

WHITE PAPER

Bispecific antibodies unleashed

From mechanisms to manufacturing and strategic collaboration for effective precision immunotherapy

Advances in disease biology have led to the burgeoning development of bispecific antibodies (BsAbs), which are synthetic proteins capable of targeting two discrete epitopes from different antigens. Their bispecific functionality offers promising therapeutic potential such that their applicability to a variety of therapies is being explored. Originally developed for the treatment of cancer, BsAbs may now benefit imaging and diagnostics, as well as disease prevention and treatment. Several bispecific antibodies have already obtained regulatory approval, while hundreds more are in development.

In this white paper, we will discuss the predominant formats and mechanisms of action of bispecific antibodies, the most common techniques for BsAbs transient production, and what is involved in their process development. We will also highlight the advantages of partnering with a CDMO with world-class service offerings, from discovery to production, and how doing so can ensure success of your BsAbs by delivering precise immunotherapy solutions and avoiding costly regulatory mistakes.

Mechanisms of action

The diversity of BsAb formats provides a variety of functional mechanisms that are suitable for a wide range of therapeutic applications. There are six common mechanisms of action that take advantage of the bifunctionality of the antibodies.¹ First and foremost, the BsAb acts as a bridge between an effector T cell and a tumor cell, as it has binding sites for both. This type of antibody is also referred to as a T cell engaging BsAb (T-BsAb). Cibisatamab is an example of a BsAb that employs this mechanism. Receptor inhibition, another important mechanism, refers to the targeting of multiple tumor receptor tyrosine kinases (RTKs) in order to disrupt their signaling pathways. Using this mechanism of action, the bispecific zenocutuzumab has been shown to be effective for certain tumors. In contrast to receptor inhibition, the BsAb acts as an agonistic antibody to activate receptor complexes in the receptor activation mechanism. This has shown promise for the treatment of obesity. In the analoging cofactor mechanism of action, the BsAb acts in place of a deficient enzyme or cofactor to stimulate a necessary activity. Potential applications of this mechanism include treatment for hemophilia A. In a different type of mechanism, one binding site of the BsAb attaches to a receptor that will carry it to its desired location where it can utilize its other binding site to activate the targeted treatment. A good example of this is connecting to the transferrin receptor (TfR) to cross the blood-brain barrier and then targeting beta secretase 1 (BACE1) to block its activity in the brain. Finally, the BsAbs act as cytokines to initiate signal transduction in the sixth mechanism of action. These BsAb surrogate agonists can be effective at inhibiting replication of a virus.

Formats of bispecific antibodies

A bispecific antibody may be produced in a multitude of possible formats, the selection of which is largely

determined according to the antigen involvement of the underlying biological causes. Generating bispecifics involves the combination of heavy and light chain variable domains (VH and VL, respectively) from different antibodies in order to assemble the desired antigen-binding sites for the specific target. However, the production is complicated by the many possible mixtures of VH and VL, which can lead to a high degree of structural diversity in which only one of the unique resultant structures is effective. Multiple platforms aim to solve this challenging issue by enhancing the pairing, and thereby generate the desired structure primarily.²

One of the main classifications of recombinant bispecific antibodies is the distinction between the presence or absence of an Fc region (full-length IgG-like or non-IgG-like, respectively). They can be further subcategorized by the number of binding sites. In IgG-like BsAbs, heterologous Fc matching results from modification of the heavy chain. The popular knobs-into-holes (KIH) platform features one heavy chain with a protruding “knob” on its surface, while the other heavy chain has a complementary “hole.” The knob and hole fit together like a lock and key, creating a stable interface between the two heavy chains.^{3,4} The DEKK platform consists of a pair of Fc variants that use salt bridges to promote heterodimerization and enhance molecular stability,⁵ while oppositely charged substitutions in the CH3 domains are utilized for heterodimer formation in the ART-Ig® platform (Asymmetric Re-engineering Technology).⁶ The orthogonal geometry of the Ortho-Fab platform helps properly align the antigen-binding fragments (Fab) domains.⁷ In the DuoBody® platform, Fab arms are dynamically recombined and exchanged between two antibodies.⁸ The symmetrical DVD-Ig (Dual Variable Domain) and FIT-Ig® (Fabs-in-tandem) molecular platforms contain four antigen binding sites capable of simultaneously acting on two different targets.⁹⁻¹¹ The XmAb® platform simplifies the generation of

bispecific antibodies by utilizing a combination of Fab and single-chain variable fragments (scFv), resulting in a simpler structure and fewer manufacturing challenges. Trifunctional antibodies (Triomabs) can interact with three different antigens. Dual Action Fab (DAF), also known as the “two-in- one” platform, utilizes phage display technology to control binding, whereas CrossMAB, Wuxibody™, and tethered-variable CL BslgG (TcBslgG) platforms all focus on correctly matching the light chains (LCs) in different ways.¹²⁻¹⁵

