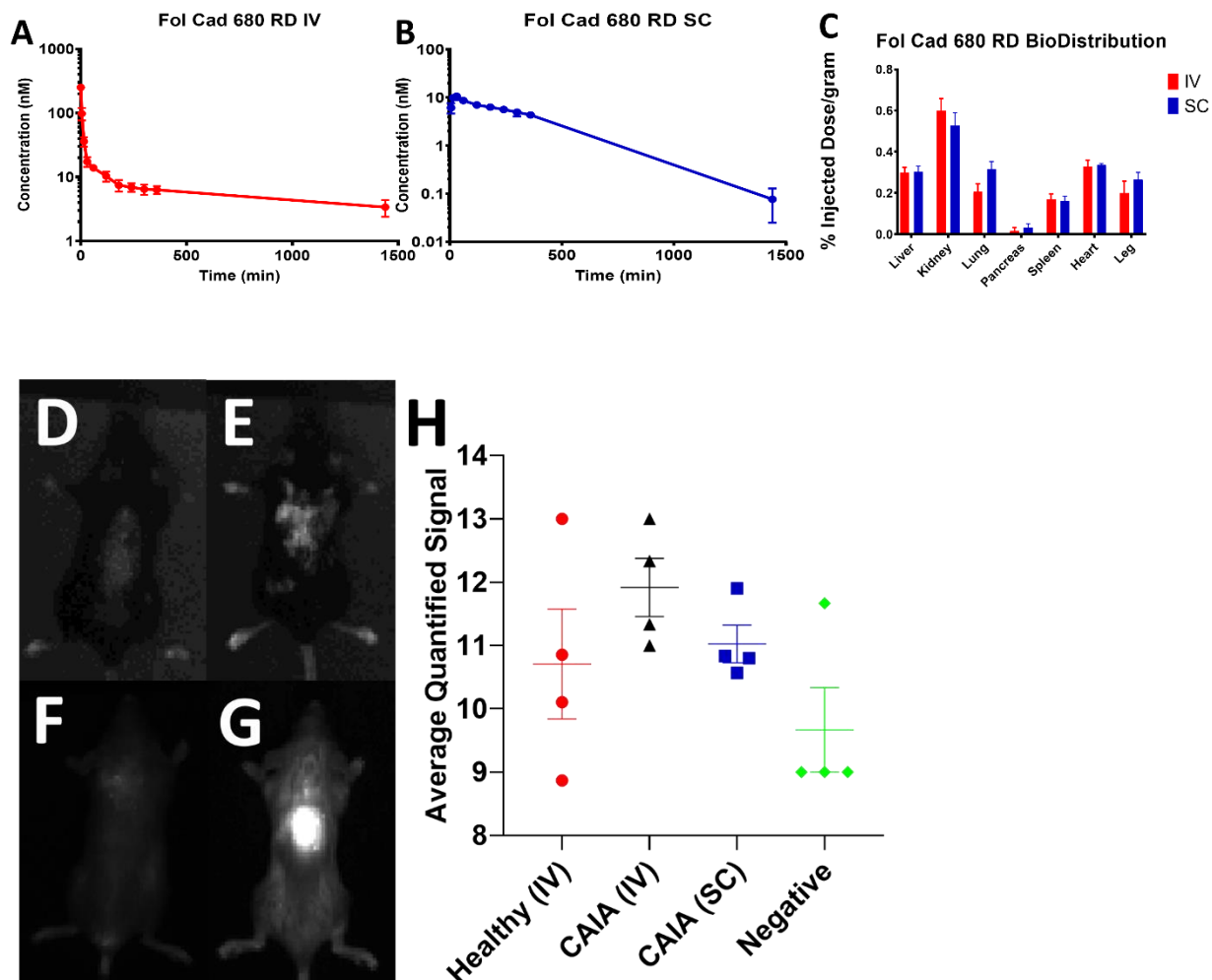


Figure 4.3 Subcutaneous and Intravenous Administration of Folate Cadaverine 680 RD after 24 hours. (A). Plasma clearance of Folate Cadaverine 680 RD administered SC. (B). Plasma clearance of Folate Cadaverine 680 RD administered IV. (C) Biodistribution was compared between both routes of administration. (H) Average signal was compared between both routes of administration and for healthy and CAIA positive mice. IVIS images taken at 24 hours post administration for uninjected (D) Healthy IV (E), CAIA IV (F) and CAIA SC (G) mice.

Biodistribution and plasma clearance data shows the Folate Cadaverine 680 RD probe was rapidly cleared, likely contributing to a lack of uptake. For these reasons, AA 680 RD and INT 800 CW were selected to continue for early detection and quantification of disease in a rat model of RA.

Early Detection and Quantification of RA

To study if NIR Imaging agents can detect RA before measurable inflammation, INT 800 CW was first dosed via IV administration in healthy rats and AIA rat models after advanced inflammation, and the signal of the joints was measured and imaged after 48 hours. Subsequent cohorts of AIA rats were injected via SC administration or PO administration and imaged and compared with their respective healthy control to establish how well signal can be detected between different administration methods. We found that signal in AIA rats is significantly higher than healthy rats by all administration methods, confirming that INT 800 CW agent can detect RA in late stages of the disease as seen in **Figure 4.4A**

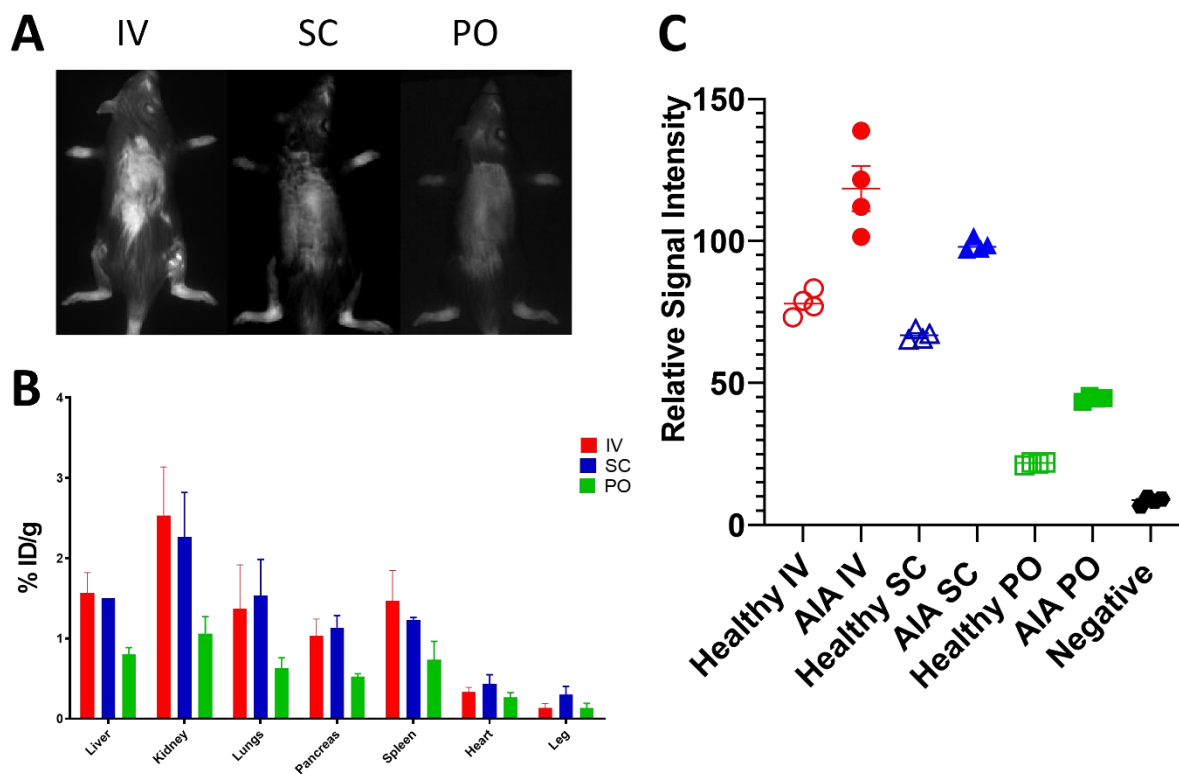


Figure 4.4 Subcutaneous Oral and Intravenous Administration of Integrin 800 CW after 48 hours in AIA rat models (A). IVIS images taken 48 hours post administration for IV, SC, and PO rats. (B) Biodistribution and average signal (C) were compared between the three routes of administration.

Next, we coadministered AA 680 and INT 800 CW in AIA rats via SC injection at earlier timepoints and compared with healthy rat signal, while simultaneously measuring rat joint inflammation. We found that significant inflammation of the joints did not occur until later stages of the disease, and that the joint signal correlated with the amount of joint inflammation. We found that the signal was higher in early stage rats relative to healthy controls, but the difference was not statistically significant. Thus, it is inconclusive if the imaging agents can be used for early detection of RA. Joint signal comparison between administration methods, SC joint signal at early time points, and joint measurements are shown in **FIGURE 4.5**

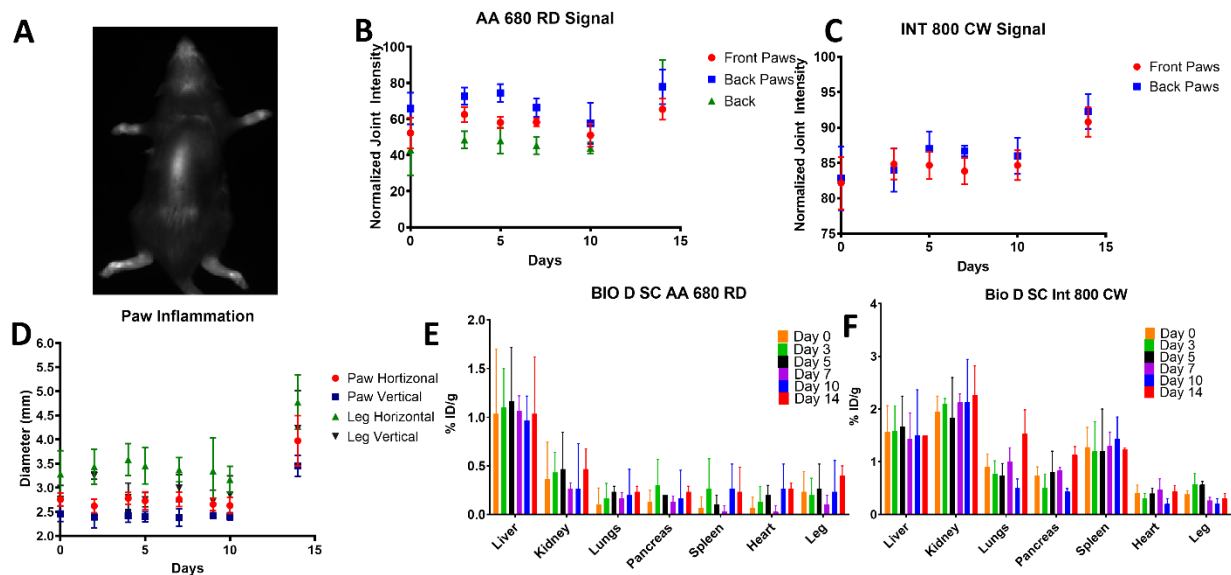


Figure 4.5 Subcutaneous Administration of Integrin 800 CW and Alendronate 680 RD after 48 hours in AIA rat models.(A) IVIS images taken 48 hours post administration for Alendronate 680 RD. Average Signal for Alendronate 680 RD (B) and Integrin 800 CW (C) and Biodistribution (E,F) were compared between earlier stages of AIA. (D) Joint inflammation was recorded over 14 days.

