

Alternative Protein Scaffolds as Novel Biotherapeutics

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Introduction: The Route to Antibody Alternatives as Biobetters

Proposed as a new type of medicine more than a century ago by Emil von Behring, initially as “antitoxins” and only later dubbed “antibodies” (Lindenmann 1984), monoclonal antibodies (MAbs) reached regulatory approval in 1986 and subsequently, in a humanized or human format, have become the most successful and fastest growing class of biopharmaceuticals with more than 30 marketed drugs today (Reichert 2012, 2014; Strohl and Strohl 2012). Antibody technology has gone through a long evolution, starting with animal sera for passive immunization against toxins. The first breakthrough with regard to developing monoclonal as opposed to polyclonal antibodies was the invention of hybridoma technology in 1975, thus allowing the preparation of monospecific antibodies obtained via immunization of rodents (Köhler and Milstein 1975). However, only one MAb derived from mouse hybridoma cell culture was successful in the clinic: Muromonab-CD3/Orthoclone (OKT3®), approved in 1986 (discontinued in 2010) as an immunosuppressant drug to reduce acute rejection in patients after organ transplantation (Strohl and Strohl 2012).

The recognition that MAbs from animal sources elicit an anti-drug immune response in patients prompted efforts to make them more alike their human counterparts. The first strategy was the construction of chimeric MAbs by combining the pair of murine variable domains, carrying the antigen-binding site, with the

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constant region of a human immunoglobulin (Ig) (Morrison et al. 1984). Since antigenic sites predominantly occur in the xenogenic constant regions, the resulting chimeric Ig proteins were well tolerated (although somewhat biased by their nature as immune suppressants), as first demonstrated with Rituximab (Rituxan[®]; anti-CD20) and Daclizumab (Zenapax[®]; anti-IL-2R α), both approved by the FDA in 1997 for treating Non-Hodgkin's lymphoma (NHL) / rheumatoid arthritis (RA) and to prevent transplant rejection, respectively (Strohl and Strohl 2012).

Following this major breakthrough, the technology of CDR-grafting was invented (Jones et al. 1986), allowing more complete humanization of MABs by further exchanging the framework regions within the variable domains with those from a human Ig. One of the first and most successful examples has been Trastuzumab (Herceptin[®]) (Carter et al. 1992), a humanized antibody directed against the HER2 cell surface receptor overexpressed on metastasizing breast cancer cells, which was approved in 1998. However, as the conformation of the six CDRs is also influenced by the framework region, this type of humanization is a tricky approach that usually necessitates introduction of additional affinity-preserving mutations via protein engineering (Carter et al. 1992). This caveat eventually stimulated the development of alternative methods for MAB humanization, for example by resurfacing (Strohl and Strohl 2012).

With the advent of phage display techniques, the age of so-called “human” MABs arose, initially by selecting recombinant antibody fragments from naïve variable gene/cDNA libraries that were cloned from non-immunized human donors. The first MAB that emerged from this technology was Adalimumab (Humira[®]), which neutralizes tumor necrosis factor α (TNF α) and received approval for the treatment of RA and Crohn's disease in 2002 (Bain and Brazil 2003; Osbourn et al. 2005). Various other experimental strategies, both *in vitro* and *in vivo*, have followed, culminating in the breeding of transgenic animals that have their own Ig gene loci replaced by corresponding human chromosomal segments, thus directly allowing the biosynthesis of human MABs—e.g. followed by cloning of the relevant V-genes from antibody producing B-cells (Lee et al. 2014; Lonberg 2005). However, it should be kept in mind that in spite of the commonly used term “human”, each MAB developed by one of these technologies is still foreign to the body of an individual who receives corresponding treatment since it exhibits novel CDR sequences (Harding et al. 2010). Hence, immunogenicity in the patient cannot be fully prevented and, in fact, with regard to immunological tolerability, it is clear that the early chimeric antibody concept already had the strongest impact (Getts et al. 2010; Strohl and Strohl 2012).

Far from over, technology development in the antibody field continues apace (cf. the Chapters in Part II of this monograph). Apart from searching for innovative MABs directed against new targets and/or novel epitopes, having greater affinities and specificities, or even agonistic activities, there are numerous ongoing approaches to improve the generic features and the Ig format as a whole (Strohl and Strohl 2012). In particular, these efforts aim at the following features: enhancing immune effector functions (usually associated with the Fc region) such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC);

prolonging (or adjusting) circulation half-life; achieving more homogeneous and functional glycosylation; arming with drugs or radioactive payloads; and creating bispecific MABs, resulting in so-called second or third generation antibodies (Beck et al. 2010). Application of these strategies to marketed MABs, while retaining the original validated epitope specificity, results in “biobetters” in the true sense. One recent example is the antibody Ado-Trastuzumab Emtansine (T-DM1, Kadcyla™), a toxic drug conjugate of Trastuzumab (Dirix et al. 2013).

Generally, the term biobetter refers to a therapeutic protein that is functionally similar (not identical) to an established biopharmaceutical but has improvements over the original in critical attributes and clinical performance (Beck 2011). However, in the case of antibodies this rather narrow definition deserves broader interpretation, especially if the focus is on the tunable antigen-binding function as dominating feature (Skerra 2003). In fact, the past two decades have given evidence that full size Igs with their characteristic Y-shaped molecular architecture are not the only suitable platform for the generation of protein reagents with novel ligand-binding properties for applications in biomedical research and human therapy. Once the technical challenges of engineering recombinant antibody fragments had been mastered, so-called alternative protein scaffolds were identified which, too, can provide specific binding sites with high affinities against a wide range of biochemical or cellular target structures (Skerra 2000a). Consequently, biobetters may be understood in this context also as advancements in the molecular format of Ig-based biologics as a class.

Functional Ig Fragments: from Fab to Domain Antibody

MABs differ from classical biologics such as hormones or cytokines insofar as the generation of new molecular entities with differing target specificities and varying mode of action is possible on the basis of the same molecular format, the one of Igs. These proteins owe their remarkable ability for molecular recognition of almost any kind of antigen as well as their utility for diverse medical applications to their unique modular format (Bork et al. 1994; Padlan 1994; Strohl and Strohl 2012). On the first level, their molecular architecture comprises two types of domains which are either of constant or of variable character. Both rest upon the same basically conserved Ig fold which is characterized by a sandwich of two β -sheets with altogether 7 or 9 strands, respectively. Several constant domains assemble to form the Fc part at the stem of the Y-shaped protein, which is responsible for most effector functions, in particular binding and/or activation of cellular receptors or complement. The two arms of the Y are called antigen-binding fragments, Fabs, which are mutually identical in natural Igs and flexibly linked to the Fc stem via the hinge region. At each tip of the Y a pair of variable domains, VH from the heavy and VL from the light chain, forms the antigen-binding site.

Within these paired variable domains, there is another level of separation between structurally conserved and variable polypeptide segments, the so-called framework

region(s) on the one hand and the hypervariable region(s) on the other. As regards the hypervariable regions there are in total six hypervariable loops, three per variable domain, also known as complementarity-determining regions (CDRs), which—upon tight association between VH and VL via their inner β -sheets—together form the combining site that intimately contacts the antigen during complex formation. Within each V-domain the three hypervariable loops are supported by the β -sandwich fold, which provides the structural framework. This discontinuous region shows high similarity in its backbone conformation, not only among the human antibody repertoire but also across species, a feature that provides the basis of the CDR-grafting technology for antibody humanization mentioned above.

This modular format explains the success of MABs as almost universal protein tools for the tight and highly specific binding of a vast number of biomedically relevant molecular structures. Obviously, the β -sheet framework is sufficiently stable to structurally support a more or less unlimited number of CDR peptide sequences and to impose a defined three-dimensional conformation on them by providing the proper geometrical constraints. As a consequence, the number of possible CDR backbone conformations, called canonical structures, seems to be restricted (Al-Lazikani et al. 1997). However, the conformational stability of the Ig fold is not indefinite. In fact, it has been shown that the folding energy of the Ig framework statistically varies about some consensus sequence, which appears to constitute the thermodynamic minimum (Steipe et al. 1994), whereas the stability of the variable domain as a whole is clearly influenced by the individual sequences of the hypervariable loops (Jung and Plückthun 1997). Interestingly, these long known structural properties of Igs also match well with observations made for the alternative protein scaffolds discussed further below.

Nevertheless, with the ever increasing application of antibodies during the last two decades, several disadvantages have become apparent. For example, MABs have a very large size and complicated composition, comprising four polypeptide chains, glycosylation of the heavy chains, at least, as well as multiple structurally relevant disulfide bonds. Thus, intact antibodies require manufacturing in eukaryotic expression systems, usually involving stably transfected mammalian cell lines, whose optimization and fermentation is laborious and costly (Steinmeyer and McCormick 2008; Werner 2004). Consequently, the exploration of alternative protein reagents that are capable of specifically recognizing and tightly binding ‘antigens’ has been stimulated, which led to the investigation of different types of antibody fragments and, eventually, even to the use of isolated Ig domains (Holliger and Hudson 2005; Skerra 1993).

Initially, the development of methods for the production and manipulation of functional Ig fragments in the bacterial host cell *Escherichia coli* (Better et al. 1988; Skerra and Plückthun 1988) enormously stimulated the field of antibody engineering (Humphreys 2003; Skerra 1993), not only with regard to research and discovery but also concerning biological drug development (Holliger and Hudson 2005; Nelson and Reichert 2009). Two major types of recombinant antibody fragments carrying the intact antigen-binding site of a MAB are commonly in use: first, the Fab fragment, which consists of the entire light chain and the N-terminal half of the

heavy chain and has been traditionally prepared by selective proteolysis of full size Igs and, second, the Fv fragment; this smallest functional antibody fragment comprises just the two variable domains and is readily accessible only via recombinant DNA technology (Riechmann et al. 1988; Skerra and Plückthun 1988).

The conventional Fv fragment is made of the paired variable regions from both Ig chains which are held together just by non-covalent forces such that its stability is limited, in particular at elevated temperatures (like body temperature), low protein concentration and in the absence of antigen. Chain dissociation may be prevented by introducing Cys residues at appropriate locations into the framework of VH and VL, resulting in a disulfide crosslink (Glockshuber et al. 1990; Reiter et al. 1996) and yielding so-called disulfide-stabilized Fv fragments (dsFv). However, the gain in stability is often accompanied by poorer expression yields, as typically observed when introducing additional Cys residues and/or disulfide bridges into recombinant proteins.

A more widely applied approach is the construction of a fusion protein comprising the two variable domains artificially linked via a flexible peptide segment, yielding the so-called single chain Fv fragment (scFv) (Bird and Walker 1991). In this manner, VH and VL are not only covalently associated, but also the difficulty on the genetic level of simultaneously coexpressing two different polypeptide chains is avoided. Nevertheless, several disadvantages have been observed with this strategy, most notably the frequently reduced antigen-binding activity as a result of connecting one N-terminus—usually close to the combining site—with the peptide linker, which can lead to conformational changes or steric hindrance during antigen binding. This is especially the case for scFvs that are originally derived from full length MAbs and not directly selected from V-gene libraries (e.g. via phage display).

