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# Bi-specific VHH Biotherapeutics: A route for treatment of Solid Tumours

Over the last 20 years antibody-based therapeutics have become vital in the treatment of a range of different cancers including both solid and liquid tumours[1,2]. Bi-specific antibodies (bsAb) refer to a single antibody or linked antibody fragments (e.g scFV or VHH) able to bind two different epitopes or antigens[3–5]. As of August 2021, only three bi-specific antibodies have been approved: blinatumomab (BiTE) for the treatment of Acute Lymphoblastic Leukemia (ALL), emicizumab for treatment of Haemophilia and amivantanab for non-small cell lung cancer[6–8]. Although solid tumours account for the majority of newly diagnosed cancer cases, there are few drugs that produce durable therapeutic benefits in patients, due to the high heterogeneity of cancer cells as well as dynamic and inhibitory tumour microenvironment (TME).

Bi-specific antibodies can provide higher binding specificity due to their two unique binding regions, which can bind different epitopes on the same protein or on two different proteins. For instance, targeting two different antigens on a single tumour cell may increase the antiproliferative effect and avoid the development of resistance. Consequently, off-site binding and associated side effects are also reduced. Target antigens can be selected with tumour heterogeneity, ligand redundancy and target synergy in mind[3,9]. Numerous formats exist and can be broadly segmented by the presence/absence of the Fc region[5,9]. Fc-free bsAbs, such as BiTEs, display better biodistribution into tumour tissues, higher potency, and less common incidence of immune-related adverse effects (irAEs). However, to achieve favourable pharmacokinetic (PK) profiles, Fc-free molecules need either continuous intravenous infusion or structural modifications to prolong their half-lives, such as PEGylation or conjugation to Human Serum Albumin (HSA), or HSA-binding moieties. Fc-containing bsAbs are characterized by longer half-lives and by their ability to elicit effector functions on immune cells, such as antibody-dependent cell cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Fc-based bsAbs display either Immunoglobulin G (IgG)-like or, more commonly, IgG-modified structures[10]. The presence of an Fc region which causes immune cell or complement engagement, is detrimental in certain therapeutic settings. For instance, immune checkpoint inhibiting antibodies, in many anti-cancer treatments, are not desired to cause target cell depletion and are chosen to be Fc-null or Fc-silenced[11].

bsAb combinations can be summarized as immune cell engagers (ICE), tumour associated antigen (TAA)-targeting, immune checkpoint blockade (ICB), or Ab-drug conjugation (bsADCs). The US FDA Bi-specific designated bsAbs into two classes, based on their mechanism of action.

**Cell bridging bsAbs** are mostly observed as immune cell engagers linking T cells or NK cells to malignant cells[12,13]. The basic anti-cancer bsAbs construct typically recognises a TAA and effector cell engager (e.g CD3 on T cells CD16 on NK cells). High tumour specificity is a fundamental pre-requisite for a TAA, as well as its cell surface density, size and mobility on tumour membranes. The greatest hinderance to the success of immune cell engagers is antigen escape through proliferation of non-TAA expressing tumour cells. The upregulation of immune checkpoints by tumour cells also represents an issue, whereby suppression of checkpoints such as the PD-1/PD-L1 axis can enhance the antitumour efficacy of BiTEs[14].

**Antigen crosslinking bsAbs** target two antigens or two receptors simultaneously to block signals of tumour cell growth/survival whilst also activating immune cells[13]. A successful example of this is amivantamab, which targets cMet and EGFR[8]. Another approach demonstrating some success in clinical development is combining immune checkpoint proteins to synergise the immune modulating functions. While this approach could potentially result in a synergistic blockade of the inhibitory receptors, it can also lead to enhanced toxicities. Interestingly, antigen-crosslinking bsAbs account for more than two thirds of drugs in clinical studies for solid tumours (Figure 1). These pairs can vary from single targets such as **HER2/HER2** (including bi-paratopic antibodies) to combinations of different immune checkpoint proteins such as **PD-1/CTLA-4, PD-1/PD-L1 or PD-L1/CTLA-4.**