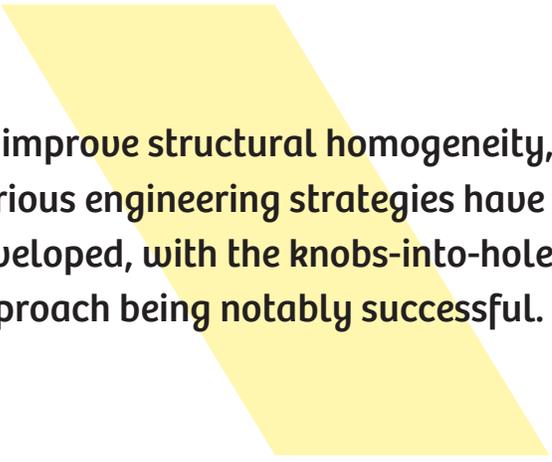
The scFv-based non-IgG-like BsAbs are of much smaller size due to their lack of an Fc segment. One of the earliest bispecific antibody platforms, the BiTE® molecule (Bispecific T-cell Engager), employs a linker between two Fvs. It showed strong efficacy despite its very short half-life. A half-life extended version, HLE-BiTE® molecules, was subsequently developed to ameliorate this issue. The DART® platform (Dual Affinity Retargeting) uses cross-pairing of VL and VH domains from different Fvs, resulting in better stability and T-cell activation compared to traditional BiTE molecules. Other notable variations are tandem diabody (TandAb) tetravalent bispecific antibody, which has two binding sites for two antigens, and bispecific nanobody (Bi-Nanobody) containing two camelid-based nanobodies that target different antigens.

Transient production for optimization and validation

Choosing the best format to be synthesized depends primarily on the properties of the target antigen as well as the preferred antibody half-life and desired mechanism of action. Furthermore, an important consideration is whether Fc effector functions are necessary, such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP). Although the presence of

the Fc region lengthens half-life and allows certain functions to take place, it may also introduce unwanted effects. As a result, strategic mutations of the Fc domain can help fine-tune the functionality of a full-length BsAb.

The number of arms of the antibody can also be engineered, which in some cases can be used to achieve greater avidity and efficacy. For example, bispecifics are sometimes constructed in which one arm targets one antigen while two other arms bind to a different antigen. These are noted as (1:2); (2:2) would be used if a total of four arms targeted two antigens. In addition, researchers are also exploring the potential of trispecific antibodies.



To improve structural homogeneity, various engineering strategies have been developed, with the knobs-into-holes approach being notably successful.

To improve structural homogeneity, various engineering strategies have been developed, with the knobs-into-holes approach being notably successful. It addresses heavy chain mispairing and reduces the number of unique structures formed, as only one heavy chain combination is formed predominantly. It is frequently combined with CrossMAB, which regulates light chain interactions, to generate the desired format. In addition, a diverse array of site-specific mutations can be employed to take advantage of steric effects or electrostatic charge interactions to aid proper assembly, stability, and purification.



LINKER ENGINEERING

In the design of single-chain variable fragment-based BsAbs, two antigen-binding fragments from two different antibodies are connected with a linker. The characteristics of linkers affect several BsAb attributes, making their proper selection critical. For example, the flexibility and hydrophobicity of the connection have a significant influence on the antibody's function. The amino acid composition affects both of these traits, while the linker length can be used to help control chain pairing.¹⁶ A combination of four glycines and one serine ($(G_4S)_n$) is frequently employed as this configuration provides flexibility and enhanced solubility without impacting immunogenicity.¹⁷ Other linker designs featuring different residues may be utilized to address solubility. Each linker must be carefully considered for the particular BsAb being produced in order to achieve the appropriate VH-VL combination and the associated functionality. The length of a linker is a critical factor that affects the VH-VL coupling. Linkers should be long enough to allow VH-VL interaction but not so long as to promote uncontrolled pairing. Therefore, they should be tailored to produce the

