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Review

The Emerging Role of New Protein Scaffold-based Agents for Treatment of Cancer

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Abstract. *In order to overcome limitations of monoclonal antibodies, new protein-based scaffolds have been designed and evaluated pre-clinically, and some of them are in clinical studies for the treatment of cancer. These entities can be placed into two categories: scaffolds which bind ligands via amino acids in exposed loops and those in which ligand binding is mediated by amino acids in secondary structures, such as β-sheet modules. Accordingly, we discuss adnectins, lipocalins, Kunitz domain-based binders, avimers, knottins, fynomers, atrimers and cytotoxic T-lymphocyte associated protein-4 (CTLA4)-based binders which fall into the first category, while darpins, affibodies, affilins and armadillo repeat protein-based scaffolds are members of the second category. In addition, we also discuss the new molecular entities as imaging tools and outline their unique characteristics in the context of multimeric and multivalent binding.*

Various monoclonal antibodies (mAbs) have been approved for the treatment of cancer, in most cases in combination with small-molecule anticancer drugs, and they are the gold standard with regard to antigen-specific protein therapeutics (1, 2). In addition, new antibody-based formats are presently undergoing preclinical and clinical evaluation (3-7). Among these formats are diabodies, multivalent, multispecific and multimodular antibodies, as well as heavy (H)-chain-based antibodies derived from camel or shark (8, 9). The latter are less hydrophobic, do not aggregate, are remarkably stable and can recognize hidden epitopes normally inaccessible to rodent and human antibodies. However, certain limitations regarding antibody-derived therapeutics have emerged. For example, due to their large size, tumor tissue penetration is a potential issue, and due to a planar binding interface, binding to grooves and catalytic sites of enzymes is difficult to achieve (8-11). Fragment crystallizable region (Fc)-mediated complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) can give rise to adverse effects. mAbs have to be produced in mammalian cells; in some cases post-translational modifications, such as specific glycosylation patterns, are required. High costs of goods, production and purification plants and often complex intellectual property issues are notable facts. Therefore the concept of a universal binding module was extended from antibodies to alternative protein frameworks referred to as scaffolds.

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General Remarks

The term protein scaffold is used either in the context of intracellular signal transduction or to define entities for protein engineering (12). In signal transduction, scaffolds can tether to multiple members of a signaling pathway, protecting them from inactivation and degradation and localize them to specific cellular compartments as crucial regulators of signaling. An example is the scaffold kinase suppressor of rat sarcoma (RAS) (KSR) and the regulation of the mitogen activated protein kinase (MAPK) pathway (13). KSR binds to many proteins, including three kinases in the signaling cascade. KSR is localized in the cell membrane during cell activation, assembles the components of the extracellular signal-regulated kinase (ERK) pathway and localizes activated ERK to the plasma membrane.

With respect to scaffolds as entities for protein engineering, which will be reviewed here, there are two basic options for the structural basis of artificial binding sites: i) binding can be mediated by one or more loops on a rigid protein structure which imitate the antibody paratope, or ii) by surface-exposed side chains of secondary structure elements. Both loop and rigid structure-mediated binding are suitable for the generation of high affinity binders. Peptides with known affinity against a target can be inserted into a

scaffold protein in order to combine characteristics of both the peptide and the scaffold protein, such as specificity, stability, and improvement of tissue penetration. Libraries can be generated based on directed randomization of selected amino acids of the scaffold. Intrinsic conformational stability of the scaffold is a prerequisite for these manipulations. Diversification and subsequent selection mimic the generation of a humoral immune response *in vitro*. Thus, in a way, these new scaffold-based molecules combine the properties of small molecules with those of antibodies (14-18). They fold properly under non-reducing conditions and can be expressed in bacteria without the need for denaturation and refolding. Even chemical synthesis is an option for the production of some of the formats. In the following, we discuss the advantages and critical issues of scaffold-based molecules, both of those with the potential for randomization of loops and those which can be randomized with regard to secondary structural elements. Representatives of the first class are atrimers, protein cytotoxic T-lymphocyte associated protein-4 (CTLA4)-based molecules, adnectins, anticalins, Kunitz domain-based binders, avimers, knottins, and fynomers; prototypes of the second class are darpins, affibodies, affilins and armadillo repeat protein-based binders.

Adnectins

Adnectins are derived from the human extracellular matrix protein fibronectin and are based on the tenth domain of fibronectin type 3 (10Fn3) (19) (Figure 1A). The functional role of 10Fn3 in human fibronectin is binding to integrins. 10Fn3 is a monomer and is composed of 94 amino acids. It is based on a β-sandwich fold comprised of seven strands connected by six loops. The scaffold is a single-domain structural homolog of the Ig-fold without disulfide bonds. Adnectins bind targets with affinity and specificity similar to antibodies, can be selected by mRNA, phage or yeast display, and can be expressed in bacterial systems. Thus, adnectins possessing affinities in the nanomolar and picomolar range have been generated (20, 21). The 10Fn3 domain has a high thermostability, with a melting temperature above 80˚C. However, due to their small size, adnectins are excreted *via* the kidneys and consequently their pharmacokinetic (PK) properties need to be improved. Resolution of the crystal structure of an adnectin binding to the epidermal growth factor receptor (EGFR) and inhibiting ligand binding revealed that non-loop residues can expand the available binding footprint (22, 23). In addition to interactions from the adnectin diversified loops, residues from the *N*-terminus and the β-strands were shown to interact with the target protein (Figure 3A). The epitope of this EGFR-specific adnectin was shown to be different from previously known EGFR antibodies. Improved tissue penetration and low immunogenicity are other potential properties of adnectins. Finally, they are well-suited for oligomerization to generate multi-functional binding molecules.

