Lipocalins are promising drug candidates, either based on their natural ligand-binding functions or as engineered 'anticalins' with novel specificities.

Keynote review:

Lipocalins in drug discovery: from natural ligand-binding proteins to 'anticalins'

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Lipocalins are a widespread family of small, robust proteins that typically transport or store biological compounds which are either of low solubility or are chemically sensitive, including vitamins, steroid hormones, odorants and various secondary metabolites. There are approximately ten different lipocalins in the human body, with the plasma retinol-binding protein being the most well known. Some lipocalins have a pathophysiological role, which opens possibilities for their use in medical applications. Furthermore, lipocalins from blood-sucking insects have evolved as scavengers for mediators of inflammation. As well as using the natural ligand-binding function, lipocalins have also been recruited as scaffolds for the design of artificial binding proteins termed 'anticalins'®. These novel proteins have potential applications as antidotes, antagonistic protein therapeutics or as target-recognition modules in a new generation of immunotoxins.

The lipocalins represent a family of functionally diverse, small proteins that comprise 160–180 amino acid residues and have weak sequence homology but high similarity at the tertiary structural level [1,2]. Members of this family have important biological functions in a variety of organisms, from bacteria to humans. The majority of lipocalins are responsible for the storage and transport of compounds that have low solubility or are chemically sensitive, such as vitamins, steroids and metabolic products [3]. The human plasma retinol-binding protein (RBP) was the first lipocalin for which a 3D structure was elucidated [4,5]; RBP transports the poorly soluble and oxidation-prone vitamin A from the liver, where it is stored as a fatty acid ester, to several target tissues. Some lipocalins appear to have a more specialized role in vertebrates [6], participating, for example, in olfaction and

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1359-6446/04/$ – see front matter PII: S1359-6446(04)03294-5

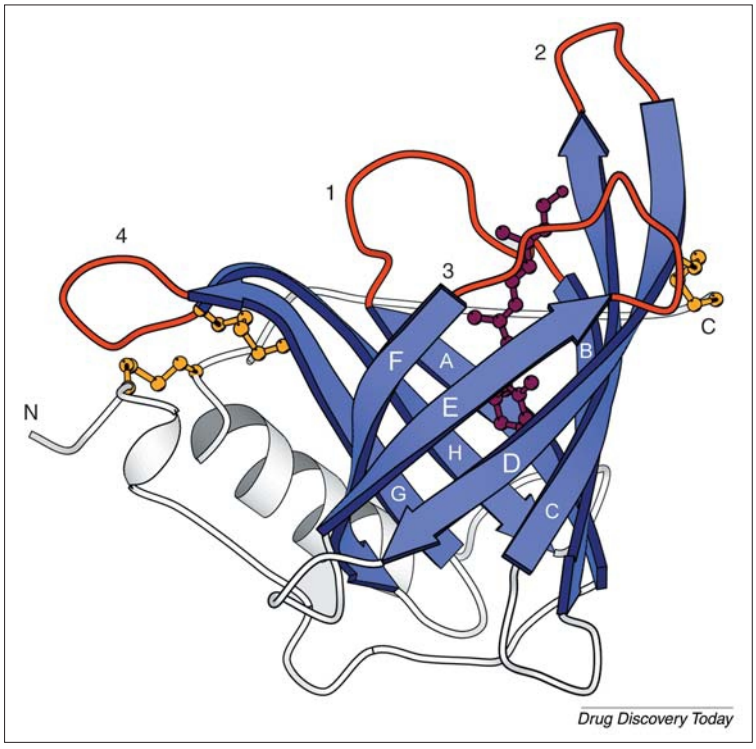

FIGURE 1

General structure of human retinol-binding protein, a prototypic lipocalin. Ribbon diagram (MolScript software) of the crystal structure of RBP (Protein Data Bank entry 1RBP) with the bound ligand retinol (ball and stick representation in magenta). The eight antiparallel strands of the conserved β-barrel structure are shown in blue with labels A to H, and the four loops, which are highly variable among the lipocalin family, are coloured red and numbered. The typical α-helix that is attached to the central β-barrel in all lipocalins, the loops at the closed end and the N- and C-terminal peptide segments are shown in grey. The three disulfide bonds of RBP are depicted in yellow.

regulation of the immune response, and even acting as an enzyme in the formation of prostaglandin D_2.

Despite their extremely poor sequence homology, the lipocalins share a structurally conserved β-barrel as their central folding motif, which consists of eight antiparallel β-strands that are arranged in a cylindrical manner (Figure 1). At the N-terminal of the protein, the β-sheet region is preceded by a coiled polypeptide segment, whereas at the C-terminal the β-sheet is appended by a characteristic α-helix and an amino acid stretch that is in an extended conformation. Within the β-barrel, the antiparallel strands (assigned A to H) are arranged in a $(+1)_7$ topology. These strands coil in a right-handed and conical manner around a central axis such that part of the backbone of strand A can form hydrogen bonds with strand H.

One end of the β-barrel is closed by the N-terminal peptide segment that traverses the base of the barrel between two short loops, which connect strands B to C and F to G, before entering into β-strand A. Dense packing of side chains in this region and within the adjacent part of the barrel leads to the formation of a hydrophobic core. At the opposite end, the conical β-barrel is typically open to solvent and provides access to a cavity. Four loops connect the strands in a pair-wise fashion and form the entrance to a cup-shaped pocket where the cognate hydrophobic ligand is bound. Hence, the term 'lipocalin' (derived from the Greek and Latin word 'calyx' meaning drinking vessel) was coined for this protein family [7]. In contrast to the highly conserved β-barrel topology, there is considerable variation in this loop region among individual members, with differences evident in the amino acid composition, conformation and length of the contributing polypeptide segments [2], which consequently gives rise to the variety of ligand specificities observed.

