


Anticalin[®] Proteins as Therapeutic Agents in Human Diseases

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Published online: 10 May 2018
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Abstract Anticalin proteins are an emerging class of clinical-stage biopharmaceuticals with high potential as an alternative to antibodies. Anticalin molecules are generated by combinatorial design from natural lipocalins, which are abundant plasma proteins in humans, and reveal a simple, compact fold dominated by a central β -barrel, supporting four structurally variable loops that form a binding site. Reshaping of this loop region results in Anticalin proteins that can recognize and tightly bind a wide range of medically relevant targets, from small molecules to peptides and proteins, as validated by X-ray structural analysis. Their robust format allows for modification in several ways, both as fusion proteins and by chemical conjugation, for example, to tune plasma half-life. Antagonistic Anticalin therapeutics have been developed for systemic administration (e.g., PRS-080: anti-hepcidin) or pulmonary delivery (e.g. PRS-060/AZD1402: anti-interleukin [IL]-4-R α). Moreover, Anticalin proteins allow molecular formatting as bi- and even multispecific fusion proteins, especially in combination with antibodies that provide a second specificity. For example, PRS-343, which has recently entered clinical-stage development, combines an agonistic Anticalin targeting the costimulatory receptor 4-1BB with an antibody directed against the cancer antigen human epidermal growth factor receptor 2

(HER2), thus offering a novel treatment option in immunoncology.

Key Points

Anticalin therapeutics offer a promising alternative to antibodies, which currently dominate biopharmaceutical drug development but have technical limitations.

Anticalin proteins are derived from natural human lipocalins and provide several benefits such as small size, robust and compact fold, pronounced target specificity, and the potential to construct fusion proteins with high formatting flexibility.

Anticalin-based biopharmaceuticals have demonstrated safety and tolerability in early clinical studies and offer both new treatment options in immunoncology and innovative routes of delivery such as inhalation.

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1 Reprogramming Natural Lipocalins for Medically Relevant Target-Binding Activities

The lipocalins constitute a protein family with many representatives in diverse phyla of life, including mammals, insects, plants and even bacteria [1]. Their members typically represent secretory monomeric proteins that share a common fold dominated by a β -barrel, which is

complemented by an α -helix that leans against its side. At one end, the β -barrel is closed by three densely packed loops and a hydrophobic core of predominantly hydrophobic side chains; at the other end, it is open to solvent. There, four loops form a binding pocket of variable size and shape [2].

The role of natural lipocalins mostly lies in the complexation of small molecules for various physiological purposes [3]. For example, retinol-binding protein (RBP) transports the poorly soluble and chemically sensitive vitamin A from its storage site in the liver through the bloodstream to various tissues. Similarly, apolipoprotein D—in plasma associated with high-density lipoprotein (HDL) particles—is involved in the transport of progesterone and arachidonic acid. Other lipocalins have scavenger functions, such as the neutrophil gelatinase-associated lipocalin [NGAL], which tightly binds certain bacterial siderophores and thus restricts iron supply to invading microbes [4]. In insects and other invertebrates, lipocalins often serve for coloration by complexing pigments, for example the bilin-binding protein (BBP) of the European butterfly *Pieris brassicae* [5]. Interestingly, blood-sucking ticks are a rich source of lipocalins that interfere with the host's innate immune system, for example by blocking complement [6] or by scavenging histamine [7] and/or releasing nitric oxide [8].

Of note, the ligand-binding activities of natural lipocalins are strictly conserved in each organism. While their predominant specificities for small molecules may be seen as complementary to the biological role of immunoglobulins, which mainly recognize protein antigens, the fixed genetic status of lipocalins distinguishes these proteins from the somatically diversified antibody repertoire that is subject to continuous generation by the immune system [9]. In the human body, 12–15 different lipocalin isotypes with distinct ligand specificities or physiological functions have been characterized [3], many of them abundant plasma proteins (much like the immunoglobulins).

Initial application of protein engineering to the lipocalins was motivated by the discovery of some structural similarity between their binding sites and those of antibodies (immunoglobulins), which comprise four variable loops in the former and six complementarity-determining regions (CDRs; “hypervariable loops”) in the latter [2, 9]. Based on this notion and utilizing the methodology of combinatorial genetic engineering that emerged during the 1990s, we explored the possibility of reshaping the binding pockets of natural lipocalins to create novel ligand-binding functions [10, 11], leading to so-called “Anticalin” proteins. Early attempts started with the BBP [10], for which a high-resolution X-ray crystallographic analysis was available at the time [5].

Employing targeted random mutagenesis of the binding site, encompassing the region of the four structurally variable loops, in combination with phage display selection and enzyme-linked immunosorbent assay (ELISA) screening enabled successful identification of BBP variants with novel specificities against several small molecules, including fluorescein and digitalis. These studies provided proof of concept that the lipocalin scaffold is suitable for generation of novel ligand specificities while maintaining a stable protein fold (for review, see Skerra [11] and Schlehuber and Skerra [12]).

