Appraisal of leucine-rich alpha-2-glycoprotein 1 (LRG1) as a target for a potential antibody–drug conjugate (ADC)

by

Faiza Javaid

Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
Declaration of Authorship

I, Faiza Javaid, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Faiza Javaid

March 2021
For mumma and daddy.
Acknowledgements

Bismillah,

I could never have imagined that the most important undertaking of my life, and indeed the most important year of my PhD would coincide with a global pandemic. But I still maintain the conviction, Alhamdulillah, that pursuing a PhD has been the greatest (albeit the most challenging) decision I have ever made in my life, and it is in no small part due to the support and friendship of the many people I have had the fortune to meet in the process. When I think of my PhD journey, what comes to mind is not merely the untold hours of growing cells and digesting antibodies, but the sounds of AoE II, Rubik's cube contests (the Chud rites of passage - neither of which I was proficient at, but was nevertheless welcomed into the Chud fold), venturing into previously unexplored corners of YouTube (notably lemonade duck and Danny’s goodbye), that weekend in Portugal, 7 days aka the Chud anthem, numerous games of Chameleon, João’s beautiful face contagious energy and the KLB space project amongst many other interesting events. I will always warmly remember everyone who has been a part of the journey.

First and foremost, I owe endless thanks to my supervisor, mentor, role model and friend (!!! It’s official) Prof. Vijay Chudasama, for agreeing to take me on as a PhD student despite my non-existent chemistry background. Never have I met someone so hardworking and so dedicated to their students. You have made my PhD every bit worth it with your unmatched support, guidance, advice and belief in me with which I have been able to make significant progress as a researcher during my time in the laboratory. I will forever be grateful for the huge amounts of knowledge you have supplied me with, for always holding me up to a high standard, without which I would not have been able to complete this work, and for all the opportunities you have provided me to learn, explore and expand my skills as a researcher. Your mentorship has been the greatest asset. I also owe a huge amount of gratitude to Prof. Stephen Moss, Prof. John Greenwood and Dr James Baker for their invaluable help and guidance during my PhD.

I’d like to thank my examiners Professor Rachel McKendry and Dr Manuel Müller for taking the time to read through this work and carry out my viva examination. I’d also like to thank my funding body, the Wellcome Trust, and my host institution UCL, for providing me with the opportunity to do a PhD.
My PhD journey would never be what it was if not for my incredible lab mates – the Chudasamas, the Bakers and the Sheppards, who I am lucky enough to call my good friends. This journey would not be complete without a few people in particular:

Antoine, the most diversely skilful person I have ever known. I have had some of the best conversations of my life with you. Thank you for being the greatest mentor, my favourite neighbour and my most cherished friend. For the many things you have taught me about science, religion, round chapatis and life. For the ‘fun fact of the day’ and for introducing me to my favourite new bragging card: origami. And most importantly, being the one guaranteed person to laugh with me. My ‘no-limit diets’ will never be the same.

Richard, the kindest person I have met. Thank you for always listening, for offering wise words of support, for the immediate sense of comfort you bring to the group, for always being up for dessert, for providing us with the best office snacks.

Calise, thank you for being the perfect introduction to the group. For being the best organic chemistry mentor. For letting me break that flask and accept when I went complete Shaggy (wasn't me). For your completely chilled out demeanour, Pret coffee breaks, but mostly for your stories and the many laughs that accompanied them.

André, for you I reserve my greatest endearments. I knew we would have something special when you gave me charge of the music on day one. Throughout my time at the KLB you have been someone I could easily share things with, and grab bubble tea with, and with you I could always guarantee an endorphin rush with those incredible bear hugs. I will forever miss our ‘secret hideout’ escapades.

João, for showing us all that it is always a good idea to leave one’s comfort zone, for your refreshing outlook on life, your energy, for sharing my looks of confusion during deep and heavy organic chemistry discussions, for being the best company during coffee breaks and for being so effortlessly cool regardless of what it was you were doing. I want to be like you when I grow up.

Marcos, I will never forget all the fun we had when we wrote our first publication together. Who knew it would get so many hits? Are we famous now?

Peter, it is so humbling and honouring to have a friend with a brain like yours. There is no doubt you will go so far in life – don’t forget us pinkies when you do (you know, because you’re the Brain).
Nehaal, for sharing my love for Bazzi, for bringing some masala (literally) to the lab and our lives, for being the poster-boy for endless streetstyle inspo, for being the greatest risk-taker I know ($$$ sneakers $$$ in the lab, mate?!), for bringing me food and for introducing me to the best tandoori chicken I have ever had. I cannot wait till you and your dad open a restaurant. I’ll be your most loyal customer.

Alex, you inspire me with your competitive attitude and breadth of ‘general’ knowledge. I’m glad that at least one of us is famous enough to have appeared on national TV.

Of course I cannot forget the veteran Chuds; Max and Dan. You will both always be fondly remembered for your colourful and unexpected personalities and infectious laughter, respectively. Max, your artistic interpretation of ‘Pierce’ Brosnan will stay with me till the end of days. Thank you both for making the group an exciting place to work, for your unique combination of hard work, sense of humour and incredible personalities which were no doubt a major part in my decision to join the group.

Roshni, my number seven/the light of my life (LOML), for being my fellow drama-loving event coordinating queen. For sharing my love (and hate) for everything ever. For the relatable memes, snowy walks for Thai food, sneaky brunch breaks and theatre dates and for being my companion at every milestone (literally) and being by my side through every slip and ‘slide’. Our chats about life and work and everything in between, our cloister walks, cake breaks are all things that have made my time during my PhD so much better.

Archie, you will always be fondly remembered for your sassy and sarcastic personality, your drive and your ability to ask all the right questions. And for THAT iconic joke.

My KLB acknowledgements would be incomplete without thanking all members of the Red Pen Fan Club: Alina, Muhammed, Usman, Yanbo and Roshi/Roshmi/Roshini, for the copious amounts of laughter and daily mischief updates. I feel at ease knowing the future of KLB banter is in your hands.

David, you helped me settle in at the Institute and your sarcasm and dad-humour made the Institute a welcome place to work. Thank you for always being open to helping – working with you has been an absolute pleasure. Camilla and Carlotta, thank you both for your help and hard work with all the animal experiments, without which this work would not be complete.
Marlene, you make the Institute a great place to be. Thank you always for your encouraging words, for listening to me ramble about my frustrations with love lab and life. For our long walks in the lift to the second floor. For the high intensity interval tamasha (HIIT) sessions. And for all the laughter.

I would like to extend my gratitude towards close friends and family whose support and love have remained constant during my adult life, and who have always pushed me to accomplish my goals.

My family was the backbone of my PhD: my brother, Saqib, supported me with long talks and laughter and always reminded me to keep going, my sister, Mehvish, has always kept me entertained with her comedic (clownish?) personality and grounded with her wisdom. My parents deserve to have the entire wings of this thesis named after them for all the support they have given me over the years. Encouraged me during the hardest times, dealt with my many mood swings and kept me sane through pep talks and endless plates of rice and bowls of fruit brought straight to my room.

Toobah, your presence in my life has always been constant. Thank you for the impromptu late-night drives, the chai runs and Turkish food cravings and car-seat karaoke sessions and the long phone calls. But most of all, for being my anchor in life. For keeping me grounded, and for being my biggest cheerleader. I love you.

Nadine, your friendship has been the best thing to ever happen in my adult life. And you being at Moorfields completely transformed how my days at the Institute would have felt. Thank you for helping me grow in ways unimaginable, for diversifying my street vocabulary and for always making me laugh, the good vibes, the many salad bowls and for making sure that we hit every hip spot in town.

Sultaan, for adding the yay to many (Fri)days. For believing in me much more than I ever did. For the jokes and the encouragement. And for telling people that I ‘make pathogens’. I deeply cherish your friendship. You know you have another book recommendation coming your way (in the form of this thesis).

Emir, you have been an incredible presence throughout my eight and some years at UCL. Your wisdom and support have helped me get through not just the bad days in the lab, but also outside. Thank you for always letting me be the funniest out of the two us. And for letting me believe I am the smartest too.
I’d like to express my love and gratitude to my best friends Huma and Naho, thank you for always lending me an ear, for your support, for believing in me and being there at all times, despite the distance of thousands of miles.

I would also like to extend thanks to the friends who I see rarely but who hold a great space in my heart and have all influenced my PhD journey in unique and irreplaceable ways: Jaewon, Sabina, Zarin and Amara.

Shoutout to Thai metro, Half cup, Coffee junction, Salad kitchen, ‘vibes’ market, ‘Korean’ market, Pizza union, UCLU beverages, Betty’s cookies (thank you Richard), Chef Antoine (and the infamous tiger cakes) and the cake fairies for keeping me fed and happy. The vending machine and the free tea and biscuits every morning at the IoO have also made sure that many a bad day turn into good ones.

Thank you to the many people who have been part of the process, encouraging me, advising me, believing in me. The undertaking of this PhD has been of immense personal significance to me. Everyone who was a part of this journey at the start, and everyone who made it to the end, I’m glad you did. Because I doubted that I would, numerous times.

I have been spoilt with the best team, the best friends, the best colleagues, the best mentors and teachers and best supervisors a girl could ask for. This really is quite a great start to my career, and I know that nothing will ever compare.

The only trick of friendship, I think, is to find people who are better than you are – not smarter, not cooler, but kinder, and more generous, and more forgiving – and to appreciate them for what they can teach you, and to try to listen to them when they tell you something about yourself, no matter how bad – or good – it might be, and to trust them, which is the hardest thing of all. But the best, as well.

Hanha Yanagihara

Ultimately, my efforts are meaningless if the stars are not aligned.
Sima Taparia, from Mumbai.
Abstract

Leucine-rich alpha-2-glycoprotein 1 (LRG1) is present abundantly in the microenvironment of many tumours where it contributes to vascular dysfunction, which impedes the delivery of therapeutics. LRG1 antibody blockade leads to vascular normalisation, and represents a novel means of improving efficacy of cancer therapeutics. The aim of this research is to evaluate whether LRG1 is a suitable target for an antibody–drug conjugate (ADC) based cancer therapy. While it is generally believed that ADCs need to internalise into tumour cells in order to liberate their payload and display optimal therapeutic activity, a growing body of evidence indicates that ADCs targeting non-internalising antigens also display potent activity. This work demonstrates that LRG1 is predominantly a non-internalising protein. Herein, the development of a novel ADC comprising the anti-LRG1 hinge-stabilised IgG4 monoclonal antibody Magacizumab coupled to the anti-mitotic payload monomethyl auristatin E (MMAE) via a cleavable dipeptide linker using the site-selective disulfide rebridging dibromopyridazinedione (diBrPD) scaffold is reported. It is demonstrated that this ADC retains binding post-modification, is stable in serum and effective in in vitro cell studies. A hLRG1-expressing B16F0 mouse melanoma cell line was developed and tumours derived from this cell line indicated localisation of Magacizumab at the site of the tumour. In vivo and in vitro studies showed that the novel extracellular LRG1-targeting ADC liberates its toxic payload, which is coupled to the antibody via a cleavable dipeptide linker, presumably upon being metabolised by appropriate proteases (e.g. cathepsin B) and provides an increase in survival when applied in mouse models of tumours when compared against standard chemotherapy and antibody alone. LRG1 targeting through this reported ADC presents a novel and effective proof-of-concept en route to improving the efficacy of cancer therapeutics.
Impact statement

Cancer is the second leading cause of mortality worldwide and accounts for one in six deaths. The disease is characterised by genetic changes that lead to uncontrolled growth and division of cells. Cancer consists of over 200 different diseases that arise from a variety of cellular defects. Consequently, a treatment that is effective against one cancer may not be effective against another. Traditionally, cancer treatment has relied upon chemotherapy; however, the side-effects of the drugs administrated are notoriously debilitating to patients due to the inability of these drugs to discriminate between healthy and diseased cells.

Recent advances in biological therapies promise a more targeted approach to cancer therapy than has been previously possible. In antibody–drug conjugate (ADC) technology, the specificity of a monoclonal antibody for its target is exploited to home an extremely cytotoxic agent into tumour cells, whereas administration of the agent alone is not suitable due to its high toxicity. The antibody acts as the drug delivery vehicle, capable of targeting cancer cells with unrivalled selectivity. The concept of using antibodies as drug delivery vehicles dates back to more than 50 years, but it has only recently developed into a major class of therapeutics.

In many diseases, including cancer, the structure and function of blood vessels becomes compromised, resulting in vessel architecture that either drives the disease or significantly contributes to it. These diseased vessels frequently permit poor access of therapeutic agents. For many years there has been considerable focus on the vasculature of solid cancers, as the formation of new blood vessels is required for their growth and dissemination. In recent years, this concept of normalising the vasculature in tumours, to improve vessel function, has gained considerable traction as this presents the opportunity to facilitate the delivery of therapeutics. This has recently led to the discovery of leucine-rich alpha-2-glycoprotein 1 (LRG1), a protein involved in vascular growth and dysfunction, as a novel therapeutic target. Accordingly, a humanised/deimmunised therapeutic antibody against LRG1 has been generated with encouraging data in safety and toxicology studies.

Herein is reported the development of an ADC comprising the aforementioned antibody, that targets the vascular destabilising protein LRG1. When tested in cell-based and
animal-based cancer models, the ADC demonstrated superior therapeutic effect when compared to the monoclonal antibody alone and similar in performance to standard chemotherapy without its adverse side effects. As such, it would be interesting to explore its impact within a clinical setting. More research is needed to determine whether therapeutic targeting of LRG1 will provide significant clinical benefit, but evidence provided within suggests the area is promising and increasingly growing.
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<tr>
<td>AcOH</td>
<td>Acetic acid</td>
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<tr>
<td>ADC</td>
<td>Antibody–drug conjugate</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AFC</td>
<td>Antibody–fluorophore conjugate</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BBS</td>
<td>Borate-buffered saline</td>
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<tr>
<td>BCN</td>
<td>Bicyclo[6.1.0]nonyne</td>
</tr>
<tr>
<td>BME</td>
<td>beta-Mercaptoethanol</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxy carbonyl (protecting group)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDR</td>
<td>Complimentary determining regions</td>
</tr>
<tr>
<td>CNV</td>
<td>Choroidal neovascularisation</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper promoted azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
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<tr>
<td>DAR</td>
<td>Drug-to-antibody ratio</td>
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<tr>
<td>DCC</td>
<td>$N,N'$-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DiBrPD</td>
<td>Dibromopyridazinedione</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
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<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>Eq</td>
<td>Equivalent(s)</td>
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<td>ESI</td>
<td>Electrospray ionisation</td>
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<td>EtOAc</td>
<td>Ethyl acetate</td>
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<td>Fab</td>
<td>Fragment antigen-binding</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
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<td>FcRn</td>
<td>Neonatal Fc receptor</td>
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<td>FDA</td>
<td>Food and drug administration</td>
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<td>FFA</td>
<td>Fundus fluorescein angiography</td>
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<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>Her</td>
<td>Herceptin</td>
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<tr>
<td>hLrg1</td>
<td>Human Leucine rich alpha-2 glycoprotein 1</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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IC$_{50}$  Half-maximal inhibitory concentration
IgG  Immunoglobulin G
IPA  Isopropanol
IR  Infrared
J  Coupling constant
kDa  Kilodalton
KI  Knock-in
K$_D$  Dissociation constant
KO  Knock-out
LCMS  Liquid chromatography mass spectrometry
LRG1  Leucine rich alpha-2 glycoprotein 1
µ  Micro
M  Molar
m  Multiplet
mAb  Monoclonal antibody
Maga  Magacizumab
Maga$_{Fab}$  Magacizumab Fab
mc  Maleimidocaproyl
Me  Methyl
MeCN  Acetonitrile
MeOH  Methanol
<table>
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<td>MMAE</td>
<td>Monomethyl auristatin E</td>
</tr>
<tr>
<td>MMAF</td>
<td>Monomethyl auristatin F</td>
</tr>
<tr>
<td>mol</td>
<td>Mole(s)</td>
</tr>
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<td>MS</td>
<td>Mass spectrometry</td>
</tr>
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<td>MWCO</td>
<td>Molecular weight cut off</td>
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<td>m/z</td>
<td>Mass to charge ratio</td>
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<td>n</td>
<td>Nano</td>
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<td>NAA</td>
<td>Natural amino acid</td>
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<tr>
<td>NHS</td>
<td>N-hydroxy-succinimide</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>PABC</td>
<td>para-aminobenzylcarbonyl</td>
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<td>PAR</td>
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<td>PD</td>
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<td>Polyethylene glycol</td>
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<td>Paraformaldehyde</td>
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<td>PNGase F</td>
<td>Peptide:N-glycosidase F</td>
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<td>Ppm</td>
<td>Parts per million</td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<td>RT</td>
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<td>Abbreviation</td>
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<td>SDS-PAGE</td>
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<td>SEM</td>
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<td>SPAAC</td>
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<td>tBu</td>
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<td>TCEP</td>
<td>Tris(2-Carboxyethyl)phosphine</td>
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<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>THF</td>
<td>Tetrahydrofuran</td>
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<td>TLC</td>
<td>Thin-layer chromatography</td>
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<td>Val-Cit</td>
<td>Valine-Citrulline</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>UAA</td>
<td>Unnatural amino acid</td>
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<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
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Chapter 1 Introduction

1.1 Cancer chemotherapy

Cancer is the second leading cause of mortality worldwide and accounts for one in six deaths.\(^1\) The disease is characterised by genetic changes that lead to unregulated cell growth, such as the activation of proto-oncogenes and inactivation of tumour suppression genes. For much of the 20\(^{th}\) century, small molecule chemotherapy has been used for the treatment of cancer and typically relies on the use of cytotoxic drugs to destroy the tumour.\(^2\) However, the side-effects of the drugs administrated are notoriously debilitating to patients due to the inability of these drugs to discriminate between healthy and diseased cells.\(^2\) In recent decades, strategies have emerged to selectively target the tumour, and exhibit the ability and potential to treat disease with superior specificity.

Small molecule cytotoxins have remarkable potency as anticancer agents but they are often limited by poor cellular selectivity. The off-target effects caused by this lack of selectivity usually lead to a narrow therapeutic window, in which there is a small difference between the maximum tolerated dose and the minimum effective concentration. However, attachment of a targeting group to the cytotoxin can offer a mechanism by which the drug is theoretically only active at the desired cell type, thereby increasing selectivity and the therapeutic window (Figure 1).\(^3\)

![Figure 1](image_url) \(\text{Figure 1} \) The difference in therapeutic window between chemotherapy and targeted drug delivery.
Recent advances in biological therapies promise a more targeted approach to cancer therapy than has been previously possible. Some targeting delivery vehicles may achieve specificity by recognising proteins that are overexpressed on the surface of cancer cells. Such targeting groups can be small molecules, peptides or monoclonal antibodies (mAbs; see Section 1.4 for more details). Although each approach has its own merits, mAbs have yielded the most clinical success. By specifically binding an antigen on a cancer cell, mAbs reduce off-target toxicities associated with conventional chemotherapy, selectively target tumour cells, and either alter their signalling patterns towards a therapeutic outcome, or direct an immune response towards the tumour cell. Antibody–drug conjugate (ADC) technology enhances the therapeutic window of its primary components, namely the targeted antibody and a cytotoxin covalently attached to the antibody (ADCs will be discussed in more detail in Section 1.5). The concept of using antibodies as drug delivery vehicles dates back to more than 50 years, but it has only recently developed into a major class of therapeutics. The antibody acts as the drug delivery vehicle, capable of targeting cancer cells with unrivalled selectivity. Although this antibody-based platform is relatively straightforward, the development of an effective ADC is remarkably challenging. The clinical development of ADCs requires careful development of several biological and pharmaceutical parameters. In particular, there are three main components of an ADC: the antibody (and antigen), the linker and the cytotoxic payload, each of which requires careful optimisation (see Section 1.5 for more details). These challenges provide an opportunity for innovation in the design of the ADC. As such, researchers have assembled an impressive array of tools to apply to ADCs, from conjugation chemistry to choice of target antigen, and this will be discussed in more detail herein. Furthermore, the work presented in this thesis will describe the evaluation of Leucine-rich alpha-2-glycoprotein 1 (LRG1), a secreted vascular destabilising protein that is abundantly present in the microenvironment of many tumours, as a new target for the development of a novel ADC.

1.2 Chemical modification of proteins

The human genome consists of ca. 25,000 genes, however owing to alternative splicing and post-translational modifications the human proteome is much more complex, giving rise to over 1 million proteins. Protein modification is widespread throughout nature and imparts immense biological diversity on the structure and function of proteins. Over the past few decades, considerable efforts have been made
by chemical biologists to explore the functions associated with these encoded proteins in greater detail. One of the ways to achieve this is through protein modification. By chemically modifying highly specific biomolecules, targeted delivery of therapeutic, diagnostic and imaging agents is now possible within biological systems of interest.

There are many applications of chemically modified proteins including in vivo tracking of fluorescently labelled protein conjugates, polyethylene glycol (PEG)ylation of proteins to extend protein half-life, and treatment of life-threatening diseases such as cancer, malaria and HIV.

Recent advances in the use of chemistry-based synthetic tools to modify proteins in the laboratory have overcome many major obstacles, the most significant of which is the need for high selectivity. It is now possible to gain good control over the precise position of modification on the protein when compared with classical methodologies that typically exhibit poor chemoselectivity. In order to achieve site-selective protein modification, reactions will have to selectively react with only one amino acid type by discriminating against all other amino acid side chains on the protein surface – and in some cases selectively reacting only one amino acid amongst many other of the amino acid residues of the same type by relying on specific local protein microenvironment effects (i.e. regioselectivity). Furthermore, these reactions must be carried out in biologically ambient conditions; aqueous media, at near physiological pH and at moderate temperatures to prevent disruption of protein function and architecture. Despite these challenges, various approaches have been developed over the years for the modification of proteins which can generally be categorised as either: (i) modification of natural amino acids (NAAs) or (ii) modification of unnatural amino acids (UAAs); each has its own merits and disadvantages. As it is of most relevance to this thesis, the former will be described in detail herein whereas information on the latter can be found in reviews by Spicer et al., Mastrobattista et al. and Bernardes et al.

1.3 Modification of natural amino acids

The modification of natural amino acids is scalable, low cost and less challenging in comparison to unnatural amino acid modification as the conditions for modification do not need to be adapted for each individual target protein. As a result, there is much interest in the development of technologies which focus on the selective modification of one of the 20 naturally occurring amino acids. The most nucleophilic residues
have been explored in this context and reagents have been developed to selectively modify cysteine, serine, lysine, histidine, tyrosine, aspartate and methionine (Figure 2).

![Figure 2 Amino acid side chains commonly targeted for chemical modification. Left to right: cysteine, serine, lysine, histidine, tyrosine, aspartate and methionine.](image)

However, the side chains of cysteine and lysine are the most nucleophilic in character,\textsuperscript{25} owing to the presence of a nucleophilic thiol and amine, respectively. As such, most of the work on the site-selective modification of natural amino acids has focused on reactions with these two amino acids; this will be discussed in more detail herein.

**1.3.1 Lysine modification**

Lysine functionalisation is a common choice for protein modification; it is highly nucleophilic (due to the primary alkyl amine side chain), high in natural abundance (for example \textit{ca.} 90 lysine residues per antibody) and has a well-defined reactivity profile.\textsuperscript{26} Of the chemically modified biologicals that have obtained US Food and Drug Administration (FDA) approval to date, most have been prepared \textit{via} the covalent modification of lysine residues.\textsuperscript{27} Due to the popularity of lysine modification over the past couple of decades,\textsuperscript{28} a large number of reagents have been developed to target this nucleophilic amine on the surface of proteins (Scheme 1). Among the most popular of these reagents are \textit{N}-hydroxy-succinimidy (NHS) esters\textsuperscript{27} and isothiocyanates (Scheme 1),\textsuperscript{29} although there are many more small molecules that have been employed in the manufacture of lysine conjugates.\textsuperscript{27,29–32}

However, when homogeneity of the bioconjugate is required, lysine residues are a suboptimal target for conjugation. Firstly, lysine is not always the most reactive centre on a protein; therefore, in the presence of amino acids with higher nucleophilicity (\textit{e.g.} cysteine), unexpected modification can occur at these residues.\textsuperscript{33} Furthermore, owing to a combination of the high natural abundance of lysines and the fact that these residues are often located on the solvent accessible surface of proteins, targeting of this amino acid results in non-site-selective modification when targeting typical modification
levels on a protein (e.g. 2-4 modifications on average per protein); modification of many sites results in poor pharmacokinetics (e.g. fast clearance). Thus, modification of lysine gives rise to a heterogeneous mixture of protein conjugates. This makes lysine modification sub-optimal for some applications. This is problematic in the development of therapeutics as the heterogeneity has a profound effect on the pharmacokinetics of the conjugates formed by modification of lysine, resulting in a decrease in treatment efficacy.
Scheme 1 Main reactions which are used for the modification of lysine.

As mentioned above, typically, one only wants to modify a few lysine residues as the modification of many can result in a decrease in solubility and issues of fast clearance owing to an increase in hydrophobicity of the conjugate.\textsuperscript{36,37} This loss in solubility is primarily due to the conversion of the charged ammonium ions to neutral moieties, resulting in a net loss of surface charges.\textsuperscript{36} Furthermore, it is not uncommon for lysines
to exist in key functional areas of proteins, such as the antigen binding site of antibodies, and modification of such lysine residues may have detrimental effects on the binding activity of the protein.\textsuperscript{38}

When modifying lysine residues, it is necessary to have very stringent control of pH and other reaction conditions to slow the rate of reaction to ensure that the modification of only the most reactive lysine takes place.\textsuperscript{39,40} Moreover, the presence of the more nucleophilic cysteine residues on the protein of interest may lead to unexpected modification at these residues.\textsuperscript{19} It is in light of these factors that cysteine makes the most promising candidate for NAA modification where a natural cysteine is available.

### 1.3.2 Modification of single cysteine

Cysteine makes an excellent target for the modification of NAAs; the thiol side chain of cysteine has the highest nucleophilicity in comparison to all other proteinogenic groups at physiological conditions.\textsuperscript{33} The high reactivity of this thiol moiety allows for rapid reactions with a variety of conjugation agents in a chemoselective manner. Whilst the total abundance of cysteine residues in a protein is 1.7\%, free cysteines occur at only 0.2\% abundance.\textsuperscript{25} The majority of cysteine residues are tied up in disulfide bonds, which are a critical part of the tertiary structure of a protein.\textsuperscript{41} This extremely low abundance of free cysteine residues in native proteins has led to the development of highly site-selective modification strategies to target these residues at a solvent accessible surface, as only a few exist to be modified.

The extreme rarity of cysteine residues on the protein surface may give rise to complications. The presence of a free solvent accessible cysteine on proteins is not common, as these cysteines often exist in their oxidised form. In the event that the amino acid sequence of a native protein does not encode a free solvent-accessible cysteine, site-directed mutagenesis is used to incorporate a single reactive cysteine at a solvent accessible site on the protein structure.\textsuperscript{42} This method offers excellent site-selectivity, as the new cysteine is the only modifiable target on the protein surface.

Whilst modification of the disulfide bonds can impact protein function, the high thiol reactivity and the higher abundance of cysteine residues liberated from reduced disulfide bonds offer promising targets for site-selective modification of native proteins. Functionalisation of a disulfide linkage may be achieved by insertion of a functional
molecule between the two cysteines.\textsuperscript{35} Thus, formation of a stable functionalised bridge between these residues enables the structure (and function) of the protein to remain intact, whilst also providing a platform for modification.

There are a number of different small molecules that have been used for the modification of single cysteine residues, as illustrated in Scheme 2. The main modes of reaction for cysteine modification are oxidation, disulfide formation and alkylation (by S\textsubscript{N}2 displacement or Michael Addition, Scheme 2),\textsuperscript{25,43–46} and the choice of reagent is underpinned by the specific application. Michael addition is a particularly attractive option for cysteine selective bioconjugation. Michael acceptors are considered soft electrophiles, and thus promote reactions with soft nucleophiles (e.g. thiols). Combined with the high nucleophilicity of the thiol on cysteine, this soft-soft interaction allows for exquisite selectivity of Michael acceptors towards cysteine residues (over other nucleophilic residues e.g. lysine).\textsuperscript{47} Maleimides and bromomaleimides have been widely

\textbf{Scheme 2} Main reactions for the modification of cysteine.
employed as Michael acceptors for the site-selective modification of cysteine.\textsuperscript{25} Maleimides can react rapidly and selectively with cysteine thiols to form succinimide adducts, and this has led to the commercial availability of a number of chemical probes linked to maleimides. Bromomaleimides react with cysteines and enable cysteine modification that is reversible under certain conditions.\textsuperscript{48} More recently, Chudasama \textit{et al.} have developed a novel class of reagents called pyridazinediones (PDs),\textsuperscript{49} which provide excellent selectivity for cysteine modification over lysine and afford conjugates that are stable over a wide range of pH (see Section 1.3.3.2). Further comments and details on these modification strategies are discussed below.

1.3.2.1 Maleimides and Bromomaleimides

A class of reagents most widely employed for the modification of cysteine residues are maleimides and many functional maleimides are available commercially. Maleimides, being good Michael acceptors, undergo a rapid and selective reaction with cysteine thiols to form succinimide adducts. However, this thiol-succinimide linkage is known to be unstable as this moiety can undergo retro-Michael degradation.\textsuperscript{50} Efforts have been made to use the hydrolysis of cysteine-succinimide conjugates to afford thiol stability (Scheme 3).\textsuperscript{51,52} Thus aiming to prevent the undesirable exchange between cysteine-succinimide conjugates and thiols on proteins present in the blood, \textit{e.g.} serum albumin. Although stable to thiols, the hydrolysed cysteine-maleimide conjugates are not obtained in high yields as the conditions for hydrolysis also promote retro-Michael deconjugation.