CT-322 is a polyethylene (PEG)ylated adnectin which binds to human vascular endothelial growth factor receptor-2 (VEGFR2) with an affinity of 11 nM, and to mouse Vegfr2 with an affinity of 250 nM, and can therefore be evaluated in mouse xenograft models (24). In contrast to the mere neutralization of vascular endothelial growth factor-A (VEGF-A), CT-322 prevents binding of VEGF-C and VEGF-D to VEGFR2 in addition to blocking the interaction with VEGF-A. Surface plasmon resonance experiments revealed no binding of CT-322 to VEGFR1 and VEGFR3. CT-322 blocked VEGF-induced VEGFR2 phosphorylation in umbilical vascular endothelial cells. In colon (Colo 205) and glioblastoma (U87) xenograft models, antitumor effects comparable to sorafenib or sunitinib were observed with 15-60 mg/kg dosing three times per week. In the U87 model, tumor growth inhibition was similar to that seen with an antibody to VEGFR2, DC101, administered at 40 mg/kg twice weekly. In an independent study, normalization of tumor vasculature and reduction of microvessel density was observed in the Colo 205 xenograft model, whereas the kidney vasculature was not affected (25). Similarly, CT 322 was evaluated in pancreatic xenograft (MiaPaCa-2) and syngeneic (Pan02) mouse tumor models at 30 mg/kg administered *i.p.* twice per week. In both models, a 50% tumor growth inhibition, as well as an *in vivo* increase of apoptosis and reduction of microvessel density, was observed (26). Clinical evaluation of CT-322 in a phase I study has shown that it can be administered safely at 2 mg/kg, i.v., weekly or biweekly (27). Minor decreases in tumor volume were observed in four out of 34 evaluable patients, and 24 patients were noted with stable disease. Plasma concentrations of CT-322 active in pre-clinical models were recorded. Plasma half-life of CT-322 was 100 h. Toxicological studies in rats and monkeys revealed drug-induced nephropathy as the major toxicity. An increase of the mean blood pressure was noted in rats and in monkeys; in addition, thickening of the epiphyseal growth plate was observed.

In order to inhibit cross-talk between EGFR and insulin-like growth factor receptor-1 (IGF1R) in cancer cells, a bispecific adnectin was designed (28). To this end, individual adnectins with affinities of 10 nM for EGFR and 1 nM for IGF1R were optimized for blocking either EGFR or IGF1R, arranged in tandem by a short linker and PEGylated. These molecules inhibited phosphorylation of EGFR and IGF1R and subsequent downstream signaling, induced receptor degradation, and inhibited proliferation of human cancer cell lines A431, H292, BxPC3 and RH41, with inhibitory concentration 50 (IC_{50}) ranging from 0.1 to 113 nM. The binding site of this adnectin on EGFR was shown to be different from that of cetuximab, panitumumab and

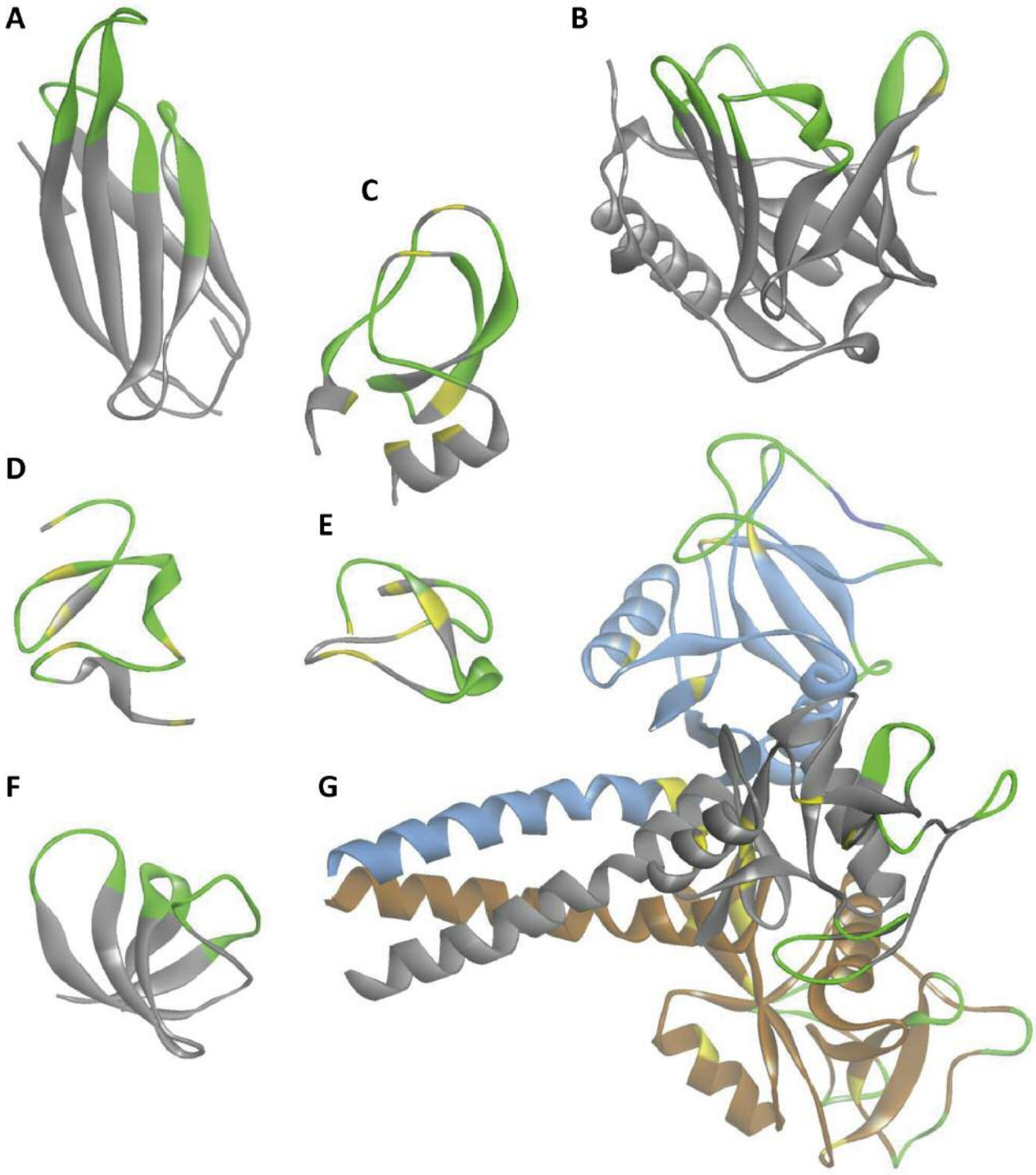

Figure 1. *Loop-based scaffold structures. Scaffold structures are extracted from the Protein DataBank (PDB, Nov. 2011) (112) (when required, models are generated based on available structure data of domains or entities of similar proteins). The scaffolds are displayed using DiscoveryStudio 31 (113) as ribbon representations to facilitate the domain type identification with the N-terminus placed either on the left or on the top for each structure. The loops that recognize the antigen are colored in green whereas the framework is indicated in grey and disulphide bridges are indicated in yellow. A: Adnectin scaffold (pdbcode: 3QWQ). B: Anticalin scaffold (pdbcode: 3BX7). C: Kunitz domain scaffold (pdb code: 1KTH). D: Avimer scaffold (pdbcode: 1AJJ). E: Knottin scaffold (pdbcode: 1CLV). F: Fynomer scaffold (pdbcode: 4AFQ). G: Atrimer scaffold (pdbcode: 1HTN). The trimeric assembly is indicated in grey, blue, and brown.*

