



Anticalins: a Novel Class of Therapeutic Binding Proteins

Anticalins, engineered human lipocalin proteins, provide a novel class of biopharmaceutical drug candidates with similar binding and recognition properties as antibodies, while offering several fundamental advantages.

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Arne Skerra (Dipl.-Ing., Dr. rer. nat.) is Founder of Pieris, Member of its Supervisory Board, and Professor at the Technische Universität München, where he heads the Institute of Biological Chemistry. He holds a Diploma degree in chemistry from the Technische Universität Darmstadt, and a PhD in Biochemistry from the Ludwig-Maximilians-Universität München. After post-doctoral studies at the MRC Laboratory of Molecular Biology in Cambridge, UK, and a group leader position at the Max-Planck-Institute for Biophysics in Frankfurt am Main, he became Associate Professor for Protein Chemistry at the TU Darmstadt. Dr Skerra started his career in the field of protein engineering, where together with Andreas Plückthun he led the biosynthesis of functional antibody fragments in *E. coli*. Since then, he has made several other important contributions to this area, including the development of the *Strep*-tag technology for the standardised purification of recombinant proteins; he is also a named inventor on numerous patents. Dr Skerra has received several fellowships and awards. Lately, his interest has focused on the structure-function analysis and functional engineering of lipocalin proteins, which laid the basis for the 'Anticalin' technology.

Human lipocalin proteins are an abundant family of natural ligand-binding and transport proteins; they exhibit four structurally hypervariable loops that form a ligand pocket, similarly to the six CDRs (complementarily determining regions) of antibodies. Using this scaffold in conjunction with targeted random mutagenesis and selection, novel binding proteins can be engineered for the specific and tight complexation of low molecular weight compounds or protein antigens – in particular, medically relevant cell surface targets. Based on recent in vitro and in vivo data, such 'Anticalins' offer three mechanisms for application in human therapy: (i) as antidotes, by quickly removing toxic or otherwise irritating compounds from the body; (ii) as antagonists, for example by binding to cellular receptors and blocking them from interaction with natural signalling molecules or vice versa; and (iii) as tissue-targeting vehicles, by addressing toxic molecules, radionuclides or enzymes to disease-related cell surface receptors. Compared with antibodies or their engineered fragments, Anticalins are much smaller, consist of a single polypeptide chain, do not require disulphide bonds and can be easily produced in microbial host cells.

Antibodies have served as universal biochemical tools for molecular recognition in biological research and

medicine for more than a century, and now constitute an accepted class of biopharmaceuticals. Currently 20% of all biotechnology drugs under development are monoclonal antibodies with a projected market of US\$30-40 billion by 2010. Humanised, or fully human monoclonal antibodies, have become particularly successful due to their high target specificity, therapeutic efficacy, safety and – consequently – low failure rate during preclinical and clinical development.

Nevertheless, several practical disadvantages persist including the high cost of manufacture, limitations in formulation and delivery, immunological side effects due to the intrinsic effector region and long circulation time, and also economic issues in conjunction with royalty stacking. Consequently, alternative protein scaffolds have attracted attention in order to address areas of significant medical need. The growing opportunity and demand for 'next generation' technologies has been demonstrated by the high valuation acquisitions seen during the last year, notably Avidia by Amgen and Domantis by GSK. At Pieris, our mission is to discover and develop Anticalins as a novel class of targeted human proteins for the diagnosis and treatment of life-threatening diseases.

Figure 1: Natural lipocalins and their use as scaffolds to engineer novel ligand binding sites.

The figure shows ribbon representation of four human lipocalins: retinol-binding protein (RBP, PDB entry 1RBP), neutrophil gelatinase-associated lipocalin (NGAL, PDB entry 1L6M), tear lipocalin (Tlc, PDB entry 1XKI) and apolipoprotein D (ApoD, PDB entry 2 HZO). Lipocalins share a conserved β -barrel of eight antiparallel β -strands (blue). The four exposed loops at its open end (red), which form the natural ligand-binding sites, exhibit high structural variability – as illustrated by a structural superposition shown to the right – and can be reshaped to generate Anticalins with novel target specificities.