With the goal of making Anticalin technology amenable to broader application in medical therapy and in vivo diagnostics, we subsequently recruited human lipocalin scaffolds, thereby reducing the risk of immunogenicity in human patients upon repeated dosing. Based on systematic biochemical and structural characterization of all known human lipocalins [3, 13], two members appeared particularly suitable for the generation of human Anticalin libraries (Fig. 1): tear lipocalin (Tlc) [14, 15], also known as lipocalin 1 (Lcn1), and the neutrophil-gelatinase associated lipocalin (NGAL), also known as lipocalin 2 (Lcn2) or siderocalin [3, 4].

Both human lipocalins were successfully employed for the preparation of advanced phage display libraries using procedures similar to those previously developed for the BBP. Furthermore, trinucleotide-based DNA synthesis was applied to allow better control of amino acid composition (and avoid stop codons) at the randomized positions [16, 17]. Using this methodology, a series of Anticalin proteins has been developed over recent years, providing smart high-affinity protein reagents directed against various targets of biomedical relevance, both for basic research and for therapeutic application.

For example, the first NGAL-derived Anticalin protein was selected against human cytotoxic T-lymphocyte-

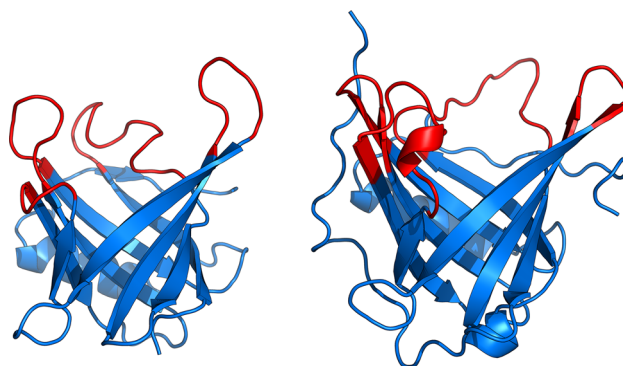


Fig. 1 Structural plasticity of the loop-based binding sites of natural human lipocalins: tear lipocalin (Tlc; Lcn1) and neutrophil-gelatinase associated lipocalin (NGAL; Lcn2), two validated scaffolds for the selection of Anticalin proteins (PDB IDs 1XKI and 1L6M, respectively; graphics prepared with PyMOL)

associated antigen 4 (CTLA-4, cluster of differentiation [CD]-152) [18], the prototypic checkpoint receptor for T cell activation [19]. After affinity maturation, the final candidate showed high affinity towards the ectodomain of the human membrane protein ($K_D = 240$ pM) as well as pronounced cross-reactivity towards murine CTLA-4. In a mouse model of *Leishmania* infection, treatment with the PEGylated Anticalin protein led to significant lowering of parasite burden in the liver and spleen, which is indicative of an enhanced cellular immune response due to efficient checkpoint receptor blockade.

After systematic optimization of the NGAL random library design [16, 20], Anticalin proteins were selected against a range of targets, e.g., lanthanide chelate complexes, including medically relevant radioisotopes [21], the extra domain B (ED-B) of oncofetal fibronectin [16, 22], prostate-specific membrane antigen (PSMA) [23], vascular endothelial growth factor (VEGF) receptor 3 (VEGFR-3) [24], the tumor cell membrane-exposed form of heat shock protein 70 kDa (Hsp70) [25], and the Alzheimer amyloid β -peptide ($A\beta$) [26].

In parallel, the toolbox for selection and screening of lipocalin variants with novel ligand specificities was expanded [17]. Beyond phagemid panning and colony-screening procedures, which were instrumental in the early days of Anticalin technology, automated high-throughput microculture screening in combination with ELISA have facilitated the quick identification of lead candidates exhibiting the desired target-binding profile. More recently, a technique was developed for the display of engineered lipocalins in an active state on the surface of live *Escherichia coli* cells via fusion with a bacterial autotransporter protein [27]. The bacterial Anticalin libraries generated in this way resemble a naïve immune B cell repertoire with clonal membrane-anchored antibodies, thus allowing the use of fluorescence-activated cell sorting (FACS) for the rapid and automated enrichment of bacterial cells that express mutant lipocalins with binding activity towards a fluorescently labeled antigen [25].

Further to these scientifically motivated studies, several disease-related Anticalin programs were launched with the goal of medical applications, predominantly in the areas of oncology and inflammation (Table 1). Anticalin-based biopharmaceuticals have shown promising results in various preclinical programs as well as early clinical studies for several indications. So far, five Anticalin therapeutic candidates have reached the clinical development stage and will be discussed in the following sections.

