Designed Ankyrin Repeat Proteins (DARPins): Binding Proteins for Research, Diagnostics, and Therapy

Andreas Plückthun

Department of Biochemistry, University of Zurich, CH-8057 Zurich, Switzerland; email: plueckthun@bioc.uzh.ch

Annu. Rev. Pharmacol. Toxicol. 2015. 55:489–511

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev-pharmtox-010611-134654

Keywords

protein engineering, directed evolution, protein scaffold, tumor targeting, sensors, virus retargeting

Abstract

Designed ankyrin repeat proteins (DARPins) can recognize targets with specificities and affinities that equal or surpass those of antibodies, but because of their robustness and extreme stability, they allow a multitude of more advanced formats and applications. This review highlights recent advances in DARPin design, illustrates their properties, and gives some examples of their use. In research, they have been established as intracellular, real-time sensors of protein conformations and as crystallization chaperones. For future therapies, DARPins have been developed by advanced, structure-based protein engineering to selectively induce apoptosis in tumors by uncoupling surface receptors from their signaling cascades. They have also been used successfully for retargeting viruses. In ongoing clinical trials, DARPins have shown good safety and efficacy in macular degeneration diseases. These developments all ultimately exploit the high stability, solubility, and aggregation resistance of these molecules, permitting a wide range of conjugates and fusions to be produced and purified.

INTRODUCTION

Motivation

Repeat proteins were devised as a new paradigm for creating binding proteins (1) using technologies that had all been established initially for recombinant antibodies. These technologies had been developed to such a level that they became, ironically, independent of the antibody molecule itself: Recombinant antibodies could be expressed functionally in *Escherichia coli* (2, 3)—a basis of all convenient engineering. Natural and semisynthetic libraries (4) as well as fully synthetic libraries (5) could be created and selected by phage display (4), ribosome display (6), and many other techniques. Because the work flow of ribosome display with its intrinsic polymerase chain reaction (PCR) step can be combined easily with random mutagenesis (i.e., enabling true directed evolution and affinity maturation, as opposed to mere selection) (7), even the somatic hypermutation had hence been replicated in the laboratory. The antibody molecule had thus become dispensable.

It therefore became possible to address the shortcomings of recombinant antibodies by creating other binding molecules. For most antibody engineering, and for most fusion proteins, fragments of antibodies such as Fab and scFv are used, but these show a high aggregation tendency, especially when linked together or when used under reducing conditions as intrabodies (8). For therapy, therefore, most recombinant antibody fragments are converted back to the IgG format (9). A novel scaffold was thus needed that would particularly address protein stability and facile expression and could thus widely open the application space.

Properties of Repeat Proteins

A suitable scaffold will have to replicate the virtues of antibodies and address their shortcomings. Repeat proteins appeared very attractive as a choice for a general binding protein. They are composed of repeat modules that stack on each other to create a compact folded domain, usually with an elongated shape (10), and use variable surface residues to create an extended target interaction interface. The conserved interfaces between the repeat units allow individual repeats to be exchanged, deleted, or inserted without destroying the tertiary structure of the domain.

After engineering work on repeat protein libraries had been well under way (1, 11, 12), Pancer and colleagues (13) reported the surprising discovery that jawless vertebrates use an adaptive immune system composed of leucine-rich repeat (LRR) proteins. Researchers had known already that most vertebrates use LRR proteins as part of their innate immune response—the family of Toll-like receptors (14)—and that LRR proteins serve similar roles in plants and insects (15–17). However, the finding that *Agnatha* (Greek: no jaws) have converted these molecules into an adaptive immune response, i.e., into a repertoire from which a specific binding protein can be selected, was a rather unexpected validation of the concept of using repeat proteins in an antibody-like manner.

Several repeat proteins have been subjected to protein engineering (reviewed in References 18 and 19). However, this review concentrates on ankyrin repeat proteins, as they have progressed the furthest toward biomedical applications.

Properties of Designed Ankyrin Repeat Proteins (DARPins) and the Design of a Library

Ankyrin repeat proteins (20, 21) are built from tightly packed repeats of, usually, 33 amino acid residues. Each repeat forms a structural unit consisting of a β-turn followed by two antiparallel α-helices (**Figure 1**), and up to 29 consecutive repeats can be found in a single protein (22). Yet ankyrin repeat domains usually consist of four to six repeats, leading to a right-handed solenoid

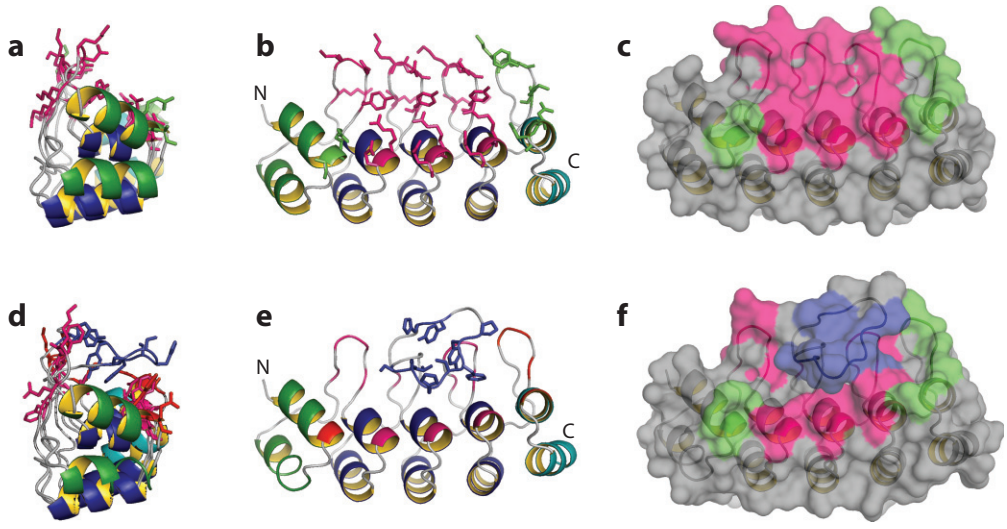

Figure 1

DARPins and LoopDARPins are depicted with different aspects of their randomized surface highlighted. DARPins consist of an N-capping repeat (*green ribbon*), several internal repeats whose number can be freely chosen (three shown here) (*dark blue ribbon*), and a C-capping repeat (*cyan ribbon*). (*a*,*b*,*c*) Classic DARPin library design, with residues in magenta randomized in the original design (11) and additional residues randomized in the caps (*green*) (31) (J. Schilling, J. Schoeppe, S. Hansen, J. Schaefer & A. Plückthun, unpublished data). Views in panels *a* and *b* are rotated by 90° about the y-axis, and the molecular surface is shown in panel *c* (in the same orientation as in panel *b*). (*d*,*e*,*f*) LoopDARPins (31) are characterized by an inserted protruding loop, with additional randomized residues shown in blue. Note that in panel *e*, only the randomized loop residues are shown with their side chains, whereas in panel *d*, side chains of all randomized residues are depicted. In panel *f*, the corresponding surfaces are colored accordingly. Abbreviation: DARPin, designed ankyrin repeat protein.

structure with a continuous hydrophobic core and a large, solvent-accessible surface (10, 23). The binding surface thereby becomes groove-like.

We chose a consensus strategy (24) to arrive at repeats that are self-compatible (and can thus be added, inserted, and shuffled) and to generate molecules with high stability and expression rate. The underlying assumption is that residues important for maintaining the fold will be more conserved and thus show up prominently in an alignment. By using an iterative process of sequence and structural analyses (11), a consensus framework was built and surface residues were identified that might potentially interact with the target—based on analogy of complexes of natural ankyrin repeat proteins with their targets. These residues were randomized, avoiding the residues Cys (to eliminate disulfide formation), Pro, and Gly (as some of the residues are located in a helix). This restriction was achieved by using trinucleotide building blocks during library generation (25).

The designed ankyrin repeat protein (DARPin) library thus comprises fixed and variable positions. The fixed positions reflect structurally important framework positions, whereas the six variable positions per repeat module reflect nonconserved, surface-exposed residues that can be potentially engaged in interactions with the target. The theoretical diversities of the DARPin libraries are 5.2×10^{15} or 3.8×10^{23} for two-module or three-module binders, respectively, and the actual sizes of the libraries are equal to the number of different molecules present. They can be estimated as 10^{12} in ribosome display (26) and 10^{10} in phage display (27).

When designing the consensus ankyrin repeats, a challenge arose regarding the first repeat (N-capping repeat or N-cap) and the last repeat (C-capping repeat or C-cap) flanking the binding modules discussed above. These should present a hydrophilic surface to the outside. Indeed, we

have observed experimentally that the presence of these caps is essential for DARPins to fold in *E. coli* (28). In the original design, both were taken from a natural protein (11). More recently, this C-cap was redesigned to make it more similar to the consensus, and the new C-cap is indeed much more resistant to thermally and denaturant-induced unfolding (28). Crystallography (29) and nuclear magnetic resonance spectroscopy experiments (30) have shown that this stabilization is due to better packing. Importantly, this robust structure has now allowed us to introduce randomized residues in the cap as well, thereby creating a larger interaction surface to bind to even more possible epitopes (31) (**Figure 1*a–c***).

