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Application of Nano-Antibodies for Cancer Immunotherapy

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Abstract

Summary By inhibiting intracellular KRAS and STAT3, SBT-100 suppresses the growth of human tumors in vivo, decreases VEGF and PD-L1 expression, inhibits IL-6 function in cancers, suppresses IL-17, GM-CSF, IFN-gamma, IL-1-alpha, and downregulates Th17 cells.

Purpose of Review Two VHH (camelid heavy chain variable region) antibodies are being used to treat patients with the thrombotic disorder and rheumatoid arthritis; however, no VHHs are currently being used to treat cancer patients. The purpose of this review is to discuss VHHs that have been developed to target intracellular oncoproteins such as KRAS and STAT3 for cancer therapy.

Recent Findings Various groups are working on optimizing cell-penetrating antibodies to target intracellular KRAS and STAT3 but are using non-VHH platforms. SBT-100 is a monomeric, bi-specific VHH that penetrates the cell membrane and BBB to give a therapeutic response against human cancers.

Keywords Nano-antibody · VHH · STAT3 · KRAS · VEGF · PD-L1

Introduction

The tumor microenvironment (TME) plays a critical role in the growth of cancers, by providing a localized area characterized by chronic inflammation and immune suppression [1–9]. Normal organs can be divided into two general categories, parenchymal cells and stroma. This categorization is also applicable to a cancerous tumor; while the parenchymal cells are the malignant cells, the stroma encompasses endothelial cells, adipocytes, mesenchymal stromal cells, fibroblasts, immune cells, and lastly acellular molecules including laminin, fibronectin, hyaluronic acid, proteoglycans, collagen, glycosaminoglycans, and other large molecules. The presence of innate and adaptive immune cells, such Treg cells, within the tumor stroma results in localized immunosuppression despite the presence of CD8+ cytotoxic T lymphocytes (CTLs) within the TME.

The important role of the TME, especially cancer-associated fibroblasts (CAFs), in the progression of non-small cell lung cancer (NSCLC) has been demonstrated, while Plava et al. have reviewed how tumor stroma mediates chemoresistance in breast cancer [10, 11]. Mesenchymal stromal cells have been found to play a central role in acquired resistance to chemotherapy. During cancer progression, serine proteases (e.g., fibroblast activation protein, urokinase-type plasminogen activator, kallikrein-related peptidases, and granzymes) are important, pleiotropic factors at the tumor-stroma interface, that interact not only with other proteases but also with growth factors, kinases, cytokines, and chemokines [12]. Sund et al. have proposed that tumor stromal interactions produce biomarkers that may have clinical applications [13]. Manipulating the stroma of a solid tumor and transforming it into an immunological target allows for host CD8+ CTLs to destroy cancer [14].

Of solid tumors, pancreatic cancer is well known to harbor a uniquely desmoplastic, immunosuppressive stroma. It has been characterized by the dense stroma with poor vascularity, limiting the ability of chemotherapeutic agents in reaching the cancer cells for maximal effect. Inhibiting signal transducer and activator of transcription 3 (STAT3) has been shown to remodel the stroma of pancreatic cancer by decreasing its dense, fibrous nature to improve vascularity within the tumor [15, 16••, 17]. STAT3 plays a critical role in oncogenesis and inflammation [18–20]. There is an abundance of evidence showing that small-molecule inhibitors of STAT3 are effective therapeutics for treating solid and hematologic

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malignancies [21–23]. RNA inhibitors of STAT3 have also been successful in treating cancers; however, both small molecules and RNA inhibitors of STAT3 have not progressed far beyond Phase I clinical trials due to toxicity or lack of clinical efficacy [24]. Similarly, nanobodies or single-domain antibodies have recently provided a new therapeutic opportunity for uniquely dense tumor stromas, such as that of pancreatic cancer and breast cancer [25–28]. In breast cancer, antibodies specifically for HER2-positive breast cancer have become new promising candidates to overcome therapeutic resistance [29–31]. Other recently developed nanobodies target intracellular antigens such as immune checkpoint molecules, growth factors, and EGFR, all well-described perpetrators of therapeutic resistance in the tumor microenvironment [32–36]. Togtema et al. demonstrate the potential for single-domain antibodies to target the human papillomavirus 16 E6 protein, a major etiological molecule of cervical cancer [37]