4.6 Discussion

Early detection of RA can improve patient morbidity by enabling therapeutic intervention before the disease can reach advanced progression. If diagnosed early enough, clinical data suggests that the disease may even be able to be cured or reversed (79, 244). MRI and ultrasound

are good imaging modalities for detection and progression of RA; however, both methods have significant limitations for widespread screening. NIRF molecular imaging has potential as a minimally invasive method for early disease detection and treatment monitoring, providing both spatial and molecular information of the joints. NIRF imaging has many key advantages over ultrasound, providing quantitative molecular information using targeted probes. NIRF imaging costs and time of acquisition is less than MRI, and does not use ionizing radiation, compared to PET and SPECT imaging.

Current clinical efforts have focused on non-targeted ICG given its FDA approval for imaging in the clinic; however, the dye is non-specific, requiring dynamic imaging to determine increased uptake in joints versus surface autofluorescence (245). More so, ICG doesn't discern between RA and other arthritic conditions that involve inflammation. Targeted coadministered imaging agents have the potential to provide a better contrast to noise ratio for more accurate diagnosis of RA and differentiation from other conditions. Current research is exploring multiple imaging agents such as ^{99m}Tc -MDP (246), PSVue 794 (247), αCD11b -APC combined with MMP750 (284), MMP-3 specific polymeric probe using NIRF (248), protease activated NIRF probes (sensitive to Cathepsin B) (245), integrins (249,250) and AP39-TSC (251). Therefore, development of new imaging agents for early detection of RA continues. However, many of these probes require intravenous delivery and do not differentiate between RA and other inflammatory diseases. In this work, we used a coadministration approach of alendronate and integrin binding agents conjugated with NIR fluorophores of different wavelengths and tested their ability for early detection and treatment monitoring of RA in AIA rat and CAIA mouse models using SC and oral delivery.

Two imaging agents in the 700 nm channel were tested - Alendronate 680 RD, Folate Cadaverine 680 RD, and two imaging agents in the 800 nm channel were tested – Integrin 800 CW and the prodrug 800 CW.

Alendronate 680 RD administered subcutaneously had an overall lower signal in the joints compared to IV delivery in mouse models due to the lower bioavailability. However, the AA 680 RD agent had sufficient signal compared to background due to the slower clearance of SC administration. Signal at the spine was significantly higher for both methods of administration, which is hypothesized to be from the higher density of hydroxyapatite in osteoclasts of the spine compared to paws (more receptors/cell) and/or thicker spinal tissue.

Folate cadaverine 680 RD showed very poor signal following SC delivery in the CAIA mouse model despite initial promising PK properties. Compared to uninjected mice, the healthy mice showed minimal signal. This is likely due to the lack of over expression of folate receptor, which is consistent with other previous conclusions (252). The CAIA mouse model had a higher signal due to folate over expression; however, it did not provide sufficient contrast compared to healthy mice, most likely due to the fast clearance and low absorption in the biodistribution, preventing the agent to sufficiently bind to the target.

In the 800 channel, the prodrug 800 CW demonstrated poor oral bioavailability. It is hypothesized that the ester group is not hydrolyzed quickly enough relative to clearance, preventing the prodrug from converting into the active ligand and reducing accumulation by cells. Integrin 800 CW proved to have moderate SC bioavailability and modest PO bioavailability in rat models, both comparable to the SC and PO bioavailability in previously established mouse models, suggesting more feasibility of this agent in future studies (114). Due

to their sufficient targeting and optical properties, the Integrin 800 CW Alendronate 680RD agents were delivered by SC and/or PO administration in a rat model of AIA.

Due to similar binding kinetics following PO administration or SC administration, it is hypothesized that Integrin 800 CW agent can be used to detect RA when delivered orally, which is confirmed in **Figure 4.4**. However, SC delivery results in a higher specific signal, lower variability, and uses less probe in AIA rat models; therefore it is an advantageous delivery method for future experiments.

It is inconclusive if AA 680 RD and Integrin 800 CW were able to detect RA at early time points due to the variability of signal in healthy mice. We found that there was a general increase in the mean signal with successive days; however, it was within range of the signal variability in healthy rats. Joint signals correlated with the amount of inflammation in rats, which validates the hypothesis that the amount of signal is relative to the amount of target over expression.

4.7 Conclusion

In conclusion, self-administration of dual channel NIR fluorescent molecular imaging agents has the potential for the early detection of rheumatoid arthritis and specific differentiation of RA from other arthritic diseases due to its low cost, target specificity, and ease of use. Initial results show the potential for early detection, but additional work is needed to confirm the earliest stages of detection. Overall, the calculated design of molecular imaging agents can be used to design agents with the potential for diagnosis and early therapeutic intervention.

Chapter 5 Concluding Remarks and Future Directions

5.1 Summary of Research Results

In this dissertation, computational approaches to study ADC and bispecific antibody distribution in tumors at different length scales were developed in order to design novel therapeutic agents with improved efficacy against heterogeneously expressed targets in cancer and used experimental techniques to develop self-administered molecular imaging agents for early detection and treatment monitoring of Rheumatoid Arthritis.

In Chapter 2, the tumor distribution of ADCs and engineered High Avidity Low Affinity antibodies was modeled using a multiscale kinetic model combining multivalent binding competition kinetics with their distribution properties in monolayer cell, spheroid, and Krogh cylinder tumor models. Using these models, a dimensionless number ψ was derived, which is a ratio between antibody competition and internalization. From these results, the ADC distribution in tumors was predicted when coadministered with HALA carrier doses at the monolayer, cellular and *in vivo* length scales at varying carrier dose concentrations, binding affinities, and cellular expression levels. The HALA antibody needed to achieve a ψ of 10 or more to partially block ADC binding to maintain good tissue penetration and Φ^2 of 1 to ensure a sufficient dose for tissue penetration. Using the model specifically for HER2 targeting, the binding affinity (K_d) needed for the HALA antibody to achieve high ADC efficacy and homogenous distribution at different cellular expression levels was predicted when administered at an 8:1 dose of HALA: ADC. From these simulations, HALA antibodies can be designed for other target systems.

In Chapter 3, the kinetic model was adapted to simulate the competition and distribution of bispecific antibodies on a cell monolayer or in an immune synapse. The model revealed that bispecific antibody binding is complex with a dependence on antibody binding affinity and avidity of each arm and receptor expression of both cell types. Moreover, the avidity effects when binding across two different cells (trans binding) are weaker than avidity effects across the same cell (cis binding), which must be included in the model. These insights help lay the groundwork for a mechanistic model to better design bispecific antibodies.

In Chapter 4, near infrared molecular imaging agents were developed for the early detection and treatment monitoring of rheumatoid arthritis. To develop an imaging agent that detects RA and can differentiate from other arthritic diseases, integrin $\alpha\text{v}\beta3$, folate receptor, and hydroxyapatite were chosen as targets because of their high expression (for integrin $\alpha\text{v}\beta3$ and folate receptor on the macrophages and hydroxyapatite on osteoclasts), fast internalization rates, and well-studied targeting ligands. The ligands selected were an integrin peptidomimetic (integrin binder) in an ethyl ester prodrug and active carboxylic acid form, folate, and alendronate for high affinity to their respective targets, low toxicity, low molecular weight, and high safety profile.

The integrin binders were conjugated to 800 CW – a fluorophore that emits in the 800nm channel, and folate and alendronate were conjugated with 680 RD, a fluorophore that emits in the 700nm channel. The imaging agents were evaluated for high bioavailability, specific targeting, and good optical properties in mouse or rat models. Two imaging agents met all 3 criteria: Integrin 800 CW and Alendronate 680 RD. Experiments continued with SC administration for rat models of Adjuvant Induced Arthritis (AIA) to ensure sufficient uptake. SC administration is similar to PO delivery in terms of absorption kinetics; however, it has less

variability. The imaging agents were tested simultaneously using co-administered SC doses of 0.1mg/kg. Alendronate 680 RD and Integrin 800 CW were dosed in AIA rats models starting from an advanced state of AIA and successively moving to earlier stages to determine the earliest time at which the signal was higher than healthy rats. Because the fluorophore excitation and emission profiles do not overlap, additional molecular information can be obtained. Both agents were found to be able to detect RA at late stages of the disease; however, it is inconclusive if the imaging agents were able to detect disease at earlier stages. Initial results showed higher signal in the diseased state even at early time points, but later replicates of healthy mice had a higher signal, resulting in overlapping error bars. While the mean signal was higher than healthy controls, they were still within the range of healthy signal, due to this increased variability.

However, a coadministration approach of two imaging agents with fluorophores in a non-overlapping optical channel demonstrated SC absorption and specific targeting for potential detection and differentiation of RA from other arthritic diseases. With this approach, patients would be able to self-administer the imaging agents 48 hours before imaging and screened immediately at the clinic for RA, preventing the need for multiple visits or the need for a trained professional for imaging.

In summary, this thesis demonstrates the advantages of using computational and experimental approaches to design anticancer therapeutics and diagnostic imaging agents, elucidating principles that can be used to shorten the development time of both.

5.2 Future Work and Directions

ADC and HALA Antibody Design

Computational modeling is becoming critical for the development and assessment of ADC efficacy in the clinic. While a coadministered dose of engineered HALA antibody with ADC is predicted to improve efficacy across different levels of cellular expression, the simulation requires *in vitro* and *in vivo* validation. Engineered HALA clones can be generated with different monovalent binding affinities and co-administered at different HALA:ADC ratios in spheroids. This can validate the “optimal,” “affinity limited,” “concentration limited,” and “over-competition” simulations in **Figure 2.6**. Upon *in vitro* validation of the spheroid simulations, the next series of experiments would include efficacy studies. Mice bearing xenograft tumors of different Her 2 expression levels can be grown and then dosed with different HALA clones and TDM1 or T-MMAE. Tumor growth curves with and without treatment in mice dosed with HALA:ADC cocktail (versus ADC or antibody alone) can be measured to evaluate the efficacy of the HALA antibodies with and without the bystander effect. After the mice are sacrificed, the tumors can be resected and used to measure biodistribution and histology to investigate the improvement of ADC penetration in the tumor tissue.