More recently, the DARPin architectural concept has been expanded (31). The proteins' concave shape, rigidity, and incompletely randomized binding surface may limit the epitopes that can be targeted by this extremely stable scaffold. Therefore, a continuous convex paratope, similar to the long CDR-H3 found in many antibodies, was introduced into the DARPin scaffold (**Figure 1*d–f***). To retain the beneficial biophysical properties of DARPins, the stem of the loop was kept constant to make it compatible with the neighboring repeats. Biophysical characterizations have indeed shown that the introduction of an elongated loop through consensus design did not decrease the stability of the scaffold. Using these design principles, a library of LoopDARPins was created, a next generation of DARPins with extended epitope-binding properties. An X-ray structure of a LoopDARPin validated the design (31). With this LoopDARPin library, binders with an affinity of 30 pM could be isolated with only a single round of ribosome display directly from the original library (31), an enrichment that so far had not been described for any scaffold or any selection technology.

Most DARPins show high thermodynamic stability against unfolding induced by heat or denaturants (32, 33) and can be brought to very high protein concentrations without aggregating. Moreover, they can be expressed at very high yield in soluble form in the cytoplasm of *E. coli*, constituting up to 30% of total cellular protein (up to 200 mg per liter of shake-flask culture). Expression in fermenters can consequently be brought to multigram quantities per liter of culture (**http://www.molecularpartners.com**).

Purification is thus straightforward, and for laboratory use, immobilized metal ion chromatography purification is the standard method used. Additional purification steps are of course required when the protein is derivatized [e.g., with polyethylene glycol (PEG) or fluorescent dyes]. For animal experiments, in which still higher purity is needed and the absence of endotoxins needs to be secured, additional washing steps and endotoxin chromatography are required and readily feasible (34). Altogether, the good manufacturing practice (GMP) production of highly pure DARPins for clinical-grade material is straightforward (**http://www.molecularpartners.com**).

Full Consensus DARPins

A series of full consensus DARPins can be obtained by converting the randomized positions also to consensus residues [using structural considerations in some cases where the choice is not clear (35)]. These molecules implicitly constitute the origin of library diversification and might be expected to have a very high stability, and indeed the experimental results support this notion. When starting from such a point of extremely high stability to create a library, many changes in the protein necessary for function but detrimental to stability can be tolerated, even in the conserved positions, and the outcome is usually still a very well-behaved protein (11, 33, 35, 36).

The full consensus DARPins express very well in *E. coli* as soluble monomers, their stability increases with length, and those with more than three internal repeats are resistant to denaturation by boiling or guanidine hydrochloride. Full denaturation requires heating in 5 M guanidine hydrochloride (35). Hydrogen/deuterium exchange experiments of DARPins with three internal

repeats indicate that some amide protons require more than a year to exchange at 37°C, highlighting the extraordinary stability of the proteins (30).

Selection Technologies for DARPin Libraries

DARPins appear to fold well under most conditions, and binding molecules can be selected from synthetic DARPin libraries with most display or selection methods. Results from ribosome display, phage display, and yeast display are summarized.

Ribosome display is a potent in vitro method to select and evolve proteins or peptides from a naive library with very high diversity to bind to any chosen target of interest (6, 7, 37, 38). A key feature of ribosome display is that, in contradistinction to most other selection technologies (26), it incorporates PCR into the procedure and thus allows a convenient incorporation of a diversification (randomization) step using a variety of error-prone procedures. Ribosome display thereby allows refinement and affinity maturation not only of defined binders but also of the whole pool during selection from a complex library, if desired (39–42). DARPin binders are apparently enriched somewhat faster than binders from a comparable scFv library in ribosome display selections (38, 43)—a finding that can be explained, for instance, by the DARPins' good folding behavior in cell-free translation and the fact that their rigid fold is less prone to instability introduced by mutations (if affinity maturation is applied). More recently, ribosome display has been automated to allow the parallel selection against 94 targets simultaneously (J.V. Schaefer, O. Scholz, T. Looser, T. Reinberg, S. Furler, M. Göransson & A. Plückthun, unpublished experiments).

Using ribosome display, DARPins have been evolved to bind various targets with affinities all the way down to dissociation constants (K_d) in the picomolar range (32, 41, 42, 44–49). This relies on designing efficient, off-rate selections, and the theoretical considerations were recently formulated (50).

Phage display can be of interest when selecting binders against targets on the surface of whole cells (whole-cell panning) (51). Because DARPins fold very fast in the cytoplasm (35), an initial problem arose: The display of DARPins using standard phage display vectors was unexpectedly low. In filamentous phage display, the protein of interest (i.e., the DARPin) is usually fused to the phage minor coat protein p3. This fusion protein is first produced as a membrane-bound intermediate by the *E. coli* Sec machinery: The major part is secreted to the periplasm, whereas the C-terminal helix of p3 still remains attached to the inner membrane before the whole fusion protein is taken up by the coat of the extruding phage.

Because DARPins appear to fold before they can be transported across the membrane via the posttranslational Sec system—the normal way of secreting *E. coli* proteins—they must be secreted via the signal recognition particle (SRP)-dependent system. This system is essentially cotranslational (52, 53). Using phagemids with SRP-dependent signal sequences, display rates of DARPins are just as high as, for example, scFv fragments with conventional Sec signal sequences. Thus, SRP phage display selection of DARPins leads to enrichment just as fast as for scFv fragments using conventional Sec-dependent phages (54). After constructing a diverse, synthetic DARPin SRP-phage library, binders with subnanomolar dissociation constants could be isolated from the phage display library without affinity maturation for a variety of targets (27).

For completeness, it should be mentioned that *E. coli* also has a third transport system, the twin arginine transport (Tat) system. However, attempts to achieve functional display of p3 fusions via the Tat route have proven unsuccessful (55–57), as the full-length p3 protein may itself be incompatible with the Tat system. Still, a truncated version of p3 can support Tat-mediated phage display (58).

More recently, yeast display (59) has also been used for DARPin selection. DARPins are displayed at rates equal to the highest display levels reported for any protein on yeast, probably because

they are well compatible with the yeast quality-control system of the endoplasmic reticulum (M. Schütz, S. de Piciotto, K.D. Wittrup & A. Plückthun, unpublished experiments). Yeast display, although limited to smaller libraries, can be used to efficiently select clones by fluorescence-activated cell sorting (FACS) for particular binding, selectivity, or fluorescent properties. The possibility of gating in FACS allows a fine-tuning of selections, thereby complementing the other display methods.

Finally, DARPins have also been selected using protein fragment complementation (bringing the two halves of dihydrofolate reductase together as a function of target recognition) (60). This is a cytoplasmic selection system, illustrating the robustness of the DARPin scaffold.

APPLICATIONS OF DARPins

DARPins in Biochemical Research

Investigation of protein regulation, i.e., inhibition and activation inside living cells, is fundamental to our understanding of how proteins are influenced by their upstream regulators. Because DARPins can be expressed in a functional form inside the cell, they form a good basis for creating biosensors. As a proof of principle, DARPins were selected by ribosome display that specifically bind to the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) in either its nonphosphorylated (inactive) or doubly phosphorylated (active, p-ERK) form (61) (**Figure 2**). They do not bind to other kinases tested. Crystal structures of complexes with two DARPins, each specific for either ERK or p-ERK, were obtained (**Figure 2*a*,*b***). Using bioluminescence resonance energy transfer, the specificity inside the cell was confirmed. In a related series of experiments, DARPins were developed as selective inhibitors of c-Jun N-terminal kinase-1 (JNK1) or JNK2 (62).

The two DARPins (specific for ERK or p-ERK) bind to essentially the same region of the kinase but recognize the conformational change within the activation loop and an adjacent area, which is the key structural difference that occurs upon kinase activation. In a follow-up study, the DARPin specific for p-ERK was derivatized with a solvatochromic merocyanine dye, whose fluorescence increases in a more hydrophobic environment, i.e., upon binding to its target (63) (**Figure 2*c*,*d***). The biosensor specifically responded to p-ERK, recognized by its conformation, but did not react to ERK or other closely related MAPKs tested. Activated, endogenous ERK could thus be selectively visualized in mouse embryo fibroblasts, revealing greater activation in the nucleus, perinuclear regions, and especially the nucleoli (63). Such conformation-sensitive biosensors will be useful tools to study many biological processes in real space and real time.

Because DARPins are very rigid molecules, they would be expected to crystallize readily with their respective targets. Many experimental results confirm this (for reviews, see References 47, 64, and 65). The facile crystal formation has greatly accelerated the process of engineering DARPins for specialized purposes, as the structural information derived from X-ray crystallography is crucial to studying the molecular interactions in atomic detail.