Camelid variable domain only immunoglobulin fragment (VHH) is a nano-antibody about 15 kilodaltons in size and measuring approximately 2.5 nm in length or about $1/10^{th}$ the size of a human IgG (Figure 1) [38–40]. These VHHs are robust molecules that are more easily produced than four-chain traditional antibodies. Because of their modularity, VHH nano-antibodies can potently target different antigens simultaneously [41, 42]. For these reasons, they are currently being pursued both diagnostic and therapeutic purposes [43, 44]. In numerous clinical trials for various indications and different antigen targets, these nano-antibodies have been found to be safe and efficacious [45–50]. Two such nano-antibodies, caplacizumab and ozoralizumab, have been approved for marketing [51•, 52]. Given their small size nano-antibodies are very attractive for their potential to develop for intracellular targets, even those that have long been considered "undruggable" such as KRAS (mammalian homolog of Kirsten RAS) and STAT3 [53–56]. Three monomeric nano-antibodies have been developed to penetrate the cell membrane and blood-brain barrier (BBB). These are SBT-100, SBT-101, and SBT-102, which are specific for either both KRAS and STAT3 (Figure 2), STAT3 only, or KRAS only, respectively [57, 58•]. SBT-100 crosses the cell membrane and BBB *in vivo* within 15 min [57]. The characterization of these novel, cell-penetrating nano-antibodies has been an ongoing process to reveal their unique properties.

Camelid VHHs: Nano-Antibodies

In the late 1980s, Dr. Raymond Hamers and his laboratory at the University of Ghent serendipitously discovered that camelids (i.e., camels, llamas, and alpacas) have slightly different antibodies than other mammals [59]. Approximately 40% of the immunoglobulins of camelids are heavy chain-only antibodies that are devoid of light chains and are approximately 90 kilodaltons [43]. Similar antibodies, named VNARs, were later identified in sharks and cartilaginous fish [60, 61]. It has been shown that the binding region of this unique camelid antibody, the variable region also called a VHH, could be genetically cleaved and produced as a recombinant protein [62]. This VHH can bind the same antigen as

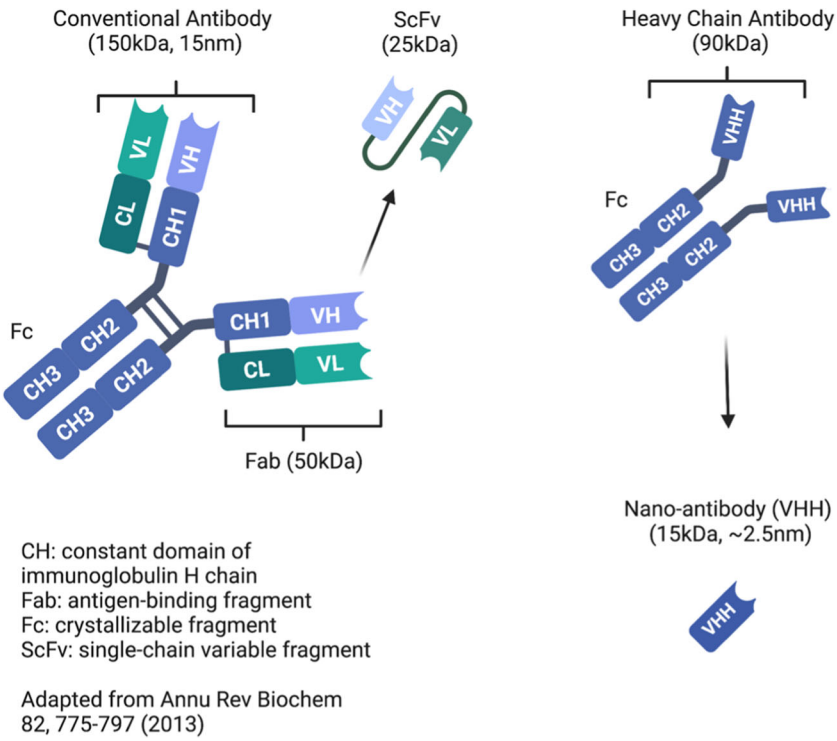

Fig. 1 Schematic representation of conventional antibodies (IgG1) containing two light (L) chains (VL and CL domains) and two heavy (H) chains (VH, CH1, CH2, CH3), and camelid antibody containing two heavy chains (VHH, CH2, CH3) only. For comparison, miniature antibodies would be scFv which are a VL and a VH covalently linked and a single camelid VHH demonstrates the small size of this nanobody (VHH)

