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REVIEW

Polyreactivity and polyspecificity in therapeutic antibody development: risk factors for failure in preclinical and clinical development campaigns

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ABSTRACT

Antibody-based drugs, which now represent the dominant biologic therapeutic modality, are used to modulate disparate signaling pathways across diverse disease indications. One fundamental premise that has driven this therapeutic antibody revolution is the belief that each monoclonal antibody exhibits exquisitely specific binding to a single-drug target. Herein, we review emerging evidence in antibody off-target binding and relate current key findings to the risk of failure in therapeutic development. We further summarize the current state of understanding of structural mechanisms underpining the different phenomena that may drive polyreactivity and polyspecificity, and highlight current thinking on how de-risking studies may be best implemented in the screening triage. We conclude with a summary of what we believe to be key observations in the field to date, and a call for the wider antibody research community to work together to build the tools needed to maximize our understanding in this nascent area.

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INTRODUCTION

Antibody-based drugs are currently the dominant biologic therapeutic modality used to modulate signaling pathways, mediate immune cell killing and/or for targeted delivery of small molecules via antibody-drug conjugates. This dominance in the biologics space is somewhat logical, as antibodies have evolved over millennia to frequently exhibit characteristics that support pharmaceutical development, such as high expression rates, high stability, high solubility, long pharmacokinetics (PK), and potent activity that is (in theory) specific to a single molecular target.[1]Despite these general beneficial characteristics, antibody therapeutics still regularly fail during development and the further that therapeutic monoclonal antibodies (mAbs) progress in preclinical and clinical development programs, the more costly failure becomes. As a result, many groups in the field have invested heavily to elucidate the factors that cause these failures and to develop screening methods to identify and eliminate those antibodies with the worst risk profiles.[2] It is now well established that failures may be caused by liabilities in molecular characteristics known as 'polyreactivity' that lead to unacceptably poor PK, potency, bioavailability or immunogenicity.[2] These investigations have generally not, however, focused on polyspecificity as a high-risk antibody characteristic for failure of drug discovery campaigns.

We believe that the relative paucity of studies that focus on polyspecificity as a development risk may be due to two main factors: 1) The historical (now outdated) assumption that monoclonality inherently equals monospecificity; and 2) The lack of rapid, affordable, and reliable methods for thoroughly investigating antibody specificity. In this review, we describe the current state of the art in monitoring antibody specificity, discuss what is known about the causes of off-target antibody binding, highlight key evidence that demonstrates the associated preclinical and clinical risks, and consider where the field may be going next in solving 'the unknowns'.

What do we mean by 'polyreactivity' versus 'polyspecificity' in therapeutic antibodies?

Firstly, we will focus on how we define the differences between 'polyreactivity' and 'polyspecificity' as they pertain specifically to mAbs intended for therapeutic use. In our estimation, these two definitions denote distinctly different issues, with distinct risk associations in clinical antibody development programs. Historically, the terms have been used somewhat interchangeably in the field and we believe the literature is now becoming mature enough that a clear delineation can be made.

In the context of the natural (endogenous) antibody repertoire, 'polyreactivity' is often defined as the ability of a mAb to bind a variety of self and foreign antigens which may be completely unrelated and is often attributed to 'a more conformationally flexible antigen binding pocket'.[3] It has been speculated that up to 50% of serum IgM in humans is polyreactive, and that polyreactivity is a highly conserved feature of the immune system. In the first line of defense against bacterial and viral pathogens, a major component of the natural antibody repertoire has been classically defined as 'polyreactive'.[4] The selection of polyreactive B cells against broadly

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neutralizing epitopes of influenza viruses was recently shown to be linked with increased binding coverage of viral strains, affinity and antibody flexibility.[5] As such, we would suggest that (*sensu stricto*), the classical definition of 'polyreactivity' in the endogenous repertoire really denotes a fundamental 'sticky' characteristic. To maximize protective potential for the host, it seems logical that the early repertoire might exhibit the ability to bind to a large variety of potential structures, with low starting affinity and low specificity, as this maximizes the potential antigen space that the repertoire can cover. Indeed, this low initial binding affinity against foreign antigens is directly compensated for by the early B-cell repertoire being predominantly generated in the large, polyvalent, highly avid, IgM format. To progress through class-switching into bivalent IgG format, with associated increases in binding affinity, T cell help is generally needed. This T cell help should be readily available for most foreign antigens, but not for self-antigens due to thymic tolerance in the host. Thereby, in a healthy individual, higher-affinity antibodies to self-antigens are generally deleted from the repertoire.[6]

Therapeutic antibodies, in contrast, are predominantly developed exogenously to humans, with exceptions including those that are isolated directly from, for example, convalescent infectious disease patients or vaccinees.[7] The development cascades that individual therapeutic antibodies go through can therefore often be distinctly different to those in the endogenous repertoire. Antibody development for therapeutic applications has been facilitated by hybridoma technology, antibody humanization, transgenic animals carrying the human IgG repertoire, single B cell screening technologies or display-based *in vitro* selection platforms.[7] Several of these technologies now enable the generation and/or designer engineering of antibodies with human sequences (a.k.a. 'fully human' antibodies). Critically, however, none of the exogenous technologies replicate the exquisite B-cell receptor editing and natural self-reactivity deselection checkpoints that occur *in vivo* in humans.[6] As a result, antibodies derived from both humanized and fully human backgrounds can exhibit unwanted low-affinity extracellular matrix and/or membrane interactions via general nonspecific chemistry. These interactions are associated with the presence of excess positive charge or hydrophobicity in the variable regions of the antibody and can have dramatic negative effects on the PK and bioavailability of antibody therapeutics.[2] It is this chemical "stickiness" that we define as 'polyreactivity' in the drug discovery setting and discussed in detail below.

Less well documented, and substantially more complex to uncover, is the phenomenon of polyspecificity, where recombinant antibodies display discrete off-target reactivity of meaningful affinity to structurally and/or functionally disparate targets. In the bivalent IgG format used by most therapeutic antibodies, very low or even high-affinity interactions with unintended host proteins may or may not be capable of affecting clinical performance. Several case studies have demonstrated that polyspecificity can cause accelerated clearance and unpredictable toxicities.[8,9] Figure 1 aims to illustrate the difference between therapeutic antibody polyreactivity and polyspecificity.

Polyreactivity as a risk factor for PK and bioavailability

Initial observations of the relationship between antibody variable domain pI and nonspecific binding were made over 20 years ago.[10–15] Modulation of v-domain charge content in recombinant immunotoxins was achieved through mutation of basic and neutral residues to neutral and acidic residues, respectively, and this was shown to substantially improve tumor-specific potency with a concomitant decrease in toxicity.[16] This was subsequently attributed to a decrease in nonspecific binding to normal tissue. This idea was further developed by Igawa and colleagues, who generated a series of anti-IL6R antibodies identical in all aspects but v-domain pI, with values ranging from that of the parental molecule at 9.3 down to 5.5.[17] Mouse pK studies with these closely related variants demonstrated that clearance rate was directly correlated with increasing pI of the variable domain. The authors speculated that this was a non-FcRn-dependent mechanism, and that lower pI conferred a net negative charge on the protein that decreased pinocytosis and elimination.

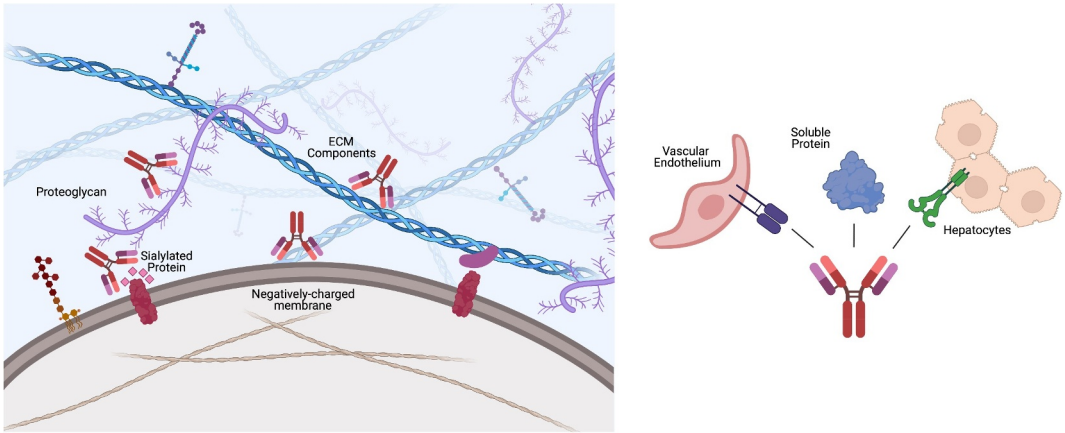

Figure 1. Polyreactivity vs Polyspecificity. (a) Excessive positive charge or hydrophobicity in antibody variable domains can lead to low-affinity, nonspecific interactions with negatively charged or hydrophobic cell membranes and ECM components across tissues. (b) Polyspecificity is defined as off-target, specific interaction with a discrete number of proteins which may or may not be related in terms of sequence or structural motifs.