Whether the cocrystallization with a DARPin would routinely allow crystal formation of proteins that otherwise do not crystallize (e.g., because they may contain flexible surface loops) will depend on whether DARPins can dominate crystal packing. Studies are under way to generate DARPins that are further optimized for this purpose, e.g., by designing rigid domain fusions to other well-crystallizing domains. The results obtained so far seem to validate this concept (A. Honegger, Y. Wu, A. Batyuk & A. Plückthun, unpublished experiments). Furthermore, rigid multidomain DARPins also hold promise for helping to solve the crystallographic phase problem by using molecular replacement with such rigid DARPin fusions.

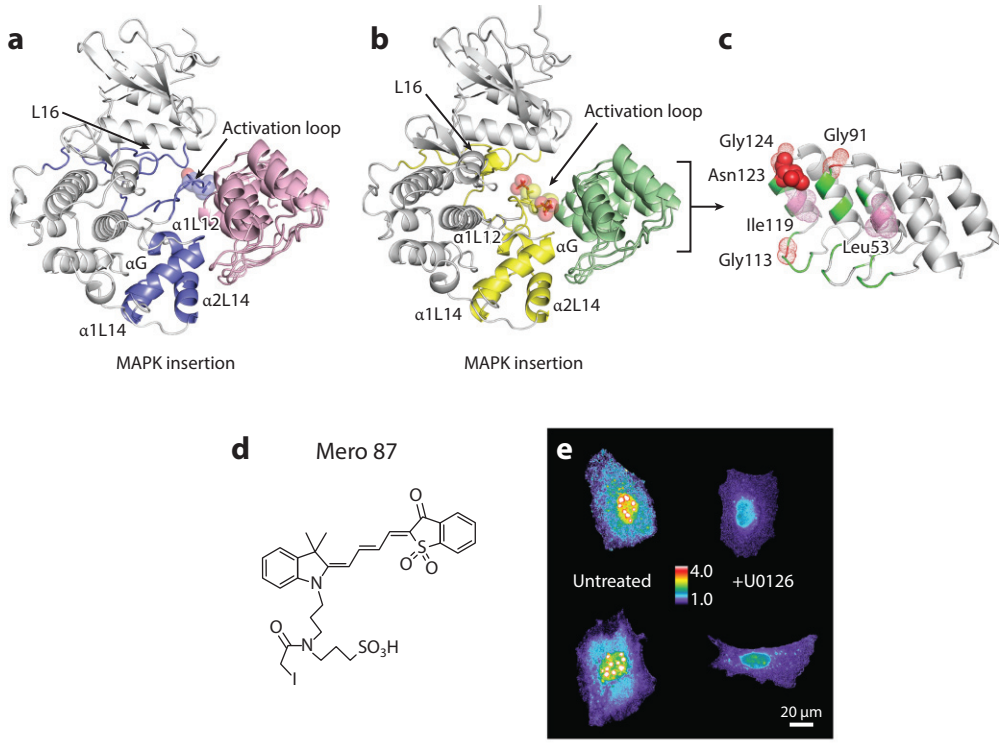

Figure 2

Development of an intracellular fluorescent sensor specific for detecting the active conformation of the kinase ERK (63). (*a*) Structure of DARPin E40 (*pink*) in complex with nonphosphorylated ERK (61). (*b*) Structure of DARPin pE59 (*green*) in complex with phosphorylated, active p-ERK (61). The DARPins recognize the activation loop, the only structural feature significantly different between the kinase forms. They also make contact with the MAPK insertion (*blue* and *yellow* for panels *a* and *b*, respectively), explaining why they are highly specific for ERK. (*c*) Ribbon structure of p-ERK binding DARPin pE59 (as in panel *b*). The area contacting p-ERK is shown in green. Residues at the edge of this area (*pink* or *red*, *numbered*) were individually changed to cysteine and derivatized with the solvatochromic dye Mero 87 shown in panel *d*. Coupling at Cys 123 (*red*) resulted in the best sensor. Once the DARPin–Mero 87 conjugate binds to p-ERK, the fluorescence intensity increases several-fold. (*e*) The sensor was microinjected into live NIH 3T3 mouse embryo fibroblasts stably expressing YPet, a derivative of yellow fluorescent protein. Ratiometric imaging (to control for cell thickness and uneven illumination) shows an increased emission ratio (sensor over YPet) in the nucleus and especially the nucleolus. This indicates the location of active p-ERK in a living cell. As expected, the inhibitor U0126 (targeting the upstream kinase MEK1/2) strongly reduces this activation of ERK. Abbreviations: DARPin, designed ankyrin repeat protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; p-ERK, phosphorylated ERK; YPet, yellow fluorescent protein for energy transfer. Reprinted from Reference 61 with permission from the US National Academy of Sciences and from Reference 63 with permission from Elsevier.

DARPins in Diagnostics

Diagnostics is still a stronghold of antibodies derived from immunized mice. This is despite the fact that, for most diagnostic purposes, there is no need for the Fc part, as typically only the binding site is required.

Because DARPins are very robust and the molecular format can be freely chosen, one can create a wide series of fusion proteins and conjugates. Thus, DARPins are interesting binders for developing new diagnostic detection systems, and they seem well poised to replace antibodies in clinical assays. An important diagnostic application is quantitative immunohistochemistry. As a

proof of principle, a DARPin specific for human epidermal growth factor receptor 2 (HER2) with picomolar affinity was compared to a US Food and Drug Administration (FDA)-approved rabbit monoclonal antibody in paraffin-embedded tissue sections in tissue microarrays (66). The HER2 gene amplification status is an important criterion to determine the optimal therapy in breast cancer. As an external reference, the HER2 amplification status was determined by fluorescence in situ hybridization. The DARPin detected a positive HER2 amplification status with similar sensitivity but significantly higher specificity than the FDA-approved antibody (66). Affinity was found to be crucial. Nonetheless, because the DARPins investigated have a picomolar K_d, it was more advantageous to use them as monovalent molecules than to increase avidity even further by making multivalent constructs—presumably because the monomers deliver more label per epitope, which is then enzymatically detected. Therefore, DARPins can have the desired specificity characteristics for diagnostic pathology.

DARPins in Tumor Targeting: Effects of Size and Affinity

Researchers have generally assumed that, to be useful in therapeutic applications, the half-life of nonantibody proteins has to be increased to approach that of IgG or serum albumin. Both of these long-lived molecules are equipped with a special recycling mechanism (67) that prevents their degradation by the reticulo-endothelial system. They exploit binding to the FcRn receptor, which recycles them back to the plasma membrane. Consequently, these properties can be transferred to the protein of interest by fusing them to the Fc part or albumin. However, this occurs at a cost: The fusion protein must then be produced in a eukaryotic host because of the disulfide-rich nature of either fusion partner.

Other approaches to extend half-life are to chemically couple the bacterially expressed DARPin with serum albumin (68) or to achieve the same by using binding proteins, by fusing the targeting DARPin to another DARPin that binds to serum albumin (**http://www.molecularpartners.com**) or the Fc part of antibodies. Serum half-life can also be extended by attaching PEG, which can be conveniently done site-specifically at a single engineered cysteine or by using bioorthogonal click chemistry (69) (see below), thereby leaving cysteine as another handle for coupling, e.g., to a small-molecule toxin, radiometal chelator, or fluorescent dye. For a PEG molecule with a nominal molecular weight (MW) of 20 kDa, the hydrodynamic properties correspond to a MW of about 250 to 350 kDa (70, 71), thus ranging beyond the size limit that can be filtered by the kidney—renal clearance being the major mechanism of short serum half-life.

But is a molecule with a long half-life always the best in vivo format? Using DARPins, the influence of affinity and size on the efficiency of targeting was systematically investigated. Point mutants of a DARPin binder to HER2 spanning affinities from 280 nM to 90 pM (different stages from directed evolution) (41, 45) were compared in unmodified form (MW 15 to 18 kDa) and PEGylated form [hydrodynamic sizes of about 250 to 350 kDa (70, 71)]. Two distinct parameter regions for efficient tumor accumulation were found.

The first parameter region is dominated by affinity: Unmodified DARPins (i.e., DARPins with a small hydrodynamic size) accumulate rather efficiently at the tumor site but do so directly proportional to affinity. This high accumulation for the smallest molecules might be at first unexpected. A value of 8% injected dose/g tissue (ID/g) was reached after 24 h for a 90-pM binder in an SK-OV-3 subcutaneous mouse xenograft model. No evidence for a barrier effect—the empirical observation that macromolecules often accumulate only at the outside of the tumor—was observed (72). The small DARPins were cleared from the blood extremely rapidly, such that very high tumor to blood ratios (60:1) were measured 24 h after injection. A lower accumulation in the tumor was seen for bivalent DARPins (measured avidity of 10 pM on cells) than for their monovalent counterparts

(which already had a K_d of approximately 90 pM), suggesting that smaller size is more important for tumor accumulation than very high avidity. When fusing a nonbinding DARPin to the anti-HER2 DARPin as a control ($K_d \approx 90$ pM), the same lowered uptake was observed as with the bivalent DARPin, pointing to a size effect (as the bivalent binding on cells had been verified). This lower accumulation is consistent with similar numbers measured for antibody scFv fragments in the same tumor model (73, 74). The scFvs have MWs similar to the two-DARPin constructs. Thus, a very small MW (smaller than scFv) can lead to efficient targeting, provided affinity is picomolar.