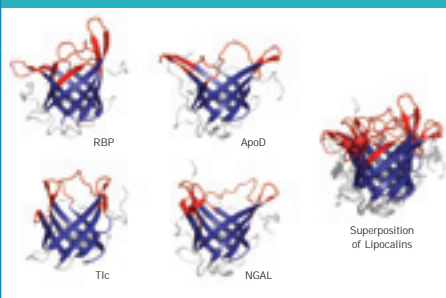
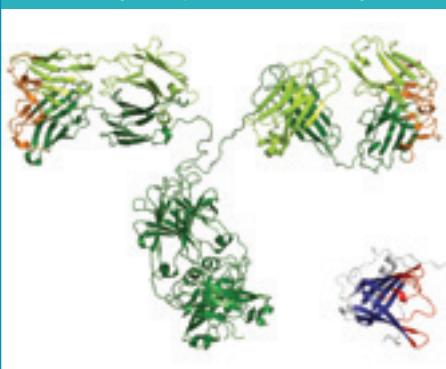


Figure 2: Comparison between an antibody (left) and a lipocalin (lower right).

Light and heavy chains of the Ig (PDB entry 1IGT) are shown in lighter and darker green, respectively, whereas hypervariable loops are coloured orange. The Lipocalin is coloured as in Figure 1.



conserved immunoglobulin (Ig) framework supports the so-called hypervariable loop region, which gives rise to the huge variety of possible antigen specificities (2). However, antibodies have a large molecular size and complex architecture, which relies on four subunits (two light and two heavy chains). In contrast, lipocalins are composed of a single polypeptide chain, comprising merely 160 to 180 residues, and their binding site is composed of four loops instead of six (see Figure 2). Consequently, they offer a small and robust scaffold for the engineering of novel binding proteins using the methods of combinatorial biochemistry.

Human lipocalins mostly occur as soluble proteins in the plasma and other tissue fluids, with concentrations of up to 1.0 mg/ml. At least some of them are freely distributed in the body, where they essentially exert a ligand buffer or transport function. This predestines this family of proteins as carrier vehicles or scavengers for

FROM NATURAL LIPOCALINS TO A SCAFFOLD FOR NOVEL LIGAND-BINDING PROTEINS

All lipocalins share a structurally conserved β -barrel as their central folding motif, which is composed of eight antiparallel β -strands that wind around a central axis (see Figure 1). At its open end, the cylindrical structure supports four loops, which form the entrance to the ligand pocket. The opposite end of the cup-shaped β -barrel is closed by short loops, and densely-packed amino acid side chains form a hydrophobic core region. Despite extremely low mutual sequence homology, the β -barrel is structurally well conserved among the 10-12 different human lipocalins, as well as family members from various other species whose three-dimensional structures are known. In contrast, the loop region around the ligand-binding site exhibits large differences, both in amino acid sequence, length and conformation of the four polypeptide segments (1), which is in agreement with the diverse ligand specificities observed for this family.

Thus, the lipocalin architecture shows a resemblance to that of antibodies, whose structurally

pharmaceutically active compounds, or even – when engineered for novel binding functions – as therapeutic drugs in their own right (3).

THE ANTICALIN PROOF-OF-CONCEPT

Initially, the structurally and biochemically well characterised bilin-binding protein (BBP) from *Pieris brassicae* (4) was employed as a lipocalin scaffold in order to tailor its binding site for ligands such as fluorescein, digoxigenin and other low molecular weight substances and peptides. A random library with altogether 16 variegated amino acid positions around the natural ligand-binding site, spread across the four loops at the open end of the β -barrel, was prepared and subjected to phage display selection against the immobilised target compounds (5).

Inspection of the primary sequences of the resulting engineered lipocalins revealed that the loop regions that had been subjected to randomisation essentially tolerated the entire set of possible side chains (1). Elucidation of the three-dimensional structure, both for the fluorescein-binding BBP variant FluA and for the digoxigenin-binding variant DigA16, revealed that the extensive alteration of side chains, affecting 10 % of all residues in this lipocalin, did not impair the β -barrel fold. The variegated loops, on the other hand, adopted dramatically altered conformations compared with the wild-type lipocalin.