While improving ADC efficacy is a significant contribution in the clinic, drug resistance remains a great challenge in ADC development. Incorporating drug resistance in computational models can offer a more informed insight in ADC efficacy, with different types of models offering specific advantages. One method is to incorporate ODEs that describe clonal tumor heterogeneity and evolution during ADC treatment (253, 254). A chief advantage of this approach is the compatibility with the current Krogh Cylinder model that incorporates pharmacokinetics of the therapeutics and emergence of drug resistance (255). Other advantages of this model are the ability to study the clonal heterogeneity and changes in time, both

numerically and theoretically (256). Additionally, models using ODEs can study changes that occur at the cellular level during ADC treatment (257). This would allow a sensitivity analysis to determine and study the intracellular proteins that most affect responsiveness of tumor cells to ADC treatment.

Another type of method that can be used is partial differential equations (PDEs) to recreate the interaction between the tumor and its microenvironment, allowing spatiotemporal dynamics of tumor growth to be studied during ADC therapy (258). Compared to traditional tumor growth models and ODEs, where the tumor is treated as a uniform mass that grows at a uniform rate, inclusion of spatiotemporal dynamics allows different cell populations to interact with ADC treatment at different response rates (drug resistant, semi-resistant, and sensitive cells). This can provide better predictions of the minimum tumor radius achieved after ADC treatment, the time of tumor regrowth, the maximum potential amount of cell death after treatment, and potential time to recovery (258, 259). Another model that should be considered is a PDE model that uses a cell population defined by the distribution of genotypic and phenotypic variables (260). This allows the study of how drug resistance arises and which processes are the primary drivers towards genetic resistance to treatment. For example, a model was developed where the susceptibility of cells to drug-induced apoptosis was a function of the amount of P-glycoprotein (P-gp) in the cell membrane. It was found that the cumulative effect of micro vesicle transport was crucial for generating the emergence of drug resistance and that Darwinian selection yielded the slowest resistance dynamics whereas Lamarckian induction and macrovesicle transfer both significantly accelerated resistance (261, 262). Through these approaches, an optimal design of therapeutic strategies to overcome drug resistance can be developed (263, 264).

Bispecific antibody Design

While some progress has been made with the bispecific antibody binding kinetic model, more work is required. One improvement would be to correlate the amount of crosslinking of bispecific antibodies to the amount of T-cell activation. With this correlation, the amount of crosslinking in the model and T-cell activation in experiments can be compared directly. One challenge with the current data set is the different binding epitopes and linker orientation. Data with identical binding epitopes and identical linkers would provide a ‘cleaner’ data set to train the model. The model also needs to include diffusion and internalization to better simulate bispecific antibody distribution in tumor spheroids, specifically changing the binding affinities of the individual binding arms, and the concentration of the antibody dose. Afterwards, the model can be adapted to include pharmacokinetics like the Krogh Cylinder model with variable cellular expression of the PD-1 and CD-137 on cells to provide better insights into how expression affects distribution in an *in vivo* scenario. Then, *in vitro* and *in vivo* studies could be conducted similarly to the HALA antibody future work to validate the simulations.

Imaging Agent Design

While it was inconclusive if Integrin 800CW or Alendronate 680 RD could detect RA at early (subclinical) time points via SC administration, it is unclear if this is due to the current imaging agents properties or if the limitation is due to the administration method. Between PO, SC, and IV administration, there is the greatest difference in joint signal between AIA and healthy controls using IV delivery, suggesting it is the most sensitive technique. Repeating the experiments (dosing at earlier days after induction of AIA) using I.V. administration would determine if preclinical RA can be detected with these agents. While using I.V. administration is not the preferred method for population screening, it would reveal whether preclinical RA

detection would require a high threshold of bioavailability, which could be addressed using formulation studies. Simultaneously, blood samples can be collected at various stages of disease progression in order to measure cytokine expression, allowing a correlation between cytokine expression and imaging signal.

A next step for RA imaging would be to evaluate if the imaging agents can be used to quantitatively measure treatment monitoring RA. Currently, RA patients are administered methotrexate (MTX) as first-line therapy, and follow up visits are used to determine if dose escalation or a change in treatment (e.g. adding a biologic) is needed. Due wide variability in patient response, determining the amount of therapy needed uses an iterative approach (121). Currently, treatment evaluation occurs between 1-3 months and may not determine complete remission (265); however, there may be a local response on the scale of days (97, 122). This would allow NIRF molecular imaging agents to identify an optimal dose without delaying treatment and potentially incurring further damage. Quantifying the change in fluorescent signal of the imaging agents following the treatment with Disease Modifying AntiRheumatic Drugs (DMARDs), it may be possible to monitor treatment response in days. Compared to non-DMARD therapies, DMARDS can reduce integrin expression (thus imaging agent probe uptake) more quickly than the 1-3 month evaluation period, allowing more accurate and rapid dose intensification. Using the imaging agents, signal could be measured following Methotrexate treatment in AIA rat models. The reduction of signal after using methotrexate in both early and advanced stages of the disease could be measured to determine a preventative and therapeutic window.

Afterwards, a next step would be to compare how the imaging agents perform with other arthritic diseases such as ankylosing spondylitis (AS). Ankylosing spondylitis is an inflammatory

disease that primarily occurs in the spine, which left untreated can cause the spine to fuse. To model AS (progressive ankylosis allele) ank/ank mice have been developed. These ank/ank mice are a useful model because they allow for Murine Progressive Ankylosis (MPA) which is a disorder that has clinical and histopathologic resemblance to human AS (266). MPA is also a nondestructive AS mouse model, which better emulates human AS, compared to more destructive AS animal models (267). Briefly, the imaging agents would be dosed at an advanced stage of MPA. Because AS is localized in the spine, it is expected that the fluorescent signal of the Alendronate 680 RD probe would be higher than the Integrin 800 CW probe. The ratio of signal between the two probes would allow researchers to better determine the type of arthritic disease.

In Conclusion, there is tremendous promise for computational tools and molecular imaging in the pharmaceutical and medical industry. Both set of tools are continually evolving, allowing new ways to design for the next generation of drugs.

Appendices

Appendix A: Supporting Information for Chapter 2

Variable	Range	Unit
F (HALA)	3.4E-10 - .0001	M
F (ADC)	1E-10 – 5.88E-8	M
k_e	2.73E-6 - .001548	s ⁻¹
Ag_0	8154 – 2.4E9	Receptors Cell ⁻¹
kd_{HALA}	4.09E-10 – 2.33E-7	M
kon_{2HALA}	2.06E-9 - .079	M ⁻¹ s ⁻¹

Table A.1: Base case parameters used for Figure 2.2C

Appendix B: Supporting Information for Chapter 4

Compound	Mobile Phase	HPLC Method	Retention Time	Expected MW	Observed MW	Purity
Int-800 CW	A: 25mM TEAA in water B: MeCN	25% B: 0-6min 25-45% B: 6-24min	10.5 min	1393	1396	95%
Prodrug Int-800 CW	A: 25mM TEAA in water B: MeCN	25% B: 0-6min 25-45% B: 6-24min	12.5 min	1421	1423.63	97%
AA-680 RD	A: .1% TFA in water B: .1% TFA in MeCN	20% B: 0-3min 20-45% B: 3-18min	6.5 min	1093	1094.88	93%
Fol-Cad-680 RD	A: .1% TFA in water B: .1% TFA in MeCN	20% B: 0-3min 20-45% B: 3-18min	8.5min	1367	1368.17	91%