2 Anti-VEGF-A Angiocal®: The First Anticalin Protein to Enter Clinical Study

The first Tlc-derived Anticalin protein with prospects for therapeutic application was selected against human vascular endothelial growth factor (VEGF)-A, a key factor of tumor angiogenesis and ocular diseases [28]. This Anticalin protein (PRS-050) tightly binds VEGF-A, with a very low K_D value of ~ 20 pM, and effectively prevents receptor binding and activation [29]. Intravitreal administration of the Anticalin protein in a rabbit model suppressed VEGF-induced blood–retinal barrier breakdown. To enable prolonged systemic neutralization of VEGF-A in vivo, the plasma half-life of the engineered lipocalin was extended by site-directed PEGylation. PEGylated PRS-050 efficiently blocked VEGF-mediated vascular permeability and growth of tumor xenografts in nude mice, which was accompanied by reductions in microvessel density.

PEGylated PRS-050 (“Angiocal”) was subjected to a first-in-human, dose-escalation phase I study in patients with advanced solid tumors [30, 31]. PRS-050 was well-tolerated when administered as a 2-h infusion at doses up to 10 mg/kg. No signs of toxicity or immunogenicity were observed, based on the absence of anti-drug antibody (ADA) in 24 patients (except for one patient [31]), including samples from six patients who had received biweekly dosing.

Table 1 Anticalin programs in various indications that have passed early clinical development

| Program | Target | Indication | Partner | Status |
|----------|-----------------------|-----------------|-------------------------------|---|
| PRS-050 | VEGF-A | Solid tumor | – | Phase I completed |
| DS-9001a | PCSK9 | Dyslipidemia | (Daiichi Sankyo) ^a | Phase I completed |
| PRS-080 | Hepcidin | Anemia | ASKA Pharmaceutical | Phase Ia/Ib completed Phase IIa launched |
| PRS-060 | IL4-R α | Asthma | AstraZeneca | Phase Ia launched |
| PRS-343 | 4-1BB/HER2 bispecific | Immuno-oncology | – | Phase Ia launched |

HER2 human epidermal growth factor receptor 2, *IL* interleukin, *PCSK9* proprotein convertase subtilisin/kexin type 9, *VEGF* vascular endothelial growth factor

^aDaiichi Sankyo discontinued development for strategic reasons and returned this Anticalin program to Pieris

Free VEGF-A was undetectable after PRS-050 dosing at levels of ≥ 0.5 mg/kg and remained so over the 3-week observation period in most patients, thus suggesting target saturation. Notably, significant reductions in circulating matrix metalloproteinase 2 (MMP-2) levels indicated an anti-angiogenic effect. Pharmacokinetic/pharmacodynamic (PK/PD) modelling, based on a measured terminal half-life of 6 days, recommended a biweekly dosing regimen for a phase II study, comparable to dosing schedules common for antibodies [32].

3 An Anti-PCSK9 Anticalin Fusion Protein for the Treatment of Metabolic Diseases

An Anticalin protein that targets proprotein convertase subtilisin/kexin type 9 (PCSK9) showed convincing pre-clinical activity in the treatment of dyslipidemia [33]. PCSK9 binds to the low-density lipoprotein receptor (LDL-R), inducing its internalization and degradation. An anti-PCSK9 antibody blocking the binding of PCSK9 to LDL-R was demonstrated to lower plasma levels of LDL cholesterol (LDL-C) and, consequently, led to fewer cardiovascular events in patients with atherosclerotic cardiovascular disease receiving standard statin therapy [34].

A corresponding Anticalin fusion protein with an albumin-binding domain (ABD), DS-9001a, also blocks PCSK9 binding to LDL-R and thus inhibits PCSK9-mediated LDL-R degradation. In cynomolgus monkeys, a single administration of DS-9001a significantly reduced serum LDL-C levels for about 3 weeks. Furthermore, administration of DS-9001a had synergistic effects with conventional statin application in murine *in vivo* models.

The genetic fusion of the anti-PCSK9 Anticalin protein to an ABD [17] significantly increased its inherent plasma half-life, as shown in rats, allowing convenient dosing in humans. The beneficial features of the small lipocalin scaffold were maintained, such as lower cost of goods by production in a microbial expression system, which is of particular interest for drugs intended for chronic treatment. Furthermore, the small size and high solubility of the Anticalin-based biopharmaceutical offers the possibility of improving treatment convenience by applying a high drug concentration in a low injection volume.

DS-9001a was investigated in a single ascending-dose phase I study in healthy volunteers. Doses up to 450 mg were injected subcutaneously in a randomized, placebo-controlled setting; hence, a favorable safety and tolerability profile was demonstrated for DS-9001a, whereas *in silico* screening indicated low immunogenic potential [35]. Importantly, PK/PD analysis revealed a dose-dependent decrease of free PCSK9 as well as LDL-C levels.