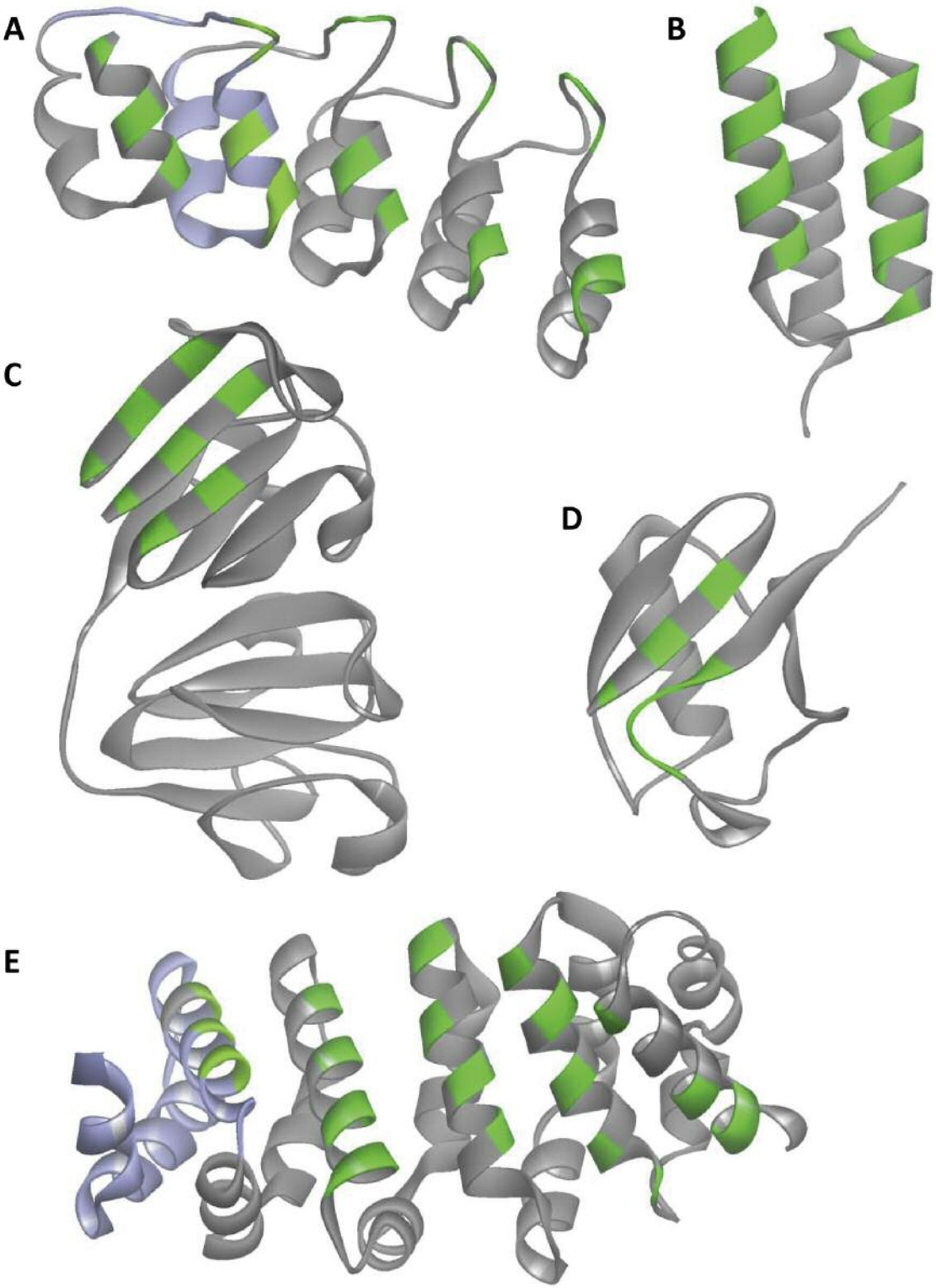

Figure 2. *Helix-/β-sheet-based scaffold structures. As for Figure 1, all scaffolds are crystal structures derived from PDB (112) and visualized using DiscoveryStudio (113). Stretches of amino acids involved in binding are indicated in green; for repeated elements, one unit repeat is indicated in violet. A: Darpin (pdbcode: 4DX5). B: Affibody (pdbcode: 1LP1). C and D: Affilins, either modified γ-B crystalline proteins (C; pdbcode, 2JDG) where the amino acid on the first β-sheets are used to bind an antigen, or based on the ubiquitin protein (D; pdbcode, 1UBQ) for which a β-sheet and a loop provide the amino acids that bind the selected antigen. E: Armadillo repeats (pdbcode: 1JDH).*