Table B.1: Purification methods and final product of Imaging agents

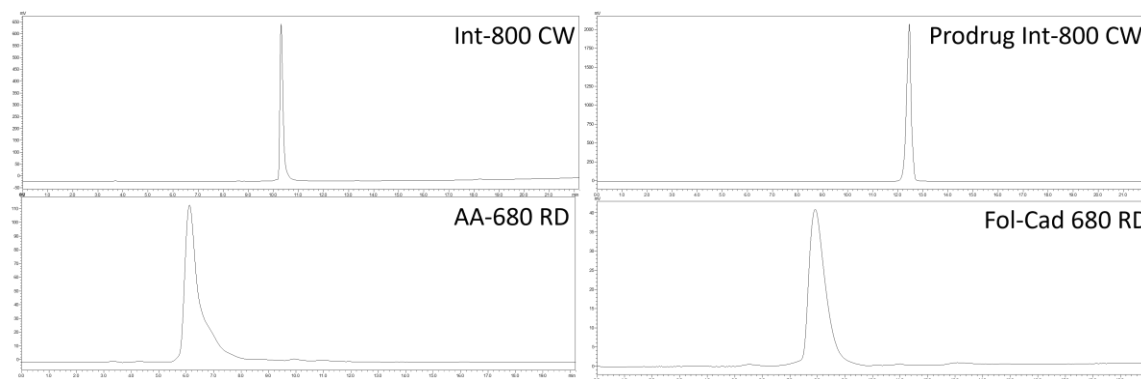


Figure B.1: HPLC Purity Traces of Imaging Agents

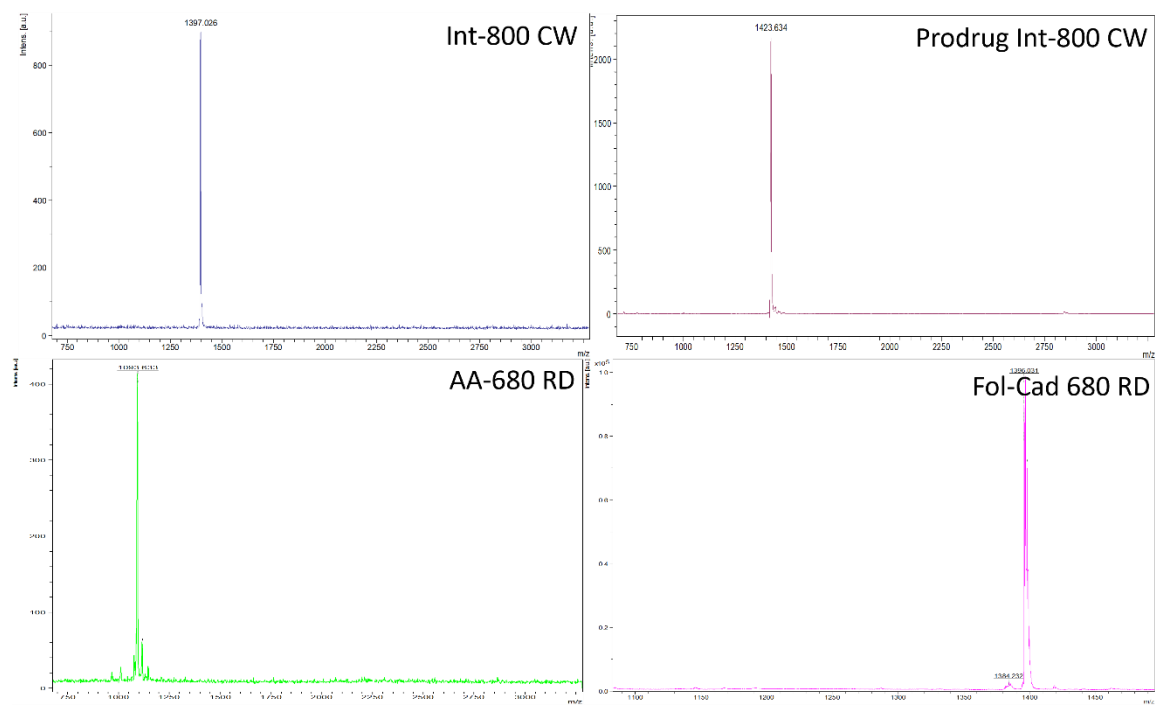


Figure B.2: Maldi Traces of Imaging Agents

Bibliography

1. Piñeros, M., Mery, L., Soerjomataram, I., Bray, F., & Steliarova-Foucher, E. Scaling up the surveillance of childhood cancer: a global roadmap. *J. Natl. Cancer Inst*, **113**, 9-15 (2021).
2. DeVita Jr, V. T., & Chu, E. A history of cancer chemotherapy. *Cancer Res.* **68**, 8643-8653 (2008).
3. Ahmed, S., Ellis, M., Li, H., Pallucchini, L. & Stein, A.M. Guiding dose selection of monoclonal antibodies using a new parameter (AFTIR) for characterizing ligand binding systems. *J Pharmacokinet Pharmacodyn* **46**, 287-304 (2019).
4. Khongorzul, P., Ling, C.J., Khan, F.U., Ihsan, A.U. & Zhang, J. Antibody-Drug Conjugates: A Comprehensive Review. *Mol Cancer Res* **18**, 3-19 (2020).
5. Murphy K, Travers P, Walport M, & Janeway C. *Janeway's immunobiology*. New York: Garland Science (2012).
6. Scott AM, Wolchok JD & Old LJ. Antibody therapy of cancer. *Nat Rev Cancer.* **12**, 278-287 (2012).
7. Hudis CA. Trastuzumab — Mechanism of Action and Use in Clinical Practice. *N Engl J Med.* **357**, 39-51 (2007).
8. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. *Nat Biotechnol.* **23**, 1147-1157 (2005).
9. Li, F., Ulrich, M., Jonas, M., Stone, I. J., Linares, G., Zhang, X., & Law, C. L. Tumor-Associated Macrophages Can Contribute to Antitumor Activity through FcγR-Mediated Processing of Antibody–Drug Conjugates Role of TAMs in ADC Antitumor Activity. *Mol. Cancer Ther.* **16**, 1347-1354 (2017).
10. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol.* **23**, 1137-1146 (2005).
11. Birrer, M.J., Moore, K.N., Betella, I. & Bates, R.C. Antibody-Drug Conjugate-Based Therapeutics: State of the Science. *J Natl Cancer Inst* **111**, 538-549 (2019).
12. McKertish, C. M. & Kayser, V. Advances and Limitations of Antibody Drug Conjugates for Cancer. *Biomedicines* **9**, (2021).
13. Schaaf, M.B., Garg, A.D. & Agostinis, P. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis* **9**, 1-14 (2018).
14. Hartung, T. Thoughts on limitations of animal models. *Parkinsonism Relat Disord*, **14**. S81-S83. (2008).
15. F. Mirasol. Research Advances are Improving ADC Development. *BioPharm International* **34** 16–18 (2021).
16. Peters C & Brown S. Antibody-drug conjugates as a novel anti-cancer chemotherapeutics. *Biosci Rep.* **35** 4-10 (2015).

17. Ilona Pysz, Paul J. M. Jackson & David E. Thurston, CHAPTER 1: Introduction to Antibody–Drug Conjugates (ADCs), *Cytotoxic Payloads for Antibody–Drug Conjugates*, 1-30 (2019).
18. Thurber, G.M., Schmidt, M.M. & Wittrup, K.D. Factors determining antibody distribution in tumors. *Trends Pharmacol Sci* **29**, 57-61 (2008).
19. Thurber, G.M., Schmidt, M.M. & Wittrup, K.D. Antibody tumor penetration: transport opposed by systemic and antigen-mediated clearance. *Adv Drug Deliv Rev* **60**, 1421-1434 (2008)
20. Thurber, G. M. & Weissleder, R. A systems approach for tumor pharmacokinetics. *PLoS One* **6**, e24696, doi:10.1371/journal.pone.0024696 (2011).
21. Cilliers, C., Guo, H., Liao, J., Christodolu, N. & Thurber, G.M. Multiscale modeling of antibody-drug conjugates: connecting tissue and cellular distribution to whole animal pharmacokinetics and potential implications for efficacy. *AAPS J* **18**, 1117-1130 (2016).
22. Thurber, G.M., Zajic, S.C. & Wittrup, K.D. Theoretic criteria for antibody penetration into solid tumors and micrometastases. *J Nucl Med* **48**, 995-999 (2007).
23. Bhatnagar, S., Deschenes, E., Liao, J., Cilliers, C. & Thurber, G.M. Multichannel imaging to quantify four classes of pharmacokinetic distribution in tumors. *J Pharm Sci* **103**, 3276-3286 (2014).
24. Rhoden, J.J. & Wittrup, K.D. Dose dependence of intratumoral perivascular distribution of monoclonal antibodies. *J Pharm Sci* **101**, 860-867 (2012).
25. Massa, S., Xavier, C., De Vos, J., Caveliers, V., Lahoutte, T., Muyldermans, S., & Devoogdt, N. Site-specific labeling of cysteine-tagged camelid single-domain antibody-fragments for use in molecular imaging. *Bioconjug Chem* **25**, 979-988 (2014).
26. Joubert, N., Beck, A., Dumontet, C. & Denevault-Sabourin, C. Antibody-Drug Conjugates: The Last Decade. *Pharmaceuticals (Basel)* **13**, (2020).
27. Beck, A., Wurch, T., Bailly, C. & Corvaia, N. Strategies and challenges for the next generation of therapeutic antibodies. *Nat Rev Immunol* **10**, 345-352 (2010).
28. Tan H, Su W, Zhang W, Wang P, Sattler M, & Zou P. Recent Advances in Half-life Extension Strategies for Therapeutic Peptides and Proteins. *Curr Pharm Des.* **24**, 4932-4946 (2018).
29. Ocean, A.J. et al. Sacituzumab govitecan (IMMU-132), an anti-Trop-2-SN-38 antibody-drug conjugate for the treatment of diverse epithelial cancers: Safety and pharmacokinetics. *Cancer* **123**, 3843-3854 (2017).
30. Nejadmoghaddam, M. R., Minai-Tehrani, A., Ghahremanzadeh, R., Mahmoudi, M., Dinarvand, R., & Zarnani, A. H. Antibody-drug conjugates: possibilities and challenges. *Avicenna J Med Biotechnol.* **11**, 3-23 (2017).
31. Chari, R. Target cancer therapy conferring specificity to cytotoxic drugs. *Acc Chem Res* **41**, 98-107 (2007).
32. Surova, O. & Zhivotovsky, B. Various modes of cell death induced by DNA damage. *Oncogene* **32**, 3789-3797 (2013).
33. Zhao, P. et al. Recent advances of antibody drug conjugates for clinical applications. *Acta Pharm Sin B* **10**, 1589-1600 (2020).
34. Yaghoubi, S. et al. Potential drugs used in the antibody-drug conjugate (ADC) architecture for cancer therapy. *J Cell Physiol* **235**, 31-64 (2020).

35. Bender, B. et al. A mechanistic pharmacokinetic model elucidating the disposition of trastuzumab emtansine (T-DM1), an antibody-drug conjugate (ADC) for treatment of metastatic breast cancer. *AAPS J* **16**, 994-1008 (2014).
36. Cilliers, C., Menezes, B., Nessler, I., Linderman, J. & Thurber, G.M. Improved tumor penetration and single-cell targeting of antibody–drug conjugates increases anticancer efficacy and host survival. *Cancer Res* **78**, 758-768 (2018).
37. Lambert, J.M. & Berkenblit, A. Antibody-Drug Conjugates for Cancer Treatment. *The Annual Review of Medicine* **69**, 191-207 (2018).
38. Garcia-Alonso, S., Ocana, A. & Pandiella, A. Trastuzumab Emtansine: Mechanisms of Action and Resistance, Clinical Progress, and Beyond. *Trends Cancer* **6**, 130-146 (2020).
39. Chen, R. et al. CD30 Downregulation, MMAE Resistance, and MDR1 Upregulation Are All Associated with Resistance to Brentuximab Vedotin. *Mol Cancer Ther* **14**, 1376-1384 (2015)
40. Yardley, D.A. et al. Quantitative measurement of HER2 expression in breast cancers: comparison with 'real-world' routine HER2 testing in a multicenter Collaborative Biomarker Study and correlation with overall survival. *Breast Cancer Res* **17**, 41 (2015).
41. Marchiò, C., Annaratone, L., Marques, A., Casorzo, L., Berrino, E., & Sapino, A. Evolving concepts in HER2 evaluation in breast cancer: Heterogeneity, HER2-low carcinomas and beyond. *Semin Cancer Biol* (2020).
42. Baxter, L.T. & Jain, R.K. Transport of Fluid and Macromolecules in Tumors I Role of interstitial pressure and convection. *Microvascular Research* **37**, 77-104 (1988).
43. Jain, R.K. Transport of Molecules, Particles, and Cells in Solid Tumors. *Annual Reviews Biomed. Eng.* **1**, 241-263 (1999).
44. Jain, R.K. Normalization of Tumor Vasculature- An Emerging concept in Antiangiogenic Therapy. *Science* **307**, 68-79 (2005).
45. Baxter, L.T. & Jain, R.K. Transport of Fluid and Macromolecules in Tumors I Role of interstitial pressure and convection. *Microvascular Research* **37**, 77-104 (1988).
46. Jain, R.K. Transport of Molecules, Particles, and Cells in Solid Tumors. *Annual Reviews Biomed. Eng.* **1**, 241-263 (1999).
47. Barbolosi, D., Ciccolini, J., Lacarelle, B., Barlesi, F. & Andre, N. Computational oncology mathematical modelling of drug regimens for precision medicine. *Nature reviews* **13**, 242-254 (2016).
48. Karolak, A. & Rejniak, K.A. Micropharmacology: An In Silico Approach for Assessing Drug Efficacy Within a Tumor Tissue. *Bull Math Biol.* **81**, 3623-3641 (2019).
49. Williams, D.P. et al. Novel in vitro and mathematical models for the prediction of chemical toxicity. *Toxicol Res (Camb)* **2**, 40-59 (2013).
50. Conrad, M., Fechner, P., Proll, G., & Gauglitz, G. Comparison of methods for quantitative biomolecular interaction analysis. *Analytical and Bioanalytical Chemistry* **414**, 661-673 (2022).
51. Wittrup, K. Dane, et al. *Quantitative fundamentals of molecular and cellular bioengineering*. MIT Press (2020).
52. Copeland RA. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*. John Wiley & Sons (2004).
53. N.J. Daka & K.J. Laidler, Temperature and pH effects on immobilized lactate dehydrogenase kinetics, *BBA - Enzymology* **612**, 305-316 (1980).