As an alternative solution, Smith \textit{et al.} sought to incorporate a bromine on the maleimide scaffold, to successfully form bromomaleimides, that react as rapidly as classical maleimides.\textsuperscript{48} However, unlike classical maleimides, upon reaction with the thiol they retain the double bond forming an unsaturated cysteine-maleimide conjugate (Scheme 3b).\textsuperscript{48} During hydrolysis, this unsaturated moiety mechanistically avoids the possibility of retro-Michael deconjugation, resulting in a quantitative yield of hydrolysed conjugates.
1.3.3 Disulfide modification

Disulfide bonds often play a critical role in the maintenance of protein stability\textsuperscript{53} and biological activity\textsuperscript{54} by providing structural integrity and conformational stability to the protein’s tertiary structure. They may be either buried within the protein folding region (\textit{i.e.} inaccessible for modification without denaturation) where they may contribute to the activity of the protein, or alternatively be situated on the solvent accessible surface where they are often involved in protein stability. As most proteins, including many proteins of therapeutic relevance, possess at least one solvent accessible disulfide bridge, these linkages present promising targets for site-selective protein modification.\textsuperscript{55} However, due to the role of these linkages in protein stability, loss of these interchain bridges as a consequence of disulfide reduction may destabilise many proteins. This is particularly the case when disulfides are the only covalent attachment between protein chains. It is therefore desirable to covalently re-link the liberated thiols upon functionalisation, and this can be achieved by a technique known as functional disulfide rebridging.\textsuperscript{56}

Functional disulfide rebridging can be achieved through the reduction of disulfide bonds, followed by incorporation of a linker to covalently reconnect the free thiols.\textsuperscript{57} By reforming a stable covalent bridge between the two cysteines, the conformational and structural stability of the protein is restored. Additionally, this bridging moiety may also carry either a functional group of interest, such as a drug or a fluorophore, or a chemical handle which may allow further protein functionalisation. However, in the instance where multiple accessible disulfides reside on the surface of the protein (such as antibodies), disulfide scrambling may occur.\textsuperscript{57,58}
Shaunak et al. were the first to report successful protein modification via native disulfide bonds, whilst retaining the biological activity and tertiary structure of the protein. They reported the synthesis of a thiol-specific enone-sulfonyl reagent. This reagent reacts with one of the two free thiols, liberated from disulfide reduction, via a conjugate addition and elimination step, followed by a second conjugate addition reaction with the second free thiol, thereby re-connecting the sulfur atoms of the cysteines via a covalent three-carbon bridge (Scheme 4).

**Scheme 4 Insertion of a three-carbon bridge into a disulfide bond.**

### 1.3.3.1 Dibromomaleimides

Recently, the Baker and Caddick groups have also demonstrated the use of dibromomaleimides as functional disulfide rebridging agents on a variety of disulfide-containing proteins. The utility of dibromomaleimides, formed by incorporation of two bromine groups on the maleimide double bond, was first demonstrated by Smith et al. by successful incorporation of this scaffold into the solvent accessible disulfide linkage of the peptide hormone somatostatin (Scheme 5). A step-wise protocol was employed where initially a mild reducing agent was used to reduce the disulfide linkage between the cysteine residues located at position 3 and 14. The subsequent addition of dibromomaleimide enabled two addition-elimination sequences to occur on reaction with the two liberated thiols from the disulfide bond, to generate a
bisthioether-maleimide bridge (Scheme 5). However, similar to bromomaleimides, a hydrolysis step is required to achieve serum stability.

**Scheme 5** Bridging of a reduced disulfide bond with dibromomaleimide.

Whilst dibromomaleimides are powerful tools in protein modification, there were still some shortcomings, which needed to be addressed. These include cross-reactivity with common reducing agents, and *in vivo* instability without hydrolysis. Additionally, whilst these reagents have been shown to work well with simple single-disulfide proteins and peptides, their use with more complex multi-disulfide systems such as antibodies frequently leads to non-native rebridging and heterogeneous mixtures. This commonly gives rise to a by-product which is referred to as ‘half-antibody’ which is formed as a consequence of antibody hinge region disulfides adopting the intra-chain conformation when rebridged (Scheme 6).

**Scheme 6** Formation of non-natively rebridged antibody.

### 1.3.3.2 Dibromopyridazinediones (PDs)

The pyridazinedione (PD) scaffold has several desirable attributes that make it a good platform for cysteine and disulfide modification. As PDs do not require a hydrolysis step to confer serum stability and have been shown to be resistant to hydrolysis at a range of pH values, they can be applied in a wider range of modification conditions.
Furthermore, in addition to having two thiol-reactive centres, PDs consist of two $N$-directed functional handles, which can be functionalised through ‘click’ reactions (Figure 3). ‘Click chemistry’ can be defined as a set of reactions that are high-yielding, regioselective, have simple reaction conditions and generate only inoffensive by-products, and are compatible with a narrow range of biorthogonal conditions.61 ‘Click chemistry’ has revolutionised the field of bioconjugation as it provides facile reaction conditions which are amenable to both biomolecules and small molecules such as fluorophores, toxins or therapeutics.61

Figure 3 PDs depicted with a range of orthogonal handles, of which two may exist on a PD at one time. A range of functional payloads has also been attached to PDs and proteins.
The ‘dual click’ strategy introduced by Maruani et al. has made it possible to achieve dual modification of proteins in a site-selective manner. Through the incorporation of a terminal alkyne handle on one nitrogen and a cyclooctyne strained alkyne on the other, two distinct moieties can be conjugated via sequential copper–free strained alkyne promoted azide–alkyne cycloaddition (SPAAC) ‘click’ on the strained alkyne and a copper promoted azide-alkyne cycloaddition (CuAAC) ‘click’ on the non-strained alkyne (Figure 4). The ‘clickable’ handles can be reacted with various reactive moieties, such as an azide-bearing fluorophore or cytotoxic payload, to generate site-selectively, dually modified antibody-PD conjugates. Furthermore, PDs are stable to common thiol reducing agents such as TCEP, which enables the use of in situ protocols and to some extent obviates issues of non-native rebridging. This will be discussed in more detail below in Section 2.3.

![Chemical structure of clickable handle](image)

**Figure 4** The dual click strategy that involves the use of strained alkyne and terminal alkynes for SPAAC and CuAAC respectively.

### 1.4 Antibodies and antibody fragments

#### 1.4.1 Antibodies

Monoclonal antibodies (mAbs) first entered the clinic about 25 years ago and have since brought about a therapeutic revolution due to their ability to target specific molecular components. A large number of mAbs have already been approved for the treatment of cancer, immune disorders and many more conditions. Currently, over 80 monoclonal antibodies have received regulatory approval for use in the US and Europe.

Antibodies are Y-shaped proteins of ca. 150 kDa (Figure 5), which are produced by highly specialised B lymphocyte cells in the blood upon activation of the immune system. Antibodies exhibit high selectivity for their target antigens, enabling them to recognise and bind to a large number of molecules. Within the B cells, antibodies undergo a variety of specialised processes that are responsible for the sequence variability that ultimately
defines their specificity to their target. Upon binding to their target antigens, antibodies have the potential to neutralise the antigen, or act as a flag for eliciting an immune response.

Due to their remarkable specificity, antibodies offer the prospect for use in a wide range of technologies. In particular, antibodies have received considerable attention as targeted therapeutics in recent years, via antibody-dependent cell-mediated cytotoxicity (ADCC), and as vehicles for drug delivery and imaging. Humanised monoclonal antibodies have already displayed impressive results when employed as therapeutics, particularly when applied to targeted cancer therapy. Compared with conventional chemotherapy approaches that lack the ability to discriminate between different cell types, this strategy results in far fewer side effects arising from ‘off-target toxicity’.

Antibodies are subdivided into different classes, also known as isotypes, which depend upon the arrangement of the heavy chain constant domains. In humans, there are five antibody classes (IgM (μ), IgD (δ), IgA (α), IgG (γ), IgE (ε)), each of which has a different role in the immune system. While IgM and IgD are early response antibodies, the IgG isotype is involved in late responses and is the major blood plasma antibody. These classes can be further subdivided into Ig allotypes, or subclasses. For example, humans have four different types of IgG (IgG1, IgG2, IgG3 and IgG4) and two different IgA (IgA1 and IgA2) with over 95% sequence similarity. Additionally, in humans there are two loci in the genome for the light chain, named kappa and lambda. The majority of therapeutics approved for clinical use are IgG based; this is unsurprising considering IgG antibodies are responsible for majority of antibody-based immunity in mammals.

In order to synthesise an effective antibody-based pharmaceutical, it is important to understand how antibodies achieve their selectivity and specificity. This becomes evident when observing the antibody’s tertiary structure in closer detail (Figure 5). IgG antibodies (as well as many other antibody classes) are composed of two identical heavy (H) chains and two identical light (L) chains, that are held together by covalent disulfide bonds. The light chains contain one variable (V_L) domain and one constant (C_L) domain, whereas the heavy chains consist of a single variable (V_H) domain and either three or four constant (C_H) domains, depending on the class of the antibody. Antibodies are incredibly diverse and have the potential to bind an infinite number of molecules. Despite their ability to bind a range of compounds, antibody variability is limited to specific regions with the
antibody variable domain, known as complementarity-determining regions (CDRs). The non-variable region of the antibody, that also includes the Fc domain remains constant amongst many humanised antibodies.

1.4.2 Antibody fragments

Through the use of prokaryotic expression systems and enzymatic digestion of full antibodies it is possible to isolate many clinically relevant antibody fragments, each with their own physicochemical properties (Figure 5).

- **Fc fragment** (fragment crystallisable). These fragments (ca. 50 kDa) comprise the C_{H2} and C_{H3} domains and part of the hinge region held together by disulfide bonds and other non-covalent interactions. The Fc fragments (typically for IgG1 and IgG2) participate in the effector function of antibodies by binding monovalently to cell surface receptors and complement proteins. The Fc region is glycosylated from the conserved Asn297 residues on the heavy chain.

- **Fv fragment** (fragment variable). This is the smallest monovalent fragment (ca. 25 kDa) produced from an immunoglobulin molecule that contains the complete antigen-binding site and therefore retains the antigen-binding specificity of a full-length antibody. It is composed of the variable regions (V_{L} and V_{H}) and contains regions of hypervariability known as the variable domain CDRs. These

\textit{Figure 5 General structure of an IgG1 antibody (the most abundant IgG subclass in human serum), with domains and fragments highlighted.}
are composed of 6 hypervariable loops of β strands that are responsible for antigen binding.\textsuperscript{73}

- **Fab fragment (fragment antigen-binding).** Fab fragments (\textit{ca.} 50 kDa) are monovalent fragments composed of the entire light chain (\(V_L\) and \(C_L\)) and part of the heavy chain (\(V_H\) and \(C_{H1}\)). They retain the binding ability to an antigen.

- **F(\(ab'\))\(_2\) fragments (\textit{ca.} 110 kDa)** contain two antibody binding fragments that are held together at the hinge \textit{via} disulfide bonds. These fragments lack most, but not all of the Fc region.

- **Fab' (\textit{ca.} 55 kDa)** fragments are formed by the reduction of F(\(ab'\))\(_2\) fragments and contain a sulfhydryl group that can be of use. Due to derivation from F(\(ab'\))\(_2\), these fragments may also contain some of the Fc portion of the antibody.

Regulated antibody digestion can be exploited to generate fragments that possess altered physicochemical features. For instance, due to their smaller size, Fab fragments obtained from enzymatic digestion of their full length counterpart (see Section 6.1) are able to penetrate tissues that are usually inaccessible to full-size antibodies.\textsuperscript{76,77} In addition to enzymatic digestion, antibody fragments can also be manufactured using expression systems. For commercial scale-up, the process of manufacturing antibody fragments is facilitated as their relatively small size and absence of glycosylation permits the use of prokaryotic expression systems, making them less costly to produce than their full-length counterparts.\textsuperscript{78}

However, these Fab fragments are not as stable as whole antibodies in human serum and have shorter circulation half-lives. This is due to the absence of the Fc domain that is involved in both the stabilization and also FcR-mediated recycling of antibodies. Half-life extension of the fragments can be achieved by several strategies, such as conjugation to albumin\textsuperscript{79,80} and PEGylation,\textsuperscript{38,81} the latter of which is seen in the FDA approved anti-TNF\(\alpha\) Fab, certolizumab pegol.\textsuperscript{82,83}

**1.5 Antibody–drug conjugates (ADCs)**

Whilst the use of monoclonal antibodies to selectively target cancers has shown impressive results, as exemplified by the success met by Rituximab\textsuperscript{TM} and Herceptin\textsuperscript{TM} amongst many others,\textsuperscript{68,84} mAb-based treatments often face several limitations to their therapeutic and diagnostic use.\textsuperscript{85} For instance, failure to deliver efficacious doses
throughout the tumour as a consequence of poor tumour penetration and heterogeneous antibody distribution are prominent drawbacks that hamper with the clinical efficacy of therapeutic antibodies, hence limiting their widespread use as therapeutics for cancer.\textsuperscript{85} Consequently, research has turned towards the development of a different type of targeted therapeutic; antibody–drug conjugates (ADCs).\textsuperscript{86} ADCs consist of a monoclonal antibody covalently attached to a potent cytotoxic agent \textit{via} linker and conjugation technologies (Figure 6).\textsuperscript{86} This technology combines the exquisite binding selectivity of antibodies and the potent toxicity of a cytotoxic drug (also referred to as the payload or warhead), whose killing potential is distinct from ADCC.

Antigen density plays an important role in determining therapeutic index of the ADC since efficient ADC targeting and delivery would be hindered by the presence of only a small number of antigenic sites.\textsuperscript{8} Therefore, the target antigen should have high and homogeneous expression on tumour cells, with low expression in healthy tissues. The antibody itself must be able to bind to tumour-associated antigens with high specificity and high affinity. Additionally, the antibody should be non-immunogenic, and this immunogenicity can be minimized with the use of chimeric and fully-humanized monoclonal antibodies.\textsuperscript{8} Hence, ADCs enable direct delivery of a toxic payload to the target cells and minimise off-target toxicity by limiting exposure of normal healthy tissues to the warhead. This in turn allows the use of drugs that would otherwise be too toxic for employment in conventional chemotherapy.\textsuperscript{42} Indeed, one of the key requirements of the effector portion of the ADC is to have cytotoxic potency in the subnanomolar range (Figure 6).\textsuperscript{87} The concept of selective delivery is by no means new and was first postulated by German scientist Paul Ehrlich in 1913.\textsuperscript{88} He conceived the magic bullet theory referring to a chemical that could be selectively delivered to the target.\textsuperscript{88} Thus ADCs satisfy the demand for weapons that would effectively target cancer cells with high specificity and precision.
1.5.1 Current Antibody–Drug Conjugates

To date, nine ADCs have received regulatory approval by the US Food and Drug Administration (FDA) (Figure 7, Table 1). Mylotarg™ (gemtuzumab ozogamicin) was the first ADC to gain regulatory approval from the FDA in 2000 for treatment of patients with relapsed CD33-positive acute myeloid leukaemia (AML).\(^{89}\) It is composed of a CD33-targeted mAb covalently attached to the DNA-alkylating agent calicheamicin via an acid-labile linker. Despite promising preliminary results, Mylotarg™ was voluntarily withdrawn from the market in 2010, as post-approval clinical trials for patients with AML did not demonstrate improved survival over standard chemotherapy and were also associated with safety concerns.\(^{90}\) Mylotarg™ was granted re-approval from the FDA in 2017 after careful reviewing of the dosing regimen.\(^{91}\)
Figure 7 Site-selective modification of antibodies. (Left) Kadcyla™, Besponsa™, and Mylotarg™ developed by the modification of lysine residues. (Right) Adcetris™, Polivy™, Padcev™, Enhertu™, Trodelvy™, and Blenrep™ developed by the modification of cysteine residues.

The second ADC to reach the market was brentuximab vedotin™ (Adcetris™) an ADC composed of a chimeric anti-CD30 mAb covalently linked to the antimitotic drug monomethyl auristatin E (MMAE) by a cleavable peptide linker. Adcetris™ received approval in 2011 for the treatment of CD30-positive Hodgkin’s lymphoma. This was followed in 2013 by approval of trastuzumab emtansine™ (Kadcyla™) for the targeted treatment of breast cancer. This is an ADC consisting of a humanised anti-HER2 mAb conjugated to the microtubule inhibitor DM-1 via a non-reducible thioether linker. In 2017, inotuzumab ozogamicin (Besponsa™)™ received approval for use against acute lymphoblastic leukaemia. It targets CD22 and employs the same linker-payload combination as Mylotarg™. Three further ADCs received approval in 2019. These include polatuzumab vedotin (Polivy™), enfortumab vedotin (Padcev™), and trastuzumab deruxtecan (Enhertu™). Polivy™ and Padcev™ comprise a humanised anti-CD79-b antibody and fully human anti-Nectin-4 antibody respectively, covalently linked to MMAE via a protease cleavable linker. Enhertu™ on the other hand consists of an anti-HER2 antibody linked to a novel topoisomerase I inhibitor payload via an enzyme cleavable tetrapeptide-based linker. Polivy™ received approval for the treatment of large B-cell lymphoma, the most common form of non-Hodgkin’s lymphoma whereas Padcev™ and Enhertu™ were approved for the treatment of bladder and breast cancer respectively. Most recently in 2020, two further ADCs have received FDA approval; these include sacituzumab goxitecan (Trodelvy™) for the treatment of triple-negative
breast cancer\textsuperscript{99} and belantamab befodotin (Blenrep\textsuperscript{TM}) for the treatment of relapsed or refractory multiple myeloma.\textsuperscript{100} Trodelvy\textsuperscript{TM} is composed of a humanised monoclonal Trop-2 antibody linked to SN-38, an active metabolite irinotecan via an acid-cleavable linker, whereas Blenrep\textsuperscript{TM} comprises an afucosylated humanised anti-B cell maturation antigen (BCMA) IgG1 antibody that is linked to the anti-mitotic MMAF via a non-cleavable maleimidocaproyl linker. In recent years, the field of ADCs has become more dynamic and it is estimated that there are currently over 60 ADCs undergoing clinical trials\textsuperscript{101} and the market for ADCs has been predicted to grow rapidly in coming years.\textsuperscript{102}

<table>
<thead>
<tr>
<th>Name</th>
<th>Date approved</th>
<th>Indication</th>
<th>Target</th>
<th>Antibody sub-type</th>
<th>Payload</th>
<th>Linker type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adcetris\textsuperscript{TM}</td>
<td>2011</td>
<td>Hodgkin’s lymphoma</td>
<td>CD30</td>
<td>IgG1</td>
<td>MMAE</td>
<td>Protease-cleavable (Val-Cit-PABC)</td>
</tr>
<tr>
<td>Kadcyla\textsuperscript{TM}</td>
<td>2013</td>
<td>Breast cancer</td>
<td>HER2</td>
<td>IgG1</td>
<td>DM1</td>
<td>Non-cleavable</td>
</tr>
<tr>
<td>Mylotarg\textsuperscript{TM}</td>
<td>2010, 2017</td>
<td>Acute myeloid leukaemia (AML)</td>
<td>CD33</td>
<td>IgG4</td>
<td>Calichaemicin</td>
<td>Acid-cleavable (Hydrazone)</td>
</tr>
<tr>
<td>Besponsa\textsuperscript{TM}</td>
<td>2016</td>
<td>Acute lymphoblastic leukaemia (ALL)</td>
<td>CD22</td>
<td>IgG4</td>
<td>Calichaemicin</td>
<td>Acid-cleavable (Hydrazone)</td>
</tr>
<tr>
<td>Polivy\textsuperscript{TM}</td>
<td>2019</td>
<td>Diffuse large B-cell lymphoma (DLBCL)</td>
<td>CD79b</td>
<td>IgG1</td>
<td>MMAE</td>
<td>Protease-cleavable (Val-Cit-PABC)</td>
</tr>
<tr>
<td>Padcev\textsuperscript{TM}</td>
<td>2019</td>
<td>Bladder cancer</td>
<td>Nectin-4</td>
<td>IgG1</td>
<td>MMAE</td>
<td>Protease-cleavable (Val-Cit-PABC)</td>
</tr>
<tr>
<td>Enhertu\textsuperscript{TM}</td>
<td>2019</td>
<td>Breast cancer</td>
<td>HER2</td>
<td>IgG1</td>
<td>Topoisomerase I inhibitor (Dxd)</td>
<td>Protease-cleavable (Gly-Gly-Phe-Gly)</td>
</tr>
<tr>
<td>Trodelvy\textsuperscript{TM}</td>
<td>2020</td>
<td>Triple-negative breast cancer</td>
<td>Trop-2</td>
<td>IgG1</td>
<td>SN-38</td>
<td>Acid-cleavable (Carbonate)</td>
</tr>
<tr>
<td>Blenrep\textsuperscript{TM}</td>
<td>2020</td>
<td>Multiple myeloma</td>
<td>BCMA</td>
<td>IgG1</td>
<td>MMAF</td>
<td>Non-cleavable</td>
</tr>
</tbody>
</table>

\textit{Table 1} FDA-approved ADCs, their approval dates, indications, targets, payloads and linkers.

By studying the FDA-approved ADCs, it is possible to associate positive and negative traits with the conjugation methods and linker technologies used for each of their development.

1.5.2 ADC mechanism of action and choice of payload

Therapeutic antibodies are commonly directed towards antigens that are expressed on the surface of tumour cells or other components of the supporting stroma. Ideally, these antigens have high expression on tumour cells or cells within the tumour environment, but much less so on healthy cells (Figure 8).
Figure 8 a) Traditional mechanism of ADC internalisation. This pathway for ADC processing involves binding of the ADC to the target antigen on the cell surface, leading to invagination and internalisation of conjugate into the early endosome. A mild drop in pH results in antibody–drug detachment from the antigen. The late endosome’s subsequent fusion with the lysosome leads to degradation of the conjugate and release of the free cytotoxic drug into the cell. The free drug can cause cell death, by a mechanism which is dependent on the type of drug used. The free drug can also diffuse out of the cell and enter the neighbouring ‘bystander’ cells, causing cell death. b) Alternatively, another route for ADC processing is independent of internalisation and involves cleavage of the ADC linker by extracellular proteases (such as cathepsin B), which are released by surrounding tumour cells in the tumour microenvironment. The free diffusible drug is released from the ADC and can then diffuse into surrounding ‘bystander’ cells causing cell death.
Effective drug design requires an understanding of the mechanisms applied to ADCs. For example, when the target antigen is abundantly and homogenously expressed on the tumour cell surface, the traditional mechanism of action is initiated by binding of the antibody to the antigen (Figure 8), which is considered crucial for cytotoxic payload release from the ADC. This mechanism involves antibody-antigen binding followed by internalisation into the cell via endocytosis and trafficking and processing along a decreasing pH gradient via the endo-lysosomal pathway. The actual site of active payload release is largely dependent upon the type of linker present.

Cell death occurs when the released payloads (generally anti-mitotic agents) disrupt DNA synthesis (e.g. calichaemicins) or microtubules (e.g. maytansinoids and auristatins) or exert RNA polymerase or topoisomerase inhibition. Cytotoxic payloads that are highly potent and selective towards the target cancer cells but exhibit low off-target cytotoxicity have mainly been used as payloads in the FDA-approved ADCs and those in clinical trials. In addition to calichaemicins (used in Mylotarg™, Besponsa™), auristatins (used in Adcetris™, Padcev™, Polivy™ and Blenrep™), maytansinoids (used in Kadcyla™) and topoisomerase I inhibitors (used in Enhertu™ and Trodelvy™) new classes of highly cytotoxic agents are also being explored for use as payloads in ADCs.

Although the majority of ADCs adopt the traditional mechanism of action (internalisation), another mode of action is also possible which does not require internalisation. In this case, cleavage of the linker and release of the cargo takes place in the extracellular tumour microenvironment (Figure 8). Thus, this negates the requirement for endocytosis and allows selection of non-internalising antigens as potential targets for ADCs. This mechanism has several advantages over the traditional endocytosis-dependent mechanism in that it provides access to a much wider range of possible target antigens, and is not dependent on high antigen expression on the cell surface and allows avoidance of the potentially inefficient cell trafficking process. Release of free drug in the tumour microenvironment may however lead to off-target toxicity and lower cell selectivity.

### 1.5.3 Linker-payload attachment

The linker-payload attachment is crucial for ADC design, as this linkage defines the nature of the active payload species and the timing of its release. The safety and efficacy
of the ADC is highly dependent upon the stability of the linker in circulation, as it must ensure that the payload is released only at the target site.

In cases where the ADC internalises upon binding, the ADC design may benefit from a non-cleavable linker which will release the drug upon proteolytic degradation of the antibody, not the linker, in the lysosome. Alternatively, ADCs may employ a cleavable linker, which can undergo cleavage by specific proteases or at a defined pH range to liberate the free drug from the ADC (Figure 8). Although the released free drug via both mechanisms can directly cause death of the target cell, it also has the potential to diffuse out of the cell to cause bystander killing, depending on the type of drug used. Bystander killing takes place when the drug is taken up by neighbouring cells, also known as bystander cells, resulting in cell death. These cells may not necessarily express the target antigen.

1.5.3.1 Non-cleavable linkers

Non-cleavable linkers do not contain a chemical trigger to cleave the linkage between the antibody and payload for release of the cytotoxic payload. Upon internalisation into the cell and trafficking to the lysosome, the proteolytic machinery in the lysosome metabolises the antibody into its constituent amino acids. The released cytotoxic species therefore consists of the cytotoxic payload, the linker and a charged amino acid appendage. Hence the success of this approach is highly dependent upon the pharmacology of the drug being unaffected by this modification. There are two commonly employed non-cleavable linkers: succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and maleimidocaproyl (mc), which are used as linkers for DM1 (maytansinoid) and monomethyl auristatin F (MMAF), respectively (Scheme 7). The FDA approved ADC Kadcyla™ contains an SMCC-DM1 linker-payload combination, whereas Blenrep™ is an example of an mc-MMAF containing ADC.
Non-cleavable linkers: these linkers do not require the presence of a chemical trigger to release the payload. Upon internalisation, the proteolytic machinery within the lysosome degrades the antibody into its constituent amino acids. The scheme represents release of the active payloads Cys-mc-MMAF and Lys-SMCC-DM1.

One of the primary benefits of the use of non-cleavable linkers over cleavable linkers is the mitigation of instability in circulation, as these linkers by design do not contain a point of potential instability and demonstrate their anti-tumour activity only upon cell internalisation and lysosomal degradation. However, non-cleavable linkers generally do not cause bystander effect due to the lack of cell permeability of the amino acid-appendaged linker-payload, causing cell kill only of tumour cells that have internalised the ADC. Such linkers are therefore mainly effective for the treatment of tumours with high and homogeneous expression of the antigen. Furthermore, this drastic modification to the payload is likely to adversely impact its cytotoxicity. For these reasons, non-cleavable linkers are less widely applicable than cleavable linkers.

1.5.3.2 Cleavable linkers
Cleavable linkers exploit specific conditions to release the payload at the target cell. These linkers can be categorised as chemically labile (disulfide-based and pH dependent) and enzyme labile (peptide-based) linkers (Scheme 8). Both modalities have been
extensively utilised in the development of many of the ADCs currently undergoing clinical trials as well as those that are currently on the market.\(^{113}\) It is commonly thought that cleavage of these linkers takes place only after selective binding and internalisation by the target cell. However, more recently it has emerged that ADC internalisation may in fact not be crucial to cause tumour cell death by cleavable linker-bearing ADCs, as these linkers can also be cleaved in the extracellular space.\(^{109}\)

**pH dependent linkers:** These linkers are stable at the neutral pH (pH 7.4) of blood in circulation but undergo hydrolysis when the ADC is trafficked to acidic endosomes (pH 5.0 – 6.0) and/or lysosomes (pH 4.5 – 5.0).\(^{116,117}\) Although this commonly takes place within the cell, some studies have shown that pH-mediated drug release can also happen extracellularly due to the acidic nature (~pH 6) of the tumour microenvironment.\(^{109}\) The FDA-approved ADCs Mylotarg™ and Besponsa™ contain an acid-sensitive N-acyl hydrazone linkage whereas Trodelvy™ contains an acid-sensitive stable carbonate linker (Scheme 8).\(^{89,95,99}\)

**Disulfide linkers:** Disulfide-based linkers are another prominent class of cleavable linker (Scheme 8). Disulfides are stable at physiological pH but are susceptible to nucleophilic attack by thiols.\(^{113}\) This linker strategy exploits the high concentration of reducing agents such as glutathione within the cytoplasm (1-10 mM) compared to outside the cell (5 µM).\(^{118}\) A disulfide bond is embedded between the drug and linker that is able to resist cleavage in circulation, however the large difference in reductive potential between the plasma and cytoplasm allows the opportunity to selectively cleave the disulfide bond to release the free payload from the ADC within the cell. Furthermore, tumour-associated oxidative stress leads to an even higher concentration of glutathione within the cell compared to healthy tissues, providing additional selectivity towards cancer cells. Methyl groups can also be included next to the disulfide bond to improve stability of the linkers in circulation.\(^{119}\)

It has been reported that internalisation of ADCs containing disulfide-based linkers may not be essential for their anti-tumour activity. Dying tumour cells release high concentrations of reductants such as glutathione into the extracellular space, which causes tumour-selective drug release. Initial payload release and the progressive death of tumour cells leads to a self-amplification effect in which each dying cell releases increasing
amounts of glutathione within the tumour microenvironment which ultimately amplifies the drug release process via degradation of disulfide linkage.\textsuperscript{120}

Disulfide linkers have been employed in the maytansinoid-based ADC mivretuximab soravtansine\textsuperscript{121} which is currently in late-stage clinical trials for folate-receptor 1 (FOLR1)-positive ovarian cancer, as well as in the FDA-approved Mylotarg\textsuperscript{TM} and Besponsa\textsuperscript{TM} alongside acid-labile linkers.\textsuperscript{89,95}

**Enzyme labile (Cathepsin B-cleavable) linkers:** Cathepsin B is a lysosomal protease that is commonly associated with tumour progression, as over-expression and activity of this protease have been observed in a variety of cancer types.\textsuperscript{122} The carboxypeptidase activity of cathepsin B allows it to preferentially recognise valine-citruline (Val-Cit) and phenylalanine-lysine (Phe-Lys) dipeptide sequences and cleave the peptide bond on the C terminal of these sequences.\textsuperscript{113} Often a para-aminobenzyl carbamate (PABC) linkage is also introduced as a self-immolative spacer between the dipeptide linker and the payload in the ADC.\textsuperscript{113} Upon binding to tumour cell surface antigens, the ADCs can internalise into cells via endocytosis and traffic to lysosomes where they can be metabolized by lysosomal proteases (such as cathepsin B). Upon proteolysis, the PABC spacer undergoes a 1,6-elimination reaction to liberate the free drug (Scheme 8). Owing to its success, the Val-Cit-PABC-MMAE linker is now licensed by Seattle Genetics under the name ‘vedotin’, present in three of the nine FDA-approved ADCs: Adcetris\textsuperscript{TM}, Polivy\textsuperscript{TM} and Padcev\textsuperscript{TM}.\textsuperscript{92,96,97}

ADCs containing cathepsin B cleavable linkers do not rely exclusively upon direct antigen-internalisation to be cleaved. In tumour cells, cathepsin B expression is not restricted to the lysosome, as these cells have elevated membrane and secreted levels of cathepsin B, which is a feature of their invasive phenotype.\textsuperscript{122,123} In such cases, extracellular cleavage of ADCs at the tumour site allows targeted delivery. Similar to disulfide-based linkers, dipeptide-linkers may also benefit from a domino effect whereby release of cathepsin B into the extracellular space caused by the initial death of a tumour cell would trigger more linker cleavage and payload release.\textsuperscript{124}
Scheme 8 Cleavable linkers. pH-dependent N-acyl hydrazone (left) and para-aminobenzyl carbonate (right) linkers. pH-dependent linkers are cleaved in an acidic environment (i.e. within the endosome or lysosome). Disulfide-containing linker: the disulfide bond is reduced by intracellular reducing agents (e.g., glutathione) to release the payload. Cathepsin B-cleavable linker: the dipeptide linker is cleaved by cathepsin B in the lysosome releasing the free payload.