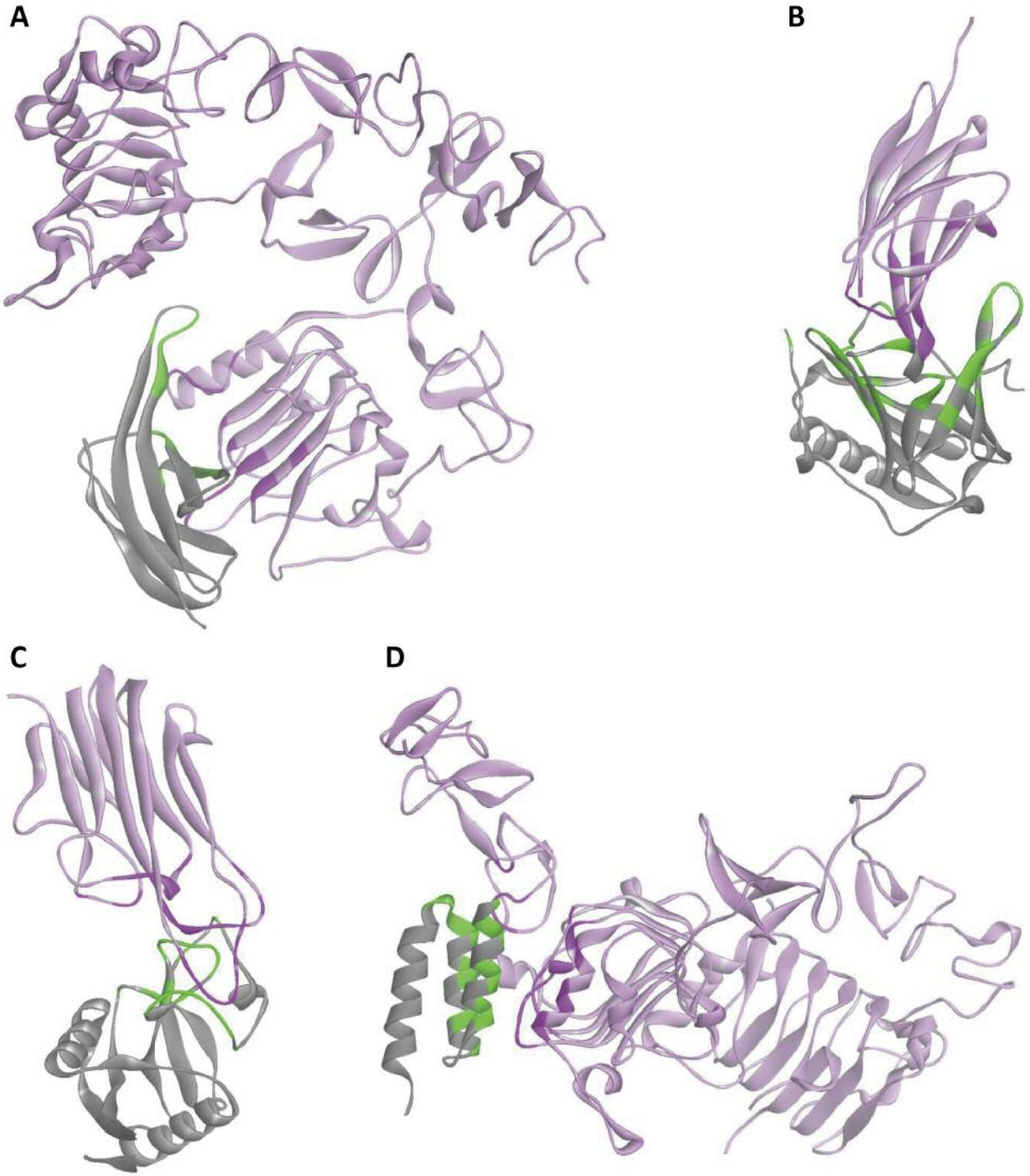

Figure 3. *Selected scaffolds in complex with their antigens. The complex crystal structures of scaffolds (grey) bound to their antigen (pink) are displayed with ribbons; the interacting amino acids are colored in green and magenta, respectively. A: Adnectin in complex with epidermal growth factor receptor (pdcode: 3QWR). B: Engineered human lipocalin bound to cytotoxic T-lymphocyte associated protein 4 (pdbcode: 3BX7). C: One binding domain of an atrimer bound to tumor necrosis factor α (pdbcode: 3L9J). D: Affibody in complex with human epidermal growth factor receptor 2 (pdbcode: 3MZW).*

nimotuzumab. *In vivo*, this bispecific adnectin inhibited growth of xenograft tumors dependent on EGFR and IGF1R signaling. Thus, treating BxPC3 xenograft tumor-bearing mice with the bispecific molecule resulted in enhanced tumor growth inhibition when compared to the corresponding monospecific EGFR and IGF1R adnectins.

Anticalins

Anticalins are molecules based on lipocalins, which are secreted proteins found in body fluids and which have evolved as ligand-binding proteins (29). The lipocalin family shares a structurally conserved β-barrel with eight anti-parallel β-strands winding around a central axis. The upper part of the lipocalins looks like a cup-shaped β-barrel with four extruding loops that form the entrance of the ligand binding pocket. Lipocalins (Figure 1B) consist of a single polypeptide chain of 160-180 amino acids. The ligand-binding site is composed of four structurally variable loops and is less complex and easier to manipulate than that of an antibody which is composed of six loops of complementarity determining regions (CDRs). A range of 16-24 randomized aminoacids per loop appears to be optimal for the design of anticalin libraries (29-32). The human lipocalin scaffold does not need further reformatting such as CDR grafting. Lipocalins are not glycosylated and do not possess disulfide bonds and can thus be produced in bacteria or yeast. In addition, lipocalins are intrinsically monovalent binders and therefore unwanted intermolecular cross-linking does not occur. They can recognize diverse epitopes on different proteins. Naturally occurring and engineered anticalins are structurally very stable with melting temperatures above 70˚C. With molecular weights in the range of 20 kDa, lipocalins are rapidly cleared by renal filtration, however, due to their small size as compared to antibodies, tissue penetration is potentially improved. For prolonged treatment, plasma half-life of anticalins can be extended by PEGylation or conjugation to polypeptides composed of proline, alanine and serine (29-32).