## TRENDS IN BI-SPECIFIC ANTIBODY DRUG DEVELOPMENT TARGETING SOLID TUMOURS

A total of 326 bsAbs span the breadth of discovery through to marketed drugs. The market analysis tool, Global Data was used to collate drugs in clinical development in the following indications: Breast Cancer;Carcinomas;Central Nervous System (CNS) Cancer;Endocrine Gland Cancer;Gastrointestinal Tract Cancer;Gynecological Cancer;Head And Neck Cancer;Lung Cancer;Male Cancer;Malignant Neoplasms;Metastatic Cancer;Neuroendocrine Cancer;Pediatric Cancer;Sarcomas;Skin Cancer;Solid Tumor;Urinary Tract Cancer;Bone Cancer;Malignant Mesothelioma;Mesenchymal Tumour.

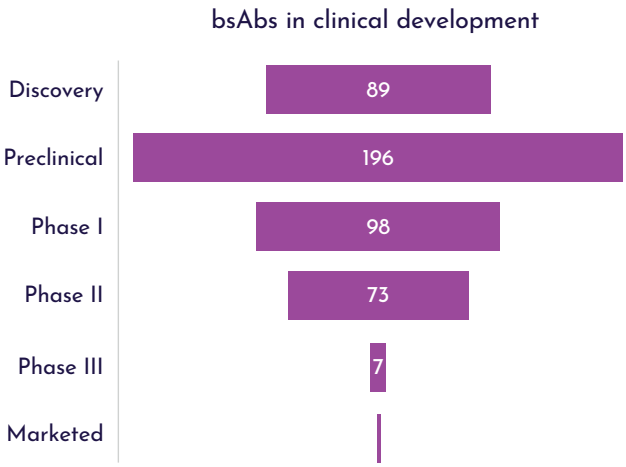



**Figure 1a:** Despite the high proportion of early phase studies of bsAbs targeting solid tumours, the attrition of drugs making it to approval increases substantially, highlighting the demand for better alternative to address inadequacies in clinical and safety criteria.

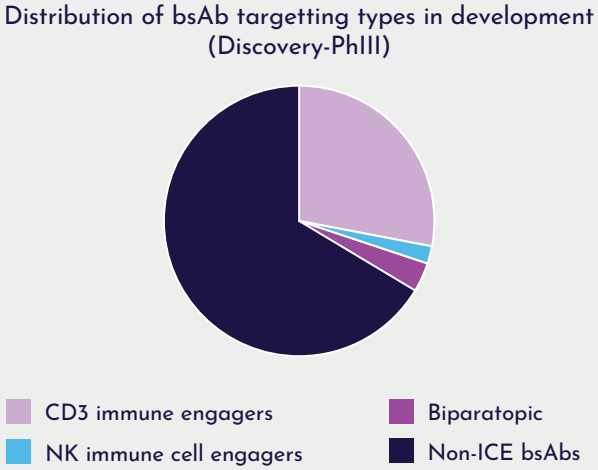



**Figure 1b:** The majority of bsAbs in clinical development encompass non-ICE targets highlighting the safety challenges of harnessing immune cells for treatment of solid tumours.

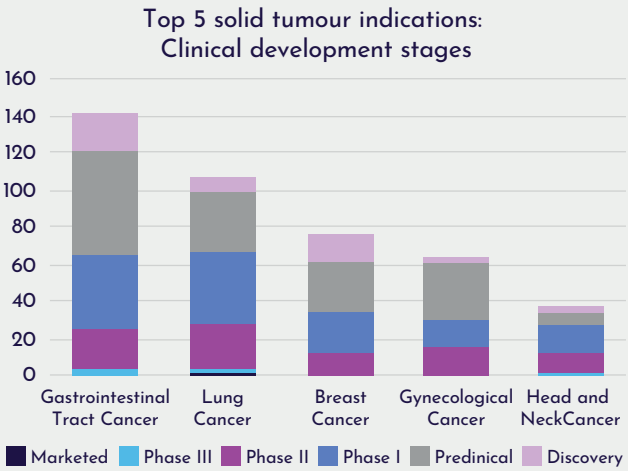



**Figure 1c:** Top five solid tumour indications of bsAbs in clinical development. The large number of studies in gastric cancer demonstrate the unmet need in this indication.