Indeed, the binding site of the lipocalins can adopt extremely different shapes. The binding site can form a wide, funnel-like opening to the solvent, as in the case of neutrophil gelatinase-associated lipocalin (NGAL) [8]. Alternatively, the loops can close over the cavity within the β-barrel and fully encapsulate the ligand, as in the mouse major urinary protein (MUP) [9]. The ligand pocket can extend deep within the hydrophobic core of the β-barrel, as is observed for RBP where the β-ionone ring of retinol becomes buried at the bottom of a narrow channel that harbours the oligo-isoprene side chain [4], or the ligand pocket can form an extended cave with several lobes close to the hydrophobic core, as noted in the structure of human tear lipocalin (Tlc) [10,11].

Because lipocalins comprise a single, small polypeptide chain that adopts a simple fold (Figure 1), this family of proteins affords several benefits for protein engineering and mass production, as well as applications in biotechnology and medicine. Many lipocalins exhibit significant thermostability and thus represent robust proteins [12]. Most natural lipocalins are of a monomeric globular nature and, particularly in the human body, are abundant components of plasma, tissue and secretory fluids. Few members of the family are glycosylated, thus lipocalins can be easily produced as recombinant proteins using bacterial expression systems [13].

Lipocalins are typically secretory proteins and as such they have a varying number of disulfide bonds. Although only one disulfide bridge is conserved in human lipocalins, connecting the C-terminal polypeptide segment to the β-barrel (Figure 1), this crosslink appears to be generally dispensable for formation of the lipocalin fold because this intramolecular bridge is absent in the *Escherichia coli* lipocalin Blc [14]. Furthermore, several human lipocalins exhibit a single unpaired Cys residue. Although the function of this residue has not always been elucidated, this amino acid occasionally forms an intermolecular covalent link to another protein. For example, apolipoprotein D (ApoD) associates with apolipoprotein A-II [15] and NGAL links to neutrophil gelatinase (also called matrix metalloproteinase IX) to varying extents [8]. However, this additional Cys residue can usually be removed by site-directed mutagenesis without a resultant loss of ligand-binding activity [16].

The binding specificity for low molecular weight compounds is well-characterized for many lipocalins. Some lipocalins have high ligand specificity, for example, RBP

is selective for retinol [4], ApoD for progesterone and arachidonic acid [16] and NGAL for Fe(III)-enterobactin [8]. By contrast, other lipocalins exhibit promiscuous ligand-binding behaviour, for example, human Tlc forms complexes with a considerable range of lipophilic molecules [10], including fatty acids, alcohols, phospholipids, glycolipids, cholesterol, retinol, arachidonic acid, lipid peroxidation products, microbial Fe(III)-siderophores [17] and even the antibiotic rifampin [18]. The ligand affinity of lipocalins is typically moderate with dissociation constants of approximately 1 μM, which is consistent with their presumed function as a physiological buffer for the bound substance. However, there are notable exceptions – NGAL with a K_d of 0.4 nM for Fe(III)-enterobactin [8] and the tick histamine-binding protein (HBP)-2 with a K_d of 1.7 nM [19].

The role of cellular receptors for the physiological function of lipocalins is less well understood [20]. Lipocalins could potentially deliver their ligand via a cell membrane receptor, possibly involving endocytosis of the whole complex, or some lipocalins might even act by themselves in cellular signalling. Circumstantial evidence exists for the specific interaction of RBP with a cell-surface receptor [20] and also for the uptake of NGAL by a receptor on kidney cells [21]. Thus far, only one specific receptor for a human lipocalin has been characterized (including cDNA sequencing and cloning) – the lipocalin-1-interacting membrane receptor (LIMR), which recognizes Tlc [22]. Therefore, it seems that lipocalins mostly act in the body as soluble proteins by way of their ligand-transport function. This makes them of interest as storage proteins, carrier vehicles for pharmaceutical compounds and as therapeutic drugs.

Broader interest in lipocalin research has only recently emerged, which is reflected in the dedication of a special issue of *Biochimica Biophysica Acta* to this protein superfamily [23], and the convening of the first international conference covering lipocalins [24]. The range of physiological processes in which lipocalins are involved, together with their beneficial molecular properties as small monomeric proteins, has also boosted their potential for medical use. This review focuses on some of the most promising future therapeutic applications of lipocalins:

- some human lipocalins are associated with pathological disorders and therefore these physiological proteins could provide targets for intervention;
- other lipocalins, in particular those of insect origin, have highly evolved binding functions for mediators of inflammation, such as histamine, and could directly serve as therapeutic drugs;
- the molecular architecture of the lipocalins (i.e. the structurally hypervariable binding site at the apex of a rigid β-barrel framework) can be exploited to engineer artificial binding proteins, termed 'anticalins', that potentially have similar medical applications to those currently emerging for therapeutic antibody fragments.

Pathophysiology of human lipocalins

Ten different lipocalins have thus far been identified in humans: RBP [25], ApoD [15,16], NGAL [8], α_1-microglobulin (protein HC) [26], complement component 8γ [27], tear lipocalin [10,11], β-trace (prostaglandin D synthase) [28], odorant-binding protein [29], α-1-acid glycoprotein (AGP or AAG) [30] and glycodelin (Gd) [31,32]. Some of these lipocalins have physiological functions that can be linked to pathological disorders. For example, single point mutations in the RBP gene, which resulted in the amino acid exchanges Ile41Asn on one allele and Gly74Asp on the second allele, led to a reduction of plasma RBP concentration to below the detection limit, inducing severe biochemical vitamin A deficiency and night blindness [33]. However, the generally mild clinical symptoms were relieved by a retinol-rich diet. Apart from that, there are at least two human lipocalins with physiological properties that make them attractive for therapeutic use – AGP and Gd.