Excessive positive charge in antibody v-domains has also been shown to directly affect FcRn-dependent pK, and this was demonstrated in a very elegant study by Schoch and colleagues comparing two anti-p40 antibodies,[18] briakinumab (generated *in vitro* from a human antibody library using phage display) and ustekinumab (generated *in vivo* from a 'humanized' mouse). Intriguingly in this case both antibodies have very similar pIs (9.6 for briakinumab, 9.3 for ustekinumab), but radically different median terminal half-lives (8 days for briakinumab, 22 days for ustekinumab). Comparative structural analyses indicated that ustekinumab shows a very even charge distribution across the v-regions. Conversely, briakinumab exhibited a large, positively charged patch dominating the variable region of the light chain. This charged patch was postulated to interact directly with an extended negatively charged region on FcRn that is not involved in standard Fc binding.[18] An FcRn affinity column with pH gradient elution was developed to mimic FcRn-IgG dissociation at physiological pH and the column retention times for the parental antibodies, as well as a series of hybrid clones that combined elements of both, were compared. Subsequent mouse PK studies demonstrated a direct correlation between FcRn elution time and *in vivo* PK, with the significantly extended retention time of briakinumab on the FcRn column being predictive of accelerated clearance *in vivo*. This study demonstrates that antibodies with high pI values do not necessarily have poor pK, but that balanced local charge distribution on the antibody variable domain surface is key.[18]

An additional interesting observation from this study lies in the very different paratope solutions that evolved for both antibodies against the same p40 antigen: *in vivo* via immunization for ustekinumab and *in vitro* via phage display for briakinumab. The iterative selection process that underpins display technologies is likely to be more susceptible to excessive charge build up in antibody complementarity-determining regions (CDRs) during engineering given the powerful influence of electrostatic interactions on affinity.[19,20] This phenomenon has recently been explored during optimization of an anti-IL21R antibody, where the epitope on IL21R represented a negatively charged patch.[21] Despite the application of multiple different selection strategies, the incorporation of aggressive deselection pressures using negatively charged molecules and the design of a variety of mutational libraries, the buildup of excessive positive charge in CDR loops could not be avoided.[21]

These studies describe the importance of high pI and net charge, but more importantly the influence of balanced charge, on polyreactivity. There are both FcRn-dependent and independent mechanisms proposed for the observed correlation between charge and clearance (Figure 2). The additional effect of excess charge on tissue distribution was explored in two studies published in 2015.[22,23] As in previous studies, Datta-Mannan and colleagues demonstrated rapid clearance of an antibody with an extensive positively charged patch, but also showed rapid accumulation of this antibody in the liver. Both peripheral and central clearance were significantly elevated, and these effects could be ameliorated through charge balancing. Peripheral clearance was postulated to occur via enhanced binding, via positive charge, to negatively charged cell membranes and associated negatively charged molecules like chondroitin, heparin and sialic acid, which are present in abundance on the vascular endothelium. *In vitro* plate-based assays measuring binding to heparin and HEK293 cell membranes directly correlated with the *in vivo* observations. These surrogate assays are exemplars of a drive across the industry to develop and implement a suite of high-throughput methods that can identify and eliminate polyreactive antibodies from the discovery pipeline at an early, and relatively inexpensive, stage of the screening process. These assays have been

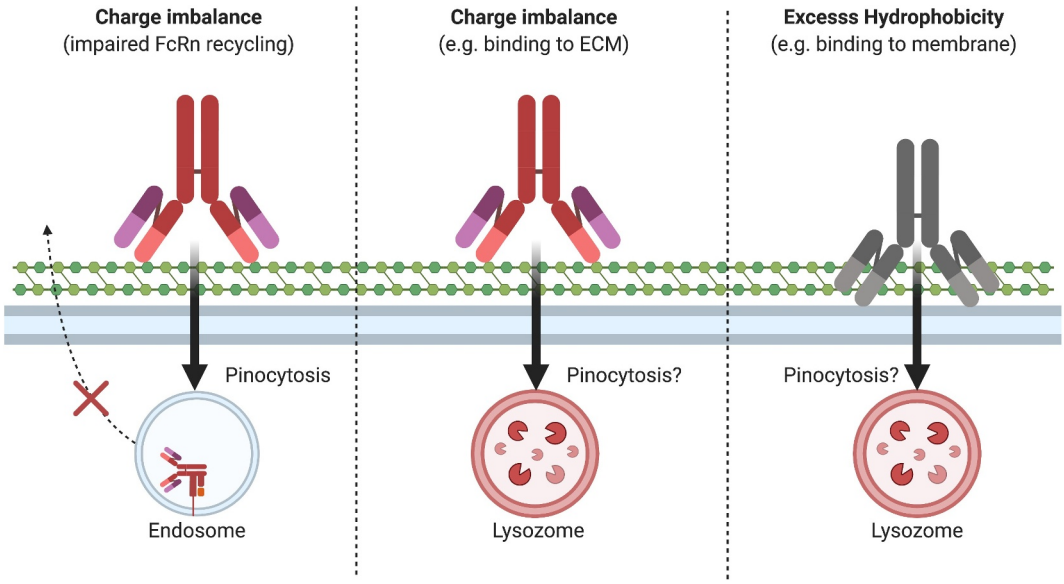

Figure 2. Off-target binding: Polyreactivity (stickiness) factors and effects. Charge imbalance, i.e., excessive positive charge in antibody variable domains, is known to cause low-affinity interactions with negatively charged membrane. Charge imbalance can potentially drive accelerated pinocytosis and/or increase the affinity of antibodies for FcRn at pH7.4, impairing recycling. Both factors combine to reduce PK and bioavailability of therapeutic antibodies. Similarly, excess hydrophobicity may cause nonspecific low-affinity binding to membranes and other hydrophobic surfaces, potentially increasing pinocytosis rates, affecting PK and biodistribution. Both charge imbalance and excess hydrophobicity can be measured using well established, rapid and affordable plate-based and chromatographic methods.

established using panels of antibodies for which *in vivo* clearance data are known, thus enabling predictions for *de novo* antibodies. The first surrogate assay that was shown to predict *in vivo* PK was a baculovirus binding assay established by Hötzel *et al.*[24] This was followed by additional ELISA-based formats that measured nonspecific binding to cell membrane preparations[25] and charge-mediated binding to negatively charged molecules including FcRn,[18] heparin,[22] DNA and insulin.[2] These assays best identify antibodies which suffer from polyreactivity caused by 'charge imbalance'.