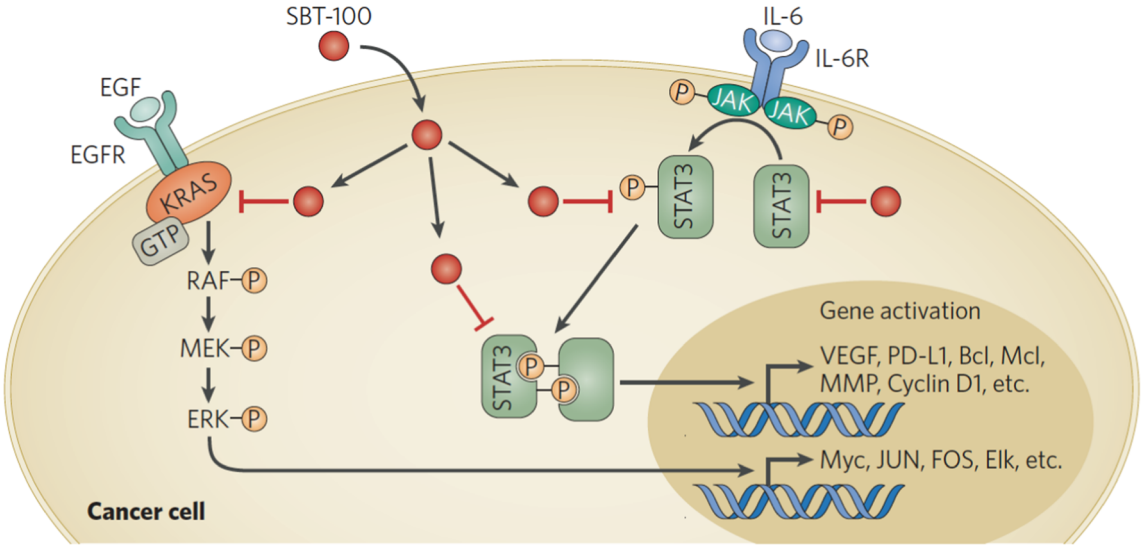

Fig. 2 Penetration of the cell membrane by SBT-100 results in rapid binding to STAT3 and KRAS. This causes a decrease in downstream effectors of KRAS. STAT3 activation, translocation to the nucleus, and binding of its DNA promotor are inhibited by SBT-100. Binding to both unphosphorylated and phosphorylated STAT3 by SBT-100 decreases total STAT3, and thus its function as a protein degrader. Ultimately both KRAS and STAT3 gene target expression is reduced

the entire camelid antibody devoid of light chains, with a VHH of only 15 kilodaltons, making it the smallest antibody in nature. After this discovery, VHHs were made against von Willebrand factor (caplacizumab), TNF-α (tumor necrosis factor-alpha) (ozoralizumab), IL-6 (interleukin-6) receptor (vobarilizumab), IL-17A/ IL-17F (ALX-0761/M1095), respiratory syncytial virus (ALX-0171), VEGF (vascular endothelial growth factor), Ang-2 (angiopoietin-2), CX3R1 (CX3C motif chemokine receptor 1), CXCR2 (C-X-C motif chemokine receptor 2), and RANKL (receptor activator of nuclear factor kappa-B ligand). These targets are all either extracellular molecules, membrane-bound proteins, or viruses. Caplacizumab is a humanized bivalent antibody fragment (VHH) which inhibits interaction between von Willebrand factor multimers and platelets. It is indicated for treatment of adult thrombotic thrombocytopenic purpura (aTTP) along with plasma exchange and immunosuppressive treatment. In patients with TTP, caplacizumab treatment was associated with quicker normalization of platelet count, decreased incidence of a composite of TTP-related death, TTP recurrence, or a thromboembolic event during the therapy [51•]. Ozoralizumab, an anti-TNFαVHH, has been approved for patients in Japan for rheumatoid arthritis. It is a trivalent humanized molecule and combines two anti-TNFα VHHs with one anti-serum albumin VHH. Ozoralizumab's clinical application is based on the results of the phase II/III clinical trial in Japan (3000-JA study). This study is a placebo-controlled, randomized, double-blind study in patients with active rheumatoid arthritis that is not effectively treated with methotrexate. In the 3000-JA study, ozoralizumab was subcutaneously administered to patients with rheumatoid arthritis in combination with methotrexate once every four weeks. Statistically significant improvement with ozoralizumab was found versus placebo. Furthermore, the Phase III clinical study in Japan (3001-JA study), where ozoralizumab was subcutaneously administered once every 4 weeks without giving methotrexate, demonstrated clinical efficacy, like that of the 3000-JA study and was well tolerated [52].