1.5.4 Conjugation technologies

The majority of payloads utilised in ADCs are highly potent, with cytotoxicity in the subnanomolar range. The number of cytotoxic molecules conjugated to each antibody, referred to as the drug-to-antibody ratio (DAR), is crucial in determining ADC potency & toxicity and therefore the distribution of DARs needs to be tightly controlled. When aiming for DARs of 2-4, which are commonly accepted drug loadings that do not impact
pharmacokinetics in a drastically negative manner, strategies used for the modification of lysine and cysteine residues offer very little regioselectivity over the modification, usually resulting in highly variable DARs.\textsuperscript{42} Although a high DAR increases potency, it also increases off-target toxicity as well as risk of aggregation and rate of drug clearance, thus reducing circulatory half-life of the ADC.\textsuperscript{125} The strategy that was adopted in the development of Kadcyla\textsuperscript{TM}, Mylotarg\textsuperscript{TM} and Besponsa\textsuperscript{TM} involved the modification of the free lysine residues on the antibody’s surface\textsuperscript{93,94} (Figure 7). However, this modification strategy leads to a heterogeneous mixture of products due to the high abundance of solvent accessible lysine residues (see Section 1.3.1). The distribution of ADCs formed in this manner have variable drug loadings (DAR 0, 1, 2, 3, 4, 5, 6, 7 etc) that have major pharmacokinetic limitations.\textsuperscript{126} Although many FDA-approved ADCs have been conjugated through lysine modification, the use of such non-selective approaches is now considered sub-optimal in the development of next generation ADCs.\textsuperscript{127} Adcetris\textsuperscript{TM}, Polivy\textsuperscript{TM}, Padcev\textsuperscript{TM}, Enhertu\textsuperscript{TM}, Trodelvy\textsuperscript{TM} and Blenrep\textsuperscript{TM} were developed by modification of cysteine residues (Figure 7).\textsuperscript{92,96,98,100,128} Cysteine conjugation is generally favoured due to the availability of fewer sites for modification, as only 8 cysteine residues are obtained by reducing the 4 accessible interchain disulfide bonds of an antibody. This modification strategy can give rise to a homogenous product if all the liberated thiols are reacted analogously, \textit{i.e.} giving 8 attachments per antibody. However, functionalisation of the thiols released upon disulfide reduction still presents the issue of uncontrolled conjugation and a heterogeneous product mixture is obtained when targeting typical DARs of 2-4, which is particularly problematic for therapeutic applications.\textsuperscript{129} Hence, the full potential of ADCs should be achieved by sophisticated linker technologies to connect the antibody to the cytotoxic drug.\textsuperscript{130} Linkers have a considerable impact on the performance of ADCs in terms of stability, pharmacokinetic properties and therapeutic window. The linker that connects the drug to the site of conjugation on the antibody must be stable in plasma to allow the toxic drug to remain attached to the antibody whilst circulating yet permit efficient release upon reaching the target site. Linkers with limited stability in circulation cause premature release of drugs in the bloodstream, resulting in a high toxicity profile.\textsuperscript{131} Most of the linking technologies in the
clinical pipeline present drawbacks, such as lack of selectivity, poor yields and/or loss of structural integrity, which limit their usability and reduce their therapeutic index.

1.5.5 Conjugation using pyridazinediones (PDs)

Conjugation chemistry governs the therapeutic window to some extent as site of conjugation can impact pharmacology. Dibromo PD derivatives provide a promising platform for functionally rebridging disulfides.\textsuperscript{132,133} As well as generally producing highly homogeneous bioconjugates, PDs provide long term blood plasma stability (without hydrolysis) and represent a versatile platform for disulfide functionalisation, without having a detectable effect on the antigen-binding ability of the parent antibody.\textsuperscript{62,132,134} Dibromo PDs have also shown their utility in a ‘plug and play’ approach using ‘dual click’ strategies – facilitated by the presence of two orthogonal $N$-directed handles (Figure 9).\textsuperscript{62} To date, the dibromo PD linker scaffold has been employed extensively, \textit{e.g.} in the generation of ADCs,\textsuperscript{132} antibody-directed photosensitisers,\textsuperscript{135} and a targeted nanotherapeutic.\textsuperscript{136}

\textbf{Figure 9} Thiol-selective dibrimopyridazinediones (diBrPDs) can be used to site-selectively modify antibodies. Various modalities can be attached via the $N$ atoms as represented by the star and circle cartoons.
Furthermore, Lee et al. reported that it is possible to reduce and rebridge the disulfide linkage in a single step. This was achieved by developing a 2-in-1 bridging reagent that combines a reducing agent (TCEP) with the PD scaffold (Scheme 9). As a result of this rapid reduction-rebridging process, the residency time of the thiols liberated from disulfide reduction is thought to be reduced, preventing the unfavourable formation of ‘half-antibody’ (Scheme 9). However, whilst this method yields antibody conjugates with high levels of homogeneity, and a desirable DAR of 4, wide-spread adoption of this method is limited due to the synthetic difficulty and poor long-term stability of the reagent without storing in the freezer under argon. Methods which allow for similar levels of homogeneity (low levels of mis-bridged species) and DAR without the associated synthetic difficulty and oxidation instability were sought after.

Scheme 9 The use of a 2-in-1 reducing and rebriding agent to achieve native disulfide rebridging.
Most recently, Bahou et al.\textsuperscript{138} reported a one-pot synthesis for the formation of the core PD scaffold and subsequent amine coupling reactions of this scaffold to enable chemical functionalisation of the PD.\textsuperscript{138} This procedure was utilised to generate a small library of functional PDs. The ability of these reagents to rebridge disulfide bonds was then optimised on the clinically relevant antibody trastuzumab (Herceptin\textsuperscript{TM}, breast cancer).\textsuperscript{139} It was found, after significant optimisation, that an \textit{in situ} protocol, in which the reducing agent (TCEP) and PD are added simultaneously, and performed at 4 °C for 16 h provided highly homogeneous antibody conjugates with a PD-to-antibody ratio (PAR) of 4.\textsuperscript{138}

1.6 The ADC target antigen

1.6.1 The role of angiogenesis in disease

The formation of new blood vessels \textit{via} angiogenesis is a key process during development and has been studied extensively to reveal the key signalling molecules and pathways involved.\textsuperscript{140} New vessels also undergo a process of stabilisation and maturation allowing them to perform their physiological functions effectively. Vascular dysfunction is a hallmark of many diseases and may manifest itself in a number of ways, and the uncontrolled growth of new blood vessels (neovascularisation) is chief among these. This can be seen in solid cancers, atherosclerosis, age-related macular degeneration (AMD) and rheumatoid arthritis.\textsuperscript{140–142}

It has been known for many decades that angiogenesis plays an important role in tumour progression as it not only supplies required oxygen, nutrients, and growth factors to the tumour, but also provides the circulatory access that enables tumour cells to metastasise.\textsuperscript{140} Blood vessels growing in a disease setting frequently have characteristics which distinguish them from normal vessels, they are haphazardly organised, poorly perfused, chaotic and leaky in nature (Figure 10), features that may be a consequence of inadequate association between pericytes and the endothelial cells.\textsuperscript{143} Limited perfusion starves the tumours of oxygen and nutrient supply and results in the formation of a hypoxic microenvironment that inhibits the immune response and also favours the selection of tumour cells that show a more aggressive phenotype, and are more metastatic and invasive in nature.

Furthermore, chaotic vessel growth hinders the delivery of anti-cancer drugs to solid tumours. Thus, the tortuous and haphazardly organised nature of tumour vessels is a major on-going impediment to the treatment of cancer (Figure 11).\textsuperscript{144}
Figure 10 Tumour vessels have abnormal structure and function. In healthy tissue, the vasculature functions normally, with pericytes providing mechanical stability to endothelial cells. In tumours, vessels exhibit functional abnormalities in which poor association between pericytes and the endothelium lead to reduced perfusion and diminished blood flow. In addition, the basement membrane becomes disrupted, allowing tumour cell intravasation.

Angiogenesis is governed by a variety of positive and negative regulatory molecules. When the net effects of the pro-angiogenic factors outweigh the effects of the anti-angiogenic factors, new blood vessels are formed in a process known as the ‘angiogenic switch’.

Traditional anti-angiogenesis ‘vessel growth blocking’ approaches antagonise the formation of new vessels and/or destroy pre-existing vessels in an attempt to starve the tumour of nutrients. A great deal is known about the factors responsible for driving developmental angiogenesis, and the same key factors are also involved in driving neovascularisation in cancer. Vascular endothelial growth factor (VEGF) and its signalling partners have been identified as key regulators of angiogenesis.

Consequently, targeting of key pro-angiogenic factors, such as VEGF,
has emerged as a therapeutic objective. Whilst approaches to block this pro-angiogenic signalling have yielded some success in increasing progression-free survival, they have had very little impact on overall survival rate.\textsuperscript{148}

\textbf{Figure 11} When the net effects of the pro-angiogenic factors outweigh the effects of the anti-angiogenic factors, new blood vessels are formed. Vessel normalisation restores the balance and results in improved delivery of drugs to solid tumours.

Thus, rather than the conventional approach of blocking tumour vessel growth and starving the tumour, focus has now shifted towards devising approaches that encourage the growth of functional vessels, or in other words promote ‘vessel normalisation’ (Figure 12).\textsuperscript{149} In order to achieve this objective, various strategies have been tested including the use of VEGF inhibitors administered at doses lower than those required to prevent neovascularisation. This has led to some success in the delivery of therapeutics to the tumour but the effect is short-lived with vessels reverting back to exhibiting a pathological phenotype after treatment.\textsuperscript{144} It has therefore been postulated that instead of targeting the drivers of angiogenesis, such as VEGF, alternative approaches should be developed that target factors that interfere with the normal process of vessel maturation.
Figure 12 A schematic illustration of the changes of the vessel wall and endothelium of normalised vessels. Vessels are stabilised by pericytes, normalised blood flow improves drug delivery, decreases hypoxia, and restoration of vessel architecture prevents tumour cell intravasation.

Research in the laboratories of Professor Steve Moss and Professor John Greenwood led to the identification of a novel therapeutic target, leucine-rich alpha-2 glycoprotein-1 (LRG1). Unlike VEGF, this molecule does not play a role in developmental angiogenesis and instead is induced only in a disease setting where it corrupts the normal process of angiogenesis.¹⁵⁰

1.6.2 LRG1 Mechanism of action

LRG1 is a highly conserved member of the leucine rich repeat (LRR) family of proteins¹⁵¹ that was originally identified as a secreted glycoprotein in the serum of healthy individuals by Haupt and Baudner.¹⁵² It has been reported to be present in the circulation at concentrations ranging from 10-50 μg/mL. Studies have since shown increased serum LRG1 concentrations in a number of inflammatory and neoplastic diseases, leading to the suggestion that LRG1 is a biomarker for these diseases.¹⁵³,¹⁵⁴ Transcriptomic analysis of pathological blood vessels isolated from the retinas of several mouse models of retinal disease showed that LRG1 was significantly elevated in these microvessels. The expression was restricted predominantly to the vascular system. In comparison to control mice, increased LRG1 expression was also observed in a mouse model of retinal vascular disease.¹⁵⁰ Furthermore, the investigation reported that LRG1 stimulates pathological
neovascularisation by modulating the activity of the transforming growth factor β (TGFβ) signalling pathway.\textsuperscript{150}

The TGFβ pathway can switch between being predominantly angiostatic to one that promotes angiogenesis.\textsuperscript{155,156} The primary determinant of the angiogenic switch is the balance between the pro-angiogenic ALK1 and anti-angiogenic ALK5 signalling pathways, with the accessory receptor endoglin (ENG) being a key regulator in promoting signalling via the ALK1 pathway. The angiogenic switch is activated upon binding of LRG1 to the TGFβ accessory receptor ENG to form a protein:protein complex which, in the presence of TGF-β1, promotes angiogenic signalling via the TβRII-ALK1-Smad1/5/8 pathway (Figure 13).\textsuperscript{150}

Wang \textit{et al}.\textsuperscript{150} also showed that in mouse models lacking LRG1, there was an attenuation of pathological blood vessel growth in a range of ocular disease models. Similarly, antibody blockade of LRG1 reduced abnormal vascular lesions.\textsuperscript{150} These observations suggest that LRG1 contributes to vessel destabilisation by corrupting the homeostatic influence of TGFβ signalling, and thus might play an important role in vessel dysfunction in a number of diseases that feature vascular abnormalities, including cancer.
LRG1 binds the TGFβ receptor endoglin, which in the presence of TGFβ promotes angiogenic signalling via the TβRII–ALK-1–Smad1/5/8 pathway.

1.6.3 LRG1 as a therapeutic target

Accumulating evidence suggests that LRG1 is involved in the growth and progression of a variety of cancer types as significantly elevated expression of LRG1 in serum and solid tumours has been found to be associated with a poor prognosis.\textsuperscript{157–162} Given the importance of neovascularisation in the pathogenesis of solid cancers, it is important to understand how LRG1 disrupts normal angiogenesis. Using two widely used tumour models, the subcutaneous LL2 lung carcinoma and B16F0 melanoma, the Greenwood and Moss group showed that compared to control mice, tumours exhibit a slower growth rate in mice lacking the Lrg1 gene and that this was associated with reduced vascular density within the tumours. Enhanced blood vessel normalisation was also observed in
mice lacking LRG1. Similar effects on blood vessel growth and characteristics were also observed in wild type mice when using a function-blocking monoclonal antibody against LRG1, called 15C4. Analysis of tumour vascularisation following LRG1 deletion and/or blockade revealed not only a reduction in the number of microvessels, but also improved vessel function. This was shown to be associated with vascular normalisation as a consequence of greater pericyte-endothelial cell association.

The high expression of LRG1 in tumours and the observed effects of LRG1 blockade on tumour vessel normalisation, together with the lack of harmful phenotype observed in LRG1 null mutant mice, make LRG1 an excellent therapeutic target. Preliminary experiments have been carried out which compared tumour growth in wild type mice dosed either with the LRG1 blocking antibody (15C4; mouse anti-LRG1 IgG1) alone, the cytotoxic drug cisplatin alone or both agents in combination. The combination therapy displayed a significant retardation of tumour growth compared to each of the agents used on their own (Figure 14). These results provide further validation of LRG1 as a therapeutic target and provide the rationale for this research. Hence in this research, the aim is to develop an ADC, to achieve both vascular normalisation and cytotoxic drug delivery with a single entity.
Figure 14 Combination therapy with anti-LRG1 antibody and the chemotherapeutic drug cisplatin leads to improved vascular normalisation, and a reduction of tumour density.
1.7 Project aims

This project involves the use of linker technology based on pyridazinedione chemistry to site-selectively modify the anti-mouse anti-LRG1 antibody 15C4, and its humanised de-immunised derivative, Magacizumab. The strategy aims to produce homogeneous or near-homogeneous antibody conjugates with a reactive handle to enable facile attachment of either a fluorophore or a drug to form an antibody–fluorophore conjugate (AFC) and antibody–drug conjugate (ADC), respectively. The development of these conjugates will require the following steps:

Formation of the linker:

- Synthesis of PD small molecules that enable site-selective antibody modification and click attachment of cargo (Chapter 2).

Antibody modification and characterisation of conjugates:

- Confirmation of homogeneity of PD-rebridged constructs (Chapter 2).
- Confirmation of PD loading and ‘clicked’ fluorophore loading by UV-Vis analysis (Chapter 2).
- Further confirmation of conjugation of PD and fluorophore to antibody by mass spectrometry (Chapter 2).
- Confirmation of retention of binding affinity to LRG1 by ELISA and serum stability studies (Chapter 3).

*In vitro* internalisation analysis:

- Generation of a stable human LRG1 transfected cell line (using B16F0 non-LRG1 melanoma cell line and human *LRG1* expression vector) to determine whether the secreted LRG1 is an internalising or non-internalising protein, and whether Magacizumab internalises upon binding its target (Chapter 3).
- *In vitro* studies to assess internalisation potential of the prepared antibody-fluorophore conjugates (Chapter 3).

Generation of the ADC:

- Selection of an appropriate linker and payload combination for generation of the ADC, based on *in vitro* studies (Chapter 4).
**In vitro and in vivo efficacy studies:**

- Synthesis of drug-linker and attachment of drug followed by the appraisal of the resultant ADC *in vitro* and *in vivo* (Chapter 4 and Chapter 5).

**Generation of the Fab fragment:**

The project will also aim to generate a Fab fragment of Magacizumab and appraise its LRG1-binding and functionality in an *in vivo* and *ex vivo* setting. The Fab will also be modified site-selectively (Chapter 6).
Chapter 2 Antibody and antibody modification

2.1 Formation of the linker

In order to appraise internalisation of the full anti-LRG1 antibody vehicle into cancer cells, the antibody platform needed to be modified with a suitable fluorophore. To achieve this, a small molecule capable of functionally rebridging the disulfide bonds of a full antibody needed to be synthesised. The thiol-selective dibromo pyridazinedione scaffold was employed for this purpose. Due to their unique structure, PDs enable robust attachment to antibodies. More specifically, the incorporation of ‘clickable’ handles in the design of the PD linker allows the covalent and controlled attachment of a fluorophore and/or a drug (Figure 15). Furthermore, in addition to producing bioconjugates with generally higher levels of homogeneity, PDs provide a serum stable (without hydrolysis) and versatile platform for the functionalisation of antibodies.

Figure 15 Dibrimopyridazinediones (diBrPDs) can be used to functionally rebridge the disulfide bonds in antibodies. Incorporation of clickable handles in their design allows attachment of a fluorophore and/or drug, represented by the star.
2.1.1 Synthesis of methyl propionic acid strained alkyne pyridazinedione

In this project, the optimised procedure reported by Bahou et al. for the synthesis of the core PD scaffold was adopted. The study began with the synthesis of an appropriate precursor for PD synthesis; methyl propionic acid di-tert-butoxycarbonyl (Boc) hydrazine 3 (Scheme 10). Methyl hydrazine 1 was Boc protected by reaction with di-tert-butyl dicarbonate to form methyl diboc hydrazine 2, which subsequently underwent base-mediated Michael addition with tert-butyl acrylate to yield methyl propionic acid diboc hydrazine 3 in 86% yield.

![Synthesis of methyl propionic acid diboc hydrazine 3.](image)

With protected hydrazine 3 in hand, synthesis of the core PD scaffold began with the in situ formation of dibromomaleic anhydride 5. Dibromomaleic acid 4 was refluxed for 30 min in acetic acid (AcOH) prior to addition of hydrazine 3, i.e. forming dibromomaleic anhydride 5 in situ (Scheme 11). Following this, reaction of 3 with dibromomaleic anhydride 5 under reflux in AcOH afforded methyl propionic acid pyridazinedione 6, in an overall yield of 61%. The acidic conditions required for PD condensation also allowed the Boc deprotection step to be accomplished in situ and the preformation of dibromomaleic anhydride 5 increased overall yield.
One of the most utilised reactions for adding functionality to functional rebridging reagents is the reaction of an amine with an activated carboxylic acid. The resultant amide bond shows excellent stability in vivo, and a great deal of toxic payloads, fluorophores, and imaging agents are commercially available as amines. Bahou et al.\textsuperscript{138} reported the synthesis of a PD harbouring an NHS ester, which reacted well with a variety of amines to form a library of amide-containing functional PDs.\textsuperscript{138} Accordingly, PD 6 was activated using \textit{N,N}'-dicyclohexylcarbodiimide (DCC) and \textit{N}-hydroxysuccinimide (NHS) to form NHS ester 7 (Scheme 12).

Bicyclononyne (BCN) derivatives are amongst the most reactive alkynes that are commercially available as they induce additional ring strain through fusion with cyclopropane, enabling a superior reaction rate when compared to plain cyclooctynes.\textsuperscript{164} BCN(endo)-PEG\textsubscript{2}-NH\textsubscript{2} was chosen as the desired ‘clickable’ handle for bioconjugation as it comprises a useful terminal primary amine, allowing amide formation in view of BCN integration in the pyridazinedione scaffold.

The NHS ester 7 was hence reacted with strained alkyne-bearing commercially available BCN(endo)-PEG\textsubscript{2}-NH\textsubscript{2} to form the strained alkyne functionalised dibromo
pyridazinedione linker 8 (Scheme 13). The presence of a strained alkyne on the resultant antibody rebridging reagent 8 would facilitate the use of ‘click chemistry’ to allow attachment of azide-bearing payloads and fluorescent dyes to the antibody for subsequent studies.

**Scheme 13** Reaction of activated PD 7 with a strained alkyne PEG2 amine to form methyl propionic acid strained alkyne amine PD 8.
2.2 Magacizumab: an anti-human LRG1 antibody

LRG1 is a serum glycoprotein synthesised primarily by the liver under normal conditions, and which is frequently upregulated at sites of pathological angiogenesis. Accumulating evidence has shown that LRG1 levels are significantly elevated in many cancers\(^\text{165-171}\). LRG1 promotes dysfunctional vessel growth via modification of the endothelial TGFβ signalling pathway\(^\text{150}\). The chaotic nature of vessels promotes a pro-oncogenic environment and limits the efficacy of systemically administered drugs. Thus, rather than the conventional approach of blocking tumour vessels and starving the tumour, focus has shifted towards devising approaches that promote the growth of functional vessels, or in other words promote ‘vessel normalisation’\(^\text{172}\). This would facilitate delivery of cytotoxic and immune therapies to the tumour site.

Consequently, attempts to neutralise LRG1 activity have led to the development of a function-blocking mouse monoclonal antibody against LRG1, called 15C4\(^\text{173}\). O’Connor et al. reported that inhibiting LRG1 with a function-blocking antibody reverses its detrimental effects on the vasculature and leads to significant restoration of normal vascular function\(^\text{163}\). These observations suggest that LRG1 is a promising therapeutic target in pathological angiogenesis.

Magacizumab is a humanised de-immunised variant of the mouse monoclonal antibody 15C4, that targets human LRG1 and prevents abnormal blood vessel formation\(^\text{173}\). In contrast to 15C4, which is an IgG1 (IgG being the isotype that denotes the structure of heavy chain constant domain), Magacizumab is an IgG4 antibody. IgG4 antibodies differ from IgG1s in that they show a reduced affinity for the Fcγ receptors and the proteins involved in the processes of ADCC and CDC. In addition to differences in the effector function, this antibody subclass also differs from IgG1 in hinge region disulfide bridge structure and stability. The rearrangement of the native IgG4 heavy chain disulfide bonds from an inter-chain disulfide into intra-chain disulfide conformation results in the presence of heavy-light-chain units also referred to as half molecules. A key determinant of this half molecule formation is the presence of a serine residue in the core hinge motif of IgG4 antibodies, Cys-Pro-\textbf{Ser}-Cys\(^\text{174}\). This is in contrast to IgG1 antibodies that possess a proline residue at this position (Cys-Pro-\textbf{Pro}-Cys\(^\text{174}\)). As a consequence, the IgG4 serine residue allows more hinge flexibility than its IgG1 counterpart and promotes the formation of a stable intra-chain disulfide bond in the hinge region (Figure 16).
The presence of serine in the IgG4 hinge region facilitates a process specific to this antibody subclass, known as Fab arm exchange (FAE) or half molecule exchange (Figure 16). This phenomenon enables two IgG4 antibodies to swap half molecules to form bispecific hybrid antibodies. However, substitution of the serine with proline has been shown to make the IgG4 hinge resemble that of an IgG1 (Cys-Pro-Pro-Cys), resulting in a drastic decrease of IgG4 half molecules detected by non-reducing SDS-PAGE.

Although the FAE of the IgG4 antibodies presents a new strategy to generate bispecific antibodies, with the ability to bind two different antigens, it presents certain issues such as reduced affinity for the antigen as well as altered binding properties and pharmacokinetic profiles of the resultant antibody, making half molecule exchange an undesirable feature in the development of therapeutic antibodies. To address these concerns, Magacizumab incorporates a stabilising serine to proline mutation (S228P, EU numbering scheme) in the core hinge region. This is consistent with all therapeutic IgG4s currently under clinical development, which have also adopted the serine to proline hinge-stabilising modification.
2.3 Antibody modification

PDs play an important role in the development of homogeneous antibody conjugates. This is due to their ability to functionally rebridge cysteine residues liberated from reduction of the disulfide bonds of native antibodies with minimal non-native disulfide re-bridging (Scheme 14).

Scheme 14 (a) An ideal disulfide bridging reaction in which interchain disulfide bonds are correctly rebridged (b) Schematic representations of the undesirable mis-bridged side-products generated with most disulfide bridging reagents.

2.3.1 Conjugation to Herceptin™

The clinically relevant IgG1 antibody Herceptin™ was chosen as a model to initially test conjugation of the prepared PD 8. Here, after reducing the disulfides with 10 equivalents (eq.) of the mild reducing agent tris-2-carboxyethylphosphine (TCEP), the liberated thiols were functionally rebrided under conditions utilising 20 eq. of PD 8 (Scheme 15). The conjugation was confirmed by SDS-PAGE and through measuring UV absorption at 335 nm (unique to the PD scaffold, see Experimental for details).
Pleasingly, this showed a desired PD to antibody ratio (PAR) of 4 showing successful modification of all solvent accessible interchain disulfide bonds (not shown).

**Scheme 15 Optimised conditions for the conjugation of PD 8 to Herceptin™.**

Following the conjugation of PD 8 to Herceptin™ under optimised conjugation conditions, the suitability of PD 8 as a bioconjugation reagent was appraised for the functional rebridging of the aforementioned target antibodies against LRG1; the mouse anti-human 15C4 and its clinically relevant humanised de-immunised variant Magacizumab.

### 2.3.2 Reduction studies

In order to avoid the use of excess reducing agent, reduction optimisation studies were carried out with both, 15C4 and Magacizumab. The four solvent accessible disulfide bonds of each antibody were reduced using the mild reducing agent TCEP. Reaction of each antibody with varying amounts of TCEP (10-150 eq.) in borate buffer (BBS) with EDTA at pH 8.0 and 37 °C was evaluated for the reduction of their interchain disulfide bonds. The excess reducing agent was removed by ultrafiltration into EDTA-containing BBS to inhibit re-oxidation of the reduced disulfides. Confirmation of reduction was achieved by SDS-PAGE and by mass spectrometry. For the purposes of analysis by mass spectrometry, excess N-methylmaleimide was added to the reduced samples to cap the liberated thiols and prevent oxidation of the reduced samples (Scheme 16). Deglycosylation of samples was achieved by incubation with the enzyme PNGase F at 37 °C for 16 h prior to analysis by mass spectrometry.
Scheme 16 Antibody reduction (BBS pH 8.0, 37 °C) with TCEP, followed by thiol capping with maleimide.

2.3.2.1 15C4
SDS-PAGE analysis revealed that 20 eq. of TCEP in borate buffer pH 8.0 with 2.5 mM EDTA was sufficient to completely reduce the four interchain disulfide bonds of 15C4 in 90 min, thus revealing heavy chains and light chains of the antibody (Scheme 17, Figure 17). These results were also confirmed by mass spectrometry (Figure 17b).

Scheme 17 Reagents and conditions for 15C4 reduction: 20 eq. TCEP for 90 min, then thiol capping with maleimide, BBS pH 8.0, 37 °C.
2.3.2.2 Magacizumab

Relative to 15C4, reduction of Magacizumab proved to be more challenging as full antibody reduction could not be achieved under the conditions used to fully reduce 15C4 (20 eq. TCEP, BBS with EDTA pH= 8.0, 90 min, 37 °C). In addition to the heavy and light chains, the SDS-PAGE gel displayed an additional band at approximately ~75 kDa for Magacizumab, which appeared to correspond to the half antibody fragment. In order to address this incomplete reduction, TCEP and dithiothreitol (DTT) were tested. TCEP is usually desirable over other reducing agents as PDs have been shown to be unreactive towards this reducing agent. Magacizumab was subjected to different amounts of DTT (10 eq., 50 eq., 100 eq. at 20 mM) and TCEP (50 eq., 100 eq., 150 eq. at 20 mM) for 4 h at 37 °C to identify the most suitable conditions for reduction. Even at lower amounts
(50 eq.), the use of DTT as a reducing agent yielded fully reduced Magacizumab (Figure 18), however due to the propensity of this agent to react with the PD, 100 eq. of 20 mM TCEP was selected as the optimal reduction conditions at this time (Figure 19). Mass spectrometry was used to confirm that under these conditions (Scheme 18, Figure 20, Figure 21), complete reduction of Magacizumab could be achieved.