The second parameter region of high tumor accumulation is given by the PEGylated DARPins. They accumulated more slowly and to an even higher extent (13.5% ID/g). As might be expected from their larger size, they were hardly cleared through the kidney, and their blood persistence was much longer, leading to smaller tumor to blood ratios. Interestingly, the importance of affinity was diminished in this format, with the DARPin of 90 pM K_d not showing a great advantage over the one with 1 nM K_d (72). Nonetheless, the low affinity construct with a K_d of 280 nM showed significantly lower uptake, excluding passive accumulation by the enhanced permeability and retention effect (75) as a major contributor.

It should be stressed that this finding of two MW optima is fully consistent with the elegant modeling studies of Wittrup and colleagues (76, 77) which all have independently and concurrently come to very similar conclusions. To rationalize these findings, a very pronounced dependence of extravasation on MW can be proposed. Thus, if extravasation is even more dependent on lower MW than the renal filtration threshold, then a molecule of intermediate MW would be filtered through the kidney and clear very rapidly but would still not extravasate very well. Importantly, a molecule of small MW needs to bind to its cell-surface receptor on the tumor very tightly, or it will be washed out rapidly. This affinity requirement is not as strong for very large, PEGylated molecules, which reside in the serum for much longer times. In contrast, medium-sized molecules (such as scFv fragments) are still being cleared through the kidney, without reaching the tumor fast enough, because of their slower extravasation.

From a series of elegant studies on quantifying tumor accumulation of mono- and multivalent scFv fragments, Adams et al. (78) have proposed that a very high affinity might be disadvantageous for efficient tumor targeting. It should be noted, however, that in these investigations, iodine was used as a label, and this is removed by dehalogenases upon internalization. Thus, high affinity (or high avidity) leading to more internalization will lead to less remaining iodine label in the tumor (79). In contrast, the DARPin study by Zahnd et al. (72) mentioned above used a residualizing Tc label (80), which will not be removed upon internalization and will thus be counted and imaged, no matter whether the protein has become internalized or whether it remains on the surface. Thus, when considering all protein molecules that have ever arrived at the tumor site, no decrease in uptake is observed with very high affinity, and no barrier effect is observed. In contrast, if one were to count only those molecules that have remained on the surface, there would indeed be a decrease with very high affinity, as a larger proportion of protein gets internalized and thus loses its (iodine) label. It is thus very important to consider the label with which these measurements are carried out before generalizing statements about tumor uptake and affinity.

Engineering Naked DARPins for Selective Tumor Killing

Human epidermal growth factor receptor-2 (HER2/ErbB2) is a receptor tyrosine kinase without a known natural ligand, directly linked to the growth of malignancies from various tissues. HER2 amplification promotes tumorigenesis (81), and human tumors and various tumor cell lines rely on HER2 signaling for their survival. Such cancer cells are often referred to as HER2-addicted (82). HER2 is the target of two FDA-approved monoclonal antibodies, trastuzumab (Herceptin) and

pertuzumab (Perjeta), as well as the antibody drug conjugate trastuzumab emtansine (T-DM1; Kadcyla) (83, 84). Nonetheless, trastuzumab resistance develops in a great number of patients, and its effect on long-term overall survival is very small at best (85).

Besides recruiting immune effector cells through the Fc part (86), the antibodies interfere with signaling. Pertuzumab binds next to the dimerization arm on subdomain II of HER2 (87) and therefore interferes with HER2/HER3 heterodimer formation, provided the HER3 is activated by its ligand heregulin (88, 89). Pertuzumab shows only moderate antitumor effects in vitro on HER2-overexpressing breast cancer cell lines (90). In contrast, trastuzumab binds to the extracellular subdomain IV of HER2, and it is thought to interfere with homodimerization of HER2 (91) (even though this is controversial) and especially heterodimerization between HER2 and unliganded HER3 in HER2-overexpressing tumors (89) (**Figure 3*a*,*b***). For this reason, it is used to treat patients with HER2-overexpressing breast cancers (92, 93).

Because the antibodies work only in conjunction with chemotherapy and because of the rapid development of resistance with subsequent remissions, new therapeutics are required that work differently. To create the modules for new modes of action, DARPins that bind to the extracellular part of HER2 were selected (41, 45). Not unexpectedly, the monovalent DARPins did not show any measurable effect on the growth of HER2-overexpressing tumor cells. However, DARPins can be joined in many orientations and in multivalent and multispecific ways, and they can all be produced and purified from *E. coli* with equal ease (e.g., 18, 94). By testing various multivalent HER2-binding DARPin constructs, binders with strong cytotoxic effects on HER2-overexpressing tumor cells could be identified. The most active constructs link a DARPin binding to domain I to one binding to domain IV with a very short linker (95).

The crystal structure of these DARPins was determined in complex with the relevant domain of HER2 (95), and because the HER2 domains, which are very rigid, superimpose perfectly onto the domains in the context of the whole HER2 extracellular region, they can be placed directly on this molecule (**Figure 3*c*,*e***), thereby delineating the exact mode of binding of the DARPins on HER2. Importantly, kinetic experiments on cells have shown that both domains are indeed engaged simultaneously, but the short linker prevents binding in an intramolecular way. This is true even if one considers that HER2 might assume a pseudotethered form (95)—for which there is no evidence but whose existence under force cannot be rigorously excluded a priori. It thus follows that the bispecific DARPins engage two molecules of HER2.

This structure then immediately explains the likely mode of action of the bispecific DARPins: To connect the DARPin binding to domain I of one HER2 molecule to the DARPin binding to domain IV of a neighboring HER2, the former HER2 extracellular domain (ECD) has to bend over, with the extracellular region moving more or less as a rigid body (**Figure 3*c*,*e***). In fact, multiple lines of evidence point to the fact that the whole extracellular region of HER2 is rather rigid (96, 97). Because HER2 is anchored by the transmembrane helix, it can move only in two dimensions within the membrane. This DARPin-induced unusual locking of HER2 molecules has several important consequences: It bends the dimerization arms of all bound HER2 molecules toward the membrane, making them essentially inaccessible, and forces the transmembrane helices and the directly connected kinase domains apart. Seminal work from Kuriyan and colleagues on the HER2 homologue epidermal growth factor receptor (EGFR) (98, 99) has shown that a mere random collision of kinases would be insufficient for mutual activation—instead, they need to be positioned, at least transiently, by the interaction mediated by the juxtamembrane peptide region, which in turn would be prevented by the forced separation of the transmembrane helices (**Figure 3**).

This model makes several important predictions, which have all been borne out by experimental support. A reversal of the order of domains in the bispecific DARPin will not enforce the bent structure well, and indeed, those inverted constructs are much less active, as are constructs with