4 A Hepcidin-Targeting Anticalin Fusion Protein for the Treatment of Anemia

PRS-080 is another Anticalin protein with prolonged circulation, this time via site-specific PEGylation, which targets hepcidin (hepatic bactericidal protein) alias liver-expressed antimicrobial peptide 1 (LEAP1). Hepcidin plays a major role in the regulation of iron metabolism [36], particularly in patients with functional iron deficiency (FID) anemia [37]. FID is a major cause of anemia of chronic disease (ACD), which particularly develops in patients with infections, inflammatory diseases, cancer, or chronic kidney disease (CKD). Routine use of erythropoietin-stimulating agents (ESAs) and high-dose intravenous iron supplementation corrects the condition in most patients; however, ESAs may result in a number of adverse clinical outcomes, and concerns about the long-term safety of intravenous iron administration have been raised [38, 39].

Hepcidin binds to ferroportin, the only iron transporter on the surface of absorptive enterocytes, macrophages, hepatocytes, and placental cells, and causes its internalization and degradation [40], thereby blocking iron export from the body's depositories and reducing its availability for erythropoiesis [37]. Consequently, antagonizing hepcidin has the potential to improve iron availability and erythropoiesis while avoiding overload with exogenous iron and, thus, may allow lower dosing of ESAs [41].

Based on findings from PK/PD modelling, the hepcidin-targeting Anticalin protein was conjugated with a 30-kDa PEG polymer to achieve a suitable plasma half-life. Considering both the *in vivo* synthesis rate of the target and the therapeutically allowable upper threshold concentration of hepcidin in the bloodstream, this pharmacokinetic optimization proved particularly important for such an Anticalin protein with scavenger function. In fact, when the Anticalin protein was coupled with PEG chains of different lengths (20, 30, and 40 kDa), a depot-like accumulation of the hepcidin–Anticalin complex was observed in cases of branched 40-kDa PEG, whereas a low steady state concentration of the complex was achieved when using the shorter and linear 30-kDa polymer. Hence, the PRS-080 biopharmaceutical development program demonstrates the benefit of tunable PK.

Data from a phase I study in healthy volunteers indicated that a single intravenous infusion of PRS-080 up to a dose of 16 mg/kg body weight was safe and well tolerated. This beneficial safety profile was confirmed in a subsequent single-administration, ascending-dose phase Ib study (up to 8 mg/kg body weight) in patients with end-stage CKD requiring hemodialysis. Notably, administration of this Anticalin protein resulted in a significant decrease in free

hepcidin concentration within 1 h after infusion, followed by robust mobilization of iron, with dose-proportional increases in both level and duration of serum iron concentration, as well as subsequent transferrin saturation [42]. Based on the encouraging data from this clinical trial, a phase IIa study was initiated to evaluate the safety and PK/PD of repeated PRS-080 administration in anemic CKD under hemodialysis (NCT03325621, ClinicalTrials.gov).

5 An IL-4-R α -Targeting Anticalin Protein in Respiratory Disease

Asthma is one of the most common respiratory diseases, with 5–10% of patients having moderate to severe conditions, which often remains uncontrolled despite high-dose inhaled corticosteroids (ICS) in combination with a long-acting beta-2 agonist (LABA)—as a second controller—and/or systemic corticosteroids [43]. A large proportion of patients with asthma have a disorder in the T helper type 2 (T_H2) pathway where the cytokines interleukin (IL)-5, IL-4, and IL-13 play an important role. Antibodies such as mepolizumab, reslizumab, or benralizumab, which target IL-5 or its receptor IL-5R α , are already approved for use in patients with severe eosinophilic asthma [44].

Notably, the cytokines IL-4 and IL-13 both signal via the IL-4 receptor α (IL-4R α) subunit, which renders IL-4R α a cornerstone of intervention. Indeed, clinical trials with biologics targeting IL-13 alone, including lebrikizumab and tralokinumab, did not yield convincing results [45]. Only dupilumab, which simultaneously targets both IL-4 and IL-13 by blocking IL-4R α , showed promising effects [46]. Dupilumab has recently completed phase III studies in severe asthma, and approval for this indication is expected in the next 12–18 months. However, a general drawback may be that all these antibodies are administered systemically via the intravenous or subcutaneous route.

Inhaled biologics, on the other hand, could offer several potential advantages. Beside much better convenience for patients, local delivery to the lung might require a significantly lower dose, which could result both in a cost of goods advantage and an expanded patient population. Lower systemic target engagement, with the potential for better tolerability, could offer further benefits. A potent IL-4R α -targeting Anticalin protein, PRS-060 alias AZD1402, is currently being developed jointly by Pieris and AstraZeneca as an inhalable biologic for the treatment of moderate to severe asthma that is not well controlled by standard of care.