Several potential applications of anticalins can be envisaged, such as antagonizing receptors by inhibition of their ligand binding site(s), or, conversely, by binding to ligands and thus circumventing interaction with their cognate receptors. Additional potential applications could be tumor-targeting of toxins, enzymes which convert prodrugs into active drugs, and radioisotopes. Finally, anticalins could be used as antidotes for neutralizing toxic or otherwise harmful components. Anticalins with high specificity and affinity can be generated against haptens, peptides and proteins. Chemical coupling of fluorescent dyes and radionuclides to anticalins *via* Lys side chains exposed on the protein surface or *via* genetically introduced Cys residues has been demonstrated (29, 30). In fact, radioconjugated anticalins have been used successfully as bioimaging reagents (29, 30). Fused, bispecific anticalins, so-called duocalins (33), can be used for example to mediate signal triggering by cross-linking of cellular receptors or to target cells of the immune system to tumors by cross-linking tumor cells with immune effector cells. A successful example is the reduction of the immune suppression exerted by the transmembrane CTLA4 (CD152) by an anticalin. CTLA4 is a cell surface receptor expressed by T-cells which interacts with B7.1 and B7.2 on tumor cells. This interaction is able to antagonize CD28-dependent stimulation of cytotoxic T-cells and thus to inhibit an antitumor T-cell response (34). Consequently, an anticalin has been identified which inhibits the interaction of CTLA4 with both B7.1 and B7.2 (35). The crystal structure of the extracellular domains of CTLA4 and the lipocalin-based binder demonstrated that all four randomized loops contributed to the binding interface. The structure of the complexed anticalin revealed a pronounced induced fit for three of the four variable loops when compared to the free, uncomplexed anticalin (Figure 3B). This conformational flexibility allows for interaction with targets differing in size and shape. Finally, an anticalin directed against VEGF (PRS-050; Pieris AG) has been derived (36). Favorable binding and affinity profile was noted by comparison with approved VEGF antagonists. A half-life extended version of this anticalin inhibited VEGF-induced angiogenesis and vascular permeability, and possessed antitumor activity in tumor xenograft models. PRS-050 is presently in clinical evaluation for neovascular diseases such as age-related macular degeneration. Lipocalin-based antagonists directed against the tyrosine kinase receptor c-met proto-oncogene product c-(MET) are under evaluation (www.pieris.ag.com).

Kunitz Domain Inhibitor Scaffold

Kunitz domain inhibitors (Figure 1C) are a class of protease inhibitors of approximately 60 amino acids in length which reversibly inhibit trypsin and other serine proteases. They contain three disulfide bonds and three loops that can be mutated without destabilization of the structural framework (37, 38). One such molecule, DX-88, a high-affinity inhibitor of human kallikrein has been identified. Kallikrein is a serine protease involved in hereditary angioedema (HAE), which is a life-threatening disorder caused by genetic deficiency of C1-esterase inhibitor. DX-88 (Ecallantide, Kalbitor) (39, 40) from Dyax Corporation is a 60-amino-acid miniprotein derived from a Kunitz domain as a scaffold with human lipoprotein-associated coagulation inhibitor (LACI) as a parent protein and was approved in 2009 for treatment of acute HAE.

Avimers

The basic scaffold for avimers (Figure 1D) is the A-domain, composed of 35 amino acids (4 kDa), containing six cysteines which form three disulfide bridges. To date, 217 A-

domains have been identified in human proteins, such as low density-related protein (LRP) and very low density lipoprotein receptor (VLDLR) (41-45). The uniform structure is stabilized by Ca binding and disulfide formation and contains 12 conserved amino acids for maintenance of the scaffold. Avimers containing up to eight A-domains have been expressed in *Escherichia coli* and properly refolded by air oxidation (45). Avimers are highly resistant to temperature-mediated denaturation. No difference with regard to aggregation, modification or degradation was observed after incubation at 50˚C to 80˚C for two weeks. With a molecular mass of only 4 kDa, the avimer scaffold is smaller than immunoglobulin domains (12 kDa), lipocalin (20 kDa), tetranectin (20 kDa) and protein A (7 kDa).

Thus, due to their small size, avimers can bind to multiple sites on a ligand. To generate specific avimers, the selection process starts with a randomized monomeric library expressed by phage display and the identification of suitable binders. Subsequently, additional randomized domains are fused to the first binder with short linkers (5 amino acids) and selection is repeated, with each of the target-binding domains recognizing a different epitope (45). This way, binders with IC_{50}s in the picomolar range were identified for interleukin 6 (IL6), c-MET, cluster of differentiation 28 (CD28), CD40L and B-cell-activating factor (BAFF) (45), while serum half-life was extended by fusing an immunoglobulin G (IgG)-binding domain to the *N*-terminus of the multimerized avimers. In contrast to antibodies directed against c-MET or CD28, no agonistic effects were observed with the corresponding avimers. This phenomenon observed with antibodies is most likely due to bivalent binding resulting in dimerization and activation of the respective receptors. A three-domain protein that binds IL6 trivalently inhibited IL6-induced cell proliferation in the low picomolar range (45). Each domain was shown to bind independently to the antigen, and the energetic contribution of each domain was demonstrated to be additive. Anti-inflammatory activity was observed in mice with such 19 kDa avimers flanked by an IgG-binding domain. These avimers also bind to cynomolgus monkey IL6, and a plasma half-life of 90 h was observed in these animals. However, potential immunogenicity remains to be investigated.

Knottins (Cysteine Knot Miniproteins)

Many knottins were isolated from the toxins of spiders, scorpions and marine cone snails, and they can act as highly specific ion channel blockers (46, 47). Their scaffold relies on an extremely stable 30-amino-acid protein fold composed of three anti-parallel β-strands connected by loops of variable length and sequence (Figure 1E). A characteristic pattern of knottins is the so-called cysteine knot. Here, Cys 1 and Cys 4, Cys 2 and Cys 5 and Cys 3 and Cys 6 are connected by disulfide bridges, respectively. Cys 3 and Cys 6 cross the macrocycle formed by the other disulfides. Cyclotides are characterized by the same interlocking arrangement of their disulfide bridges, but are circular molecules based on an additional loop that interconnects the amino- and carboxy-termini resulting in head-to-tail cyclization. The pseudoknot cysteine topology is responsible for the extraordinary thermic, proteolytic and chemical stability of the knottins. For example, knottins can be boiled in 1 N HCl or 1 N NaOH without loss of structural or functional integrity. The proteolytic stability opens the potential for oral administration. However, the immunogenicity of these molecules remains to be investigated.