54. Srinivasan, Bharath. A guide to the Michaelis–Menten equation: steady state and beyond. *The FEBS Journal* (2021).
55. Elena V. Emelyanova. Graphical Approach to Compare Concentration Constants of Hill and Michaelis-Menten Equations. *Journal of Biotechnology and Biomedical Science* **1**, 94-99 (2018).
56. Gesztelyi, Rudolf et. Al. The Hill equation and the origin of quantitative pharmacology. *Archive for History of Exact Sciences*. 66 427–438 (2012).
57. Resat, Haluk, Linda Petzold, & Michel F. Pettigrew. "Kinetic modeling of biological systems." *Computational systems biology* 311-335 (2019)
58. Bocci, G. et al. ADME-Space: a new tool for medicinal chemists to explore ADME properties. *Sci Rep* **7**, 1-13 (2017).
59. Shah, D.K. & Betts, A.M. Towards a platform PBPK model to characterize the plasma and tissue disposition of monoclonal antibodies in preclinical species and human. *J Pharmacokinet Pharmacodyn* **39**, 67-86 (2012).
60. Vassal, G. et al. Prospective validation of a novel IV busulfan fixed dosing for pediatric patients to improve therapeutic AUC targeting without drug monitoring. *Cancer Chemother Pharmacol* **61**, 113-123 (2008).
61. Jin, F. et al. Population pharmacokinetic modeling of idelalisib, a novel PI3Kdelta inhibitor, in healthy subjects and patients with hematologic malignancies. *Cancer Chemother Pharmacol* **77**, 89-98 (2016).
62. Shamsi, M., Mohammadi, A., Manshadi, M.K.D. & Sanati-Nezhad, A. Mathematical and computational modeling of nano-engineered drug delivery systems. *J Control Release* **307**, 150-165 (2019).
63. Singh, A.P., Shin, Y.G. & Shah, D.K. Application of Pharmacokinetic-Pharmacodynamic Modeling and Simulation for Antibody-Drug Conjugate Development. *Pharm Res* **32**, 3508-3525 (2015).
64. Gabrielsson, Johan, and Daniel Weiner. "Non-compartmental analysis." *Computational toxicology*. 377-389 (2012).
65. Singh, A.P. et al. Evolution of Antibody-Drug Conjugate Tumor Disposition Model to Predict Preclinical Tumor Pharmacokinetics of Trastuzumab-Emtansine (T-DM1). *AAPS J* **18**, 861-875 (2016).
66. Goldman, D. Theoretical models of microvascular oxygen transport to tissue. *Microcirculation* **15**, 795-811 (2008).
67. Vasalou, C., Helmlinger, G. & Gomes, B. A mechanistic tumor penetration model to guide antibody drug conjugate design. *PLoS One* **10**, (2015).
68. Lewin Group. *The Value of Diagnostics Innovation, Adoption and Diffusion into Health Care*. Ava Medical .
69. Agdeppa ED & Spilker ME 2009. A Review of Imaging Agent Development. *AAPS J.* **11**, 286-299 (2009).
70. Trouw LA & Mahler M. Closing the serological gap: promising novel biomarkers for the early diagnosis of rheumatoid arthritis. *Autoimmun Rev.* **12**, 318–322 (2012).
71. Birnbaum, Howard, et al. Societal cost of rheumatoid arthritis patients in the US. *Current medical research and opinion* **26**, 77-90 (2010).
72. Cutolo, Maurizio, Kitas, George D., van Riel & Piet L.C.M. Burden of disease in treated rheumatoid arthritis patients: Going beyond the joint. *Seminars in Arthritis and Rheumatism*. 43, 479–488 (2014).

73. Balbir-Gurman Alexandra, Yigla, Mordechai, Nahir, Abraham Menahem & Braun-Moscovici, Yolanda. Rheumatoid pleural effusion. *Seminars in Arthritis and Rheumatism*. **35**, 368–378 (2006).
74. Aviña-Zubieta JA, Choi HK, Sadatsafavi M, Etminan M, Esdaile JM, & Lacaille D. Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. *Arthritis Rheum*. **59**, 1690–1697 (2008).
75. Saag KG, Teng GG & Patkar NM. American College of Rheumatology 2008 recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. *Arthritis Rheum*. **59**, 762–84 (2008).
76. Upchurch KS & Kay J. Evolution of treatment for rheumatoid arthritis. *Rheumatology*. **51**, 28-36 (2012).
77. Singh JA et. al. 2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. *Arthritis care & research* **64**, 625-639 (2012).
78. Lipsky PE. Are new agents needed to treat RA? *Nature reviews Rheumatology*. **5**, 521-522 (2009).
79. Finckh A, Liang MH, van Herckenrode CM, & de Pablo P. Long-term impact of early treatment on radiographic progression in rheumatoid arthritis: A meta-analysis. *Arthritis Care Res* **55**, 864–872 (2006).
80. National Academies of Sciences, Engineering, and Medicine. *Improving diagnosis in health care*. National Academies Press, 2016
81. Kgoebane, Kgomo, et al. "The role of imaging in rheumatoid arthritis." *SA Journal of Radiology* **22**, 1-6 (2018).
82. Hall EJ, & Brenner DJ. Cancer risks from diagnostic radiology. *Br J Radiol*. **81**, 362–378 (2008).
83. Mota HLM, Laurindo IMM, dos Santos Neto LL, et al.. Imaging diagnosis of early rheumatoid arthritis. *Rev Bras Rheumatol*. **52**, 757–766 (2012).
84. Nishimura K, et. al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Annals of Internal Medicine*. **146**, 797–808 (2007).
85. Imai MDT, Takahashi MDK, Fukura MDH, & Morishita MDY. Measurement of Cardiac Output by Pulse Dye Densitometry Using Indocyanine Green A Comparison with the Thermodilution Method. *Anesthesiology* **87**, 816-822 (1997).
86. Cooke AR, Harrison DD & Skyring AP. Use of indocyanine green as a test of liver function. *Am J Dig Dis*. **8**, 244-250 (1963).
87. Spaide RF, Klancnik JM, Jr & Cooney MJ. Retinal vascular layers imaged by fluorescein angiography and optical coherence tomography angiography. *JAMA Ophthalmology*. **133**, 45-50 (2015).
88. Santos-Moreno, Pedro, et al. Quantifying Potential Cost-Savings Through an Alternative Imaging-Based Diagnostic Process in Presumptive Seronegative Rheumatoid Arthritis. *CEOR* **13**, 519 (2021).
89. Westbrook JI, Rob MI, Woods A, et al. Errors in the administration of intravenous medications in hospital and the role of correct procedures and nurse experience. *BMJ Quality & Safety*. **20**, 1027-1034 (2011).
90. Ong, W. M., and S. Subasini. Medication errors in intravenous drug preparation and administration. *Med J Malaysia* **68**, 52-57 (2013).

91. Kemp SF, Lockey RF & Simons FE, World Allergy Organization ad hoc Committee on Epinephrine in A. Epinephrine: the drug of choice for anaphylaxis. A statement of the World Allergy Organization. *Allergy*. **63**,1061-1070 (2008).
92. Anderson JA, & Adkinson NF, Jr. Allergic reactions to drugs and biologic agents. *Jama*. **258**, 2891-2899 (1987).
93. Bhattacharya S. The facts about penicillin allergy: a review. *J Adv Pharma Tech & Res*. **1**, 11-17 (2010).
94. Macdougall IC. Strategies for iron supplementation: oral versus intravenous. *Kidney international Supplement*. **69**, S61-66 (1999).
95. Smolen JS, & Steiner G. Therapeutic strategies for rheumatoid arthritis. *Nature reviews Drug discovery*. **2**, 473-488 (2003).
96. Niewold TB, Harrison MJ & Paget SA. Anti-CCP antibody testing as a diagnostic and prognostic tool in rheumatoid arthritis. *QJM : Mon J Assoc of Physicians*. **100**, 193-201 (2007).
97. Neurath MF et. al. Methotrexate specifically modulates cytokine production by T cells and macrophages in murine collagen-induced arthritis (CIA): a mechanism for methotrexate-mediated immunosuppression. *Clin. Exp. Immunol*. **115**, 42-55 (1999).
98. Jin, Jing-fen, et al. The optimal choice of medication administration route regarding intravenous, intramuscular, and subcutaneous injection. *Patient preference and adherence* **9**, 923 (2015).
99. Reisz, Julie A., et al. Effects of ionizing radiation on biological molecules—mechanisms of damage and emerging methods of detection. *Antioxid*. **21**, 260-292 (2014).
100. Ahmed, T., & Bader M. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. *Drug Des. Dev.***10**, 483-490 (2016).
101. Elgazzar, Abdelhamid H., & Nafisa Kazem. Biological effects of ionizing radiation. *The pathophysiologic basis of nuclear medicine*. 715-726 (2015).
102. Wilson B, Jermyn M, & Leblond F. Challenges and opportunities in clinical translation of biomedical optical spectroscopy and imaging. *J Biomed. Opt.* **23**, 30-40 (2018).
103. Weissleder R. A clearer vision for in vivo imaging. *Nat Biotech* **19**, 316-317 (2001).
104. Ntziachristos V, Ripoll J & Weissleder R. Would near-infrared fluorescence signals propagate through large human organs for clinical studies? *Opt Lett*. **27**, 333-335 (2002).
105. Frangioni JV 2003. In vivo near-infrared fluorescence imaging. *Curr Opin in Chem Biol*. **7**, 626-634 (2003).
106. Pansare VJ, Hejazi S, Faenza WJ, & Prud'homme RK. Review of Long-Wavelength Optical and NIR Imaging Materials: Contrast Agents, Fluorophores, and Multifunctional Nano Carriers. *Chem Mater* **24**,812-827 (2012).
107. van Dam GM, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor- α targeting: first in-human results. *Nat Med* **17**, 13-15 (2011).
108. Hilderbrand SA & Weissleder R. Near-infrared fluorescence: application to in vivo molecular imaging. *Curr Opin Chem Biol* **14**,71-79 (2010).
109. Doak, B. C., Over, B., Giordanetto, F. & Kihlberg, J. Oral druggable space beyond the rule of 5: Insights from drugs and clinical candidates. *Chem. Biol*. **21**, 1115– 1142 (2014).
110. Zhang, M. Q. & Wilkinson, B. Drug discovery beyond the 'rule-of-five'. *Curr. Opin. Biotechnol*. **18**, 478–488 (2007).