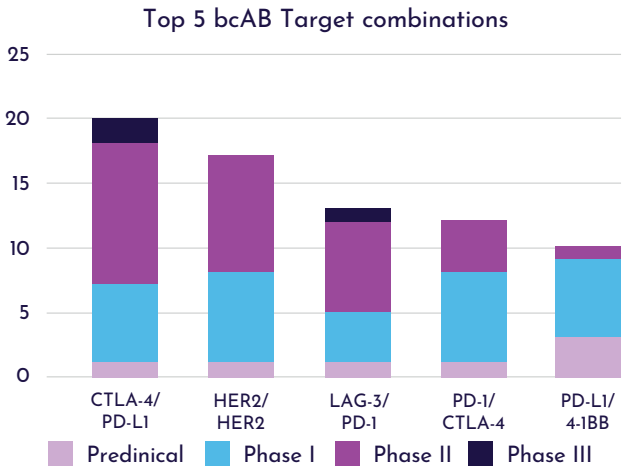



**Figure 1d:** Top five target combinations among bsAbs in development. There are also a significant proportion of CD3/Undisclosed target bsAbs in development which have not been included in the graph.

## BI- AND MULTI-SPECIFIC VHH ANTIBODIES

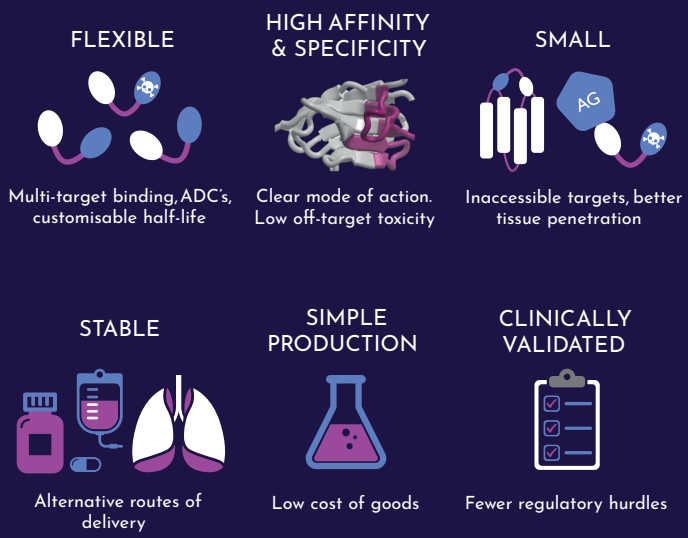
The bispecific VHH connects the VHH regions of two or more heavy chain only antibody molecules to achieve multi-specific binding. The products of this construct are small, robust proteins that possess the advantage of high biophysical stability and increased tissue permeability in vivo[15,16]. VHH are characterised by the absence of an Fc region, thus further formatting such as Fc-silencing is not required, making them ideal formats for these therapeutic settings[11,17]. VHH multi- and bsAbs in clinical development for the treatment of solid tumours are detailed in the table below, confirming them as a clinically validated and accepted format of next generation biotherapeutics in solid tumours (Table 1).

| Drug | Sponsor | Domain Properties | Target | Indication | Status |
|---|---|---|---|---|---|
| M1095 | Avillion / Merck KGaA (Ablynx) | VHH Bi-specific | IL-17A IL-17F | Psoriasis | Phase IIb |
| BI836880 | Boehringer Ingelheim (Ablynx) | VHH Bi-specific | VEGF, Ang2 | NSCLC | Phase II |
| HPN424 | Harpoon Therapeutics | VHH-scFv Tri-specific | PSMA | Prostate cancer | Phase II |
| HPN-328 | Harpoon Therapeutics | VHH-scFv Tri-specific | DLL3 | Neuroendocrine Tumors | Phase II |
| HPN-536 | Harpoon Therapeutics | VHH-scFv Tri-specific | MSLN | Epithelial Ovarian Cancer | Phase II |