The scFv format also shows other undesired properties, such as low folding efficiency upon expression in *E. coli*, enhanced aggregation and, importantly, a pronounced tendency to form oligomers (Arndt et al. 1998; Demarest and Glaser 2008; Power et al. 2003), which can significantly hamper bioprocess development. Although numerous attempts were made to vary the length and amino acid sequence of the peptide linker and to alter the mutual order of the VH and VL domains within the fusion protein, these caveats have not been fundamentally alleviated and, therefore, laborious individual optimization is needed (Strohl and Strohl 2012). This may explain why, despite their flourishing in biomedical research for more than 25 years, no scFv has been approved as a biopharmaceutical to date (Nelson 2010). The currently most advanced scFv under clinical development (beside an immunotoxin fusion protein discussed below) is a PEG-linked conjugate with doxorubicin-charged liposomes targeting HER2, MM-302 (Merrimack, <http://merrimackpharma.com>), that has entered phase II/III studies for the treatment of breast cancer (Reichert 2015; Nielsen et al. 2002; Wickham et al. 2010).

Still, there were attempts to generate scFvs with elevated stability, for example using the so-called Immuna[®] screening platform (ESBAtch, <http://www.esbatech.com>) which involves the generation of stable humanized single-chain Fv fragments from rabbit monoclonal antibodies (Borras et al. 2010). A few scFv candidates, directed against TNF α (ESBA105) and VEGF were developed up to clinical phase

II for topical application on the eye (Strohl and Strohl 2012; Thiel et al. 2013). Further development was pursued in the form of the PENTRA[®]body technology (Delenex, <http://www.delenex.com>) for local application in dermatology, where the anti-TNF α scFv (DLX105) appeared to induce a clinical response in psoriasis patients after intradermal administration (Tsianakas et al. 2014).

In the area of antimicrobial therapy, Efungumab (Mycograb[™]) (Pachl et al. 2006), which binds to the heat shock protein 90 of *Candida albicans*, and Aurograb[™] (NCT00217841), an scFv directed against a surface protein of methicillin resistant *Staphylococcus aureus*, were brought to advanced clinical stages before their development was discontinued. Efungumab formed aggregates in solution, was contaminated with host proteins, and administration was associated with cytokine release while no treatment benefit was seen (EMEA 2007). In fact, reevaluation of its in vitro activity indicated that the presumed antifungal potentiation of amphotericin B was a nonspecific effect that was also seen with a wide range of unrelated proteins (Richie et al. 2012). Aurograb was discontinued following a review of phase II data showing lack of efficacy (Nelson 2010).

Other scFvs were clinically exploited as part of fusion proteins, either with distinct functional modules or in bispecific form, as will be discussed in greater detail further below. For example, Darleukin (L19-IL2) is a fusion protein between the scFv L19 selected from a phage display library against the extra-domain B (ED-B) of oncofetal fibronectin (a splice form specific for neovasculature described below) and the cytokine interleukin-2 (IL-2) to trigger immunopotentiating and antineoplastic activities. This immunocytokine has been developed as combination therapy with dacarbazine for the treatment of melanoma in several clinical studies up to phase II (Philogen, <http://www.philogen.com>). The same L19 scFv moiety is also under clinical investigation in different formats and/or combination therapies, for example as TNF α fusion protein, as well as for radio-immunotherapy (RIT). While initially L19 was described as a high-affinity scFv having a remarkable K_D in the low picomolar range (Pini et al. 1998), a significantly diminished potency was measured when reformatted to a Fab (Gebauer et al. 2013). Indeed, detailed inspection of the scFv revealed its propensity to form a stable homodimer, and this form showed much enhanced ED-B binding activity (most likely due to an avidity effect) in a head to head comparison with both the isolated monomeric scFv and the inherently monovalent Fab.

In a similar fusion protein approach, an immunotoxin (VB4-845) has been developed by combining a humanized anti-EpCAM scFv with a truncated fragment of *Pseudomonas* exotoxin A (Premsukh et al. 2011). This biological drug candidate is in phase II clinical trials (Viventia, <http://www.viventia.com>) and has shown promising results in the treatment of loco-regional tumors such as non-invasive-bladder cancer (Vicinium[™]) as well as recurrent refractory cancer of the head and neck (Proxinium[™]). Notably, to allow repeated systemic dosing, VB4-845 was recently reformatted as a Fab fragment fused to a deimmunised version of the plant proteotoxin bouganin via a furin-cleavable linker (VB6-845) (Cizeau et al. 2009; Entwistle et al. 2012).

The currently most advanced recombinant immunotoxin utilizing an Fv moiety is Moxetumomab pasudotox (CAT-8015 or HA22), which is in phase III clinical

trials (Reichert 2013). This fusion protein comprises PE38, a catalytically active fragment of *Pseudomonas* exotoxin A, fused to an anti-CD22 Fv. Originally, this program had started with an scFv fragment derived from the anti-CD22 MAb RFB4; however, to increase protein stability an interchain disulfide bond was introduced instead of the peptide linker, thus converting the Fv moiety into the dsFv format (Kreitman and Pastan 2011). In a phase I trial for refractory hairy-cell leukemia (HCL), Moxetumomab pasudotox showed a high response rate and a remarkable number of complete remissions, despite some residual immunogenicity (Kreitman et al. 2012).

A clearly more robust functional antibody fragment is the Fab, comprising the entire light chain and the variable domain and first constant domain of the heavy chain (also known as Fd fragment). Due to the additional tight packing between the pair of constant domains from both chains, compared with the association between VH and VL alone, and an interchain disulfide bond that is usually located close to the C-termini, the Fab is a structurally well-defined protein with high stability similar to a full size Ig. Thus, Fabs were considered early on for medical applications wherein either an Fc effector region is not needed or one of the usually beneficial features of intact MAbs is detrimental in the clinical context, for example the long circulation or the bivalency which may cause cellular signaling via receptor clustering. The first Fabs for clinical use were prepared according to the classical route by proteolysis from polyclonal antibodies elicited in immunized sheep and have served as antidotes to treat acute intoxications since decades (Holliger and Hudson 2005). Also, several Fabs were approved as diagnostic reagents for radio-immuno imaging of tumors via single-photon emission computed tomography (SPECT) in vivo: ^{99m}Tc -Sulesomab (LeukoScan) and ^{99m}Tc -Bectumomab (LymphoScan) (Mendler et al. 2014).

The first Fab that was approved for therapy by the FDA in 1994 was Abciximab (RheoPro[®]), which binds to the platelet glycoprotein IIb/IIIa (integrin $\alpha 2\text{b}/\beta 3$) on blood platelets (Knight et al. 1995), thus inhibiting their aggregation in cardiovascular disease. Notably, this Fab is produced by papain cleavage from a full length chimeric MAb. In contrast, Ranibizumab (Lucentis[®]) was the first fully recombinant Fab produced in *E. coli* approved by the FDA in 2006 for treating wet age-related macular degeneration (AMD) by intravitreal injection (Rosenfeld et al. 2006). Ranibizumab (Y0317), which binds and neutralizes vascular endothelial growth factor (VEGF-A), was derived from the same parent mouse antibody as the humanized Ig Bevacizumab (Avastin[®]), previously developed for antiangiogenic cancer therapy, in a process involving in vitro affinity maturation via CDR mutagenesis and phage display (Chen et al. 1999).

The strictly monovalent nature and the lack of immunological effector functions are usually considered advantages of the Fab format, in particular if just the blocking of a biomolecular disease target or use as antidote is intended. However, the much shorter plasma half-life compared with full size MAbs, due to the smaller size and lack of endosomal recycling as explained in the next Chapter of this book, is a general disadvantage which hampers the broader clinical application of this protein class. One approach to circumvent this problem is PEGylation, as exemplified by

Certolizumab pegol (CDP-870, Cimzia[®]), a bacterially produced Fab directed against TNF α conjugated to a branched PEG chain of 40 kDa via a single Cys residue that was introduced into the hinge region (Melmed et al. 2008; Rose-John and Schooltink 2003). Cimzia is approved since 2008 for the treatment of Crohn's disease and RA (Blick and Curran 2007; Goel and Stephens 2010).

The number of marketed Fabs is still rather limited (Nelson 2010), despite previous optimistic expectations (Nelson and Reichert 2009). However, there is a robust clinical pipeline for this class of Ig fragments, often as PEGylated versions, with examples from major pharma and biotech companies: anti-TNF α Afelimomab (AZD9773, MAK195F, CytoFab[™]) for the treatment of severe sepsis; a PEGylated Fab directed against the type III secretion system of *Pseudomonas aeruginosa* (KB-001); anti-Factor D Lampalizumab (RG-7417) for treatment of dry AMD; anti-C5 Fab LFG316A against the same condition as well as other inflammatory eye diseases; the PEGylated humanised Fab CDP7657 developed as a monovalent CD40L antagonist for the treatment of systemic lupus erythematosus; and a humanized Fab (Idarucizumab, BI655075) destined as an antidote for dabigatran etexilate mesylate (Pradaxa[®]) to reverse its anticoagulation effect (Brennan 2014; Reichert 2015; Strohl and Strohl 2012).

Apart from scFv and Fab, another class of small Ig fragments known as domain antibodies (dAbs) or Nanobodies has attracted attention (Holliger and Hudson 2005). These artificial binding proteins comprise an isolated variable domain and exhibit a binding site made of just three CDRs. Consequently, such single-domain Ig fragments (sdIgs) cannot be derived from intact MAbs but must be generated by different methods. This approach is complicated by the fact that an unpaired variable Ig domain usually exposes a significant hydrophobic surface area to solvent, which is otherwise shielded by association either with the second variable domain from the other Ig chain or, as often seen for light chains, via formation of so-called Bence Jones homo-dimers (Stevens et al. 1991). Especially in the case of isolated heavy chain variable domains (VH) this fundamental property normally causes aggregation (Davies and Riechmann 1994; Glockshuber et al. 1990) and/or non-specific adsorption.

Natural antibodies that are devoid of light chains were initially identified as an Ig subclass in camels (Hamers-Casterman et al. 1993; Muyldermans et al. 2001; Wiecek 2010). In such "heavy-chain" antibodies, which are composed of a pair of heavy chains that lack the C_H1 domain and sometimes have an extended hinge region, the VH domains have apparently evolved to stay soluble without hetero- or homo-dimerization. These camelid VH domains (dubbed VHH), which are found in camels, dromedaries, and also in llamas, reveal elevated surface hydrophilicity in the region that faces the VL domain in ordinary Igs. In addition, they have a much longer CDR-H3, which often participates in an additional disulfide bridge within the VH domain, thus partially shielding this interface region from solvent (Conrath et al. 2005; Muyldermans 2013).

Engineered camelid VHH domains (detached from the constant region) are exploited for disease diagnosis and therapy (Siontorou 2013) as Nanobodies[®] (Ablynx, <http://www.ablynx.com>). As these sdIgs intrinsically have a very short

plasma half-life, the corresponding drug candidates are usually developed as fusion proteins to enlarge size and retard kidney filtration. This involves combining either several copies of the same domain or domains with different specificities, including one that confers affinity to serum albumin in order to prolong circulation via endosomal recycling, a mechanism further explained in the following Chapter. Notably, the latter strategy also provides a general targeting activity in the case of cancer and inflammatory diseases as these tissues are known to actively take up albumin (Merlot et al. 2014; Tijink et al. 2008).