Functionalization of knottins is achieved by loop grafting, the transfer of a loop mediating a biological function onto the knottin scaffold (48). Thus, a peptide involved in VEGF-A antagonism was grafted into a cyclotide framework, resulting in molecules possessing biological activity with regard to *in vitro* VEGF-A antagonism at low micromolar concentrations. Obviously, further affinity optimization is required to achieve the performance of antibody-based antagonists which inhibit VEGF-A at subnanomolar concentrations (49, 50). Knottin-based receptor activation through receptor dimerization has been achieved for stimulation of thrombopoiesis at an effective concentration of 100 pM. Here, chemical conjugation of the antagonistic monomers resulted in the generation of agonistic dimers that were almost as potent as natural thrombopoietin with respect to megakaryocyte colony formation from human bone marrow mononuclear cells (51). Another example is the generation of integrin-binding knottins. A six-residue loop of the agouti-related protein (AgRP) was replaced by a loop containing an RGD-tripeptide with randomized flanking sequences and displayed on the surface of *Saccharomyces cerevisiae*. Variants with antibody-like affinity for the αvβ3 receptor in the subnanomolar to 15 nM range were isolated using high-throughput fluorescence-activated cell sorting (52). A similar approach making use of a trypsin-inhibitor based scaffold resulted in knottins with high affinity (10-30 nM) for tumor vasculature-related integrins, such as αvβ3, αvβ5 and αvβ1 (53). Optical and positron-emission tomography (PET) imaging demonstrated better uptake and biodistribution of fluorescence label-tagged knottins in a murine glioblastoma xenograft model than of a cyclic pentapeptide which is currently under clinical development. Finally, due to the small size of knottins, chemical synthesis is also feasible. Non-proteinogenic amino acids can be introduced for chemical conjugation and for improvement of biological activity or plasma half-life (54).

Fynomers

Fynomers (Figure 1F) are a scaffold derived from amino acids 83-145 of src-homology domain 3 (SH3) of the tyrosine kinase fyn (55). The scaffold consists of two anti-parallel β-sheets and

two flexible loops. FYN-SH3 domains are fully conserved between man, mouse, rat and gibbon (56, 57). Chicken and xenopus laevis SH3-fyn domains differ from their human counterpart at only one or two positions, respectively. SH3 domains bind to proline-rich peptides containing a PXXP core-binding motif (58). A human fynomer library containing more than one billion individual clones was derived by combinatorial mutation of residues in the flexible loops. The loop residues were randomized by polymerase chain reaction (PCR) using partially degenerate primers (59, 60). FYN-SH3 derived proteins binding to the extra-domain B (EDB) of fibronectin were isolated from the above-described library. EDB is a 91-amino-acid type III homology domain inserted into fibronectin by alternative splicing (61). The domain has been identified as a marker for tumor angiogenesis, and is undetectable in normal tissues but abundantly expressed in many types of solid tumor. Clone D3, one of the FYN-SH3 derived EDB binders, stains vascular structures in tumor sections (62). D3 is a monomeric binder (dissociation constant: 8.5×10^{-8} M), does not contain Cys residues, and is thermostable (melting point of 70.5˚C). Biodistribution experiments using mouse xenograft models demonstrated that D3 selectively accumulated in the tumor site. Neither wild-type Fyn-SH3 nor D3 were immunogenic in mice after intravenous injection (62). Thus, fynomer clone D3 was identified as a molecule possessing the potential for targeted delivery of bioactive agents to the tumor vasculature. Three therapeutic derivatives of antibody L19 specific for the EDB of fibronectin are presently undergoing clinical trials for targeted cytokine delivery (63).

Atrimers

The underlying scaffold for atrimers (Figure 1G) is tetranectin, a blood plasma protein (64). Tetranectin is a member of the C-type lectin family which is characterized by the C-type lectin domain (CTLD) (65, 66). It is composed of three identical chains possessing a C-terminal trimerizing coil-coil region. The formation of the homotrimer leads to an apparent 100-fold increase in affinity for its cognate ligand, which is probably due to an avidity effect based on the three-fold clustering of the CTLDs. Each CTLD (67) has five loop regions, 6-9 amino acids in length, which mediate binding specificity. The sequence of these loops can be changed without perturbing the overall structure. Accordingly, monomeric CTLDs can be displayed on phage libraries for the selection of binders to specific targets, but trimeric versions were used for later applications (68, 69). Atrimers with molecular weights ranging between 60-70 kDa are much smaller than antibodies, are not glycosylated and might be endowed with better tissue penetration in comparison to antibodies. Lysine-rich, unstructured regions are potentially suitable for conjugation to payloads. Based on the characteristics as outlined, atrimers might be particularly suited to interact with trimeric targets such as tumor necrosis factor (TNF), receptor activator for nuclear factor κB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL), which have to trimerize as a prerequisite for their biological activity. Potentially critical are symmetry-related restrictions due to avidity generated by the trimer as a quaternary structure. For example, phage-library based randomization of loops 1 and 4 in the CTLD and subsequent optimization has allowed to identify a TNFα antagonist with a 200-fold improvement of biological activity after switching to the trimeric format (69). X-Ray analysis of the TNF/TNF-R antagonist complexes have revealed structural changes in the CTLD, but outside of the randomized loops no substantial changes were noted (69). Figure 3C shows the structure of a monomeric CTLD and monomeric TNFα. The first lead compound of Anaphore Incorporation, ATX 3105, blocks binding of IL23 to its receptor and thus inhibits its activation and, subsequently, inflammation. Similarly, a death receptor 4 (DR4) atrimer which induces cell death in tumor cells is under development (70).

CTLA4-based Binders

CTLA4 is an inhibitory cell surface receptor which is expressed on activated T-cells and interacts with CD80 (B 7.1) and CD86 (B 7.2) on antigen-presenting cells (71). CTLA4 is a 44 kDa homodimeric protein; the monomers consist of an extracellular immunoglobulin variable (V)-like domain possessing three loops similar to CDRs connected by a stalk peptide to transmembrane and intracellular SH2 domains. The rationale for consideration of CTLA4 as a possible scaffold for protein engineering was its structural similarity to camel immunoglobulin V-region domains (72, 73) which can display an extended polypeptide loop. The stalk region contains a cysteine residue allowing disulfide-linked dimerization (74). Somatostatin, a 14 residue intra-disulfide-linked peptide hormone was inserted into CDR1 or CDR3 of human CTLA4 V-domains as a fusion protein with pIII coat protein of bacteriophage fd and shown to bind specifically to surface receptor somatostatin type 4 (SST4) (75). Similarly, a phage-display library of the extracellular domain of CTLA4 with CDR3 as the permissive site was evaluated by replacing nine amino acids with XXX-RGD-XXX at the apex of the loop, resulting in binding to human integrin αvβ3. Another potential permissive site was identified diametrically opposite the natural CDR, indicating the potential for construction of bispecific molecules (76).