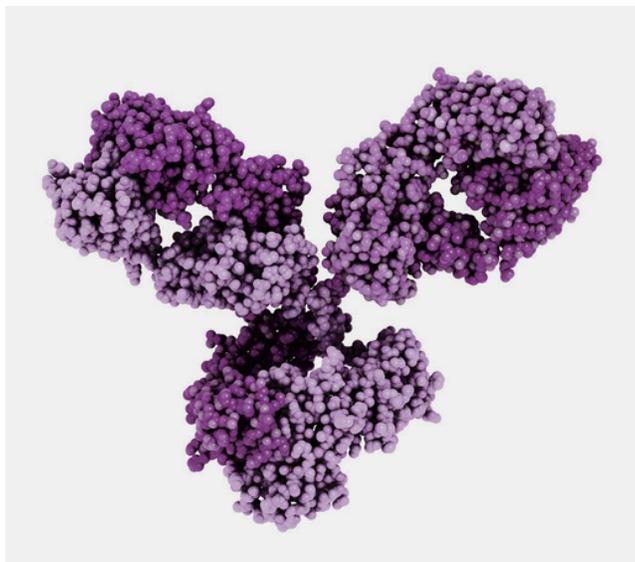
desired conformation and valence for the specific application. In BiTE[®] constructs, homologous domains are joined via long linkers, while short amino acid chains connect the heterologous variable fragments. In contrast, the DART[®] platform features extremely short linkers in order to minimize unfavorable pairing. BiTE BsAbs employ a flexible polypeptide linker which allows the binding sites to access receptors as the antibody re-targets T cells to tumor cells. BiTE molecules such as blinatumomab are small, which increases their mobility to reach the targets but also leads to rapid clearance from the body.¹⁸ As such, they require lengthy intravenous dosages at high concentrations. In addition, BiTE antibodies may experience complications with aggregation and stability.

DART-based antibodies, however, may ameliorate some of these issues. In DART BsAb design, VH and VL from different chains are joined in a complementary fashion via a short linker, such that VH-VL cross-pairs with VL-VH, making its behavioral properties more IgG-like. A disulfide bridge is engineered at the C-termini where cysteines have been added to improve stability and retain the necessary orientation.¹⁹ Lower effective

concentrations, decreased aggregation rates, and longer potency have been observed with DART molecules compared to BiTE antibodies.

STABILITY

As they are the primary constituents of non-IgG-like BsAbs, it is important for single-chain variable fragments to be stable. There are several approaches to accomplish this, such as altering their chains via loop grafting or mutagenesis. In loop grafting, both stability and humanization are attained by attaching the complementarity determining regions (CDRs) for the antigen onto a stable framework.²⁰⁻²⁴ This technique can also be used to introduce other desired properties to the molecule. In contrast to loop grafting, mutagenesis entails site-specific mutation or directed protein evolution. While the evolution approach is time-consuming and effortful, site-specific mutation is more straightforward. Mutations toward particular amino acids may increase stability via the consensus sequence route.^{23,25-27} Alternatively, other amino acids could be used to form disulfide bonds,²⁸⁻³¹ hydrogen bonds,^{32,33} or adjust hydrophobicity.^{23,31,33} Stability issues of bispecifics may also be addressed during expression and production, as several strategies (including adjusting the expression environment) may help with solubility and misfolding issues.



TRANSIENT EXPRESSION

Chinese hamster ovary (CHO) cells have long been the standard expression system for biologic molecules, including bispecific antibodies, despite the unique challenges posed by these molecules such as size and mispairings. Significant progress has been made in recent years on transient transfection in CHO systems. Some of the advancements have benefited BsAb production as well. Optimized transfection reagent, various enhancers, and enriched nutritional feed potentially improve the yield of the bispecific antibodies. Temperature and other conditions also have a significant effect on the expression and aggregation as well.³⁴ IgG-like asymmetric bispecific antibodies are particularly challenging to express because of the mispaired variants. This issue might be mitigated by varying the DNA ratio of different chains of bispecific antibody during the transfection and adjusting the production duration.