α-1-Acid glycoprotein

AGP, also referred to as orosomucoid, is a highly glycosylated, negatively charged lipocalin with a molecular weight of 41–43 kDa that is found in many vertebrates [30]. Under physiological conditions, AGP has a stable, high-serum concentration of ~1 g l^{-1}. On tissue injury, inflammation or infection, the levels of AGP in serum can markedly increase, which led to its classification as an acute phase protein. Therefore, AGP has been widely used in clinical practice as a biochemical marker for the diagnosis and follow-up of patients with all kinds of inflammatory disease, such as rheumatoid arthritis and inflammatory bowel disease [34]. More recently, the serum level of AGP has attracted interest as a predictor of response and a major objective prognostic factor of survival for patients with non-small cell lung cancer who received docetaxel chemotherapy [35].

AGP exhibits immunomodulatory and anti-inflammatory properties *in vitro* and *in vivo*, with the glycosylation pattern of AGP being a crucial determinant of these activities [30]. With respect to possible therapeutic applications, AGP enhances the capillary barrier function in different rodent models of shock, thereby maintaining the perfusion of vital organs and conferring a protective effect [36]. Addition of AGP to resuscitation fluid has proven beneficial in a rat model of soft tissue trauma and haemorrhagic shock. Consequently, this lipocalin could serve as an adjunct to prevent capillary leakage in patients undergoing major traumatic injury: direct interaction of AGP with pores on the endothelial cell lining would reduce the amount of plasma water filtrated into the surrounding tissues [37].

Glycodelin

Human Gd, also known as placental protein 14 (PP14), is a lipocalin of 180 amino acids [38] that exists in three

isoforms with different glycosylation patterns: GdA is present in female amniotic fluid; GdF is found in female follicular fluid; and GdS is detected in male seminal plasma. Investigations with purified GdA revealed the presence of a GdA-binding site on sperm. Moreover, the binding of GdA to this site inhibits the sperm–egg interaction and thus GdA exhibits contraceptive activity *in vitro* [39]. Contraceptive activity was also shown for GdF [40] and, taken together with the observed time- and site-specific occurrence of GdA and GdF, a general role of Gd in the regulation of fertilization has been suggested [41].

Furthermore, GdA is an immunosuppressive protein that has a direct impact on T-cell activity [42] and B-cell proliferation [43], and also induces apoptosis of T-cells [44]. Physiologically, this behaviour could have a role in preventing the mother from rejecting her antigenically foreign embryo or foetus [45]. Finally, GdA and GdS that have been chemically modified with 3-hydroxyphthalic anhydride can block the binding site for the surface glycoprotein 120 of HIV on the T-cell coreceptor CD4, which in turn might inhibit HIV transmission [46]. The apparent therapeutic potential of GdA as a contraceptive agent, and possibly also as an antiviral, has inspired the development of a large-scale production method for the properly glycosylated isoform [47].

Insect lipocalins with therapeutic potential

Blood-sucking arthropods have developed several strategies to overcome the mechanisms used by a host to protect itself against invasion. The saliva of most of these insects contains at least one compound that inhibits platelet-aggregation, one substance that interferes with the blood coagulation cascade and one agent that mediates vasodilatory effects [48]. This armament often includes lipocalins, which have evolved sophisticated functions that prove advantageous to the insect [49]. As a consequence of their specialized activities in haemostasis, some of these lipocalins could be of therapeutic value.

Triabin

One of the first lipocalins to gain attention as a potential drug is triabin, which is produced by the blood-sucking insect *Triatoma pallidipennis* [50]. The protein consists of 142 amino acids and shares the overall lipocalin fold, with the exception that the position of β-strands B and C is reversed in the β-barrel [51]. Thus, it exhibits an 'up-up-down-down' topology, which differs from the strictly antiparallel β-sheet conformation of other lipocalins. Triabin forms a non-covalent 1:1 complex with thrombin and inhibits platelet aggregation by blocking the substrate-binding site of this serine protease.

Although triabin has a remarkably low inhibitory constant for thrombin (3 pM) and inhibits thrombin-induced platelet aggregation *in vitro* as effectively as the well-known thrombin inhibitor hirudin, residual activity of the protease has been experimentally observed, even with an excess of the lipocalin. This phenomenon was attributed to some degree of structural flexibility of the triabin–thrombin complex, which enables thrombin activity and thus leads to the partial degradation of fibrinogen, one of the substrates of thrombin [50,51]. However, this residual fibrinogen-cleaving activity was proposed to be beneficial for clinical applications because of the less stringent anticoagulant intervention compared with standard medication [51,52]. Triabin is currently subject to preclinical development by Paion (http://www.paion.de), which has recently acquired this drug candidate.