**Table 1: Summary of VHH bsAbs in clinical development** (clinicaltrials.gov)

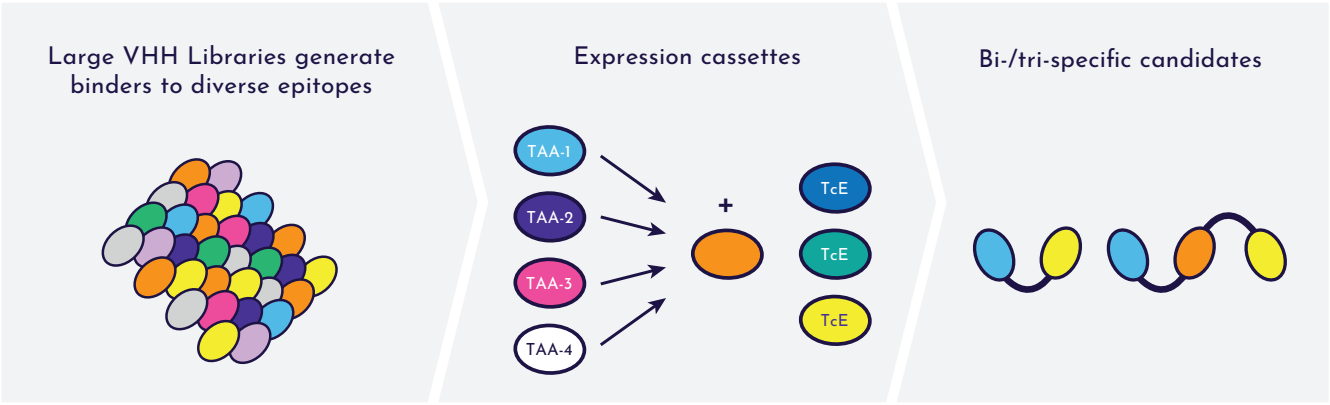
## ADVANTAGES OF VHH TO TARGET SOLID TUMOURS

VHH can be engineered to tune their binding selectivity and specificity for antigen targeting whilst their favourable biophysical properties simplify development and manufacturing. Their small size also confers advantages for tuning the immune synapse, the interface between antigen-presenting cells and T-cells, to optimise potency and minimise toxicity of T-cell redirecting immunotherapies. At only 15kDa, VHH's small size makes them advantageous for targeting therapeutically important but challenging targets[18]. VHH-only bi-specifics offer improved targeting and tissue penetration for solid tumours[19]. VHH can also be combined with conventional antibodies to create novel bi-specifics. VHH domains are also typically less immunogenic than other single chain constructs such as scFv because of their high homolog¬y with human VH genes and absence of exposed hydrophobic regions making them less potent immune targets. This has identified them as potentially excellent building blocks for novel next-generation biotherapeutics.




## ISOGENICA VHH BI- AND MULTI-SPECIFIC PLUG-AND-PLAY PLATFORM

Isogenica have developed fully synthetic, *in vitro* plug-and-play cassette-based approach to efficiently generate combinations of bi- and tri-specific VHH and have exemplified this in *in vitro* studies.




- Isogenica's libraries ensure standardised flanking sequences that can be easily slotted into bi- and tri-specific expression cassettes
- Cross-species specific anti-albumin VHH binds to protein A to simplify downstream CMC
- Production in mammalian expression systems and scaled-up purification facilitates developability and functional testing, including T-cell activation