Two advanced clinical candidates are Caplacizumab and Ozoralizumab (Ablynx). Caplacizumab (ALX-0081 and ALX-0681, respectively, for i.v. and s.c. administration) is a bivalent Nanobody, with two domains fused in tandem, directed against the A1 domain of von Willebrand Factor (vWF) that has successfully completed phase II trials in hematology to treat the rare disorder thrombotic thrombocytopenic purpura (TTP) (Bartunek et al. 2013; Holz 2012). Ozoralizumab (ATN103 or PF-5230896) is a bivalent Nanobody comprising two anti-TNF α modules which are linked to a third Nanobody that binds to human serum albumin to extend plasma half-life. A phase IIa study demonstrated a statistically significant improvement of disease scores compared with placebo in patients with active RA (Comer et al. 2012). Similarly, a monovalent anti-IL-6 receptor (IL-6R) Nanobody (ALX-0061) comprising a bispecific fusion protein with a second Nanobody that confers binding activity towards albumin has completed phase IIa studies and is developed for the treatment of inflammatory diseases like RA (Van Beneden et al. 2014). Two additional Nanobodies are part of phase I programs: ALX-0171 is a trivalent Nanobody (three identical copies consecutively fused to each other) that neutralizes Respiratory Syncytial Virus (RSV) and offers potential for inhalational administration to treat pulmonary infections; ALX-0761 constitutes a bispecific half-life extended Nanobody that neutralizes both IL-17A and IL-17F—comprising an N-terminal IL-17F specific moiety, a C-terminal moiety that binds both IL-17A and F and a central module conferring albumin affinity—which has shown efficacy in a cynomolgus monkey model of RA (Vanheusden et al. 2013).

Beyond therapeutic applications, Nanobodies have attracted attention for *in vivo* imaging (Chakravarty et al. 2014; Vaneycken et al. 2011). In this setting, their small size and rapid renal elimination allows high tumor to blood contrast already shortly after administration. The lead optimized HER2-specific Nanobody 2Rs15d was prepared in current-good-manufacturing-practice grade, labeled with ^{68}Ga via a 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) derivative and assessed in biodistribution and positron emission tomography/computed tomography (PET/CT) studies using a murine SK-OV-3 tumor xenograft model, resulting in high-specific-contrast imaging of HER2-positive tumors with no observed toxicity (Xavier et al. 2013). ^{68}Ga -NOTA-2Rs15d is under development for first-in-human clinical trials.

Another type of sdIg has been derived from the human immune system as so-called domain antibodies, dAbs (Connelly 2005), by employing cloned repertoires of human V-genes together with stringent selection protocols to achieve high protein solubility, apart from target-binding activity (Holt et al. 2003). So far, two products have advanced to the clinical trial stage. An anti-TNF α dAb-Fc fusion protein

(Placulumab, ART-621, CEP-37247) (Gay et al. 2010) was tested in a phase II clinical study (NCT00928317) for the treatment of stable plaque psoriasis and RA (Strohl and Strohl 2012). Another dAb (dubbed AlbuAb™), which was selected to bind with affinities in the mid nM range to serum albumin from various species, was initially employed to create a fusion protein with the interleukin-1 receptor antagonist (IL-1Ra, also known as Anakinra), thus resulting in an immunomodulator for RA treatment that showed extended plasma half-life in a mouse model (Holt et al. 2008). AlbuAb was also combined with some bioactive peptides, in particular the orexigenic peptide YY (PYY) and Exendin-4, a clinically approved GLP-1 mimetic (Bao et al. 2013). A recombinant fusion protein of AlbuAb with Exendin-4 (GSK2374697) was investigated in a phase I study in normal and obese healthy volunteers (Hodge et al. 2013).

An alternative source of naturally occurring sDIGs are the so-called Ig novel antigen receptors (IgNARs) of shark (Kovaleva et al. 2014). Antigen-specific VNAR fragments of these receptors have similar structure and properties as VHH from camels and llamas (Stanfield et al. 2004; Streltsov et al. 2004), and they can be cloned as isolated proteins either from immunized animals or selected *in vitro* from combinatorial libraries. However, despite some efforts to exploit the shark VNAR format for therapeutic applications by biotech companies, e.g. AdAlta (<http://adalta.com.au>) and Haptogen/Wyeth (<http://www.wyeth.co.za>), clinical applications have not thus far been publicized (Zielonka et al. 2015).

The Concept of Alternative Protein Scaffolds

sDIGs offer a kind of conservative approach for generating MAb biobetters insofar as the methods for their selection (*in vivo* or *in vitro*) as well as their biophysical properties are related to conventionally engineered antibody fragments, except that they do not rely on the characteristic light/heavy chain association of natural human Igs. However, it is still not fully clear whether all sDIGs behave as truly monomeric proteins (Schiefner et al. 2011). At least in some cases, X-ray structural analysis has indicated a mode of dimerization in the crystal packing that resembles either the VH/VL pairing of classical Igs or the structurally analogous light chain homo-association that is typical for Bence-Jones proteins (George et al. 2014; Jespers et al. 2004b). Thus, like for scFvs, intrinsic oligomerization and aggregation propensities may affect bioprocess development and/or biological drug formulation and must be carefully assessed in each instance.

Interestingly, however, proteins with non-Ig fold can also be furnished with novel binding sites. This has been achieved using the methodology of combinatorial biotechnology, that is site-directed random mutagenesis in combination with phage display or other molecular selection techniques (Rothe et al. 2006; Skerra 2003). The resulting class of novel alternative binding reagents has also become known as engineered “protein scaffolds” (Skerra 2000a), illustrating the fact that a

structurally rigid natural protein is utilized either to modify an existing binding site towards a prescribed target or to implement a new one. This development has triggered a paradigm shift since antibodies have no longer to be considered as a unique class of binding reagents in biomedical research or biotechnology (Sheridan 2007; Skerra 2003) and, eventually, it has enabled the concept of biobetters discussed in this Chapter.

Usually, a protein scaffold suitable for therapeutic applications may be derived from either of two sources: (1) a robust and small monomeric soluble protein (e.g. a lipocalin or a Kunitz protease inhibitor—for details on the latter, see (Fiedler and Skerra 2014; Nixon et al. 2014)) or (2) a stably folded domain excised from a cell surface (e.g. protein A or fibronectin) or cytoskeletal protein (e.g. ankyrin). Compared with antibodies or their recombinant fragments, such protein scaffolds promise practical advantages, in particular elevated stability and high production yield in microbial expression systems, together with an independent intellectual property situation (Fiedler and Skerra 2014).

As these novel binding proteins are obtained via biomolecular engineering *in vitro*, with the primary goal to achieve tight and highly specific target-binding activity, they can also be subjected to additional selection schemes in order to address other desired properties such as solubility, thermal stability and protease resistance (Jermutus et al. 2001; Jespers et al. 2004a; Jung et al. 1999; Sieber et al. 1998). Resulting protein reagents are expected to provide beneficial properties for subsequent drug development and manufacturing as a built-in feature. Since the effort to generate and/or develop such alternative binding proteins still is greater than that for preparation of a conventional antibody (or a recombinant Ig fragment), most of the ongoing activities in this area are directed towards therapeutic use—instead of aiming at research or *in vitro* diagnostic reagents—hence offering the chance of a high return on investment from a business perspective. This has led to a series of corresponding biobetter formats developed by biotech companies, some of which have already been adopted by the pharmaceutical industry, as will be described in detail in the following sections.

So far, more than 50 different protein scaffolds have been proposed during the past two decades and these approaches have been extensively summarized in several recent reviews (Binz et al. 2005; Gebauer and Skerra 2009; Gill and Damle 2006; Hey et al. 2005; Mintz and Crea 2013; Nuttall and Walsh 2008; Wurch et al. 2012). This field emerged in the late 1990s with research on three different protein scaffolds, initially: (1) the Z-domain of *staphylococcal* protein A, a three-helix bundle of 58 residues, providing an interface for protein binding on two of its α -helices, leading to the Affibodies[®] (Nord et al. 1995); (2) the 10th extracellular domain of human fibronectin III (¹⁰FN3), which adopts an Ig-like β -sandwich fold (94 residues) with two to three exposed loops but lacks the central disulfide bridge, leading to the Monobodies (Koide et al. 1998), subsequently also dubbed Adnectins[™]; (3) the lipocalins, comprising a diverse family of soluble β -barrel proteins that naturally form binding sites for small ligands with four structurally variable loops, resulting in the Anticalins[®] (Beste et al. 1999).

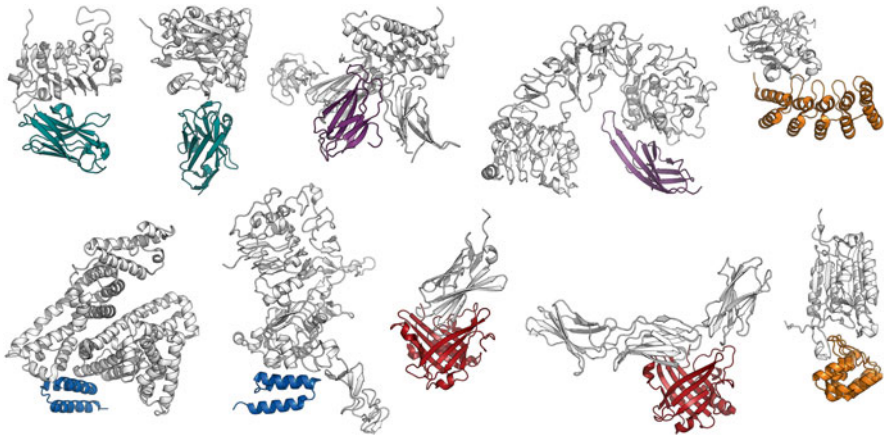


Fig. 1 Structural comparison between single domain Ig fragments and advanced alternative protein scaffolds (shown from left to right in *different colors*), each in complex with a biomedically relevant target protein (*light grey*): Nanobody (*green*) and EGFR fragment (PDB entry: 4KRL); Nanobody (*green*) and ricin (4LHQ); AdNectin (*violet*) and IL23 heterodimer (3QWR); AdNectin (*violet*) and EGFR extracellular region (3QWQ); DARPin (*orange*) and HER2 fragment (4HRL); Affibody (albumin binding domain; *blue*) and albumin (1TF0); Affibody (*blue*) and HER2 extracellular region (3MZW); Anticalin (*red*) and CTLA-4 extracellular region (3BX7); Anticalin (*red*) and ED-B as part of a three-domain fibronectin fragment (4GH7); DARPin (*orange*) and caspase 3 (2XZT)

Whereas the Affibodies were initially derived from a bacterial protein, Adnectins and Anticalins are both based on endogenous human proteins. In contrast, the designed ankyrin repeat proteins, DARPins, were more recently proposed as an alternative scaffold as result of a protein design effort. In this case an artificial consensus sequence was derived from a series of known ankyrin repeat proteins, which are present in virtually all species and provide a rigid interface arising from modules with typically two anti-parallel α -helices and a β -turn as the underlying principle (Binz et al. 2003). Together, these four scaffolds (Fig. 1) currently constitute the most advanced approaches in this field, each with multiple target specificities exemplified in basic research as well as preclinical studies and at least one drug candidate tested in the clinic (Gebauer and Skerra 2009).

Otherwise, the field of engineered protein scaffolds has undergone some consolidation and appears to focus on those few that may potentially yield products with commercial value. In particular, technical demands at the outset of biopharmaceutical drug development left behind many protein scaffolds that were once proposed in an academic setting, but never matured beyond an initial model case study (Gebauer and Skerra 2009). As result, only few scaffold technologies, in particular those mentioned above, have successfully expanded after first in vitro proof of concept and now see increasing application toward medical use, as discussed in the following sections.

Adnectins

The fibronectin type III domain (FN3), a small (10 kDa) autonomous folding unit found in the abundant extracellular matrix proteins fibronectin and tenascin, as well as in a large variety of other multidomain cell adhesion proteins (Hohenester and Engel 2002), was one of the first protein scaffolds employed for generating novel binding sites. The FN3 structure closely resembles the Ig domain fold, exhibiting three exposed loops, termed BC, DE, and FG, analogous to the CDRs described further above. In contrast, the FN3 domain lacks the central disulfide bond which normally connects the sandwich of β -sheets. Despite their conserved structure, individual FN3 domains show considerable sequence divergence, which hinted at their tolerance for mutations introduced in order to implement desired binding functions (Koide et al. 1998).

