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Review

Designing antibodies as therapeutics

Paul J. Carter[1,*] and Arvind Rajpal[1,*]
[1]Genentech, Inc., South San Francisco, CA 94080, USA
*Correspondence: pjc@gene.com (P.J.C.), rajpala@gene.com (A.R.)
https://doi.org/10.1016/j.cell.2022.05.029

SUMMARY

Antibody therapeutics are a large and rapidly expanding drug class providing major health benefits. We provide a snapshot of current antibody therapeutics including their formats, common targets, therapeutic areas, and routes of administration. Our focus is on selected emerging directions in antibody design where progress may provide a broad benefit. These topics include enhancing antibodies for cancer, antibody delivery to organs such as the brain, gastrointestinal tract, and lungs, plus antibody developability challenges including immunogenicity risk assessment and mitigation and subcutaneous delivery. Machine learning has the potential, albeit as yet largely unrealized, for a transformative future impact on antibody discovery and engineering.

INTRODUCTION

Over 100 antibody-based therapeutics are now approved for the treatment of a plethora of serious human diseases and in some cases transforming the lives of patients (Kaplon et al., 2022). The number of antibody therapeutics is growing rapidly with 6–13 approvals per year since 2014 by the US Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA) (Kaplon et al., 2022). The majority of approved antibodies are in IgG format, although several alternatives are emerging, including antibody-drug conjugates (ADCs) and assorted antibody fragments including domain antibodies such as nanobodies, as well as bispecific antibodies (BsAbs), IgG mixtures, and antibody fusion proteins (Figure 1A). Antibodies are most commonly used for the treatment of cancer, autoimmunity, and chronic inflammatory diseases (Figure 1B). However, antibody therapeutics are being extended to a broader range of human maladies including infectious diseases, hematology, neurology, ophthalmology, metabolic diseases, musculoskeletal diseases, and transplantation. There are currently $\geq$13 common targets with $\geq$3 approved antibodies each (Figure 1B). The vast majority of antibodies are administered by either intravenous infusion (i.v.) or subcutaneous (s.c.) injection (Figure 1B).

Recent approvals—some via conditional or emergency use authorizations—include multiple different antibody products targeting SARS-CoV-2, including three different antibody mixtures (estevimab plus bamlanvimab, casirivimab plus imdevimab, and tixagevimab plus cilgavimab) (Corti et al., 2021; Kaplon et al., 2022) (antibodysociety.org). Some of these antibodies are less effective against a SARS-CoV-2 variant of concern widespread in early 2022 (Omicron). Nevertheless, the speedy discovery and clinical evaluation of these anti-SARS-CoV-2 antibodies are an exceedingly impressive accomplishment and represent the fastest ever development of any antibody therapeutics to date. This is a testament to the decades of prior work in enabling technologies to develop antibody therapeutics in conjunction with the extraordinary efforts of all those involved in the response to the global public health crisis posed by COVID-19. An additional antibody that was already approved for other uses and recently approved for use in COVID-19 is the anti-IL-6R antibody, tocilizumab.

The development of antibody therapeutics commonly starts with a therapeutic hypothesis for intervention in the pathobiology of disease. Antibodies are then discovered and then routinely further engineered to support one or more mechanisms of action (MOAs) to test the therapeutic hypothesis. The modern toolbox of antibody discovery technologies includes many robust routes to antibodies from humans and other species using immunized animals, *in vitro* display technologies, and machine learning as reviewed elsewhere (Laustsen et al., 2021). Common MOAs for antibodies include the following: ligand blockade, receptor blockade, receptor downregulation, target cell depletion, receptor agonism (signaling induction), and soluble target antigen clearance/catabolism (Carter and Lazar, 2018).

We review the design of antibody therapeutics by selecting a few emerging directions and unsolved problems where future progress has the potential to provide broad benefit. This article is organized around three major themes. First, enhancing antibodies for cancer therapy building upon major successes with antibodies in oncology. There still remains plenty of room for further improvement that is inspiring much innovation in antibody design. Second, targeting antibody delivery to selected organs and tissues, including the brain, gastrointestinal (GI) tract, and lungs, represents a major unmet challenge that if ultimately solved may rewrite medical textbooks. Third, we discuss two developability challenges: the risk of unwanted immunogenicity, which can make or break antibody drugs, and the use of s.c. delivery that can benefit patients in ways that include greater convenience and enhanced quality of life.

Enhancing antibodies for cancer therapy

The treatment of cancer has been one of the greatest success stories with antibody therapeutics with 46 approvals as of May 2022 (Figure 1B) and many lives extended or saved. The

Figure 1. Snapshot of marketed antibody therapeutics

(A) Molecular format for antibody therapeutics approved for any indication highlighting formats of antibodies approved for use in oncology (*). Antibody variable and constant domains are represented by lighter and darker tones, respectively.

(B) Metrics for antibody therapeutics that are fully approved and currently (May 2022) marketed in the USA and/or Europe. IgG mixtures include co-formulated IgG such as the anti-SARS-CoV-2 antibodies, casirivimab, and imdevimab but not separately formulated antibodies that are approved for use in combination, such as trastuzumab plus pertuzumab. Common antibody targets are defined here as ones for which there are ≥3 antibodies on the market. Antibodies are counted for each category in which they belong. For example, rituximab is included under both oncology and A&I disease areas; tocilizumab is scored for both i.v. and s.c. delivery; and blinatumomab is listed under both bispecifics and fragment formats. Biosimilars and approved antibodies that were subsequently withdrawn from

(legend continued on next page)

predominant current format for anticancer antibodies is IgG (n = 28) including ≥6 with Fc point mutations or glycan modifications (low or no fucose) to enhance their cytotoxic effector functions including antibody-dependent cellular cytotoxicity (ADCC). Alternative formats for approved anticancer antibodies include eleven ADCs and two bispecifics (blinatumomab and amivantamab). Common targets for antibodies in cancer include B cell lineage markers such as CD19 and CD20, growth factor receptors (epidermal growth factor receptor [EGFR] and human epidermal growth factor receptor 2 [HER2]), immune checkpoint inhibitors (programmed cell death protein 1 [PD-1] and programmed death-ligand 1 [PD-L1]), and an angiogenic growth factor (vascular endothelial growth factor [VEGF]) (Figure 1B).

These successes with antibodies in oncology, particularly for the treatment of hematologic malignancies, have motivated intense effort to develop next-generation anticancer antibodies with an enhanced response rate or duration. For some anticancer antibodies, it may be desirable or necessary to improve the safety profile including the therapeutic index (TI): the ratio of antibody doses that causes toxic versus therapeutic effects. One strategy to improve safety is by increasing the selectivity of the antibody for tumors over normal tissue that may also express antigen or by increasing the efficiency of tumor uptake. An additional goal with future anticancer antibodies is the mitigation of innate or acquired resistance to treatment.

Beyond the formats represented by antibodies approved for cancer treatment (indicated by * in Figure 1A), a plethora of alternative approaches are being pursued including several that have reached clinical trials with representative examples shown in Figure 2. Here, we focus on ADCs, bispecifics, activatable antibodies for selective activation in tumors, IgM, and IgG hexamers. Many excellent reviews cover other promising approaches toward next-generation anticancer antibodies including intratumoral (ITU) immunotherapy (Table 1) and immunocytokines (Neri, 2019; Runbeck et al., 2021). Most, if not all, approved antibody-based drugs utilize recombinant antibody production. Also beyond the scope of this article are cell-based therapies that utilize surface antibody fragments for targeting, including chimeric antigen receptor T cells (CAR-T) and natural killer cells (CAR-NK). Lipid-encapsulated mRNA-based vaccines have been successfully developed and broadly deployed in response to COVID-19. Lipid-encapsulated mRNA that encodes antibodies is now starting to be evaluated in early clinical trials (August et al., 2021).

Antibody-drug conjugates

Beyond IgG, ADCs are the second most common format for anticancer antibodies with 11 approvals (Figure 1B) and >80 ADCs in clinical development (Dean et al., 2021). ADCs are complex molecules combining the targeting ability of antibodies with a cytotoxic payload connected by a cleavable or non-cleavable linker. Technologies allowing for site-specific (instead of less precise interchain disulfide cysteine or random lysine residue) conjugation are increasingly utilized to optimize structure-activity relationships (SARs), leading to more homogeneous and better-characterized drug substances (Walsh et al., 2021). Many excellent reviews focus on specific ADC topics including targeting (Damelin et al., 2015), linker chemistry (Bargh et al., 2019; Tsuchikama and An, 2018), novel payload design (Thurston and Jackson, 2019) and conjugation methods, and ADC characterization (Tumey, 2020).