Transcription Factors as Drug Targets in Cancer and Autoimmune Disease

Transcription factors have long been sought as targets for drug development, but due to their intracellular location, this has been an arduous goal. Signals from interferons are mediated via the transcription factor STAT1, while the other STAT transcription factors play important role in T cell differentiation, embryogenesis, growth, and tissue differentiation [63–65]. Specifically, STAT3 has been implicated in regulating the production of T helper cells, such as Th17 cells [66, 67]. Other transcription factors such as RORγt, Gata3, Foxp3, Bcl-6, and Tbet play critical roles in the differentiation of naïve CD4+ T cells into Th1, Th2, Th17, and Treg cells [68, 69••, 70–71]. Similarly, Yang et al. demonstrated that STAT3 promotes Th17 helper cell differentiation through transcriptional regulation of RORγt. In this study, STAT3 deficiency led to the upregulation of Tbet and Foxp3 expression in T cells [66, 72, 73].

With our group's recently developed nano-antibody, SBT-100, STAT3 transcriptional function is inhibited through direct non-covalent binding to the nano-antibody, and this subsequently downregulates RORγt. In vivo, this has been shown to reduce levels of autoimmune CD4+, IL-17+ T cells, and CD4+ RORγt +T cells [74, 75]. Our group has recently

shown that SBT-100-mediated STAT3 inhibition impairs RORγt transcriptional activity, thereby reducing levels of autoreactive CD4+IL17+ T cells [76]. In the experimental autoimmune uveitis (EAU) model, there exists a significant increase in IL-17 expressing Th17 cells and IFN-gamma expressing Th1 cells, in the eyes, draining lymph nodes, and spleen of the affected animals. With SBT-100 nano-antibody treatment targeting STAT3, it was observed that much lower percentages of Th1 and Th17 cells were present in these tissues. Like EAU induced by active immunization, EAU induced by the adoptive transfer method was also significantly more severe in the PBS control group versus the treatment group which received SBT-100 that targeted STAT3. Similar to the well-established role of Th17 cells in the etiology of many autoimmune diseases, the percentage of T cells expressing the Th17 master transcription factor (RORγt) was significantly decreased with SBT-100 therapy. These studies present new therapeutic opportunities to pursue nano-antibody-specific targeting of transcription factors for the treatment of a myriad of autoimmune diseases [77, 78].

Other transcription factors that have been implicated in oncogenesis and fostering a tumor-promoting microenvironment include NF-κB (nuclear factor kappa B) and CREB (cAMP response element-binding protein). The role of NF-κB in cancer progression, specifically through its induction of inflammation, proliferation, survival, and metastasis, has been extensively studied in various cancers [79]. Interestingly, NF-κB signaling has been shown to engage in crosstalk with STAT3 and other transcription factors, such as AP-1 and p53. The role of NF-κB in the progression of colorectal cancer has been explored by Jana et al., where they discovered a mechanistic link between metastatic activin and NF-κB signaling [80]. In pancreatic adenocarcinoma (PDAC), cyclic AMP response element-binding protein (CREB) has been shown to promote cancer progression in the setting of tobacco-related carcinogenesis [81]. Similarly, in PDAC, CREB transcriptional activity has recently been implicated in epithelial to mesenchymal plasticity of tumor cells, suggesting various roles for this pleiotropic transcription factor [82•].

The role of STAT3 in cancer pathogenesis has been a long-time topic of interest and an elusive target [21–23]. Specifically, cancers as diverse as breast cancer, colorectal cancer, lung cancer, pancreatic cancer, prostate cancer, ovarian cancer, leukemias, and many others have STAT3 involved in their growth, proliferation, metastasis, and resistance to chemotherapy and radiation therapy [83–99]. This also occurs in pediatric cancers such as retinoblastoma, Wilms tumors, and leukemias [100–102]. Common mechanisms that activate STAT3 in these malignancies include IL-6/JAK/STAT3 autocrine loop, EGFR mutation, Src activation, miRNAs, and chromosomal translocation resulting in BCR/ABL fusion protein. Our group has demonstrated that the nano-antibody SBT-100 binds STAT3 with nanomolar affinity, inhibits the production of STAT3 gene products, suppresses the growth of eleven different human cancers in vitro, and inhibits various human cancers growth in vivo using an athymic xenograft mouse model. VEGF and PD-L1 are two well know genes transcribed by STAT3 [91, 103, 104]. Incubating SBT-100 with retinal epithelial cells that continuously produce VEGF resulted in a statistically significant reduction in VEGF protein in the supernatant of the cultured retinal epithelial cells. This was demonstrated as quickly as 12 h, and a single dose of SBT-100 resulted in a significant reduction in VEGF production over 48 h. Using the human TNBC MDA-MB-231cultured with the nano-antibody SBT-100, PD-L1 expression was significantly decreased within 24 h as demonstrated by immunohistochemical staining techniques and confocal microscopy. The staining reduction of PD-L1 in the MDA-MB-231 cells paralleled the reduction in activated pSTAT3 and total STAT3 in these cells. A similar reduction in PD-L1 expression was shown using the human osteosarcoma cell line SJSA-1. When these cells were cultured with SBT-100 for 48 h, a four-fold reduction of PD-L1 expression was noted using flow cytometry. These studies demonstrate that a nano-antibody such as SBT-100, which binds the STAT3, results in the downregulation of two critical genes in cancer growth and maintenance.