111. Giménez, B G et al. Evaluation of blockbuster drugs under the rule-of-five. *Die Pharmazie* **65**, 148-52 (2010).
112. Zhang, L., Bhatnagar, S., Deschenes, E. & Thurber, G.M. Mechanistic and quantitative insight into cell surface targeted molecular imaging agent design. *Sci. Rep.* **6** 25-42 (2016).
113. Yi, X., Wang, F., Qin, W., Yang, X. & Yuan, J. Near-infrared fluorescent probes in cancer imaging and therapy: an emerging field. *Int. J. Nanomedicine* **9**, 13-47 (2014).
114. Bhatnagar, Sumit, et al. Oral and Subcutaneous Administration of a Near-Infrared Fluorescent Molecular Imaging Agent Detects Inflammation in a Mouse Model of Rheumatoid Arthritis. *Sci Rep* **9**,46-61 (2019).
115. Yazici Y, & Abramson SB. Rheumatoid arthritis treatment and monitoring of outcomes-where we are in 2007? *Bull NYU Hosp Jt Dis.* **65**, 300-305 (2007).
116. Sokka T, Haugeberg G, & Pincus T. Assessment of quality of rheumatoid arthritis care requires joint count and/or patient questionnaire data not found in a usual medical record: examples from studies of premature mortality, changes in clinical status between 1985 and 2000, and a QUEST-RA global perspective. *Clin Exp Rheumatol.***25**, 86-97 (2007).
117. Grigor C, Capell H, Stirling A, et al. Effect of a treatment strategy of tight control for rheumatoid arthritis (the TICORA study): a single-blind randomised controlled trial. *Lancet.* **364**, 263-269. (2004).
118. van der Kooij SM, Goekoop-Ruiterman YP, & de Vries-Bouwstra JK. Probability of continued low disease activity in patients with recent onset rheumatoid arthritis treated according to the disease activity score. *Ann Rheum Dis.* **67**, 266-269. (2008).
119. Mottonen T, Hannonen P, Leirisalo-Repo M. Comparison of combination therapy with single-drug therapy in early rheumatoid arthritis: a randomised trial. FIN-RACo trial group. *Lancet.* **353**, 1568-1573 (1999).
120. Verstappen SM, Jacobs JW, van der Veen MJ, et al. Intensive treatment with methotrexate in early rheumatoid arthritis: aiming for remission. Computer Assisted Management in Early Rheumatoid Arthritis (CAMERA, an open-label strategy trial). *Ann Rheum Dis.* **66**, 1443-1449. (2007).
121. Smolen JS, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. *Ann rheum dis.* **73**, 492-509 (2014).
122. Lange F, Bajtner E, Rintisch C, Nandakumar KS, Sack U, & Holmdahl R. Methotrexate ameliorates T celldependent autoimmune arthritis and encephalomyelitis but not antibody induced or fibroblast induced arthritis. *Ann rheum dis.* **64**, 599-605 (2005).
123. Dean, A. Q., Luo, S., Twomey, J. D. & Zhang, B. Targeting cancer with antibody-drug conjugates: Promises and challenges. *MAbs* **13**, 1951427, doi:10 (2021).
124. Joubert, N., Beck, A., Dumontet, C. & Denevault-Sabourin, C. Antibody-Drug Conjugates: The Last Decade. *Pharmaceuticals (Basel)* **13**, doi:10.3390/ph13090245 (2020).
125. McKertish, C. M. & Kayser, V. Advances and Limitations of Antibody Drug Conjugates for Cancer. *Biomedicines* **9**, (2021).
126. Polakis, P. Antibody Drug Conjugates for Cancer Therapy. *Pharmacol Rev* **68**, 3-19 (2016).
127. Junutula, J. R. *et al.* Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat Biotechnol* **26**, 925-932 (2008).
128. Donaghy, H. Effects of antibody, drug and linker on the preclinical and clinical toxicities of antibody-drug conjugates. *MAbs* **8**, 659-671 (2016).

129. Ponte, J. F. *et al.* Antibody Co-Administration Can Improve Systemic and Local Distribution of Antibody-Drug Conjugates to Increase. *Mol Cancer Ther* **20**, 203-212, (2021).
130. Yamada, K., Yuji I. Recent Chemical Approaches for Site- Specific Conjugation of Native Antibodies: Technologies toward Next- Generation Antibody–Drug Conjugates. *ChemBioChem* **20**, 2729-2737 (2019).
131. Zhou, Q. Site-Specific Antibody Conjugation for ADC and Beyond. *Biomedicines* **5**, 64-70 (2017).
132. Sadiki A. *et al.* Site-specific conjugation of native antibody. *Antib Ther.* **3**, 271-284 (2020).
133. Matsuda, Y., Mendelsohn, B. A. Recent Advances in Drug–Antibody Ratio Determination of Antibody–Drug Conjugates. *Chem. and Pharm. Bulletin*, **69**, 976-983, (2021).
134. Behrens, Christopher R *et al.* Antibody-Drug Conjugates (ADCs) Derived from Interchain Cysteine Cross-Linking Demonstrate Improved Homogeneity and Other Pharmacological Properties over Conventional Heterogeneous ADCs. *Molecular pharmaceuticals* **12**, 3986-3998. (2015).
135. Walsh, Stephen J., *et al.* Site-selective modification strategies in antibody–drug conjugates. *Chem. Soc. Rev.* **50**, 1305-1353 (2020).
136. van Geel, Remon *et al.* Chemoenzymatic Conjugation of Toxic Payloads to the Globally Conserved N-Glycan of Native mAbs Provides Homogeneous and Highly Efficacious Antibody-Drug Conjugates. *Bioconjug. Chem.* **26**, 2233-2242 (2015).
137. Nessler, I. *et al.* Increased Tumor Penetration of Single-Domain Antibody-Drug Conjugates Improves. *Cancer Res* **80**, 1268-1278 (2020).
138. Deonarain, M. P. *et al.* Small-Format Drug Conjugates: A Viable Alternative to ADCs for Solid Tumours? *Antibodies (Basel)* **7**, (2018).
139. Deonarain, M. P. & Xue, Q. Tackling solid tumour therapy with small-format drug conjugates. *Antib Ther* **3**, 237-245 (2020).
140. Wang, B., Gallolu Kankanamalage, S., Dong, J. & Liu, Y. Optimization of therapeutic antibodies. *Antib Ther* **4**, 45-54, (2021).
141. Annunziata, C. M. *et al.* Phase 1, open-label study of MEDI-547 in patients with relapsed or refractory solid tumors. *Invest New Drugs* **31**, 77-84 (2013).
142. Bennett, G. *et al.* MMAE Delivery Using the. *Mol Cancer Ther* **19**, 1385-1394 (2020).
143. Lee, N. K., Su, Y., Bidlingmaier, S. & Liu, B. Manipulation of Cell-Type Selective Antibody Internalization by a Guide-Effector Bispecific Design. *Mol Cancer Ther* **18**, 1092-1103 (2019).
144. Cilliers, C., Menezes, B., Nessler, I., Linderman, J. & Thurber, G. M. Improved Tumor Penetration and Single-Cell Targeting of Antibody-Drug Conjugates Increases Anticancer Efficacy and Host Survival. *Cancer Res* **78**, (2018).
145. Singh, A. P. *et al.* Antibody Coadministration as a Strategy to Overcome Binding-Site Barrier for ADCs: a Quantitative Investigation. *AAPS J* **22**, 28-35 (2020).
146. Menezes, B., Cilliers, C., Wessler, T., Thurber, G. M. & Linderman, J. J. An Agent-Based Systems Pharmacology Model of the Antibody-Drug Conjugate Kadcyla to Predict Efficacy of Different Dosing Regimens. *AAPS J* **22**, 29-40 (2020).
147. Menezes, B., Linderman, J. J. & Thurber, G. M. Simulating the Selection of Resistant Cells with Bystander Killing and Antibody Coadministration in Heterogeneous HER2 Positive Tumors. *Drug Metab Dispos*, (2021).

148. Crothers, D. M. & Metzger, H. The influence of polyvalency on the binding properties of antibodies. *Immunochemistry* **9**, 341-357 (1972).
149. Khera, E. *et al.* Quantifying ADC bystander payload penetration with cellular resolution using pharmacodynamic mapping. *Neoplasia* **23**, 210-221 (2021).
150. Khera, E., Cilliers, C., Bhatnagar, S. & Thurber, G. M. Computational transport analysis of antibody-drug conjugate bystander effects and payload tumoral distribution: implications for therapy. *Mol Sys Des Eng* **3**, 73-88 (2018).
151. Bartelink, I. H. *et al.* Tumor Drug Penetration Measurements Could Be the Neglected Piece of the Personalized Cancer Treatment Puzzle. *Clin Pharmacol Ther* **106**, 148-163 (2019).
152. Bostrom, J., Haber, L., Koenig, P., Kelley, R. F. & Fuh, G. High affinity antigen recognition of the dual specific variants of herceptin is entropy-driven in spite of structural plasticity. *PLoS One* **6**, 78-87 (2011).
153. Landry, J. P., Ke, Y., Yu, G. L. & Zhu, X. D. Measuring affinity constants of 1450 monoclonal antibodies to peptide targets with a microarray-based label-free assay platform. *J Immunol Methods* **417**, 86-96 (2015).
154. Tang, Y. *et al.* Regulation of antibody-dependent cellular cytotoxicity by IgG intrinsic and apparent affinity for target antigen. *J Immunol* **179**, 2815-2823 (2007).
155. Hendriks, B. S., Opresko, L. K., Wiley, H. S. & Lauffenburger, D. Coregulation of epidermal growth factor receptor/human epidermal growth factor receptor 2 (HER2) levels and locations: quantitative analysis of HER2 overexpression effects. *Cancer Res* **63**, 1130-1137 (2003).
156. Austin, C. D. *et al.* Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. *Mol Biol Cell* **15**, 5268-5282 (2004).
157. Thurber, G. M. & Wittrup, K. D. Quantitative spatiotemporal analysis of antibody fragment diffusion and endocytic consumption in tumor spheroids. *Cancer Res* **68**, 3334-3341 (2008).
158. Baker, J. H. *et al.* Direct visualization of heterogeneous extravascular distribution of trastuzumab in human epidermal growth factor receptor type 2 overexpressing xenografts. *Clin Cancer Res* **14**, 2171-2179 (2008).
159. Hilmas, D. E. & Gillette, E. L. Morphometric analyses of the microvasculature of tumors during growth and after x-irradiation. *Cancer* **33**, 103-110 (1974).
160. Yuan, F. *et al.* Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res* **55**, 3752-3756 (1995).
161. Ferl, G. Z., Kenanova, V., Wu, A. M. & DiStefano, J. J. A two-tiered physiologically based model for dually labeled single-chain Fv-Fc antibody fragments. *Mol Cancer Ther* **5**, 1550-1558 (2006).
162. Green, E. L. Biology of the laboratory mouse. (1966).
163. Maass, K. F., Kulkarni, C., Betts, A. M. & Wittrup, K. D. Determination of Cellular Processing Rates for a Trastuzumab-Maytansinoid Antibody-Drug Conjugate (ADC) Highlights Key Parameters for ADC Design. *AAPS J* **18**, 635-646 (2016).
164. Pruijn, F. B., Patel, K., Hay, M. P., Wilson, W. R. & Hicks, K. O. Prediction of tumour tissue diffusion coefficients of hypoxia-activated prodrugs from physicochemical parameters. *Australian journal of Chemistry* **61**, 687-693 (2008).