The clinical exploration of payloads beyond traditionally used anti-mitotic tubulin disruptors and DNA damaging agents such as topoisomerase I (Goldenberg and Sharkey, 2019; Modi et al., 2020), DNA alkylating agents (Jeffrey et al., 2013; Staben et al., 2020), RNA polymerase II, BCL-xL inhibitor, and toll-like receptor (TLR) agonists are ongoing (Dean et al., 2021). With this clinical experience, there is an increasing appreciation for the role of bystander killing to mitigate the reduction in efficacy due to the heterogeneity of tumors as well as mechanisms of innate and acquired resistance (Drago et al., 2021; Jabbour et al., 2021).

Although six out of eleven approved ADCs are for the treatment of hematological malignancies, the majority of ADCs currently in clinical trials are targeted toward solid tumor indications (Dean et al., 2021; Figure 1). Interest to treat solid tumor indications with ADCs has grown with their approval and success in multiple different cancer types: trastuzumab emtansine (breast), trastuzumab deruxtecan (breast and gastric), sacituzumab govitecan (breast and urothelial), enfortumab vedotin (urothelial), and tisotumab vedotin (cervical). However, careful considerations of combinations of ADCs with immunotherapy in preclinical (Müller et al., 2015) and clinical (Matulonis et al., 2018) settings will be critical, as immunotherapy becomes the standard of care in an increasing number of different cancer types.

Beyond target choice and biological context, the following are emergent ADC design principles. First is addressing the requirements of specificity, minimal threshold for antigen expression, and internalization of targets by engineering antibodies with high affinity, bispecific (Maruani, 2018), and biparatopic (DaSilva et al., 2021; Kast et al., 2021) binding. Most ADCs employ the IgG1 scaffold including one, namely, belantamab mafodotin (Tai et al., 2014) that is afucosylated for enhancement of ADCC. Others are exploring antibody fragments or other smaller proteins to enhance penetration into solid tumors (Deonarain and Yahioglu, 2021), balancing the potential benefits of uptake and penetration with lower systemic exposure. Second is reconsidering the pursuit of ultrapotent cytotoxic payloads with the recent success of a high drug:antibody ratio (DAR) of moderately potent topoisomerase I inhibitor-based ADCs in solid tumors. The expectation is that these high DAR ADCs, with camptothecin-derived payloads, may allow for sufficient tumor delivery with low normal tissue toxicity, incorporate bystander effects, and not be substrates for efflux pumps such as multi-drug resistance mutation 1 (MDR1) (Nakada et al., 2016). Non-traditional

the market are excluded from this analysis. Also excluded are antibody products that have received emergency use or conditional authorization but not yet full approval. Sources: www.antibodysociety.org, the full prescribing information for approved antibody therapeutics and "Antibodies to Watch in 2022" (Kaplon et al., 2022).

ADCs, antibody-drug conjugates; A&I, autoimmunity and inflammatory diseases; dsFv, disulfide-stabilized Fv fragment; ImmTAC, immune mobilizing monoclonal T cell receptors against cancer; PEG, polyethylene glycol; PE-38, truncated form of *Pseudomonas* exotoxin; scFv, single-chain Fv fragment; TCE, T cell engager; and TCR, T cell receptor.

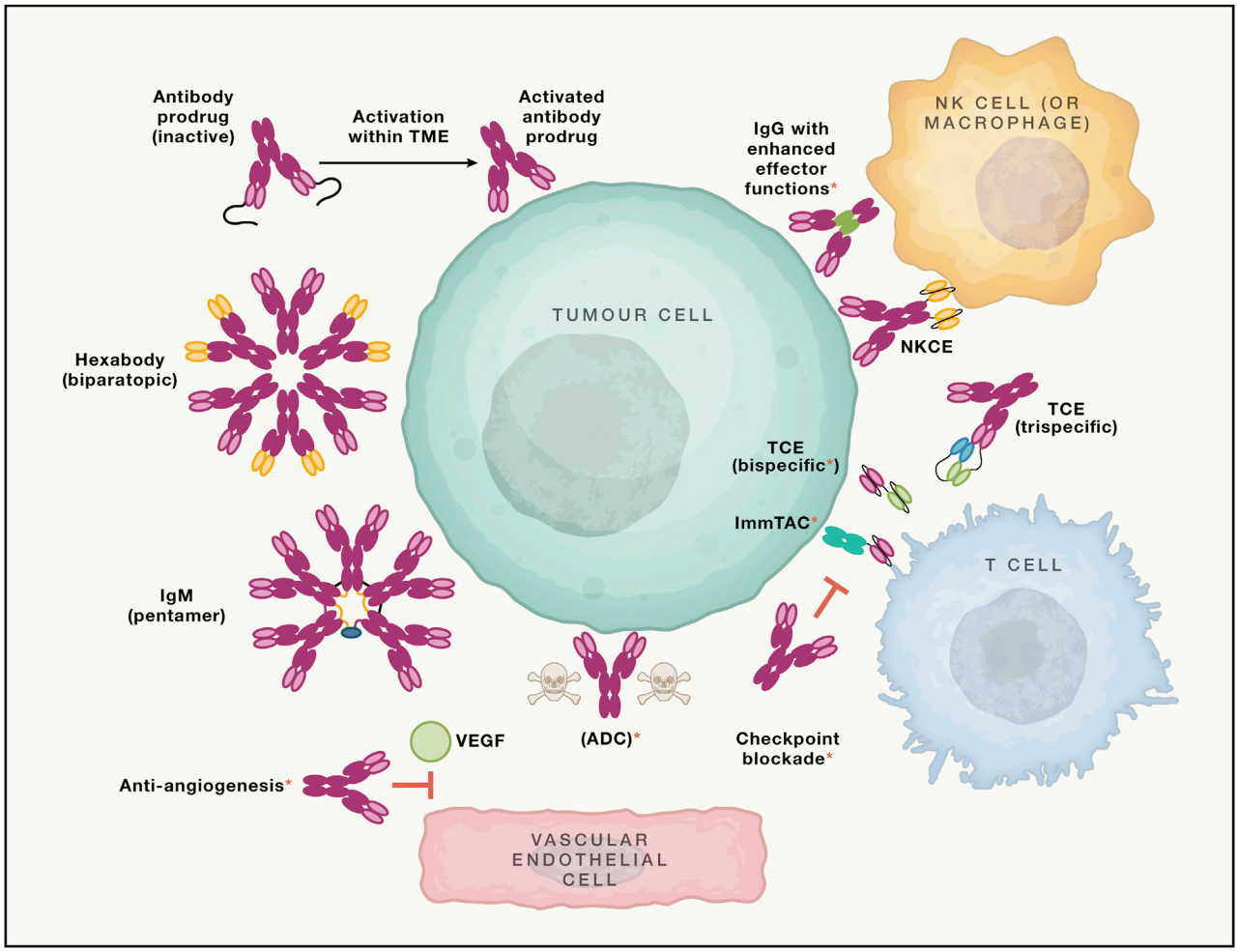

Figure 2. Selected approved (*) and clinical-stage antibody-based therapeutics for oncology

Triggers for activatable antibodies within the TME include tumor-associated proteases to remove peptide or protein masks from antibody prodrugs (shown). Additional triggers for antibody activation (not shown) include mildly acidic extracellular pH or high concentrations of extracellular ATP (Table 2). Checkpoint blockade includes antibodies targeting PD-1, PD-L1, and CTLA-4. The approved anti-angiogenic, bevacizumab, binds to VEGF and blocks interaction with its receptors (Flt-1 and KDR) on the surface of vascular endothelial cells. An additional approach, not shown for simplicity, is the use of antibody-based therapeutics to deplete Treg within the TME (Huang et al., 2021).