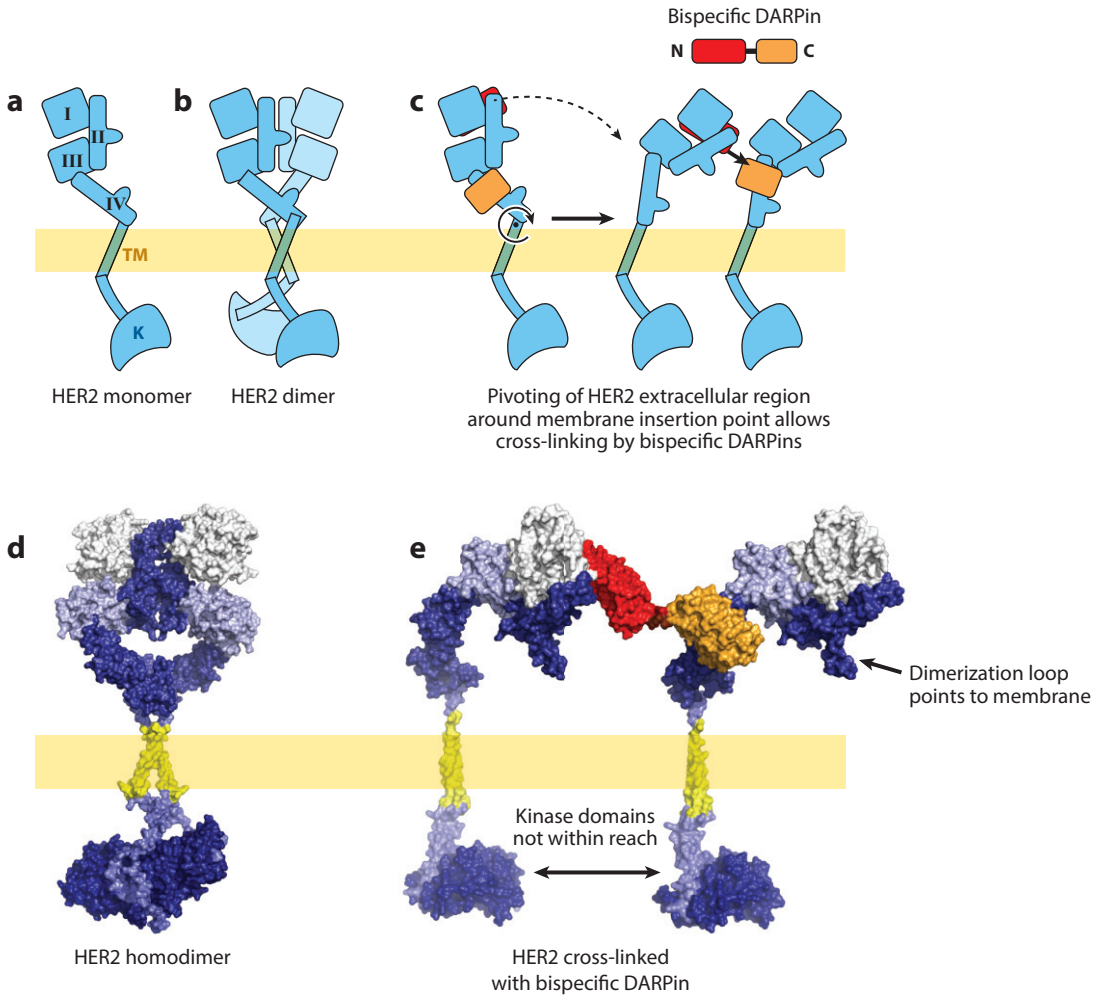

Figure 3

Mechanism of action of bispecific DARPins to induce apoptosis in HER2-addicted tumor cells. (*a*) HER2 monomer with the extracellular domains (roman numerals), the transmembrane domain (TM), and the kinase domain (K) indicated. (*b*) HER2 dimer interacting via the dimerization loop. (*c*) Action of the bispecific DARPin (schematically shown on the top right in *red* and *orange*). On the left HER2 monomer, the two monovalent DARPins (*red* and *orange*) are located as deduced from the individual crystal structures of the complexes with the extracellular domains of HER2 (95). When the two DARPins are linked, as in the orientation shown on the right, the only way that both can remain bound to HER2 is if the whole extracellular region bends over, as HER2 can only move within the plane of the membrane. Bending of the whole extracellular region is possible because there are a few disordered residues in HER2 next to the membrane, providing a likely pivot. In contrast, the whole extracellular region is assumed to be rather rigid (see main text). (*d*) Model of the HER2 homodimer, based on the experimental monomer structure and the dimer structure of the EGFR. (*e*) Detailed model of the DARPin-induced, signaling-inactive state providing a pan-HER or panErbB inhibition, corresponding to the illustration in panel *c*. The tilting of the whole extracellular region results in removal of the dimerization loop from possible interactions and pushes the transmembrane helices, and thus the kinase domains, apart from each other; this abolishes stable contacts and thereby prevents the kinases from *trans*-phosphorylation. HER2 is therefore removed from participating in signaling by an agent acting from the outside of the cell. Other abbreviations: C, C terminus; DARPin, designed ankyrin repeat protein; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor-2; N, N terminus. Reprinted from Reference 95 with permission from Elsevier.

longer linkers. Mixtures of monovalent DARPins and mixtures of homobivalent constructs are all inactive, emphasizing that it is the disengagement of the kinase in the inactive complex that is the key (95).

The bispecific DARPins turn out to be pan-HER inhibitors. They lead to a loss of phosphorylation from both HER2 and HER3, whereas trastuzumab only leads to HER3 dephosphorylation, with no effect on HER2 dephosphorylation (R. Tamaskovic, M. Schwill, C. Jost, D.C. Schaefer, G. Nagy-Davidescu, A. Honegger & A. Plückthun, unpublished data). A detailed investigation of the DARPins' interference with signaling has clarified how the DARPins achieve this, and what the decisive differences are to the effects of trastuzumab and pertuzumab on signaling. As a consequence, the net effect of the described DARPin treatment is the robust induction of apoptosis in all HER2-overexpressing cell lines and tumors investigated, with no measurable effect on cardiac myocytes (R. Tamaskovic, M. Schwill, C. Jost, D.C. Schaefer, G. Nagy-Davidescu, A. Honegger & A. Plückthun, unpublished data). In contrast, trastuzumab—always given together with chemotherapy—has led to cardiotoxicity in a significant percentage of patients (100).

By creating a trap for HER2, in which the receptor is bent over and kinases are unable to interact, all signaling from HER2 complexes is obstructed, leading to a pan-HER inhibition. The outlined strategy may be the first rational approach to engineer cell-specific apoptosis based on a structurally and mechanistically understood principle yet without using a toxin with potential off-tumor side effects. It thus has the potential to avoid resistance because of the DARPins' receptor-mediated cytotoxic and not their cytostatic action.

DARPins as a Delivery Vehicle in Tumor Therapy

Despite many open questions in EGFR-family biology, most receptors outside this family are understood even significantly less well and do not yet lend themselves to the type of structure-based engineering described for HER2. In these cases, the DARPin needs to deliver a payload, and the main advantage of this platform is that it maintains its favorable biophysical properties as a fusion protein.

Two DARPin examples, both using the epithelial cell adhesion molecule (EpCAM) as the target, are discussed below to illustrate their potential. EpCAM is a homophilic cell adhesion molecule of 291 amino acids, consisting of a large glycosylated and disulfide-bonded ECD, a single transmembrane helix, and a short cytoplasmic domain (101, 102). The latter can be cleaved off by intramembrane proteolysis as a result of specific cell-cell contacts and might, as a result, travel to the nucleus to drive tumor and stem cell proliferation.

EpCAM is an attractive tumor-associated target, as it is expressed at low levels on basolateral cell surfaces of only some normal epithelia, whereas high levels of homogeneously distributed EpCAM are detectable on cells of epithelial tumors. Recently, EpCAM was also identified as a marker of cancer-initiating cells (101, 102).

The favorable properties of EpCAM for cancer therapy are currently exploited in Phase II clinical trials with an immunotoxin where an antibody scFv fragment is fused to *Pseudomonas aeruginosa* exotoxin A (PE40/ETA) (103–106). This scFv-based immunotoxin had been developed (104) before the DARPin technology was invented.

To exploit the favorable properties of DARPins, an EpCAM-specific DARPin was produced as a fusion toxin with ETA and expressed in soluble form in the cytoplasm of *E. coli* (107) in excellent yield. Whereas the DARPin has no cysteines, the disulfides in the toxin part formed spontaneously, and the protein was monomeric. The DARPin-ETA fusion was highly cytotoxic against various EpCAM-positive tumor cell lines with IC_{50} values less than 0.005 pM. Upon systemic

administration in athymic mice, the DARPin-ETA fusion efficiently localized to EpCAM-positive tumors and resulted in a strong antitumor response in tumor-bearing mice, using two different EpCAM-positive cell lines, leading to complete regressions in some animals (107).

The facile chemical modification of DARPins could also be used to couple them to PEG in a bioorthogonal way at a defined position, thus not interfering with the thiols of ETA (69). The non-natural amino acid azidohomoalanine (Aha), a methionine analog, can be incorporated in methionine-auxotrophic *E. coli*. It allows strain-promoted click PEGylation with suitable strained alkynes and thus does not require the use of toxic Cu(I). Despite an increased hydrodynamic radius resulting from the polymer, the fusion toxin demonstrated high EpCAM-binding activity and retained cytotoxicity in the femtomolar range. Pharmacological analysis in mice unveiled an almost 6-fold increase in the elimination half-life and a more than 7-fold increase in the area under the curve (AUC) compared to non-PEGylated DARPin-ETA, which translated directly into increased and longer-lasting effects on established tumor xenografts (69).

Among the targeted toxins, besides the fusion proteins with protein toxins, antibodies with chemical drug conjugates are showing important progress in the clinic (108). Here, DARPins can also be used as building blocks, as their properties can be exploited for site-specific orthogonal coupling to two different moieties, a toxin and a component for half-life extension. In this manner, EpCAM-targeting DARPin Ec1 was genetically modified with a C-terminal cysteine for conjugation of monomethylauristatin F (MMAF) (68), and at the same time functionalized at the N terminus by introducing Aha during production in *E. coli*. Because DARPins do not contain cysteines and can easily be made methionine-free, either residue can be incorporated at any place in the protein. Aha was linked to dibenzocyclooctyne-modified mouse serum albumin (MSA) for half-life extension using strain-promoted click chemistry (68). The conjugate MSA-Ec1-MMAF, assembled in high yields as a pure and stable drug conjugate, increased the serum half-life from 11 min to 17.4 h, resulting in a more than 22-fold increase in the AUC (68). DARPins can thus be formatted for facile modular assembly of drug conjugates with improved pharmacokinetic performance for tumor targeting.