Where once the focus for antibody engineering lay in humanization of v-domains to improve immunogenicity risk profile, and/or affinity optimization to improve potency, the field has latterly embraced the idea of 'developability' being equally important in a successful preclinical to clinical transition. Therapeutic antibodies undergo a number of stresses and mechanical strains during the scale-up to manufacturing process, including fluctuations in pH, temperature, concentration and buffer conditions, any of which may have a negative impact on protein stability.[26] Aside from polyreactivity, antibody self-association and aggregation propensity has emerged as more than just a bioprocessing factor. These issues can also affect antibody specificity, pK and efficacy.[27] A phage display-derived anti-nerve growth factor antibody was successfully affinity optimized to 69 pM K_D, but with this came a concomitant increase in viscosity and aggregation, with significantly increased retention on an SEC column that resulted from nonspecific interaction with the column matrix.[27] These post-optimization issues were localized to three surface-exposed hydrophobic amino acids that had been introduced during engineering and were shown to drive nonspecific interaction in the baculovirus binding ELISA, nonspecific tissue binding in a broad tissue cross-reactivity study, and ultimately accelerated pK *in vivo*. It is clear from this study that polyreactivity can be driven by both charge and hydrophobicity, and that these properties should be screened for across a range of assays to ensure these diverse but overlapping phenotypes can be uncovered at an early stage of development.[27]

A comprehensive study from Jain et al. characterized a broad panel of antibodies from across the clinical landscape in a diverse set of developability assays, including polyreactivity.[28] Critically, this study demonstrated the value of this type of screening in predicting successful progression of an antibody through late-stage development, showing that a strong polyreactivity profile is one of several risk factors that could potentially combine to increase the likelihood of failure in clinical development. Importantly, the Jain et al. study[28] provided insights into the strong polyreactivity characteristics of some high-profile late-stage clinical development failures, such as bococizumab, a humanized and affinity matured anti-PCSK9 antibody that suffered from short PK, poor biodistribution after sub-cutaneous dosing and high immunogenicity in man.[29–31]

Polyspecificity: preclinical evidence for accelerated clearance and toxicity

As outlined above, we are now much better informed as a field on how to detect and avoid polyreactivity at an early stage of the development process.[2,32] In contrast, due to the historical lack of clear examples, we have struggled with the more complex issue of specific off-target reactivity to highly disparate targets in the human and preclinical animal test species proteomes, which is the phenomenon we suggest should be defined as 'polyspecificity'. As a result, there remains a lack of understanding in how, when, and why to screen for such a phenotype, and a lack of predictability in how off-target reactivities might affect behavior *in vivo*.

As early as 1990, Stern and colleagues reported that a monoclonal anti-β-amyloid peptide antibody exhibited binding to circulating human fibrinogen in peripheral blood, in addition to the clots from plasma and purified fibrinogen.[33] The techniques used in this study included immunoblotting and peptide ELISAs, and the authors concluded that this cross-reactivity was due to conformational homologies between the two proteins, urging caution in the use of such antibodies for diagnostic purposes. However, this type of polyspecificity can also have important implications for therapeutic antibody development. For example, in a study by Bumbaca *et al.*, an anti-fibroblast growth factor receptor 4 (FGFR4) antibody derived from a murine hybridoma was characterized *in vitro* post-humanization and shown to have the same binding and functional characteristics as the parental chimeric molecule.[9] Surprisingly, the humanized version of the antibody demonstrated rapid clearance, poor target tissue biodistribution and limited efficacy in a human xenograft mouse model. Immunoprecipitation (IP) followed by mass spectrometry identified specific off-target binding to mouse complement component 3 (C3), a highly abundant soluble serum protein. The authors subsequently eliminated this off-target reactivity through affinity optimization, via mutation and selection on FGFR4 (captured) in the presence of high-concentration mouse C3 (in solution). While no structural work was carried out as part of this study, it was speculated that additional loop flexibility or plasticity could have been introduced during CDR grafting as part of the initial humanization process.[9]

The Bumbaca et al. study highlighted the potential for polyspecificity to cause rate-limiting PK and efficacy problems during the preclinical characterization of potential antibody-based therapeutics.[9] Of even greater concern is the potential for off-target reactivities to cause toxicity-driven adverse events. Santostefano and colleagues showed that an antibody against a soluble target resulted in thrombocytopenia, platelet activation, reduced blood pressure and transient loss of consciousness in cynomolgus monkeys.[34] Interestingly, other antibodies that were derived during the same lead discovery campaign did not have the same effects, and the platelet activation that was seen for this antibody was only observed with primary cells from macaque species and not from other non-human primates or human cells *ex vivo*. Further investigation suggested that this was a case of species-specific off-target reactivity that required interaction of the antibody with a target expressed on platelets via the antigen-binding fragment (Fab), in addition to engagement of FcγRIIa via the Fc to induce sufficient cross-linking for platelet activation.[34] A follow-on study to this work also noted a similar species-specific platelet activation and acute thrombocytopenia with antibody CH12.[34] In this case, the authors invested heavily in identifying the off-target protein that was responsible for the observed effects. Traditional IP

methods were not successful, perhaps hinting at the lower affinity of the antibody with the off-target protein. A complex age-grouped proteomics analysis was undertaken and after a series of statistical and empirical analyses, integrin αIIbB3 was identified as the culprit off-target antigen.

In an interesting parallel to the Stern et al. study mentioned above,[33] a recent study by Loberg et al. also reported an anti-β-amyloid antibody (ABT-736) with off-target reactivity.[8] The development of ABT-736 was discontinued due to severe toxicity observed in cynomolgus monkey studies. These toxicities included infusion reactions, ataxia, emesis, tremors and/ or decreased body temperature, and thrombocytopenia. The authors conducted extensive follow-up investigations that identified high-affinity binding of ABT-736 to monkey and human plasma protein platelet factor 4 (PF-4), which is known to be involved in heparin-induced thrombocytopenia (HIT) in humans. The authors also derived a second high-affinity antibody to β-amyloid (h4D10), which did not bind PF-4 or other unintended targets and was found not to drive toxicity in monkeys. The authors concluded that this finding supported the hypothesis that ABT-736 toxicity was not target-related but driven by polyspecificity. They further concluded that "thorough screening of antibody candidates for nonspecific interactions with unrelated molecules at early stages of discovery can eliminate candidates with polyspecificity and reduce potential for toxicity caused by off-target binding."[8]

Clinical observations of toxicity driven by polyspecificity

As outlined above, it has become clear that polyspecificity is more than just a laboratory artifact and can cause rate-limiting difficulties in preclinical antibody drug development. Despite these extensive preclinical findings, until recently there has been no clear 'smoking gun' that connects polyspecificity with adverse events in the clinic. This is unsurprising, given that full proteomic screening for specificity analysis (depicted in Figure 3) has not been part of the classical antibody development cascade until very recently and is still only regularly reported by a small number of companies. As a result, there has never been any systematically generated data to link off-target reactivity to a clinical phenotype. In addition, it remains relatively rare for a series of biologic drugs to be developed against the same protein target, where they have essentially the same mode of action and where they are thought to differ only in their affinities, relative potencies, and exact epitope specificities. The proliferation of companies prosecuting multiple anti-programmed cell death protein 1 (PD1) antibodies in the clinic has enabled the identification of one clear example of clinical polyspecificity: camrelizumab.

The anti-PD1 antibody SHR-1210 (camrelizumab) exhibits potent PD1 antagonism in man, but uniquely among PD(L)1 pathway drugs, it causes capillary hemangioma in most patients dosed.[31,35,36] Hemangioma is a benign tumor in which blood vessels hyper-proliferate in the skin and potentially in the liver and other organs.[37] In a recent study, proteomic screening using a cell microarray library of >5,500 human proteins successfully identified that SHR-1210 binds not only human and monkey PD1, but also human vascular endothelial growth factor receptor 2 (VEGFR2), FZD5 and ULBP2.[38] The study further concluded that SHR-1210 stimulates vascular neogenesis (leading to hemangioma) through potent agonism of the pro-angiogenic receptor VEGFR2. Subsequently, in an unrelated study, a research group running clinical trials on SHR-1210 published data corroborating that SHR-1210 does indeed bind human VEGFR2, with an estimated affinity in the μM range.[39] Importantly, it has since been shown that salvage therapy with the potent VEGFR2 antagonist apatinib ameliorates the hemangioma effect of the antibody.[40] This finding