Targeting Cytokines of the Tumor Microenvironment

Pleiotropic cytokines have an abundant presence within the TME and play a critical role in promoting growth, proliferation, anti-apoptosis, angiogenesis, metastasis, and immune suppression. These cytokines can come from tumor cells, immune cells, and other stromal cells, impacting neighboring cells in a paracrine or autocrine fashion. One such cytokine is interleukin-6 (IL-6), a potent pro-inflammatory molecule that, after binding to its cell surface receptor (IL-6R), recruits intracellular Janus kinase (JAK) to the cytoplasmic portion of the receptor, it is then phosphorylated. This subsequently recruits STAT3 to the cytoplasmic portion of IL-6R and phosphorylates STAT3 at its tyrosine residue at amino acid position 705 (Tyr705) and sometimes the serine residue at amino acid position 727 (Ser727). pSTAT3 then dimerizes and translocates via chaperone proteins to the nucleus where the pSTAT3 dimer bind to STAT3 motifs on the DNA promoters of various target genes, such as PD-1 (programmed cell death protein-1), PD-L1 (programmed cell death protein ligand-1), VEGF, IL-17, BCL, MCL, cyclin D, survivin, Myc, MMP) [103, 104]. SBT-100 binds to both unphosphorylated and phosphorylated STAT3; this nanomolar binding affinity causes inhibition of STAT3 phosphorylation and prevents translocation of IL-6-activated pSTAT3 dimers to the nucleus of Hep-2 cells and pancreatic cancer cells, blocking the ability of IL-6 activated STAT3 from binding to the STAT3 DNA

promoter [57, 58•]. To the best of our knowledge, this is the only STAT3 inhibitor that blocks all these three steps in STAT3 activation and function.

Another key cytokine in cancer growth and metastasis is the vascular endothelial growth factor (VEGF). VEGF stimulates angiogenesis, enhancing tumor growth and metastasis. When retinal epithelial cells, which constitutively produce VEGF, are incubated with SBT-100, VEGF protein production is significantly reduced by 12 h for up to at least 48 h. Intracellular SBT-100 binds to pSTAT3, thereby preventing VEGF transcription [58•]. Unlike trastuzumab which binds to VEGF in the extracellular space, SBT-100 prevents VEGF production at the transcriptional level. Wei et al. have shown that STAT3 activation regulates VEGF expression by directly binding to the promoter, to control angiogenesis and metastasis in pancreatic cancer [91].

Other relevant cytokines such as IL-17, GM-CSF, IFNγ, and IL-1α are inhibited by SBT-100, and this has been demonstrated by Dr. Egwuagu's laboratory at the National Eye Institute at the NIH [76]. Using an autoimmune uveitis murine model, the administration of SBT-100 significantly impaired disease development by inhibiting CD4+ Th1 and CD4+ Th17 cells and their cytokines. Mouse splenocytes were re-stimulated ex vivo with the retinal antigen that produced the autoimmune uveitis and adoptively transferred into healthy mice. After 12 days of examination, it was determined that no autoimmune uveitis was transferred to the healthy mice, demonstrating long-acting suppression of pathogenic CD4+ Th1 and CD4+ Th17 cells and their cytokines by SBT-100 nano-antibody [76].

Overcoming Immune Suppression: Targeting PD-L1 and Regulatory T Cells

PD-1 and PD-L1 are both transcriptional targets of STAT3 [104]. Blocking PD-1 on the extracellular surface of T cells by nivolumab and pembrolizumab has been a tremendously successful immunotherapy treatment of many cancers such as melanoma, renal cancer, bladder cancer, Hodgkin lymphoma, head and neck cancer, stomach cancer, cervical cancer, and others. Similarly, anti-PD-L1 antibodies, such as atezolizumab and avelumab, have also been successful immunotherapy treatments by blocking PD-L1 on the cell surface. Atezolizumab is a monoclonal antibody of the IgG1 isotype that is completely humanized. It is used to treat TNBC, NSCLC, urothelial carcinoma, hepatocellular carcinoma, and small-cell lung cancer. Avelumab is also a monoclonal antibody of the IgG1 isotype too that is completely humanized. It is used to treat urothelial carcinoma, and renal cell carcinoma [105, 106].