ADC, antibody-drug conjugate; NK, natural killer; NKCE, natural killer cell engager; TCE, T cell engager; TME, tumor microenvironment; and Treg, regulatory T cells.

cytotoxic payloads such as kinesin spindle protein inhibitors (Lerchen et al., 2018), immune activators (for example, TLR agonists; Ackerman et al., 2020), and nucleic acids (for example, siRNA; Dovgan et al., 2019) are being explored to target diseases that are otherwise difficult to treat due to lack of appropriate exposure and/or systemic toxicity of these payloads. Third, in addition to the preferential release of payloads in tumors, linkers are also being designed to improve the solubility of hydrophobic payloads and pharmacokinetics of corresponding ADCs (Bargh et al., 2019). Fourth, the site of conjugation can dictate potency and exposure. Site-specific conjugations allow for tunable SAR as well as more homogeneous ADCs that can be better characterized and monitored (Walsh et al., 2021). Fifth, antibody prodrugs employing protease activation for antigen binding may mitigate on-target/off-tumor toxicity (Lin and Sagert, 2018). In addition, the accumulated experience with safety profiles of payloads is informing clinical practice on how to best dose and manage the toxicities of ADCs (Masters et al., 2018).

Beyond oncology, ADCs are being evaluated in several additional areas of medicine. For example, ADCs are being generated to deliver glucocorticoid receptor modulators, nuclear receptor agonists, and phosphodiesterase inhibitors to treat inflammatory disorders, antibiotics to treat methicillin-resistant *S. aureus* infections, and bisphosphonates for osteoporosis (Leung et al., 2020).

Bispecific, trispecific, and multispecific antibodies

Therapeutic applications of bispecifics to date have primarily focused on oncology as discussed below, with a growing number of applications in other disease areas. The number of

Table 1. Activatable antibodies

Activation trigger	Activation type	Selected disclosed targets	Potential methods for measurement of triggers in human tumors	Selected references
Proteases (e.g., MMP2, MMP9, and MMP 13)	prodrug (irreversible)	clinical: CD71[a], CD166[a], CTLA4, PD-L1 preclinical: PD-1, HER2, EGFR, EpCAM, CD19, CD20, CD3, αvβ3	immunohistozymography	(Kavanaugh, 2020; Vasiljeva et al., 2020)
Mildly acidic extracellular pH	conditional (reversible)	clinical: AXL[a], ROR2[a], CTLA4, CD47 preclinical: EpCAM, Her2, Nectin-4, CD73, CD3, VISTA	AcidoCEST MRI	(Chang et al., 2021; Jones et al., 2017)
High extracellular ATP concentration	conditional (reversible)	clinical: CD137 preclinical: IL-6R, PD-1	not currently reported	(Kamata-Sakurai et al., 2021; Mimoto et al., 2020)

[a]Activatable antibody-drug conjugates.

bispecifics in clinical development has soared to ~200 with the majority of them in phase 1 oncology trials. A broad range of bispecific and multispecific topics have been covered in several excellent recent reviews (Labrijn et al., 2019; Nie et al., 2020). Bispecifics have been categorized by formats including Fc-content, mechanism of action, and disease indications. Additionally, bispecifics have been binned into obligate, which require the co-engagement of both antigens for activity, and combinatorial concepts. The obligate concepts include molecules that bridge cells (in *trans*), inhibit/activate receptors (in *cis*), cofactor mimetics, or piggyback to access otherwise poorly accessible compartments (Labrijn et al., 2019). The bridging of T and target cells with concomitant activation by CD3 engagement (TCEs, T cell engagers) is the largest category among obligate concepts with an emerging group of bispecifics and trispecifics using CD16A (Ellwanger et al., 2019) and NKp46 (Gauthier et al., 2019) to bridge NK cells with target cells (NKCE, NK cell engager) (Figure 2). In the combinatorial concept, bispecifics that address multiple immune checkpoint receptor targets are a majority and seek to address the growing need for combinations in immunotherapy.

The discovery of BsAb is highly empirical with the geometry of formats, epitope, binding affinity, and valency dictating that multiple molecules be made and tested. This is illustrated with complex formats such as the so-called 2 +1 bispecifics (valency of two and one for the first and second specificity, respectively) or targets (particularly in the case of obligate concepts) requiring a large number of molecules to be generated to find optimal combinations (Sampei et al., 2013). Bispecifics may also present major manufacturing challenges including cell line generation, purification, and analytical characterization. For example, efficient bispecific IgG production often requires robust antibody engineering solutions to facilitate the efficient formation of heavy-chain heterodimers and cognate heavy/light-chain pairs, as well as favorable developability characteristics (Wang et al., 2019), including the assessment and mitigation of immunogenicity risk (Kroenke et al., 2021).

The striking anti-tumor activity of blinatumomab (anti-CD19/CD3) (Bargou et al., 2008) ultimately led to the approval of this bispecific TCE for acute lymphoblastic leukemia. More broadly, the success of blinatumomab reinvigorated the field of TCEs with numerous such bispecifics advancing into clinical trials for both hematologic malignancies and solid tumors (Zhou et al., 2021). However, the progress in solid tumor indications with TCEs has been hampered by the lack of differential tumor:normal target expression leading to on-target/off-tumor toxicity and heterogeneous target expression. Additional challenges with TCEs for solid tumors include the immunosuppressive environment of tumors impacting the quantity and quality of T cells in the tumor and size of lesions impeding the penetration of TCEs. Although the importance of tumor-specific targets, with minimal normal tissue expression (with on-target/off-tumor toxicity that is monitorable and reversible), is recognized, protein engineering approaches to engender avidity-based targeting (with a 2 + 1 format) to differentiate between low normal from high tumor expression (Slaga et al., 2018) or preferential tumor binding by protease-cleavable masks to expand the TI are also being considered (Hsiue et al., 2021; Panchal et al., 2020).

A minimum anticipated biological effect level (MABEL) approach may be required to establish a low phase 1 starting dose for agonist antibodies such as TCEs, leading to slow dose escalation. Additional clinical challenges with TCEs include the common need to mitigate cytokine release syndrome (for example, by modified dosing schedule and anti-inflammatory medication) plus monitoring for potential neurotoxicity (Kamperschroer et al., 2020; Lim et al., 2021). The major mechanisms of resistance for TCE bispecifics in oncology are attributed to antigen loss (Braig et al., 2017) or immunosuppressive factors, such as regulatory T cells and the upregulation of immune checkpoint receptors.

Many solid tumors have limited T cells (“cold” tumors), and those present display an exhausted/anergic phenotype, thus leading to the exploration of costimulation in combination with TCEs in preclinical models (Chiu et al., 2020; Skokos et al., 2020). These studies demonstrate that costimulation can enhance TCE efficacy with increased T cell activation and proliferation and lead to durable responses by inducing memory (Chiu et al., 2020). In another preclinical study, the efficacy of TCEs was correlated to the resident number of T cells in the tumor with limited contribution from peripheral T cell infiltration. They also observed significant activity in the triple combination of

anti-PD-1, CD137 (4-1BB)-agonist, and TCE in cold tumors with low numbers of resident T cells. This effect was enhanced with Treg depletion using anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4) (Belmontes et al., 2021).

Bifunctional fusion proteins as well as BsAb are being used to recruit T cells to kill tumor cells by targeting MHC class II complexes with tumor-associated neoantigens derived from intracellular targets. For example, a fusion protein format that is known as immune-mobilizing monoclonal T cell receptors (TCRs) against cancer (ImmTAC) (Figures 1A and 2) combines an engineered TCR genetically fused to an anti-CD3 single-chain Fv fragment (scFv) fragment (Liddy et al., 2012). Clinical validation of the ImmTAC technology was recently achieved with the approval of tebentafusp, targeting gp100 peptide bound to HLA-A*02:01, for uveal melanoma. In addition, progress is being made in targeting mutated public neoantigens derived from intracellular molecules, like p53 and RAS, presented in the context of MHC class I (Douglass et al., 2021).

Bispecifics are gaining increasing clinical use beyond oncology. For example, emicizumab (anti-factor IXa/X) is approved for the treatment of hemophilia A, whereas faricimab (anti-VEGF/Ang-2) is approved in ophthalmology for the treatment of neovascular age-related macular degeneration. In neurology, bispecifics (anti-target/transferrin receptor) are being used to facilitate uptake into the brain (Terstappen et al., 2021). In infectious diseases, bispecifics are being applied to gain broad protection against pathogens like *P. aeruginosa* (Nie et al., 2020). In autoimmunity, applications of bispecifics include dual blockade of proinflammatory cytokines. For example, romilkimab (anti-IL-4/IL-13) recently completed a phase 2 study in diffuse systemic sclerosis (NCT02921971).