**Figure 18** SDS-PAGE analysis of Magacizumab reduction by DTT in borate buffer pH 8.0 at 37 °C for 4 h. M = molecular weight marker; Lane 1 – native Magacizumab; Lane 2 – 10 eq. of DTT; Lane 3 – 50 eq. of DTT; Lane 4 – 100 eq. of DTT.
Figure 19 SDS-PAGE analysis of Magacizumab reduction by TCEP in borate buffer pH 8.0 at 37 °C for 4 h. M = molecular weight marker; Lane 1 – native Magacizumab; Lane 2 – 50 eq. of TCEP; Lane 3 – 100 eq. of TCEP; Lane 4 – 150 eq. of TCEP.

Scheme 18 Reagents and conditions for Magacizumab reduction: 100 eq. TCEP for 4 h, then thiol capping with maleimide, BBS pH 8.0, 37 °C.
Figure 20 a) Non-deconvoluted and b) de-convoluted LCMS data for native Magacizumab 15 (deglycosylated).

Figure 21 Non-deconvoluted and deconvoluted LCMS data for Magacizumab 16 reduced with 100 eq. 20 mM TCEP and capped with maleimide (deglycosylated) displaying a) light chain and b) heavy chain fragments.
2.3.3 Conjugation studies

2.3.3.1 15C4

A one-pot approach was adopted for the attempted functional rebridging of 15C4 with PD 8. In this approach, the reducing reagent and bridging agent were added to the antibody at the same time (Scheme 19).

To this effect, 15C4 (13) was incubated with 20 eq. of PD 8 and 20 eq. of TCEP in BBS pH 8.0. The reaction was performed at 4 °C, in accordance with the protocol described by Bahou et al. Delightfully, this led to the formation of an antibody construct 17 that had correctly rebridged disulfide bonds, with a PAR of ca. 4, by SDS-PAGE and UV-Vis spectroscopy respectively (Figure 22).

After this, the site-selectively modified antibody 17 was functionalised with a commercially available azide-fluorophore (Alexa Fluor™ 488 Azide). The presence of a cyclooctyne strained alkyne handle on PD 8 enabled conjugation of the fluorophore via a
copper-free strain promoted alkyne-azide cycloaddition (SPAAC) ‘click’ reaction to yield antibody-fluorophore conjugate 18 (AFC 18; Scheme 19b). Confirmation of a fluorophore loading of ca. 4 was obtained by UV-Vis spectroscopy (Figure 22).

**Figure 22** Conjugation of PD 8 to 15C4. Figure showing UV-vis data of 15C4 a) before and b) after functionalisation with Alexa Fluor™ 488 azide. c) SDS-PAGE analysis; M= Molecular weight marker; Lane 1 – Native 15C4; Lane 2 – 15C4 reduced with 20 eq. 20 mM TCEP; Lane 3 – PD-rebridged 15C4 (conjugate 17); Lane 4 – 15C4-AF488 conjugate (conjugate 18).

### 2.3.3.2 Magacizumab

Although most of the commonly employed reducing agents have the propensity to cross-react with disulfide bridging reagents, it has previously been demonstrated that PDs are amenable to both stepwise (reduction, followed by a wash step to remove unreacted reducing reagent prior to rebridging) and in situ (one-pot reduction and rebridging) protocols. This is due to minimal cross-reactivity with TCEP under the reaction conditions. However, due to the very high concentration of TCEP required for the complete reduction of Magacizumab, it was envisaged that a stepwise conjugation...
protocol in which reduction is performed first, followed by removal of the reducing agent and addition of the bridging reagent, would be better suited (Scheme 20). Notably, Bahou et al. have reported that adoption of this procedure results in the formation of small amounts of ‘half-antibody’ isomer.138,179

Scheme 20 a) Sequential reduction and rebridging of Magacizumab, followed by b) SPAAC ‘click’ functionalisation with Alexa Fluor™ 488 Azide to form antibody–fluorophore conjugate 20 (AFC 20).

Nevertheless, reduction of Magacizumab was achieved by incubation of the antibody 15 with 100 eq. of TCEP (as described in Section 2.3.2.2), followed by purification via ultrafiltration to remove the reducing agent, and subsequent addition of PD 8 to rebridge the disulfide bonds of Magacizumab. UV-Vis analysis revealed a desired PAR of ca. 4. However, somewhat as expected, SDS-PAGE analysis revealed that the stepwise reaction of TCEP followed by addition of PD yielded two major antibody conjugate isomers. The higher molecular weight species was the major product and is attributed to the natively rebridged antibody, whereas the middle band indicates a product of non-native bridging of the antibody hinge region, resulting in a small amount of ‘half-antibody’ (Figure 23).179–182 Nevertheless, reports by Bahou et al. demonstrated that when PDs are
used to form an abundance of the ‘half-antibody’ species, no effect on antigen binding is observed.\textsuperscript{179}

Following this, Alexa Fluor\textsuperscript{TM} 488 Azide was ‘clicked’ onto site-selectively modified antibody 19 to yield antibody-fluorophore conjugate 20 (AFC 20; Scheme 20). Confirmation of a fluorophore loading of ca. 4 was obtained by UV-Vis spectroscopy and mass spectrometry. The effect of temperature on the bridging efficiency was also examined. The stepwise protocol was utilised; with 100 eq. of TCEP and 20 eq. of PD 8. Incubation for 16 hours at either 4 °C or 21 °C displayed no significant temperature dependence on the overall composition of the final construct, generating ~26% and ~31% (calculated using ImageJ software, see Conjugation methods) ‘half-antibody’ product respectively (Figure 23).
Figure 23 Conjugation of PD 8 to Magacizumab. Figure showing UV-Vis data of Magacizumab a) before and b) after functionalisation with Alexa Fluor™488 azide; c) SDS-PAGE of a PD 8 modified Magacizumab by step-wise protocol. M = Marker;
Lane 1 – Reduced control; Lane 2 – Rebridged Magacizumab 19 at 21 °C; Lane 3 – Rebridged Magacizumab 19 at 4 °C; Lane 4 – AFC 20 at 21 °C (~31% ‘half-antibody’); Lane 5 – AFC 20 at 4 °C (~26% ‘half-antibody’); d) Non-deconvoluted and e)
2.4 Conclusion

Due to their chemoselectivity, PDs enable robust attachment to antibodies. In addition to producing conjugates with generally higher levels of homogeneity, antibody-PD conjugates provide serum stable bioconjugates and a versatile platform for the functionalisation of antibodies.\(^\text{132}\) In view of this, PD-strained alkyne 8 was employed as the choice of linker in this study.\(^\text{138}\) The incorporation of a ‘clickable’ stained alkyne handle in the design of this PD linker enables the covalent and controlled attachment of an azide-bearing fluorophore (or a drug for later studies) by SPAAC, allowing chemical conjugation under aqueous conditions without the need for toxic catalysts. Conjugation efficiency of the antibody functional rebridging by PD 8 could be confirmed by SDS-PAGE and UV-Vis spectroscopy, taking advantage of the characteristic absorbance of PDs at 335 nm. Alexa Fluor\textsuperscript{TM} 488 azide was selected as an appropriate fluorophore to ‘click’ onto the antibody-PD conjugates. The presence of an azide handle facilitated ‘click’ reaction with the strained alkyne on the PD moiety. Moreover, due to having a maximum absorbance at a distinct wavelength to the PD, reaction with this fluorophore enabled confirmation that the PD loading obtained by UV-Vis was a good measure of loading on an antibody as the ‘click’ proceeded completely. These results provide proof of concept for the ability to site-selectively modify an IgG1 and IgG4-based antibody (15C4 and Magacizumab respectively) in a controlled manner using the PD scaffold.
Chapter 3 *In vitro* validation of antibody conjugates

The development of therapeutically effective conjugates is a highly intricate process. Therefore, modification of antibodies with small molecules may have major consequences on protein function and stability whilst in circulation. The mode of action at cellular or molecular level is complex, with each step adding an additional layer of difficulty. Thus, several factors, activity and stability-related, are key when designing an ADC:

- **Antigen Binding.** It is important that the binding affinity of the conjugated antibody to its target antigen is retained after modification. Ideally, attachment of the cytotoxic payload to the antibody should not disturb its binding potential to enable selective accumulation and retention of the ADC at the tumour site.

- **Stability in circulation.** The linker that connects the antibody to the cytotoxic payload in the ADC should be stable in circulation to allow release of the cytotoxic payload in a target-specific manner. Premature release of the cytotoxic drug in the bloodstream would indiscriminately damage healthy tissue, causing off-target toxicity, thereby lowering the therapeutic index of the ADC.8

- **Internalisation potential and delivery mechanism.** Sufficient intracellular concentration of the drug needs to be achieved for an ADC to be effective. It is therefore important to explore the internalisation potential of ADCs. Antibody binding to its target antigen traditionally results in internalisation of the ADC-antigen complex by receptor-mediated endocytosis to enable release of the payload within the cell.103 Ideally, the antigen for an effective ADC must be highly expressed on the surface of the cell, however the correlation between the efficacy of the ADC and density of the target antigen on the cell surface remains to be determined. This is due to variable antigen expression across different tissue types and differences in ADC binding and internalisation, which together have an impact on drug uptake and release within the tumour cells. Although most ADCs focus on targets that are located on the membrane of tumour cells and rely on
internalisation, there is an emerging class of ADCs that are not dependent upon
internalisation to release their payload, and can release their payload by
extracellular cleavage of the linker in the tumour environment.\textsuperscript{183}

3.1 Antigen binding activity of bioconjugates: ELISA

3.1.1 15C4

To assess whether the antigen-binding affinity of the mouse monoclonal antibody 15C4
to LRG1 was impacted upon conjugation to PD 8 and ‘click’ functionalisation with
Alexa Fluor\textsuperscript{TM} 488 azide, an ELISA was performed. An ELISA is an assay used to
measure the strength with which a protein (typically an antibody) binds to its
complementary fragment (or antigen).\textsuperscript{184} As the targeted inter-chain disulfide bonds are
distal from the antigen binding site, it was rationalised that this modification should have
minimal impact on antibody/antigen binding. Conjugate 18 (15C4-488) was prepared
freshly before the study and evaluated against native 15C4. Binding to LRG1 was
assessed by measuring absorbance at 450 nm and plotting the values against the antibody
concentration, to obtain a standard curve. Encouragingly, the conjugate was shown to
have comparable binding activity to its unmodified counterpart (Figure 24). These tests
were performed in triplicate and the results gave a good indication that conjugation of
15C4 to PD 8 followed by ‘click’ functionalisation does not affect the binding affinity of
15C4 to LRG1.
As Magacizumab is the humanised derivative of the mouse monoclonal anti-LRG1 antibody 15C4, this makes Magacizumab clinically more relevant when considering the long-term objective of generating a LRG1-targeting ADC. Henceforth, all modifications will be discussed in the context of Magacizumab as the antibody portion of the ADC.

### 3.1.2 Magacizumab

The impact on LRG1 binding of Magacizumab following disulfide modification with PD 8 and subsequent modification with Alexa Fluor™ 488 was also assessed via ELISA. Binding was assessed by measuring absorbance at 450 nm and plotting the values against the antibody concentration, to obtain a standard curve. As expected, Magacizumab-Alexa Fluor™ 488 20 (also referred to as Maga-488 throughout this report) was shown to have comparable binding activity to native Magacizumab (Figure 25). These experiments were performed in triplicate and the results give good indication that PD conjugation and azide functionalisation of Magacizumab have no effect on its ability to bind its target antigen, human LRG1 (hLRG1).
Figure 25 Binding activity to hLRG1 of PD-rebridged and ‘click’-functionalised Magacizumab (conjugate 20) compared with native Magacizumab. Experiments were performed in triplicate (n=3). Data are shown as mean ± SEM.

3.2 Stability in circulation

The stability of the Magacizumab-PD conjugate was examined in blood serum (Figure 26). PD 8 was conjugated to Magacizumab and then ‘click’ functionalised with Alexa Fluor™ 488 azide to give Maga-488 (conjugate 20), as described in section 2.3.3.2. The conjugate was incubated in human blood serum for 7 days at 37 °C. Samples were taken at defined intervals (day 1, 2, 4 and 7) and transfer of fluorescence from conjugate 20 to serum albumin was determined by HPLC-SEC. Consistent with previous findings, the PD-rebridged conjugate displayed minimal transfer of fluorescence to serum albumin over the period of incubation, indicating that the conjugate would be stable in vivo. This demonstrates a key advantage in the use of a PD linker over the classical maleimide chemistry, whereby the ADC typically undergoes reaction with blood thiols, which leads to the transfer of the drug to thiol-bearing proteins such as serum albumin and may lead to off-target toxicity. Hence, the stability of the conjugate in serum for 7 days is not only proof of the likely minimal off-target effects for a PD-substituted
antibody *in vivo*, but also highlights the robustness of PD chemistry for the development of ADCs.

![Figure 26 Serum stability analysis of Magacizumab-Alexa Fluor 488 by HPLC-SEC.](image)

### 3.3 Internalisation analysis

The selection of an appropriate linker-payload combination for the development of an ADC is largely dependent on the ability of the antibody-antigen complex to internalise into a target cell. Since LRG1 is a novel target, it was not known whether this secreted protein would internalise into cells when complexed to or not complexed to Magacizumab. Thus, in order to evaluate the internalisation potential of LRG1 and LRG1-Magacizumab, a pair of cancer cell lines were required: a native cell line that expressed no detectable hLRG1 and a transfected cell line that expressed hLRG1 highly.

#### 3.3.1 Establishing a hLRG1-expressing cell line

It was important to first establish a model cancer cell line that expresses the hLRG1 protein. In view of previously conducted studies into the effect of LRG1 on vessel destabilisation, the B16F0 mouse melanoma cell line was selected as the parent cell line. A variant of this cell line that is stably transfected with a plasmid containing the human *LRG1* gene was to be developed.
In order to distinguish non-transfected cells from cells that had taken up plasmid DNA, selective screening needed to be performed. This is generally achieved by selection using a drug when an appropriate drug-resistance marker is included in the plasmid containing the DNA. The plasmid that was used for transfection of the human \textit{LRGI} gene incorporated the neomycin (neo) resistance gene, that would allow transfected cells to be grown under selection with neomycin analogue G418.

Before beginning the process of selection of transfected cells, it was important to determine the amount of drug that would be necessary to kill cells that had not successfully taken up the gene of interest. As this amount varies greatly among cell types, it was therefore optimised prior to initiating the transfection process. A cell-kill curve was plotted to determine the optimal concentration of G418 that would be sufficient to kill non-transfected non-resistant cells. Therefore, B16F0 cells were treated with increasing concentrations of G418 diluted in growth medium, ranging from 0 mg/mL to 2 mg/mL, for 10 days. During this time, selection media was replaced every 2 days, and on the final day the number of cells alive was counted and plotted as a percentage of living cells (Figure 27). The results indicated that 1 mg/mL of G418 was sufficient to kill most of the non-transfected cells, and this concentration was within the range of G418 concentrations recommended for this cell type (0.4 – 1 mg/mL) by the manufacturer.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Antibiotic kill curve. Determining the optimal concentration of G418 required to kill non-transfected B16F0 cells, expressed as percentage survival of cells as measured on day 10 of culture.}
\end{figure}
3.3.1.1 Quantitative polymerase chain reaction (qPCR)

In order to determine whether cells were transfected successfully, quantitative polymerase chain reaction (qPCR) was performed to assess the level of expression of the desired gene; human LRG1. To do this, cell lysates were obtained from non-transfected and transfected B16F0 cells. RNA was isolated from the lysates and transcribed to cDNA, which was then amplified by qPCR to examine expression levels of human LRG1. Relative gene expression was determined against the expression of a selected house-keeping gene. Mouse Gapdh (mGapdh) was used as the house-keeping gene. The results showed that there was a considerable increase in transcript for human LRG1 in transfected cells in comparison to non-transfected cells. Mouse Lrg1 levels were also assessed and not found to be significantly different between the two cell lines (Figure 28).

![Figure 28](image)

**Figure 28** qPCR was used to determine expression levels of mouse Lrg1 (left) and human LRG1 (right) in transfected and non-transfected cells. Expression values were normalised to expression of the housekeeping gene mouse Gapdh. Data are shown as mean ± SEM. Experiments were performed in triplicate (n=3) and statistical analysis performed using Student’s t test (ns = not significant, *** = P ≤ 0.001).

3.3.1.2 Western blotting

Having established the presence of the LRG1 mRNA, it was important to assess whether human LRG1 was being expressed in the transfected cells at the protein level. Protein
samples for western blot analysis were isolated by two routes; from the cell lysates and from the supernatants – as LRG1 is a secreted protein. The samples were probed with an antibody specific for human LRG1. Western blot analysis showed the presence of a closely spaced polypeptide doublet with a molecular weight of about 50 kDa in the supernatants of B16F0-hLRG1 (transfected) cells but no comparable band in wild type B16F0 (non-transfected) cells (Figure 29a). The bands at 50 kDa most likely correspond to glycosylation variants of human LRG1. The lysates of transfected cells also displayed a clear band for hLRG1 that was absent in control LAN-1 cells (Figure 29b). The presence of a band in the lysate of wild type B16F0 is most likely due to cross-reactivity of the antibody with the mouse LRG1 protein (also reported by other members of the group when using this antibody). Densitometric analysis, which involved normalising expression against a loading control (mouse GAPDH), confirmed that hLRG1 expression in B16F0-hLRG1 cells was considerably higher than in wild type B16F0 cells (Figure 29c). A human neuroblastoma cell line (LAN-1) was used as a negative control as qPCR data (performed by Dr Camilla Pilotti, data not shown) showed no expression for hLRG1 (Figure 29).

Figure 29 Western blot analysis of hLRG1 expression in B16F0 cells. Supernatants and cell lysates were probed using a rabbit anti-hLRG1 antibody. Representative western blot of a) supernatants and b) cell lysates as separate experiments. c) Densitometric analysis of hLRG1 levels of the western blot performed using cell lysates. Expression was normalised against mouse GAPDH. LAN-1, a human neuroblastoma cell line was
used as negative control as qPCR data confirmed no expression of LRG1 (data not shown).

3.3.2 Analysis of Magacizumab cell internalisation

Having successfully established a cell line that expresses hLRG1, the fate of Magacizumab was next explored by performing an *in vitro* internalisation assay. The aim of the study was to ascertain whether Magacizumab and/or hLRG1 (or a complex thereof) are internalised, as this was an important consideration for designing an appropriate drug linker for the efficient release of the payload at the target site. As it was not known whether secreted hLRG1 alone is internalised following adherence to the tumour cell surface, *via* its association with TGFβ family receptors or whether Magacizumab bound to this hLRG1 could stimulate internalisation, an *in vitro* assay was employed.

For this purpose, Magacizumab was conjugated to an azide derivative of Alexa Fluor™ 488 *via* SPAAC to form Maga-488 20, as described previously. The construct was then incubated with hLRG1-positive B16F0 mouse melanoma cells and the parent non-transfected B16F0 cells as a control. Initially, incubation of the conjugate with cells was performed at 4 °C, this is a temperature which would allow antibody-LRG1-cell binding but not internalisation. The temperature was then increased to 37 °C to permit internalisation of the conjugate. However, analysis by confocal microscopy revealed that under these *in vitro* conditions no signal was detectable for Maga-488 (conjugate 20), either attached to the cell surface or internalised into the cells (Figure 30).
Figure 30 Internalisation analysis of Maga-488 (conjugate 20) by confocal microscopy.

a) hLRG1-positive and hLRG1-negative B16F0 cells incubated with Maga-488 at 4 °C; b) hLRG1-positive and hLRG1-negative cells incubated with Maga-488 at 37 °C. Phalloidin was used to stain actin and DAPI was used to stain nuclei. Scale bar, 50 µm.

To rule out the possibility of any errors in experimental design, a positive control was performed using the transferrin receptor ligand, transferrin. The transferrin receptor is a membrane glycoprotein that is responsible for mediating iron uptake from transferrin, a plasma glycoprotein. Iron uptake from transferrin involves the binding of transferrin to
the transferrin receptor, internalisation of transferrin via receptor-mediated endocytosis and the release of iron within the acidic endosome. Transferrin receptors are expressed on the surface of all cells, with particularly high expression in rapidly dividing cells, such as cancer cells or cells in culture.\textsuperscript{187} Thus, tracking the internalisation of transferrin under the conditions described would provide information about success of the assay.

Labelled transferrin (transferrin-555) was kindly provided by Dr Tim Burgoyne of the UCL Institute of Ophthalmology for these positive control experiments. As with Maga-488 (conjugate 20), B16F0 cells were initially incubated with transferrin-555 at 4 °C and then the temperature raised to 37 °C to encourage internalisation. It was clear upon visualisation by confocal microscopy that incubation at 37 °C resulted in cytoplasmic localisation of the labelled transferrin (Figure 31). These findings indicated that the methodology used to investigate the internalisation of Magacizumab was indeed appropriate and confirmed that Magacizumab does not apparently bind and subsequently internalise into B16F0 cells that secrete hLRG1.

These data suggest that Magacizumab binds to hLRG1 and may prevent it from binding to its molecular partners and, perhaps also entering the cell. In order to determine whether LRG1 internalisation is blocked by Magacizumab, or indeed if LRG1 is a non-internalising or non-cell surface binding protein, hLRG1 was labelled and the internalisation assay performed again. Labelling of hLRG1 was performed using an Alexa Fluor\textsuperscript{TM} 555 protein labelling kit. The presence of a succinimidyl ester moiety on the Alexa Fluor\textsuperscript{TM} dye enables reaction with primary amines of proteins to form stable dye–protein conjugates. Once labelled, hLRG1-555 was purified into PBS.
**Figure 31** Internalisation analysis of transferrin-555 by confocal microscopy (positive control). Incubation of transferrin-555 with hLRG1-positive and hLRG1-negative B16F0 cells at 37 ºC. Phalloidin was used to stain actin and DAPI was used to stain nuclei. Scale bar, 50 µm.

hLRG1-positive B16F0 cells were incubated with labelled hLRG1 (hLRG1-555) alone or in the presence of Maga-488 20. It was rationalised that if Magacizumab acts by preventing internalisation of hLRG1, then in the absence of the Magacizumab conjugate, hLRG1 internalisation or cell surface binding would occur. However, analysis by confocal microscopy revealed (Figure 32) that despite the absence of Maga-488 20, no signal for hLRG1 could be observed, indicating that under these conditions hLRG1 is non-internalising or non-cell surface binding, or at the very least is a poorly internalising or cell surface binding protein. These observations suggest that under these conditions the hLRG1 detected earlier in western blotting remains free in the supernatant, and that the protein identified in the lysate is cytoplasmic and not located at the cell surface.
This is the first time that the fate of LRG1 post-secretion has been determined. Having established that secreted LRG1 is predominantly extracellular, and that consequently LRG1 bound Magacizumab will most likely remain in the extracellular space, an appropriate linker strategy must be employed for effective ADC design. Even in the event of other cancer cell types being able to internalise LRG1, the proportion available in the extracellular space for an anti-LRG1 ADC to target will far outweigh that available internally.

3.4 Conclusion

By assessing the in vitro activity of the antibody–fluorophore conjugates, in particular the Magacizumab conjugate, it was demonstrated that even after conjugation to a large construct, PD-modified conjugates show significant retention of binding to LRG1 and demonstrate stability in serum, as evidenced by minimal transfer of fluorescence from Maga-488 to albumin when incubated in human serum for 7 days. Furthermore, fluorophore modification of Magacizumab and LRG1 enabled visualisation of
internalisation ability of the antibody and antigen respectively, which are important considerations when designing an ADC. It was demonstrated that LRG1 is predominantly a non-internalising and non-cell surface binding protein, which is an important finding when considering selection of an appropriate linker-payload combination for the ADC to be developed.

Despite the commonly held belief that the effectiveness of ADCs is dependent upon internalisation via a cell-surface antigen, there are several difficulties that are encountered with this approach. For instance, this approach limits ADC targets to proteins that are internalised upon ADC binding. In some cases, despite existing on the cell surface or the tumour environment, ADC targets do not internalise. Recently, a type of ADC that does not rely upon internalisation has been investigated.\textsuperscript{124,183} These non-internalising ADCs often target antigens that are structural components of the tumour extracellular matrix.\textsuperscript{124,183} Studies provide increasing evidence to indicate that ADCs with particular linker/drug combinations can exert cell killing through other mechanisms.\textsuperscript{113} Non-internalising ADCs offer a way to release the cargo independent of internalisation into the target cell. This strategy takes advantage of the high local concentrations of various enzymes specifically in the tumour microenvironment that can cleave the linkage between the payload and antibody to release the payload. To this end, a non-internalising hLRG1-targeting ADC bearing an enzyme cleavable linker was to be developed.
Chapter 4 Generation of the hLRG1-targeting ADC

4.1 Non-internalising ADCs

Traditionally, upon binding to its target antigen an ADC-antigen complex internalises via receptor-mediated endocytosis to enable intracellular release of a toxic payload. In recent years however, there has been a considerable shift from the belief that ADCs are strictly dependent upon selective binding and internalisation of the antibody into tumour cells to release their toxic payload in order for them to be effective. For example, it has emerged that internalisation is not essential to cause tumour cell death. Through the use of extracellular cleavable linker-bearing ADCs, non-internalising ADCs have shown great promise. Indeed, it has been shown that targeting ADCs to components in the tumour extracellular space or to non-internalising tumour markers has considerable therapeutic activity.

Evidence of potent preclinical activity in cancer models has been reported for non-internalising ADC products directed against a number of targets including fibrin and collagen IV, as well as splice variants of tenascin-C, which are all components of the tumour extracellular matrix.

4.1.1 Choice of ADC linker-payload

Having demonstrated the inability of Magacizumab to internalise into B16F0 cells, it was rationalised that a non-internalising ADC bearing an extracellular cleavable linker could display anti-cancer activity by liberating its payload in the tumour extracellular space upon binding to hLRG1 in this region.

Cleavable linkers, in particular the valine-citrulline (Val-Cit) dipeptide linkers, have been reported to be stable in circulation in vivo, and have also been employed in a vast majority of the ADCs on the market and in clinical trials (see Section 1.5). In 2017, researchers in the Neri laboratory reported the development of non-internalising ADCs bearing Val-Cit-PABC linkers to target components in the extracellular space that were able to exhibit potent anti-cancer activity in murine cancer models. They also showed that ADC stability and activity could be altered significantly upon single amino acid
substitution of the dipeptide linker, whereas substitution of the cleavable dipeptide linker with a non-cleavable linker completely eradicated the anti-cancer activity of the ADC in vivo, providing evidence that the anti-tumour activity of their non-internalising therapeutics was dependent upon the presence of the vital cleavable linker.

There are several core requirements that must be met when selecting an appropriate payload for an ADC; the payload should have cytotoxic potency in the subnanomolar range, it should contain an appropriate functional handle to allow conjugation to the antibody, and the payload must be stable and soluble under physiological conditions. The majority of ADCs currently on the market use microtubule inhibitors such as auristatins and maytansinoids as payloads. Auristatins cause cell death (apoptosis) by inhibiting microtubule polymerisation which blocks the progression of the cell cycle during mitosis resulting in cell cycle arrest. The antimitotic payload monomethyl auristatin E (MMAE) has been successfully employed in FDA-approved ADCs Adcetris™, Padcev™ and Polivy™ alongside Val-Cit dipeptide linkers. Tumour cells often overexpress certain proteolytic enzymes and these can be exploited for the activation of ADCs in tumour therapy. The cleavable dipeptide linkers allow successful release of the active payload upon processing by lysosomal proteases such as cathepsin B.

It was therefore decided that the hLRG1-targeting ADC would incorporate a linker-payload combination consisting of the antimitotic payload MMAE with an azide handle adjacent to an enzymatically cleavable dipeptide valine-citrulline linker equipped with a self-immolative para-aminobenzylcarbonyl (PABC) spacer and a solubilising PEG3 moiety (N3-PEG3-vc-PABC-MMAE, Figure 33). The incorporation of the azide group in the payload moiety satisfied the requirement for the presence of a functional handle on the drug to enable facile ‘click’ conjugation to the Magacizumab-PD conjugate to form the desired ADC. The PABC spacer hydrolytically decomposes upon decarboxylation, spontaneously releasing the free drug MMAE (Figure 33).
4.1.2 Cathepsin B in the B16F0 melanoma model

The rationale for the construction of cathepsin B cleavable Val-Cit linkers is dependent upon several distinct characteristics. Cysteine cathepsins belong to the papain family of cysteine proteases that are usually highly concentrated in endosomes and lysosomes where they are involved in proteolytic processes. The human genome encodes 11 cathepsins that largely overlap in specificities.\textsuperscript{197} The roles of cathepsins are not strictly limited to endo-lysosomal compartments and they have been reported to be found in the cytoplasm, nucleus and extracellular space.\textsuperscript{198} Their extracellular localisation is common in different pathological conditions. Indeed, cathepsins are elevated during inflammation which accompanies many diseases, including cancer.\textsuperscript{199} The role of cysteine cathepsins, in particular cathepsin B in cancer, was first demonstrated some 40 years ago.\textsuperscript{200} As a
consequence of their elevated activity in the extracellular space during disease, cathepsins have received much attention for the development of therapeutic and diagnostic applications. In particular, targeted drug delivery approaches largely benefit from the over-expression of cathepsin B in cancer. ADCs act as prodrugs that become active only after cathepsin B-mediated release of the free drug.\textsuperscript{199} This differential expression of cathepsin B between healthy and disease cells contributes to the stability of the cathepsin B cleavable Val-Cit linkers in circulation.