Our group has shown that the nano-antibody SBT-100, a camelid VHH, penetrates the cell membrane and binds pSTAT3 preventing it from transcribing PD-L1 in TNBC (MDA-MB-231) and osteosarcoma (SJSA-1). There was a four-fold reduction of PD-L1 expression on the surface of SJSA-1 within 48 h as demonstrated by flow cytometry [58•]. This shows the feasibility of using nano-antibodies for immunotherapy against checkpoint molecules. One potential benefit of combining SBT-100 with checkpoint inhibitors is that SBT-100 may reduce or prevent autoimmune disease and inflammatory conditions associated with checkpoint inhibitors. By binding pSTAT3, SBT-100 has been shown in vivo to inhibit CD4+ Th17 and inflammatory cytokines IL-17, GM-CSF, IFNγ, and IL-1α [76].

Furthermore, in immunocompetent mice, treatment with SBT-100 decreased the number of Treg cells [76]. In addition to the downregulating checkpoint molecules, SBT-100 may eliminate localized immune suppression within the tumor by reducing Treg cells and allowing CD8+ CTLs to destroy the cancer. This is the first demonstration that one single antibody can reduce PD-L1 and Treg cells, acting as a dual immunotherapy mechanism.

SBT-100, SBT-101, and SBT-102: Inhibition of KRAS and STAT3

Gordon et al. suggest that two-thirds of the most desirable targets in medicine are inside the cell. They explain how anti-DNA autoantibodies found in systemic lupus erythematosus (SLE) patients can penetrate cells [107•]. Harnessing this mechanism of action, they argue, can potentially accelerate drug development against intracellular targets. Shin et al. developed a large traditional antibody (IgG1) that binds and inhibits KRAS in cancers and penetrates the cell membrane, which has been developed but has not progressed beyond the preclinical stage. This antibody, RT11, when internalized into the cytoplasm. selectively binds to activated GTP-bound forms of different oncogenic Ras mutants, thus preventing downstream signaling and cancer-promoting behavior. Aftabizadeh et al. have produced cell penetrating acetylated STAT3, c-Myc, and gp130 targeting peptides by linking phosphorothioated polymer backbone to peptides. These cell-penetrating peptides efficiently enter cells and block the activation of the targets and their gene targets [108]. The goal of developing a cell-penetrating antibody or peptide which can effectively treat diseases like cancer and yet be safe has been tried by many groups for decades. We utilized the camelid VHH molecule platform to generate several cell-penetrating nano-antibodies. The SBT-100 nano-antibody is a monomeric VHH which is bi-specific for KRAS and STAT3. Below we describe its properties and efficacy against cancer targets.

Using Surface Plasmon Resonance (SPR), specifically BIAcore binding studies, SBT-100 was found to bind human STAT3 (KD=2.24×10^{-8}), KRAS (4.20×10^{-9}), and mutant KRAS(G12D) ($1.50\text{x}10^{-8}$), the most common mutation of

KRAS. As negative controls, SBT-100 did not bind to HRAS or 12-lipoxygenase. SBT-101 binds STAT3 but not KRAS, and SBT-102 binds KRAS (3.22×10^{-9}) and mutant KRAS(G12D) (1.48×10^{-7}) but not STAT3 [58•]. Furthermore, using co-immunoprecipitation and western blot studies with human and murine cancer cell lysates, SBT-100 was found to bind human and murine STAT3 which have approximately 99% homology at the amino acid level. SBT-100 also was found to bind unphosphorylated and phosphorylated STAT3. This is an important finding because there is an increasing body of literature demonstrating that unphosphorylated STAT3 forms dimers and activates many of the same genes activated by pSTAT3 [57].

The nanomolar binding affinity of SBT-100 and SBT-102 resulted in the inhibition of KRAS GTPase activity in a cell-free assay from Promega. Here the degree of inhibition was comparable to the inhibition by polyclonal antibodies against KRAS. In vitro, human triple-negative breast cancer (TNBC), MDA-MB-231[KRAS(G13D) mutation], and human pancreatic cancer, PANC-1[KRAS(G12D) mutation] were cultured with SBT-100. SBT-100 decreased pERK1/2 in both cell lines by western blot [58•]. In addition, cancer cell growth inhibition as measured using an MTT assay was used to determine SBT-100 anti-cancer potential. Growth of eleven human cancer cell lines TNBC (MDA-MB-231, MDA-MD-468, MDA-MB-453), HER-2 amplified breast cancer (BT474), ER+PR+ breast cancer (MCF-7), glioblastoma (U87), osteosarcoma (SJSA-1), fibrosarcoma (HT-1080), pancreatic cancers (PANC-1 and BxPC3), and metastatic prostate cancer (DU145)) were significantly ($p<0.001$) suppressed within 3 days.