FN3 random libraries were based on the 10th type III (10 FN3) domain from human fibronectin to generate antibody (or, more precisely, sdIg) mimics initially by randomizing the BC and FG loops and, later, also the DE loop (Koide et al. 1998; Lipovsek et al. 2007; Xu et al. 2002). The first variant of this scaffold that had a significant, though still moderate affinity ($K_D \approx 5 \mu\text{M}$) for ubiquitin as a model target was selected from a phagemid display library and called “Monobody”. In combination with mRNA display technology, this scaffold was subsequently commercialized as “Trinectin” and continued as “Adnectin™” (Lipovsek 2011).

The design of the 10 FN3-based random libraries was successively improved using insights from synthetic antibody libraries, including loop length variation and bias in amino acid composition within the binding site, which eventually allowed the generation of high-affinity binding proteins against various targets (with K_D values down to the pM range) (Hackel et al. 2008, 2010; Hackel and Wittrup 2010; Koide et al. 2007). Furthermore, the 10 FN3 scaffold was investigated for the potential to utilize all six loops—at both ends of the β -sandwich structure—to implement binding activities (Batori et al. 2002). No consensus has yet emerged as to which loop design strategy is most effective, although some studies indicate that the BC and FG loops, which are topologically equivalent to the CDRs H1 and H3 of a VH domain, are most important for target binding (Koide et al. 2007). Recently, an alternative recognition mode was described that utilizes a concave binding site on the surface of the β -sandwich (Gilbreth and Koide 2012; Ramamurthy et al. 2012). Hence, the wedge-shaped 10 FN3 scaffold offers the possibility to address either cleft-like epitopes, e.g. on interleukin 23 (IL-23), or those with a convex shape, e.g. on epidermal growth factor receptor (EGFR) (Fig. 1), depending on the interface.

In 2006 the first Adnectin, Pegdinetanib (CT-322, BMS-844203, Angiocept), which specifically inhibits the vascular endothelial growth factor receptor 2 (VEGF-R2), i.e. the primary receptor in mediating tumor angiogenesis, entered clinical trials (Bloom and Calabro 2009; Dineen et al. 2008; Mamluk et al. 2010). Pegdinetanib, whose penultimate Cys residue (no. 93) is modified with a doubly methoxy-PEG-derivatized (40 kDa) maleimide, binds VEGF-R2 *in vitro* with a K_D value of 11 nM (Mamluk et al. 2010). The first in human study confirmed the

favorable pharmacokinetics of the PEGylated protein scaffold, showing slow plasma clearance with $\tau_{1/2}$ of 3–4 days (Tolcher et al. 2011). Notably, the majority of patients developed anti-drug antibodies (ADAs), which appeared to be specifically directed against the engineered binding loops, as no cross-reactivity with the original 10 FN3 scaffold was seen. However, no adverse events associated with the ADA response appeared in this phase I trial (Tolcher et al. 2011). Nevertheless, a novel design of the Adnectin libraries aiming at eliminating immunogenic hot spots was recently published (Davis et al. 2013). In this approach, key areas within the β -strand B, the BC loop and β -strand C were kept as wild-type sequence while modifications were introduced into other regions of the scaffold to achieve high affinity target binding. Pegdinetanib was subsequently evaluated in multiple phase II trials to assess its applicability in a range of cancers including non-small cell lung cancer, glioblastoma multiforme and metastatic colorectal cancer.

Recently, a phase I clinical study of a second Adnectin (BMS-986089), a myostatin inhibitor developed to enhance muscle growth in indications such as muscular dystrophy or sarcopenia in older people (Cload et al. 2014), was initiated (BMS, <http://www.b-ms.co.uk/research/pipeline>). Myostatin is a secreted protein and member of the TGF- β family that is produced primarily in skeletal muscle cells, preventing muscle cell growth and differentiation, thus being of great interest as a therapeutic target in myopathies (Dschietzig 2014).

Other Adnectins in preclinical development include a fusion protein between fibroblast growth factor 21 (FGF21) and an “Adnectin pharmacokinetic enhancer” (AdPKE) as a potential therapeutic agent in diabetes. In FGF21-AdPKE the Adnectin moiety confers binding activity towards human as well as monkey serum albumin, thus extending the very short circulation half-life of human FGF21 from 4 to 96 h in cynomolgus monkeys (Klöhn et al. 2013), potentially offering once weekly subcutaneous dosing in human patients (Mukherjee 2013). The second Adnectin in advanced preclinical stage, BMS-938790 (likely in a PEGylated form), is a potent inhibitor of the inflammatory cytokine IL-23, whose increased levels are associated with several autoimmune diseases such as RA, multiple sclerosis (MS) and Crohn’s disease (Das Gupta 2014).

Apart from this 10 FN3 scaffold, similar domains derived from the same fibronectin or related extracellular matrix proteins were also employed to generate scaffold libraries. For example, so-called PronectinsTM were developed on the basis of the 14th type III domain of fibronectin utilizing a bioinformatics approach (Mintz and Crea 2013). The Pronectin phage display library (Cappuccilli et al. 2014) was designed after analysis of the natural loop diversity found in thousands of non-redundant human FN3 sequences and comprises a combination of three sublibraries, each having mutations in only two of the exposed BC, DE and FG loops (Protelica, <http://www.protelica.com>).

Another related concept was recently adopted during development of the Centyrins (Jacobs et al. 2012). In this case, random libraries were built on an artificial consensus sequence derived from the tenascin FN3 domains. Not only the CDR-like loops but also exposed side chains on some of the β -strands were randomized to generate novel binding proteins, e.g. against human hepatocyte growth factor

receptor (HGFR, c-MET), murine IL-17A and rat TNF α (Diem et al. 2014). Centyrins also offer potential as bispecific reagents (for broader discussion see further below), for example c-MET \times EGFR, engineered as a fusion protein of two Centyrin modules that are additionally linked to an albumin-binding domain (Klein et al. 2013).

Affibodies

Affibody molecules are small (7 kDa) artificial binding proteins originally derived from protein Z (Nilsson et al. 1987), an isolated engineered IgG-binding domain of the “protein A” found on the bacterial cell surface of *Staphylococcus aureus*. Combinatorial phage display libraries were generated by targeting random mutagenesis at residues exposed on the first two α -helices of the three-helix bundle scaffold in a region that is naturally involved in binding to the Fc part of antibodies (Nord et al. 1995, 1997). To date, a large number of Affibodies have been described, not only as research or purification reagents, but also exerting binding activities towards various medically relevant protein targets, e.g. insulin, fibrinogen, transferrin, TNF α , IL-8, gp120, CD28, IgA, IgE, IgM, EGFR, HER2 (Löfblom et al. 2010) and, recently, ErbB3/HER3 (Kronqvist et al. 2011). One Affibody, Z_{HER2:342}, specific for the breast cancer target HER2, constitutes probably the most intensely studied protein of this class (>200 hits in PubMed; <http://www.ncbi.nlm.nih.gov>). This Affibody, which after extensive in vitro maturation shows extraordinary affinity for its target ($K_D=22$ pM) (Orlova et al. 2006), will be discussed further below.

Affibodies have been predominantly exploited for use in diagnostic settings, especially molecular imaging, where they offer beneficial features. The small size of this scaffold ensures rapid tissue penetration, fast blood clearance and, moreover, allows production via solid-phase peptide synthesis, permitting the direct site-specific incorporation of various chemical functionalities such as fluorescent probes or chelating groups for radioactive metal ions. The generally high stability, independent of disulfide bonds, in concert with the high solubility of most Affibodies further enable these molecules to resist even harsh chemical labelling conditions while retaining binding activity. Nevertheless, some Affibodies may adopt molten globule structures in the absence of their target proteins, probably due to the extensive mutagenesis of secondary structural elements (Wahlberg et al. 2003). Also, one Affibody was described that surprisingly adapts to the hairpin conformation of its target, the Alzheimer amyloid- β peptide, by dimerizing and forming a four-stranded intermolecular β -sheet, thus giving rise to an extended groove (Hoyer et al. 2008).

Up to now, Affibodies were developed in the clinic mainly as tracers for HER2-specific molecular imaging. In 2010, the ⁶⁸Ga- or ¹¹¹In-radiolabeled chelator-functionalized Affibody ABY-002 (DOTA-Z_{HER2:342-pep2}) demonstrated for the first time that it is possible to localize via SPECT or PET/CT imaging even very small HER2-positive cancer lesions in patients with recurrent metastatic breast cancer and, notably, as early as 2 h after injection of the tracer (Baum et al. 2010). Imaging

with ABY-002 was also successful in a patient that had already received Herceptin treatment. Indeed, ABY-002 binds to an epitope distinct from those of Trastuzumab (Herceptin[®]) and Pertuzumab (Perjeta[®]) (Eigenbrot et al. 2010) and, hence, this tracer may be useful for monitoring changes in HER2 expression during therapeutic intervention with these antibodies.

Compared to the current standard of tumor imaging, ¹⁸F-FDG PET, which indicates increased glucose metabolism, the uptake of ABY-002 in some metastatic lesions was distinct, either due to differing levels of HER2 expression or to the limited sensitivity of ¹⁸F-FDG PET/CT in early-stage cancer (Baum et al. 2010). However, this study also showed that the Affibody was mainly cleared via the liver, which obscured imaging of metastases in this organ. To address this issue, the scaffold of ABY-002 was reengineered by substituting surface-exposed side chains outside of the target interface to increase hydrophilicity, to achieve higher thermal and chemical stability, and to reduce unwanted binding to Ig as well as normal tissues, thus effecting elimination predominantly via the kidney (Feldwisch et al. 2010). The resulting anti-HER2 Affibody, Z_{HER2:2891}, had an optimized surface that, despite the considerable deviation from the parental scaffold, fully retained tumor targeting functionality in vitro and in vivo in a mouse xenograft model (Ahlgren et al. 2010).

The clinical safety and efficacy of Z_{HER2:2891} for HER2 imaging in metastatic breast cancer was recently evaluated (Sörensen et al. 2014). In contrast to the parental molecule ABY-002 (DOTA-Z_{HER2:342-pep2}), the new lead candidate ABY-025 ([MMA-DOTA-Cys61]-Z_{HER2:2891}-Cys) can also be produced (apart from peptide synthesis) by recombinant expression in *E. coli* (Feldwisch et al. 2010) and, via a C-terminally introduced Cys residue (Z_{HER2:2891}-Cys), coupled to the chelator maleimido-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). The resulting radiolabeled molecule, ¹¹¹In-ABY-025, allowed diagnosis of HER2 expression in seven patients while lacking the disadvantage of ABY-002, as the highest uptake of the new Affibody was seen in the kidneys (Sörensen et al. 2014). Probably as a benefit of the reengineered scaffold of ABY-025, and in good agreement with previous animal experiments (Ahlgren et al. 2010), no anti-ABY-025 antibodies were observed after single administration (100 µg). In one patient, molecular imaging with ABY-025 in conjunction with ¹⁸F-FDG PET enabled detection of a converted receptor status on metastases despite a HER2-positive primary tumor, and, in another patient, a brain lesion was diagnosed that was not seen via previous ¹⁸F-FDG PET alone (Sörensen et al. 2014).