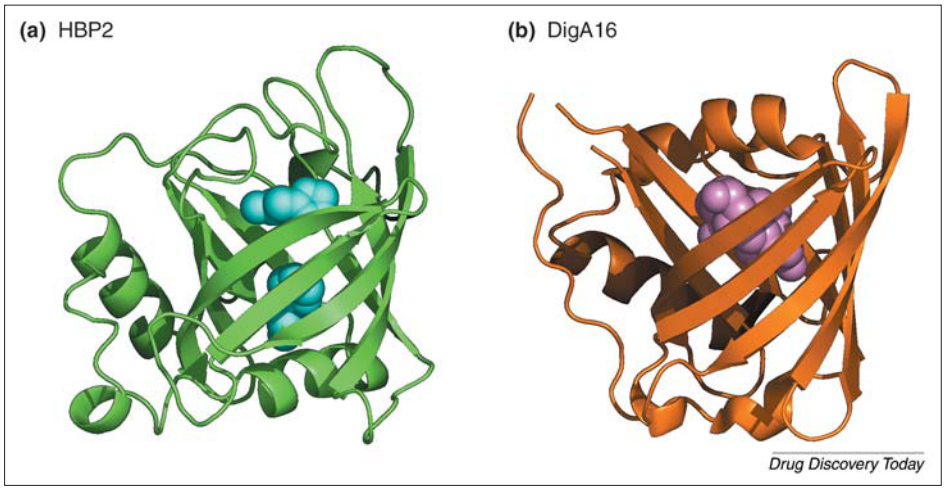

FIGURE 2

A natural (Histacalin) and an engineered lipocalin (Digicalin) with therapeutic potential. Ribbon diagram (PyMOL software) of the crystal structures of **(a)** HBP2 (Protein Data Bank entry 1QFT) and **(b)** DigA16 (Protein Data Bank entry 1LKE). The insect lipocalin HBP2 binds two histamine ligands (cyan), one at the open end (apex) and one buried within the lower part of the β-barrel. The anticalin DigA16 complexes with the steroid digoxigenin in a typical manner at the open end of the β-barrel, amid the four structurally variable loops (loop four was not fully resolved in the electron density and thus the tip is omitted).

Tick histamine-binding protein

The 171 amino acid tick histamine-binding protein (HBP2) present in the saliva of *Rhipicephalus appendiculatus* constitutes an exceptional member of the lipocalin family of proteins in that it provides two distinct binding sites for its cognate ligand histamine (Figure 2). One of the ligand pockets resides at a position analogous to the cavities found at the open end of the β-barrel in other lipocalins, and the binding affinity of this site for histamine is ~2 nM [19]. A second, lower-affinity binding site is located at the opposite end of the β-barrel, which is usually closed by dense side-chain packing in other lipocalins. HBP2 lacks the N-terminal 3_{10}-helix, a structural element that is common to other lipocalins, but instead incorporates a unique α-helix that shields the second cavity from solvent.

Histamine interferes with the attachment and successful feeding of blood-sucking ticks [53], thus, to prevail over this defence mechanism, these insects have had to develop an antihistamine strategy. Histamine, the decarboxylation product of histidine, is secreted predominantly by mast cells and basophils in response to tissue damage. This compound not only facilitates the entry of wound repair factors through enhancement of vascular permeability but is also the key mediator of inflammatory and allergic reactions.

Consequently, the histamine-scavenging capacity of HBP has prompted its application for the treatment of allergic disorders, particularly conjunctivitis and rhinitis. HBP is currently undergoing clinical development by Evolutec (http://www.evolutec.co.uk). As the anti-inflammatory drug candidate rEV131, recombinant HBP reduced the airway resistance in a mouse asthma model as effectively as the conventionally prescribed corticosteroid budesonide [54]. In a lipopolysaccharide-induced murine model for acute respiratory distress syndrome resistant to corticosteroids, rEV131 reduced bronchoconstriction as well as neutrophil recruitment and activation. Furthermore, preliminary data from a combined Phase I and Phase II clinical trial indicate safety and pharmacological activity of this lipocalin in the treatment of human allergy, where it has proven beneficial against ciliary and conjunctival redness, sneezing and running nose (unpublished).

Nitrophorin

Nitrophorins (NPs) are a family of haem-containing lipocalins with a molecular weight of approximately 20 kDa and are present in the saliva of the haematophagous kissing-bug *Rhodnius prolixus* [55]. The NPs comprise a group of four proteins, NP1–NP4, which are capable of delivering nitric oxide (NO) and of complexing with histamine. On release from the lipocalin in the skin of the host, NO binds to guanylate cyclase, which ultimately results in smooth muscle relaxation and vasodilation [56]. The NPs use a Fe(III) haem prosthetic group buried in the lipocalin pocket to complex either histamine or NO, both of which coordinate with the central ion [55,57]. Binding of NO is stronger at the pH of the saliva of the insect (~ pH 5.0) than at the pH of the host tissue (~ pH 7.5), which facilitates the release of the signal molecule at the feeding site, thus generating a vacant binding pocket ready for complexation of the proinflammatory histamine. The vasodilatory properties of NPs render them potential therapeutic agents for cardiovascular disease.

Platelet aggregation inhibitor-1

Another type of lipocalin found in the saliva of the haematophagous kissing-bug is the *R. prolixus* platelet aggregation inhibitor-1 (RPAI-1) [58]. Experimental evidence suggests that RPAI-1 functions as an efficient scavenger for ADP *in vivo* and *in vitro* at low nucleotide concentrations with an apparent affinity of ~50 nM. ADP is a nucleotide released by injured cells and a potent inducer of platelet aggregation [59]. In concert with an apyrase that is also present in the saliva of *R. prolixus*, RPAI-1 has been shown to inhibit ADP-dependent platelet-rich plasma aggregation by collagen, which ultimately enables the insect to obtain a steady blood meal [60]. As a result of its ADP-neutralizing properties, RPAI-1 could be envisaged as a therapeutic compound for immediate interventions in acute cardiovascular indications, for example, after angioplasty.

The concept of anticalins as a novel class of therapeutic proteins

Several observations, both on the structural level and from biochemical experiments, support the notion that the β-barrel structural element of the lipocalins represents a rigid folding unit [2]. Despite the varying sequences and diverse binding functions of the natural lipocalins, the backbone conformation of the β-barrel is highly conserved throughout the lipocalins. Hence, this β-barrel structure can support loops with highly variable lengths, sequences and conformations at its open end (Figure 1). This is analogous to the mode in which antibodies present their six hypervariable loops (complementarity-determining regions) on top of a structurally conserved framework. However, compared with antibodies, lipocalins exhibit several biotechnological advantages because they are smaller in size, are composed of a single polypeptide chain and they exhibit a simpler set of four hypervariable loops that can be more easily manipulated at the genetic level.