Activatable antibodies

Some anticancer antibodies, including checkpoint inhibitors, present serious safety issues, including on-target/off-tumor toxicities (Segal et al., 2017). Safety risks with antibodies may be exacerbated by factors that include target antigen expression on normal tissues, the use of highly potent formats such as ADCs and TCEs, and the inefficient localization of antibodies to tumors. *A priori*, the safety risk associated with normal tissue expression of the target might be mitigated by designing activatable antibodies with little or no antigen-binding activity in circulation and selective activation in the tumor microenvironment (TME) and/or tumor-associated draining lymph nodes (dLNs) (Figure 2).

Antibodies have been designed for activation by diverse triggers (Lucchi et al., 2021) including three common ones highlighted in Table 2. Antibody prodrugs are designed for irreversible activation involving cleavage by tumor-associated proteases. Alternatively, conditionally active antibodies have been designed for reversible activation by triggers such as mildly acidic pH or high ATP concentration (Table 1). In developing activatable antibodies, it is highly desirable, perhaps necessary, to establish methods to assess the activation trigger in human tumors. Heterogeneity of the trigger in tumors may lead to inefficient activation thereby limiting efficacy. Additionally, if the trigger is present at non-tumor sites, it may lead to unwanted activation and give rise to toxicity. More research is needed to understand antibody activation triggers in human tumors and non-tumor tissue and establish biomarkers for patient and lesion stratification. It remains to be seen if the irreversibility of activation of antibody prodrugs is an advantage or disadvantage, for example, by conferring activity in associated dLNs or distant sites in normal tissue, respectively. Similarly, reversible activation may be beneficial in restricting activity to tumor sites or undesirable if the activity is also needed in dLNs.

The most extensively explored approach to antibody prodrugs is masking of the antigen-binding site with a peptide (or protein) that is typically genetically fused to the antibody via a peptide linker (Kavanaugh, 2020). The linker is designed to be cleaved by one or more proteases associated with the TME (Table 1). The mask is engineered to attenuate or prevent antigen binding and following linker cleavage dissociate efficiently to fully activate the antibody for antigen binding. $\geq$13 different targets for protease-activatable antibody prodrugs have been reported including immune checkpoint inhibitors, an immune costimulatory molecule, growth factor receptors, B cell lineage markers, and a T cell antigen (CD3) for incorporation into TCEs (Table 1).

The most extensively tested protease-activatable antibody prodrugs so far use Probody technology (Kavanaugh, 2020). The first reported Probody was a prodrug of the anti-EGFR antibody, cetuximab (Desnoyers et al., 2013). The anti-EGFR Probody showed attenuated antigen binding that was fully restored upon activation by proteases. The anti-EGFR Probody was activated in tumors in mice and gave rise to comparable efficacy as cetuximab. In non-human primates the EGFR Probody was tolerated at much higher doses than the cetuximab parent antibody. $\geq$4 Probodies have reached clinical development including anti-PD-L1 (CX-072) (Table 1). CX-072 behaves as a prodrug including circulating in a predominantly masked form with evidence for activation within the TME (Kavanaugh, 2020). Similarly, CX-2029, an anti-transferrin receptor (CD71) Probody-drug conjugate in a phase 1 clinical study, was found to circulate predominantly (>90%) intact, suggesting that activation at the tumor (or other anatomical sites) and subsequent release to circulation are not significant issues (Johnson et al., 2021). A bispecific TCE prodrug, CX-904 (anti-EGFR/CD3), recently entered a phase 1 clinical trial (NCT05387265). Optimization of the linkers with Probodies may be necessary for efficient activation by proteases within the TME while minimizing the likelihood of unwanted activation by proteases at other sites. Assessment of the presence of active proteases in human tumors is possible with biopsies using immunohistozymography (Vasiljeva et al., 2020).

Engineering conditionally active antibodies for pH-dependent antigen binding was first developed to facilitate antibody recycling and extend pharmacokinetic half-life (Chaparro-Riggers et al., 2012; Igawa et al., 2010). More recently, a similar strategy was utilized to develop antibody prodrugs with enhanced tumor/normal tissue selectivity (Chang et al., 2021). This approach exploits the observation that the extracellular pH of the TME can be slightly acidic (pH $\sim$ 6.4–7.0) and slightly below that of surrounding normal tissue (Hao et al., 2018). Measuring the extracellular pH of the TME tumors in patients may be possible using emerging technologies such as AcidoCEST magnetic resonance imaging (MRI) (Jones et al., 2017). However, the clinical feasibility of this approach appears doubtful given the very high concentration of the contrast agent, such as iopamidol, needed.

Several pH-dependent anti-CTLA4 antibody prodrugs were engineered using point mutations (mainly Asp and Glu) in the complementarity-determining regions. The anti-CTLA antibody prodrugs have only weak binding at pH 7.4 that was reversibly increased at pH 6.0 (Chang et al., 2021). Anti-CTLA4 antibody prodrugs showed efficacy in human CTLA4 transgenic mouse tumor models comparable with the parent antibody and enhanced safety in non-human primates in combination with an anti-PD-1 antibody. ≥2 pH-dependent antibody prodrugs in ADC format have reached early clinical trials (Table 1). The pH-dependent antigen binding may help mitigate on-target/off-tumor toxicity of these ADCs, but it seems unlikely to alleviate off-target toxicity that often defines the maximum-tolerated dose for ADCs (Polakis, 2016).

Another activatable antibody technology for enhancing tumor/normal tissue selectivity is the ATP switch (Kamata-Sakurai et al., 2021; Mimoto et al., 2020). This technology relies on extracellular ATP concentration being elevated in the TME (~100 μM) and barely detectable elsewhere as observed in tumor-bearing mice (Pellegatti et al., 2008). This high extracellular concentration of ATP in tumors likely reflects the release of intracellular ATP by multiple processes, including apoptosis and necrosis of cancer cells. Agonistic antibodies to CD137 have been unsuccessful in the clinic due to systemic toxicity and/or limited efficacy (Segal et al., 2017). An elegant engineering strategy was used to develop an ATP-switch antibody (STA551) that bound tightly and minimally to CD137 in the presence or absence of ATP, respectively (Kamata-Sakurai et al., 2021). STA551 had robust anti-tumor activity in mice, and unlike a non-switch anti-CD137 antibody, it was well-tolerated in non-human primates at high doses. STA551 is now in a phase 1 clinical trial. The development of methods to measure the concentration of extracellular ATP in human tumors is highly desirable, perhaps necessary, for the clinical development of ATP-switch antibodies.

IgM and IgG hexamers

Recent years have seen a resurgence in interest in developing antibody therapeutics using alternative isotypes to IgG including IgM (Keyt et al., 2020), IgA (Sterlin and Gorochov, 2021; van Tetering et al., 2020), and IgE (Chauhan et al., 2020) for oncology and other disease areas. The most advanced of these approaches are IgM (Keyt et al., 2020) and IgG hexamers (HexaBody technology; de Jong et al., 2016) that mimic some properties of IgM as discussed below.

Only ~20 IgM antibodies have been tested in clinical trials to date with very limited success (Keyt et al., 2020), reflecting in part that IgM are much more complex molecules than IgG. . Specifically, IgG, IgM pentamers and IgM hexamers have 4, 21, or 24 polypeptide chains, respectively, and typically 2, 51, or 60 N-linked glycosylation sites, respectively (Keyt et al., 2020). IgM are more difficult to engineer and express at a small scale for preclinical research than are IgG. Even more challenging is the large-scale production of IgM under good manufacturing practice (GMP) conditions to enable clinical development. Although GMP expression, purification, and characterization of IgM remains hard, recent years have seen sufficient progress in these areas to enable more extensive clinical testing of IgM (Keyt et al., 2020).