Prior to construction of the hLRG1-targeted ADC, the presence of cathepsin B in the tumour microenvironment was ascertained using fluorescence microscopy. To do this, tumours derived from subcutaneously grafted hLRG1-expressing B16F0 melanoma cells in mice were immunofluorescently stained for cathepsin B using a goat anti-mouse cathepsin B antibody and subjected to fluorescence microscopy analysis. Parallel experiments were performed, where tumour sections were stained with the anti-cathepsin B antibody with and without membrane permeabilisation of the tumour sections. The permeabilisation step allows the antibody to enter cells and is crucial when the antibody epitope is intracellular, but dispensable when the epitope is expressed on the cell surface or the extracellular space. Hence this comparison would enable determination of whether cathepsin B is limited to being expressed within tumour cells. In both cases, signal for cathepsin B could be seen throughout the tumour sections with much more pronounced labelling in areas where there was cell death, as indicated by lack of nuclear staining with DAPI (Figure 34). These results confirm that in pathological conditions cathepsin B is expressed extracellularly.

The presence of cathepsin B in the microenvironment of these tumours encouraged the development of an ADC bearing an antibody linked to its cytotoxic drug via a cathepsin B-sensitive Val-Cit dipeptide linker. These ADCs would selectively deliver the cytotoxic payload to the tumour environment and rely on extracellular cathepsin B to initiate some free MMAE drug release, which would cause tumour cell death. The subsequent release of high concentrations of cathepsin B from the dead cell would facilitate cleavage of the dipeptide linkage on other ADCs to release more payload, leading to a self-propelling cycle of more and more free drug release.
Figure 34 Cathepsin B is present in the tumour microenvironment of B16F0 melanoma tumour models with or without permeabilisation of tumour sections. Nuclei were stained using DAPI. Scale bar, 50 µm.

4.2 Generation of the anti-LRG1 ADC

Having already established a method for the site-selective modification of Magacizumab with a fluorophore to form an antibody–fluorophore conjugate (AFC 20, see Chapter 2), the process was optimised for modification with a cytotoxic drug to form an ADC. MMAE has been successfully employed in many of the ADCs on the market and in late stage clinical trials, its cell permeability is essential in the design of a non-internalising ADC and it also possesses the favourable property of mediating killing of surrounding cells via the bystander effect. It was thus chosen as the toxic payload for the anti-hLRG1 non-internalising ADC.

Having already developed a strained alkyne PD that successfully site-selectively modified Magacizumab (see Section 2.3.3), N3-PEG3-vc-PABC-MMAE was chosen as the linker for the non-internalising ADC as the presence of an azide handle would facilitate a SPAAC reaction with PD 8. In light of previous experiments conducted in the group, ‘click’ conjugation of a site-selectively modified antibody bearing strained alkyne ‘click’ handles with hydrophobic azide molecules presented issues (unpublished results); a
common (general) problem in the field. Hence it was reasoned that the conjugation reaction to form the ADC could be made more efficient by performing a ‘pre-click’ reaction where the disulfide-reactive PD-strained alkyne 8 is SPAAC ‘clicked’ with the azide-linked MMAE prior to site-selective conjugation to Magacizumab resulting in more efficient use of MMAE. Previous work in the group has also shown that direct conjugation of a hydrophobic PD is feasible and reproducible highlighting another advantage of PD chemistry for antibody modification. As with previously labelled Magacizumab conjugates, quantification of drug loading could be determined by UV-Vis analysis, by taking advantage of the characteristic absorbance of PDs at 335 nm. This approach is substantiated by the results obtained by ‘click’ functionalisation of Magacizumab-PD 17 conjugate with Alexa Fluor™ 488 azide.

4.2.1 Optimisation studies

4.2.1.1 Conjugation of PD to Magacizumab: pre-click vs post-click strategy

Prior to proceeding with conjugation of the payload to Magacizumab, studies were performed to compare efficiency of the pre-click approach, where the azide-strained alkyne cycloaddition reaction between the payload and PD takes place before conjugation to Magacizumab, to the post-click approach (described in Section 2.3.3.2). Alexa Fluor™ 488 azide was used to mimic the payload moiety. For the pre-click approach, PD 8 was reacted with Alexa Fluor™ 488 azide in a 1 to 1.1 ratio respectively. LCMS analysis revealed that all the PD had been consumed in the ‘click’ reaction, to give PD-linked Alexa Fluor™ 488 21 (Figure 35). Following this, reduced Magacizumab was rebridged using 20 equivalents of PD-Alexa Fluor™ 488 (PD 21) to form the AFC 22 (Scheme 21).
Scheme 21 Schematic representation of Magacizumab-AF488 prepared via the pre-click strategy. a) shows the pre-click reaction between PD 8 and Alexa Fluor™ 488 azide to form PD 21; b) sequential reduction and rebridging of Magacizumab with PD 21 to form the antibody–fluorophore conjugate 22 (AFC 22).

Experiments comparing the pre-click and post-click strategies were performed in parallel, and success of conjugation was determined by UV-Vis analysis and by SDS-PAGE, which revealed a desired FAR of ~4 for both methods (Figure 36).
**Figure 36** Comparison of AFCs prepared via pre-click and post-click strategies. a) SDS-PAGE analysis of products. Lane M – marker, Lane 1 – Native Magacizumab, Lane 2 – Pre-click AFC 22, Lane 3 – post-click ADC 20; b) UV-Vis analysis of products.

### 4.2.1.2 Generation of Magacizumab PD-MMAE ADC 24

Having demonstrated the merit of the pre-click approach using Alexa Fluor™ 488 azide, the reaction of PD 8 with MMAE-azide was appraised using a 1 to 1.1 molar ratio of PD 8: MMAE-azide in DMSO to yield PD-MMAE 23 (Scheme 22a). A slight excess of MMAE-azide was used to achieve complete consumption of PD 8.
Scheme 22 Schematic representation of the Magacizumab ADC. a) SPAAC ‘click’ functionalisation (the pre-click strategy) reaction between the strained alkyne bearing PD 8 and azide-linked MMAE to form PD 23; b) sequential reduction and rebridging of Magacizumab with pre-clicked PD 23 (drug) to form antibody–drug conjugate 24 (ADC 24).

LCMS analysis revealed that all the PD had been consumed in the ‘click’ reaction, furnishing disulfide reactive PD-linked MMAE 23 and a small excess of (unreacted) MMAE-azide (Figure 37). The conjugation of PD-MMAE 23 with Magacizumab was next appraised. In view of the need to scale up the ADC to evaluate efficacy, several distinct parameters were investigated for the formation of the ADC. The different reaction conditions that were tested for conjugation of pre-clicked PD 23 to Magacizumab to form the ADC included varying reaction temperature, equivalents of PD 23 used for rebridging and the final DMSO concentration (Scheme 22b). UV-Vis analysis was used to assess PAR (Table 2).

Table 2 Reaction conditions for conjugation of PD-MMAE 23 to Magacizumab to form Magacizumab PD-MMAE ADC 24. PAR represents PD (and hence drug) loading on the antibody.
It was found that all reaction conditions tested yielded ADCs with a PAR of ~4 (Table 2, Figure 38). In the interests of scaling up the ADC reaction for cytotoxicity and therapy studies, the conditions that used the lowest equivalents of PD (10 eq.), had the lowest final DMSO% (6%) in the ADC and gave a high PAR were taken forward for large-scale reactions (Reaction 6). An Alexa Fluor™ 488 azide ‘click’ control was also performed on this prepared ADC to demonstrate that all clickable handles on the functionally rebridged antibody had reacted with MMAE-azide and that no, or negligible, unreacted strained alkyne was present and to corroborate that the value for PD loading reflected drug loading. This gave a minimal fluorophore readout of 0.2 (Figure 39). This is in line with the fluorophore readout value that is obtained upon incubation of Alexa Fluor™ 488 azide with PD-rebridged antibody that lacks a strained alkyne clickable handle, e.g. dibromo diethyl pyridazinedione 25 which has been washed the same number of times (see Conjugation methods for details).

*Figure 38 UV-Vis analysis of ADC products prepared according to Table 2. The UV-Vis trace was used to calculate PD (drug) loading as reported in Table 2.*
Figure 39 UV-Vis analysis of ADC (reaction 6) after incubation with Alexa Fluor™ 488 azide (control experiment). The UV-Vis trace was used to calculate PD and fluorophore loading (PAR = 3.7, FAR = 0.2).

The reaction to generate ADC 24 was successfully scaled up for in vitro and in vivo studies using the optimised conjugation conditions (Figure 40a), with success of conjugation further confirmed by UV-Vis and SDS-PAGE (Figure 40b), and retention of binding affinity of ADC 24 to hLRG1 was also demonstrated by ELISA (Figure 40c).
Figure 40 a) Conditions for generation of Magacizumab PD-MMAE ADC 24; b) SDS-PAGE analysis of ADC products. Lane 1 – marker, Lane 2 – Native Magacizumab, Lane 3 – reduced Magacizumab, Lane 4 – Magacizumab PD-MMAE ADC 24, Lane 5 – reduced Magacizumab PD-MMAE ADC 24 (reduction performed with 100 eq. TCEP at 37 °C; c) ELISA showing binding activity of Magacizumab PD-MMAE ADC 24 and Magacizumab to LRG1. ELISA experiments were performed in triplicate (n=3). Data are shown as mean ± SEM.

4.3 Cytotoxicity studies

The in vitro cytotoxic potency of Magacizumab PD-MMAE ADC 24 was evaluated in hLRG1-positive and hLRG1-negative B16F0 cell lines. Initially, both cell lines were exposed to MMAE and exhibited a comparable reduction in cell viability at a similar concentration of the toxic payload, with IC50 values of 0.2 nM and 0.1 nM for hLRG1 positive and negative B16F0 cell lines respectively (Figure 41).
Figure 41 Inhibition of cell proliferation in hLRG1 positive (blue) and hLRG1 negative (grey) B16F0 cell lines with MMAE. Experiments were performed in triplicate (n=3). Data are shown as mean ± SEM.

Following this, the potency of PD-MMAE ADC 24 was evaluated against Magacizumab and vc-MMAE-N$_3$ using the hLRG1-positive cell line. As predicted, Magacizumab alone had little influence on cell viability. Due to the non-internalising nature of the ADC, incubation of ADC 24 alone with cells did not impact cell viability, as release of payload from the ADC requires the presence of an adequate concentration of a specific protease in the extracellular environment (Figure 42). Similarly, vc-MMAE-N$_3$ also had no cytotoxic effect on cells as the absence of dipeptide-cleaving protease prevents the release of free MMAE to exert its cytotoxic effect (Figure 42).
Following the above, a novel assay was designed for determining the potency of the non-internalising ADC 24. In order to mimic the tumour microenvironment and trigger the release of cytotoxic drug from the ADC, serial dilutions of ADC 24 were prepared in culture medium containing activated cathepsin B (Figure 42).\textsuperscript{183} This protease is highly abundant in the extracellular space of solid tumours\textsuperscript{123} and can cleave the dipeptide Val-Cit linkage promoting self-immolative release of free MMAE. As cathepsin B is preferentially active in an acidic environment,\textsuperscript{201} the pH of the culture medium was adjusted accordingly, this also reflects the acidity of the tumour environment.\textsuperscript{202} Gratifyingly, in the presence of exogenous cathepsin B, ADC 24 showed potency against the hLRG1-positive cell line.

The effect of combination therapy on cell viability was also investigated. This involved treating cells with a therapeutic combination comprising the hLRG1-targeting ADC 24 and the chemotherapeutic agent cisplatin. The rationale of this combined therapy approach was to induce tumour cell death by the chemotherapeutic agent, resulting in increased amounts of cathepsin B being released from dead cells, which would amplify the liberation of free drug from ADC 24 and lead to an increased reduction in cell viability. First, the susceptibility of hLRG1-positive and hLRG1 negative B16F0 cell lines to cisplatin was determined (Figure 43). Both cell lines were sensitive to cisplatin,
showing a comparable reduction in cell viability, with IC$_{50}$ values of 10 µM and 18 µM for hLRG1 positive and negative B16F0 cell lines respectively (Figure 43).

![Graph showing inhibition of cell proliferation in hLRG1 positive and negative B16F0 cell lines with cisplatin.](image)

**Figure 43** Inhibition of cell proliferation in hLRG1 positive (magenta) and hLRG1 negative (grey) B16F0 cell lines with cisplatin. Experiments were performed in triplicate (n=3). Data are shown as mean ± SEM.

In the presence of a sub-optimal amount of cisplatin (Figure 43, dotted line), ADC 24 reduced cell viability significantly, affording an IC$_{50}$ value of 15 pM (Figure 42). In comparison to the combination approach, ADC 24 (plus cathepsin B) showed relatively moderate toxicity with IC$_{50}$ values of 0.9 nM. These results demonstrate that the drug release process could be improved with progression of cancer cell death, which is amplified in the presence of an additional chemotherapeutic agent such as in the case of cisplatin.

### 4.4 Conclusion

In view of the fact that angiogenesis occurs infrequently in healthy adult individuals but is a hallmark feature of many solid cancer types, it would be reasonable to develop an ADC that targets the tumour vasculature. In line with this, the expression of LRG1 is limited to disease tissue, where it destabilises tumour vessels and thus primes the endothelium for pathological angiogenesis, making it an ideal target for the development of an ADC. In this chapter, the generation of a novel class of ADC has been described. It
targets the pro-angiogenic protein LRG1 and releases the cytotoxic drug *via* a mechanism that does not rely upon the internalisation of the antibody. The presence of cathepsin B in the tumour environment was ascertained using confocal microscopy and the cathepsin B-cleavable dipeptide linker was selected for development of this ADC alongside the antimitotic agent MMAE as the payload. Using *in vitro* cell viability assays, it was demonstrated that Magacizumab PD-MMAE ADC 24 could release its cytotoxic payload in the extracellular environment. This could be achieved either in the presence of added extracellular cathepsin B, or upon initiation of tumour cell death (caused *in vitro* by treatment of cells with a chemotherapeutic agent) and subsequent release of high concentrations of protease - which would aid cleavage of the dipeptide Val-Cit linkage and release of the anti-mitotic drug MMAE from the ADC.
Chapter 5 In vivo analysis of the hLRG1-targeting ADC

ADC drugs that release their cytotoxic payloads extracellularly, i.e. non-internalising ADCs, offer distinctive therapeutic advantages over ADCs that employ the traditional internalisation route to release their payload within the cell.\textsuperscript{16,124,183} It is often difficult for antibodies to reach all cells within a solid tumour mass (typically <1% of the injected dose of antibody localises to tumours).\textsuperscript{203} Drugs released in the tumour extracellular space may diffuse into tumour cells. The majority of antibodies used to synthesise ADCs target internalising antigens that are situated on the cell surface membrane of tumour cells. Although in principle targeting of internalising antigens should offer an additional level of selectivity for the development of ADC products, in practice, these targets may be suboptimal in terms of in vivo pharmacodelivery, furthermore, antigen loss by the tumour cells may lead to resistance.\textsuperscript{204} The novel ADC prepared in Chapter 4, which is based on the hLRG1-targeting antibody Magacizumab, is potentially a good candidate, as it incorporates the linker-payload combination of approved biopharmaceuticals (Adcetris\textsuperscript{TM}, Polivy\textsuperscript{TM}, Padcev\textsuperscript{TM}) and as the target antigen is highly expressed in a number of human cancers with low expression in healthy tissues.\textsuperscript{166–171}

5.1 In vivo target engagement

Having demonstrated that site-selectively modified Magacizumab (Maga-488 20) retained its ability to bind to its target antigen in vitro via ELISA (see Section 3.1), it was next evaluated whether Magacizumab is able to bind to hLRG1 and localise to tumours in vivo. Human LRG1 knock-in (KI) mice were injected subcutaneously with hLRG1-negative or hLRG1-positive B16F0 mouse melanoma cells (generated previously, see Section 3.3.1) into the lower back and left to develop into tumours. When the tumours became palpable, labelled Maga-488 20 was injected into the peritoneum and left for 4 h. Mice were then sacrificed and the tumours and livers excised for analysis by fluorescence microscopy. Analysis of tumour tissues revealed selective localisation of Maga-488 20 in hLRG1-expressing tumours in contrast to the non-hLRG1 expressing tumours (Figure 44). Furthermore, microscopic analysis of liver tissue revealed no signal
for Maga-488 20 in the liver (Figure 45), indicating that Magacizumab is specifically targeted at the tumour. LRG1 is primarily synthesised by the liver and secreted into circulation,152 however as the dipeptide Val-Cit linker employed in ADC 24 is sensitive to cleavage by lysosomal proteases (e.g. cathepsin B) which are commonly observed to have overexpression and extracellular activity exclusively within the tumour microenvironment,205,206 it was not expected to cause any off-target toxicity.

This is the first-time target engagement of the humanised anti-LRG1 antibody Magacizumab to LRG1 has been demonstrated in vivo in a cancer model. Proof of engagement of Magacizumab to hLRG1-expressing tumours encouraged investigation of the in vivo therapeutic potential of the hLRG1-targeting ADC 24.

**Figure 44** Magacizumab-488 20 accumulates in tumours expressing hLRG1. Sections collected from mice receiving a single injection of Maga-488 at a dose of 100 µg. After AFC 20 was in circulation, tumours were excised, sectioned and subjected to immunofluorescence staining. Blood vessels were stained using anti-CD31 (red). Scale bars, 100 µm.
Figure 45 Magacizumab-488 20 does not accumulate in livers of mice expressing hLRG1. Liver sections were collected from hLRG1 knock-in mice that received a single injection of Maga-488 20 at a dose of 100 µg. Livers were excised from mice bearing hLRG1-positive and hLRG1-negative tumours, sectioned and subjected to immunofluorescence staining. Blood vessels were stained using anti-CD31 (red). Scale bars, 50 µm.

5.2 In vivo therapy studies

Following the favourable results of the in vitro cytotoxicity studies of ADC 24 and the in vivo study demonstrating target engagement of the humanised anti-LRG1 antibody Magacizumab to an experimental hLRG1 expressing tumour, an in vivo study was conducted to evaluate the efficacy of hLRG1-targeting ADC 24 in tumour-bearing mice (Figure 46). A fundamental aspect of the use of ADCs as anti-cancer agents is to limit the potential for off-target toxicity. However, ADCs can exert unexpected dose-limiting toxicities, which can be mediated via any of the components of the drug.207 ADC 24 was therefore compared to standard treatment to ensure that the toxicity for the ADC was equivalent or less than that of standard treatment.207
Hence, a study was conducted where the novel ADC 24 was compared against a number of treatments including a sub-optimal dosing regimen of the native parent antibody Magacizumab and a maximum tolerated dose of the chemotherapeutic agent cisplatin. In order to obtain more insights into the susceptibility of ADC products to proteolytic cleavage, ADC 24 was also investigated in combination with the chemotherapeutic agent cisplatin. The study also included an untreated control. In accordance with most ADC therapy studies, treatment in this study was initiated when tumours were palpable in order to be clinically relevant (Figure 46).

Figure 46 Therapy study to assess ADC 24 in vivo in combination with other treatments. Cells were injected subcutaneously into the lower back of mice. Therapy was initiated when tumours reached 0.1 cm$^3$. Animals were treated by intraperitoneal injection of treatments every 7 days for 3 weeks, and tumour volumes and mouse weights were measured regularly. Mice were sacrificed when tumours reached 1.5 cm$^3$.

Magacizumab and ADC 24 were administered at 20 mg/kg and cisplatin at 2.5 mg/kg via intraperitoneal (i.p.) injection once every 7 days for a total of three doses for mice in the treatment groups. This dosing regimen is in line with other interchain disulfide bridging ADCs.$^{132,208}$ Tumours were measured and mice were weighed regularly throughout the duration of the study. Measurements of tumour volumes showed a significant tumour growth retardation for mice receiving ADC 24, cisplatin and combination therapy (ADC 24 + cisplatin) (Figure 47a, $P<0.01$, two-way ANOVA with Dunnett’s multiple comparison test). Survival analysis reflected this result and revealed that treatment led to
a significant prolongation of survival for mice that received cisplatin (P<0.05), ADC 24 (P<0.01) and ADC 24 + cisplatin (P<0.01) (Figure 47b).

Magacizumab alone resulted in a non-significant reduction in tumour growth (Figure 47a). The lack of effectiveness contrasts with a previous report showing significant tumour inhibition following LRG1 blockade with the parent antibody 15C4. This is most likely due to the sub-optimal dose of 20 mg/kg of Magacizumab used in this study and the less frequent administration. However, ADC 24 administered on its own and in combination with cisplatin gave a pronounced tumour growth delay even after receiving just a single treatment dose, and extended overall survival from 24 days as seen for untreated mice to 30 and 34 days respectively (Figure 47b). These results are comparable to the survival of mice that received the chemotherapeutic agent cisplatin on its own. When mice were treated with a therapeutic combination of ADC 24 and cisplatin, the effect of ADC 24 was amplified, although not significantly. Cisplatin-induced tumour cell death in these mice could have resulted in increased amounts of appropriate proteases (e.g. cathepsin B) being released into the tumour environment, which would then amplify the release of more free MMAE from the non-internalising ADC 24 and lead to an increase in survival. Although all treatments were very well tolerated throughout the duration of the study, with no significant weight loss observed (Figure 47c), alterations in weight were detected in 20-40% of all mice receiving cisplatin either as a monotherapy or in combination with the ADC. These mice displayed between 10% and 15% weight loss (mice were sacrificed if weight loss exceeded >15%) and also appeared to show signs of fatigue which could be attributed to the off-target toxicity caused by cisplatin. Overall, this is an exciting result as this novel ADC exhibited similar performance to an existing chemotherapeutic without the off-target effects associated with non-targeted delivery of cisplatin.
**Figure 47** Therapeutic efficacy of ADC 24 in a mouse melanoma model. 6-11 week old hLRG1 knock-in mice bearing subcutaneous hLRG1-expressing B16F0 tumours were treated intravenously with 20 mg/kg of ADC 24 (n=9), 20 mg/kg of Magacizumab.
(n=9), 2.5 mg/kg of Cisplatin (n=8) or left untreated (n=9). Treatment was performed once every 7 days for a period of three weeks. Therapy was initiated when tumours reached a size of 0.1 cm³. a) Data represents mean tumour volumes, expressed as mean ± SEM (**P<0.01, two-way ANOVA with Dunnett’s multiple comparisons). Tumour growth curves are displayed until day 16, when the first mouse was sacrificed; b) Survival curves of treatment and control groups; prolongation of survival for mice that received cisplatin (*P<0.05), ADC 24 (**P<0.01) and combination therapy (***P<0.01) as determined by the Log rank test; c) Body weight variations of the mice during therapy. No detectable weight loss was observed.

Tumour sections were also probed for apoptosis using the DNA double-strand break marker γ-H2AX, which indicates apoptosis-induced DNA damage. Tumour sections were co-stained with DAPI to enumerate nuclei. The percentage of nuclei with γ- was measured and an increase in apoptosis was observed in ADC-treated mice compared to untreated and Magacizumab-treated tumours. This suggests that the cytotoxic component of the ADC leads to increased cell death in the tumour relative to Magacizumab alone (Figure 48). The accumulation of DNA damage in ADC-treated tumours could also be indicative of a delay in the recruitment of DNA repair proteins as a consequence of microtubule destabilisation caused by MMAE. Indeed it has been shown that stability of microtubules is required for intracellular trafficking of DNA repair proteins in response to DNA damage.²⁰⁹
Figure 48 Analysis of apoptosis using the DNA double-stranded break marker γ-H2AX. DNA double strand breaks detected with antibody against γ-H2AX (red). Scale bar, 50 μm. Data represents percentage of γ-H2AX-positive nuclei, expressed as mean ± SEM (***P<0.01, One-way ANOVA on ranks with Dunn’s multiple comparisons).

Untreated, n= 5; Magacizumab, n=5, ADC 24, n= 5.

Although the Val-Cit-PABC-based linkers have been shown to exhibit long term instability in rodent plasma studies, which may impact rodent-based pre-clinical studies, it is noted that this is often in a way that reduces the ‘true’ efficacy of the ADC, which could explain the lower than expected efficacy of ADC 24. Carboxylesterase 1C (Ces1C), a serine protease, has been identified as the sole enzyme that is responsible for this instability of the linker. Consequently, testing the efficacy of ADC 24 using Ces1C knock-out mice could further improve the tumour survival. Nevertheless, the in vivo therapy studies conducted using the Val-Cit containing hLRG1-targeting ADC 24 demonstrate that the ADC is active in vivo, especially when compared to various control groups. Moreover, it is demonstrated that on this novel target, the ADC exhibited similar efficacy to an existing chemotherapeutic without the off-target effects associated with non-targeted delivery of cisplatin, and that combination therapy could be an interesting strategy to increase the potency of this non-internalising ADC.
ADC 24 was also investigated using a different dosing regimen (Figure 49). This involved initiating treatment before the tumours became palpable, instead of when the tumours reached 0.1 cm³, as evaluated previously and as is common with most ADC studies reported in literature. Hence, efficacy of ADC 24 under this new dosing regimen was evaluated against mice that received no treatment. Due to their observed side-effects in mice (i.e. weight loss, fatigue), treatment groups containing cisplatin were not included in this study. As described previously, ADC 24 was evaluated in C57 hLRG1 KI mice, bearing subcutaneously grafted hLRG1-positive B16F0 tumours. As a control, a group of mice (n=6) received no treatment. Treatment was initiated 4 days after tumour cell inoculation, when tumours were not yet palpable (Figure 49). Mice were dosed at 20 mg/kg via i.p. injection once every 7 days for a duration of three weeks (n=6).

**Figure 49** In vivo assessment of ADC 24 using an alternative treatment schedule. Cells were injected subcutaneously into the lower back of mice. Therapy with ADC 24 was initiated 4 days after tumour cell injection at 20 mg/kg by intraperitoneal injections. Animals were treated every 7 days for 3 weeks, and tumour volumes and mouse weights were measured regularly. Mice were sacrificed when tumours reached 1.5 cm³.

Measurements of tumours in both groups revealed tumour growth retardation for the ADC group, which was superior compared to the untreated group, however this effect was not significant (P > 0.05, Unpaired t test) (Figure 50a). ADC 24 was very well tolerated, with no detectable weight loss, and led to a significant prolongation of survival for mice that received treatment (P<0.05, Log rank test), with the ADC-receiving mice outliving the
untreated mice by 10 days (Figure 50b,c). However, as noted previously, despite exhibiting substantial anti-tumour activity, the hLRG1-targeting ADC 24 was not curative. This could be remedied in future studies by further modification of the dosing regimen, and dosing more frequently in line with the dosing schedules of many of the non-internalising ADCs. Modifications to the dosing regimen would need to be managed so as to limit off-target toxicity while enhancing the anti-tumour efficacy of the ADC. A dose escalation study could also prove useful in determining the maximum tolerated dose of the ADC.
Figure 50 In vivo analysis of ADC 24 in a mouse melanoma model. 6-8 week old hLRG1 knock-in mice bearing subcutaneous hLRG1-expressing B16F0 tumours were treated with 20 mg/kg of ADC 24 (targeting LRG1) or left untreated (n=6 per group).
Treatment was performed once every 7 days for a period of three weeks. Therapy was initiated 4 days after tumour cell inoculation. a) Data represents mean tumour volumes, expressed as mean ± SEM (Unpaired t test); b) Survival curves of treatment and control groups; substantial prolongation for mice which received ADC 24 (*P<0.05, Log rank test). Median survival: untreated = 22 days, ADC 24 = 28 days; c) Body weight variations of the mice during therapy. No detectable weight loss was observed.

5.3 Conclusion

Herein, the direct interaction and therapeutic potential of the humanised antibody Magacizumab against the novel target antigen hLRG1 has been demonstrated in vivo in a cancer model. The ADC developed in this report against this novel target does not rely upon internalisation to release its payload. Current FDA-approved ADCs target tumour cell surface markers and rely on ADC internalisation into the cells for drug delivery. Instead, the approach reported herein relies on targeting the novel extracellular vasculopathic factor LGR1, offering coverage of a wide range of tumours as LRG1 is induced in disease lesions where it disrupts the angiogenic process through interfering with vessel maturation. B16F0 tumour-bearing wild type mice were treated with Magacizumab, ADC, cisplatin or a combination of both ADC and cisplatin. As monotherapies, ADC and cisplatin each elicited a reduction in tumour volume and increase in survival. In combination, however, the effect was potentiated, and survival analysis revealed increased survival of mice receiving the combination of treatments. Although the ADC was not curative, which could in part be explained by the non-internalising nature of LRG1, and potentially be remedied by more frequent dosing in future studies, this is the first experimental demonstration that an extracellular hLRG1-targeting ADC synthesised using the pyridazinedione scaffold can be used to mediate antitumor activity in vivo. The ADC presented here allows the progressive amplification of drug release, presumably as tumour cell death mediates cathepsin B release to the surrounding tissue. This effect is amplified upon administration of a therapeutic combination of ADC and chemotherapeutic agent cisplatin. Overall, the proof-of-concept findings of this study provide novel opportunities for cancer therapy.
Chapter 6 Generation of Magacizumab Fab

6.1 Fab fragments

It is possible to selectively digest the antibody molecule into fragments that have discrete characteristics. Fab fragments (ca. 50 kDa) are a type of antibody fragment that have received a lot of attention due to their small size and ease of synthesis. At present, there are at least 17 known antibody fragments that have either been approved for marketing or are in clinical trials for a range of indications. Three Fab fragments have been approved by the FDA for marketing in the US (Table 3), abciximab (ReoPro®), ranibizumab (Lucentis®), and certolizumab pegol (Cimzia®).