Hence, it was demonstrated that the high structural plasticity of the binding site provided by the lipocalin protein architecture generally enables the generation of novel ligand-binding proteins with antibody-like properties, thus termed 'Anticalins'. The therapeutic potential of such engineered lipocalins became readily apparent during these early studies. An affinity-improved variant of the digoxigenin-binding Anticalin with a sub-nanomolar dissociation constant, dubbed Digical, proved to completely reverse intoxication by digitalis in an established guinea pig model, thus demonstrating *in vivo* efficacy as an antidote (3).

ANTICALINS DIRECTED AGAINST MEDICALLY RELEVANT PROTEIN TARGETS

Most of the molecular structures that are currently considered relevant targets for biopharmaceuticals constitute extracellular proteins or cell surface receptors. Consequently, Anticalin libraries were specifically developed for the recognition of protein antigens instead of haptens. In addition, to reduce immunogenic side effects upon prolonged treatment, these libraries were constructed on the basis of human lipocalins – in

particular ApoD, NGAL and Tlc (3) (see Figure 1). To this end, 16-24 amino acid residues located at exposed positions, close to the tips of the four hypervariable loops, were subjected to random mutagenesis in order to allow tight contact formation with a macromolecular target, which cannot penetrate as deeply into the ligand-binding site as a small molecule. Using these libraries, Anticalins have been successfully selected against a variety of disease-related protein antigens, including several immunological receptors such as CTLA-4 and soluble growth factors such as VEGF. Typically, high selectivity and affinities in the sub-nanomolar range have been achieved.

Recently, the crystal structure of the complex between a cognate Anticalin and the extracellular domain of cytotoxic T lymphocyte antigen No 4 (CTLA-4) was solved, demonstrating that a macromolecular protein 'antigen' can effectively be bound at the cup-shaped binding-site of an engineered lipocalin, whose natural counterparts almost exclusively recognise low molecular weight substances. All four randomised loops of NGAL – which had served as a scaffold in this case – contribute to the specific complex formation, thus validating the design of the Anticalin library.

CTLA-4 (CD152) is an activation-induced, transmembrane T cell co-receptor with an inhibitory effect on T cell-mediated immune responses. CTLA-4 antagonises CD28-dependent co-stimulation of T cells, whereby CTLA-4 and CD28 share the same counter-receptors on antigen-presenting cells, B7.1 and B7.2. Notably, the bound Anticalin shields the CTLA-4 epitope that is involved in the interaction with B7.1 and B7.2. Indeed, an antagonistic activity of the Anticalin toward CTLA-4 was confirmed in several *in vitro* cell culture tests, where T cell proliferation was stimulated in a comparable manner as with commercially available antibodies against the same target. Thus, the CTLA-4 specific Anticalin (PRS-010) provides a promising drug candidate for the immunotherapy of cancer (3). Its lack of immunological effector functions should limit off-target toxicity because only the antagonistic activity is needed. In fact, this is the case for many interesting targets involved in the regulation of the immune response, inflammation and neoangiogenesis.

Another drug candidate is an Anticalin with strong antagonistic activity towards vascular endothelial growth factor (VEGF). VEGF is a well characterised mediator of tumour angiogenesis and other neovascular diseases such as age-related macular degeneration (AMD). The selected Anticalin (PRS-050) exhibits a favourable binding and activity profile in direct comparison with

currently approved VEGF antagonists. A half-life extended version of the Anticalin demonstrated excellent efficacy in three animal models assessing VEGF-induced enhanced vascular permeability, angiogenesis and anti-xenograft tumour activity. As immunological effector functions appear to be irrelevant for biomedical activity, an Anticalin with proven VEGF-antagonistic function should provide an interesting alternative to full size antibodies, especially in the light of its presumably better tissue penetration.

THE ANTICALIN DISCOVERY ENGINE







Founded in 2001 in Freising (near Munich, Germany), Pieris is the exclusive beneficiary of the Anticalin patent estate and has the freedom to operate to develop and commercialise Anticalin-based products. An integrated technology process and strong in-house expertise allows the company to deliver high-quality product candidates in a time-span of 10-12 months. Typically, this process covers the initial design phase of a project, Anticalin lead discovery, affinity maturation for lead optimisation, and full functional *in vitro* and *in vivo* characterisation of highly purified Anticalin product candidates. Anticalins can be efficiently produced in microbial host systems such as *E. coli* and yeast, which permit fast growth and simple up-scaling. The Anticalin technology has been validated *in vivo* with currently three proprietary therapeutic programmes in early preclinical development. In addition, the future potential for companion diagnostics could be demonstrated.