Darpins

Designed ankyrin repeat proteins, darpins, are artificial scaffolds based on human ankyrin repeat domain proteins which are abundant intracellular adaptor molecules binding

to various proteins with different biological consequences, such as inhibition of complex formation. Darpins (Figure 2A) are composed of two to four randomized, genetically fused repeats which are flanked by *N*- and *C*-terminal-capping repeats which are essential for efficient folding and for avoiding aggregation (77-80). Darpin repeats consist of 33 amino acids and are composed of two α-helices and a β-sheet. As a consequence, darpins possess a molecular mass of between 14 and 21 kDa, which is about 10% of the size of an antibody. Randomized darpin libraries have been established, and binders can be selected by ribosome or phage display technologies (81). *In vivo*, darpins are characterized by rapid clearance and good tissue penetration due to their small size and the lack of Cys residues. Because of their large surface area, darpins have a potentially larger binding area than typical globular proteins. Darpins are very stable proteins, the midpoints of denaturation range between 66˚C and 95˚C, depending on the number of repeats. They are cleared by the kidneys and rapidly removed from the circulation. In proof-of-concept experiments, darpins have been generated against proteases, kinases and membrane proteins. They can be labeled radioactively, conjugated with small molecule toxins, or fused to cytokines or cytotoxic proteins. A single darpin can be directed against different epitopes on the same target, or against several targets. For example, it was shown that a bispecific darpin can bind to two epitopes on the IgE receptor simultaneously, thus blocking binding of IgE to its receptor efficiently (82).

Numerous applications in oncology have been reported. For example, a darpin directed against epithelial cellular adhesion molecule (EpCAM) was fused to a truncated version of *Pseudomonas* exotoxin (83). *In vitro,* IC_{50} values between 0.005 pM and 0.7 pM for EpCAM-positive tumor cells were observed, while 10,000-fold higher IC_{50}s for EpCAM-negative tumor cells were noted. These darpin-toxin conjugates were also shown to possess *in vivo* efficacy, however, their short plasma half-life of 11 min is a critical issue. In another experiment, two pairs of EGFR-specific darpins were genetically fused to form a tetravalent, bispecific darpin which possessed similar or even greater *in vitro* potency on tumor cells than cetuximab (78). Next, a darpin directed against human epidermal growth factor 2 (HER2) was isolated by ribosome display technology, and was shown by FACS analysis and immunohistochemistry to share its epitope with trastuzumab (84). Affinity maturation of this molecule by error-prone PCR and ribosome display resulted in a darpin with four mutations in framework positions and an improved binding affinity of 90 pM. X-Ray crystallography demonstrated that one of these mutations, His to Tyr at position 52, altered the intra-repeat hydrogen-bonding pattern which caused a significant change in the relative disposition of the repeat subdomains resulting in an enhanced on-rate of the mutated darpin. This agent might have use as an imaging agent due to its rapid clearance of less than one hour after intravenous injection. Another potential application for darpins is based on their ability to act as bispecific adapters for targeting oncolytic viruses to tumors. This was achieved with a darpin consisting of four domains arranged in tandem with three of the domains being specific for the trimeric knob at the end of the protruding adenoviral fibers and the fourth binding to HER2 as the tumor target (85). Finally, the most advanced darpin is a VEGF-A binder (MPO 112) which is presently under clinical investigation in patients with age-related macular degeneration and macular edema (86).

Affibodies

The scaffold for affibodies (Figure 2B) is derived from the B-domain in the Ig-binding region of *Staphylococcus aureus* protein A (87). It is based on a non-cysteine three-helix bundle domain. A single mutation was introduced into helix 2 to obtain increased chemical stability and loss of Fab binding activity. The surface interacting with antigen is made up by two randomized α-helices, and differs significantly from the corresponding surface in immunoglobulins which is generated by six variable peptide loops. Diversity is created by randomization of solvent accessible residues in helices 1 and 2. Nine of these residues participate in the native interaction with Fc (88). Binders can be selected from phage libraries displaying the small (6 kDa) three-helix bundle domain. Due to the small size, functional affibody proteins can also be produced by chemical synthesis. Affibodies are highly soluble, stable, rapidly removed from the circulation, and can be conjugated with toxins and fluorophores, radioactive labels, or chemical groups for immobilization. Due to their small size, effective tissue penetration is expected. The first affibody molecule evaluated *in vivo* was directed against HER2 and is a dimeric form of the molecule Z HER2:4 which has an affinity constant of 50 nM and recognizes an epitope different from that of trastuzumab (Figure 1C). A dimeric version was created because low nanomolar or subnanomolar affinity are a precondition for molecular imaging. Using the radiolabeled, dimerized Z HER2:4, HER2 expression in xenografts could be visualized as soon as six hours after injection, and a tumor-to-blood radioactivity ratio of more than 10 was noted eight hours after administration. Further refinement resulted in the isolation of a monomeric high-affinity molecule with a Kd of 22 pM, designated Z HER2:342 (89). High contrast images of HER2-positive xenograft tumors were obtained six hours after injection. Z HER2:342 did not induce phosphorylation of HER2 and inhibited phosphorylation of phospholipase Cγ and cell migration. Similarly, an affibody-based, 111-In labeled, tracer binding to EGFR with an affinity of 50 nM in its dimeric form was described (90). This molecule accumulated in tumors, and a tumor-to-blood