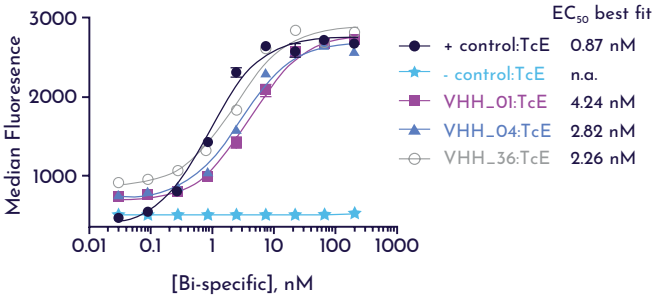
## BI-SPECIFICS MODEL TAA:TCE BI-SPECIFIC MOLECULES BIND SPECIFICALLY TO TARGETS AND EFFECTIVELY KILL TAA+ BREAST CANCER CELL LINE

Anti-TAA primary monomers were selected from Isogenica's VHH antibody library. Primary monomers were screened for specific binding to designated recombinant protein and cells and optimal SEC profile. Leads VHH_01, VHH_04 and VHH_36 represent unengineered primary monomers with desired characteristics. Using a well characterised TcE (T cell Engager) scFv, these monomers were formatted, in-house, into bi-specific molecules using an in-house acceptor vector cassette. In all cases, specific binding to target cells was retained for both TAA and TcE arms (Figure 2).

We demonstrate that these molecules are able to specifically bind their targets (Figure 2) and to effectively induce tumour cell killing (Figure 3) with potency values comparable to that of the positive control, an scFv from a clinically approved mAb against the same TAA target. Furthermore, killing is directly associated with activation of TcE+ effector cells thus demonstrating functionality of the TcE arm (Figure 4).

**A**

Binding of TAA:TcE bi-specific molecules to $TAA^+$ breast cancer cell line




**B**

Binding of bi-specific molecules to $TcE^+$ cell line

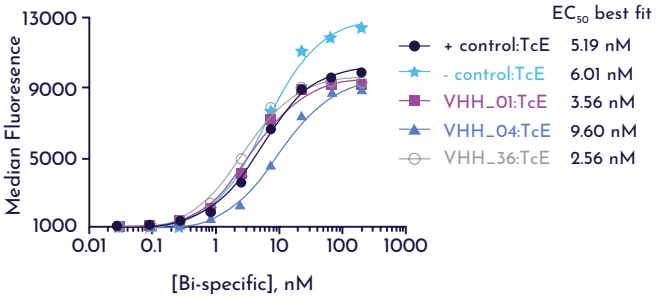



**Figure 2 - Cell binding of in-house designed and produced TAA:TcE bi-specific model molecules.** Sequential dilutions of each bi-specific molecule, and corresponding controls, were incubated with respective target cell line. Cell bound bi-specifics were detected by flow cytometry, using an anti-His antibody. A) binding of bi-specifics to TAA+ breast cancer cell line, B) binding of bi-specifics to TcE+ T-cell cell line. Specific binding was confirmed by testing these molecules in $TAA^{-ve}$ breast cancer cell line and $TAA^{-ve}$ T-cell cell line; no binding was detected (data not shown). Data represents average of 2 wells. (TcE – scFv of validated T cell Engager; + control – scFv of clinically validated anti-TAA Ab; - control – VHH specific for Hen Egg Lysosyme; n.a. – not applicable).

A tumour cell killing assay was established using a reliable TAA-expressing cell line and PBMCs from human donors. This demonstrated effective tumour cell killing with potency values comparable to that of the positive control, an scFv from a clinically approved mAb against the same TAA target, with EC50s in the low pM range (Figure 3). Furthermore, killing was directly associated with activation of TcE+ effector cells thus demonstrating functionality mediated by the TcE arm (Figure 4). In both cell killing and T cell activation assays, coupling the TcE arm to a negative control molecule (an anti-lysozyme VHH on the same scaffold) elicited no response, further reinforcing the immune synapse between target and effector cells as the mode of action for cell killing.