Anti-HER2 Affibodies were also labeled with radionuclides suitable for RIT. Generally, the small size and rapid pharmacokinetics (Orlova et al. 2009), which is compatible with very short-lived positron emitting radionuclides like ⁶⁸Ga, is particularly advantageous for molecular imaging, especially if compared to imaging with ⁸⁹Zr-Trastuzumab, whose optimal time point to assess tumor accumulation is 4–5 days post injection (Dijkers et al. 2010). However, pronounced renal reabsorption of the Affibody radioconjugates and, thus, high levels of kidney exposure constitute a potential safety problem for therapeutic applications. Two approaches were evaluated to alleviate this problem for Affibodies: first, reducing renal uptake by

modifying the pharmacokinetics via fusion to a second Affibody module that effects reversible binding to albumin (Orlova et al. 2013; Tolmachev et al. 2007, 2009b) and, second, decreasing renal retention of radioactivity by means of modified labeling chemistry (Orlova et al. 2010; Tolmachev et al. 2009a).

Notably, use of serum albumin as a drug carrier is often not only paired with longer circulation half-lives but also favors tumor targeting as albumin is known to preferentially localize to malignant versus normal tissue (Kratz 2008; Merlot et al. 2014). In the novel Affibody construct, ^{111}In -ABY-027, the same target-specific molecule used for ABY-025 was fused to a small, high-affinity albumin-binding domain, ABD₀₃₅, an engineered bacterial three-helix bundle protein with similar structure as the Z-domain (Jonsson et al. 2008), and site-specifically radiolabeled. In a mouse xenograft model ^{111}In -ABY-027 showed a prolonged kinetic profile and significantly lower renal accumulation of radioactivity together with an increase in the delivered dose to the tumor compared to previous anti-HER2 Affibody reagents (Orlova et al. 2013). The impact of the nature and position of the radionuclide chelator within the protein molecule on the biodistribution of Affibodies was demonstrated using Z_{HER2:342} with different radiolabels, including mercaptoacetyl-containing peptide-based chelators for $^{99\text{m}}\text{Tc}$ -labelling. In this case, the use of more hydrophilic chelators resulted in a switch from predominantly hepatobiliary excretion to renal excretion, which significantly improved image contrast in the abdominal area (Tolmachev and Orlova 2009).

While most of the molecular imaging studies were performed with Affibodies specific for HER2, this approach was recently complemented by Affibodies targeting other important members of the transmembrane receptor tyrosine-kinase family such as EGFR (Malmberg et al. 2011; Tolmachev et al. 2010) and PDGF-R β (Strand et al. 2014; Tolmachev et al. 2014), which are both overexpressed in many malignancies.

Anticalins

Anticalins are antibody mimetics with designed ligand-binding properties derived from the natural scaffold of the lipocalins, a family of compact, soluble proteins that are abundant in human plasma and other body fluids and serve to transport or scavenge low molecular weight molecules. Lipocalins are found in many organisms including vertebrates, insects, plants and even bacteria (Åkerström et al. 2006). These functionally diverse small proteins, with a single polypeptide chain of 160–180 residues, generally form complexes with chemically sensitive biological compounds, in particular, vitamins, steroids, signaling molecules, lipids and other secondary metabolites. In humans, more than 12 different lipocalins have been identified thus far (Breustedt et al. 2006; Schiefner and Skerra 2015). Due to their high abundance in blood and their capacity for binding various physiologically active compounds, even some natural lipocalins have been considered for

therapeutic use. In this regard, not only endogenous human lipocalins attracted attention, but also lipocalins from blood-sucking insects with medically relevant binding activities as well as low immunogenicity in humans.

For example, human neutrophil gelatinase-associated lipocalin (NGAL, also known as lipocalin 2, Lcn2, or as siderocalin), which tightly binds the catechol-type siderophore Fe^{III}-enterochelin/enterobactin (Goetz et al. 2002) as well as other siderophores of mycobacteria, was proposed for anti-infective drug development (Holmes et al. 2005). Apart from its potential use as antibacterial agent, its therapeutic application for renal protection from ischemia-reperfusion injury was suggested—based on the notion that NGAL also mediates iron trafficking in kidney epithelial cells (Mori et al. 2005). Furthermore, two insect lipocalins have attracted interest for clinical use: the histamine-binding protein (HBP) from the saliva of the tick *Rhipicephalus appendiculatus* (Paesen et al. 1999) and the C5 complement inhibitor (OmCI) from the soft tick *Ornithodoros moubata* (Barratt-Due et al. 2011; Fredslund et al. 2008).

In vivo efficacy was demonstrated for recombinant HBP (rEV131) in preclinical studies, where it was found to inhibit murine allergic asthma (Couillin et al. 2004), and this lipocalin was further evaluated up to a phase II clinical trial (NCT00353964). Preclinical studies of recombinant OmCI, initially termed rEV576, showed effective inhibition of complement and significantly reduced weakness in vivo in two rat models of experimentally acquired myasthenia gravis (Soltys et al. 2009). Other preclinical studies of OmCI demonstrated a protective effect against neural injury in an in vitro mouse model of the Miller Fisher syndrome (Halstead et al. 2008) as well as reduction of excessive inflammatory reactions associated with severe forms of Influenza A virus infections in mice (Garcia et al. 2013). OmCI, meanwhile named Coversin (Varleigh Immuno Pharmaceuticals, <http://www.vipimmunopharma.com>), has been evaluated in a phase I clinical study (Weston-Davies et al. 2013).

The central folding motif of the lipocalins is a structurally highly conserved β -barrel: eight antiparallel β -strands wind around a central axis and form an almost cylindrical cup-shaped structure. The bottom of this β -barrel is closed by short loops, and a hydrophobic core is formed there by densely packed amino acid side chains. At the open end, a set of four loops forms the entrance to the ligand pocket. While in general the amino acid sequence homology among the lipocalins is extremely low (Flower 1996), these four loops additionally exhibit large differences in their lengths and backbone conformations, in line with the diverse ligand specificities seen for the different family members (Skerra 2000b; Schiefner and Skerra 2015). Thus, in principle, the ligand-binding sites of lipocalins with their four structurally hypervariable loops resemble those of Igs with their set of six CDRs. However, a major advantage of the lipocalin scaffold is its simple composition of just one rather short polypeptide chain. Thus, biotechnological production and purification of lipocalins and their engineered variants is much easier and more cost-effective than antibody production, especially in connection with an *E. coli* expression system.

The peculiar ligand-binding site of the lipocalins with its (outside the Ig superfamily) unprecedented structural plasticity has prompted the engineering of novel

antigen-binding proteins with antibody-like properties that were named “Anticalins” (Beste et al. 1999; Skerra 2001). Employing the methods of combinatorial protein design to alter the four hypervariable loops at the entrance to the ligand pocket as well as adjoining β -strand regions in a directed manner, artificial lipocalins with novel ligand specificities for prescribed targets can be generated (Gebauer and Skerra 2012). Meanwhile, several different natural lipocalin scaffolds for which three-dimensional structures became available have been utilized to generate Anticalins (Richter et al. 2014): e.g. the bilin-binding protein (BBP) from the butterfly *Pieris brassicae*, the human apolipoprotein D (ApoD), human lipocalin 1 (Lcn1, also known as tear lipocalin, Tlc) and human lipocalin 2 (Lcn2, NGAL).

In the case of BBP, a blue pigment protein that naturally protects insects from oxidative stress (Schmidt and Skerra 1994), the first Anticalins with novel specificities were selected via phage display from a semi-synthetic random library to bind the dye fluorescein (Beste et al. 1999), the plant steroid digoxigenin (Schlehuber et al. 2000) and a phthalic ester plasticiser (Mercader and Skerra 2002). These findings demonstrated the high tolerance of the lipocalin scaffold for introduction of artificial side chains into the binding site on a wider scale and, with the help of site-directed mutagenesis studies and crystal structure analyses (Korndörfer et al. 2003a, b; Vopel et al. 2005), confirmed the fundamental similarity with the combining site of antibodies.

Building on this knowledge, Anticalins were subsequently designed on the basis of human lipocalins, in particular Lcn1 and Lcn2, with the goal to ensure high tolerability to patients upon therapeutic application (Mendler and Skerra 2013). Again, these Anticalins were generated via combinatorial protein engineering in an in vitro process that in principle resembles the humoral immune response against an antigen (Gebauer and Skerra 2012). One notable aspect of this technology is that due to the large differences between natural members of the lipocalin family each scaffold involves an individually optimized random library design. This pertains to choosing the most suitable set of residues to be targeted for mutagenesis, whereby the total number of randomized positions is generally limited according to theoretical considerations (Richter et al. 2014). In case of the Lcn2 scaffold it was nicely demonstrated that the library design can be optimized in a few iterative cycles, also taking advantage of X-ray structural information (Gebauer et al. 2013). The third generation Lcn2-based Anticalin library resulting from this endeavor is highly potent in yielding binding proteins against a wide range of biomolecular targets, showing high affinities in the nM to pM range directly after phage display selection from the naive library (Richter et al. 2014) while still offering the potential for further improvement via in vitro affinity maturation.

Anticalins have been selected against a series of disease-relevant protein antigens. An early example was the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, CD152), which has attracted considerable interest as target of immune-checkpoint inhibitors (Pardoll 2012). In agreement with its function as a negative regulatory T-cell co-receptor, the lead Anticalin showed potent blocking activity and, consequently, an immunostimulatory effect on the T-cell response in an animal model of infectious disease (Schönfeld et al. 2009).

Furthermore, this Anticalin has shown potential for the immunotherapy of cancer in preclinical studies (Pieris, <http://www.pieris-ag.com>). Another protein-specific Anticalin recognizes a disease-specific splice variant of the extracellular matrix protein fibronectin (Fn) exhibiting the extra-domain B (ED-B) (Gebauer et al. 2013). Since in adults ED-B-positive, so-called oncofetal Fn is almost exclusively expressed during tumor angiogenesis, apart from wound healing and some other pathophysiological states that involve neovascularization, it has emerged as a promising marker for various cancers and constitutes a validated target for *in vivo* imaging (Kaspar et al. 2006), as already mentioned further above. Cognate Anticalins show specific staining of ED-B positive cells in immunofluorescence microscopy and, notably, allow the detection of primary glioblastoma multiforme in human patients (Albrecht et al. [submitted](#)).

Beside Lcn2-based Anticalins, other human lipocalin scaffolds have been successfully applied to generate highly specific and functionally active binding proteins against medically relevant targets. The human Lcn1/Tlc scaffold served to develop an Anticalin that effectively blocks VEGF-A (Chekhonin et al. 2013) and constitutes a drug candidate for the treatment of solid cancers. This Anticalin, dubbed Angiocal[®] (PRS-050; conjugated with 40 kDa branched PEG), has been investigated as inhibitor of tumor angiogenesis in a first-in-human phase I trial, demonstrating safety and high tolerability as well as a terminal half-life ($\tau_{1/2}$) ranging from 5.5 to 7.0 days (Mross et al. 2013). Importantly, PRS-050 appeared to lack immunogenicity, based on the absence of an ADA response in 24 patients with post-baseline samples available, including six patients who received biweekly dosing and one who had received altogether 17 doses. Based on the encouraging data from this repeat dose escalating study, Angiocal shows promise for phase II clinical trials.

Recently, another Anticalin based on the Lcn1/Tlc scaffold (PRS-110; conjugated with 40 kDa branched PEG) was shown to act as a highly potent and specific c-Met antagonist with both ligand-dependent and ligand-independent activity in mouse xenograft models (Olwill et al. 2013). Moreover, a radiolabeled version of this PEGylated Anticalin, ⁸⁹Zr-PRS-110, allowed visualization of c-Met tumor expression via *in vivo* imaging in mice (Terwisscha van Scheltinga et al. 2014).