In a cell-based functional assay, PRS-060/AZD1402 demonstrated in vitro potency comparable to that of dupilumab. Moreover, this Anticalin protein showed significantly higher in vitro potency than pitrakinra, an IL-4

mucin that also antagonizes IL-4R α and was in clinical testing via the inhalation route. Notably, PRS-060/AZD1402 has demonstrated proof of concept as well as feasibility for pulmonary delivery in a disease model of humanized mice that only express the human version of the receptor and its respective cytokines. PRS-060/AZD1402 can be nebulized in high yield and without aggregation using approved devices [47], thus enabling inhaled delivery directly to the lungs. This Anticalin drug candidate is currently subject to clinical testing in a first-in-human phase I study in healthy volunteers (NCT03384290, ClinicalTrials.gov).

6 Anticalin Fusion Proteins as Novel Multispecific Agents in Immuno-Oncology

Multispecific Anticalin-based fusion proteins can be used to pursue innovative therapeutic strategies in immuno-oncology, particularly by addressing the “immunological synapse” that can form upon contact between an immune cell and a cancer cell at their interface. This may enhance activation of tumor-specific T cells near the tumor site, thereby avoiding some of the toxicities usually observed with peripheral T-cell activation in healthy tissues. In recent years, immuno-oncological approaches have significantly changed cancer therapy, and several corresponding biopharmaceuticals are currently being evaluated for the treatment of a broad range of human tumors. Monoclonal antibodies targeting inhibitory checkpoint molecules such as CTLA-4 and programmed cell death protein 1 (PD-1) were the first to show promising therapeutic outcomes with durable response or cure in different cancer types, demonstrating immunotherapy as a viable approach [48–50]. Despite these encouraging results, many patients have shown only minimal benefit, thus providing strong motivation for alternative treatment strategies, particularly in the light of a growing number of known costimulatory receptors, such as 4-1BB, CD27, CD40, Ox40, or GITR [51].

Among those, 4-1BB (CD137) is a member of the tumor necrosis factor receptor super-family (TNFRS) and offers a compelling therapeutic target as it plays a central role in the regulation of the immune response. 4-1BB is mainly expressed on activated CD8⁺ and CD4⁺ T cells, activated B cells, and natural killer (NK) cells, whereas its ligand, 4-1BBL, is constitutively expressed on antigen-presenting cells (APCs) [52, 53]. Data from in vivo murine models [54] and human ex vivo assays [55] as well as results from adoptive T-cell therapy [56] have demonstrated the benefit of 4-1BB co-stimulation for the elimination of tumors. Activation of 4-1BB requires higher-order cell surface receptor clustering, which occurs under physiological

conditions by binding of the trimeric 4-1BBL on APCs. Of note, soluble trimeric 4-1BBL cannot trigger activation of 4-1BB. This sophisticated mode of action may explain why the success of monoclonal antibodies targeting 4-1BB that are currently in clinical development remains limited [52].

Biotherapeutics addressing this costimulatory pathway must effectively activate the 4-1BB receptor, but their activation should be restricted to the tumor and its microenvironment (TME) to reduce systemic effects and unwanted toxicity. PRS-343 is an Anticalin–antibody fusion protein that targets both 4-1BB and the well-known breast cancer antigen human epidermal growth factor receptor 2 (HER2) at the same time. PRS-343 was designed to activate 4-1BB on T cells by clustering the receptor at the tumor site but not in the periphery, thus enhancing the T-cell receptor-mediated activity and effecting tumor destruction (Fig. 2).

The two building blocks of PRS-343 are an Anticalin protein selected against 4-1BB, which binds in a ligand-independent manner, and an Fc-silenced version of the HER2-targeting antibody trastuzumab [57, 58]. The formatting flexibility offered by the Anticalin technology enabled the generation of various Anticalin–antibody fusion formats as depicted in Fig. 3. The Anticalin protein was genetically fused to either the N- or the C-terminus of the antibody heavy or light chain, thereby resulting in different geometries of the fusion protein, wherein the antibody and Anticalin binding sites cover a range of distances concerning the T-cell target on the one hand and the tumor antigen on the other.

All four formats of the bispecific fusion protein bound to 4-1BB and HER2, respectively, with nearly identical affinities compared to the parental building blocks and were capable of binding both targets simultaneously [59]. Furthermore, all constructs showed comparable beneficial biophysical properties and PK profiles in animals. Notably, however, HER2-dependent agonistic engagement of 4-1BB in ex vivo T cell activation assays was found to depend on the geometry of the bispecific protein. The genetic fusion of the Anticalin protein to the C-terminus of the heavy chain of modified trastuzumab (PRS-343) appeared to be more potent than the other three formats.