DARPins have also been explored for the delivery of small interfering RNA (siRNA). Although much research has been carried out on the biological function and application of siRNA for tumor control, the efficient organ- and cell-specific uptake of nucleic acids remains a major challenge for gene-targeted cancer therapies. An anti-EpCAM DARPin was used as a carrier for siRNA complementary to the Bcl-2 mRNA, an antiapoptotic factor overexpressed in many cancers (109). To achieve complexation of siRNA, the DARPin was genetically fused to protamine, a positively charged protein that is probably unstructured, and about four to five molecules of siRNA could be bound per protamine. This could be exploited to result in a significant sensitization of EpCAM-positive MCF-7 cells toward doxorubicin. Indeed, this sensitization was not observed in EpCAM-negative cells, indicating that siRNA uptake is receptor dependent (109). This direct delivery of naked oligonucleotides to particular cells will have to be evaluated in comparison to delivery with engineered viruses (see the next section).

DARPins for Viral Retargeting

Viral retargeting to predefined organs and cells is clearly one of the grand challenges of future medicine. Recently, the first human gene therapy, an adeno-associated virus (AAV) encoding the gene lipoprotein lipase to treat a genetic deficiency in this enzyme, alipogene tiparvovec (Glybera), has received European Medicines Agency approval (110). Here, the virus's natural broad tropism is exploited to target muscles, as Glybera is injected just once into the muscle of the lower extremities, where it is taken up by myocytes.

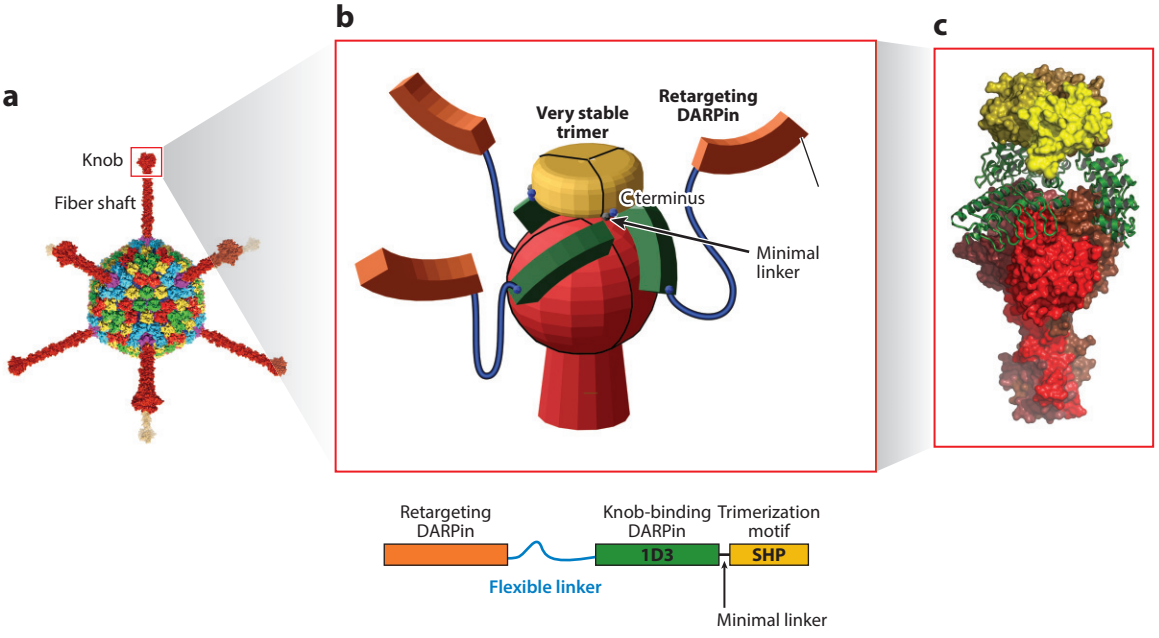

Figure 4

Adapter strategy for retargeting adenovirus (115). (*a*) Atomic model of adenovirus 5, highlighting the fibers (*red*) that extend from the viral capsid and consist of fiber shaft and knob domains, which were used for DARPin selection. (*b*) The adenovirus adapter. The gene is shown at the bottom, and the protein can be made in *Escherichia coli*, where it assembles to a soluble and fully functional trimer. The knob-binding DARPin (*green*) is fused on one side to the phage SHP protein, which forms a very stable trimer (*yellow*) that is kinetically stable against dissociation (116). At the other end, the knob-binding DARPin is connected via a flexible linker (*blue*) to a retargeting DARPin (*orange*) that binds to a cell surface receptor. This trimeric clamp shows no measurable dissociation from the virus over 10 days. A detailed model is shown in panel *c*, based on the solved crystal structures of SHP alone and knob-binding DARPin in complex with the knob domain. Abbreviations: DARPin, designed ankyrin repeat protein. Reprinted from Reference 115 with permission from the US National Academy of Sciences.

There are three main challenges to generalizing viral delivery: (*a*) to specifically target the virus to the cells of interest and to spare other tissues or organs; (*b*) to deliver a payload that is effective for the desired application; and (*c*) to evade the immune system, at least for as long as necessary to carry out the desired treatment. Adenoviruses (Ads) are a family of nonenveloped viruses that contain a double-stranded DNA genome, which remains episomal—currently perceived as an important safety factor—and they have been developed for gene therapy (111), genetic immunizations (112), and molecular-genetic imaging (113). Their large genome makes it possible to encode sophisticated genetic constructs and multiple genes.

Many strategies of retargeting are currently being pursued, including fusions with viral coat proteins or covalent attachments to the coat (114). With DARPins, another approach became possible, with a bispecific adapter that can be produced in *E. coli* (42, 115) (**Figure 4**). A series of adapters was developed that bind to the virus with such high affinity that they remain fully bound for more than 10 days, block its natural receptor-binding site, and mediate interaction with a surface receptor of choice. The adapter contains two fused modules, both consisting of DARPins, one binding to the fiber knob of adenovirus serotype 5 (Ad5) and the other binding to a cell surface receptor of choice, e.g., various tumor markers. By solving the crystal structure of the complex of the trimeric knob with three bound DARPins, computer modeling could be used to

design a link to a trimeric protein of extraordinary kinetic stability, the capsid protein SHP from the lambdoid phage 21 (116). A module was thereby derived that binds the knob like a trimeric clamp (**Figure 4*c***). When this clamp was fused with DARPins of varying specificities (**Figure 4*b***), it enabled Ad5-mediated delivery of a transgene in a HER2-, EGFR-, or EpCAM-dependent manner with transduction efficiencies comparable to or even exceeding those of Ad itself. With these adapters, all efficiently produced in *E. coli* and readily scaled up, Ads can be converted rapidly to new receptor specificities using any ligand as the receptor-binding moiety. Prefabricated Ads with different payloads can thus be retargeted readily to many different cell types of choice.

Lentiviral vectors lead to stable integration and transgene expression in nondividing cells. Cell entry is dependent on two viral glycoproteins, hemagglutinin (H) and fusion protein (F) (117). By using lentiviral vectors expressing MV-H and MV-F from measles virus (MV), and by specifically creating a variety of fusions of MV-H to HER2-specific DARPins, infection of HER2-expressing cells could be obtained (118). All H-DARPin fusion proteins tested were expressed efficiently on the cell surface and incorporated into lentiviral vectors at a more uniform rate than different scFvs tested, perhaps because of the more robust folding of the DARPin within the fusion protein. The lentiviral vectors only transduced HER2-positive cells, whereas HER2-negative cells remained untransduced. The location of the epitope may be important, as the highest titers were observed with one particular anti-HER2 DARPin binding to the membrane-distal domain I of HER2; lower titers were found for a DARPin binding to domain IV of HER2, which is closer to the membrane. When these DARPin-carrying viral vectors were applied systemically in a mouse tumor xenograft model, gene expression was observed exclusively in HER2-positive tumor tissue, whereas control vectors mainly transduced cells in spleen and liver (118). Thus, DARPins constitute a promising route to engineer the specificity of lentiviral vectors for therapy.

In a similar manner, MV has been reengineered with DARPins (119). The MV attachment protein was fused to DARPins, simultaneously ablating entry through the natural receptors. DARPin-targeted viruses were strongly attenuated in off-target tissue, thereby enhancing safety, but completely eliminated tumor xenografts, albeit only after intratumoral injection. Because DARPins can be linked without disturbing their folding, a virus could be generated that simultaneously targets two different tumor markers. The bispecific virus retained the original oncolytic efficacy while providing proof of concept for a strategy to counteract issues of resistance development (119).