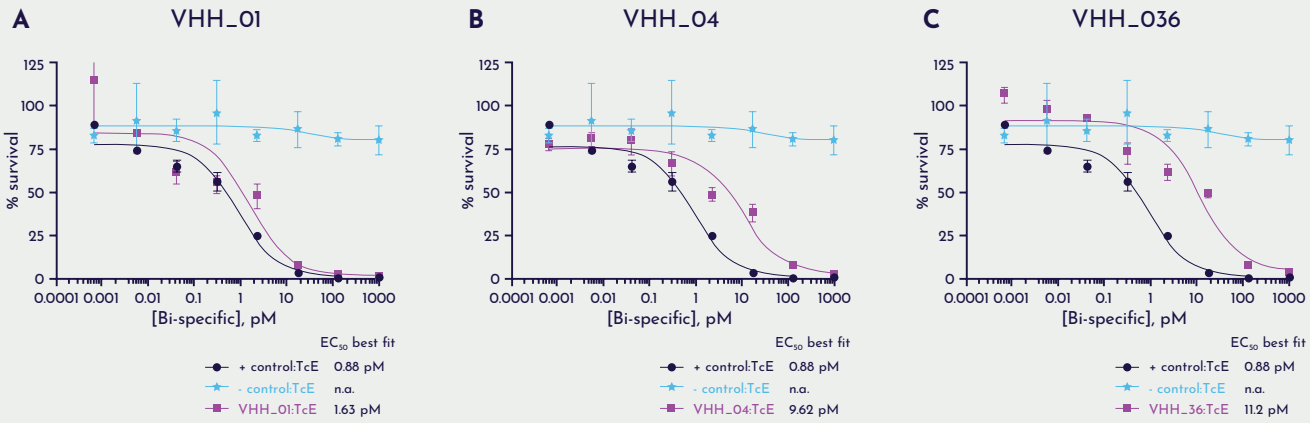



**Figure 3 - % survival of TAA+ breast cancer cell line.** % survival of TAA+ breast cancer cell line after 72h co-culture with PBMCs at a 10:1 E:T ratio and sequential dilutions of each bi-specific molecule. At 72h PBMCs were removed, target cells washed and % survival measured using an LDH assay. % survival was calculated in relation to the average signal of wells containing PBMCs + target cells only. Data representative of 2 independent donors. Graphs depict % survival of TAA+ breast cancer cell line when incubated with A) VHH_01:TcE bi-specific; B) VHH_04:TcE bi-specific, C) VHH_36:TcE bi-specific. (TcE – scFv of validated T cell Engager; + control – scFv of clinically validated anti-TAA Ab; - control – VHH specific for Hen Egg Lysosyme; n.a. – not applicable).