165. Krol, A., Maresca, J., Dewhirst, M. W. & Yuan, F. Available volume fraction of macromolecules in the extravascular space of a fibrosarcoma: implications for drug delivery. *Cancer Res* **59**, 4136-4141 (1999).
166. Jain, R. K. & Baxter, L. T. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. *Cancer Res* **48**, 7022-7032 (1988).
167. Drago, J. Z., Modi, S. & Chandarlapaty, S. Unlocking the potential of antibody-drug conjugates for cancer therapy. *Nat Rev Clin Oncol* **18**, 327-344 (2021).
168. Nessler, I., Menezes, B. & Thurber, G. M. Key metrics to expanding the pipeline of successful antibody-drug conjugates. *Trends Pharmacol Sci* **42**, 803-812 (2021).
169. Lucas, A. T., Moody, A., Schorzman, A. N. & Zamboni, W. C. Importance and Considerations of Antibody Engineering in Antibody-Drug Conjugates Development from a Clinical Pharmacologist's Perspective. *Antibodies (Basel)* **10** (2021).
170. Sun, X. *et al.* Effects of Drug-Antibody Ratio on Pharmacokinetics, Biodistribution, Efficacy, and Tolerability of Antibody-Maytansinoid Conjugates. *Bioconjug Chem* **28**, 1371-1381, (2017).
171. Lu, G. *et al.* Co-administered antibody improves penetration of antibody-dye conjugate into human cancers with implications for antibody-drug conjugates. *Nat Commun* **11**, 56-67 (2020).
172. Csizmar, C. M. *et al.* Multivalent Ligand Binding to Cell Membrane Antigens: Defining the Interplay of Affinity, Valency, and Expression Density. *J Am Chem Soc* **141**, 251-261 (2019).
173. Ong, G. L., Marria, V. & Mattes, M. J. The fate of antibodies and their radiolabels bound to tumor cells in vitro: the effect of cross-linking at the cell surface and of anti-idiotypic antibodies. *Cancer Immunol Immunother* **39**, 325-331 (1994).
174. Lin, J. & Sagert, J. in *Innovations for Next-Generation Antibody-Drug Conjugates* Springer (2018).
175. Li, F. *et al.* Tumor-Associated Macrophages Can Contribute to Antitumor Activity through FcγR-Mediated Processing of Antibody-Drug Conjugates. *Mol Cancer Ther* **16**, 1347-1354 (2017).
176. Ogitani, Y. *et al.* DS-8201a, A Novel HER2-Targeting ADC with a Novel DNA Topoisomerase I Inhibitor, Demonstrates a Promising Antitumor Efficacy with Differentiation from T-DM1. *Clin Cancer Res* **22**, 5097-5108 (2016).
177. Khera *et al.* Cellular-Resolution Imaging of Bystander Payload Tissue Penetration from Antibody-Drug Conjugates *Mol Cancer Ther.* (2022).
178. Donaghy H. Effects of antibody, drug and linker on the preclinical and clinical toxicities of antibody-drug conjugates. *MAbs* **28**, 659-671 (2016).
179. Gorovits, B., Krinos-Fiorotti, C. Proposed mechanism of off-target toxicity for antibody–drug conjugates driven by mannose receptor uptake. *Cancer Immunol Immunother* **62**, 217–223 (2013).
180. Strop P, Tran TT, Dorywalska M, *et al.* RN927C, a Site-Specific Trop-2 Antibody-Drug Conjugate (ADC) with Enhanced Stability, Is Highly Efficacious in Preclinical Solid Tumor Models. *Mol. Cancer Ther.* **15**, 2698-2708 (2015).
181. Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, *et al.* Drug resistance in cancer: an overview. *Cancers (Basel)* **6**,1769-1792 (2014).

182. Holohan C, Van Schaeybroeck S, Longley DB, & Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* **13**,714-726 (2013).
183. Wang X, Zhang H & Chen X. Drug resistance and combating drug resistance in cancer. *Cancer Drug Resist* **2**, 141-160 (2019).
184. Ma J, Mo Y, Tang M, et al. Bispecific Antibodies: From Research to Clinical Application. *Front Immunol.* **12**,616-626 (2021).
185. Alfarouk KO, Stock CM, Taylor S, Walsh M, Muddathir AK, et al. Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. *Cancer Cell Int* **15**, 71-84 (2015).
186. Hunter, F.W., Barker, H.R., Lipert, B. *et al.* Mechanisms of resistance to trastuzumab emtansine (T-DM1) in HER2-positive breast cancer. *Br J Cancer* **122**, 603–612 (2020).
187. Hormone Therapy for Breast Cancer Fact Sheet - NCI
188. Bayat Mokhtari, Reza et al. Combination therapy in combating cancer. *Oncotarget* **8**, 38022-38043 (2017).
189. Albain KS et al. Gemcitabine plus Paclitaxel versus Paclitaxel monotherapy in patients with metastatic breast cancer and prior anthracycline treatment. *J Clin Oncol.* **26**, 3950–3957 (2008).
190. Mokhtari RB, Kumar S, Islam SS, Yazdanpanah M, Adeli K, Cutz E, & Yeger H. Combination of carbonic anhydrase inhibitor, acetazolamide, and sulforaphane, reduces the viability and growth of bronchial carcinoid cell lines. *BMC Cancer.* **13**, 378-385 (2013).
191. Hanahan D, Bergers G, & Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *J Clin Invest.* **105**, 1045–1047 (2000).
192. Gorski DH, et al. Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res.* **59**, 3374–3378 (1999).
193. Henricks, Linda M et al. The use of combinations of monoclonal antibodies in clinical oncology. *Cancer treatment reviews* **41**, 859-867 (2015).
194. Ma J, Mo Y, Tang M, Shen J, Qi Y, & Zhao W. Bispecific Antibodies: From Research to Clinical Application. *Fron. Immunol.* **12**, 616-626 (2021).
195. Bargou R, et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science.* **321**, 974–977 (2008).
196. Weiner LM, et al. Binding and cytotoxicity characteristics of the bispecific murine monoclonal antibody 2B1. *J Immunol Res.* **151**, 2877–2886 (1993).
197. Dahlén, Eva et al. Bispecific antibodies in cancer immunotherapy. *Therapeutic advances in vaccines and immunotherapy* **6**, 3-17 (2018).
198. Varela MA. Identification of sequences common to more than one therapeutic target to treat complex diseases: simulating the high variance in sequence interactivity evolved to modulate robust phenotypes. *BMC Genomics.* **16**, 530-40 (2015).
199. Mazor, Yariv, et al. Insights into the molecular basis of a bispecific antibody's target selectivity. *MAbs.* **7**, 3-19 (2015).
200. Puhalla S, Bhattacharya S, & Davidson NE. Hormonal therapy in breast cancer: a model disease for the personalization of cancer care. *Mol Oncol.* **6**, 222-236. (2012).
201. Esfahani K, Roudaia L, Buhlaiga N, Del Rincon SV, Papneja N, & Miller WH Jr. A review of cancer immunotherapy: from the past, to the present, to the future. *Curr Oncol.* **27**, 87-97 (2020).

202. Koury J, Lucero M, Cato C, et al. Immunotherapies: Exploiting the Immune System for Cancer Treatment. *J Immunol Res.* **9**,52-64 (2018).
203. Shenfield, G M. Fixed combination drug therapy. *Drugs* **23**, 462-480. (1982).
204. Wang Q, Chen Y, & Park J. Design and Production of Bispecific Antibodies. *Antibodies (Basel).* **8**, 33-43 (2019).
205. Liddell, Eryl. Antibodies. *The immunoassay handbook.* Elsevier, 245-265 (2013).
206. *Biophysical Characterization of Bispecific Antibodies - Dynamic Biosensors.*
207. Kareva, Irina, et al. Bispecific antibodies: A guide to model informed drug discovery and development. *Heliyon* **7**, 76-89 (2021).
208. Karagiannis, P., Singer, J., Hunt, J. et al. Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER2/*neu*-positive tumour cells. *Cancer Immunol Immunother* **58**, 915–930 (2009).
209. Fu, Wenyan et al. Insights into HER2 signaling from step-by-step optimization of anti-HER2 antibodies. *mAbs* **6**, 978-990 (2014).
210. Lakayan, Dina et al. Affinity profiling of monoclonal antibody and antibody-drug-conjugate preparations by coupled liquid chromatography-surface plasmon resonance biosensing. *Anal Bioanal Chem.* **410**, 7837-7848 (2018).
211. *DATASHEET Methods to Determine the Binding of Trastuzumab to HER2.*
212. Guram, Kripa, et al. A threshold model for T-cell activation in the era of checkpoint blockade immunotherapy. *Front. Immunol* **10**, 491-502 (2019).
213. Ward, Steve. Raising the threshold for T-cell activation. *Trends Immunol.* **22**, 597-611 (2001).
214. Nicholson, Lindsay B., Ana C. Anderson, & Vijay K. Kuchroo. Tuning T cell activation threshold and effector function with cross-reactive peptide ligands. *Int Immunol.* **12**, 205-213 (2000).
215. Antonella, V. & Lanzavecchia, A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273**, 104-106 (1996).
216. Zikherman J., & Au-Yeung, B. The role of T cell receptor signaling thresholds in guiding T cell fate decisions. *Curr opin Immunol.* **33**, 43-48 (2015).
217. Berrouet, C., et al. Comparison of Drug Inhibitory Effects in Monolayer and Spheroid Cultures. *Bull Mat Biol* **82**, 1-23 (2020).
218. Thorarensen, A., et al. The advantages of describing covalent inhibitor in vitro potencies by IC50 at a fixed time point. IC50 determination of covalent inhibitors provides meaningful data to medicinal chemistry for SAR optimization. *Bioorg Med Chem.* **29**, 58-65 (2021).
219. Bhuvaneswari, R. Evaluation of Otoacoustic Emissions, Brainstem Evoked Response Audiometry and Serum Levels of Anticyclic Citrullinated Protein Antibodies in patients with Active Rheumatoid Arthritis. *Diss. Madras Medical College, Chennai*, 2017.
220. Narváez JA, Narváez J, De Lama E & De Albert M. MR imaging of early rheumatoid arthritis. *Radiographics.* **30**, 143–165 (2010).
221. Bongartz T, Halligan CS, Osmon DR, et al. Incidence and risk factors of prosthetic joint infection after total hip or knee replacement in patients with rheumatoid arthritis. *Arthritis Care & Research.* **59**, 13-17 (2008).
222. Boesen, M, et al. MRI quantification of rheumatoid arthritis: current knowledge and future perspectives. *Euro J Radiol.* **71**, 189-196 (2009).