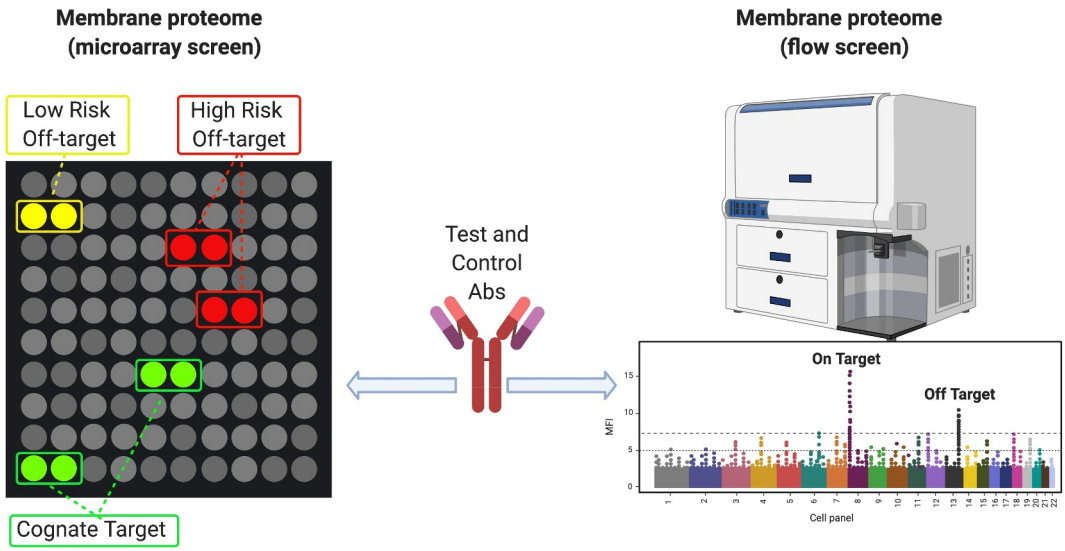

Figure 3. Off-target binding: Methods for identifying Polyspecificity. Two main methods are currently used for screening the human membrane proteome. Microarray screening may be performed using purified proteins or via retro-transfection, leading to presentation of native conformation protein on human cell membranes. Similarly, high-throughput flow cytometric screening may be performed e.g., on cells or virus-like particles that have been transfected to express human membrane proteins. In both cases, test and control mAbs are examined for their ability to bind their cognate target (On Target hit) or unrelated proteins (Off Target hit). Off Target hits are subsequently verified via orthogonal assays and decisions on risk profile are made.

appears to 'close the circle', confirming that the mechanism by which the antibody causes angiomas is indeed *via* VEGFR2 agonism.

We believe that the findings outlined above are 'food for thought' for the therapeutic antibody community. Had camrelizumab been the first and only PD1 molecule to undergo clinical study, the assumption might have been that hemangioma is simply an unexpected side effect of PD1 pathway inhibition. Interestingly, the original mouse-derived progenitor of camrelizumab exhibited the same polyspecific interactions, also driving VEGFR2 agonism.[38] Such classical hybridoma or B-cell screening-derived antibodies are assumed to be 'deselected' for auto-reactivity due to immune tolerance mechanisms, but this is only true for the host species proteome in which the antibody was developed and not for the human one. Indeed, as researchers often try to generate antibodies in animals where the homology is relatively high between human and immune host orthologs of the target, immunization regimes will often deliberately drive to break tolerance. In this derived scenario, it may be the case that 'all bets are off' regarding specificity of the resulting antibodies. We would therefore argue that the ultimate question for any antibody moving into formal preclinical development should not be 'Which technology platform was applied?', but rather 'How rigorously has it been characterized?'

The camrelizumab story is unlikely to be unique, and unexpectedly poor PK, biodistribution or side effect profiles in human may be driven by unidentified polyspecificity. An open question to the field is 'How many adverse events and failures in both the lab and the clinic are a result of a failure to address potential polyspecificity?' An important factor to consider is that we are currently only beginning to measure those things that we can measure. The technologies currently at hand, which are designed to effectively examine specificity across the proteome (human proteome arrays, high-throughput flow cytometric screening, etc.), sample only the human receptor proteome and even that is sampled incompletely. Current methods also cannot effectively sample compound epitopes spanning two or more multimerizing proteins or receptor-ligand interactions. Moreover, it is hypothetically possible that common 'off target' events might be observed in such technologies, where many antibodies could show binding to the same human proteins, but more due to the inherently 'sticky' nature of the protein displayed in the technology than the actual reactivity of the antibody. As such, we do not yet have any fully realized technologies that can give us comprehensive insights into specificity within the human proteome and (at the time of writing) we have no ability to measure similar off-target polyspecific interactions in the proteomes of cynomolgus or rhesus monkeys, mouse, rat, dog, mini pig and other species regularly used in critically important preclinical studies of antibodies. Clearly, technological advances that enable efficient and cost-effective screening of human and preclinical species proteomes are required. The application of such a technology would facilitate predictive screening that would reduce failures in the preclinical space.

Structural mechanisms believed to mediate antibody polyspecificity

While our understanding of antibody polyspecificity remains nascent, some early insights have been gleaned on the structural drivers of multi-target recognition. These factors may be separated into 3 classes. The first of these is molecular mimicry, where an antibody can bind to proteins with no immediately obvious overall sequence identity, but a few critical epitope residues that may be mirrored in a completely unrelated protein. This is exemplified by the work of Tucker *et al.*, which describes the isolation of a panel anti-GLUT4 antibodies using a combination of *in vivo* chicken immunization coupled with *in vitro* phage display and selection.[41] A standard screening triage was adopted, first measuring binding of lead antibodies to virus-like particles expressing GLUT4, followed by confirmatory analysis on HEK-293-GLUT4 cells. LM052 displayed high affinity binding to membrane-expressed GLUT4, but did not bind to the closely related human GLUT1, GLUT2, GLUT3 or to mouse GLUT1. However, a membrane protein array screen of 4,571 receptors demonstrated low affinity but dose-dependent, specific binding to Notch-1. Overall sequence homology between these proteins is <7%, but the core epitope residues recognized by LM052 on GLUT4 (61-LGXXGP-66) are mirrored almost exactly in a disulfide-constrained loop in Notch-1 (91-LGXXGP-96). The study concludes that off-target binding to sequence and structurally unrelated proteins should be tested on a case-by-case basis and certainly cannot be predicted *a priori*.[41] Indeed, the risk of identifying antibodies that are polyspecific could be considered higher for viral and bacterial targets given that molecular mimicry is a common feature of infectious agents in evading the host immune response. There are several examples of this in the literature, including anti-HIV antibodies that bind cardiolipin,[42] anti-Dengue NS1 antibodies that recognize human LYRIC protein,[43] and antibodies against the group A streptococcal M protein that also interact with myosin.[44]

The second molecular mechanism that can potentially drive polyspecificity is best described as CDR plasticity, a phenomenon by which the antibody paratope may adopt multiple conformations.[45,46] For example, 4E10 is a broadly neutralizing anti-HIV antibody that targets a highly conserved linear epitope in the membrane-proximal region of the envelope glycoprotein. Analysis of B-cell development in 4E10 heavy-chain knock-in mice demonstrated that 4E10 recognizes self-antigen and was largely deleted from the repertoire.[45] However, subsequent characterization using immunofluoresence and surface plasmon resonance methods discounted a broadly polyreactive phenotype for the antibody with only low affinity, weak interactions for phospholipid head groups being accommodated by a dramatic restructuring of the central combining site in the absence of complexed ligand (mediated in part by an unusually long H-CDR3 loop). In a search for novel autoantigens, the group used a phage-displayed peptide library representing the complete human proteome in overlapping 36-mers. Of the top 5 hits identified, 3 represented closely related type 1, 2 and 3 inositol triphosphate receptors that all shared a conserved peptide sequence motif. This sequence is completely divergent from the 4E10 core epitope on the MPER, hinting at alternative

molecular interaction mechanisms that can be accommodated by a flexible combining site. The potential for H-CDR3 loop conformational flexibility has also been modeled in a recent study describing discrete conformation-dependent charge states in an antibody with a long HCDR3 loop.[47] In this case, a pH-dependent change between H-CDR3 "open" and "closed" conformations was driven by local pKa changes in the side chains of Asp-100 and Asp-118, which are located at the base of the H-CDR3 loop.

The third example of polyspecificity does not rely on common motifs or conformational flexibility but describes interaction of an antibody with two unrelated proteins with high affinity simply through differential engagement of the VL and VH CDRs. In other words, single antibodies may have multiple (potentially overlapping) functional paratopes, depending on the protein they are binding. The *in vitro* evolution of an antibody that deliberately encodes for this 2-in-1 specificity was described very elegantly by Fuh and colleagues using trastuzumab (Herceptin®) as a starting point.[48] Herceptin has been shown to mediate interaction with human epidermal growth factor receptor 2 (Her2) primarily through the heavy chain. Mutagenesis in the light chain identified several variants that had acquired high-affinity binding to a second unrelated antigen, VEGF. Structural and functional analysis demonstrated that the interactions between one of these variants, bH1-44, and the two entirely unrelated antigens are distinct and characterized by a simple conformational adaptation of the central combining site. Remarkably, two mutations in the heavy chain of bH1-44 were sufficient to knock out binding of Her2 while retaining high-affinity interaction with VEGF. Similarly, 2 alanine substitutions in the light chain of bH1-44 knocked out VEGF interaction with no impact on Her2 engagement.[48] While this work describes directed evolution toward the desired 2-in-1 modality, it is striking that even minimal mutation can result in such striking specificity differences. These mutagenesis approaches are commonplace in antibody optimization campaigns. Interaction with the antigen of interest is monitored throughout the process, but how many of these new mutations have the potential to mediate interaction with unrelated antigens?