In vivo efficacy of SBT-100 against MDA-MB-231 was established using tumors that had growth in athymic nude mice for approximately 3 weeks and had reached a volume of 80-100 mm^3. As a monotherapy, SBT-100 inhibited the growth of this TNBC with a KRAS(G13D) mutation with a dose of 1 mg/kg/day [57]. Significant suppression of this tumor was seen even with dosing as low as 0.5 mg/kg/day (unpublished data). After a 14-day treatment period, no further administration of SBT-100 was given, yet no increase in tumor volume was noted for another 7 days.

Despite the almost ubiquitous KRAS(G12D) mutation, pancreatic cancer is considered to be a KRAS-independent malignancy [109]. This makes it a more difficult cancer to treat, so here gemcitabine was combined with SBT-100 for in vitro and in vivo testing. Combining SBT-100 with gemcitabine in vitro demonstrated synergism in inhibiting the growth of PANC-1, a human pancreatic cancer cell line. In vivo, this combination gave an additive effect in suppressing tumor growth. Here again, the PANC-1 tumor was grown for approximately three weeks in athymic nude mice until the tumors were between 100-150 mm^3. Compared to the control group, the gemcitabine treatment group had 14.93% tumor suppression, the SBT-100 treatment-only group had 19.17% tumor suppression, and the combination of the gemcitabine and SBT-100 treatment group had 31.52% tumor suppression. This demonstrated that SBT-100 combined with a traditional chemotherapeutic drug could augment its therapeutic effect [58•]

Many older traditional chemotherapeutic drugs that are highly effective in inhibiting cancer growth produce numerous untoward effects such as nausea, vomiting, diarrhea, bone marrow suppression, weight loss, hair loss, organ failure, and death. One such drug is doxorubicin which is used against solid tumors, leukemias, and lymphomas. In a study with human osteosarcoma (SJSA-1), the doxorubicin group had excellent tumor suppression but only 28% of the mice survived the 3-week study. Another group received the same dose of doxorubicin but also received SBT-100, here the same degree of tumor suppression was achieved; however, the survival of the mice treated increased to 71%. This study suggests that the doxorubicin-associated toxicities were killing the mice, and by some unknown mechanism, SBT-100 was protecting the mice from these toxicities. It has been shown that inhibiting STAT3 augments doxorubicin efficacy against lung adenocarcinoma, breast cancer, and melanoma [110–112].

Overcoming Therapeutic Resistance

Many traditional chemotherapeutic drugs such as doxorubicin, gemcitabine, cisplatin, and many others become ineffective in treating cancers because variants of these cancers upregulate STAT3. This pathway then becomes a key driver of the malignancies. Using SBT-100 in combination therapy with traditional chemotherapeutic drugs may reduce the development of such escape variants that are resistant to these chemotherapeutic drugs.

Pathway-targeting drugs such as erlotinib, gefitinib, vemurafenib, lapatinib, and others produce great results in the treatment of cancer patients but ultimately are made ineffective by acquired drug resistance (ADR). It has been shown that many drug-treated "oncogene-addicted" cancers are involved in a positive feedback loop which results in Stat3 activation [113]. This ultimately results in cancer cell survival and limits overall drug effectiveness. This was found in cancer cells driven by diverse activated kinases. These include EGFR, HER2, ALK, MET, and mutant KRAS. In addition, this group showed MEK suppression led to autocrine activation of Stat3 via the FGF receptor and JAK kinases. Blocking MEK pharmacologically along with JAK and FGFR promoted tumor regression. Their data demonstrated that inhibition of a Stat3 feedback loop can increase the response to a broad spectrum of drugs that target pathways of oncogene addiction [113].