This indicates that the geometry of the bispecific construct and particularly the distance between the two distinct binding sites are key for optimal tumor-localized activation of co-stimulatory receptors [59]. Furthermore, this demonstrates the value of Anticalin technology for the generation of innovative bi- and multispecific biopharmaceuticals. Several ex vivo assays and murine in vivo tumor models have confirmed the local activation of T cells in a tumor-specific manner and the lack of systemic 4-1BB activation, therefore conferring a manageable risk profile.

Thus, PRS-343 has the potential to offer a therapeutic alternative for patients with HER2-positive malignancies, including breast, bladder, and gastric tumors. PRS-343 has entered clinical development as a first-in-class bispecific Anticalin–antibody fusion protein functioning as a tumor-targeted immune-costimulatory 4-1BB agonist. The recently launched phase I trial is designed to determine the safety, tolerability, and potential anti-cancer activity of

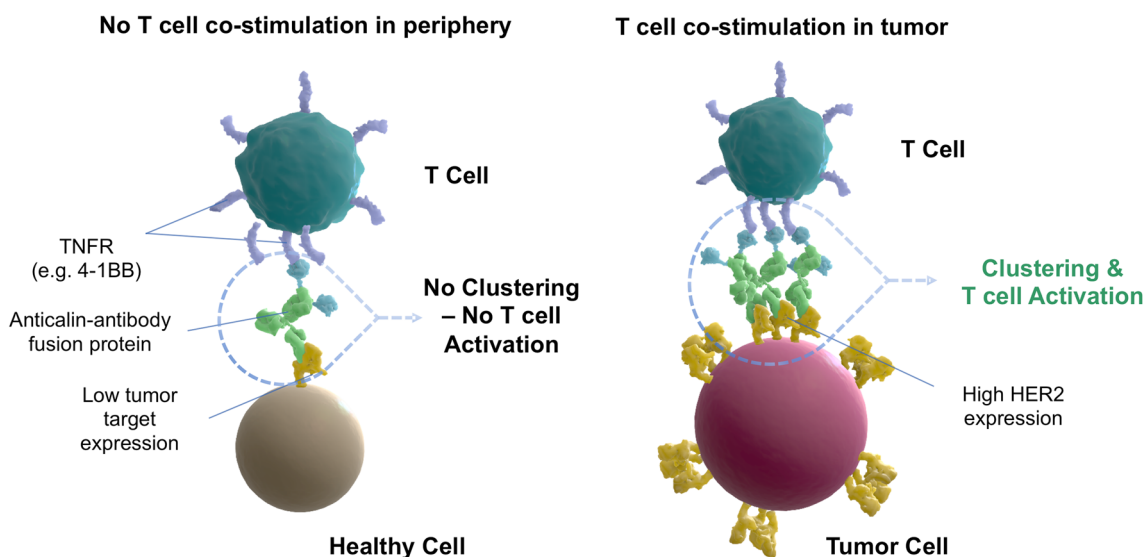
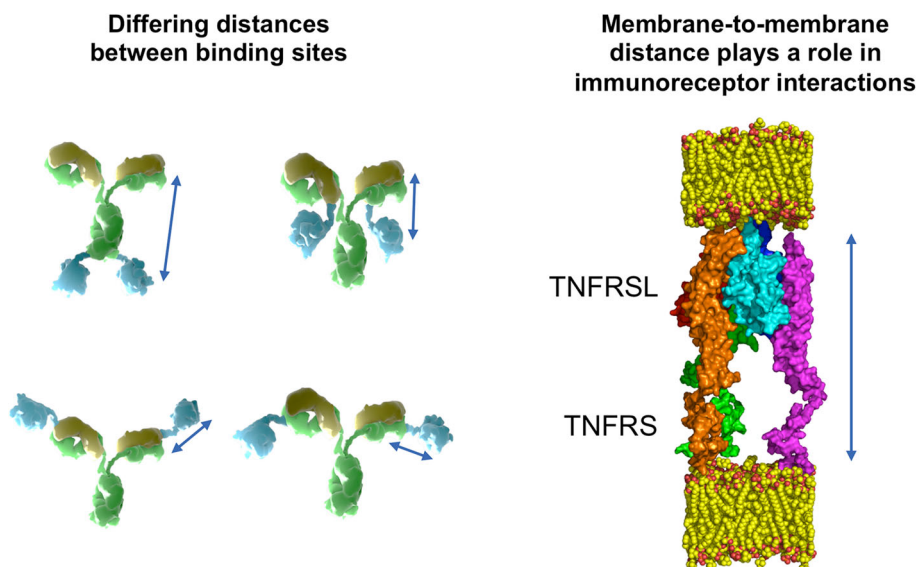