Finally, AAV has also been retargeted with DARPins by using a fusion to the VP2 protein on AAV capsids ablated for natural primary receptor binding. DARPin-AAV vectors delivered a suicide gene to tumor tissue and substantially reduced tumor growth without causing fatal liver toxicity (120).

DARPins in the Clinic

DARPins have been selected against human vascular endothelial growth factor VEGF-A with single-digit picomolar affinity (121) for the treatment of diabetic macular edema (DME) and age-related macular degeneration (AMD) (122). To facilitate preclinical and clinical development, DARPins were chosen that showed cross-reactivity with VEGF-A of several mammalian species. Intravitreally injected DARPin penetrated into the retina and reduced fluorescein extravasation in a rabbit model of vascular leakage. In addition, topical DARPin application was found to diminish corneal neovascularization in a rabbit suture model and to suppress laser-induced neovascularization in a rat model.

To evaluate the safety and bioactivity of MP0112 (AGN-150998, abicipar pegol), the anti-VEGF DARPin, a Phase I/II, open-label, multicenter, dose-escalation trial was carried out in

patients with DME (123) to assess safety, aqueous MP0112 levels, change in best-corrected visual acuity (BCVA), and foveal thickness measured by optical coherence tomography. The DARPin showed long residence in the eye: One week after the injection of 0.4 mg, the concentration was 555 nM and was >10 nM in 3 of 4 patients 12 weeks postinjection. Thus, even at this modest injected dose, DARPin concentrations in the aqueous humor remain above the half-maximal inhibitory concentration after 8–12 weeks. Median BCVA improvement at week 12 was 4, 6, and 10 letters in cohorts receiving 0.04 mg, 0.15 mg, and 0.4 mg, respectively.

Initial data have been released from a parallel AMD trial (124, 125), a Phase I/II, open-label, multicenter, dose-escalation trial. The clinical study with DARPin MP0112 assessed the safety and preliminary efficacy in treatment-naive patients for 16 weeks. The MP0112 wet AMD study (124, 125) consisted of different dose-ascending cohorts, with patients receiving a single dose of MP0112 (from 0.04 to 3.6 mg) as intravitreal injections. MP0112 was safe and well tolerated. Visual acuity scores were stable or improved compared with baseline for ≥4 weeks following injection; both retinal thickness and fluorescein angiography leakage decreased in a dose-dependent manner. At the end of the 16 weeks' follow-up, all patients had stable or increased visual acuity. At the 4-week visit, only 4 of 10 (40%) patients who received 1.0 or 2.0 mg required rescue therapy. Of patients in the higher-dose cohorts who did not require rescue treatment, 83% (5/6) maintained reductions in central retinal thickness through week 16. Thus, the higher MP0112 doses show potential for quarterly dosing for the treatment of wet AMD.

DARPin MP0112 (AGN-150998, abicipar pegol) therefore represents a very promising, new, anti-VEGF treatment option with potential in various retinal diseases. It directly shows that a benefit for the patient can be achieved with proteins engineered to have very good biophysical properties, combined with very high target affinity and specificity.

DARPins in Other Approaches

For space reasons, other approaches of potential therapeutic significance can be mentioned only briefly. DARPins have been used to investigate the steps of apoptosis, either to control the regulators of the Bcl-2 family (31, 126) or those of caspases (46, 127). Tubulin polymerization and depolymerization control many critical aspects of the cell, and DARPins have been selected that cap the microtubule plus end. They stop polymerization and help give structural insight into tubulin and kinesin motors (128, 129). These are all intracellular targets, nota bene, and the activities of the DARPins can be fully exploited only once gene delivery, such as with engineered viruses, or efficient protein delivery to the cytoplasm becomes a reality.

DARPins have also been selected to bind to IgE or its receptor FcεRIα (130–132), and they might be further developed to interfere with the allergic response. Another DARPin target has been gp120 of HIV, whose binders may be used to eventually develop novel modes of action for HIV entry inhibitors (133). These additional examples further illustrate how modular the DARPin system is and that many of the approaches described in all sections of this review can be combined into new strategies.

Immunogenicity

Very few aspects of therapeutic proteins have been as hotly debated as the issue of immunogenicity. Because there are few certain facts, speculations are invited, alleging immunogenicity or the lack of it, depending on which side of the fence one is on with regard to a particular molecule or protein class.

Any type of protein, including fully human antibodies in human patients, can be immunogenic, as found for adalimumab (Humira), for example (134). Conversely, each individual case is still

almost impossible to predict. Different studies have reached different conclusions over whether there is a connection between an immune response and reduced clinical efficacy of this fully human antibody (summarized in Reference 134), because, for instance, the antibody might become neutralized.

DARPins show essentially no aggregation propensity, making a T cell–independent immune response highly unlikely. DARPins devoid of experimental T cell epitopes can be obtained from selections, as the constant parts do not carry them, and those sections containing randomized residues can be tested in the form of overlapping peptides against human T cells.

The nonzero risk of immunogenicity when administering any therapeutic protein whatsoever must be balanced by a clear patient benefit, e.g., by novel modes of action, as exemplified in the apoptosis-inducing HER2-binding molecules in **Figure 3**. Clearly, the whole field of therapeutic antibodies and therapeutic proteins eagerly awaits truly predictive animal models of immunogenicity in humans—better yet, in vitro models—and ultimately a complete understanding with the ability to control it.

CONCLUSIONS AND PERSPECTIVES

Today, three classes of molecules have been developed successfully into drugs: (*a*) small molecules, (*b*) whole antibodies, and (*c*) other engineered proteins. Although ever more members of the first two classes are being discovered and developed, their principal mode of action is unlikely to change: Small drug-like molecules can and will typically enter any cell, and their specificity is given entirely by a high affinity for the protein of choice and a low affinity for most other proteins. Antibodies can be engineered to achieve any desired cell specificity via a surface receptor interaction or to titrate out components from the serum, but they cannot efficiently get access to cytoplasmic targets that would require them to first cross a membrane. Both can be combined as in antibody-drug conjugates, but the delivered quantities limit this to the most active toxins, with a very narrow therapeutic window.

However, therapeutic intervention will not end there. We can chart out desirable strategies for the future that will, however, critically depend on engineering proteins beyond what is possible with immunoglobulins and small molecules. Examples are strategies to achieve receptor inactivation by novel cross-linking, engineering viruses for cell-specific uptake to use the body's cells for producing therapeutic proteins, and designing proteins entering not only endosomes but also the cytoplasm. Obviously, the delivered proteins must remain folded there. This would make many undruggable targets druggable, as binding proteins for any target can already be made today.

It thus appears that protein engineering will have a major role to play in the development of future medicines. We just have to be in it for the long haul.

DISCLOSURE STATEMENT

The author is a cofounder and shareholder of Molecular Partners AG, which is commercializing DARPin technology.

ACKNOWLEDGMENTS

The author would like to thank many former and current members of his laboratory, mentioned in the references, for their pivotal contributions to the research described. He is grateful to Drs. Birgit Dreier, Christian Jost, Karola Schlinkmann, Nikolas Stefan, Wouter Verdurmen, and Jakob Stüber for critical reading of the manuscript and many helpful suggestions. Financial support

has been obtained from, among others, the Swiss National Science Foundation, the European Research Council, the Swiss Cancer League, the National Center for Competence in Research in Structural Biology, and the European Union.

LITERATURE CITED

1. Forrer P, Stumpp MT, Binz HK, Plückthun A. 2003. A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett.* 539:2–6
2. Skerra A, Plückthun A. 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* 240:1038–41
3. Glockshuber R, Malia M, Pfitzinger I, Plückthun A. 1990. A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* 29:1362–67
4. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. 1994. Making antibodies by phage display technology. *Annu. Rev. Immunol.* 12:433–55
5. Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, et al. 2000. Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* 296:57–86
6. Hanes J, Plückthun A. 1997. In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA* 94:4937–42
7. Hanes J, Jermutus L, Weber-Bornhauser S, Bosshard HR, Plückthun A. 1998. Ribosome display efficiently selects and evolves high-affinity antibodies in vitro from immune libraries. *Proc. Natl. Acad. Sci. USA* 95:14130–35
8. Ewert S, Honegger A, Plückthun A. 2004. Stability improvement of antibodies for extracellular and intracellular applications: CDR grafting to stable frameworks and structure-based framework engineering. *Methods* 34:184–99
9. Plückthun A, Moroney SE. 2005. Modern antibody technology: the impact on drug development. In *Modern Biopharmaceuticals*, ed. J Knäblein, pp. 1147–86. Weinheim: Wiley-VCH
10. Kobe B, Kajava AV. 2000. When protein folding is simplified to protein coiling: the continuum of solenoid protein structures. *Trends Biochem. Sci.* 25:509–15
11. Binz HK, Stumpp MT, Forrer P, Amstutz P, Plückthun A. 2003. Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J. Mol. Biol.* 332:489–503
12. Stumpp MT, Forrer P, Binz HK, Plückthun A. 2003. Designing repeat proteins: modular leucine-rich repeat protein libraries based on the mammalian ribonuclease inhibitor family. *J. Mol. Biol.* 332:471–87
13. Pancer Z, Amemiya CT, Ehrhardt GR, Ceitlin J, Gartland GL, Cooper MD. 2004. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* 430:174–80
14. Aderem A, Ulevitch RJ. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782–87
15. Heidrich K, Blanvillain-Baufumé S, Parker JE. 2012. Molecular and spatial constraints on NB-LRR receptor signaling. *Curr. Opin. Plant Biol.* 15:385–91
16. Pancer Z, Cooper MD. 2006. The evolution of adaptive immunity. *Annu. Rev. Immunol.* 24:497–518
17. Waterhouse RM, Povelones M, Christophides GK. 2010. Sequence-structure-function relations of the mosquito leucine-rich repeat immune proteins. *BMC Genomics* 11:531
18. Boersma YL, Plückthun A. 2011. DARPins and other repeat protein scaffolds: advances in engineering and applications. *Curr. Opin. Biotechnol.* 22:849–57
19. Jost C, Plückthun A. 2014. Engineered proteins with high specificity. *Curr. Opin. Struct. Biol.* 27:102–12
20. Bork P. 1993. Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? *Proteins* 17:363–74
21. Li J, Mahajan A, Tsai MD. 2006. Ankyrin repeat: a unique motif mediating protein–protein interactions. *Biochemistry* 45:15168–78
22. Walker RG, Willingham AT, Zuker CS. 2000. A *Drosophila* mechanosensory transduction channel. *Science* 287:2229–34