Moving beyond the standard antibody format, it remains unknown what the frequency of polyspecificity issues are in the myriad antibody-derived modalities from disparate technologies that are currently intended for clinical development. If polyspecificity is more common than is currently recognized in the field, it could drive unexpected and severe toxicities in newly-developed antibody-based immunotherapy modalities with extremely potent cell-killing mechanisms of action, such as antibody-drug conjugates,[49] CD3-targeting bispecifics,[50] or CAR-T.[51] As with so many aspects of therapeutic antibody discovery, deep, systematic study of antibody specificity is lacking and something that the field would clearly benefit from.

Structural heterogeneity and dynamics: post-translational modifications as a potential risk factor for polyreactivity and polyspecificity?

One of the cardinal principles of chemistry, biology, and medicine, is that chemical structure determines properties and functions. For proteins, the primary structure (i.e., amino acid sequence) intrinsically determines the higher order structures. Therapeutic proteins such as antibodies are almost exclusively recombinant and expressed from clonal cell lines containing a single antibody gene sequence, but recombinant antibodies are still heterogeneous to some extent, due to structural changes in protein processing during cell culture (e.g., ribosomal errors, disulfide scrambling), or chemical stress during purification procedures and even formulation and storage.[52] These intrinsic heterogeneities in chemistry are known as post-translational modifications (PTMs). For antibodies, myriad PTMs have been documented, such as deamidation of asparagine, isomerization of aspartic acid,[53] sulfation of tyrosine, oxidation of aromatic residues, peptide cleavage (clipping), glycosylation and even crosslinking.[54] As a result, therapeutic antibodies are highly heterogenous in their chemical compositions, including the so-called "charge variants" and "sequence variants"(variants in which the exact amino acid sequence has not been fully faithfully replicated, due to fidelity errors during expression).[55]

A more precise definition of intrinsic protein heterogeneity is the newly coined term "proteoforms", which designates all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and PTMs.[56] These proteoforms are known to alter various chemo-physical properties of proteins, including aggregation and binding affinity to the intended targets. Critically, proteoform-altering characteristics such as tyrosine sulfation and glycosylation in antibody variable domains have been shown to be critical determinants of antibody binding to cognate targets.[57,58] Indeed, fascinating examples have also been described in which soluble cofactors such as calcium ions and heme may be critically required for antibody target binding.[59,60] These findings suggest that modification of the antibody paratope–epitope interaction can be mediated by PTMs of the side-chains and/or soluble factors, which may lead to dramatic changes in function.[61] However, as mentioned above, few studies have explored the impact of these proteoforms on polyreactivity and polyspecificity.

Many PTMs drastically alter the structure of proteins. For instance, deamidation of asparagine generates both aspartic and isoaspartic acids, thereby converting a neutral amide into a negatively charged carboxylate.[62] More importantly, all natural peptide bonds are of alpha-linkage, but isoaspartic acid confers a beta-peptide linkage, which has been shown to markedly alter backbone conformation, higher-order structures, proteolysis,[63] antigen presentation and immunogenicity.[64,65] These chemical events are somewhat analogous to the modulation of local variable domain and CDR loop chemistry and structure by DNA-encoded mutations introduced during affinity maturation, which is also intrinsic to achieving more stable binding to antigen. It seems plausible that such mutations also have the potential to alter specificity. It follows therefore, that PTMs in CDRs may also have the potential to modulate changes in specificity, as the antibody paratope is inherently changed by the PTM. Thus, we hypothesize that antibody polyspecificity may not always be intrinsic to a given molecule, but may be inducible.

To reduce and control the PTM-driven heterogenicity of antibodies, considerable efforts have been spent on optimizing upstream and downstream processes, such as cell culture

optimization, purification, and formulation. Mildly acidic conditions are often used in formulation to slow down deamidation during storage.[52] Importantly given the long half-lives of antibodies, PTMs may also occur *in vivo*, with repeated pH stress during endothelial recycling and exposure to circulating soluble enzymes such as proteases. It has been shown that antibodies can exhibit up to 20% deamidation post-administration.[66] Trastuzumab has been shown to undergo deamidation within both CDRs and constant regions. Though formulation can help to maintain deamidation at relatively low levels, degradation can be observed *in vivo* within a matter of days.[67] As a result, therapeutic antibody candidates are routinely studied under challenge of *in vitro* and/or *in vivo* stress conditions to identify any highly modifiable 'risk' positions in the protein sequence. However, for the "stressed samples", the common parameters being examined are still limited to stability and binding affinity to the cognate target. As testing for polyreactivity and polyspecificity becomes a routine part of antibody therapeutic discovery, it seems plausible that PTM investigations may become integral to understanding whether an antibody has intrinsic or inducible specificity problems.

Screening for polyspecificity as part of a standard antibody discovery campaign

Ideally polyspecificity screening should be an integrated part of the drug discovery process. But how and where to implement? As discussed earlier, there are crucial risks in delaying polyspecificity screening until undesirable preclinical or clinical observations emerge. However, screening for polyspecificity on the final lead or candidate molecule also carries risks – in this instance, disruption to the drug discovery process is inevitable because the identification of off-target liabilities at this stage necessitates thorough examination of the fundamental properties of the off-target itself, and detailed *in vitro* characterization of the interaction of the lead molecule with the human off-target protein and relevant orthologues. In some cases, *in vivo* studies may also be warranted to assess the impact of off-target binding on the PK of the lead molecule.

Polyspecificity screening at a much earlier stage in the drug discovery process creates different risks, primarily in generating potentially misleading data. Screening before lead optimization is generally not advisable since affinity maturation and other engineering steps that modify the variable domain sequence could well alter both the on- and off-target binding profile. Screening at an early stage also creates an issue simply in terms of feasibility due to challenges with generating sufficient quantities of high-quality material for large panels of molecules.

Therefore, as highlighted in Figure 4, we believe a sweet spot for incorporating polyspecificity screening in the drug discovery process exists *prior to* selection of the final lead but *after* completion of lead optimization. By incorporating polyspecificity screening at this stage, ideally using a small panel (n ≤ 10) of epitope-diverse molecules, key decision-making data are generated to enable selection of the final lead. This approach is exemplified by observations that off-target binding is specific to a small proportion of molecules within a diverse panel, as would be expected.[8,34] Furthermore, if polyspecificity is identified sufficiently early in the drug discovery process, this feature can potentially be successfully 'engineered out' of a single favored lead, as described in the next section.[9,21,38]

So, my antibody is polyspecific – what now?

All is not necessarily lost if polyspecificity is identified. It has been shown through comprehensive molecular engineering that the antibody paratope may be refined to successfully remove off-target binding. Having demonstrated that SHR-1210 exhibited a polyspecific phenotype, Finlay et al. used a rapid, in-depth screen of the CDR amino acid tolerance of SHR-1210 to both human germline and non-germline

Discovery Phase	Rationale to Screen	Feasibility	Risks	Summary
Hit Identification (HT Screen, 1000+ mAbs)	**Low** Too early: mAbs may not be final affinity-matured / engineered variants and are unlikely to be in final therapeutic format	**Low** Polyspecificity screening for very large numbers of mAbs not feasible currently	**Medium** Polyspecificity screening at this stage could be misleading since affinity-maturation and engineering can alter the profile	**Too early to screen:** issues with feasibility and potentially misleading data.
Lead Optimization (detailed functional and biophysical screening, ≤100 mAbs)	**Strong** mAbs are affinity-matured / engineered variants in final format	**Medium** Feasible if a favoured clone from each epitope bin can be selected; confirmatory screen on final lead may be required	**Low** Screening all available epitope bins likely to identify most potential off-target risks	**Strong rationale to screen.**
Cell Line Development (≤10 mAbs)	**Strong** mAbs are affinity-matured / engineered variants in final format	**High** Feasible to screen small panels of mAbs	**Low-Medium** Variable risk dependent on epitope diversity (e.g. higher risk if panel is all derived from same parental mAb)	**Strong rationale to screen and highly feasible. Low risk** if screening a diverse panel of molecules.
Final Lead / Candidate (1 mAb)	**Medium** Potentially too late: mAb is affinity-matured / engineered variant in final format but significant risk if polyspecific binding is observed.	**High** Feasible to screen final lead/candidate	**Medium** Polyspecific binding identified at this stage could be a significant risk that can only be discharged in the clinic	**Too late to screen:** significant risks if polyspecific binding is observed.