Fig. 2 The concept of costimulatory T cell engagement. Following binding of the Anticalin–antibody fusion protein to the tumor cell and interaction with a T cell in its vicinity, the clustering of the costimulatory tumor necrosis factor receptor (TNFR) 4-1BB provides a local co-activation signal to the latter, thus further enhancing its T

cell receptor-mediated activity and leading to tumor destruction. As T cells in the periphery should not get activated, toxic side effects are expected to be manageable. *HER2* human epidermal growth factor receptor 2

Fig. 3 Anticalin technology offers a broad molecular formatting flexibility as the distance between antibody and Anticalin binding sites varies between types of fusion proteins. Testing various geometries provides the possibility of matching the distance of the antibody/ Anticalin binding sites with the distance between the binding partners in the immunological synapse, here shown for a member of the costimulatory tumor necrosis factor receptor superfamily (TNFRS) and its respective ligand (TNFRSL)



PRS-343 in patients with advanced or metastatic HER2-positive solid tumors for which standard treatment options are unavailable, or no longer effective or tolerated, or where the patient has refused standard therapy (NCT03330561, ClinicalTrials.gov).

The concept of tumor-localized activation of co-stimulatory checkpoint proteins can easily be exploited further by (a) combining the 4-1BB agonistic Anticalin protein with other tumor-targeting agents or (b) combining Anticalin proteins directed against other co-stimulatory targets with tumor-targeting Anticalin proteins or antibodies. One such example is PRS-342, a genetic fusion of the 4-1BB-targeting Anticalin with a glypican 3 (GPC-3)-targeting Anticalin protein and a silenced Ig Fc moiety. GPC-3 is an oncofetal antigen with almost no expression in normal adult tissue but increased expression in multiple cancers, including hepatocellular carcinoma, Merkel cell carcinoma, and melanoma [60, 61].

7 Prospects for Anticalin® Technology

Anticalin technology offers a mature toolset for the selection of modified human lipocalins with specificities for prescribed targets of interest, ranging from small molecules over peptides to proteins [20]. The affinities of the resulting Anticalin proteins are competitive for or even better (particularly for low-molecular-weight ligands) than those accessible by antibody technology and range from typically low nanomolar—after initial selection from a naïve random library—to mid-picomolar, usually after affinity maturation via partial mutagenesis and selection under conditions of increasing stringency [17]. Apart from ligand affinity and specificity, Anticalin proteins can be tailored for other

beneficial biochemical or biophysical properties such as solubility and folding stability. A crucial advantage of Anticalin proteins for medical applications is their low immunogenic potential, as they are derived from soluble human lipocalins that are abundant in plasma or other body fluids and only deviate by a limited number of amino acid exchanges from their natural counterparts.

As outlined above, several Anticalin therapeutics have entered early clinical development and have so far been well tolerated with no signs of overt immunogenicity after repeated administration. However, generally all therapeutic proteins carry a risk of inducing an immune response, including even fully human proteins such as insulin or coagulation factors [62] and humanized or so-called human antibodies [63]. Notably, Anticalin proteins differ in fewer positions (typically around 20 mutated residues) from the endogenous lipocalin scaffold compared with the set of grafted foreign CDR residues (approximately 60) in a typical humanized antibody [64].

Causes for immunogenicity are diverse and not yet fully understood. Potential T or B cell epitopes within the amino acid sequence or posttranslational modifications at the primary structural level, physical aggregates or sub-visible particles may elicit ADA, and even extrinsic factors such as the administration route or patient characteristics play a role [65, 66]. Nevertheless, there is a gap between the prediction of immunogenicity and the clinical outcome [67]; in fact, even regulatory authorities concede that drug immunogenicity can only be fully assessed after late-stage clinical testing and/or market approval [68].

In silico analysis tools to predict potential T cell epitopes, although known to overestimate immunogenicity risk, combined with in vitro immunogenicity assays are commonly used to rank protein candidates during drug

development. In the context of Anticalin technology, bioinformatics tools to screen for potential T cell epitopes and posttranslational modification sites in the Anticalin sequence are routinely applied and complemented by in vitro immune cell assays as well as biophysical characterization to select Anticalin candidates with a low predicted risk for immunogenicity. Indeed, several Anticalin therapeutics that have entered early clinical development were so far well tolerated.

Anticalin proteins are small biomolecules with a robust structure, which makes them an ideal scaffold for development as inhaled drugs for the treatment of respiratory diseases [47], among other applications. By using well-established half-life extension methods (e.g., PEGylation or fusion with an ABD), the inherently short in vivo circulation of Anticalin proteins can be prolonged to meet pharmacological requirements and even extended to a half-life approaching that of antibodies, if necessary [32].