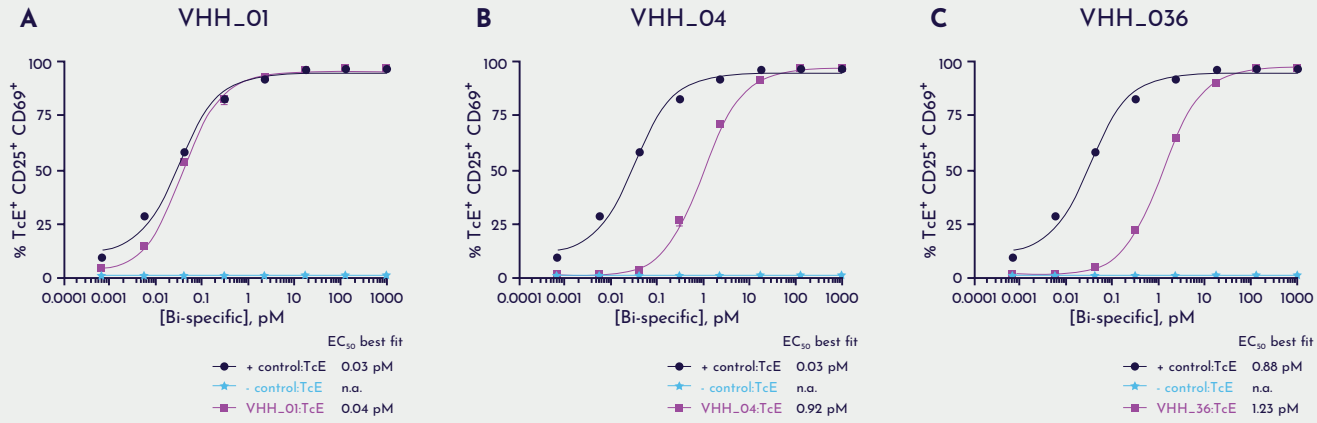



**Figure 4 - Activation of TcE+ PBMCs.** Activation of TcE+ PBMCs after 72h co-culture of TAA+ cell line with total PBMCs at a 10:1 E:T ratio and sequential dilutions of each bi-specific molecule. At 72h PBMCs were removed, and stained for TcE, CD25 and CD69. Cells gated on TcE+ signal were evaluated for expression of both CD25 and CD69 surface molecules by Flow Cytometry. Activation of PBMCs is represented by increased % of cells expressing the surface activation markers CD25 and CD69. 1 when PBMCs and TAA+ breast cancer cell line incubated with A) VHH_01:TcE bi-specific; B) VHH_04:TcE bi-specific, C) VHH_36:TcE bi-specific. Data representative of 2 independent donors. (TcE – scFv of validated T cell Engager; + control – scFv of clinically validated anti-TAA Ab; - control – VHH specific for Hen Egg Lysosyme; n.a. – not applicable).

In this case study, it has been demonstrated that Isogenica's VHHs are highly modular and can be assembled in a cassette-like manner to create biologically functional bsAbs. These have been shown to perform comparably in vitro to clinically approved equivalent molecules built on those already approved clinically, bridging the immune synapse to elicit tumour cell killing. Further modifications could also be implemented to overcome their relatively short serum half-lives in relevant therapeutic applications. With the upward trend of next-generation antibody formats in clinical development, it is clear VHH are a clinically accepted antibody format with great potential to change the landscape of antibody therapeutics in solid tumours.

Solid tumours account for more than 90% of cancers and durable therapies remain remain a huge unmet need, demonstrated by the high number of antibodies in clinical development. Bi-specific antibodies provide a possible solution as therapeutics in solid tumours, as they can target multiple epitopes, leading to higher binding specificity and lower off-site binding. VHH are highly modular, have no Fc region, which is advantageous in certain settings, and can penetrate tumours more deeply due to their small size. Safety and tolerability complications, such as cytokine release syndrome are a substantial obstacle for many antibody drugs. With VHH however, due to their highly modular nature, aspects such as tumour evasion and off-target toxicity can be facilitated by increasing specificity through through targeting multiple tumour antigens simultaneously. Therefore, combining intrinsic VHH advantageous traits with the bi-specific format, VHH bsAbs offer an accepted solution against hurdles most often encountered with bsAbs more traditional mAb-style formats solid tumours.

## READY TO START YOUR NEXT PROJECT?

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## GLOSSARY

1. BsAb - bi-specific antibody
2. BiTE - bi-specific T Cell Engager
3. ALL - Acute Lymphoblastic Leukemia
4. irAEs - immune related adverse effects
5. PEG - Polyethylene Glyco
6. HSA - human serum albumin
7. ADCC - Antibody-Dependent Cell Cytotoxicity
8. CDC - Complement-Dependent Cytotoxicity
9. TAA - Tumour Associated Antigen
10. ICE - Immune Cell Engagement
11. ICB - Immune Checkpoint Blockade
12. FDA - Food & Drug Administration
13. bsADC - Bi-specific Antibody Drug Conjugate
14. TME - Tumour Microenvironment
15. EGFR - Epidermal Growth Factor Receptor
16. HER2 - Human Epidermal Growth Factor Receptor 2
17. PD-1 - Programmed Death Protein 1
18. PD-L1 - Programmed Death Ligand 1
19. CTLA-4 - Cytotoxic T Lymphocyte Associated protein 4
20. SEC - Size Exclusion Chromatography
21. TcE - T Cell Engager
22. ScFv - Singe chain variable fragment
23. Nanobody™ - Single-domain VHH antibody trademarked by Ablynx

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