Although a vast number of differing antibodies is created in the course of an immune response by way of somatic gene rearrangement and hypermutation events [61], there are no physiological mechanisms for functional variation of the lipocalins, which were apparently optimized during evolution to serve specialized tasks. It is probable that there are just ten different lipocalins in the human organism that exhibit distinct binding functions and have similar orthologues in other mammals or vertebrates. For example, Human RBP and the porcine protein differ by only 12 amino acid residues, which are all remote from the retinol-binding site [4,13].

Anticalins: engineered lipocalins with antibody-like binding function

The lipocalin architecture appears to be well-suited to the implementation of novel binding activities via combinatorial protein design. The structurally variable part of the four loops is particularly attractive in this respect because this protein architecture results in discontinuous stretches of the polypeptide chain being in close proximity to each other, and hence an extended interface for the target can be formed. The bilin-binding protein (BBP) from *Pieris brassicae* [62,63] served as a model lipocalin in initial studies to create artificial binding sites with prescribed ligand specificities. Sixteen amino acid positions located within the four loops at the open end of the β-barrel and

adjoining regions of the β-strands were subjected to targeted random mutagenesis using a PCR assembly strategy [64,65]. These residues were chosen because their position in the loops is such that their side chains surround the natural ligand, biliverdin IXγ, when complexed with BBP [63].

The resulting molecular random library with ~4×10^8 independent members was subjected to selection towards several immobilized low molecular weight compounds using the method of phagemid display. Thus, BBP variants that specifically recognize fluorescein [64], digoxigenin [66], phthalic acid esters [67] and doxorubicin (Christian Kirchner and Arne Skerra, unpublished) were obtained. Observed affinities were in the nM range and thus higher than those known for many lipocalins with their natural ligands. Sequence analysis showed that the chosen amino acid positions have high tolerance for side chain replacement [2]. Furthermore, structural analyses revealed that the β-barrel architecture remained intact despite random mutagenesis, whereas the loop region exhibited considerable backbone plasticity [68,69]. Indeed, conformational response of the loops to the amino acid substitutions and concomitant rearrangement of aromatic side chains within the β-barrel led to binding sites with completely novel shapes.

As a result, a new class of engineered binding proteins with antibody-like ligand-binding function emerged, which was designated 'anticalins' [65]. The mechanism of complex formation of the anticalins with low molecular weight ligands is similar to the interaction between antibodies and haptens, with the exception that the ligand can be buried more deeply in the lipocalin-binding pocket [68,69]. Shape complementarity is principally generated through aromatic side chains, with specific interactions being formed by appropriately placed hydrogen bond donors or acceptors, and occasionally these interactions are mediated by buried water molecules.

Notably, in the binding site of the digoxigenin-binding anticalin DigA16, the bound steroid ligand is sandwiched between one Trp and two Tyr side chains, which is analogous to a monoclonal antidigoxin antibody; this similarity provides an example of 'convergent *in vitro* evolution' [68]. In addition, a His side chain at the base of the ligand pocket of DigA16 displays an induced fit on complex formation with digoxigenin [68], an effect that has up to now been regarded as typical for antibodies. Taken together, the lipocalin scaffold has proven to be useful for the generation of novel binding proteins with high affinity and specificity.

Two strategies have recently been used to extend the anticalin concept. First, apart from the BBP of insect origin, several human lipocalins were recruited as scaffold proteins for random mutagenesis in their loop region and selection of cognate binding proteins, including ApoD [70], NGAL and Tlc (unpublished). Second, corresponding anticalin libraries were generated to enable recognition of macromolecular protein targets. Because proteins have larger molecular dimensions than small biochemical compounds, they cannot penetrate into the ligand-binding site and thus different amino acid positions in the loops were randomly mutated compared with the mutagenesis described for BBP.

Consequently, side chains at more exposed positions, close to the tips of the four loops at the open end of the β-barrel, were subjected to random mutagenesis when a protein was the designated ligand [70]. Following this strategy, a panel of anticalins based on human scaffold proteins with specificities for several protein targets, including disease-related cell surface receptors such as cytotoxic T lymphocyte-associated antigen (CTLA)-4, CD4, CD22, CD25 and CD33, with affinities in the low nM range were successfully selected (Steffen Schlehuber, Arne Skerra and co-workers, unpublished). Currently, the anticalin technology is being exploited by PIERIS (http://www.pieris.biz) to develop novel biopharmaceuticals for the treatment of cardiovascular diseases and cancer.

Anticalins directed against low molecular weight compounds as antidotes

The digoxigenin-binding anticalin DigA16 was the first promising engineered lipocalin for therapeutic use against digitalis intoxications. Digoxigenin is the aglycon of digoxin, a well-known secondary plant metabolite with cardioactive function. Digoxin, and to a lesser extent the closely related compound digitoxin, has a prominent role in the treatment of ventricular tachyarrhythmias and congestive heart failure [71]. All cardioactive steroids are potent and highly specific inhibitors of the Na^+/K^+-ATPase located in the cellular plasma membrane, thereby exerting sympatholytic or positive inotropic effects.

Unfortunately, there is a narrow margin between therapeutic and toxic doses of the pharmacological compound. In the past, the treatment of digoxin toxicity was largely supportive and aimed at inhibiting the absorption of the drug or counteracting its cardiac effects. In cases of extreme digoxin overdoses, standard supportive therapy has limited effectiveness, and the overdose often has a fatal outcome. The need for secure treatment for severely intoxicated patients led to the development of digoxin-specific antibody Fab fragments such as Digibind® (GlaxoSmithKline) and DigiFab™ (Protherics), which have been well-characterized in clinical trials in terms of pharmacokinetic properties and ligand affinity [72].