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Protein format</th>
<th>Major indication</th>
<th>Year of approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReoPro® (Abciximab)</td>
<td>GPIIb/IIIa on platelets</td>
<td>Chimeric Fab</td>
<td>Cardiovascular disease</td>
<td>1994</td>
</tr>
<tr>
<td>Lucentis® (Ranibizumab)</td>
<td>VEGF-A</td>
<td>Humanised Fab</td>
<td>Wet Age-related macular degeneration (AMD)</td>
<td>2006</td>
</tr>
<tr>
<td>Cimzia® (Certolizumab pegol)</td>
<td>TNF-α</td>
<td>Pegylated humanised Fab</td>
<td>Rheumatoid arthritis</td>
<td>2009</td>
</tr>
</tbody>
</table>

*Table 3 FDA-approved Fab fragments available on the market.*

Fabs consist of the variable regions of both heavy and light immunoglobulin chains (Figure 5, Section 1.4.2) and therefore retain the capacity to bind to the antigen. They also lack a non-specific binding Fc region and contain a native single disulfide bond that can be site-selectively modified. Due to their smaller size, Fab fragments have several characteristics that offer them superiority over their full-length counterparts, such as improved tissue penetration and broader biodistribution. Furthermore, the smaller size of these monovalent fragments permits them to be manufactured in microbial systems such as yeast or *E. coli* making production cheaper and faster. The use of Fabs provides access to highly desirable antibody fragments that can be site-selectively modified, obviates the requirement for any protein engineering, and can exploit the large number of commercial full antibodies that have already been developed against multiple targets as they can be prepared from native full antibodies.
6.1.1 Digestion of Magacizumab to form Magacizumab Fab

Antibody fragments can be generated in a facile manner by the enzymatic digestion of full-length antibodies.\textsuperscript{62,138,216} When IgG molecules are incubated with the enzyme papain, usually in the presence of a reducing agent as the enzyme is inactive in its oxidised form, papain cleaves in the upper hinge region of the antibody.\textsuperscript{219} This yields two individual Fab fragments and one Fc fragment which can be separated by using protein A purification (Scheme 23). Protein A purification relies on the specific and reversible binding of the antibody Fc region to an immobilised protein A ligand and allows for purification of the Fab fragment. Given the inherent advantages of antibody Fab fragments over their full-length counterparts, Fab fragments of Magacizumab were to be prepared, and the route of enzymatic digestion using papain was to be adopted.

![Scheme 23 Fab generation by papain digestion and protein A purification.](image)

Initially the digestion of Magacizumab to its corresponding Fab fragment was appraised using a papain digestion protocol where the digestion of Magacizumab was performed using a thiol-free protocol. Papain was pre-reduced/activated with reducing agent DTT, at an enzyme/IgG ratio of 1/10 (wt/wt) before incubating with Magacizumab for different amounts of time. At all incubation times tested, the protocol yielded undigested Magacizumab as determined by SDS-PAGE (Figure 51).
Magacizumab was subsequently reacted with 1/50 and 1/10 (enzyme/IgG ratio, wt/wt) of immobilised papain in the presence of a different reducing agent; Cysteine-HCl. At high concentration of enzyme (1/10 papain, enzyme/IgG ratio, wt/wt) the digestion of Magacizumab yielded a Fab fragment, determined by SDS-PAGE. Following this, Magacizum Fab (MagaFab) was isolated from the resultant mixture via protein A purification (Scheme 24) with a yield of 11%. Although low yielding, analysis of the digest by SDS-PAGE and LCMS revealed highly pure MagaFab as the sole product (Figure 52).

Scheme 24 Magacizumab Fab (MagaFab) generation by papain digestion and protein A purification. Reagents and conditions: (i) Papain (1/10, papain/Magacizumab, wt/wt), Cysteine buffer pH 7.0, 37 °C, 24 h.
6.1.2 Antigen binding activity of MagaFab: ELISA

In order to determine whether the antigen-binding activity to hLRG1 was impacted by digestion of Magacizumab to MagaFab, an ELISA was performed against full length Magacizumab. MagaFab 26 was prepared freshly before the study and evaluated against undigested Magacizumab 15. Binding was assessed by measuring absorbance at 450 nm and plotting the values against the antibody concentration, to obtain a standard curve. Gratifyingly, MagaFab was shown to have comparable binding activity to its full-length counterpart (Figure 53). These tests were performed in triplicate and the results gave good indication that digestion of Magacizumab to form Fab did not notably affect its binding to hLRG1. Furthermore, binding affinity of MagaFab was assessed by surface plasmon resonance (SPR) analysis and ELISA (using the Langmuir binding isotherm to quantify the dissociation constant ($K_D$); performed by David Kallenberg, Department of Cell Biology, UCL Institute of Ophthalmology). Data from SPR analysis shows that while MagaFab has a 10-fold reduction in binding affinity, it still binds hLRG1 with high affinity (Table 4). The binding affinity of MagaFab was found to be comparable to Magacizumab binding to human LRG1 by ELISA (Table 4).
Figure 53 Binding activity to LRG1 of MagaFab 26 compared with full-length Magacizumab 15. Experiments were performed in triplicate (n=3). Data are shown as mean ± SEM.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>K_d - ELISA</th>
<th>K_d - SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fab</td>
<td>Human LRG1</td>
<td>5.78 × 10^{-10} M</td>
<td>4.39 × 10^{-9} M</td>
</tr>
<tr>
<td>Magacizumab</td>
<td>Human LRG1</td>
<td>1.45 × 10^{-10} M</td>
<td>2.81 × 10^{-10} M</td>
</tr>
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</table>

Table 4 Table displaying comparison of the affinity of Magacizumab Fab and Magacizumab to human LRG1 by ELISA (using the Langmuir binding isotherm to quantify the dissociation constant (K_d)) and surface plasmon resonance (SPR). These were performed by David Kallenberg, Department of Cell Biology, UCL Institute of Ophthalmology. (Manuscript in preparation).

6.2 Fab and angiogenesis

Having demonstrated that after digestion of full-length Magacizumab, the resultant antibody fragment retains binding to LRG1, the functional integrity of MagaFab was then assessed. The impact of MagaFab on LRG1-mediated angiogenesis was investigated using *in vitro*, *in vivo* and *ex vivo* models of angiogenesis.
6.2.1 The impact of MagaFab on angiogenesis: *In vitro* model

In the *in vitro* model of angiogenesis, human endothelial cells were cultured with human fibroblasts. The fibroblasts secrete a complex extracellular matrix, which becomes remodelled into a 3D environment over 7-14 days. During this time, the endothelial cells reorganise to form tubules that resemble microvessels formed during angiogenesis. The assay is responsive to factors that either stimulate or inhibit angiogenesis. Previously, a Matrigel human umbilical vein endothelial cell (HUVEC) tube-formation assay performed by Wang *et al*.\(^{150}\) showed that supplementation of media with recombinant human LRG1 significantly increased tube formation and branching, whereas an use of anti-LRG1 antibody showed a significant blockade of tube formation. The inhibitory effect of Magacizumab on tube formation has also recently been demonstrated.\(^{173}\) In light of these findings, the tube formation assay was performed using MagaFab to determine the functional integrity of the truncated antibody *in vitro*. In order to draw valid conclusions from this assay, an appropriate Fab control had to be established. A commercially available Fab control was purchased from Novus Biologicals. Mass spectrometry and SDS-PAGE analyses were used to confirm the identity of the control Fab and an ELISA was performed to assess binding of the control Fab to hLRG1 (Figure 54). The control Fab did not display any binding to LRG1 and therefore proved to be an appropriate control for the functional assays of angiogenesis.
For the co-culture assay, endothelial cells were cultured for 11 days during which they were treated with control Fab as well as increasing concentrations of MagaFab (70 pM, 700 pM and 70 nM) diluted in growth medium (Figure 55a). On day 11, cells were fixed and stained with an antibody against human CD31, an integral membrane glycoprotein that is concentrated at the junctions between endothelial cells, making it an ideal marker for vascular analysis. In the co-culture assay, angiogenesis was assessed by measuring two parameters: sprouting expressed as total tubule length and branching expressed as the number of junctions. A significant reduction in tubule growth and branching was observed in the wells treated with MagaFab compared to control Fab (Figure 55). The impact of MagaFab on tubule branching and length can be seen in Figure 55b. There was a modest but significant decrease in tube formation and branching following treatment with 700 pM MagaFab compared to Fab control. A similar reduction in growth and branching could also be seen when the concentration of Fab was increased to 70 nM. These results demonstrate the functional integrity of Fab in an in vitro setting and show
that blocking the activity of hLRG1 using MagaFab increases vessel normalisation by reducing vascular growth.

**Figure 55** (a) A schematic diagram of the co-culture assay. Cultured cells were treated with various concentrations of MagaFab and control Fab over 11 days. On day 11, cells were fixed and stained with anti-CD31 antibody; (b) Comparison of tubule growth and branch formation from cells treated with control Fab and Magacizumab Fab. All values are expressed as mean ± SEM. of n = 3 independent experimental groups. *P<0.05; **P<0.01; (c) Representative phase-contrast images of vessels treated with Fab/control and stained with anti-CD31 antibody.

### 6.2.2 The impact of MagaFab on angiogenesis: Ex vivo model

The capacity of MagaFab to influence angiogenesis was validated in an ex vivo setting via a foetal metatarsal explant assay using tissues extracted from hLRG1 knock-in (KI) mice. This assay provides a unique tool for studying angiogenesis in a physiologically relevant environment that is representative of in vivo blood vessel formation. This assay permits evaluation of the effects of various external agents on angiogenesis, such as antibodies, growth factors, inhibitors etc. Therefore, in order to investigate whether MagaFab, similar to its full-length counterpart, had any impact on angiogenesis, an ex vivo embryonic mouse metatarsal assay was performed. Metatarsal bones were harvested from mouse embryos at day 17.5 (harvesting performed by Dr Camilla Pilotti, Department of Cell Biology, Institute of Ophthalmology, UCL) and cultured in wells pre-coated with gelatine, for 11 days. The concentrations of MagaFab used for this assay were chosen based on the results of the metatarsal angiogenesis assay previously performed with full length
Magacizumab, where a dose-dependent reduction in angiogenesis could be observed with increasing concentrations of Magacizumab.\textsuperscript{173} Hence, metatarsal bones were treated with Maga\textsubscript{Fab}, at concentrations equivalent to those used in the Magacizumab study (6 nM, 13 nM, 33 nM), and with control Fab, diluted in growth medium supplemented with foetal bovine serum (FBS) and penicillin/streptavidin. After this time, metatarsals were fixed and stained with anti-CD31 antibody, an endothelial cell marker, as demonstrated in Figure 56a. Lastly, Figure 56b shows the quantification of angiogenesis. Vessel outgrowth and branching in explanted metatarsals showed a 46% reduction when treated with 6 nM Fab, compared to controls. When treated with 13 nM and 33 nM of Fab, metatarsals displayed a 50% and 44% reduction in vessel branching and growth respectively. This is in contrast to assays performed with full length Magacizumab where a dose dependent reduction in angiogenesis was observed at these concentrations, with a maximum reduction of 40% is seen upon treatment with 33 nM of Magacizumab. In the case of Maga\textsubscript{Fab}, maximal effect can be observed at a lower concentration. This might indicate that at lower concentrations Maga\textsubscript{Fab} is more effective at normalising vasculature compared to the full-length antibody. Nevertheless, these results are promising and provide compelling evidence that not only does the Fab retain binding activity to hLRG1, but also has a significant functional impact \textit{ex vivo}. 
Figure 56  a) Vessel outgrowth in the metatarsal assay is attenuated by MagaFab; b) Quantification of vessel growth and branching from metatarsals treated with control Fab and Magacizumab Fab (6 nM, 13 nM and 33 nM) shows reduced angiogenesis in the latter. All images shown are representative and values are expressed as mean ± SEM. of n = 3 independent experimental groups. (One-way ANOVA, ****P<0.0001, **P<0.01.

6.2.3 The impact of MagaFab on angiogenesis: In vivo model

Having demonstrated that MagaFab inhibits angiogenesis using in vitro and ex vivo models, the therapeutic potential of MagaFab was evaluated in vivo by studying vascular leakage which is a primary defect associated with diseased vessels in several forms of retinal vascular disease. Wet AMD is characterised by choroidal neovascularisation (CNV)\textsuperscript{221} and is associated with leakage of fluid and blood from abnormal vessels, features that are reproducible in the well-characterised laser-induced CNV mouse model.\textsuperscript{222} The CNV model consists of a laser injury to the Bruch’s membrane resulting in the growth of new blood vessels from the choroid into the subretinal space, which then leads to the development of a lesion at the site of laser injury (Figure 57). The progression of angiogenesis post-laser induction is similar to that seen in the neovascular form of AMD (wet AMD). This model has been extensively used to study molecular mechanisms
responsible for driving angiogenesis as well as for the evaluation of efficacy of new drugs for future use.\textsuperscript{223} Furthermore, a prerequisite for many of the anti-angiogenic drugs entering human trials involves intravitreal administration of these agents into mouse CNV models and indeed, the first generation of anti-VEGF drugs used to manage wet AMD today were developed in this way.\textsuperscript{224}

\textbf{Figure 57} Schematic representation of laser-induced choroidal neovascularisation (CNV). Laser injury to the Bruch’s membrane causes sprouting of vessels into the sub-retinal space, leading to the formation of a lesion at the site of the laser injury. (RPE - retinal pigment epithelium).

It has been shown that upon induction of CNV lesions in the eyes of a mouse, there is a significant increase in LRG1 expression in both the retina and retinal pigment epithelium (RPE)/choroid.\textsuperscript{150} This makes it a good model to study LRG1-mediated angiogenesis. Hence, the effect of intravitreal delivery of Maga\textsubscript{Fab} on blood vessel leakage was investigated using the mouse model of laser-induced CNV.

CNV was induced in human \textit{LRG1} KI mice and fundus fluorescein angiography (FFA) was used to determine the permeability of the lesion. Mice were anaesthetized and
received intravitreal injections of various doses of either MagaFab or control Fab, immediately following the laser burn. After 7 days (to allow the vascular lesions to form) the mice were again anaesthetized and examined by FFA. This method involves injection of a fluorescein dye immediately before imaging the eye. Early phase FFA images (taken 90 s after injection) reflected the degree of vascularization at the site of the lesion, while late phase images (taken 7 min after injection) demonstrated fluorescein leakage at the lesion, indicating hyperpermeability at the lesion (FFA was performed by David Kallenberg, Department of Cell Biology, Institute of Ophthalmology, UCL). Comparison of the areas of fluorescence in both the early phase and late phase images permitted quantification of the leakage ratio (Figure 58). Compared to eyes injected with control Fab, intravitreal injection of as little as 0.20 µg of MagaFab showed a significant reduction in vascular leakage. Hence these results show that intravitreal delivery of MagaFab leads to improved vessel function by decreasing permeability of the vessels, and highlights that the abnormal architecture of the vessels is associated with an increased concentration of LRG1.

Figure 58 Fundus fluorescein angiographic (FFA) analysis of choroidal neovascularization (CNV) lesions following an intravitreal injection of MagaFab. a) A schematic diagram of the laser-induced CNV mouse model. b, c) CNV lesions were analysed by FFA at day 7 after treatment. Images were taken 90 s after injection of dye
(early FFA) and 7 min after injection of dye (late FFA) and data are presented as leakage ratio. CNV leakage is significantly reduced in eyes treated with 0.2 µg and 1.0 µg MagaFab compared to IgG controls (one-way ANOVA followed by Tukey’s multiple comparison test, *P<0.05 and **P<0.01). Arrows indicate the lesions of CNV. All images shown are representative and values are expressed as mean ± SEM. of n = 3 independent experimental groups.

Thus, using in vivo, ex vivo and in vitro models of angiogenesis, these results demonstrate that fragmentation of Magacizumab to its corresponding Fab fragment does not impact its functionality, and in fact, the smaller size of Fab may be responsible for a more pronounced effect on vascular normalisation compared to the full-length antibody. These findings suggest MagaFab would be a suitable candidate as an anti-angiogenesis agent.

6.3 Modification of MagaFab

6.3.1 Reduction study

As with full-length Magacizumab, a reduction study was carried out with MagaFab 26 to investigate the amount of reducing agent that is necessary to reduce the single disulfide bond available (Scheme 25). SDS-PAGE revealed that 100 equivalents of TCEP in borate buffer pH 8.0 with 1 mM EDTA at 37 °C, was sufficient to fully reduce the single disulfide bond in 4 h, thus revealing the heavy chain (observed mass 23887 Da) and light chain (observed mass 23576 Da) of reduced MagaFab 27 (Figure 59).

![Scheme 25 Reagents and conditions for MagaFab reduction: 100 eq. TCEP for 4 h, BBS pH 8.0, 37 °C.](image)
Figure 59  a) SDS-PAGE analysis of MagaFab reduction by TCEP in borate buffer pH 8.0 at 37 °C for 4 h. M = molecular weight marker; Lane 1 – native MagaFab 26; Lane 2 – Reduction of MagaFab using 100 eq. of TCEP to yield heavy and light chains; b,c) Non-deconvoluted and deconvoluted LCMS data for b) heavy and c) light chain of reduced MagaFab 27.

6.3.2 Conjugation study

In accordance with the conjugation studies performed using full-length Magacizumab, a step-wise approach was also adopted for the functional rebridging of MagaFab with PD 8. After removal of excess TCEP by diafiltration, it was shown that 5 equivalents of ‘clickable’ PD 8 was sufficient to rebridge MagaFab 26 to afford the desired MagaFab conjugate 28 (Scheme 26). The conjugation was confirmed by SDS-PAGE and UV-Vis analysis (Figure 60b).
Scheme 26 a) Sequential reduction and rebridging of MagaFab, followed by b) SPAAC ‘click’ functionalisation with Alexa Fluor™ 488 azide.

Having successfully incorporated the PD moiety into the Fab disulfide, excess unbound small molecules were removed by diafiltration and Alexa Fluor™ 488 azide was then ‘clicked’ onto the modified Fab fragment 28 to yield Fab-fluorophore conjugate 29 (Figure 60, Scheme 26). Confirmation of a fluorophore loading of ~1 was obtained by UV-Vis spectroscopy. Additionally, Sulfo-Cyanine5.5 azide was successfully ‘clicked’ on to the PD-rebridged Fab fragment 28 to yield conjugate 30. Confirmation of successful ‘clicking’ of the azide fluorophore was obtained by UV-Vis spectroscopy, again revealing a fluorophore-to-Fab ratio (FFR) of ~1 (Figure 60).
Figure 60 a) SPAAC ‘click’ functionalisation of functionally rebridged MagaFab 26 with Sulfo-Cyanine 5.5 to form conjugate 30; b) SDS-PAGE of a PD 8 modified MagaFab by sequential protocol. M – marker; Lane 1 – Native MagaFab 26; Lane 2 – Rebridged MagaFab 28; Lane 3 – MagaFab 29 click-functionalised using Alexa Fluor™ 488 Azide; Lane 4 – MagaFab 30 click-functionalised using Sulfo-Cyanine 5.5 azide.

6.3.2.1 Antigen-binding activity of modified Fab: ELISA

To appraise the impact of interchain disulfide modification on LRG1 binding, the binding profile of MagaFab conjugates 29 and 30 was evaluated against unmodified MagaFab 26 using ELISA as previously described (Section 6.1.2). Encouragingly, comparable binding affinity to LRG1 was observed (Figure 61). This indicates that site-selective modification does not impact the affinity of the modified fragment to hLRG1, and therefore permits further use of the conjugates in in vivo studies.
Figure 61 Binding activity to LRG1 of MagaFab 26 compared with MagaFab 29 and MagaFab 30, click-functionalised using Alexa Fluor™ 488 azide and Sulfo-Cyanine5.5 respectively. Experiments were performed in triplicate (n=3). Data are shown as mean ± SEM.

6.3.3 In vivo imaging analysis of conjugates

In order to appraise the binding potential of MagaFab conjugates to LRG1 in vivo, CNVs were induced in human LRG1 knock-in (KI) and knock-out (KO) mice (negative control). One week after the laser injury animals received intravitreal injections of freshly prepared MagaFab-Cy5.5 conjugate 30; 3 h post-injection the mice were culled. The dissected RPE-choroid were stained for Claudin-5 (endothelial cells) and counterstained with Dapi (nuclear stain). Confocal microscopy (Figure 62) showed binding of Fab conjugates to LRG1 in KI mice around the claudin-5 positive vascular lesions. LRG1 binding was not observed in the Lrg1 KO mice, demonstrating specificity of the conjugated Fab for hLRG1. Significantly, this demonstrated that attachment of a
fluorophore to the Fab via site-selective modification of the single interchain disulfide bond enabled visualisation of MagaFab target engagement in vivo, with the Fab sequestered exclusively to the sites of neovascularisation.

Figure 62 Imaging LRG1 using a site-selectively modified Fab fragment against LRG1 (conjugate 30). Eyes of human LRG1 knock-in (KI) and Lrg1 knock-out (KO) mice were lasered and the lesions were allowed to grow for 7 days. After this time, eyes received intravitreal injections of MagaFab conjugate 30 (LRG1; red). Once fixed, eyes were stained for Claudin-5 (endothelial cells; green) and Dapi (nucleus; blue). Scale bar, 30 µm.
6.4 Conclusion

Given that antibody Fab fragments lack potentially inflammatory Fc domains and may also be delivered at a higher molar dose due to their lower molecular weight, the Fab fragment of Magacizumab was prepared using papain digestion of the full-length antibody. Investigation of the affinity of MagaFab by ELISA demonstrated that MagaFab and Magacizumab exhibited indistinguishable binding characteristics to LRG1, and that LRG1 binding by MagaFab was unaltered upon targeted conjugation of a fluorophore azide. In functional assays in hLRG1 KI mice, comparable dose-dependent inhibition of vascular leakage to that observed for Magacizumab was observed. Furthermore, injection of MagaFab-Cy5.5 into mouse eyes with laser-induced lesions revealed that target engagement occurs in vivo, with the Fab sequestered exclusively to the sites of neovascularisation. MagaFab was also effective in the mouse foetal metatarsal assay, using bones from hLRG1 KI mice a significant inhibition of tubule growth and numbers of junctions was observed. In summary, in this chapter the development and characterisation of the Fab fragment of Magacizumab has been reported that inhibits both angiogenesis and vascular leakage in ex vivo and in vivo mouse models, without any detectable toxicity or inflammatory reaction in the latter.
Chapter 7 Conclusions and future work

7.1 ADC work

7.1.1 ADC work: Summary

ADCs have the potential to improve the treatment of tumours over conventional chemotherapy as they allow the use of toxins with orders of magnitude greater potencies to be delivered in a selective manner. Current FDA-approved ADCs target tumour cell surface markers and rely on ADC internalisation into the cells for drug delivery. There are however, a number of non-internalising ADCs in clinical trials that do not rely upon ADC internalisation to exert their anti-tumour activity. Consequently, the approach reported herein relies on targeting the novel extracellular vasculopathic factor LGR1, offering coverage of a wide range of tumours as LRG1 is induced in disease where it causes disruption of angiogenesis through interfering with the process of vessel maturation. It is well understood that in order for ADCs to deliver their full potential, sophisticated conjugation strategies to connect the drug to the linker are required. Pyridazinediones (PDs) can functionally rebridge cysteine residues liberated upon reduction of interchain disulfide bonds and were used as antibody conjugation method in this project in view of their favourable properties in terms of reproducibility, homogeneity, serum stability and exemplification in vitro and in vivo. By applying the disulfide-rebridging PD scaffold to the hinge-stabilised hLRG1-targeting IgG4 antibody Magacizumab, it was demonstrated that this ADC retains binding post-modification, displays excellent serum stability and is effective in in vitro cell viability assays.

LRG1 is abundantly present in the tumour microenvironment and continuously secreted by tumour cells making it easily accessible which can greatly improve accumulation of the macromolecular ADC at the site of disease. In this work, it is demonstrated LRG1 and Magacizumab-LRG1 are predominantly non-internalising, and that a Magacizumab-fluorophore conjugate localises at the tumour site in an in vivo model. The work shows that upon binding to the novel target LRG1 in the tumour environment, the ADC liberates its toxic payload and shows, in vivo, enhanced tumour activity relative to antibody alone and similar anti-tumour activity when compared against standard chemotherapy but with fewer side-effects. The ADC presented here allows the
progressive amplification of drug release, which is linked to the antibody via a cleavable dipeptide linker, presumably upon being metabolised by appropriate proteases (e.g. cathepsin B) which are released into the surrounding tissue upon induction of tumour cell death. This effect is amplified upon administration of a therapeutic combination of ADC and cisplatin.

Figure 63 Illustration of the mechanism of action of the non-internalising ADC targeted against LRG1 developed in this project. Upon ADC binding to LRG1, the drug, which is linked to the antibody via a cleavable dipeptide linker, is progressively released upon being metabolised by appropriate proteases (e.g. cathepsin B) which are released into the surrounding tissue upon induction of tumour cell death.

7.1.2 ADC work: Significance

In brief summary, in this work is described the development of a novel non-internalising ADC against a secreted, non-cell surface-binding protein that is present abundantly in the microenvironment of many tumours. Current ADC approaches are generally limited to efficiently internalising receptors, which are typically only overexpressed in a subset of solid tumour patient populations. The key impact of this work stems from facilitating and inspiring other researchers to expand their scope of thinking of what is an appropriate ADC target to include non-internalising proteins that are present in high abundance in the
extracellular matrix of solid tumours. Exploring fundamentally new types of targets such as these could have a significant impact in the field as having enough appropriate targets for ADC-based cancer treatments remains one of the, if not most, critical bottlenecks in the field. Enabling such targets to be applicable in ADC therapy, as is exemplified in this thesis from a ground-level up of appraising a new target, will thus have fundamental consequences. Overall, the proof-of-concept findings of this study provide novel opportunities for cancer therapy. They are especially significant in the context of the need for finding novel ADC targets (in general) but are particularly pertinent in this study as the target exploited here is a common feature of virtually all types of aggressive solid cancers (i.e. LRG1 in abnormal angiogenesis).

7.1.3 ADC work: Limitations

This work explores the efficacy of the hLRG1-targeting ADC 24 using only a single model; the B16F0 mouse melanoma subcutaneous graft model. In previous work, it has been demonstrated that Lrg1 transcript is detected within the tumours of the LLC Lewis lung carcinoma subcutaneous graft model, the KPC model of pancreatic ductal adenocarcinoma (PDAC), and models of colorectal cancer (CRC). As such, the in vivo therapy analysis of ADC 24 should be expanded to include these models.

Although ADC 24 was well tolerated in in vivo therapy studies, the distribution of the ADC has not explicitly been determined. It is therefore difficult to comment on the off-target toxicity caused with the ADC with certainty. As such, it would be important to conduct a biodistribution analysis, which would involve radiolabelling of the antibody construct and injecting in mice to see where ADC 24 localises in mice, and ascertain the off-target toxicity of the ADC.

One of the drawbacks of the use of the Val-Cit-PABC-based linkers, despite their excellent stability in human plasma, is their long term instability in rodent plasma studies, owing exclusively to the presence of Ces1C in rodents. It is noted that this is often in a way that reduces the ‘true’ efficacy of the ADC, which could explain the lower than expected efficacy of ADC 24. Consequently, testing the efficacy of ADC 24 using Ces1C knock-out mice could further improve the tumour survival.
7.1.4 ADC work: Future outlook

Typical treatment plans for the treatment of cancers in the clinic consist of administering repeated cycles of drug at as high a dose as possible, without causing unacceptable toxicity. There exists a clear dose-response relationship between treatment and tumour regression in preclinical studies (e.g. in mice) and in the clinical setting. It is therefore important to conduct a dose-escalation study in order to determine the maximum tolerated dose, which is the result of a balance between efficacy and toxicity. Hence an important next step in this project would be to conduct a dose-response study using ADC 24 in mouse models of human B16F0 tumours in order to establish the optimal dose and/or schedule of ADC 24 needed to attain regression of tumour growth in vivo.

It has long been recognised that tumours have the ability to evade immune recognition by eliciting powerful immunosuppressive signals that prevent an effective immune response. In recent years there has been interest in reversing this negative immune response regulation through the use of immune checkpoint inhibitors such as anti-PD1. There are now a variety of ongoing clinical trials of combinations of ADCs, including Kadcyla™ and Adcetris™, with antibodies that interfere with the PD-1–PD-L1 interaction. Recent findings reported by the Moss and Greenwood groups demonstrate that LRG1 antibody blockade results in profound enhancement of immune co-therapies, namely the immune checkpoint inhibitor anti-PD1. Given the observed functional efficacy of ADC 24 in the mouse models of cancer, and in view of these findings it would be interesting to employ a dual targeting approach for the targeting of hLRG1 (via the use of ADC 24) and PD-1, through combination therapy.

7.2 Fab work

7.2.1 Fab work: Summary

In addition to cancers, upregulation of angiogenesis is also a prominent feature of the neovascular form of AMD (also referred to as wet AMD) and diabetic eye disease. This has driven the search for, and development of, anti-angiogenic therapies for the treatment of the pathological changes to the vasculature of the eye that are characteristic of such conditions. For clinical application in ophthalmology there are advantages in using Fab fragments rather than full-length antibodies. Accordingly, as the eye is a site of immune
privilege it may be especially sensitive to innate immune responses so Fab fragments, which lack the Fc portion, will prevent potential activation through Fc receptor engagement. In addition, the lower molecular weight of Fab fragments may theoretically enable them to be delivered at a higher molar dose which, in spite of less favourable pharmacokinetics, will prolong the presence of an effective dose within the eye. In this work, MagaFab was generated and it was observed that it retained the binding affinity of the full-length parent antibody and was functionally efficacious in experimental models of angiogenesis. Reports that LRG1 expression is elevated in the vitreous of patients with wet AMD and diabetic eye disease\textsuperscript{150,233,234} provide a rationale for taking MagaFab into clinical trials in these patient groups.