CUSTOM DESIGN OF FUNCTIONALITY, VALENCY AND PHARMACOKINETICS

Natural as well as engineered lipocalins are quickly cleared by renal filtration due to their small size of ca 20 kDa if they circulate as monomeric proteins. When conjugated with radioactive isotopes for *in vivo* diagnostic purposes, these properties should lead to images of high contrast soon after administration. Nevertheless, for medical indications that require prolonged treatment, the simple architecture and robustness of the lipocalin scaffold facilitates the preparation of fusion proteins or site-directed conjugates to modulate clearance by established methodologies.

Several techniques are available to extend the plasma half-life of Anticalins – for example, by production of fusion proteins with serum albumin, with an albumin-binding domain or peptide, or via PEGylation. Anticalins display both their N- and C-termini in an accessible manner and remote from the binding site, which differs from the situation with scFv fragments of antibodies, where the N-terminus often forms part of the

Figure 3: Anticalins offer a broad range of attributes supporting their potential as biopharmaceuticals

Aspects	Attributes	Pieris
Affinity/specificity	 Bind diverse spectrum of targets Affinity in pM range High binding selectivity	Anticalins® with therapeutic product potential readily isolated
Small size	 Broad interface for target binding within 20kDa protein	Ideal combination of target binding and tissue penetration properties
Adaptable	 N- and C-terminal fusions (for example effector domains, dual targeting) Site-directed conjugation (for example PEG, toxins, chelates)	Custom design of functionality, valency and pharmacokinetics
Robust	 High thermal stability High aqueous solubility Long shelf-life High pH resistance	Diverse range of formulation and delivery options as products
<i>E. coli</i> /yeast production	 Binding independent of glycosylation Binding independent of disulfide bonds GMP manufacture in development	Versatile and cost-effective manufacturing options
Intellectual property	 Anticalins® outside mAb patent claims No third party royalty obligations	Attractive pharmacoeconomics

paratope. Thus, Anticalins are well-suited for fusion with other functional domains without compromising their engineered binding activity.

Several types of Anticalin fusion proteins have been prepared, including fusion with another Anticalin, resulting in a so-called Duocalin leading to dual binding specificity (6). A dimeric binding mode utilising either a Duocalin that has twice the same specificity, or a fusion protein between an Anticalin and a dimerisation domain, may be employed to enhance binding avidity, to accelerate the rate of internalisation for a cell surface receptor or to exert an agonistic mode of action via its clustering. Fusion proteins of Anticalins that address a specific receptor on solid tumours with enzymes that generate a cytotoxic compound from an inactive precursor (prodrug) might be of special interest as an alternative to antibody-directed enzyme prodrug therapy (ADEPT).

CONCLUSION AND OUTLOOK

Anticalins provide binding sites with high structural plasticity and an extended molecular interface with their prescribed targets, comparable in size to that of conventional antibodies. Thus, Anticalins with high specificity and affinity can be readily generated against hapten, peptide and protein targets. As Anticalins are derived from human lipocalin scaffolds, further reformatting – such as by CDR-grafting during humanisation of antibodies – is not required. Their monovalent binding activity decreases the risk of unwanted intermolecular cross-linking of cellular receptor targets, which is desired in many cases.

Available structural and functional data suggest that Anticalins are able to recognise a diverse set of epitopes on different target proteins and therefore offer considerable potential as antagonistic reagents in general. Compared with antibodies, Anticalins provide several practical advantages because they are much smaller, consist of a single polypeptide chain, do not require glycosylation or disulphide bonds, exhibit robust biophysical properties and can easily be produced in microbial expression systems.

Since their structure-function relations are well understood, rational engineering of additional functions such as site-directed PEGylation or fusion with functional effector domains, dimerisation modules or with a second Anticalin can be readily achieved. Dual targeting is of particular interest as this is difficult to accomplish with monoclonal antibodies. Furthermore, intellectual property rights on the development of Anticalin-based drugs are well-defined and independent of antibody technology, thus allowing free access to clinically validated molecular targets of high commercial interest (see Figure 3).

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Note

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