IgM antibodies have outperformed IgG in a few preclinical settings, fueling renewed interest in the clinical evaluation of IgM. The high valency of IgM may lead to extensive cross-linking of cell surface receptors—a potentially desirable property for agonist antibodies. For example, a pentameric anti-DR5 IgM (IGM-8444) showed more potent induction of cancer cell apoptosis *in vitro* than did the corresponding anti-DR5 IgG (Wang et al., 2021). Importantly, IGM-8444 did not kill primary human hepatocytes *in vitro*. In contrast, a tetravalent nanobody agonist of DR5 showed unexpected hepatotoxicity in a phase 1 clinical study (Papadopoulos et al., 2015). IGM-8444 also showed anti-tumor activity in mouse xenograft studies that was further enhanced by a BCL-2 inhibitor, or by cytotoxic chemotherapy. IGM-8444 is currently in a phase 1 trial in solid tumors (NCT04553692). Preclinical evidence suggests that IgM also warrant clinical evaluation for the treatment of COVID-19. Specifically, reformatting an anti-SARS-CoV-2 spike protein IgG (IgG-14) into an IgM pentamer (IgM-14) increased the *in vitro* potency in neutralizing SARS-CoV-2 by 230-fold (Ku et al., 2021). Nasally administered IgM-14 demonstrated therapeutic efficacy against SARS-CoV-2 in a mouse model (Ku et al., 2021). IgM-14, now known as IGM-6268, recently started a phase 1 trial in healthy volunteers as an intranasal and intraoral spray (NCT05160402).

As an alternative approach to IgM, Fc point mutations have been used to endow IgG1 with the ability to efficiently hexamerize upon antigen binding on the surface of cells (Diebolder et al., 2014)—HexaBody technology (de Jong et al., 2016). The manufacture of HexaBody molecules is based on well-established capabilities with IgG and thus appears to be simpler than for the more complex IgM molecules. Applications of HexaBody technology include more efficient complement-dependent cytotoxicity (CDC) than IgG1 (Cook et al., 2016; de Jong et al., 2016) as well as receptor agonism (Cook et al., 2016; van der Horst et al., 2021). For example, an anti-death receptor 5 (DR5) HexaBody (GEN1029) was developed from an equimolar mixture of two different IgG1 antibodies that bind non-competitively to two different epitopes on DR5 (Overdijk et al., 2020). GEN1029 has potent agonist activity to DR5 that is independent of FcγR-mediated cross-linking plus potent *in vivo* anti-tumor activity. A phase 1/2 clinical trial of GEN1029 (NCT03576131) for the treatment of malignant solid tumors was recently terminated for reasons that are not yet disclosed. Two HexaBody molecules are currently in early clinical development, namely, GEN3014 (anti-CD38) for multiple myeloma (NCT04824794) and GEN3009—a biparatopic antibody (DuoHexaBody) directed against two non-overlapping epitopes on CD137 (Oostindie et al., 2020)—for B cell non-Hodgkin lymphoma (NCT04358458).

Antibody delivery to selected organs and tissues

A major emerging theme with antibody therapeutics is the delivery of antibodies to selected organs and tissues (Table 2). In this section, we focus on three of these delivery areas that are in their infancy but have high potential if successful, namely the brain, GI tract, and lungs. Efficient delivery of antibodies to the eye for serious ophthalmic diseases has been achieved with intravitreal (IVT) injection as demonstrated by the anti-VEGF antibody fragments, ranibizumab, and brolucizumab (Table 2; Mandal et al.,

Table 2. Delivery of antibodies to selected organs and tissues

Organ or tissue (site-specific delivery methods)	Approved Ab (selected other drugs)	Potential clinical applications	Potential benefits	Major challenges for Ab drugs	Possible solutions	Selected reviews
Brain (IT)	aducanumab, pabinafusp alfa	brain cancers, neurodegenerative diseases	more effective treatment options for CNS diseases	highly inefficient uptake of Ab from systemic circulation into brain due to the BBB.	very high Ab doses (>50 mg/kg) medical devices for IT delivery, bispecific Ab to facilitate transcytosis across the BBB, Ab or fusion proteins with super-stoichiometric MOAs	(Terstappen et al., 2021)
Lungs (inhalation)	none (dornase alfa)	COPD, lung cancer, asthma, emphysema, respiratory infections, IPF, cystic fibrosis	mitigate toxicities from systemic delivery, more effective delivery to lumen of lungs	Ab inactivation by proteases or sheer stress in aerosolization, inefficient mucus permeation particularly in obstructive lung diseases, immunogenicity of denatured Ab	Ab engineering to improve stability, use of Ab fragments including nanobodies, nebulizers to reduce sheer stress	(Fröhlich and Salar-Behzadi, 2021)
Gastrointestinal tract (oral)	none ($\geq$13 proteins and peptides)	GI disorders plus broad range of systemic diseases	mitigate toxicities from systemic delivery or circumvent need for parenteral delivery	Ab inactivation by low pH and proteases, inefficient release of Ab at desired site in gut, inefficient uptake from gut lumen into systemic circulation, dose limited by pill burden	Ab engineering plus enteric coating to enhance stability and facilitate release at desired place in gut, use of Ab fragments, enzyme inhibitors, permeation enhancers, microdevices to deliver Ab from gut lumen into circulation	(Madani et al., 2020; Perry and McClements, 2020; Wright et al., 2020; Zhu et al., 2021)
Eye (IVT, surgically implanted device)	ranibizumab, brolucizumab, and faricimab (aflibercept)	ophthalmic diseases	more effective treatment for ophthalmic diseases	highly inefficient uptake of Ab from systemic circulation into eye due to BRB, small IVT dosing volumes (20–100 μL), need for Ab with favorable high concentration biophysical properties and long-term stability	IVT delivery, surgically implanted refillable port delivery systems, Ab fragments, half-life extension for less frequent dosing	(Mandal et al., 2018)
Tumor (ITU)	none (oncolytic virus)	solid tumors, e.g., melanoma, head and neck, breast, prostate and colorectal cancers	mitigate toxicities from systemic delivery, lower dose required, use tumor as its own vaccine	tumor accessibility, poor retention of Ab within tumor, need for action at a distance (abscopal effect)	intratumoral immunotherapy, engineered binding to TME components for enhanced tumor retention	(Champiat et al., 2021; De Lombaerde et al., 2021)

Ab, antibody; BBB, blood brain barrier; BRB, blood retinal barrier; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; IPF, idiopathic pulmonary fibrosis; IT, intrathecal; ITU, intratumoral; IV, intravenous, IVT, intravitreal; MOAs, mechanisms of action, SC, subcutaneous; and TME, tumor microenvironment.

2018). Recently, faricimab became the first BsAb (anti-VEGF/Ang2) to be approved for an ocular indication as well as the first bispecific approval using Crossmab technology (Surowka et al., 2021). As for oncology, ITU injection of checkpoint inhibitor antibodies is being evaluated to focus the action of antibodies at the tumor while reducing systemic exposure as reviewed elsewhere (Champiat et al., 2021; De Lombaerde et al., 2021).

Toward the efficient delivery of antibodies to the brain

Although antibodies, such as ocrelizumab, have made a significant impact on neurological diseases like multiple sclerosis, the efficient delivery of antibodies into the central nervous system (CNS) for the effective treatment of neurodegenerative and other brain diseases remains a major challenge. Indeed, the delivery of systemically administered antibodies and protein molecules to the brain is highly inefficient due to the presence of the blood-brain barrier (BBB) (Pardridge, 2019). This results in much lower concentrations of administered proteins in the brain than in the plasma, estimated as 0.01%–0.35% using a variety of different experimental methodologies (Atwal et al., 2011; Garg and Balthasar, 2009; Pardridge, 2019; Shah and Betts, 2013). This inefficient delivery of proteins into the brain may make it especially difficult to engage targets such as tau and amyloid beta (Abeta) that likely require large and stoichiometric amounts of drug to engender a pharmacodynamic effect. Very high antibody doses—up to 100 mg/kg in the case of the anti-leucine-rich repeat and Ig containing Nogo receptor interacting protein-1 (LINGO-1) antibody, opicinumab (NCT01864148)—have been used to increase brain uptake, but this dosing strategy appears impractical from a drug manufacturing perspective.

More elegantly, several approaches have been tested to enhance the BBB penetration of antibodies as summarized in a recent review (Terstappen et al., 2021). Among these, receptor-mediated transcytosis (RMT) has been extensively used to increase the exposure of antibodies in the CNS. Receptors for transferrin, insulin, insulin-like growth factor, and low-density lipoprotein are often used to piggyback targeting agents into the brain. Although some of these efforts increased levels of antibodies and other protein therapeutics in the brain by 10- to 20-fold, the absolute levels remain low (<2% of plasma).