Like the natural lipocalins and as mentioned above for the BBP platform, Anticalins derived from a human scaffold, in particular Lcn2, can also tightly bind low molecular weight substances, i.e. hapten-like targets. This has been shown for an Anticalin developed with pM affinity against a metal-chelate complex comprising a lanthanide ion, e.g. Y^{III}, and a derivative of diethylenetriamine pentaacetic acid (DTPA) (Eggenstein et al. 2013; Kim et al. 2009), which offers a promising reagent for *in vivo* pretargeting radioimmuno therapy and diagnostics. Hapten-specific Anticalins based on the BBP scaffold have been investigated for therapeutic applications as well. The fluorescein-specific Anticalin FluA was used, after genetic fusion to an scFv fragment recognizing the Fn extra-domain A as angiogenesis marker, for pretargeted payload delivery in a mouse tumor xenograft model (Steiner et al. 2013). Also, an affinity-improved digoxigenin-binding Anticalin (DigiCal) showed promising results in a rat study as antidote to treat digitalis intoxication (Eyer et al. 2012).

High affinity Anticalins with blocking activity were also selected against peptides, for example the A β peptide (Rauth et al. [in preparation](#)), which plays a crucial role in the pathophysiology of Alzheimer's disease, and hepcidin (Hohlbaum et al. [2011](#)), a negative regulator of iron homeostasis relevant in anemia (Ruchala and Nemeth [2014](#)). The development of Anticalins against the hepcidin peptide has been funded by the European Commission via the EUROCALIN Consortium (EUROCALIN, <http://www.eurocalin-fp7.eu>) to promote clinical investigation. PRS-080, an anti-hepcidin Anticalin designed to treat anemia, has entered a phase I clinical trial in 2014 (Pieris). Generally, the lipocalin scaffold appears to offer the most versatile platform to engineer binding proteins against a wide range of target molecules with different sizes, shapes and biomolecular properties (Fig. 1), including the potential to create multispecific or multifunctional fusion proteins (e.g. so-called Duocalins). Furthermore, it is the only type of scaffold for which natural representatives have been subjected to biopharmaceutical development.

DARPinS

The so-called designed ankyrin repeat protein (DARPin) scaffold emerged from a consensus design approach (Binz et al. [2004](#); Forrer et al. [2004](#)) based on the highly abundant natural ankyrin repeat proteins, ARPs (Grove et al. [2008](#); Mosavi et al. [2002](#)). ARPs are found, for example, as cytoskeletal proteins, transcriptional initiators, cell cycle regulators and signal transducers mostly in the cellular cytoplasm, where they mediate protein-protein interactions. This protein class exhibits a rigid and also modular fold comprising characteristic repeat units of 33 amino acids, each with two anti-parallel α -helices that are mutually connected by a short loop and are linked to the next unit by an extended β -turn. Natural ARPs usually contain four to six of these repeats (while this number can rise up to ≥ 24 , e.g. for ankyrin itself), which are stacked on each other to form a compact, overall rod-shaped domain, also called linear solenoid (Fig. 1). To generate DARPinS, random amino acid substitutions are introduced into the β -turn and the first α -helix of each module, usually employing three repeat units (depending on the DARPin library), together with N- and C-terminal capping units. Typically, six potential target interaction positions per internal repeat are mutated in the artificial ARP consensus sequence.

From such combinatorial libraries DARPinS with high affinities have been selected, normally via ribosome display, against a variety of proteins, including several medically relevant targets: for example, HIV gp120 (Mann et al. [2013](#); Schweizer et al. [2008](#)), EpCAM (Stefan et al. [2011](#)), Alzheimer amyloid- β peptide (A β) (Hanenberg et al. [2014](#)) and, as will be described in greater detail below, VEGF-A (Stahl et al. [2013](#)). Since the advantageous biophysical properties of the parental ankyrin scaffold such as high-level expression, solubility, and stability are often retained in these DARPinS, they are also well suited for the generation of multispecific constructs (Molecular Partners, <http://www.molecularpartners.com>). Indeed, the therapeutic potential of DARPinS in the area of cancer therapy is supported

by the development of bispecific and/or biparatopic DARPins (Jost et al. 2013) as well as toxin fusions (Simon et al. 2014).

Furthermore, methods were established to extend the intrinsically very short plasma half-life of DARPins (Zahnd et al. 2010), which appears to be only few minutes in cynomolgus monkeys (Klöhn et al. 2013). To this end, DARPins were site-specifically functionalized using unique Cys residues or, alternatively, through incorporation of non-natural amino acids via metabolic pressure in order to allow selective conjugation with PEG, serum albumin and, optionally, with cytotoxins (Moody et al. 2014; Simon et al. 2013, 2014). An interesting concept of DARPins directed against tumor-specific cellular surface receptors such as HER2, EGFR and EpCAM aims at gene therapy: different DARPIn-targeted viruses were shown to efficiently discriminate between tissues, potentially offering cell type-specific *in vivo* gene delivery (Friedrich et al. 2013; Munch et al. 2013). Notably, viral envelope proteins fused with DARPins showed superior incorporation to corresponding fusion proteins with scFvs, which are typically used for this kind of approach (Munch et al. 2011), probably owing to the more robust nature of this non-Ig scaffold.

The lead clinical candidate DARPIn, Abicipar pegol (initially developed as MP0112, after reformulation named AGN-150998), is a potent antagonist of VEGF-A and inhibits all relevant subtypes with $IC_{50} \leq 10$ pM (Binz et al. 2014; Klöhn et al. 2013; Stahl et al. 2013). Abicipar pegol is currently tested in two human eye diseases related to neovascularization: age-related macular degeneration (AMD), a painless eye condition that leads to the gradual loss of central vision, and diabetic macular edema (DME) (Campochiaro et al. 2013). In two phase I/II clinical trials, MP0112 was shown to be well tolerated and to efficiently neutralize VEGF-A after a single intravitreal injection. However, application of the DARPIn was also associated with adverse events such as ocular inflammation in most of the patients, probably caused by impurities from the bacterially produced drug. MP0112 was subsequently reformulated into AGN-150998 (EudraCT 2011). Although the sponsor has not yet publicized which modifications were made, it appears that the formulation of AGN-150998 comprises a DARPIn covalently linked to a single molecule of methoxy maleimide polyethylene glycol 20 (mPEG20), possibly even containing a modified Fc part of human Ig (CROSS 2011; INN 2012).

Safety and efficacy of AGN-150998 were recently tested in a phase II clinical trial (REACH study), where the reformulated drug proved to be at least as effective as monthly dosed Ranibizumab (Lucentis), the standard of care for wet AMD, while showing longer duration of action (NCT01397409; Reuters 2014). However, in contrast to Ranibizumab, a humanized high affinity anti-VEGF Fab produced in *E. coli* (Rosenfeld et al. 2006) mentioned already further above, patients treated with AGN-150998 still experienced ocular inflammation, although to a lesser extent than in phase I (Campochiaro et al. 2013). Improvements to the manufacturing process for AGN-150998 are expected before commencing phase III studies (Bloomberg 2013).

Apart from this monospecific protein drug, the dual-antagonistic DARPIn MP0260, which is directed both against VEGF-A and platelet-derived growth factor B (PDGF-B), is under development for the treatment of wet AMD and related conditions (Molecular Partners). Considering that another anti-VEGF/anti-PDGF combination therapy of wet AMD, i.e. Fovista® with Ranibizumab, had already

shown superior efficacy in a phase II trial compared to Lucentis monotherapy (Boyer 2013), a bispecific “all-in-one” drug such as MP0260 might prove beneficial. However, in light of the potential complications and burden for the patient associated with repeated intravitreal drug injections for the treatment of chronic ocular diseases, an alternative route of administration would be highly desirable. Yet, only preclinical data were published so far with regard to the potential application of DARPins as eye-drops (Stahl et al. 2013).

Bispecific Constructs

Engineered protein scaffolds are no longer regarded as alternatives to conventional MABs just for the sake of intellectual property or other commercial aspects; rather, there is increasing recognition that they, together with robust Ig fragments, may enable unique therapeutic modes of action more easily owing to their small size and facile manipulation, possibly extending to bioprocess development. One area of great interest (Garber 2014) is the preparation of bispecific binding proteins, in particular by way of the modular fusion approaches mentioned above. In fact, despite the long history of bispecific MABs (Milstein 2000)—starting with hybrid hybridoma (quadroma) technology—this classical format still poses a challenge with regard to biomanufacturing, even though several strategies were recently developed to drive heterodimerization of the two halves of the Y-shaped Ig molecule more efficiently (Strohl and Strohl 2012).

One successful strategy that makes use of scFv fragments is the bispecific T-cell engager format, BiTE[®] (Bäuerle and Reinhardt 2009), directing the patient’s T-cell immune response to cancer cells by creating a physical link. This concept arose from the notion that bispecific MABs which redirect non-cognate T-lymphocytes to tumor cells were found to be generally more efficient in triggering cell killing or elimination than conventional MABs of the same target specificity (Riethmüller 2012; Weiner and Hillstrom 1991). To provide a simpler molecular format, BiTEs are composed of two flexibly linked scFvs, fused in tandem, one binding to CD3 as part of the T-cell receptor (TCR) complex and the other one to a predefined surface antigen on the tumor cell, for example CD19 on B-cell lymphomas. The small size of BiTEs allows bringing target and effector cells in close proximity, thereby enabling the formation of a cytolytic synapse and triggering tumor cell killing—a process that is normally seen only between T-cells and antigen presenting cells (Dreier et al. 2003; Hoffmann et al. 2005; Offner et al. 2006).

In the BiTE approach, systemic cytokine release and toxicities are low or negligible, probably as a result of the monovalent format, which does not activate the CD3 receptor on non-engaged T-cells in the absence of target cells (Brischwein et al. 2007). So far, BiTEs have been generated against several tumor-associated target molecules such as CD19, CD33, EpCAM, HER2, CEA, ephrin A2 (EphA2), and melanoma-associated chondroitin sulfate proteoglycan (MCSP) (Bäuerle et al. 2009).

Blinatumomab (AMG103, formerly MT103 or MEDI-538) (Löffler et al. 2000) was the first genetically engineered bispecific antibody that entered clinical trials

and constitutes the most advanced BiTE (Garber 2014). Besides CD3, it targets the B-cell surface marker CD19 which is ubiquitously expressed throughout all stages of B-cell differentiation and, in particular, present on virtually all tested malignant B-lineage cells in acute lymphoblastic leukemia (ALL) (Raponi et al. 2011). This biological drug candidate has been tested in combination with chemotherapy in a phase III trial in ALL patients who were not cured by chemotherapy alone and were given a poor prognosis (NCT02013167). A preceding large phase II study had confirmed antileukemic activity of Blinatumomab alone in a difficult-to-treat population with relapsed/refractory ALL (Topp et al. 2014). Considering these encouraging results, the FDA designated Blinatumomab in July 2014 as breakthrough therapy for ALL and, furthermore, this compound received orphan drug designation for the treatment of non-Hodgkin's lymphoma (NHL) (Viardot et al. 2013). Blinatumomab was approved by the FDA in the end of 2014 as Blincyto™ for treating a rare form of B-cell ALL (Sheridan 2015).

Another BiTE (AMG330) targets CD33, a cell surface marker of acute myeloid leukemia (AML), i.e. the most frequent form of acute leukemia in adults. Ex vivo studies of AMG330 with primary AML cells revealed potent cytotoxicity, showing efficient T-cell activation and expansion even in samples with low CD33 expression. Moreover, AMG330 did not modulate surface expression of CD33, suggesting that long-term exposure to the BiTE, as in the clinical application of Blinatumomab, should not diminish expression of the receptor (Krupka et al. 2014; Laszlo et al. 2014). Given the fact that all previous attempts to introduce novel targeted therapies for AML have failed to date—ultimately including the anti-CD33 antibody-drug conjugate (ADC) Mylotarg®, which had been approved in 2000 but was subsequently withdrawn from the market due to safety issues and limited benefit (Strohl and Strohl 2012)—it will be interesting to see if this BiTE may offer a more potent treatment.