223. Lopez-Ben R, Bernreuter WK, Moreland LW, & Alarcón GS. Ultrasound detection of bone erosions in rheumatoid arthritis: a comparison to routine radiographs of the hands and feet. *Skeletal Radiol.* **33**, 80-84 (2004).
224. Hoving JL, Buchbinder R, Hall S, et al. A comparison of magnetic resonance imaging, sonography, and radiography of the hand in patients with early rheumatoid arthritis. *J Rheumatol.* **31**, 663-675 (2004).
225. Mountz JM, Alavi A, Mountz JD: Emerging optical and nuclear medicine imaging methods in rheumatoid arthritis. *Nat Rev Rheumatol.* **8**, 719-728 (2012).
226. Mohajerani P, Meier R, Noel PB, Rummeny EJ, & Ntziachristos V. Spatiotemporal analysis for indocyanine green-aided imaging of rheumatoid arthritis in hand joints. *J Biomed Opt* **18**,17-29 (2013).
227. Wunder A, Tung CH, Muller-Ladner U, Weissleder R & Mahmood U 2004. In vivo imaging of protease activity in arthritis: a novel approach for monitoring treatment response. *Arthritis Rheum.* **50**, 2459-2465 (2004).
228. Schafer VS, Hartung W, Hoffstetter P, Berger J, Stroszczynski C, & Muller M Quantitative assessment of synovitis in patients with rheumatoid arthritis using fluorescence optical imaging. *Art Res Ther.* **15**,124-130 (2013).
229. Benson RC, & Kues HA. Fluorescence properties of indocyanine green as related to angiography. *Phys med and biol.* **23**, 159-163. (1978).
230. Dziekan T, Weissbach C, Voigt J, Ebert B, Macdonald R, & Bahner M. Detection of rheumatoid arthritis by evaluation of normalized variances of fluorescence time correlation functions. *J. biomed opt.* **16**, 6-15(2011).
231. Kisten Y, Gyori N, Af Klint E, Rezaei H, Levitsky A, & Karlsson A. Detection of clinically manifest and silent synovitis in the hands and wrists by fluorescence optical imaging. *RMD open* **1**, 6-12 (2015).
232. Werner SG, Langer HE, Schott P, Bahner M, Schwenke C, & Lind-Albrecht G. Indocyanine green-enhanced fluorescence optical imaging in patients with early and very early arthritis: a comparative study with magnetic resonance imaging. *Arthritis Rheum.* **65**, 3036-3044 (2013).
233. Jackisch C, Muller V, Maintz C, Hell S & Ataseven B. Subcutaneous Administration of Monoclonal Antibodies in Oncology. *Geburtshilfe und Frauenheilkunde.* **74**, 343-349 (2014).
234. Utz KS, Hoog J, Wentrup A, Berg S, Lammer A, Jainsch B & Waschbisch A Patient preferences for disease-modifying drugs in multiple sclerosis therapy: a choice-based conjoint analysis. *Ther. Adv. Neurol. Disord* **7**, 263-275 (2014).
235. Hope-Ross M, Yannuzzi LA, Gragoudas ES, Guyer DR & Slakter JS. Adverse reactions due to indocyanine green. *Ophthalmology* **101**, 529-533 (1994).
236. Bottinor W, Polkampally P, & Jovin I. Adverse reactions to iodinated contrast media. *Int. J. Angiol.* **22**,149-154 (2013).
237. Ryu JH, Lee A, Chu JU, Koo H, Ko CY, Kim HS, Yoon SY, Kim BS & Choi K. Early diagnosis of arthritis in mice with collagen-induced arthritis, using a fluorogenic matrix metalloproteinase 3-specific polymeric probe. *Arthritis Rheum.* **63**, 3824-32 (2011).
238. Henne WA, Rothenbuhler R, Ayala-Lopez W, Xia W, Varghese B & Low PS. Imaging sites on infection using a ^{99m}Tc-labeled folate conjugate targeted to folate receptor positive macrophages. *Mol Pharm.* **9**,1435-1440 (2012).

239. Anzola-Fuentes, L. K., et al. Somatostatin receptor scintigraphy in patients with rheumatoid arthritis and secondary Sjögren's syndrome treated with Infliximab: a pilot study. *EJNMMI research* **6**, 49-54 (2016).
240. Bhatnagar, S. *et al.* Oral Administration and Detection of a Near-Infrared Molecular Imaging Agent in an Orthotopic Mouse Model for Breast Cancer Screening. *Mol Pharm*, **15**, 1746-1754 (2018).
241. Antonov, A *et al.* $\alpha V\beta 3$ integrin regulates macrophage inflammatory responses via PI3 kinase/Akt-dependent NF- κ B activation. *J. Cell Phys.* **226**, 469-476 (2011).
242. Oliveira S, Cohen R, Stigter-van Walsum M, van Dongen G, & Elias SG. PMvB A novel method to quantify IRDye800CW fluorescent antibody probes ex vivo in tissue distribution studies. *EJNMMI research* **2**, 50-55 (2012).
243. Burgers LE, Allaart CF, Huizinga TWJ, & van der Helm-van Mil AHM 2017. Brief Report: Clinical Trials Aiming to Prevent Rheumatoid Arthritis Cannot Detect Prevention Without Adequate Risk Stratification: A Trial of Methotrexate Versus Placebo in Undifferentiated Arthritis as an Example. *Arthritis Rheum* **69**, 926-931 (2017).
244. Alander JT, Kaartinen I, Laakso A, Patila T, Spillmann T & Tuchin VV A review of indocyanine green fluorescent imaging in surgery. *Int. J. biomed imaging* **94**, 79-85 (2012).
245. Bender AT, Spyvee M, Satoh T, Gershman B, Teceno T, Burgess L & Kumar V. Evaluation of a candidate anti-arthritic drug using the mouse collagen antibody induced arthritis model and clinically relevant biomarkers. *Am J Transl Res* **5**, 92-102 (2013).
246. Chan MM, Gray BD, Pak KY & Fong D. Non-invasive in vivo imaging of arthritis in a collagen-induced murine model with phosphatidylserine-binding near-infrared (NIR) dye. *Arthritis Res Ther.* **17**, 50-58 (2015).
247. Cho H, Bhatti FU, Yoon TW, Hasty KA, Stuart JM, & Yi AK. Non-invasive dual fluorescence in vivo imaging for detection of macrophage infiltration and matrix 163 metalloproteinase (MMP) activity in inflammatory arthritic joints. *Biomed opt express* **7**, 1842-1852 (2016).
248. Ryu JH, Lee A, Chu JU, Koo H, Ko CY, Kim HS, Yoon SY, Kim BS, & Choi K,. Early diagnosis of arthritis in mice with collagen- induced arthritis, using a fluorogenic matrix metalloproteinase 3-specific polymeric probe. *Arthritis Rheum.* **63**, 3824- 3832 (2011).
249. Garrood T, Morrison M, Shivapatham D, Chaabo K & Ul-Hassan F. Whole-Body Synovial Uptake of a 99m-Tc-Labelled RGD Peptide is Highly Correlated with Power Doppler Ultrasound [abstract]. *Arthritis Rheum.* **67** (2015).
250. Zhu Z, Yin Y, Zheng K, Li F, Chen X, Zhang F & Zhang X. Evaluation of synovial angiogenesis in patients with rheumatoid arthritis using (6)(8)Ga-PRGD2 PET/CT: a prospective proof-of-concept cohort study. *Ann Rheum Dis.* **73**, 1269-1272 (2014).
251. Vollmer S, Vater A, Licha K, Gemeinhardt I, Gemeinhardt O, & Voigt J. Extra domain B fibronectin as a target for nearinfrared fluorescence imaging of rheumatoid arthritis affected joints in vivo. *Mol Imaging* **8**, 330-340 (2009).
252. Hu, Y, et al. Depletion of activated macrophages with a folate receptor-beta-specific antibody improves symptoms in mouse models of rheumatoid arthritis. *Arthritis Res Ther* **21**, 1-14 (2019).
253. Michelson, S, & Leith, J. Tumor heterogeneity: a review of the theory. *Drug News Perspectives.* **6**, 655-658 (1993).
254. Panetta, J. A mathematical model of drug resistance: heterogeneous tumors. *Math biosci* **147**, 41-61 (1998).

255. Hickey, E. Tools to define the relevance of PK/PD parameters to the efficacy, toxicity and emergence of resistance of antimicrobials. *Current opinion in drug discovery & development* **10**, 49-52 (2007).
256. Gaffney, EA. The application of mathematical modelling to aspects of adjuvant chemotherapy scheduling. *J. mat biol* **48**, 375-422 (2004).
257. Lebedeva, G. et al. Model-based global sensitivity analysis as applied to identification of anti-cancer drug targets and biomarkers of drug resistance in the ErbB2/3 network. *Eur. J. of Pharm Sci.* **46**, 244-258 (2012).
258. Jackson, T.L., & Byrne H. A mathematical model to study the effects of drug resistance and vasculature on the response of solid tumors to chemotherapy. *Mat biosci.* **164**, 17-38 (2000).
259. Lampropoulos, I., Charoupa M. & Kavousanakis M. Intra-tumor heterogeneity and its impact on cytotoxic therapy in a two-dimensional vascular tumor growth model. *Chem Eng Sci.* **11**, 77-92 (2022).
260. Chisholm, R.H., et al. Emergence of drug tolerance in cancer cell populations: an evolutionary outcome of selection, nongenetic instability, and stress-induced adaptation. *Cancer res.* **75**, 930-939 (2015).
261. Alvarez-Arenas A., Belmonte-Beitia J., Calvo G., & Podolski-Renic A. Interplay of Darwinian Selection, Lamarckian Induction and Microvesicle Transfer on Drug Resistance in Cancer. *Sci Rep.* **9**, 93-100 (2019).
262. Craig, M. et al. Engineering in Medicine To Address the Challenge of Cancer Drug Resistance: From Micro- and Nanotechnologies to Computational and Mathematical Modeling. *Chem rev.* **121**, 3352-3389 (2021).
263. Pouchol, C., et al. Asymptotic analysis and optimal control of an integro-differential system modelling healthy and cancer cells exposed to chemotherapy. *Journal de Mathématiques Pures et Appliquées.* **116**, 268-308 (2018).
264. Bouchnita, A., et al. A multiscale model to design therapeutic strategies that overcome drug resistance to tyrosine kinase inhibitors in multiple myeloma. *Mat biosci.* **319**, 108-128 (2020).
265. Wakefield RJ, D'Agostino MA, Naredo E, Buch MH, Iagnocco A, & Terslev L. After treat-to-target: can a targeted ultrasound initiative improve RA outcomes? *Ann. Rheum. Dis.* **71**, 799-803.
266. Zhang, Y., et al. Animal models of ankylosing spondylitis. *Curr rheum rep.* **4**, 507-512 (2002).
267. Braem, K. & Rik J.L. Insights into the pathophysiology of ankylosing spondylitis: contributions from animal models. *Joint Bone Spine.* **79**, 243-248 (2012).