High-throughput transient production platforms allow for the rapid and efficient expression of bispecific antibodies in various formats, making it possible to screen and test a large number of potential candidates. This approach not only speeds up the drug discovery process but also ensures that the most promising candidates are identified and validated quickly, ultimately leading to the development of more effective bispecific antibodies for therapeutic use. In certain instances, transiently produced bispecific antibodies can be purified to a high degree of monomeric content with low levels of endotoxin, which makes them suitable for direct use in toxicology studies.

Process development

As a program enters the drug development stage, production quantities, product quality, and program timelines need to be evaluated. Work typically begins with developing a stable cell line to produce large quantities of material. Process development work

is required to maximize the yields and to ensure product consistency from batch to batch. A critical first step is to prioritize understanding all product-related challenges and address them early in the design phase. It should be recognized that the correct assembly of antibody fragments is compulsory for large-scale production. Given the complexity of BsAbs, it should be determined at the outset that ramping up to commercial scale is both practical and economically feasible.

CELL LINE DEVELOPMENT

CHO cells are the prevailing cell line utilized for bispecific antibody production. In general, generating a stable CHO cell line involves optimization of the transfection method (including the number of plasmids) which is then followed by clone selection. At least two plasmids for heavy chains and one or two plasmids for light chain are recommended. Expressing the heavy and light chains on separate plasmids is advantageous, as it allows easy adjustments of the plasmid ratio.³⁵ The clonal cell line that produces the highest concentration of the desired structure is then selected from the transfectant pool.³⁶ Optimizing the plasmid ratio and then choosing the best clone leads to a more refined bispecific molecule. Note that it is critical to have analytical assays already established to identify the product-related impurities prior to selecting a clone. The titer and product quality/activity are important considerations.

The cell line development process typically takes approximately six months to complete. More advanced technologies that utilize single cell seeders/robotics alongside *in situ* imaging systems can shorten the cell line generation timelines. Average production titers for standard monoclonal antibodies can range from 3–6 g/L titers. Due to the pairing complexity of BsAbs, production levels can be noticeably lower than monoclonal antibodies. Selection of a high-quality cell line will drastically assuage the subsequent process development work.

UPSTREAM PROCESS DEVELOPMENT

The type of bioreactor and culture mode can significantly impact the purity of the BsAb. Similar to the production of other molecules, the standard process for scale-up employs stir tanks. The traditional fed-batch culture is the most cost-effective approach, whereas perfusion continuously provides fresh medium while removing harmful cellular byproducts. It should be noted that if the antibody is unstable in the production conditions, perfusion may be the more suitable culturing method, as it minimizes the antibody's interactions with degradation-causing components. Generally, the stability of the molecule must be understood to determine the optimal culturing method and duration. Material and labor costs should be considered as well.

Media and feed are critical for obtaining the highest yields of an antibody. Various combinations may make significant improvements in the expression levels of the BsAb. Measuring the changing concentration of medium components during culturing allows for the understanding of the metabolic needs and can be used to determine proper feed rates and feed strategies. Parameters such as pH, temperature, and oxygen levels influence cell growth, expression levels, and product quality. Platforms with microbioreactors enable the high-throughput screening of culture conditions which will rapidly determine the optimal culturing conditions for BsAbs at earlier stages in development. Working with bioreactors of a singular platform is conducive to developing a scalable process.

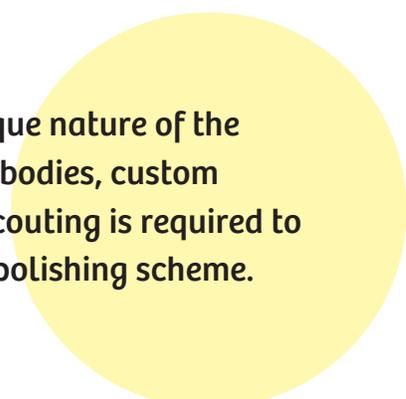
DOWNSTREAM PROCESS DEVELOPMENT

Downstream processing ensures the purity and quality of the bispecific antibody. Even with strategies to reduce the generation of BsAb-related side products during cell culturing and production, their creation cannot be completely avoided.