The success of this bispecific format triggered the design of other scFv-based constructs with similar size and valence. A competing technology is the dual-affinity re-targeting (DART®) molecule (Johnson et al. 2010), a heterodimer composed of two polypeptide chains, each comprising a VH domain from one Fv fused to a VL domain from another one, thus resembling the classical Diabody format (Holliger et al. 1993). However, DARTs are covalently linked by a C-terminal interchain disulfide bond, which makes them independent of the stability of the natural VL/VH association and avoids domain exchange, which is known to affect conventional Diabodies (Johnson et al. 2010).

In head-to-head in vitro experiments comparing the DART and BiTE formats with the same Fv sequences, the CD19xCD3 DART was 4–60 fold more potent in redirecting cell killing (Moore et al. 2011). This was surprising insofar as the DART protein displayed only a twofold higher affinity for each target molecule. Consequently, this platform has been developed for different targets (MacroGenics, <http://www.macrogenics.com>). The first DART that entered phase I clinical study is MGD006 which binds the tumor target CD123 and redirects, via its CD3 specificity, T-cells against corresponding leukemic cells, resulting in the clearance of AML blasts in vitro and in vivo (Al Hussaini et al. 2013). Furthermore, the DART

MGD007, directed against gpA33, is under development for the treatment of colorectal cancer and other gastrointestinal tumors (MacroGenics).

The relatively short biological half-life of BiTEs and DARTs, which is only a few hours in humans (Hoffman and Gore 2014), requires an appropriate dosing schedule and/or formulation to ensure lasting activation of T-cells against target cells. Blinatumomab, for instance, is administered as continuous intravenous infusion over four weeks to achieve sufficient steady-state plasma levels of the drug.

In comparison, a more favorable pharmacokinetic profile has been described for TandAb[®]s (Kipriyanov 2009; Kipriyanov et al. 1999; Klöhn et al. 2013). In this format, two pairs of scFvs with different specificities are genetically fused in tandem to self-assemble in a head-to-tail fashion, thus forming a tandem Diabody structure (Kontermann 2012). By individually engineering the lengths and compositions of the linker sequences that join the four Fv moieties, the individual V regions can be forced to specifically pair with their counterparts in the second polypeptide chain, thus stabilizing the bispecific protein (Le Gall et al. 2004). With their higher molecular weight (110 kDa) the molecular size of TandAbs is slightly above the renal threshold for first-pass kidney clearance and their half-life in non-human primates is between 3 and 23 h, depending on the dosage (Klöhn et al. 2013). Exhibiting binding activity for both the T-cell effector CD3 and the disease target CD19, the TandAb AFM11 was demonstrated to induce killing of tumor cells in preclinical studies, without any cytotoxicity toward target-negative cells (Zhukovsky et al. 2012b, 2014). Also, compared to similar BiTE and DART reagents, the tetravalent AFM11 showed increased avidity for both CD19 and CD3 as well as higher potency in preclinical experiments (McAleese and Eser 2012; Zhukovsky et al. 2014).

As in classical MAbs, bivalent binding activity is expected to lead to higher avidity and also efficacy, but this must be evaluated for each target with great care. Interestingly, application of elevated concentrations of AFM11 to T-cells in the absence of target-positive cells caused down-modulation of the CD3/TCR-complex following an initial T-cell boost (Zhukovsky et al. 2012a, b), a phenomenon that has not been reported for monovalent BiTEs and DARTs. A phase I clinical study with AFM11 in patients with CD19-positive B-cell malignancies was started in 2014 (NCT02106091). The most clinically advanced TandAb AFM13 (CD30xCD16A), which recruits host natural killer cells and macrophages via CD16A (FcγRIIIa) to kill CD30-positive cells in both B- and T-cell malignancies (Rajkovic et al. 2012), has been scheduled for a phase II study after it was demonstrated to be well tolerated and had shown anti-tumor activity in advanced Hodgkin lymphoma patients (Affimed, <http://www.affimed.com>). Like BiTEs and DARTs, TandAbs are also under development for the treatment of solid tumors (e.g. AFM21; EGFRvIIIxCD3).

In the context of bispecific biobetters, beyond the Ig fragments and alternative scaffolds discussed so far, there are also some miscellaneous approaches that deserve mention, especially as these have already reached the clinic or are close to it. For example, Fcab[™]s are engineered (homo-dimeric) Fc fragments that carry mutated loop sequences at the C-terminal tip of the C_{H3} domain, thus conferring target specificity while retaining FcRn binding as well as immunological effector functions (Wozniak-Knopp et al. 2010). A HER2-specific drug candidate, FS102

(Batey et al. 2013), is expected to reach a phase I investigation in the near future (F-star, <http://www.f-star.com>). Notably, this technology forms a link between antibody fragments and alternative scaffolds insofar as the binding site in the Fc fragment is generated via combinatorial design in a manner typical for non-Ig proteins. In an extension of the Fcab technology the mAb²TM format comprises a full-length MAb in which the natural Fc region has been replaced by an Fcab (Woisetschläger et al. 2013). Such bispecific MAbs can bind (bivalently) two different antigens at the same time, with corresponding drug candidates in the discovery to preclinical stage in immuno-oncology.

Furthermore, Fynomers[®] are non-Ig scaffolds based on the SH3 domain of the human intracellular Fyn tyrosine kinase, which carries two flexible loops suitable for randomization (Grabulovski et al. 2007). The FynomAb[®] technology platform involves fusion of several Fynomers with an Ig Fc region, thus creating a hybrid homodimeric construct. The lead product COVA322, a bispecific anti-TNF α /anti-IL17A FynomAb (Covagen, <http://covagen.com>), is in a phase Ib/IIa study for psoriasis (NCT02243787).

Conclusions and Outlook

Both recombinant antibody fragments and engineered binding proteins based on non-Ig scaffolds have emerged as biobetters which complement and, in the future, may even supersede MAbs for various applications in medical therapy (Table 1). With regard to Ig-derived binding proteins, either as recombinant fragments of existing MAbs or directly selected from cloned V-gene libraries, the Fab format is currently most successful, with several established biopharmaceuticals and a growing number of drug candidates in the clinical pipeline (Nelson and Reichert 2009). While their stably monovalent behavior provides a clear mechanistic benefit in a range of therapeutic settings, the predominant disadvantage of Fabs is their short circulation in the blood, which they share with all other platforms discussed in this Chapter. With Cimzia there is already a clinically approved version that shows an *in vivo* half-life comparable to MAbs, achieved with the help of PEGylation. Further conjugates of this kind are under development, both for Fabs and for most of the alternative scaffolds. However, PEGylation, with its potential burden of a non-biodegradable synthetic polymer, will likely be replaced by more advanced technologies, for example by albumin-binding peptides (Dennis et al. 2002) or by PASylation (Schlapschy et al. 2013), as will be discussed in the next Chapter.

Despite its long history and broad use in basic science, the scFv format is less likely to become a successful therapeutic drug class by itself, especially with regard to systemic application. The reason lies in the generally poor biochemical behavior and inferior stability at the level of protein folding and solubility (Demarest and Glaser 2008). The scFv format appears more promising as part of fusion proteins such as bispecific antibody reagents, e.g. the BITEs, or if linked to cytotoxic enzymes as recombinant immunotoxins. Nevertheless, even in these settings the

Table 1 Engineered protein scaffolds in clinical development

Scaffold, generic name	Target(s)	Molecule	Sponsor	Clinical trials identifier	Name of trial	Phase, start, status
Adnectin: CT-322, BMS-844203, Pegdimetanib, Angiocept	Vascular endothelial growth factor receptor 2 (VEGF-R2)	PEGylated Adnectin; engineered human ¹⁰ FN3 domain, coupled via its penultimate C-terminal cysteine residue to a branched 40 kDa PEG molecule	Adnexus/ Bristol-Myers Squibb	NCT00374179	CT-322 in treating patients with advanced solid tumors and non-Hodgkin's lymphoma	Phase 1, Sep 2006, completed
				NCT00768911	CT-322 in combination with radiation therapy and temozolomide to treat newly diagnosed glioblastoma multiforme	Phase 1, Oct 2008, closed
				NCT00562419	CT-322 in treating patients with recurrent glioblastoma multiforme and combination therapy with irinotecan	Phase 2, Nov 2007, closed
BMS-962476	Proprotein convertase subtilisin/kexin type 9 (PCSK9)	PEGylated Adnectin; engineered human ¹⁰ FN3 domain, coupled via its penultimate C-terminal cysteine residue to a branched 40 kDa PEG molecule	Bristol-Myers Squibb	NCT00851045	Ph II trial of a novel anti-angiogenic agent in combination with chemotherapy for the second-line treatment of metastatic colorectal cancer	Phase 2, Feb 2009, completed
				NCT00850577	Ph II of a novel anti-angiogenic agent in combination with chemotherapy for the treatment of non-small cell lung cancer	Phase 2, Feb 2009, terminated
				NCT01587365	Single ascending dose safety study of BMS-962476 in healthy subjects and patients with elevated cholesterol on statins	Phase 1, May 2012, completed

(continued)

Table 1 (continued)

Scaffold, generic name	Target(s)	Molecule	Sponsor	Clinical trials identifier	Name of trial	Phase, start, status
BMS-986089	Myostatin	Engineered human ¹⁰ FN3 domain	Bristol-Myers Squibb	NCT02145234	Single and multiple ascending-dose study to evaluate the safety, tolerability, immunogenicity, pharmacokinetics and pharmacodynamics in healthy adult and elderly subjects	Phase 1, May 2014, ongoing
Affibody: ABY-025	Her2	Radioconjugate composed of the affibody Z _{4HER2:2891} -Cys, conjugated via its C-terminal cysteine to maleimide-DOTA and labeled with radioisotope ¹¹¹ In or ⁶⁸ Ga	Biomedical Radiation Sciences, Swedish Cancer Society	NCT01216033 NCT01858116	Exploratory study of breast cancer with ABY025 (ABY0125); drug: ¹¹¹ In-ABY-025 PET study of breast cancer patients using [68Ga]ABY-025	Phase 1, Oct 2010, completed Phase 1, Apr 2013, ongoing
Anticlin: PRS-050, Angiocal	Vascular endothelial growth factor (VEGF-A)	Engineered human tear lipocalin (Tlc), conjugated to 40 kDa PEG	Dorte Nielsen, Herlev Hospital, Denmark Pieris	NCT02095210 NCT01141257	HER2 PET imaging in breast cancer patients using [68Ga]ABY-025 Study of Angiocal® in patients with solid tumors, investigating safety, tolerability, blood concentration of study drug	Phase 1, Apr 2013, ongoing Phase 1, May 2010, completed
PRS-080	Hepcidin	Engineered human neutrophil gelatinase-associated lipocalin (NGAL), conjugated to 30 kDa PEG	Pieris		Anemia of chronic disease (ACD)	Phase 1, Dec 2014, ongoing