7.2.2 Fab work: Future outlook

Given the observed functional efficacy of the Fab fragment of Magacizumab in the mouse models of retinal vascular disease, one can also envisage the benefits of employing a dual targeting approach for the targeting of LRG1 and VEGF, since they represent distinct signalling pathways involved in driving neovascularisation. This can be achieved either through combination therapy or the generation of a bispecific.\textsuperscript{235} A precedent for this strategic approach exists in faricimab, a bispecific that targets Ang2 and VEGF, and that is currently in clinical trials in diabetic macular edema.\textsuperscript{236,237}

It has been shown that the combination of anti-mouse LRG1 (15C4) and anti-VEGFR2 (DC101) in CNV in wild-type mice resulted in a greater reduction in lesion volume as compared to single treatment.\textsuperscript{150} With this as the premise, and using the dibromopyridazinedione scaffold for the construction of homogeneous bispecific antibody-conjugates as reported by Maruani et al.,\textsuperscript{238} it would be interesting to generate a Fab fragment of the full-length anti-mouse VEGFR2 (DC101) and combine this with the Fab fragment of anti-human LRG1 Magacizumab (MagaFab) to form a bispecific, (Figure 64) which could then be tested for efficacy by inducing CNV in human \textit{LRG1} knock-in mice. These mice are a good model to test the bispecific as they express human LRG1 and mouse VEGFR-2 to which MagaFab and the Fab of DC101 can be targeted respectively.
Figure 64 Pyridazinedione-based method for the generation of a dually functionalised bispecific, consisting of the Fab fragment of the anti-hLRG1 antibody Magacizumab and the Fab fragment of the anti-mouse VEGFR2 antibody DC101.

Furthermore, progress with the generation of Fab has resulted in an industrial collaboration for the large-scale generation of Fab. The generation of the Fab fragment of Magacizumab has also opened up the avenue for structural studies of the Fab-antigen complex and this has resulted in a collaboration with the group of Hartmut Luecke at the University of Oslo. Recently, they have been able to successfully determine the high-resolution crystal structure of Magacizumab Fab both on its own (Figure 65) and in complex with a 15 amino acid peptide that corresponds to the LRG1 epitope (Figure 66). Due to the glycosylation of the LRG1 protein, which may cause problems with crystallisation, efforts are underway to obtain LRG1 in a deglycosylated form in order to obtain a crystal structure of the Fab in complex with LRG1.
**Figure 65** Structure of MagaFab obtained by X-ray crystallography (data obtained by the Luecke group in Oslo).

**Figure 66** Superposition of LRG1 model (green) over the 15-mer peptide (violet) and MagaFab (orange), including the 90° view. The star represents a flexible and exposed loop of the Fab that may be involved in recognition of LRG1. Adapted from Gutiérrez-Fernández et al., (Luecke group, Oslo), manuscript in preparation.
Appendix A Experimental

General Experimental

All chemical reagents were purchased from Sigma Aldrich, ThermoFisher Scientific, Molecular probes, Lumiprobe, Alfa Aesar, Acros, New England Biolabs and Medchem Express. Compounds and solvents were used as received. Petrol refers to petroleum ether (b.p. 40–60 °C). Chemical reactions were monitored using thin layer chromatography (TLC) on pre-coated silica gel plates (254 μm) purchased from VWR. Flash column chromatography was carried out with pre-loaded GraceResolv\textsuperscript{TM} flash cartridges on a Biotage\textregistered Isolera Spektra One flash chromatography system (Biotage\textregistered). \textsuperscript{1}H NMR spectra were obtained at 600 MHz or 700 MHz. \textsuperscript{13}C NMR spectra were obtained at 150 MHz or 175 MHz. All results were obtained using Bruker NMR instruments, the models are as follows: Avance III 600, Avance Neo 700. All samples were run at the default number of scans and at 21 °C. Chemical shifts (δ) for \textsuperscript{1}H NMR and \textsuperscript{13}C NMR are quoted on a parts per million (ppm) scale relative to tetramethylsilane (TMS), calibrated using residual signals of the solvent. Where amide rotamers are the case, and when possible, only the chemical shifts of the major rotamer has been assigned and areas underneath all rotameric peaks have been considered for the integral intensity calculations. Coupling constants (J values) are reported in Hertz (Hz) and are reported as J_{H-H} couplings between protons. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR spectrometer operating in ATR mode. Melting points were measured with Gallenkamp apparatus and are uncorrected.
Chemical products

Di-tert-butyl-1-methylhydrazine-1,2-dicarboxylate

To a solution of methyl hydrazine 1 (1.14 mL, 21.7 mmol) in IPA (16 mL), was added drop wise di-tert-butyl dicarbonate (11.85 g, 54.3 mmol) pre-dissolved in CH$_2$Cl$_2$ (12 mL) over 30 min. The reaction was then stirred at 21 °C for 16 h. Following this, the solvents were removed in vacuo and the crude residue purified by flash column chromatography (0% to 20% EtOAc/petrol) to afford di-tert-butyl-1-methylhydrazine-1,2-dicarboxylate 2 (4.48 g, 18.2 mmol, 84%) as a white solid m.p. 58–62 °C (lit. m.p. 55-56 °C). $^1$H NMR (600 MHz, CDCl$_3$, rotamers) δ 6.41–6.16 (m, 1H) 3.11 (s, 3H), 1.47–1.46 (m, 18H). $^{13}$C NMR (150 MHz, CDCl$_3$, rotamers) δ 155.9 (C), 81.3 (C), 37.5 (CH$_3$), 28.3 (CH$_3$). IR (solid) 3315, 2981, 1702 cm$^{-1}$. 

![Diagram of the reaction process]
$^1$H and $^{13}$C NMR data for di-tert-butyl-1-methylhydrazine-1,2-dicarboxylate.
Di-tert-butyl-1-(3-(tert-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate

To a solution of di-tert-butyl 1-methylhydrazine-1,2-dicarboxylate 2 (3.75 g, 15.2 mmol) in tert-butanol (25 mL), was added 0.5 mL of 2 M NaOH and the reaction mixture stirred at 21 °C for 10 min. After this, tert-butyl acrylate (6.63 mL, 45.67 mmol) was added to the solution and the reaction mixture was heated under reflux for 72 h. The solvent was then removed in vacuo and the crude residue purified by flash column chromatography (0% to 20% EtOAc/petrol) to afford di-tert-butyl-1-(3-(tert-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate 3 (4.73 g, 12.6 mmol, 86%) as a clear oil. $^1$H NMR (700 MHz, CDCl$_3$, rotamers) $\delta$ 3.84–3.53 (m, 2H), 3.06–2.98 (m, 3H), 2.57-2.45 (m, 2H), 1.47–1.43 (m, 27H). $^{13}$C NMR (175 MHz, CDCl$_3$, rotamers) $\delta$ 171.1 (C), 155.5 (C), 154.5 (C), 81.1 (C), 44.7 (CH$_3$), 36.7 (CH$_2$), 34.2 (CH$_2$), 28.4 (CH$_3$)). IR (thin film) 2976, 2933, 1709 cm$^{-1}$. 

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$^1$H and $^{13}$C NMR data for di-tert-butyl-1-(3-(tert-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate.
3-(4,5-Dibromo-2-methyl-3,6-dioxo-3,6-dihydropyrazin-1(2H)-yl) propanoic acid\textsuperscript{138}

\[ 
\begin{align*}
\text{AcOH} & \xrightarrow{\text{Reflux}} \quad 4 \\
\text{Boc} & \xrightarrow{\text{AcOH} \text{ Reflux}} \quad 5 \\
\text{AcOH} & \xrightarrow{\text{Reflux}} \quad 6
\end{align*}
\]

Dibromomaleic acid 4 (1.82 g, 6.68 mmol) was dissolved in AcOH (75 mL) and heated under reflux for 30 min. To this solution, was added di-\text{tert}-butyl-1-(3-(\text{tert}-butoxy)-3-oxopropyl)-2-methylhydrazine-1,2-dicarboxylate 3 (3.00 g, 8.01 mmol) and the reaction heated under reflux for a further 4 h. After this time, the reaction mixture was concentrated in vacuo with toluene co-evaporation (3 \times 30 \text{ mL}, as an azeotrope) and the crude residue purified by flash column chromatography (50\% to 100\% \text{EtOAc/petrol} (1\% \text{AcOH})) to afford 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyrazin-1(2H)-yl) propanoic acid 6 (1.44 g, 4.05 mmol, 61\%) as a yellow solid. \textbf{m.p.} 139–142 °C (lit. m.p. 140–144 °C).\textsuperscript{138} \textbf{\textsuperscript{1}H NMR} (700 MHz, MeOD) \( \delta \) 4.44 (t, \( J = 7.3 \text{ Hz} \), 2H), 3.69 (s, 3H), 2.75 (t, \( J = 7.3 \text{ Hz} \), 2H). \textbf{\textsuperscript{13}C NMR} (175 MHz, MeOD) \( \delta \) 173.8 (C), 154.8 (C), 154.5 (C), 136.7 (C), 136.4 (C), 44.9 (CH\textsubscript{3}), 35.4 (CH\textsubscript{2}), 32.6 (CH\textsubscript{2}). \textbf{IR} (solid) 3044, 1725, 1606, 1570 cm\textsuperscript{-1}. 

\textsuperscript{138}
$^1$H and $^{13}$C NMR data for 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoic acid.
2,5-Dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoate

\[
\begin{align*}
\text{Br} & \text{N} \text{N} \text{O} \text{O} \\
\text{Br} & \text{N} \text{N} \text{O} \text{O} \\
& \text{OH} \\
\text{DCC} & \text{THF} \\
\text{ii)} & \text{OH} \\
\text{O} & \text{N} \text{N} \text{O} \\
\end{align*}
\]

To a solution of 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoic acid 6 (700 mg, 1.97 mmol) in THF (20 mL), pre-cooled to 0 °C, was added \( N,N' \)-dicyclohexylcarbodiimide (445.7 mg, 2.16 mmol). The homogenous solution was then stirred at 0 °C for 30 min. Following this, was added \( N \)-hydroxysuccinimide (249 mg, 2.16 mmol) and the reaction stirred at 21 °C for a further 16 h. The newly formed heterogenous mixture was then filtered and the filtrate concentrated in vacuo. Purification of the crude residue by flash column chromatography (30% to 100% EtOAc/petrol) afforded 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoate 7 (70 mg, 0.15 mmol, 8%) as a white solid. m.p. 101–104 °C (lit. m.p. 100–104 °C).

\(^{1}H\) NMR (700 MHz, CDCl\(_3\)) \( \delta \) 4.48 (t, \( J = 6.9 \) Hz, 2H), 3.68 (s, 3H), 3.10 (t, \( J = 6.9 \) Hz, 2H), 2.85 (s, 4H). \(^{13}C\) NMR (175 MHz, CDCl\(_3\)) \( \delta \) 168.7 (C), 166.0 (C), 153.4 (C), 153.2 (C), 136.9 (C), 135.3 (C), 43.0 (CH\(_2\)), 35.3 (CH\(_3\)), 29.1 (CH\(_2\)), 25.7 (CH\(_2\)). IR (solid) 2992, 1814, 1782, 1735, 1634, 1576 cm\(^{-1}\).
$^1$H and $^{13}$C NMR data for 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoate.
((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanamido)ethoxy)ethoxy)ethyl) carbamate

To a solution of 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl) propanoate 7 (70 mg, 0.15 mmol) in MeCN (10 mL) was added N-[(1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl]-1,8-diamino-3,6-dioxaoctane (50 mg, 0.15 mmol) and the reaction stirred at 21 °C for 16 h. After this time, MeCN was removed in vacuo and the crude residue dissolved in CHCl₃ (50 mL), and washed with water (2 × 30 mL) followed by saturated aq. K₂CO₃ (30 mL). The organic layer was then dried (MgSO₄) and concentrated in vacuo. Purification of the crude residue by flash column chromatography (0% to 10% MeOH/EtOAc) afforded ((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanamido)ethoxy)ethoxy)ethyl) carbamate 8 (73 mg, 0.11 mmol, 72%) as a yellow oil. ¹H NMR (600 MHz, CDCl₃, rotamers) δ 7.84 (s, 0.3H, 25-NH), 6.38 (s, 0.6H, 25-NH), 5.78 (s, 0.3H, 4-NH), 5.24 (s, 0.7H, 4-NH), 4.44 (t, J = 6.6 Hz, 2H, 29-CH₂), 4.14–4.12 (m, 2H, 1-CH₂), 3.73–3.71 (m, 3H, 38-CH₃), 3.60–3.44 (m, 12H, 5-CH₂, 6-CH₂, 8-CH₂, 9-CH₂, 11-CH₂, 12-CH₂), 2.62 (t, J = 6.6 Hz, 2H, 27-CH₂), 2.29–2.20 (m, 6H, 17-CHH, 18-CH₂, 21-CH₂, 22-CHH), 1.61–1.57 (m, 2H, 17-CHH and 22-CHH), 1.35–1.32 (m, 1H, 41-CH), 0.96–0.94 (m, 2H, 23-CH and 24-CH).
$^{13}$C NMR (150 MHz, CDCl$_3$, rotamers) δ 169.1 (C-26), 157.0 (C-3), 153.1 (C-31/34), 153.0 (C-31/34), 136.6 (C-33), 135.5 (C-32), 99.0 (C-19 and C-20), 70.4 (CH$_2$-9), 70.3 (CH$_2$-8), 69.7 (CH$_2$-6 and CH$_2$-11), 63.0 (CH$_2$-1), 44.6 (CH$_2$-29), 40.8 (CH$_2$-5), 39.6 (CH$_2$-12), 35.1 (CH$_3$-38), 34.1 (CH$_2$-27), 29.3 (CH$_2$-17), 29.2 (CH$_2$-22), 21.6 (CH$_2$-18 and CH$_2$-21), 20.2 (CH$_2$-15 and CH$_2$-16), 17.9 (CH-14), 14.3 (CH). IR (thin film) 3329, 2920, 2858, 1708, 1630, 1572, 1534 cm$^{-1}$. 
$^{1}H$ and $^{13}C$ NMR data for ((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)propanamido)ethoxy)ethoxy)ethyl) carbamate.
Chemical Biology

General Remarks

Reagents and solvents were purchased from commercial sources and used as supplied. All buffer solutions were prepared with double-distilled water (ddH$_2$O) and filter-sterilised. Borate-buffered saline (BBS) was made up of 25 mM sodium borate, 25 mM sodium chloride and 0.5 mM EDTA at pH 8.0. Phosphate buffered saline (PBS) was made up of 140 mM sodium chloride and 12 mM sodium phosphates at pH 7.4. Phosphate-buffered saline for SEC-HPLC was 140 mM NaCl, 100 mM sodium phosphates and 0.02% sodium azide at pH 7.0. Solutions of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 20 mM were prepared in ddH$_2$O. Filtration of particulates was carried out through Spin-X 0.22 µm cellulose acetate filters. Ultrafiltration was carried out in vivaspin 500 polyethersulfone (PES) membrane concentrators with a molecular weight cut-off (MWCO) of 10,000 Da.

15C4 is a mouse monoclonal IgG1 full length antibody directed against hLRG1. The antibody was developed in-house using conventional mouse immunisation and hybridoma technology. 15C4, originally in PBS pH 7.4, was buffer exchanged completely for borate buffer pH 8.0 via ultrafiltration (MWCO 10,000 Da) prior to bioconjugation reactions. Concentration was determined by UV-Vis absorbance as described.

Magacizumab is a humanised IgG4 full length antibody directed against hLRG1. The antibody was obtained in its clinical formulation (15 mM sodium citrate, pH 6.9, 6.75% sucrose, 0.05% polysorbate 80, 104.5 mg/mL) from Abzena, dissolved in sterile water and then buffer exchanged completely for borate buffer pH 8.0 via ultrafiltration (MWCO 10,000 Da) prior to bioconjugation reactions. Concentration was determined by UV-Vis absorbance as described.

SDS-PAGE

Non-reducing glycine-SDS-PAGE 12% acrylamide (10% for Fab) gels were performed following standard lab procedures. A 6% stacking gel was used and a broad-range molecular weight marker (10-250 kDa, Prestained PageRuler Plus Protein Standards, ThermoScientific) was run alongside the samples to estimate protein weights. Samples (10 µL at ~ 6 µM) were mixed with loading buffer (2 µL, composition for 5 × SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tri buffer pH 6.8, 2 mg bromophenol blue in 10 mL),
heated at 75 °C for 5 min, and centrifuged at 16,000 RPM for 5 min. Samples were subsequently loaded into the wells in a volume of 5 µL. All gels were run at a constant current of 30 mA for 40 minutes using 1 × SDS running buffer. Gels were stained using a modified Coomassie stain (25 g ammonium sulfate, 250 mg Coomasie G-250, 8.8 mL 85% ortho-phosphoric acid, 50 mL ethanol, made up to a total of 250 mL with d.d. H₂O) at 21 °C for 16 h. Quantification of bands was performed using ImageJ.

**UV-Vis spectroscopy**

UV-Vis spectroscopy was used to determine protein concentrations and payload to antibody ratios, using a nanodrop ND-2000 spectrophotometer operating at 21 °C. Baseline correction was performed using sample buffer as a blank. Extinction coefficients for proteins (at A₂₈₀) and payloads (at A_max) are listed below. A correction factor was applied in the event that the conjugated payload had a competing absorption at A₂₈₀, which is listed in the table below. Pyridazinedione to antibody ratio (PAR) and fluorophore to antibody ratio (FAR) values were calculated by comparing concentrations of the payloads and the protein (calculated with corrected A₂₈₀ values).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Extinction Coefficient ε₂₈₀ (M⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15C4</td>
<td>210,000</td>
</tr>
<tr>
<td>Magacizumab</td>
<td>230,690</td>
</tr>
<tr>
<td>Magacizumab Fab</td>
<td>70,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Payload</th>
<th>Extinction Coefficient (M⁻¹cm⁻¹)</th>
<th>Correction Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ε₂₈₀</td>
<td>ε₃₃₅</td>
</tr>
<tr>
<td>Pyridazinedione</td>
<td>2,275</td>
<td>9,100</td>
</tr>
<tr>
<td>AlexaFluor™ 488 azide</td>
<td>8,030</td>
<td>-</td>
</tr>
</tbody>
</table>

Calculation of the molecule over antibody ratio, r, follows the formula below:

\[ r = \frac{(A_\lambda)/\varepsilon_\lambda}{(A_{280} - \sum_\lambda \times C F_\lambda \times A_\lambda)/\varepsilon_{280}} \]

With A_λ the absorbance at wavelength λ, and ε_λ the extinction coefficient of the molecule of interest.
LCMS analysis – Method 1

LCMS was performed using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD). All samples were diluted in deionised water and run with the following parameters. Column: Hypersil Gold C4, 1.9 μm, 2.1 μm × 50 μm. Mobile Phase: 95:5 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid) Gradient over 4 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.6 mL/min. MS Mode: ES+. Scan Range: m/z = 150 – 2000. Scan time: 0.25 s. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the appropriate range.

LCMS analysis – Method 2

Molecular masses of proteins were measured using an Agilent 6510 QTOF LCMS system (Agilent, UK). Agilent 1200 HPLC system was equipped with an Agilent PLRP-S, 1000A, 8 μM, 150 mm × 2.1 mm column. 10 μL of a protein sample (diluted to 0.2 mg/mL in d.d. H2O) was separated on the column using mobile phase A (water-0.1% formic acid) and B (acetonitrile-0.1% formic acid) with an eluting gradient (as shown below) at a flow rate of 300 μL/min. The oven temperature was maintained at 60 °C. Full antibody samples (100 μL, 1.0 mg/mL, in ammonium acetate buffer, 0.2 M, pH 6.8) were deglycosylated by incubating with PNGase F (1 μL, New England BioLabs) at 37 °C for 24 h.

LCMS mobile phase gradient for A/B elution

<table>
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<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
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</tr>
<tr>
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<td>25</td>
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Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 3500 V, a gas temperature at 350 °C, a dry gas flow rate at 10 L/min and a nebulizer of 30 psig. MS Tof was acquired under conditions of a fragmentor at 350 V, a skimmer at 65 V and an acquisition rate at 0.5 spectra/s in a profile mode, within a scan range between 700 and 4500 m/z. The data was then analysed by deconvoluting a spectrum to a zero-charge mass spectrum using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00. Deconvoluted spectra were avoided where possible in the quantification of conjugates due to differing ionisation tendencies between species with significantly different masses.

**Enzyme-linked immunosorbent assay (ELISA) against human LRG1**

ELISA was used in order to determine the binding affinity of modified antibody conjugates for their antigen, human LRG1. The assay was performed as follows: a 96-well Nunc Maxisorp plate was coated overnight at 4 °C with human LRG1 (50 µL of a 4 µg/mL solution in PBS). Next, the coating solutions were removed and each well washed with 0.1% Triton ×100 in PBS (wash buffer) three times. Then, the wells were coated with a 3% BSA solution in PBS (200 µL) for 1 h at 21 °C. After this time, the wells were emptied and washed with wash buffer 3 times. Modified and non-modified antibody conjugates were diluted in PBS yielding the following concentrations (ng/mL): 280, 140, 70, 35, 17.5, 8.75, 4.38, 2.19, 1.09, 0.547, 0.273. Wells were coated with the dilution series solutions, each in triplicate, and incubated for 2 h at 21 °C. Then, the solutions were removed, and the wells washed with wash buffer 6 times. The detection antibody; anti-human IgG HRP conjugated (Sigma; 1:10,000) was added and incubated for 1 h at 21 °C. Then, the solutions were removed, and the wells washed 6 times with wash buffer. Finally, equal amounts of substrate A (stabilised hydrogen peroxide) and substrate B (stabilised tetramethylbenzidine) (ELISA substrate reagent kit; R&D Systems) were premixed and added to each well (50 µL). After ca. 20 min the reaction was stopped by the addition of 25 µL of 2 N sulfuric acid. Absorbance was measured at 450 nm. PBS-only controls were included in each experiment. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average. Graphpad Prism was used to create the dose-response curve using the log(Inhibitor) vs. response -- Variable slope with the following equation:
\[ Y = Bottom + \frac{Top - Bottom}{1 + (10^{(\log EC_{50} - X}) - X)Hillslope} \]

**Alexa Fluor™ 488 conjugate serum stability**

Alexa Fluor™ 488 antibody–fluorophore conjugate (AFC) 20 was prepared as a 0.2 mg/mL solution in PBS 140 mM sodium chloride, 12 mM sodium phosphates and 2 mM sodium azide at pH 7.4. The conjugate was diluted with 50% of human blood serum to give a final concentration of 0.1 mg/mL of AFC and 1 mM sodium azide. A single aliquot (50 µL) was taken, flash frozen and stored at -80 °C. The remaining solution was incubated at 37 °C under shaking (300 rpm) and under the cover of light. Aliquots (50 µL) were taken at 1, 2, 4 and 7 days, flash frozen and stored at -80 °C. Aliquots were thawed, spin-filtered (0.22 µm filter) and diluted 100× with elution buffer. Samples (20 µL) of diluted aliquots were analysed by SEC-HPLC on a TSK gel G3000SWXL (7.8 mm × 30 cm) column connected to an Agilent 1200 HPLC system equipped with a 1200 series diode array detector and a fluorescence detector. Samples were eluted using PBS 140 mM NaCl, 100 mM sodium phosphates and 0.02% sodium azide at pH 7.0 as mobile phase at a flow rate of 0.5 mL/min. over 30 min. Fluorescence was detected with an excitation wavelength of 495 nm and emission wavelength of 525 nm.

**Ellman’s test**

A 1 mM solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman’s reagent) was prepared in PBS. The assay was performed by mixing 5 µL of this solution with 5 µL of reduced Magacizumab at 20 µM and diluting with PBS (60 µL). The solution was incubated at 20 °C for 2 min before measuring absorption at 280 nm (protein concentration) and 412 nm (2-nitro-5-thiobenzoic acid). For baseline correction, a sample of 5 µL of the 1 mM Ellman’s reagent diluted with 65 µL PBS was used as a blank. The sulfhydryl per antibody ratio (SAR) was calculated as follows with \( \varepsilon_{412} = 14150 \text{ M}^{-1} \text{ cm}^{-1} \) for 2-nitro-5-thiobenzoic acid and \( \varepsilon_{280} = 210000 \text{ M}^{-1} \text{ cm}^{-1} \) for Magacizumab, where \( A_{280} \) and \( A_{412} \) define the absorption measured at 280 nm in the absence of Ellman’s reagent and at 412 nm in the presence of the reagent respectively.
Conjugation methods

In situ reduction of 15C4 and reaction with PD 8 at 2 °C to form conjugate 18

To a solution of 15C4 13 (20 µM, 100 µL) in BBS (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) was added PD 8 (2 µL, 20 mM in DMSO, 20 eq.) and subsequently TCEP (2 µL, 20 mM in ddH2O, 20 eq.), and the reaction mixture incubated at 2 °C overnight under mild agitation (300 rpm). The excess reagents were removed by repeated diafiltration into fresh PBS buffer (140 mM sodium chloride, 1 mM potassium phosphate monobasic and 8 mM sodium phosphate dibasic at pH 7.4) using diafiltration columns (GE Healthcare, 10,000 Da MWCO), and the volume was corrected to 100 µL. The samples were analysed by SDS-PAGE and UV-Vis spectroscopy, which was used to determine a pyridazinedione to antibody ratio (PAR) of 4.13. Following this, Alexa Fluor™ 488 Azide (Molecular Probes) (1 µL, 20 mM in DMSO, 10 eq.) was added and the reaction mixture was incubated at 21 °C for 4 h. Excess fluorophore was removed by repeated diafiltration into fresh PBS (140 mM sodium chloride, 1 mM potassium phosphate monobasic and 8 mM sodium phosphate dibasic at pH 7.4) using diafiltration columns (GE Healthcare, 10,000 Da MWCO). Following this, sample analysis by SDS-PAGE and UV-vis spectroscopy revealed conversion to the desired antibody-PD Alexa Fluor™ 488 Azide bioconjugate 18 with a fluorophore to antibody ratio (FAR) of 3.33. A TCEP reduction control was also performed on 15C4: TCEP (1 µL, 20 mM in ddH2O, 20 eq.) was added to 15C4 (20 µM, 50 µL) in BBS and the reaction mixture was incubated at 37 °C for 1 h.
SDS-PAGE analysis of conjugate 18; M – Molecular weight marker; Lane 1 – Native unmodified 15C4; Lane 2 – 15C4 reduced with 20 eq. 20 mM TCEP; Lane 3 – PD-rebridged 15C4 17; Lane 4 – 15C4-AF488 conjugate (conjugate 18).

UV-Vis data for PD-rebridged 15C4.
UV-Vis data for Alexa Fluor™ 488-clicked 15C4.
Step-wise reduction of Magacizumab and reaction with PD 8 at 21 °C to form conjugate 20

To a solution of Magacizumab 15 (20 µM, 100 µL) in borate buffer (BBS; 25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) was added TCEP (10 µL, 20 mM in ddH2O, 100 eq.), and the reaction mixture incubated for 4 h at 37 °C under mild agitation (300 rpm). After this time excess TCEP was removed by diafiltration into fresh BBS buffer using PD Minitrap G-25 columns (GE Healthcare) and the concentration was corrected to 20 µM. PD 8 (2 µL, 20 mM in DMSO, 20 eq.) was added to the solution of reduced Magacizumab at 21 °C and the solution incubated for 16 h. Excess reagents were removed by repeated diafiltration into fresh PBS buffer (140 mM sodium chloride, 1 mM potassium phosphate monobasic and 8 mM sodium phosphate dibasic at pH 7.4) using diafiltration columns (GE Healthcare, 10,000 Da MWCO), and the volume was corrected to 100 µL. The samples were analysed by SDS-PAGE and UV-Vis spectroscopy, which was used to determine a PAR of 3.8. Following this, Alexa Fluor™ 488 Azide (Molecular Probes) (1 µL, 20 mM in DMSO, 10 eq.) was added and the reaction mixture was incubated at 21 °C for 4 h. Excess fluorophore was removed by repeated diafiltration into fresh PBS using diafiltration columns (GE Healthcare, 10,000 Da MWCO). Following this, sample analysis by SDS-PAGE and UV-vis spectroscopy revealed conversion to the desired antibody-PD Alexa Fluor™ 488 Azide bioconjugate 20 with a fluorophore to antibody ratio (FAR) of 3.80. Expected mass by LCMS (method 2): 149,692 Da. Observed mass by LCMS (method 2): 149,684 Da.
A TCEP reduction control was also performed on Magacizumab: TCEP (5 µL, 20 mM in ddH₂O, 100 eq.) was added to Magacizumab (20 µM, 50 µL, 0.02 µmol) in BBS and the reaction mixture was incubated at 37 °C for 4 h.

SDS-PAGE analysis of conjugate 20; Lane M – Molecular weight marker; Lane 1 – Magacizumab reduced with 100 eq. 20 mM TCEP; Lane 2 – PD-rebridged Magacizumab 19: Lane 3,4 – Maga-AF488 conjugate (conjugate 20).
Densitometry traces of a) natively rebridged and b) non-natively rebridged antibody–fluorophore conjugate (AFC) at 21°C (above) and 4°C (below).

### Conjugation performed at 21°C

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<td>b</td>
<td>31</td>
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Quantification of a) natively rebridged and b) non-natively rebridged antibody–fluorophore conjugate (AFC) at 21°C by densitometry analysis.

### Conjugation performed at 4°C

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<tr>
<td>b</td>
<td>26</td>
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Quantification of a) natively rebridged and b) non-natively rebridged antibody–fluorophore conjugate (AFC) at 21°C by densitometry analysis.
UV-Vis data for PD-rebridged Magacizumab.

UV-Vis data for Alexa Fluor™ 488 -clicked Magacizumab.

LCMS (method 2) data for Maga-488 20:
Preparation of PD-MMAE

To a solution of PD 8 (4 µL, 20 mM in DMSO, 1.08 mol) was added N₃-PEG₃-vc-PABC-MMAE (120 µL, 10 mM in DMSO, 1.20 mol). The reaction mixture was left to react at 21 °C overnight. The product was analysed by LCMS to reveal the formation of PD 23 with complete consumption of limiting reagent PD 8. Whilst excess unreacted N₃-PEG₃-VC-PABC-MMAE was observed by LCMS, this species is likely to be unreactive towards proteins, and so further purification was not conducted prior to conjugation to Magacizumab.