Whereas these commercially available polyclonal antibody preparations are raised as intact immunoglobulins in sheep, the Fc component, which has the majority of the antigenic determinants, is removed from the actual product (i.e. the Fab fragments) to reduce the antigenic threat to patients compared with the full size antibodies. However, there is still the danger of severe anaphylactic reactions, also as a consequence of residual papain in the preparation, which is introduced during the proteolytic Fc cleavage step. Patients with known allergies

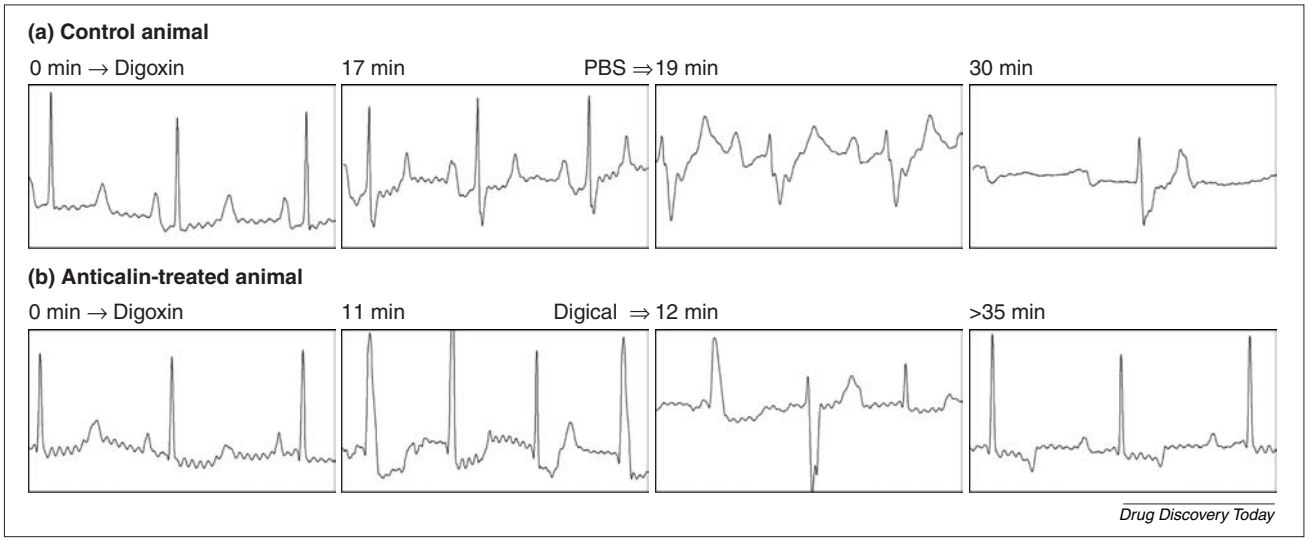

FIGURE 3

Therapeutic effect of Digical, an engineered lipocalin based on BBP, in an animal model of digitalis intoxication. Serial samples from continuous ECG are shown for two guinea pigs that were intravenously treated with digoxin. After onset of toxic symptoms, one guinea pig (lower series) was given the anticalin DigA16(H86N) and the other received PBS (upper series): first time point, normal ECG at the start of the experiment; second time point, first signs of individual but characteristic aberrations of the ECG at 11 and 17 min after injection of a lethal dose of digoxin (270 μg). At this time point, one animal received a bolus injection of 16.4 mg of the anticalin, whereas the control animal was injected with PBS; third time point, during the course of the experiment, increased acute toxicity effects were observed for both animals, even after anticalin treatment; and fourth time point, 24 min after injection of the anticalin, almost full reversion of the pathological ECG became apparent for the anticalin-treated animal, which was maintained throughout the remainder of the experiment (102 min). By contrast, the control animal deteriorated, with a fatal outcome 30 min after administration of the toxic digoxin dose. Abbreviations: ECG, electrocardiogram; PBS, phosphate-buffered saline.

are particularly at risk, as are individuals who have previously received other antibodies from sheep or related animals. Thus, the availability of digoxin-binding agents that are not derived from animal immunoglobulins and that have not been subjected to *in vitro* processing could provide a major advantage in the therapy of digitalis intoxications.

DigA16 was constructed in a two-step procedure from the BBP lipocalin scaffold [66]. First, the variant DigA, which binds digoxigenin with a K_d of ~300 nM, was selected from a BBP library having 16 randomly mutated amino acids in the four loops with side chains in close proximity to the natural ligand-binding site [64]. Affinity maturation was subsequently performed by selectively randomizing residues in the first loop only, leaving the mutated residues in the other three loops fixed. Next, the resulting library was again subjected to screening against digoxigenin. This approach led to the variant DigA16, which binds digoxigenin with a tenfold lower K_d of ~30 nM [66].

DigA16 has high specificity for the steroid ligand because it does not demonstrably bind the structurally related cardiac steroid ouabain, whereas antibodies raised against digoxigenin usually cross-react with ouabain. Crystallographic analyses of the apo form of DigA16 and the DigA16–digoxigenin complex reveal a structural explanation for this difference in binding behaviour (Figure 2) [68]. Almost 95% of the ligand surface is buried in the deep lipocalin pocket, and a pronounced structural complementarity with the steroid moiety is achieved by hydrophobic contacts and specific hydrogen-bonding partners.

Attempts to increase the digoxigenin-binding activity further involved a combinatorial approach according to the previously employed 'loop-walking' randomization strategy [12] and a strategy of rational protein design based on the crystal structure of DigA16 (Alexander Peim and Arne Skerra, unpublished). Remarkably, these approaches yielded several point mutants of DigA16 exhibiting affinities as low as 800 pM for digoxin and 600 pM for digitoxin. These results show that novel ligand-binding proteins with medically relevant affinity and specificity can be obtained on the basis of a lipocalin scaffold.

Indeed, the affinities of these anticalins directed against digoxigenin are sufficient for use as an effective digitalis antidote. Preliminary animal studies in a guinea pig model [73] revealed complete reversal of toxicity effects of a digoxin overdose, as monitored by electrocardiogram (Figure 3), after administration of slightly more than stoichiometric amounts of the anticalin [74]. Furthermore, application of 25 mg kg^{-1} of the DigA16 anticalin to mice did not cause any acute toxicity effects during a time period of two weeks. Hence, the concept of therapeutic application of anticalins as antidotes against toxic small molecules has been proven in an animal system.