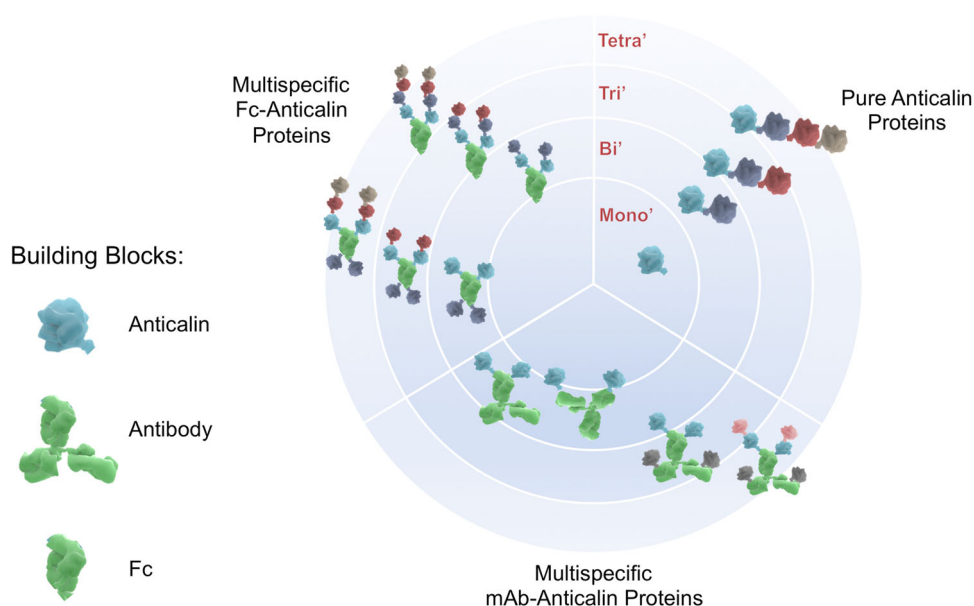
The molecular architecture of Anticalin proteins, comprising a single polypeptide chain that folds into a stable eight-stranded β -barrel with exposed N- and C-termini, neither of which are part of the binding site, makes them ideal building blocks to generate bi- and even multispecific fusion proteins, thus offering novel therapeutic modalities. In this manner, Anticalin proteins can be genetically fused with other proteins, including Anticalin proteins of the same or different specificity [69], thereby generating multivalent, multiparatopic and multispecific proteins (Fig. 4).

For example, the possibility of fusing one or more Anticalin proteins to the Ig Fc portion was exploited to implement Fc effector functions or to increase plasma

half-life. Beyond that, Anticalin proteins can even be fused to entire antibodies, thereby adding another target specificity to existing therapeutic antibodies and thus creating novel modes of action, as exemplified by PRS-343. Further combinations of Anticalin-based fusion proteins are conceivable, addressing diverse tumor antigens and co-stimulatory or inhibitory checkpoint targets, thus offering novel treatment options. The formatting flexibility of Anticalin proteins enables adaption of the geometry of the binding sites in such bi- and multispecific biopharmaceuticals to optimally fit the respective target combination in immuno-oncology. In addition, the ability to develop Anticalin therapeutics as inhaled biologics should provide considerable benefits for patients with respiratory diseases.

In conclusion, Anticalin proteins offer differentiation potential compared with standard-of-care therapies, including antibodies, in several therapeutic areas and have shown promising results in both preclinical and early clinical development in various indications. The nature of lipocalins as endogenous human plasma proteins provides a unique benefit and makes Anticalin proteins attractive, also in comparison with other potential protein drugs based on non-immunoglobulin scaffolds (such as Adnectins, Affibodies, or DARPin; for a recent review see Gebauer and Skerra [70]). Importantly, multiple Anticalin-based biopharmaceuticals have proven safety and tolerability in early clinical development. In summary, Anticalin proteins provide a novel class of protein drugs that may complement and even surpass conventional antibodies in many areas.

Fig. 4 Formatting opportunities for Anticalin proteins. Anticalin therapeutics can be developed as stand-alone small biologics but can also be used as building blocks to generate multispecific fusion proteins. Multispecific Anticalin-based biologics are accessible by mutually fusing several Anticalin proteins or by fusing Anticalin proteins to antibodies or an Ig Fc part. *mAb* monoclonal antibody



Compliance with Ethical Standards

Funding No sources of funding were used to conduct this study or prepare this manuscript. Open access publication of this article was funded by Pieris Pharmaceuticals GmbH.

Conflicts of interest Arne Skerra is founder and shareholder of Pieris Pharmaceuticals. Christine Rothe is an employee of and holds ownership interest in Pieris Pharmaceuticals. The authors have no other conflicts of interest that are directly relevant to the content of this study.

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