Figure 4. When to screen for polyspecificity? For each discovery phase, the rationale, feasibility, and risks of polyspecificity screening were evaluated. A sweet spot for incorporating polyspecificity screening in the drug discovery process exists prior to selection of the final lead/candidate but after completion of lead optimization. By incorporating polyspecificity screening at this stage, ideally using a small panel (n ≤ 10) of epitope-diverse molecules, key decision-making data are generated to enable selection of the final lead.

mutations.[41] This process identified several novel antibodies with globally improved pharmacological properties, including fully ablated binding to VEGFR2, FZD5 and ULBP2. This paratope refinement effect was mediated principally by extensive germlining of the light chain, coupled with minor alterations in amino acid content in the heavy chain.

The Finlay et al. and Bumbaca et al. studies both suggest that it is possible in some cases to remove off-target binding via molecular engineering.[9,38] In contrast, the recently published study by Campbell et al. clearly demonstrates that the off-target binding properties of antibodies may be intrinsic, may require disproportionate effort or even be impossible to fix, and efforts to refine the paratope to ameliorate either polyspecificity or polyreactivity are entirely empirical.[21] Every antibody is inherently unique and no attempt to refine any paratope is guaranteed to work. The devil, as ever, is in the detail.

A call to arms

This article has focussed on the described literature to highlight and summarize seven key observations that we believe to be important. First, polyreactivity and polyspecificity are potential causes of failure in antibody development programs either at the preclinical or, worse, at the clinical stage as they may impair PK, pharmacodynamics, biodistribution and safety. Second, *in vivo* generation of mAbs (e.g., in mice) does not preclude the generation of polyspecific antibodies, and many of the highest-profile failures in development were animal-derived antibodies. Third, very specific off-target interaction can occur in the absence of any obvious sequence or structural similarity to the target proteins. Fourth, off-target binding events can be limited to single interactions observed in individual species, or broadly across species used in preclinical studies that are often key to the progress of drug discovery programs. Fifth, straightforward classical biochemical approaches may not be sensitive enough to identify low affinity off-target interactions that are then amplified by receptor density and avidity effects of antibody binding at the cell surface. Sixth, the investigation of species-specific off-target binding has historically only been performed after dramatic and mechanistically surprising observations in animal studies or, worse, in man. Seventh, our understanding of the clinical influence, molecular causes, and frequency of occurrence of polyspecificity in drug discovery campaigns is nascent and requires substantial further study.

These observations demonstrate that our limited ability (as a field) to identify polyspecificity in early antibody drug leads may lead to increased inefficiency in the antibody drug discovery process. We believe that this argues strongly for the development of improved workflows and supporting technologies to eliminate or, at a minimum, 'flag' antibodies that suffer from off-target reactivity to proteins from human and preclinical test species. For the development of improved workflows to be efficient, they will ideally work across both the human and research animal species proteomes, be rapid, cost-effective to perform, and applicable early enough in the drug discovery process to maximize their value. These technologies may be purely experimental, or a combination of artificial intelligence-driven in silico and in vitro approaches. Indeed, we note with great interest the recent in-roads being made for proteome-wide structure prediction[68] and for computational analyses of antibody specificity.[69,70] Whether experimental, computational, or hybrid approaches will achieve these lofty goals will require rigorous, well-controlled studies at scale. We openly appeal to the wider antibody community to stop, collaborate, and listen.

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Abbreviations

mAb, monoclonal antibody; PK, pharmacokinetics.

References

1. Granier C, De Guillebon E, Blanc C, Roussel H, Badoual C, Colin E, Saldmann A, Gey A, Oudard S, Tartour E, et al. Mechanisms of action and rationale for the use of checkpoint inhibitors in cancer. ESMO Open. 2017;2(2):e000213. doi:10.1136/esmoopen-2017-000213.
2. Avery LB, Wade J, Wang M, Tam A, King A, Piche-Nicholas N, Kavosi MS, Penn S, Cirelli D, Kurz JC, et al. Establishing in vitro in vivo correlations to screen monoclonal antibodies for physicochemical properties related to favorable human pharmacokinetics. MAbs. 2018;10(2):244–11. doi:10.1080/19420862.2017.1417718.
3. Notkins AL. Polyreactivity of antibody molecules. Trends Immunol. 2004;25(4):174–79. doi:10.1016/j.it.2004.02.004.
4. Van Regenmortel MH. Specificity, polyspecificity, and heterospecificity of antibody-antigen recognition. J Mol Recognit. 2014;27(11):627–39. doi:10.1002/jmr.2394.
5. Guthmiller JJ, Lan LY, Fernandez-Quintero ML, Han J, Utset HA, Bitar DJ, Hamel NJ, Stovicek O, Li L, Tepora M, et al. Polyreactive broadly neutralizing b cells are selected to provide defense against pandemic threat influenza viruses. Immunity. 2020;53(6):1230–44 e5. doi:10.1016/j.immuni.2020.10.005.
6. McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. Nat Rev Immunol. 2011;12(1):24–34. doi:10.1038/nri3128.
7. Strohl WR. Editorial (thematic issue: modern therapeutic antibody drug discovery technologies). Curr Drug Discov Technol. 2014;11(1):1–2. doi:10.2174/15701638111011401241602560.
8. Loberg LI, Chhaya M, Ibraghimov A, Tarcsa E, Striebinger A, Popp A, et al. Off-target binding of an anti-amyloid beta monoclonal antibody to platelet factor 4 causes acute and chronic toxicity in cynomolgus monkeys. MAbs. 2021;13(1):1887628. doi:10.1080/19420862.2021.1887628.
9. Bumbaca D, Wong A, Drake E, Reyes IAE, Lin BC, Stephan J-P, et al. Highly specific off-target binding identified and eliminated during the humanization of an antibody against FGF receptor 4. mAbs. 2014;3(4):376–86. doi:10.4161/mabs.3.4.15786.
10. Pavlinkova G, Booth BJ, Batra SK, Colcher D. Radioimmunotherapy of human colon cancer xenografts using a dimeric single-chain Fv antibody construct. Clin Cancer Res. 1999;5:2613–19.
11. Pavlinkova G, Beresford GW, Booth BJ, Batra SK, Colcher D. Pharmacokinetics and biodistribution of engineered single-chain antibody constructs of MAb CC49 in colon carcinoma xenografts. J Nucl Med. 1999;40:1536–46.