For this reason, the downstream processing for bispecific antibodies is more challenging than for mAbs, due to the additional steps needed to remove these product-related impurities that are structurally similar to the target antibody. The platform approaches used for monoclonal antibodies are therefore not entirely applicable to bispecifics. There is no single methodology for the purification of bispecific antibodies; protocols must be tailored to the particular molecule of interest. The BsAb downstream process entails capture and polishing, virus inactivation, and two orthogonal viral reduction steps. It must also follow regulatory guidelines from the FDA for bispecifics, which are similar to monoclonal antibodies.

After the harvest is clarified, multiple chromatographic procedures are typically employed for purification of the BsAb. First, the capture step enriches the target protein using a variety of methods. Affinity resins are highly effective at selectively capturing the target protein while separating process-related impurities such as cell debris, DNA, and host-cell proteins (HCPs). The choice of affinity resin used for capture is critical, as its specificity and binding affinity must be suitable for the target product in order to obtain maximum yield with minimal process impurities. The elution profile of product-related impurities should be evaluated during the capture phase. More cost-effective resins, such as ion exchange resins, can also be applied as capture steps; however, additional scouting work is required. After the initial capture, polishing steps are required to further reduce the process-related impurities (i.e., host cell protein, residual DNA) and product-related impurities (i.e., soluble aggregates, mispaired species, and fragments). Given the unique nature of the bispecific antibodies, custom purification scouting is required to establish the polishing scheme. The physical properties of the bispecific, such as its isoelectric point (pI), hydrophobicity, and the effects of alternative affinity

tags, must be considered during the scouting process to identify the most effective purification condition. Although size exclusion chromatography (SEC) is frequently used at bench-top scales, it is not always capable of separating all of the undesired chain-pairing variants from the desired product. Additionally, it is not an economically scalable process, thus not recommended for commercial-scale manufacturing. Ion exchange chromatography (IEX) has been used successfully for the removal of mispaired species based on the differences in net surface charges of the molecules in the post-capture mixture. Mixed-mode resins, alternative affinity-based resins, and hydrophobic interaction chromatography (HIC) may also be employed, depending on the characteristics of the BsAb.



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The stability of the BsAb may create additional challenges in developing the downstream purification process. An understanding of the BsAb's tolerances for salt, pH, and specific buffering components will aid in finding conditions that successfully stabilize the final product at intermediate hold steps. Additional excipient and pH screening may be required to identify conditions that stabilize the target protein. Viral inactivation commonly uses a low pH hold. Excipients may be added to help stabilize the BsAb in the harsh environment; however, in cases where the antibody is not amenable to acidic conditions, solvent-detergent treatment may be used. It is important to note, however, that any of these modifications may impact viral inactivation.



ANALYTICAL CHARACTERIZATION

The production of bispecific antibodies calls for the development of assays to characterize their impurities, size (including aggregate and fragment determination), and activity/potency. Product-related impurities, such as aggregates, fragments, and mispaired molecules, pose a unique challenge due to the many structural variations that result from the BsAb production. The misassembled species are similar enough to the desired target that they may be very difficult to detect and quantify. Therefore, these contaminants must be well understood so that they may be differentiated from the target product using sensitive analytical tools. In some instances, specific regions of the molecules have been modified to yield different characteristics from the target product, such as hydrophobicity or pI, in order to streamline their separation.³⁷ In addition to the product-specific impurities, assays are also necessary to measure the residual levels of process-related impurities such as HCPs, DNA, and affinity ligand leachates. Well-established immunoassays are commercially available for characterizing the purity of the produced samples. Commonly used techniques for analyzing BsAbs include capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and size exclusion ultra-performance liquid chromatography (SE-UPLC). These methods provide information on the purity and size of the

BsAbs, which are critical to ensure the molecular integrity of the BsAb product. In CE-SDS, proteins are denatured and separated by molecular weight to track a BsAb's misassembled variants and fragments. SE-UPLC is a chromatographic method commonly used for monomeric content characterization that separates antibodies based on their size, with larger molecules eluting earlier than smaller molecules. In addition to these methods, hydrophobic interaction chromatography UPLC (HIC-UPLC) and reverse-phase UPLC (RP-UPLC) can be used as an orthogonal purity assay to provide complementary data on the BsAbs' purity and identify impurities, based on its hydrophobicity. Since mispaired species may have different isoelectric points and charged states, IEX can aid in identifying the distinct charged species present in a given sample. Capillary isoelectric focusing (ciEF) can also be used to detect mismatched chains, similar to IEX.