Anticalins directed against protein targets as antagonistic agents

Anticalins that recognize cell-surface receptors or soluble protein ligands, particularly if based on human lipocalin scaffolds, could be of use as specific protein drugs in the

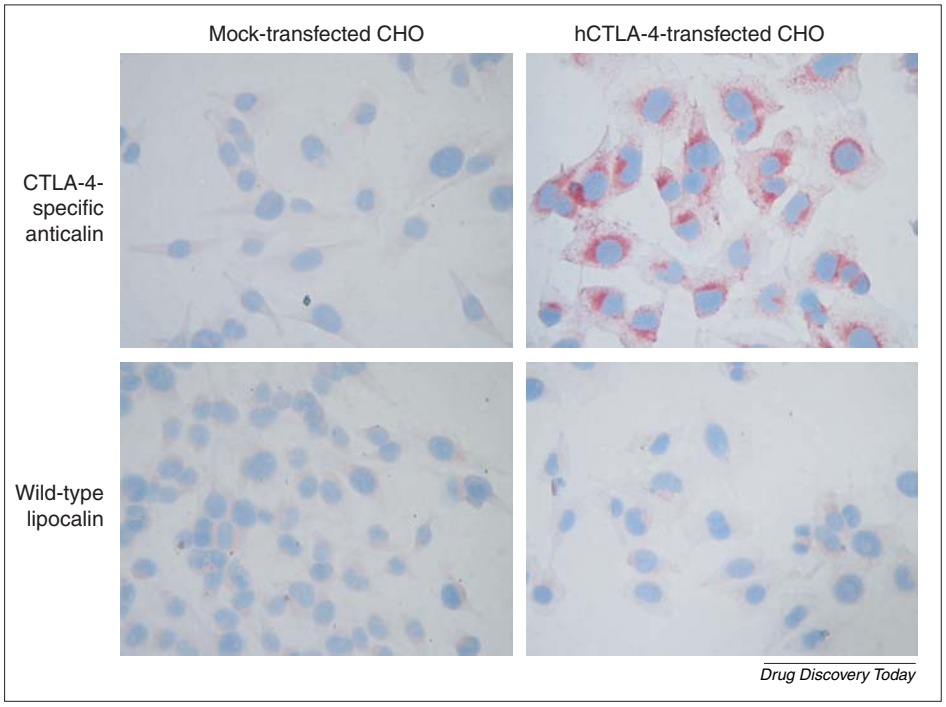

FIGURE 4

Specific recognition of human CTLA-4 by an engineered lipocalin based on NGAL. CHO cells transfected with a cDNA encoding human CTLA-4 or mock-transfected CHO cells were grown on glass slides and fixed with acetone. After treatment with 0.5% saponin, the slides were incubated over night at 4°C, either with a fluorescein-labelled hCTLA-4-specific anticalin or with the wild-type lipocalin that had been labelled by the same method (both at a concentration of 12 μg ml^{-1} in PBS). Bound lipocalin was detected by incubation with mouse antiFITC IgG, followed by incubation with alkaline phosphatase-conjugated rabbit antimouse IgG and Fast Red staining in conjunction with haematoxylin counterstaining. Abbreviations: CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate.

therapeutic treatment of tumours and autoimmune and cardiovascular diseases. For example, CTLA-4 is crucial for immunomodulation and has attracted attention as a T-cell surface protein target for cancer immunotherapy [75]. T-cells receive their primary activating signal through specific interaction between the T-cell receptor and a major histocompatability complex-bound peptide epitope on antigen-presenting cells (APCs). In addition, costimulatory receptors on the T-cell, such as CD28, must interact with cognate costimulatory ligands expressed by the APC, namely CD80 (B7.1) and CD86 (B7.2), to prevent clonal anergy [76].

Two to three days after activation, the T-cell surface protein CTLA-4 (CD152) is upregulated and can bind to CD80 and CD86 on APCs in a competitive manner, but at 10–100 times higher avidity than CD28 [76,77], thereby effecting T-cell deactivation. The balance between stimulatory and inhibitory signals ultimately determines the overall T-cell response to pathogens. Suspending this balance by blockade of the physiological interaction between CTLA-4 and its counter-receptors undermines the corresponding negative regulatory function. Accordingly, application of antibodies that bind to human CTLA-4 and block interaction with CD80 were effective in cancer treatment in preclinical [78], as well as clinical [79,80], studies.

Consequently, CTLA-4 was chosen as a protein target for the development of an antagonistic anticalin. A mutant lipocalin library derived from human NGAL, with 20 randomized amino acid positions in the loop region and a complexity of ~2×10^{10}, was used [81]. Selection against human CTLA-4–Ig, a soluble fusion protein of the extracellular region of the target receptor with an Ig Fc fragment [82], resulted in several anticalins with differing epitope specificities. Among these, one candidate specifically blocked the interaction between the recombinant CTLA-4 and its physiological ligands CD80 and CD86 in various binding experiments, such as ELISA and BIACORE, as well as in functional *in vitro* cell culture assays (Gabriele Matschiner, Andreas Hohlbaum, Evelyn Braungart, Stefan Trentmann, Peter Söhlemann, Steffen Schlehuber and Arne Skerra, unpublished). Fluorocytometry and immunocytochemical staining of adherent Chinese hamster ovarian cells stably transfected with human CTLA-4 cDNA (Figure 4) were used to demonstrate that this anticalin recognizes the native target protein.