An anti-transferrin receptor antibody fusion protein withiduronate-2-sulfatase (JR-141, now known as pabinafusp alfa; Sonoda et al., 2018) provides the strongest evidence to date for the use of RMT to facilitate the brain uptake of a protein therapeutic (Giugliani et al., 2021). Preclinically, pabinafusp alfa reduced the accumulation of its glycosaminoglycan substrates both in peripheral tissues as well as in the brains of engineered mice, consistent with successful delivery across the BBB. Clinically, pabinafusp alfa lowered glycsoaminoglycans in both the periphery and the CNS (cerebrospinal fluid) with evidence for both somatic and neurocognitive efficacy (Giugliani et al., 2021), leading to approval for Hunter syndrome in Japan. In contrast, enzyme replacement therapy using recombinant iduronate-2-sulfatase (idursulfase alfa) improved somatic but not neurocognitive symptoms (Giugliani et al., 2021).

RMT using the transferrin receptor for enhanced brain uptake is being increasingly widely used. For example, a brain penetrant progranulin fusion protein demonstrated the preclinical *in vivo* rescue of a lysosomal storage disorder (Logan et al., 2021). RMT approaches may be impacted by non-BBB expression of the receptors affecting peripheral exposure, differential expression across species and disease states confounding clinical translation, and potential lysosomal degradation of drugs. Many alternative approaches are being explored, each with its own advantages and limitations, including neurotropic viruses, nanoparticulate systems, focused ultrasound, and intrathecal (IT) and intraparenchymal delivery (Terstappen et al., 2021).

Most approved antibodies for neurological indications are for disorders with an autoimmune etiology such as multiple sclerosis and myasthenia gravis. The recent approval of four different antibodies targeting either calcitonin gene-related peptide (CGRP) (eptinezumab, fremanezumab, and galcanezumab) or its receptor (erenumab) for migraine is an exception and indicative of the growing list of diseases and mechanisms that are being explored in neurology (Gklinos et al., 2021). Despite several molecules targeting amyloid beta and tau, there has been limited success in clinical trials for Alzheimer's disease (AD). The anti-Abeta antibody, aducanumab, was rejected by the EMA for mild cognitive impairment and dementia due to AD over efficacy and safety concerns but was approved—albeit controversially—by the FDA (Mahase, 2021). Aducanumab may suffer from limited uptake due to the approval based on surrogate outcome biomarkers and the high cost of treatment. This underscores the severe need for clinically meaningful biomarkers and the difficulty in the clinical development of molecules to address neurodegenerative diseases.

Oral delivery of antibodies

A "holy grail" for antibody therapeutics is oral delivery to obviate the need for invasive, albeit highly successful, i.v. and s.c. routes of administration. Potential benefits of oral delivery include improved quality of life for patients plus greater compliance with therapy and reduced healthcare costs. Additionally, oral administration of antibodies offers a potential local delivery option for GI tract maladies that may mitigate safety risks associated with systemic exposure. The numerous challenges and potential solutions to oral delivery of protein therapeutics including antibodies have been comprehensively reviewed elsewhere (Madani et al., 2020; Perry and McClements, 2020; Wright et al., 2020; Zhu et al., 2021). Here, we highlight major challenges for oral antibody delivery and progress toward overcoming them.

Significant obstacles to the oral delivery of antibodies include instability due to degradation by digestive and microbial enzymes and denaturation by the broad range of pH (~pH 1.5–8.0). The major physical barriers to oral delivery include mucus and epithelial cells lining the GI tract, exacerbated by the relatively short and variable time for transit through the gut. Pharmaceutical challenges to oral delivery include the need for sophisticated formulation to maintain the physical and chemical stability of antibodies plus facilitate their absorption or release at the desired site of action. From a practical standpoint, the pill burden tolerable by patients may set an upper limit on antibody doses for oral delivery, rather than commercial considerations such as the cost of goods.

Multiple different approaches are being used in combination to provide at least some mitigation for the many of the obstacles

associated with oral delivery of antibodies and other proteins. For example, proteolytic stability has been improved by the use of antibody fragments such as domain antibodies in conjunction with antibody engineering (Roberts et al., 2021). Chemical modification such as PEGylation has been used to enhance the stability of proteins (Lawrence and Price, 2016). Enteric coating can prevent antibody release in the stomach (pH ~ 1.5–2.5) with the dissolution of the coating and release at the higher pH in the small intestine (pH ~ 6–8) (Maderuelo et al., 2019). Protease inhibitors can attenuate protein degradation by digestive enzymes (Zhu et al., 2021). Broad approaches to tackling mucus include mucus-penetrating and mucoadhesive systems such as hydrogels (Zhu et al., 2021). Additional approaches used include so-called permeation enhancers to increase intestinal permeability to proteins (Zhu et al., 2021). Microdevices including gastric-autoinjectors have been designed to deliver protein therapeutics from the gut lumen into circulation and show promise in preclinical animal models (Abramson et al., 2022). Ionic liquids and deep eutectic solvents are showing some promise for oral delivery of antibodies as evidenced by preclinical studies in rats showing delivery of an anti-tumor necrosis factor (TNF) antibody into the intestinal mucosa as well as systemic circulation (Angsantikul et al., 2021). A key outstanding question is the safety profile of long-term treatment with protease inhibitors, absorption enhancers, and autoinjectors.

Several orally delivered proteins and peptides have been approved for therapeutic use—mainly for local rather than systemic delivery—providing some proof of concept for this route of administration (Zhu et al., 2021). The majority of these molecules are enzymes or agonists, suggesting that careful selection of target or molecule design may be key to maximizing the efficacy of the limited amount of drug that crosses the epithelial barrier similar to the emerging strategies for brain delivery. In contrast, few antibody-based drugs have ever been evaluated for oral delivery and none are yet approved. To our knowledge, immunogenicity has not posed a significant limitation to the development of orally delivered antibodies, but this question remains open and warrants further investigation. Antibodies currently in active development with oral administration include the anti-CD3 IgG, foralumab, in a phase 1B clinical trial in Crohn's disease (NCT05028946).

Inhalation of antibodies

The delivery of antibodies by inhalation offers significant opportunities for the treatment of serious lung diseases. Inhalation delivery may achieve high local concentrations of antibodies in the respiratory airways and mitigate safety risks from systemic exposure (Table 2). No antibodies and very few protein drugs have been approved for inhalation delivery to date (Fröhlich and Salar-Behzadi, 2021). Major challenges and possible solutions for inhalation delivery of antibodies are summarized below with more extensive coverage elsewhere (Fröhlich and Salar-Behzadi, 2021; Hickey and Stewart, 2022; Parray et al., 2021). Nebulizers are the most common delivery devices for lung delivery of proteins, although dry powder inhalers are also being used.

Insufficient stability of antibodies is a major obstacle to inhalation delivery including denaturation by shear stress in nebulization and degradation by proteases in the lung. Compounding the stability problem is the paucity of stabilizing excipients approved for use for inhalation delivery (Strickley and Lambert, 2021) and the increased immunogenicity of antibodies if denatured. Obstructive airway diseases or poorly ventilated regions of the distal lung may impair pulmonary function in ways that reduce the efficiency of inhalation delivery. For example, the antibody therapeutic may then need to permeate mucus that is more prevalent in diseases such as cystic fibrosis than in healthy lungs. Additionally, the particle or droplet size for delivery may need to be optimized and tightly controlled to enable the delivery of a sufficient dose of antibody to the desired location in the lung (Fröhlich and Salar-Behzadi, 2021). Specifically, large particles of >10-μm aerodynamic diameter are deposited mainly in the nose, mouth, pharynx, and larynx. Smaller particles (1–5 μm) can reach deep into the lungs whereas particles of <1 μm are typically exhaled without significant accumulation (Liang et al., 2020).

A common approach to mitigate the stability challenges with inhaled antibodies has been the use of small antibody fragments including Fab and domain antibodies such as nanobodies (Fröhlich and Salar-Behzadi, 2021) as well as small engineered proteins including anticalins (Deuschle et al., 2021). In other cases, Fc fusion proteins are being used to enable transcytosis of the therapeutic across the lung epithelium via the neonatal salvage receptor, FcRn (Liang et al., 2020). Nebulizers are being improved to reduce shear stress and control droplet size (Fröhlich and Salar-Behzadi, 2021). The conjugation of proteins to polyethylene glycol (PEGylation) may improve mucus penetration and stability against proteases (Liang et al., 2020). Antibodies may be more amenable to electrospray drying at lower temperatures (70°C) than to conventional spray drying, thereby enabling pulmonary delivery by dry powder inhalers (Mutukuri et al., 2021). Lipid nanoparticles warrant exploration as a potential way to deliver antibodies to the lung with potential benefits of reduced dose and immunogenicity plus extended half-life (Parray et al., 2021; Sousa et al., 2017).