12. Pavlinkova G, Beresford G, Booth BJ, Batra SK, Colcher D. Charge-modified single chain antibody constructs of monoclonal antibody CC49: generation, characterization, pharmacokinetics, and biodistribution analysis. Nucl Med Biol. 1999;26(1):27–34. doi:10.1016/S0969-8051(98)00075-4.
13. Colcher D, Pavlinkova G, Beresford G, Booth BJ, Batra SK. Single-chain antibodies in pancreatic cancer. Ann N Y Acad Sci. 1999;880 (1 CELL AND MOLE):263–80. doi:10.1111/j.1749-6632.1999.tb09531.x.
14. Colcher D, Goel A, Pavlinkova G, Beresford G, Booth B, Batra SK. Effects of genetic engineering on the pharmacokinetics of antibodies. Q J Nucl Med. 1999;43:132–39.
15. Beresford GW, Pavlinkova G, Booth BJ, Batra SK, Colcher D. Binding characteristics and tumor targeting of a covalently linked divalent CC49 single-chain antibody. Int J Cancer. 1999;81(6):911–17. doi:10.1002/(SICI)1097-0215(19990611)81:6<911::AID-IJC12>3.0.CO;2-O.
16. Onda M, Nagata S, Tsutsumi Y, Vincent JJ, Wang Q, Kreitman RJ, et al. Lowering the isoelectric point of the Fv portion of recombinant immunotoxins leads to decreased nonspecific animal toxicity without affecting antitumor activity. Cancer Res. 2001;61:5070–77.
17. Igawa T, Tsunoda H, Tachibana T, Maeda A, Mimoto F, Moriyama C, Nanami M, Sekimori Y, Nabuchi Y, Aso Y, et al. Reduced elimination of IgG antibodies by engineering the variable region. Protein Eng Des Sel. 2010;23(5):385–92. doi:10.1093/protein/gzq009.
18. Schoch A, Kettenberger H, Mundigl O, Winter G, Engert J, Heinrich J, et al. Charge-mediated influence of the antibody variable domain on FcRn-dependent pharmacokinetics. Proc Natl Acad Sci U S A. 2015;112(19):5997–6002. doi:10.1073/pnas.1408766112.
19. Nguyen H, Lan PD, Nissley DA, O'Brien EP, Li MS. Electrostatic interactions explain the higher binding affinity of the CR3022 antibody for SARS-CoV-2 than the 4A8 antibody. J Phys Chem B. 2021;125(27):7368–79. doi:10.1021/acs.jpcb.1c03639.
20. Tiller KE, Li L, Kumar S, Julian MC, Garde S, Tessier PM. Arginine mutations in antibody complementarity-determining regions display context-dependent affinity/specificity trade-offs. J Biol Chem. 2017;292(40):16638–52. doi:10.1074/jbc.M117.783837.
21. Campbell SM, DeBartolo J, Apgar JR, Mosyak L, McManus V, Beyer S, et al. Combining random mutagenesis, structure-guided design and next-generation sequencing to mitigate polyreactivity of an anti-IL-21R antibody. MAbs. 2021;13(1):1883239. doi:10.1080/19420862.2021.1883239.
22. Datta-Mannan A, Thangaraju A, Leung D, Tang Y, Witcher DR, Lu J, et al. Balancing charge in the complementarity-determining regions of humanized mAbs without affecting pI reduces non-specific binding and improves the pharmacokinetics. MAbs. 2015;7(3):483–93. doi:10.1080/19420862.2015.1016696.
23. Datta-Mannan A, Lu J, Witcher DR, Leung D, Tang Y, Wroblewski VJ. The interplay of non-specific binding, target-mediated clearance and FcRn interactions on the pharmacokinetics of humanized antibodies. MAbs. 2015;7(6):1084–93. doi:10.1080/19420862.2015.1075109.
24. Hotzel I, Theil FP, Bernstein LJ, Prabhu S, Deng R, Quintana L, et al. A strategy for risk mitigation of antibodies with fast clearance. MAbs. 2012;4(6):753–60. doi:10.4161/mabs.22189.
25. Xu Y, Roach W, Sun T, Jain T, Prinz B, Yu TY, et al. Addressing polyspecificity of antibodies selected from an in vitro yeast presentation system: a FACS-based, high-throughput selection and analytical tool. Protein Eng Des Sel. 2013;26(10):663–70. doi:10.1093/protein/gzt047.
26. Lahlou A, Blanchet B, Carvalho M, Paul M, Astier A. Mechanically-induced aggregation of the monoclonal antibody cetuximab. Ann Pharm Fr. 2009;67(5):340–52. doi:10.1016/j.pharma.2009.05.008.
27. Dobson CL, Devine PW, Phillips JJ, Higazi DR, Lloyd C, Popovic B, Arnold J, Buchanan A, Lewis A, Goodman J, et al. Engineering the surface properties of a human monoclonal antibody prevents self-association and rapid clearance in vivo. Sci Rep. 2016;6 (1):38644. doi:10.1038/srep38644.
28. Jain T, Sun T, Durand S, Hall A, Houston NR, Nett JH, Sharkey B, Bobrowicz B, Caffry I, Yu Y, et al. Biophysical properties of the clinical-stage antibody landscape. Proc Natl Acad Sci U S A. 2017;114(5):944–49. doi:10.1073/pnas.1616408114.
29. Ridker PM, Revkin J, Amarenco P, Brunell R, Curto M, Civeira F, Flather M, Glynn RJ, Gregoire J, Jukema JW, et al. Cardiovascular efficacy and safety of bococizumab in high-risk patients. N Engl J Med. 2017;376(16):1527–39. doi:10.1056/NEJMoa1701488.
30. Ridker PM, Tardif JC, Amarenco P, Duggan W, Glynn RJ, Jukema JW, et al. Lipid-Reduction variability and antidrug-antibody formation with bococizumab. N Engl J Med. 2017;376(16):1517–26. doi:10.1056/NEJMoa1614062.
31. Mo H, Huang J, Xu J, Chen X, Wu D, Qu D, Wang X, Lan B, Wang X, Xu J, et al. Safety, anti-tumour activity, and pharmacokinetics of fixed-dose SHR-1210, an anti-PD-1 antibody in advanced solid tumours: a dose-escalation, phase 1 study. Br J Cancer. 2018;119 (5):538–45. doi:10.1038/s41416-018-0100-3.
32. Kohli N, Jain N, Geddie ML, Razlog M, Xu L, Lugovskoy AA. A novel screening method to assess developability of antibody-like molecules. MAbs. 2015;7(4):752–58. doi:10.1080/19420862.2015.1048410.
33. Stern RA, Trojanowski JQ, Lee VM. Antibodies to the beta-amyloid peptide cross-react with conformational epitopes in human fibrinogen subunits from peripheral blood. FEBS Lett. 1990;264 (1):43–47. doi:10.1016/0014-5793(90)80760-G.
34. Santostefano MJ, Kirchner J, Vissinga C, Fort M, Lear S, Pan WJ, et al. Off-target platelet activation in macaques unique to a therapeutic monoclonal antibody. Toxicol Pathol. 2012;40(6):899–917. doi:10.1177/0192623312444029.
35. Zhang Y, Sun J, Tan M, Liu Y, Li Q, Jiang H, et al. Species-specific involvement of integrin alphaiibbeta3 in a monoclonal antibody CH12 triggers off-target thrombocytopenia in cynomolgus monkeys. Mol Ther. 2018;26(6):1457–70. doi:10.1016/j.ymthe.2018.04.005.
36. Teng Y, Guo R, Sun J, Jiang Y, Liu Y. Reactive capillary hemangiomas induced by camrelizumab (SHR-1210), an anti-PD-1 agent. Acta Oncol. 2019;58(3):388–89. doi:10.1080/0284186X.2019.1567935.
37. Boye E, Olsen BR. Signaling mechanisms in infantile hemangioma. Curr Opin Hematol. 2009;16(3):202–08. doi:10.1097/MOH.0b013e32832a07ff.
38. Finlay WJJ, Coleman JE, Edwards JS, Johnson KS. Anti-PD1 'SHR-1210′ aberrantly targets pro-angiogenic receptors and this polyspecificity can be ablated by paratope refinement. MAbs. 2019;11 (1):26–44. doi:10.1080/19420862.2018.1550321.
39. Lickliter JD, Gan HK, Voskoboynik M, Arulananda S, Gao B, Nagrial A, Grimison P, Harrison M, Zou J, Zhang L, et al. A first-in-human dose finding study of camrelizumab in patients with advanced or metastatic cancer in Australia. Drug Des Devel Ther. 2020;14:1177–89. doi:10.2147/DDDT.S243787.
40. Li W, Wei Z, Yang X, Huang G, Han X, Ni Y, Wang J, Meng M, Zou Z, Wen Q, et al. Salvage therapy of reactive capillary hemangiomas: apatinib alleviates the unique adverse events induced by camrelizumab in non-small cell lung cancer. J Cancer Res Ther. 2019;15(7):1624–28. doi:10.4103/jcrt.JCRT_997_19.
41. Tucker DF, Sullivan JT, Mattia KA, Fisher CR, Barnes T, Mabila MN, et al. Isolation of state-dependent monoclonal antibodies against the 12-transmembrane domain glucose transporter 4 using virus-like particles. Proc Natl Acad Sci U S A. 2018;115 (22):E4990–E9. doi:10.1073/pnas.1716788115.
42. Haynes BF, Fleming J, St Clair EW, Katinger H, Stiegler G, Kunert R, Robinson J, Scearce RM, Plonk K, Staats HF, et al. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science. 2005;308(5730):1906–08. doi:10.1126/science.1111781.