<table>
<thead>
<tr>
<th>Compound</th>
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<td>PD 8</td>
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<tr>
<td>N₃-PEG₃-VC-PABC-MMAE</td>
<td>1353</td>
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<tr>
<td>PD 23</td>
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</table>

LCMS (method 1) data for click reaction of PD 8 with N₃-PEG₃-VC-PABC-MMAE, including controls:

LCMS data of PD 8:
LCMS data of N3-PEG3-VC-PABC-MMAE:

FJ102_1 64 (1.191) Cm (61:68) 1: Scan ES+ 9.10e6

FJ103_1 94 (1.755) Cm (90:99) 1: Scan ES+ 3.28e7

FJ103_1 1: Scan ES+ TIC 3.92e9
LCMS data of reaction of PD 8 with N\textsubscript{3}-PEG\textsubscript{3}-VC-PABC-MMAE showing formation of PD 23 and complete consumption of PD 8:
Protocol comparison for the conjugation of Magacizumab with PD-MMAE 23 to form ADC 24

Reaction conditions are in the table below

To a solution of Magacizumab (20 µM, 100 µL, 0.002 µmol) in BBS pH 8.0 was added TCEP (20 mM, 10 µL, 100 eq.) and the reaction incubated at 37 °C for 4 h under mild agitation (300 rpm). After this, excess TCEP was removed by ultrafiltration (10,000 Da MWCO) and the reduced Magacizumab concurrently transferred into fresh BBS pH 8.0. Following this, PD-MMAE 23 (6.25 mM, 10 eq. or 20 eq., in DMSO) was immediately added to the reduced Magacizumab solution, thus giving a solution with a final DMSO content of 6% or 8% (v/v). The reaction was incubated for 16 h at 21 °C or 37 °C. After this, excess reagents were removed by ultrafiltration (10,000 Da MWCO) into PBS pH 7.4 to afford the modified Magacizumab PD-MMAE ADC 24 in PBS at a final concentration of 20 µM. Average PAR by UV-Vis is reported in the table below.

An Alexa Fluor™ 488 click control was performed on the Magacizumab PD-MMAE ADC 24 (reaction 6) to ascertain whether all clickable handles on the functionally rebridged ADC had reacted with MMAE-azide: Alexa Fluor™ 488 (10 mM, 1 µL, 20 eq.) was added to Magacizumab PD-MMAE ADC 24 (20 µM, 50 µL) in PBS. Sample analysis by and UV-Vis spectroscopy gave an FAR of 0.2.
Reaction conditions for conjugation of PD-MMAE 23 to Magacizumab to form Magacizumab PD-MMAE ADC 24. PAR represents PD (and hence drug) loading on the antibody.

Reaction of Magacizumab with a PD lacking a strained alkyne clickable handle (dibromo diethyl pyridazinedione 25), followed by incubation with azide bearing Alexa Fluor™ 488 azide:
UV-Vis data for reaction of Magacizumab with dibromo diethyl pyridazinedione 25, bearing no ‘clickable’ handles, followed by incubation with Alexa Fluor™ 488 azide. 

\[ \text{FAR} = 0.13 \]

**Conjugation of Magacizumab with PD-MMAE 23 to form ADC 24**

To a solution of Magacizumab 15 (20 μM, 3.3 mL) in BBS pH 8.0 was added TCEP (330 μL, 20 mM in ddH2O, 100 eq.) and the reaction incubated at 37 °C for 4 h under mild agitation (300 rpm). After this, excess TCEP was removed by ultrafiltration (10,000 Da MWCO) and the reduced Magacizumab concurrently transferred into fresh BBS pH 8.0. Following this, PD-MMAE 23 (6.25 mM, 106 μL, 10 eq., in DMSO) was immediately added to the reduced Magacizumab solution, thus giving a solution with a final DMSO content of 6% (v/v). The reaction was incubated at 37 °C for 16 h. After this, excess reagents were removed by ultrafiltration (10,000 Da MWCO) into PBS pH 7.4 to afford the modified Magacizumab PD-MMAE ADC 24 in PBS at a final concentration of 20 μM. Yield 90%, average PDAR by UV-Vis was 4.0. A TCEP reduction control was
also performed on Magacizumab PD-MMAE ADC 24 to demonstrate that all solvent accessible disulfide bonds were functionally rebridged: TCEP (5 µL, 20 mM in ddH$_2$O, 100 eq.) was added to Magacizumab PD-MMAE ADC 24 (20 µM, 50 µL) in BBS. Additionally, an Alexa Fluor™ 488 click control was also performed on Magacizumab PD-MMAE ADC 24 to demonstrate that that all clickable handles on the functionally rebridged ADC had reacted with MMAE-azide: Alexa Fluor™ 488 (1 µL, 10 mM in DMSO, 20 eq.) was added to Magacizumab PD-MMAE ADC 24 (20 µM, 50 µL) in PBS and the reaction mixture was incubated at 21 °C for 4 h. Excess fluorophore was removed by repeated diafiltration into fresh PBS using diafiltration columns (GE Healthcare, 10,000 Da MWCO).

**SDS-PAGE analysis of ADC 24**; Lane 1 – Molecular weight marker; Lane 2 – Native unmodified Magacizumab; Lane 3 – Magacizumab reduced with 100 eq. 20 mM TCEP; Lane 4 – ADC 24) ADC reduced with 100 eq. 20 mM TCEP.
UV-Vis data for Magacizumab

UV-Vis data for Magacizumab PD-MMAE ADC 24
UV-Vis data of Magacizumab PD-MMAE ADC incubated with Alexa Fluor\textsuperscript{TM} 488
Cell Biology

Cell culture

B16F0 mouse melanoma cells (ATCC) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with glucose (4.5 g/L), sodium pyruvate (110 mg/L), 10% Fetal bovine serum (FBS), penicillin (100,00 U/L) and streptomycin sulphate (100 mg/L). Transfected B16F0 cells were also cultured as described above and additionally supplemented with G418 antibiotic (1.0 mg/mL). All cultures were maintained at 37 °C in 5% CO₂ and checked to be clear of contamination. For cell culture experiments, sterile PBS was purchased from Thermofisher.

Human Lrg1 expression in mammalian cells

Plasmid was received from XiaoMeng (Singapore). Competent MC1061 E. coli cells were transformed with the construct, single colonies were picked, the DNA was purified by miniprep (Qiagen) and the sequence verified by Sanger sequencing. In preparation for mammalian cell transfection, the plasmid (pcDNA3.1-hLrg1) was purified by maxiprep (Qiagen EndoFree Plasmid Maxi Kit).

In brief, B16F0 cells (0.5 × 10⁶ cell/mL, plated 24 h previously) were transfected with 14 μg of plasmid, using Lipofectamine 2000 (Thermofisher) according to the manufacturer’s instructions. 24 h after transfection, the plasmid/Lipofectamine solution was removed and the medium was replaced with fresh cell growth medium without added antibiotics. 48 h following transfection, cells were diluted to a density of 0.5 × 10⁶ cell/mL and cultured in fresh cell growth medium containing G418 (1.0 mg/mL) antibiotic (Sigma) to select for transfected cells only. After several selections, transfected cells were isolated.

Quantitative Polymerase Chain Reaction (qPCR)

RNA from transfected and wild type B16F0 cell lines was extracted using the RNeasy mini kit (Qiagen) and analysed for quality using the 4200 TapeStation (Agilent). cDNA was synthesised using the QuantiTect Reverse transcription kit (Qiagen). qPCRs were run on the QuantStudio 6 Flex System (Applied Biosciences) using Power SYBR Green PCR.
Master Mix (ThermoFisher). All the reactions performed were run in triplicate in a 96-well plate and a housekeeping gene (mouse Gapdh) was used to normalise the results. Normalised expression values were obtained using the formula $2^{-\Delta C_t}$, where $C_t$ represents cycle threshold values. Delta (Δ) $C_t$ values were calculated for each gene subtracting the $C_t$ value of the house keeping gene from the $C_t$ value of the target gene. Mouse gapdh was used as the housekeeping gene. Student’s t test was used to calculate significance.

Primer sequences used were as follows: Mouse Gapdh: forward, 5’-ACTGAGGACCAGGGTGTCTCC-3’; reverse, 5’-CTGTAGCGGCTATTCATTGTCATACC-3’. Mouse Lrg1: forward, 5’-CCATGTCAGTGCGCAGATTC -3’; reverse, 5’ AAGAGTGAGAGGTGGAAGAG -3’. Human LRG1: forward, 5’- CAGCGACCAAAAAAGCCCAAG -3’; reverse, 5’-ATTTCGCGAGGTGGT GTA -3’.

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$qPCR$ components

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</table>

$qPCR$ program
Sample preparation for western blotting

Acetone precipitation of hLRG1 from B16F0 cells

Supernatants were collected from transfected and wild type B16F0 cells and to the supernatants were added four volumes of acetone cooled to 0 °C. The mixture was incubated at -20 °C for 60 min, followed by spinning at full speed in a centrifuge for 10 min. After this, the supernatant was carefully discarded and excess acetone was allowed to evaporate for 30 min. The pellet was resuspended in 100 µL of 1 × SDS sample buffer ready to be analysed by western blotting.

Cell lysis

Cells were washed with PBS and lysed on ice by addition of 1 × SDS sample buffer (500 µL for a 10 cm diameter plate). Cell lysates were centrifuged at 12000 rpm for 5 min and the supernatants were mixed with sample buffer ready to be analysed by western blotting.

Western blotting

Protein samples were boiled with sample buffer for 5 minutes at 90 °C. Protein samples along with 12 µL protein ladder (Dual colour precision plus protein standards, Biorad) were loaded onto and run on 10% precast polyacrylamide mini-gels (Biorad). The gels were run at 100 V for 90 min and then transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare) by running at 400 mA for 60 min, immersed in transfer buffer (100 mL 10X Tris Glycine + 200 mL methanol + 700 mL water). Membranes were blocked for 60 min at room temperature with TBS-T buffer (Tris-Buffered Saline: 50 mM Tris, 150 mM NaCl, adjusted to pH 7.6, 0.1% Tween-20 (Sigma) and 3% BSA to saturate non-specific binding sites. Primary antibodies were diluted in blocking buffer at 4 °C with gentle agitation. Membranes were washed 3 × 5 min in TBST. Horseradish peroxidase conjugated secondary antibodies were diluted in TBST-3% BSA and applied for 1 h at room temperature with gentle agitation. Following incubation, the membranes were washed with TBS-T, treated with ECL (Pierce ECL 2 Western Blotting Substrate) and developed using X-ray films.
<table>
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<table>
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</table>

*Antibodies for western blotting.*

**Preparation of Alexa 555-labelled hLRG1**

hLRG1 was labelled using the Alexa Fluor 555 protein labelling kit (ThermoFisher), according to the instructions provided by the manufacturer. Briefly, 50 μL of 1 M sodium bicarbonate buffer (pH 8.3) was added to 500 μL (500 μg in PBS) of hLRG1, followed by incubation with the reactive dye in the vial for 1 h at room temperature. Excess dye was removed by applying the reaction mixture to a PD-10 column (Amersham Biosciences). The internalisation of Alexa 555-labeled hLRG1 (10 μg/mL) was then assessed via the internalisation assay described below.

**In vitro internalization analysis by confocal microscopy**

hLRG1-transfected and non-transfected B16F0 cells on coverslips at 70% confluency were incubated with labelled constructs at 10 μg/ml for 1 h at 4 °C and then at 37 °C. Cells were washed three times with PBS to remove unbound antibodies followed by fixation with 4% formaldehyde for 15 min at room temperature. Coverslips were permeabilised and blocked as described previously. Actin was detected with phalloidin-568 (Invitrogen) and DAPI was used to stain cell nuclei.

As a positive control, cells were incubated as above with Transferrin-555 (obtained from Tom Burgoyne, UCL Institute of Ophthalmology) at 4 °C and then incubated at 37 °C. Cells were fixed as above and stained with phalloidin-680 for actin and DAPI for nuclear staining.
**In vitro cytotoxicity assessment**

*In vitro* cytotoxicity of compounds was evaluated in hLRG1-positive and wild type B16F0 cell lines by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colourimetric assay. Briefly, 5×10⁴ cells were seeded in 96-well plates and incubated at 37 °C overnight. Cells were then exposed to a range of concentrations of the test compounds diluted in growth medium at 37 °C as follows: MMAE (0-100 nM, 72 h), Cisplatin (0 – 66 µM, 48 h), Magacizumab (0-100 nM, 72 h), N₃-PEG₃-vc-PABC-MMAE (0-100 nM, 72 h) and ADC 24 (0-100 nM, 72h). Following each treatment, cells were washed twice with PBS and the medium was replaced with growth medium free of phenol red as phenol red may interfere with the final assay results. MTT reagent (12 mM) was then added to each well and cells were incubated for 4 h at 37 °C, followed by the addition of DMSO and further incubation at 37 °C for 1 h. Optical density (OD) was measured at 540 nm. The percentage of viable cells was calculated as follows.

\[
\text{Cell viability} \, (\%) = \left( \frac{\text{OD}_{\text{treated cells}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{untreated cells}}} \right) \times 100
\]

ADC 24 + Cathepsin B[^183]: 3.3 µL of Cathepsin B (human liver, Sigma-Aldrich, 13.8 µM) was added to 11.7 µL sodium acetate buffer (2.2 M, pH = 5.8), 24 µL 30 mM DTT and 1 µL 500 mM EDTA. The resulting mixture was activated by incubation at room temperature for 15 min, then 360 µL sodium acetate buffered medium (pH 6.0) was added and to this solution was added ADC 24 (100 µL at 10 µM). Cells were treated with this ADC 24 + Cathepsin B mixture as described above for ADC 24 (0-100 nM, 72 h at 37 °C).

ADC 24 + Cisplatin: Cells were initially incubated with 5 µM of Cisplatin for 48 h. Following this, serial dilutions of ADC 24 (0-100 nM) were prepared in growth medium and cells were treated as described for ADC 24 (72 h at 37 °C).

**Tumour models**

C57BL/6 mice were purchased from Charles River Laboratories. Human LRG1 knock-in mice were generated by the Moss and Greenwood laboratories as described[^173]. Single-cell suspensions of 1×10⁶ B16F0 cells (human-LRG1 transfected and wild type cells) were injected subcutaneously into the back of Lrg1⁺/⁺ C57BL/6 mice in 100 µL PBS. Mice were randomised by age prior to inoculation. Tumours were measured at defined intervals using calipers and tumour volume was calculated using the formula:
V = 4\pi/3 (1/2 length × 1/2 width × 1/2 height)

Mice were sacrificed at the end of the experiment, or when tumours reached a maximum of 1.5 cm³ or weight loss exceeded 15% of the total body weight. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act and the Animal Welfare and the Ethical Review Bodies of the UCL Institute of Ophthalmology. Tumour cell inoculation, treatment by intraperitoneal (i.p.) injection, and mouse sacrifice were carried out by Dr Carlotta Camilli and Dr Camilla Pilotti (UCL Institute of Ophthalmology). Tumour growth and body weight measurements were performed with help from Jack Blackburn (UCL Institute of Ophthalmology).

In vivo tumour localisation analysis of Magacizumab-488 20

Lrg1+/+ C57BL/6 mice were injected subcutaneously into the lower back with single-cell suspensions of 1 ×10⁶ hLRG1-transfected or wild type B16-F0 cells in 100 µL PBS. Tumours were allowed to grow, and on day 12 post tumour cell inoculation mice were subjected to Magacizumab-AF488 (conjugate 20) by a single intraperitoneal injection of 100 µg of the conjugate. Mice were sacrificed 4 h after injection. Tumours were excised and fresh frozen on dry-ice and embedded in optimal-cutting-temperature medium (OCT).

In vivo efficacy assessment

Therapy studies

Therapy study 1

Lrg1+/+ C57BL/6 mice (age, 6–11 wk; weight, 19-31 g) were injected subcutaneously into the lower back with single-cell suspensions of 1 ×10⁶ hLRG1-transfected B16F0 cells in 100 µL PBS. Animals were randomised and allocated to the following groups prior to treatment: (1) Untreated (n=9); (2) Magacizumab (n=9); (3) Cisplatin (n=8); (4) Magacizumab PD-MMAE ADC 24 (n=9); (5) ADC 24 + Cisplatin (n=8). Tumours were caliper-measured and therapy was initiated when tumour volumes reached 0.1 cm³. Magacizumab and ADC 24 were administered at a dose of 20 mg/kg, and cisplatin at
2.5 mg/kg. All treatments were administered by a single intraperitoneal injection every 7 days for 3 weeks. Tumour volumes were measured, and mouse weights were monitored throughout the duration of the study. In addition to weight loss, disease progression was also evaluated qualitatively by observation of behaviour and muscle wasting. Tumour growth curves and survival curves were used to evaluate treatment efficacy.

**Therapy study 2**

*Lrg1*+/+ C57BL/6 mice (age, 6–8 wk; weight, 18-25 g) were injected subcutaneously into the lower back with single-cell suspensions of 1 ×10⁶ hLRG1-transfected B16F0 cells in 100 µL PBS. Therapy was initiated 4 days post tumour cell inoculation, before tumours became palpable. Animals were allocated randomly to the following groups (n=6 per group): (1) Untreated and (2) Magacizumab PD-MMAE ADC 24. ADC 24 was administered by intraperitoneal (i.p.) injection at a dose of 20 mg/kg, every 7 days for 3 weeks. Tumour volumes were measured, and mouse weights were monitored throughout the duration of the study. Tumour growth curves and survival curves were used to evaluate treatment efficacy.

**Immunofluorescence studies**

Subcutaneous B16F0 tumour models were fresh frozen on dry ice in Optimal cutting temperature compound (OCT). Contiguous frozen tissue sections were cut at a thickness of 10 µm for therapy studies and 30 µm for localisation studies and stored at -80 °C. Sections were fixed in 4% paraformaldehyde for 15 min at room temperature or 100% methanol for 5 min at -20 °C, depending on antibodies used. After this, sections were washed with PBS and permeabilised with 0.1% Tween in PBS for 10 min. Sections were blocked in 1% BSA prior to overnight incubation with primary antibody diluted in blocking buffer at 4 °C. Sections were washed in 0.01% Tween-20 in PBS and incubated with secondary antibodies for 1 h at room temperature. Sections were then washed and mounted using Dako mounting medium. Antibodies used to label mouse endothelium were anti-CD31 (Dianova), mouse cathepsin B was labelled using an anti-cathepsin B polyclonal antibody (Invitrogen) and apoptosis was measured using an anti-γ-h2ax antibody (Abcam). Alexa-fluor labelled secondary antibodies were from Thermofisher.
Sections were imaged using a Zeiss 710 confocal microscope. Maximum intensity projections of z-stacks were analysed using NIS elements software (Nikon).

**Statistical analysis**

Statistical analysis was performed using Graphpad Prism version 6.0 for Windows, Graphpad software (La Jolla California USA, www.graphpad.com). Error bars and statistical tests used for each experiment are indicated in the figure legends. Data are expressed as mean ± standard error of the mean (SEM). A $P$ value of less than or equal to 0.05 was considered statistically significant (non-significant $P>0.05$; $*P≤0.05$; $**P≤0.01$; $***P≤0.001$; $****P≤0.0001$).
Magacizumab Fab chapter

Magacizumab Fab

Magacizumab Fab (Maga\textsubscript{Fab}) generation by papain digestion and protein A purification. Reagents and conditions: Papain (1/10, papain/Magacizumab, wt/wt), Cysteine buffer pH 7.2, 37 °C, 24 h.

Preparation of Magacizumab Fab (Maga\textsubscript{Fab}) using papain

Magacizumab 15 (75.4 mg/mL) was reacted with a 1/10 amount (wt/wt) of immobilised papain (250 µg/mL of gel) by incubation for 24 h at 37 °C whilst shaking (1100 rpm), in a buffer containing 20 mM sodium phosphate monobasic, 10 mM disodium EDTA and 80 mM cysteine·HCl (pH 7.2). The cysteine·HCl was incorporated immediately before Magacizumab digestion, and the buffer was adjusted to the correct pH. After this time, the resin was separated from the digest using a filter column and washed with PBS (pH 7.0) three times. The digest was combined with the washes and the buffer was exchanged completely for PBS (pH 7.4) using diafiltration columns (10,000 Da MWCO) and the volume adjusted to 2 mL. The sample was then applied to a NAb protein A column (Thermo Scientific). The Fab fraction, Maga\textsubscript{Fab} 26, was eluted according to manufacturers’ protocol, the column washed three times with PBS (pH 7.4) and the Fc fraction 27, eluted four times with 0.2 M glycine·HCl, pH 2.5, which was neutralised with 10% of the volume of a 1 M Tris, pH 8.5 solution. The Maga\textsubscript{Fab} fraction was combined with the washes and both Fab and Fc solutions were buffer exchanged into PBS using diafiltration columns (10,000 Da MWCO). The digests were analysed by SDS-PAGE and LCMS to reveal formation of Maga\textsubscript{Fab}: observed mass 47,461 Da. The concentration of
MagaFab 26 was determined by UV/VIS using a molecular extinction coefficient of $\varepsilon_{280} = 70000 \text{ m}^{-1}\text{.cm}^{-1}$.

a) SDS-PAGE of digested Magacizumab. M – marker; Lane 1 – Magacizumab; Lane 2 – MagaFab; b) Non-deconvoluted and c) deconvoluted LCMS data for MagaFab 26.
Preparation of MagaFab conjugates

To a solution of Magacizumab Fab 26 (20 µM, 200 µL) in BBS (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) was added TCEP (20 µL, 20 mM in dH₂O, 100 eq.), and the reaction mixture incubated for 4 h at 37 °C under mild agitation (300 rpm). After this time excess TCEP was removed by diafiltration into fresh BBS buffer using PD Minitrap G-25 columns (GE Healthcare) and the concentration was corrected to 20 µM. PD 8 (1 µL, 20 mM in DMSO, 5 eq.) was added to the solution of reduced MagaFab at 21 °C and the solution incubated for 16 h. Excess reagents were removed by repeated diafiltration into fresh PBS buffer (140 mM sodium chloride, 1 mM potassium phosphate monobasic and 8 mM sodium phosphate dibasic at pH 7.4) using diafiltration columns (GE Healthcare, 10,000 Da MWCO), and the volume was corrected to 200 µL. The samples were analysed by SDS-PAGE and UV-Vis spectroscopy. Pyridazinedione to Fab ratio (PFR) was determined to be 0.99. Following this, Alexa Fluor™ 488 azide or Sulfo-Cyanine5.5 Azide (1 µL, 20 mM in DMSO, 5 eq.) was added
and the reaction mixture was incubated at 21 °C for 4 h. Excess fluorophore was removed by repeated diafiltration into fresh PBS using diafiltration columns (GE Healthcare, 10,000 Da MWCO). Following this, sample analysis by SDS-PAGE and UV-vis spectroscopy revealed conversion to the desired MagaFab-PD Alexa Fluor™ 488 conjugate 29 with a fluorophore to Fab ratio (FFR) of 0.91 and MagaFab-PD Sulfo-Cyanine5.5 conjugate 30 with a FFR of 0.93.

**Activity of MagaFab by ELISA**

Binding affinity of Magacizumab Fab to LRG1 was determined by ELISA. A 96-well Maxisorp plate was coated overnight at 4 °C with LRG1 (50 µL of a 4 µg/mL solution in PBS). Next, the coating solutions were removed and each well washed with 0.1% Triton X-100 in PBS (wash buffer) three times. Then, the wells were coated with a 3% BSA solution in PBS (200 µL) for 1 h at 21 °C. After this time, the wells were emptied and washed with wash buffer 3 times. Magacizumab and Magacizumab Fab were diluted in PBS to the following concentrations (ng/mL): 280, 140, 70, 35, 17.5, 8.75, 4.38, 2.19, 1.09, 0.547, 0.273. Wells were coated with the dilution series solutions, each in triplicate, and incubated for 2 h at 21 °C. Then, the solutions were removed, and the wells washed with wash buffer 6 times. The detection antibody; anti-human IgG (Fab-specific) HRP conjugated (1:40,000) was added and incubated for 1 h at 21 °C. Then, the solutions were removed, and the wells washed 6 times with wash buffer. Finally, equal amounts of substrate A (stabilised hydrogen peroxide) and substrate B (stabilised tetramethylbenzidine) (ELISA substrate reagent kit; R&D Systems, DY999) were premixed and added to each well (50 µL). After 20 min, the reaction was stopped by the addition of 25 µL of 2 N sulfuric acid. Absorbance was measured at 450 nm.
Functional assays using Fab

Co-culture tube formation assay

The angiogenesis assay was performed according to manufacturer’s instructions (Cellworks). Briefly, human umbilical vein endothelial cells (HUVECs) and human fibroblasts were seeded, and the plate was incubated at 37 °C in 5% CO2 overnight. Cells were treated with MagαFab (70 pM, 700 pM and 70 nM), and an IgG Fab control (Novus Biologicals, NBPI-97020) (at 70 nM) diluted in growth medium. Medium was replaced every two days for 11 days of culture, during which time the endothelial cells reorganise to form tubules that resemble microvessels formed during angiogenesis. Cells were washed with PBS and fixed with 70% ethanol pre-cooled to 0 °C for 30 min at room temperature, then immunostained mouse anti-human CD31 (Cellworks). Cells were imaged using a TiE Nikon Inverted Fluorescence Microscope. Angiogenesis was quantified using Angiosys (TCS Cellworks). The total tubule length for the treated samples was normalised to equivalent controls. Three independent experiments were carried out with a mean of 12 wells being analysed for each treatment. One-way ANOVA was used to determine the statistical significance between groups.

Mouse foetal metatarsal angiogenesis assay

Metatarsal bones were isolated from mouse embryos at day 17 – 18 and dissection was performed in sterile-filtered 10% heat inactivated foetal bovine serum (FBS) (Gibco) in PBS. 24-well plates were coated with sterile-filtered 0.1% gelatine (Sigma) diluted in 1 × PBS. Each bone was plated in the middle of the well with α-MEM medium (MEM α Med 1X w/Earle’s Salts Glut Nucleosides, Invitrogen) supplemented with 10% FBS and 100 U/mL Penicillin and 100 mg/mL Streptomycin (Gibco) and incubated overnight at 37 °C and 5% CO2 (performed by Dr Camilla Pilotti, Department of Cell Biology, Institute of Ophthalmology, UCL). The metatarsals were left undisturbed for 3 days allowing them to adhere to the wells. On day 3, 5, 7 and 9 the metatarsals were treated with MagαFab (6 nM, 13 nM and 33 nM) and IgG Fab control (Novus Biologicals) at 33 nM, diluted in α-MEM medium. On day 11 the conditioned medium was collected and stored at -80 °C for future analysis. Bones were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. Non-specific binding of antibodies was inhibited
by addition of blocking buffer (10% BSA and 0.1% Triton X-100 (Sigma) in PBS) for 1 h at room temperature. The blocking buffer was replaced with primary antibody (rat anti-mouse CD31, 1:100) diluted in 5% BSA and 0.1% Triton in PBS and left overnight at 4 °C. Excess antibody was washed off with 0.1% Tween-20 in PBS and then replaced with 5% BSA and 0.1% Triton in PBS containing the secondary antibody (Alexa Fluor™ 488 Goat anti-rat IgG, 1:1000) for 2 h at room temperature. Four washes were done with 0.1% Tween in PBS for 1 h.

Bones were imaged using a TiE Nikon Inverted Fluorescence Microscope. Angiogenesis was quantitated using Angiosys (TCS Cellworks). The number of vessel branches and the total tubule length for the treated samples was normalized to equivalent controls. Metatarsals from three independent litters were used and one-way ANOVA with multiple comparisons was performed to determine statistical significance between test groups.

**Laser-induced choroidal neovascularization (CNV)**

Laser-induced choroidal neovascularization (CNV) and quantification using fundus fluorescein angiography (FFA) was performed (by Alex Hoeh, Department of Cell Biology, Institute of Ophthalmology, UCL) as described previously. Each eye received three laser lesions at two to three disk diameters away from the optic nerve head with a slit-lamp-mounted diode laser system (Keeler microlase Windsor, UK; wavelength: 810 nm; laser settings: 210 mW power, 100 ms duration, 100 μm spot diameter). In the MagaFab angiogenesis study, human Lrg1 KI mice received intravitreal injections of various doses of either MagaFab or control Fab, immediately following the laser burn (by Dr David Kallenberg, Department of Cell Biology, Institute of Ophthalmology, UCL). Antibody was delivered at a concentration of 100 μg/mL (each in 1 μL). Seven days after injury, mice were anaesthetized and examined by FFA. Images from the early phase (90 s after fluorescein injection) and late phase (7 min after injection) were obtained using a Micron III retinal imaging microscope with appropriate filters (Phoenix research labs, Pleasanton, USA). The pixel area of CNV-associated hyperfluorescence was quantified for each lesion using ImageJ version 1.44i image analysis software (NIH, Bethesda, MD). Images were blinded for analysis and lesion perimeters were hand drawn to measure pixel area positive for fluorescein. One-way ANOVA was used to test statistical significance between antibody treatment groups.
Statistical analysis

Statistical analysis was performed using Graphpad Prism version 6.0 for Windows, Graphpad software (La Jolla California USA, www.graphpad.com). Error bars and statistical tests used for each experiment are indicated in the figure legends. Data are expressed as mean ± standard error of the mean (SEM). A $P$ value of less than or equal to 0.05 was considered statistically significant (non-significant $P>0.05$; $*P\leq0.05$; $**P\leq0.01$; $***P\leq0.001$; $****P\leq0.0001$).
Appendix B Publications


   - **Publication derived from work presented in Chapter 6**


   - **Publication derived from work presented in Chapter 1-5**

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