BITE: AMG103, MT103, MEDI-538, Blinatumomab, Blincyto™	CD19xCD3	Bispecific immunoglobulin format comprising a single-chain fusion of an anti-human CD19 mouse monoclonal scFv fragment with an anti-human CD3 mouse monoclonal scFv fragment	National Cancer Institute (NCI)	NCT02101853	Blinatumomab in treating younger patients with relapsed B-cell acute lymphoblastic leukemia	Phase 3, Aug 2014, ongoing
			Amgen	NCT02013167	Ph 3 trial of blinatumomab versus investigator's choice of chemotherapy in patients with relapsed or refractory ALL	Phase 3, Dec 2013, ongoing
			National Cancer Institute (NCI)	NCT02003222	Combination chemotherapy with or without blinatumomab in treating patients with newly diagnosed BCR-ABL-negative B lineage acute lymphoblastic leukemia	Phase 3, Dec 2013, ongoing
DART: MGD006	CD123xCD3	Bispecific and disulfide-stabilized immunoglobulin format, comprising a humanized Fv heterodimer made of the chains V _{L2} + V _{H1} and V _{L1} + V _{H2}	MacroGenics	NCT02152956	Safety study of MGD006 in relapsed/refractory AML	Phase 1, May 2014, ongoing
	Vascular endothelial growth factor (VEGF-A)	Alternative scaffold domain based on ankyrin repeats, conjugated via cysteine 135 to a single linear 20 kDa PEG	Molecular Partners/Allergan	NCT01086761 NCT01042678	Study of MP0112 intravitreal injection in patients with wet age related macular degeneration Study of MP0112 intravitreal injection in patients with diabetic macula edema	Phase 1, Mar 2010, terminated Phase 1, Feb 2010, terminated
DARPin: MP0112, AGN-150998			Allergan	NCT01397409	Evaluation of AGN-150998 in exudative age-related macular degeneration (AMD)	Phase 2, Sep 2011, completed

(continued)

Table 1 (continued)

Scaffold, generic name	Target(s)	Molecule	Sponsor	Clinical trials identifier	Name of trial	Phase, start, status
Nanobody: ALX-0081, (ALX-0681), anti-vWF Nanobody, Caplacizumab	human von Willebrand factor (A1 domain; vWF)	Biparatopic immunoglobulin format, comprising a single-chain VHH-linker-VHH fusion of humanized <i>Lama glama</i> monoclonal variable heavy chain (VHH) fragments	Ablynx	NCT01020383	Comparative study of ALX-0081 versus GPIIb/IIIa inhibitor in high risk percutaneous coronary intervention (PCI) patients	Phase 2, Sep 2009, completed
			Ablynx	NCT01151423	Study to assess efficacy and safety of Anti-von Willebrand Factor Nanobody in patients with acquired thrombotic thrombocytopenic purpura (TTP)	Phase 2, Sep 2010, completed
			Ablynx	NCT01020383	Comparative study of ALX-0081 versus GPIIb/IIIa inhibitor in high risk percutaneous coronary intervention (PCI) patients	Phase 2, Sept 2009, completed
ALX-0061	Human IL-6R and human serum albumin (HSA)	Bispecific immunoglobulin format, comprising a single-chain VHH ₁ -linker-VHH ₂ fusion of an anti-human IL-6R humanized <i>Lama glama</i> monoclonal VHH fragment with an anti-HSA humanized <i>Lama glama</i> VHH fragment	Ablynx	NCT01284569	Study to assess safety and efficacy of anti-interleukin 6-receptor (IL6R) Nanobody in Rheumatoid Arthritis (RA) patients	Phase 1/2, Mar 2011, completed
Ozoralizumab ATN-103, PF-5230896	Human TNF α (cachectin) and human serum albumin (HSA)	Bispecific, trivalent immunoglobulin format, comprising a single-chain VHH _{TNF} -linker-VHH _{HSA} -linker-VHH _{TNF} fusion of two anti-human TNF α and a single anti-HSA humanized <i>Lama glama</i> monoclonal VHH fragment	Ablynx	NCT01063803	Study evaluating long-term safety of ATN-103 in subjects with Rheumatoid Arthritis	Phase 2, Feb 2010, completed

ALX-0171	Anti-human respiratory syncytial virus (RSV)	Trivalent immunoglobulin format, comprising a single-chain VHH-linker-VHH-linker-VHH fusion of humanized <i>Lama glama</i> monoclonal VHH fragments	Ablynx	NCT01483911	ALX-0171 phase I study, evaluating single ascending dose and multiple dose in healthy male volunteers	Phase 1, Dec 2011, completed
			Ablynx	NCT01875926	ALX-0171 phase I pharmacokinetic study in healthy male volunteers	Phase 1, Jun 2013, completed
			Ablynx	NCT01909843	ALX-0171 safety study in adults with hyperresponsive airways	Phase 1, Aug 2013, completed
TandAb: AFM11	CD19xCD3	Bispecific, tetravalent immunoglobulin format, comprising a humanized Fv homodimer made of the chains V _{H1} -linker-V _{L2} -linker-V _{H2} -linker-V _{L1}	Affirmed Therapeutics	NCT02106091	Safety study to assess AFM11 in patients with relapsed and/or refractory CD19 positive B-cell NHL or B-precursor ALL	Phase 1, Apr 2014, ongoing
AFM13	CD30xCD16A	Bispecific, tetravalent immunoglobulin format, comprising a humanized Fv homodimer made of the chains V _{H1} -linker-V _{L2} -linker-V _{H2} -linker-V _{L1}	Affirmed Therapeutics	NCT01221571	A study to assess AFM13 in patients with Hodgkin lymphoma	Phase 1, Oct 2010, completed

labile VH/VL pairing and the presence of two crucial disulfide bonds often cause problems during biosynthesis and bioprocess development. In this context the advantages of truly single-domain binding proteins, including sdIgs and alternative scaffolds, are brought to light, which likely will replace scFv fragments as target-specific fusion partners in the long run.

Currently, Nanobodies are the most broadly developed class of sdIgs with regard to molecules in the clinic and disease-relevant target specificities. In contrast to dAbs, Nanobodies are generated from immunized animals and, although it is claimed that their immunogenic potential in patients is low, still a certain risk prevails due to the set of conserved mutations that are necessary to maintain solubility and folding stability of the camelid VHH format but that are distinct from the human germline V-gene sequences. Also, it is interesting to note that, at least for therapeutic applications, Nanobodies are not employed as single domain proteins per se but are usually fused with other Nanobody modules. Apart from achieving higher avidity for the biomedical target in case of the biparatopic constructs, the resulting larger size generally slows down kidney filtration, even if not incorporating an albumin-specific Nanobody module to further extend plasma half-life.

In some sense, sdIgs of this kind bridge conventional antibody technology, including their antigen-binding fragments, to the alternative scaffolds with their fully synthetic binding sites. The non-Ig scaffold that is structurally most closely related to dAbs or Nanobodies is the Adnectin, whose fold still remotely belongs to the Ig superfamily but shows important deviations, in particular the missing central disulfide bond. Nevertheless, Adnectins exhibit three CDR-like loops in homologous regions at one end of the wedge-like β -sandwich, which can be randomized in order to create Adnectin libraries suitable for in vitro selection against prescribed targets. Consequently, Adnectins are likely to show a similar preference for pocket-like epitopes as has been discussed for Nanobodies (Gebauer and Skerra 2009). While the number of publicized development programs has remained low, there is still considerable academic research on this protein scaffold in progress (Gilbreth and Koide 2012; Hackel and Wittrup 2010). Of note, the Adnectin scaffold constitutes an isolated domain from the abundant extracellular matrix protein fibronectin and, thus, it does not occur as such in the body. Consequently, there has been concern about the exposure of immunological neoepitopes in Adnectins. However, in clinical trials conducted so far, ADA responses, though detected, were mostly directed against the engineered loops (Tolcher et al. 2011).

Like the FN3 format, the scaffold for Affibodies is an isolated domain excised from the larger extracellular region of a cell surface protein, in this case the Z domain of protein A from *Staphylococcus aureus*. As this small three-helix bundle does not offer a set of spatially contiguous loops, exposed side chains in two neighboring α -helices were chosen for random mutagenesis. This leads to a rather flat interface for complex formation, fixed by the underlying secondary structure. A problem inherited by this scaffold is its bacterial origin, which poses a risk with regard to ADA formation. Even though a deimmunized version has been developed recently (Feldwisch et al. 2010), it is probably wise to apply this class of alternative binding proteins preferentially either to in vivo imaging, where only minute protein

amounts are administered and multiple doses are not required, or to highly immune-suppressed patients.

DARPin are based on an engineered consensus sequence from the ankyrin repeat proteins and, albeit the fold differs, this scaffold is characterized by a conformationally rigid secondary structure not unlike Affibodies. This may be considered an advantage and explains the stability and beneficial biochemical behavior of many DARPins. Generally, it appears that this scaffold with its rather flat binding site is optimally suited to recognize protein targets (Binz et al. 2004; Boersma and Plückthun 2011); in fact, similar to Nanobodies, DARPins have found use as reagents for co-crystallisation of sensitive proteins to promote their X-ray structural analysis (Bukowska and Grütter 2013). To make DARPins also amenable to small molecule or peptide targets, so-called loop-DARPins were recently designed (Schilling et al. 2014). However, it remains to be seen if this concept is useful for wider application, especially with regard to biopharmaceutical development. Notably, so far, DARPins have only been studied in phase II clinical trials for local application in the eye, which is a relatively immune-privileged organ.

Anticalins distinguish themselves from the other non-Ig scaffolds discussed here as they are derived from natural soluble plasma proteins abundant in the human body. This is not the only feature they share with Igs. In addition, their natural binding site, which is made of four structurally hypervariable loops mounted on a sandwich-like, almost circular arrangement of β -strands, shows fundamental similarity to the CDRs of MAbs. However, there are also two key differences: first, natural lipocalins prefer small molecules as ligands and, second, they are not subject to genetic mechanisms of somatic recombination and hypermutation like antibodies. Nevertheless, the methodology of combinatorial protein design has closed this gap, and Anticalins have convincingly demonstrated the ability to address the full spectrum of conceivable medically relevant targets, i.e. proteins (both soluble and as part of cell surface receptors), peptides and, naturally, haptens, similar to their endogenous counterparts.

The preparation of Fc fusion proteins, which is discussed more broadly in the next Chapter of this book, has become popular for most of the non-Ig scaffolds and even for scFv fragments, in this case yielding so-called small modular immunopharmaceuticals (SMIPs) (Hayden-Ledbetter et al. 2009). This concept has been carried further by creating fusions between engineered binding proteins specific for one target and a full length MAb that carries its own specificity for another target, as exemplified by the mAb² platform (Woisetschläger et al. 2013). These approaches form another link between the more conservative antibody engineering field and the presumably more innovative area of alternative scaffolds, indicating that at least from the industry perspective, both territories are on their way to optimize novel biopharmaceuticals by incorporating the best properties of each platform. However, with increasing complexity of the resulting constructs (Kontermann 2012), which also creates challenges for bioprocess development and quality control, the rationale behind the molecular design is not always clear, especially if compared to the classical bispecific antibody format (Milstein 2000).

In fact, it has to be questioned whether the diverse constructs that are currently in discussion really provide for biobetters in a functional sense. From the protein

design perspective, it may be worthwhile examining more critically the features as well as benefits and drawbacks of the individual concepts to generate binding proteins before combining them in a hasty manner. A fast and successful biological drug development program is most likely based on a simple molecular design. This is probably easiest to realize for the construction of antagonists which just block a cell surface receptor or a signaling molecule, and there are already numerous examples of that in the alternative scaffold field. Another area with future potential for biobetters could be their combination with toxic drugs, as an alternative to the currently flourishing ADCs. Here, the non-Ig scaffolds may provide decisive advantages, including (1) the capability for site-directed conjugation with the drug in defined stoichiometry while retaining high target-binding activity, (2) possibly better endosomal escape into the cytoplasm of the target cell, enhancing cytotoxic effects, and (3) lower liver toxicity due to distinct routes of clearance.

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