43. Liu IJ, Chiu CY, Chen YC, Wu HC. Molecular mimicry of human endothelial cell antigen by autoantibodies to nonstructural protein 1 of dengue virus. J Biol Chem. 2011;286(11):9726–36. doi:10.1074/jbc.M110.170993.
44. Cunningham MW, McCormack JM, Fenderson PG, Ho MK, Beachey EH, Dale JB. Human and murine antibodies cross-reactive with streptococcal M protein and myosin recognize the sequence GLN-LYS-SER-LYS-GLN in M protein. J Immunol. 1989;143:2677–83.
45. Finton KA, Larimore K, Larman HB, Friend D, Correnti C, Rupert PB, Elledge SJ, Greenberg PD, Strong RK, et al. Autoreactivity and exceptional CDR plasticity (but not unusual polyspecificity) hinder elicitation of the anti-HIV antibody 4E10. PLoS Pathog. 2013;9(9):e1003639. doi:10.1371/journal.ppat.1003639.
46. James LC, Roversi P, Tawfik DS. Antibody multispecificity mediated by conformational diversity. Science. 2003;299(5611):1362–67. doi:10.1126/science.1079731.
47. Lan W, Valente JJ, Ilott A, Chennamsetty N, Liu Z, Rizzo JM, Yamniuk AP, Qiu D, Shackman HM, Bolgar MS, et al. Investigation of anomalous charge variant profile reveals discrete pH-dependent conformations and conformation-dependent charge states within the CDR3 loop of a therapeutic mAb. MAbs. 2020;12(1):1763138. doi:10.1080/19420862.2020.1763138.
48. Bostrom J, Yu S-F, Kan D, Appleton BA, Lee CV, Billeci K, Man W, Peale F, Ross S, Wiesmann C, et al. Variants of the antibody herceptin that interact with HER2 and VEGF at the antigen binding site. Science. 2009;323(5921):1610–14. doi:10.1126/science.1165480.
49. Rios-Doria J, Harper J, Rothstein R, Wetzel L, Chesebrough J, Marrero AM, et al. Antibody-drug conjugates bearing pyrrolobenzodiazepine or tubulysin payloads are immunomodulatory and synergize with multiple immunotherapies. Cancer Res. 2017;77(10):2686–98. doi:10.1158/0008-5472.CAN-16-2854.
50. Root A, Cao W, Li B, LaPan P, Meade C, Sanford J, Jin M, O'Sullivan C, Cummins E, Lambert M, et al. Development of PF-06671008, a highly potent anti-P-cadherin/Anti-CD3 Bispecific DART molecule with extended half-life for the treatment of cancer. Antibodies. 2016;5(1):6. doi:10.3390/antib5010006.
51. Bonifant CL, Jackson HJ, Brentjens RJ, Curran KJ. Toxicity and management in CAR T-cell therapy. Mol Ther Oncolytics. 2016;3:16011. doi:10.1038/mto.2016.11.
52. Manning MC, Dk C, Bm M, Rw P, Ds K. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27:544–75.
53. Liu S, Moulton KR, Auclair JR, Zhou ZS. Mildly acidic conditions eliminate deamidation artifact during proteolysis: digestion with endoprotease Glu-C at pH 4.5. Amino Acids. 2016;48(4):1059–67. doi:10.1007/s00726-015-2166-z.
54. Liu M, Zhang Z, Cheetham J, Ren D, Zhou ZS. Discovery and characterization of a photo-oxidative histidine-histidine cross-link in IgG1 antibody utilizing ^{18}O-Labeling and mass spectrometry. Anal Chem. 2014;86(10):4940–48. doi:10.1021/ac500334k.
55. Borisov OVAM, Carroll JA. Brown PW Sequence variants and sequence variant analysis in biotherapeutic proteins. ACS Publications. 2015;2:63–117.
56. Smith LM, Kelleher NL. Consortium for Top Down P. Proteoform: a single term describing protein complexity. Nat Methods. 2013;10(3):186–87. doi:10.1038/nmeth.2369.
57. Huang CC, Lam SN, Acharya P, Tang M, Xiang SH, Hussan SS, et al. Structures of the CCR5 N terminus and of a tyrosine-sulfated antibody with HIV-1 gp120 and CD4. Science. 2007;317(5846):1930–34. doi:10.1126/science.1145373.
58. van de Bovenkamp FS, Derksen NIL, Ooijevaar-de Heer P, van Schie KA, Kruithof S, Berkowska MA, et al. Adaptive antibody diversification through N-linked glycosylation of the immunoglobulin variable region. Proc Natl Acad Sci U S A. 2018;115(8):1901–06. doi:10.1073/pnas.1711720115.
59. Wojciak JM, Zhu N, Schuerenberg KT, Moreno K, Shestowsky WS, Hiraiwa M, et al. The crystal structure of sphingosine-1-phosphate in complex with a Fab fragment reveals metal bridging of an antibody and its antigen. Proc Natl Acad Sci U S A. 2009;106(42):17717–22. doi:10.1073/pnas.0906153106.
60. Dimitrov JD, Roumenina LT, Doltchinkova VR, Mihaylova NM, Lacroix-Desmazes S, Kaveri SV, et al. Antibodies use heme as a cofactor to extend their pathogen elimination activity and to acquire new effector functions. J Biol Chem. 2007;282(37):26696–706. doi:10.1074/jbc.M702751200.
61. Kanyavuz A, Marey-Jarossay A, Lacroix-Desmazes S, Dimitrov JD. Breaking the law: unconventional strategies for antibody diversification. Nat Rev Immunol. 2019;19(6):355–68. doi:10.1038/s41577-019-0126-7.
62. Clarke S. Aging as war between chemical and biochemical processes: protein methylation and the recognition of age-damaged proteins for repair. Ageing Res Rev. 2003;2(3):263–85. doi:10.1016/S1568-1637(03)00011-4.
63. Ni W, Dai S, Karger BL, Zhou ZS. Analysis of isoaspartic Acid by selective proteolysis with Asp-N and electron transfer dissociation mass spectrometry. Anal Chem. 2010;82(17):7485–91. doi:10.1021/ac101806e.
64. Doyle HA, Mamula MJ. Autoantigenesis: the evolution of protein modifications in autoimmune disease. Curr Opin Immunol. 2012;24(1):112–18. doi:10.1016/j.coi.2011.12.003.
65. Moss CX, Matthews SP, Lamont DJ, Watts C. Asparagine deamidation perturbs antigen presentation on class II major histocompatibility complex molecules. J Biol Chem. 2005;280(18):18498–503. doi:10.1074/jbc.M501241200.
66. Liu YD, van Enk JZ, Flynn GC. Human antibody Fc deamidation in vivo. Biologicals. 2009;37(5):313–22. doi:10.1016/j.biologicals.2009.06.001.
67. Schmid I, Bonnington L, Gerl M, Bomans K, Thaller AL, Wagner K, Schlothauer T, Falkenstein R, Zimmermann B, Kopitz J, et al. Assessment of susceptible chemical modification sites of trastuzumab and endogenous human immunoglobulins at physiological conditions. Commun Biol. 2018;1(1):28. doi:10.1038/s42003-018-0032-8.
68. Tunyasuvunakool K, Adler J, Wu Z, Green T, Zielinski M, Zidek A, Bridgland A, Cowie A, Meyer C, Laydon A, et al. Highly accurate protein structure prediction for the human proteome. Nature. 2021;596(7873):590–96. doi:10.1038/s41586-021-03828-1.
69. Robert PA, Akbar R, Frank R, Pavlović M, Widrich M, and Snapkov I, et al. A billion synthetic 3D-antibody-antigen complexes enable unconstrained machine-learning formalized investigation of antibody specificity prediction. BioRxiv. 2021.
70. Mason DM, Friedensohn S, Weber CR, Jordi C, Wagner B, Meng SM, Ehling RA, Bonati L, Dahinden J, Gainza P, et al. Optimization of therapeutic antibodies by predicting antigen specificity from antibody sequence via deep learning. Nat Biomed Eng. 2021;5(6):600–12. doi:10.1038/s41551-021-00699-9.