Clinical trials with several different inhaled antibody fragments have been initiated but most discontinued for insufficient efficacy, although it is not clear whether this is due to poor choice of target or antibody, unfavorable pharmacokinetics, or insufficient delivery (Fröhlich and Salar-Behzadi, 2021; Liang et al., 2020). Current antibody clinical trials with oral inhalation include CSJ117, an anti-thymic stromal lymphopoietin (TSLP) Fab for asthma (phase 2, NCT04882124, NCT04410523, and NCT04946318). The pressing need for additional therapies for COVID-19 is driving innovation in many aspects of drug development. Indeed, a plethora of antibodies in different formats are under development for the treatment of COVID-19 (Kaplon et al., 2022) including some by inhalation delivery (Fröhlich and Salar-Behzadi, 2021; Liang et al., 2020). Representative antibodies targeting SARS-CoV-2 include the nanobody, Nb 11–59 (preclinical; Gai et al., 2021), and the IgM, IGM-6268, for intranasal and intraoral delivery (phase 1, NCT05160402). Additionally, the anti-CD3 IgG, foralumab, is being evaluated for the intranasal treatment of COVID-19 (phase 2, NCT04983446).

Selected development challenges in the design of antibody therapeutics

The term "developability" for antibodies has grown to encompass a broad set of desirable drug-like properties that span their

feasibility of manufacture, stability in storage, ease of administration, and favorable pharmacological behavior in patients, excluding target binding (Jain et al., 2017). Much progress has been made in recent years in understanding and mitigating antibody developability challenges. For example, Wittrup and colleagues conducted a seminal survey of the developability properties of 137 clinical-stage antibodies, including 48 antibodies approved for therapeutic use (Jain et al., 2017). The amino sequences from these antibodies were used to construct isotype-matched IgG1 antibodies, which were expressed, purified, and evaluated in 12 different *in vitro* assays. The distributions of the observed antibody properties were then used empirically to define boundaries of drug-like behavior that may be useful in selecting future antibody-drug candidates (Jain et al., 2017). This approach is analogous to the Lipinski rule of five that has proved so valuable in the development of small-molecule drugs (Lipinski et al., 2001). Subsequently, Deane and colleagues (Raybould et al., 2019) developed five computational metrics to guide the selection of antibody clinical candidates. This and other computational approaches to antibody developability assessment are potentially complementary to experimental methods and, particularly for sequenced-based methods, have the advantage of much greater throughput (Khetan et al., 2022). Below we focus on two important developability challenges, namely, immunogenicity risk assessment and mitigation and engineering antibodies for s.c. delivery, where further progress may have a broad impact on the future of antibody therapeutics.

Immunogenicity risk assessment and mitigation of antibody therapeutics

The collective ingenuity of antibody engineers and drug developers has led to an impressive suite of molecular engineering tools for enhancing the existing properties of antibodies or endowing them with new activities to enhance their clinical potential (Carter and Lazar, 2018). However, such engineering may increase the risk of unwanted immune responses (i.e., immunogenicity) in patients, including the development of anti-drug antibodies (ADAs).

The variable incidence of ADAs, including neutralizing antibodies, serum titer, and duration, influences their clinical impact with clinical sequelae ranging from indiscernible to severe. The effect of ADAs can include loss of efficacy of the therapeutic antibody, formation of immune complexes with the therapeutic antibody leading to accelerated clearance, reduced safety, and even the termination of clinical trials. For example, repeat doses of the humanized anti-proprotein convertase subtilisin/kexin type 9 (PCSK9) antibody, bococizumab, in a phase 3 clinical trial led to the development of ADAs in ~48% of patients after one year, attenuating the therapeutic benefit and leading to discontinuation of clinical development (Ridker et al., 2017). In contrast, two human antibodies targeting PCSK9 had a much lower incidence of ADAs—alirocumab (5.5% ADAs) and evolocumab (0.3% ADAs) as reported in their respective US prescribing information—and were approved for the treatment of hypercholesterolemia.

Immunogenicity risk assessment is an exceedingly complex problem that is impacted by a myriad of product-, patient-, and treatment-related factors that confound the systematic dissection of each individual parameter (Tourdot and Hickling, 2019). Multiple *in silico*, *in vitro*, and *in vivo* experimental methods are being used to assess the immunogenicity risk of protein therapeutics, focusing on T cell-dependent generation of ADAs as extensively reviewed elsewhere (Ducret et al., 2022; Ulitzka et al., 2020). *In silico* methods include forecasts of T cell epitopes based upon predicted peptide binding to MHC class II. *In vitro* methods include T cell activation assays, MHC-associated peptide proteomics (MAPPs), and dendritic cell uptake. *In vivo* methods attempt to recapitulate a human response in an animal—typically mouse—model. Each method has its strengths and limitations, and they are commonly used in combination, albeit without yet a clear consensus on how best to do so (Ducret et al., 2022).

Preclinical immunogenicity risk assessment is becoming more widely practiced for protein drugs and has recently become required by the FDA and the EMA. One key concept in immunogenicity risk assessment is the use of clinical-stage proteins as benchmarks to establish correlates between different assay methods and clinical ADA rates. Preclinical protein drug candidates are then compared with the benchmark molecules to assess their clinical ADA risk. However, the detection of ADAs is highly dependent on the sensitivity and specificity of the method that has been used for ADA analysis in clinical samples. Moreover, ADA assay results may be influenced by several factors, including sample handling, timing of sample collection, and underlying disease. For these reasons, comparison of ADA incidences between protein therapeutics may be misleading. An additional problem is that immune modulators, including TCEs, may interfere with some *in vitro* assays and require alternative approaches.

The highly immunogenic antibody, bococizumab (Ridker et al., 2017) (see above), has several unfavorable developability properties (Jain et al., 2017). Mammalian display was used to identify bococizumab variants with improved biophysical properties that correlated with reduced immunogenicity risk as judged by T cell activation and other *in vitro* assays (Dyson et al., 2020). The humanization of antibodies is particularly well suited to machine learning and may reduce immunogenicity risk (Marks et al., 2021; Prihoda et al., 2022). Antibody clinical candidates can be ranked for immunogenicity risk and lower risk variants advanced. In addition, candidates with predicted immunogenicity risk can be engineered to reduce their risk. For example, the design of emicizumab included *in silico* prediction and subsequent removal of T cell epitopes (Sampei et al., 2013), which may have contributed to the low ADA rate reported for this bispecific (Oldenburg et al., 2017). In contrast, a moderate to high incidence of ADAs has been reported for several other bispecifics in the clinic (Akpalu et al., 2019; Hellmann et al., 2021; Jimeno et al., 2019; Staton et al., 2019), which begs the as yet unanswered question as to whether the immunogenicity risk for bispecifics is inherently higher than for monospecific antibodies. Emicizumab notwithstanding, it remains to be seen if other highly engineered proteins, including antibodies, can be routinely developed as successful therapeutics without being stymied by ADAs elicited in patients.

Engineering antibodies for subcutaneous delivery

Over 30 antibodies have been approved for s.c. delivery (Figure 1B) for non-oncologic indications such as autoimmunity and chronic inflammatory diseases. s.c. delivery of antibodies

has multiple advantages and some disadvantages over i.v. delivery (Viola et al., 2018), as discussed below. A central problem in the s.c. delivery of antibodies is that of maximizing the dose by increasing the antibody concentration and/or increasing the injection volume (Jiskoot et al., 2022). Recent progress in addressing limitations of s.c. delivery is anticipated to encourage even broader adoption of this attractive route of administration.