Notably, after one cycle of affinity maturation a K_d value in the 10 nM range was achieved, which is comparable with the affinity of the humanized antibody that has been investigated for tumour immunotherapy in animal models [78]. However, in contrast to this antibody, the anticalin exhibits full cross-reactivity between human and murine CTLA-4, which provides a clear benefit for preclinical studies. Investigation of this anticalin in appropriate animal models could reveal potential pharmacokinetic or pharmacodynamic advantages that might arise from the differing architecture of anticalins compared with therapeutic antibodies, particularly regarding their smaller size and lack of the Fc region. Taken together, the successful generation of an anticalin that recognizes native CTLA-4 illustrates that engineered lipocalins should be useful as antagonistic reagents for disease-related cell-surface receptors.

Conclusions and prospects

The lipocalin protein family, either as natural proteins or as artificial ligand-binding proteins with engineered specificities (i.e. anticalins), constitutes a promising novel class of potential biopharmaceuticals. In principle, corresponding protein drugs should have applications in several medical therapies.

Antidotes against signalling molecules or toxic compounds

Insect-derived lipocalins, most notably HBP, that exhibit natural binding activity for mediators of inflammation

could be of use for therapeutic applications and are currently undergoing clinical development. Moreover, some human lipocalins, NGAL and Tlc, were shown to exert antimicrobial activity as a result of their affinities for iron-siderophores and thus might be useful as anti-infective agents. The anticalin DigA – or its derivatives – proved to be effective against digitalis intoxication in an animal model. Although this medical indication corresponds to a niche market, its acute nature and the limited availability of alternative treatments should enable rapid regulatory approval. More importantly, this same principle of action could be applicable to the development of anticalins for potentially harmful signalling molecules, such as prostaglandins (e.g. thromboxane). Hypothetically, the same strategy could be used to construct reversing agents for small molecule therapeutics, for example, against pentasaccharides that serve as anticoagulants, which can cause bleeding under particular circumstances [83].

Carriers for drug delivery

Instead of using the ligand-binding activity of natural or engineered lipocalins for the specific depletion of small effector molecules, such proteins could also prove effective carriers in the transport of poorly soluble or quickly degradable substances, which is the natural role of RBP, for example. Several tumour drugs, such as paclitaxel and doxorubicin, suffer from poor solubility [84] and severe side effects [85]. An anticalin with binding activity for doxorubicin has recently been developed. If coupled or fused to a recognition module for a tissue-specific cell-surface receptor – either an antibody fragment or a corresponding anticalin, hence resulting in a so-called 'duocalin' [86] – the carrier concept could be extended to drug targeting strategies.

Membrane receptor antagonists

Anticalins directed against the active site of a cell-surface receptor, which interfere with the natural ligand interaction, should prove useful reagents to either stimulate or inactivate a corresponding cellular response. The anticalin that blocks CTLA-4 on T-cells provides an example of this potential application. Thus far, full-size antibodies have been mostly used for such purposes. However, their application involves potential disadvantages associated with poor tissue penetration and non-specific binding via the Fc region, although antibodies might provide the benefit of an avidity effect because of the bivalent binding capacity for a cell-surface antigen; in this regard, comparison of the much smaller and monomeric lipocalin scaffold with antibodies will prove interesting.

Membrane receptor agonists

In principle, lipocalins could also be engineered to mimic a natural receptor ligand and elicit the corresponding intracellular signal; however, there is no appropriate example of the successful application of this strategy currently available. Furthermore, this approach will be hindered by the requisite to select for a molecule that not only recognizes the correct epitope on the cell-surface receptor but also triggers the relevant conformational change.

Molecular recognition units for targeting of cytotoxic compounds

Anticalins that specifically recognize a tumour surface marker could be useful for drug targeting approaches when coupled either to small molecule cytotoxic agents or to potent protein toxins [87]. In this context, they could replace the single chain antibody fragment (scFv) component of recombinant immunotoxins and hence afford an advantage in terms of their nature as stably folded monomeric proteins.

General considerations

Today, recombinant or engineered lipocalins appear to have a promising future as drugs for human therapy. Several preclinical studies are currently in progress with anticalins, for example, as an antidote for digitalis and as a blocking reagent for CTLA-4 (PIERIS), and there is at least one anticalin (HBP2) that is already in clinical trials (Evolutec). These investigations will provide information about aspects of pharmacokinetics and immunogenicity of this novel class of biopharmaceuticals. Available data on the pharmacokinetics of lipocalins indicate that these proteins have a serum half-life below one hour, which is similar to the behaviour of single chain antibody fragments, and they are predominantly cleared via the renal route. Such rapid pharmacokinetics could prove to be advantageous for applications as antidotes. However, for medical indications that require prolonged treatment, established techniques are available to extend the serum half-life of therapeutic proteins, for example, by modification with polyethylene glycol [88].

Immunogenicity is an issue relevant to all biopharmaceuticals [89]. For single acute dosing, as in the case of the digitalis antidote, immunogenicity is less relevant. In general, the choice of human lipocalins as scaffolds for the generation of anticalins should minimize problems in this area. Some lipocalins, such as those from animal skin or excretions, are known to be potent allergens, but their T-cell epitopes are well characterized [90] and are absent from endogenous human members of this protein family. Notably, insect-derived lipocalins, particularly those from blood-sucking species, have apparently evolved to evade an efficient immune response and should therefore also be suitable for therapeutic use.

In conclusion, several investigations have shown that the lipocalin scaffold itself does not elicit any acute toxicity effects. In particular, the human lipocalins – at least when engineered for removal of the typical free Cys residue – appear to behave as stable and almost biochemically inert plasma proteins that are tolerated at

high concentrations in the body. Thus, they should have similar properties to, for example, Ig Fab fragments. However, the much simpler molecular architecture of the anticalins (i.e. composition of a singular short polypeptide chain and potential lack of disulfide bonds), together with the high folding stability, promises much easier production and safe application as a biopharmaceutical drug of the future.

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