radioactivity ratio of 9 was measured four hours after injection. Conversely, a HER2-directed affibody albumin-binding domain fusion protein was shown to associate with human serum albumin which resulted in an *in vivo* half-life corresponding to that of albumin, a 25-fold reduction in kidney clearance and a 3- to 5-fold increase in tumor tissue concentration (90). The structure of an affibody in complex with HER2 is shown in Figure 3D. Since HER2 and EGFR are co-expressed in several types of tumors, and co-expression correlates with a poor prognosis, a bispecific affibody directed against these targets was generated (91, 92). Binders were selected by affinity maturation and fused to each other with a glycine-serine linker. By linking the two binders, the N-terminal HER2-binding moiety was not affected with regard to binding properties, whereas the C-terminal EGFR-binding moiety had a lower association rate. Detailed binding studies suggested that this bispecific affibody is able to interact simultaneously with both target molecules. Based on these results, a new set of HER2/EGFR bispecific molecules, with a 30-fold higher affinity compared to binding to cells containing only one of the receptors, were generated. Bispecific, tetravalent affibodies mediating dual EGFR and HER2 binding were also constructed by covalently linking duplicated HER2- and EGFR-directed affibodies. The size of such tetravalent molecules is approximately 28 kDa, similar to monovalent scFv. These tetravalent fusion proteins were shown to mediate cell ligation as demonstrated with A431 and SKBR-3 tumor cells. Affibody molecules with different affinities for EGFR and HER2 were generated to investigate the possibility for cooperative and therefore more selective binding. An affitoxin comprising a HER2-specific affibody combined with a truncated version of *Pseudomonas* exotoxin A (93, 94) was shown to bind to HER2 with nanomolar affinity and to kill HER2-positive cells with IC_{50} values three orders of magnitude lower than the corresponding HER2-negative cells, and was able to eradicate BT-474 breast cancer xenograft tumors. Finally, affibodies are also useful tools for the prediction and monitoring of responses to therapy and for the detection of recurrencies (follow-up therapy) (94-96). Taken together, affibodies are scaffolds enabling flexible engineering of antigen binders with regard to multimerisation, generation of multispecificity, fusion with effector proteins and site-specific modifications.

Affilins

Affilins are derived from human γ-B crystallin or human ubiquitin (Figure 2C and D). γ-B Crystallin is a 20 kDa protein composed of 176 amino acids. It consists of two domains connected by a linker and functions exclusively as a structural protein in the eye lens, no interactions with other molecules have been found (97). Ubiquitin consists of 76 amino acids, which form three and a half α-helices and five β-strands (98). Both γ-B crystallin and ubiquitin-derived scaffolds have a binding region which is located in a β-sheet structure; the γ-B-crystallin β-sheet structure was engineered to form a universal binding site. For γ-crystallin, six surface amino acids are suitable for modification (amino acids 2, 4, 6, 15, 36 and 38), similarly, in ubiquitin, eight surface exchangeable residues can be manipulated (amino acids 2, 4, 6 and 62 to 66) without dramatic change of the structural backbone and with a negligible loss of stability (99-101). By randomization of eight codons, binders for estradiol/testosterone, human IgFc and pro human nerve growth factor with dissociation constants in the nanomolar range were generated. The identified binders were resistant to 8 M urea, stable at pH values ranging from 1 to 9, and the structure remained native at temperatures of up to 75˚C in the presence of 7 M urea. Expression yields of up to 200 mg protein from 1 l cultures and a final yield of up to 100 mg after a two-step purification were achieved.

Armadillo Repeat Proteins

Armadillo domains were identified first in the *Drosophila melanogaster* segmentation polarity gene armadillo, which is homologous to β-catenin (102, 103). The armadillo domain (Figure 2E) consists of 42 amino acids repeats and can interact with peptides or parts of proteins in extended conformation (104-106). The armadillo domain forms a right-handed superhelix; every repeat is composed of three α-helices (H1, H2, H3), and several domains stack to form the compact domain. Special repeats are present at the *N*- and *C*-termini, respectively, thereby protecting the hydrophobic core from exposure to solvent (107). The superhelical armadillo domain winds around the bound peptide thus forming a double-helical complex similar to a DNA double strand. Each dipeptide unit of the bound peptide is specifically recognized by one repeat of the armadillo domain. Dissociation constants ranging between 10 and 20 nM for ligands have been reported (108). Any given number of repeats can be directly combined to extend recognition to much larger peptide sequences. The amino acid residues in armadillo repeats involved in binding are mostly located on helix 3. Peptide-specific repeats can be combined without performing additional selection (109). The armadillo scaffold was systematically improved by designing internal modules and capping repeats based on molecular dynamics, nuclear magnetic resonance spectroscopy and X-ray-based structural analysis (110). Library generation was based on expression of the repeats in the cytoplasm of *E. coli* as monomers. A library based on six randomized positions of the repeat was prepared and making use of ribosome display, binders to the 13 amino acid random-coil peptide neurotensin were identified (108). The selected binder possessed a moderate affinity (7 μM at 4˚C) and was optimized by further selection techniques (108).

Concluding Remarks

Clinical proof-of-concept for new scaffold-based therapeutics was demonstrated by the approval of Kalbitor in 2009 which is a rationally designed Kunitz domain-derived inhibitor of human plasma kallikrein for the treatment of HAE. In addition, the clinical evaluation of several alternative scaffolds as antibody mimetics is ongoing. These new scaffolds might be able to fill a significant gap, since the ability of mAbs to bind to grooves or catalytic pockets was identified as one of their limitations (111). The potential advantage of improved tumor and tissue penetration due to their smaller size in comparison to mAbs is counteracted, however, by the need for their modification by fusion to half-life increasing entities in order to achieve extended target exposure. On the other hand, efficient tumor penetration and fast plasma clearance are important prerequisites for agents for tumor imaging. Bi- and multispecific molecules based on such new scaffolds are also emerging which have the potential for increased tumor specificity and possibly also more efficient blockage of ligand- and receptor-based target molecules. Cross-linking of receptors resulting in clustering might result in accelerated endosomal degradation and inhibition of several pathways such as MAPK and Pl3K signaling, thus counteracting possible mechanisms of drug resistance as a result of receptor inactivation. Efficient induction of apoptosis of tumor cells based on mechanisms as outlined above is a further prospect of these new agents. In addition, the small size of these bi- or multispecific agents might enable binding to epitopes which are too close to each other to be accessible for targeting with bispecific antibodies due to spatial restrictions imposed by the hinge region. Coupling of the new scaffold-based entities to cytotoxic payloads is another perspective.

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