A major advantage of s.c. over i.v. delivery of antibodies is much more rapid administration: only a few minutes for s.c. versus up to several hours for i.v. delivery (Viola et al., 2018). Furthermore, s.c. administration at home is possible for some antibody drugs—even by patients in some cases—and may lead to greater patient convenience and compliance plus lower healthcare costs. A major challenge with s.c. delivery of antibodies is the common need to formulate antibodies at high concentrations—up to 200 mg/mL for some currently approved antibodies—to enable the delivery of the desired antibody dose in a small injection volume that is typically 0.5–2.0 mL (Strickley and Lambert, 2021). Such high antibody concentrations are not possible with all antibodies, as they may promote untoward self-interactions that result in high viscosity, aggregation, precipitation, gelation, or opalescence (Kingsbury et al., 2020). Although s.c. doses of up to 400 mg per administration are sometimes possible, this may still be insufficient for some applications. Another limitation is that antibodies with known or anticipated skin toxicity (for example, anti-EGFR) do not appear well suited to s.c. delivery.

Ideally, antibodies are screened during the discovery process for favorable high concentration properties if needed. This is challenging with sizeable numbers of antibodies because of the large amounts of antibody protein—tens of milligrams—commonly needed to assess high concentration behavior. Emerging methods such as charge-stabilized self-interaction nanoparticle spectroscopy (Starr et al., 2021) suggest that measurements of antibody self-association at low concentration may be predictive of high concentration behavior. This may allow much larger scale antibody screening early in antibody discovery to identify antibodies with favorable properties at high concentration. The application of machine learning for the prediction of favorable high concentration properties of antibodies offers an additional approach to identifying antibodies that are potentially suitable for s.c. delivery (Arslan et al., 2021; Lai et al., 2021). Beyond the discovery process, antibodies, including bispecifics, can sometimes be engineered to reduce their viscosity while preserving their antigen-binding affinity (Tilegenova et al., 2020). Additional and potentially complementary strategies to mitigate or circumvent the high viscosity of high concentration antibodies include the addition of formulation excipients such as NaCl or arginine-HCl to attenuate inter-molecular interactions (Strickley and Lambert, 2021).

The maximal volume for s.c. delivery—typically $\leq$2 mL (Strickley and Lambert, 2021)—has been increased for a few antibodies by co-formulation with recombinant human hyaluronidase PH20 (rHuPH20) (Knowles et al., 2021; Locke et al., 2019). Hyaluronidase hydrolyzes hyaluronan locally thereby allowing s.c. delivery of volumes up to 15 mL (Knowles et al., 2021). $\geq$4 antibodies co-formulated with rHuPH20 have been approved for oncologic indications, namely, trastuzumab, trastuzumab with pertuzumab, rituximab, and daratumumab (Knowles et al., 2021). Alternative emerging methods for s.c. delivery of greater quantities of antibodies include a medical device known as large-volume patch injector (Lange et al., 2021). Multiple s.c. injections at different sites are precedented by casirivimab plus imdevimab that recently received emergency use authorization for the treatment of COVID-19, albeit with i.v. infusion as the strongly recommended route of administration.

Systemic circulation of antibodies following s.c. delivery occurs primarily via uptake by the lymphatic system (Viola et al., 2018). In contrast, there is conflicting evidence for the role of direct uptake of s.c. antibodies across capillary endothelia mediated by FcRn (Datta-Mannan et al., 2020). The bioavailability of an antibody following s.c. delivery is the fraction of active antibody that reaches systemic circulation. The bioavailability of antibodies after s.c. delivery is incomplete and varies over ~2-fold from 49% to 96%, as evidenced by our review of prescribing information for all antibodies approved up until May 2022. Historically, the s.c. bioavailability of antibodies has been difficult to predict preclinically (Datta-Mannan et al., 2020). However, the study of related antibody variants suggests that s.c. absorption and bioavailability may be enhanced by reducing local positive charge, lowering hydrophobic matrix interactions, increasing thermal stability, and reducing thermally induced aggregation (Datta-Mannan et al., 2020). Tools showing some promise in predicting antibody bioavailability include an s.c. injection site simulator instrument (“Scissor”) (Bown et al., 2018) and machine learning (Lou and Hageman, 2021).

As for pharmacokinetics, s.c. delivery of an antibody leads to a lower peak serum concentration (C_{max}) and longer time to achieve C_{max}, compared with i.v. administration (Bittner et al., 2018). This may be an advantage or disadvantage for s.c. delivery depending upon the specific therapeutic application. Regarding immunogenicity, the evidence is mixed: s.c. delivery of biologics may lead to higher, similar, or lower immunogenicity than i.v. delivery (Jarvi and Balu-Iyer, 2021).

Longer-term opportunities with antibody therapeutics

Future clinical opportunities with antibodies include pursuit of the “high-hanging fruit” such as targets that are difficult to hit, poorly understood, or previously “undruggable” (Carter and Lazar, 2018). For example, efficient intracellular delivery of antibodies would greatly expand the range of targets that are druggable with antibodies but remains exceedingly difficult to achieve (Niamsuphap et al., 2020). Machine learning is predicted to transform biomedicine (Goecks et al., 2020) and has much potential, as yet largely unrealized, for the development of antibody therapeutics as mentioned throughout this review and in the section below.

Applications of computational protein design to antibody discovery and engineering

There is an increasing role of computational protein design in antibody discovery and optimization (Sormanni et al., 2018). The combination of deep sequencing of antibody repertoires with associated functional data can be used to train novel machine learning-based models for affinity maturation, humanization, and developability (Marks and Deane, 2020; Pertseva

et al., 2021). These methods offer opportunities to unify different considerations and design stages into joint models as well as transfer knowledge across many different sources.

Antibody structure prediction, from repertoire sequences, requires addressing the challenges of VH/VL pairing and CDR-H3 loop modeling. Experimental and computational advances have enabled progress in both (DeKosky et al., 2016; Ruffolo et al., 2022). The prediction of antibody-antigen interactions remains a challenge, and the representation of the interacting molecular surfaces displaying geometric and chemical features may be beneficial in scoring complementarity (Gainza et al., 2020) and docking. Many approaches to specificity and affinity optimization using computational design (Liu et al., 2020; Mason et al., 2021) have been demonstrated, with some specifically attempting to address epistasis in the mutational landscape (Adams et al., 2019). The rapid progress in computational antibody design suggests that *de novo* antibody design may be achievable in the near future.

In designing antibodies as therapeutics, it is often desirable to optimize several different parameters including affinity, potency, and developability or at least optimize one parameter without degrading another parameter. Such empirical optimization has commonly been done sequentially, which can be both time consuming and resource intensive. Moreover, optimization of individual antibody properties may lead to unintended degradation of other attributes. For example, the affinity maturation of an anti-respiratory syncytial virus (RSV) antibody, palivizumab, led to unwanted binding to a rat protein and rapid clearance in cotton rats that was resolved by further engineering (Wu et al., 2007) to create motavizumab. Computational approaches to multi-objective antibody optimization, the so-called ''pareto optimization'' can have a significant benefit with faster timelines for therapeutics (Kuroda and Tsumoto, 2020).

CONCLUDING REMARKS

Progress in developing antibody therapeutics in recent years has been astounding with 79 approvals in the last decade alone (Kaplon et al., 2022). The repertoire of different formats for approved antibody therapeutics has grown large (Figure 1A) and can only expand further given the many additional approaches in clinical development including some illustrated in Figure 2. The impressively rapid development of antibodies to treat COVID-19 illustrates significant successes in responding to a global health crisis as well as ongoing technological innovation with antibody therapeutics and their delivery (Corti et al., 2021; Kaplon et al., 2022). We previously offered a perspective on opportunities with next-generation antibody therapeutics (Carter and Lazar, 2018). Here, we have provided an updated view that highlights some of the many areas of innovation with antibodies that have seen substantial progress in recent years. This bodes well for a future where antibody therapeutics provide even greater benefit to the lives of patients, including emergent pathogens

ACKNOWLEDGMENTS

We thank the following Genentech colleagues for providing input on this manuscript: Jasi Atwal, Richard Bonneau, James Koerber, Gail Phillips, Valerie Quarmby, Karthik Nagapudi, Christoph Spiess, Mark Wilson, Luke Xie, Zhengmao Ye, and Jonathan Zarzar. Additionally, we thank the following external colleagues for their input: Bruce Keyt, Tomoyuki Igawa, and Janine Schuurman. The authors apologize to researchers whose work we were unable to cite owing to space limitations.

DECLARATION OF INTERESTS

Both authors are current employees of Genentech, a member of the Roche Group, and Roche stockholders. The Roche Group develops and commercializes therapeutics including antibodies.

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