Furthermore, liquid chromatography-mass spectrometry (LC-MS) is a powerful tool for the characterization of bispecific antibodies, enabling the identification of BsAbs and their variants, as well as the detection of any post-translational modifications or impurities that could affect drug safety and efficacy. Although LC-MS can be expensive and require complex operations, it is still considered one of the primary techniques for the qualitative identification of the target molecule and impurities.

Aside from product-related impurities, assays are necessary for evaluating other quality attributes of the BsAb, such as potency, affinity, and specificity. However, some of these tests are complicated by the very nature of bispecific molecules. Potency assays are a major challenge for any program, but for BsAbs, the complex mechanism of action and the molecule's dual targeting characteristic make development of a bioassay especially difficult. In addition, it must be demonstrated that the novel antibody is capable of binding two specific targets simultaneously. Immunogenicity is another concern

for bispecifics, as the more engineering that goes into a molecule, the less it will resemble a native protein which could induce an undesired immune response. *In silico* analyses and polyspecificity assays at an early stage can be used to guide the selection of bispecific antibodies to reduce the propensity for rapid antibody mediated clearance in humans. Safety assessments may be further complicated by the targeting of more than one antigen. The various requisite tests must overcome the numerous challenges of their design while complying with FDA guidelines.

SUMMARY

Bispecific antibodies show incredible promise for immunotherapy as they bind to two different antigens, thereby enhancing therapeutic efficacy. This dual functionality can be tailored for precise immunogenic targeting, making BsAbs a powerful budding alternative to conventional therapies. Unfortunately, the synthesis of these bispecific molecules is complicated by the multitude of product-related impurities, such as mispaired species. Strategies for inhibiting their formation and promoting selective pairing have been developed. However, even with the optimal design, some level of undesired product-related variants is nonetheless observed. Therefore, purification processes must include steps to remove these particular contaminants. Generally, the best outcomes stem

from the combination of optimized production and the downstream processes to remove the impurities. Analytical monitoring throughout the process and downstream testing of the product are essential for ensuring the quality of the novel antibody. Development of testing is complicated by the complexity of the bispecific molecule and must be deliberated to verify that the tests fulfill the needs of the program and meet FDA guidelines.



Curia's role in bispecific antibody development and production

At Curia, we understand the intricacies of bispecific antibody discovery and production. Our experts have deep experience across discovery research, production, and development. For many years, we have been helping clients with antibody discovery, variable region sequencing and cloning, transient production, stable cell line development, extended characterization and formulation.

A key differentiator is our ability to start working with you as early as the discovery stage and employ our proprietary high-throughput transient production system to quickly generate candidates for evaluation. This allows you to rapidly assess a wide range of potential options and distinguish our offerings from those of our competitors. Curia's high-throughput gene-to-protein (GTP) set-up facilitates efficient execution of this approach, thereby saving time and expediting results.

Our high-throughput 96 block production (HTP-96) services are particularly convenient for rapid and cost-effective screening of BsAbs. Moreover, we offer companion intact mass and CE-SDS analysis to provide insight into bispecific mispairings and other product-related impurities. Additional characterization of antibodies is also available, such as affinity analysis, epitope binning, FcR binding, and *in silico* predictions (sequence liability and immunogenicity analysis). We work side-by-side with you as you select leads for further evaluation,

continuing to supply and characterize your promising BsAb candidates. We have successfully provided transiently produced materials for toxicology studies. Finally, we can scale with you into the clinic for GMP production. Our decades of GMP manufacturing proficiency have empowered our biopharmaceutical partners to cross the finish line smoothly.

Curia provides a true end-to-end partnering experience with integrated teams of experts to ensure seamless transitions between phases as we provide best-in-class, customizable solutions for your project.

Curia provides a true end-to-end partnering experience with integrated teams of experts to ensure seamless transitions between phases as we provide best-in-class, customizable solutions for your project. This streamlining saves time and cost while reducing risk. The "one team" approach fosters effective collaboration and delivers meaningful results with the greatest efficiency. From discovery to clinic, our scientific expertise and advanced technology platforms can enable you to reduce costs, shorten timelines, and achieve better outcomes.

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