It has been shown that JAK/STAT3 controls lipid metabolism, which promotes breast cancer stem cells (BCSCs) and the ADR of cancer [114]. Blocking JAK/STAT3 inhibits BCSC self-renewal and expression of many lipid metabolic genes, which includes carnitine palmitoyltransferase 1B

(CPT1B). This gene encodes the very important enzyme for fatty acid beta-oxidation (FAO). Furthermore, mammary-adipocyte-derived leptin increases STAT3-induced CPT1B expression and FAO function in BCSCs. Data derived from human breast cancer indicates that the STAT3-CPT1B-FAO pathway promotes cancer cell stemness and ADR. Later this same group demonstrated that increased FAO induced by activated STAT3 in CD8+ T effector cells is important for obesity-associated breast tumor progression [115]. It was shown that ablating T lymphocyte Stat3 or treatment with a FAO blocker in obese mice which spontaneously develop breast tumors had reduced FAO. This increased glycolysis and CD8+ T effector cell functions which subsequently resulted in the suppression of breast tumor development.

It has been shown that an inverse relationship exists between MEK and STAT3 in pancreatic tumor cells [116]. Upon MEK inhibition, pSTAT3 was upregulated, and paradoxically, STAT3 inhibition increased the phosphorylation of MEK in tumor cells. This inverse relationship emphasizes the importance of bi-specific blockade of intracellular KRAS and STAT3 pathways, which can be achieved with inhibitors such as SBT-100. By directly forming non-covalent bonds between KRAS and pSTAT3, SBT-100 would block this inverse relationship by inhibiting both pathways at once.

The new KRAS(G12C) inhibitors, sotorasib and adagrasib, are the first two molecules to effectively block the function of this KRAS-activating mutation in cancer patients, primarily in NSCLC and less effectively in CRC [117, 118]. This targeting of the "undruggable" KRAS is a major milestone in cancer therapy. Unfortunately, like other inhibitors of the MAPK pathway, resistance to the sotorasib and adagrasib seems to be developing too. Though unknown at this time, it would be interesting if STAT3 upregulation turns out to be the mechanism of acquired drug resistance to KRAS (G12C) inhibition.

Discussion

The majority of desirable targets for drug development and treatment of diseases are inside the cell, therefore it is essential that therapeutics be developed to target these intracellular molecules such as mutated proteins, transcription factors, and overexpressed proteins. Creating therapeutic antibodies has revolutionized medical treatment but has been limited to treating extracellular targets and cannot reach intracellular targets due to the large size of these antibodies. The development of camelid VHHs has led to the commercialization of two therapeutic VHHs which also target extracellular proteins involved in human diseases. We have utilized the VHH nano-antibody platform to develop cell-penetrating single-domain antibodies that target KRAS and STAT3 to give a therapeutic response in human cancers, ophthalmic diseases, and autoimmune diseases. The benefits of these VHH nano-antibodies include their safety in patients because of their short serum half-life and high homology, greater than 90%, with human VH region of immunoglobulins. No significant toxicity has been associated with these VHHs in clinical trials.

The main nano-antibody discussed in this review is SBT-100 which is a monomeric VHH that is bi-specific for KRAS and STAT3. By binding and inhibiting mutated KRAS and the transcription factor STAT3, SBT-100 has broad efficacy against many human cancers. In addition to penetrating the cell membrane, SBT-100 crosses the BBB rapidly in less than 15 min in vivo. By binding mutated KRAS, SBT-100 inhibits its GTPase activity, reduces downstream P-ERK production, and suppresses cancer cell proliferation. All of this results in human cancer tumor growth inhibition in vivo. SBT-100's binding of STAT3 causes a significant decrease in VEGF and PD-L1 which are transcribed by STAT3. SBT-100 blocks IL-6-mediated STAT3 activation and translocation to the nucleus of cancer cells, and binding of the STAT3 promoter. Furthermore, in an EAU model at the NIH, it was demonstrated that SBT-100 suppresses Th1 and Th17 autoimmune cells and key inflammatory cytokines such as IL-17, IFN-gamma, GM-CSF, and IL-1-alpha. Some of these cytokines and IL-6 play an important role in the TME and help suppress the host immune response against the tumor. Treatment of approximately two hundred mice and rabbits with SBT-100 has resulted in no deaths, no weight loss, and no other signs of toxicity. SBT-100 is a prototype and first-in-class cell penetrating nano-antibody which provides proof of concept that targeting intracellular proteins for a therapeutic effect is possible and safe.

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Writing, review, and revision of the manuscript: SS, SPS.
Administrative, technical, or material support: SS, SPS.
Study supervision: SS, SPS, ASP.

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Declarations

Ethics Approval and Consent to Participate Not applicable

Consent for Publication Consent granted for publication of original figures

Conflict of Interest Sunanda Singh and Ashutosh S. Parihar are both employed by and shareholders of Singh Biotechnology.

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- •• Of major importance

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