23. Sedgwick SG, Smerdon SJ. 1999. The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem. Sci.* 24:311–16
24. Forrer P, Binz HK, Stumpp MT, Plückthun A. 2004. Consensus design of repeat proteins. *ChemBioChem* 5:183–89
25. Virnekäs B, Ge L, Plückthun A, Schneider KC, Wellnhofer G, Moroney SE. 1994. Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucleic Acids Res.* 22:5600–7
26. Plückthun A. 2012. Ribosome display: a perspective. *Methods Mol. Biol.* 805:3–28
27. Steiner D, Forrer P, Plückthun A. 2008. Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. *J. Mol. Biol.* 382:1211–27
28. Interlandi G, Wetzel SK, Settanni G, Plückthun A, Caflisch A. 2008. Characterization and further stabilization of designed ankyrin repeat proteins by combining molecular dynamics simulations and experiments. *J. Mol. Biol.* 375:837–54
29. Kramer MA, Wetzel SK, Plückthun A, Mittl PR, Grütter MG. 2010. Structural determinants for improved stability of designed ankyrin repeat proteins with a redesigned C-capping module. *J. Mol. Biol.* 404:381–91
30. Wetzel SK, Ewald C, Settanni G, Jurt S, Plückthun A, Zerbe O. 2010. Residue-resolved stability of full-consensus ankyrin repeat proteins probed by NMR. *J. Mol. Biol.* 402:241–58
31. Schilling J, Schoeppe J, Plückthun A. 2014. From DARPins to LoopDARPins: novel LoopDARPin design allows the selection of low picomolar binders in a single round of ribosome display. *J. Mol. Biol.* 426:691–721
32. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, et al. 2004. High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat. Biotechnol.* 22:575–82
33. Kohl A, Binz HK, Forrer P, Stumpp MT, Plückthun A, Grütter MG. 2003. Designed to be stable: crystal structure of a consensus ankyrin repeat protein. *Proc. Natl. Acad. Sci. USA* 100:1700–5
34. Tamaskovic R, Simon M, Stefan N, Schwill M, Plückthun A. 2012. Designed ankyrin repeat proteins (DARPins): from research to therapy. *Methods Enzymol.* 503:101–34
35. Wetzel SK, Settanni G, Kenig M, Binz HK, Plückthun A. 2008. Folding and unfolding mechanism of highly stable full-consensus ankyrin repeat proteins. *J. Mol. Biol.* 376:241–57
36. Binz HK, Kohl A, Plückthun A, Grütter MG. 2006. Crystal structure of a consensus-designed ankyrin repeat protein: implications for stability. *Proteins* 65:280–84
37. Mattheakis LC, Bhatt RR, Dower WJ. 1994. An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. USA* 91:9022–26
38. Hanes J, Jermutus L, Plückthun A. 2000. Selecting and evolving functional proteins in vitro by ribosome display. *Methods Enzymol.* 328:404–30
39. Hanes J, Schaffitzel C, Knappik A, Plückthun A. 2000. Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat. Biotechnol.* 18:1287–92
40. Luginbühl B, Kanyo Z, Jones RM, Fletterick RJ, Prusiner SB, et al. 2006. Directed evolution of an anti-prion protein scFv fragment to an affinity of 1 pM and its structural interpretation. *J. Mol. Biol.* 363:75–97
41. Zahnd C, Wyler E, Schwenk JM, Steiner D, Lawrence MC, et al. 2007. A designed ankyrin repeat protein evolved to picomolar affinity to Her2. *J. Mol. Biol.* 369:1015–28
42. Dreier B, Mikheeva G, Belousova N, Parizek P, Boczek E, et al. 2011. Her2-specific multivalent adapters confer designed tropism to adenovirus for gene targeting. *J. Mol. Biol.* 405:410–26
43. Dreier B, Plückthun A. 2010. Ribosome display, a technology for selecting and evolving proteins from large libraries. *Methods Mol. Biol.* 687:283–306
44. Amstutz P, Binz HK, Parizek P, Stumpp MT, Kohl A, et al. 2005. Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. *J. Biol. Chem.* 280:24715–22
45. Zahnd C, Pécorari F, Straumann N, Wyler E, Plückthun A. 2006. Selection and characterization of Her2 binding-designed ankyrin repeat proteins. *J. Biol. Chem.* 281:35167–75
46. Schweizer A, Roschitzki-Voser H, Amstutz P, Briand C, Gulotti-Georgieva M, et al. 2007. Inhibition of caspase-2 by a designed ankyrin repeat protein: specificity, structure, and inhibition mechanism. *Structure* 15:625–36

47. Huber T, Steiner D, Röthlisberger D, Plückthun A. 2007. In vitro selection and characterization of DARPins and Fab fragments for the co-crystallization of membrane proteins: the Na^+-citrate symporter CitS as an example. *J. Struct. Biol.* 159:206–21
48. Veesler D, Dreier B, Blangy S, Lichière J, Tremblay D, et al. 2009. Crystal structure and function of a DARPin neutralizing inhibitor of lactococcal phage TP901-1: comparison of DARPin and camelid VHH binding mode. *J. Biol. Chem.* 384:30718–26
49. Stefan N, Martin-Killias P, Wyss-Stoeckle S, Honegger A, Zangemeister-Wittke U, Plückthun A. 2011. DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display and engineered for multivalency. *J. Mol. Biol.* 413:826–43
50. Zahnd C, Sarkar CA, Plückthun A. 2010. Computational analysis of off-rate selection experiments to optimize affinity maturation by directed evolution. *Protein Eng. Des. Sel.* 23:175–84
51. Siva AC, Kirkland RE, Lin B, Maruyama T, McWhirter J, et al. 2008. Selection of anti-cancer antibodies from combinatorial libraries by whole-cell panning and stringent subtraction with human blood cells. *J. Immunol. Methods* 330:109–19
52. Fekkes P, Driessen AJ. 1999. Protein targeting to the bacterial cytoplasmic membrane. *Microbiol. Mol. Biol. Rev.* 63:161–73
53. Bibi E. 2011. Early targeting events during membrane protein biogenesis in *Escherichia coli*. *Biochim. Biophys. Acta* 1808:841–50
54. Steiner D, Forrer P, Stumpp MT, Plückthun A. 2006. Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display. *Nat. Biotechnol.* 24:823–31
55. Dröge MJ, Boersma YL, Braun PG, Buining RJ, Julsing MK, et al. 2006. Phage display of an intracellular carboxylesterase of *Bacillus subtilis*: comparison of Sec and Tat pathway export capabilities. *Appl. Environ. Microbiol.* 72:4589–95
56. Paschke M, Höhne W. 2005. A twin-arginine translocation (Tat)-mediated phage display system. *Gene* 350:79–88
57. Nangola S, Minard P, Tayapiwatana C. 2010. Appraisal of translocation pathways for displaying ankyrin repeat protein on phage particles. *Protein Expr. Purif.* 74:156–61
58. Speck J, Arndt KM, Müller KM. 2011. Efficient phage display of intracellularly folded proteins mediated by the TAT pathway. *Protein Eng. Des. Sel.* 24:473–84
59. Pepper LR, Cho YK, Boder ET, Shusta EV. 2008. A decade of yeast surface display technology: Where are we now? *Comb. Chem. High Throughput Screen.* 11:127–34
60. Amstutz P, Koch H, Binz HK, Deuber SA, Plückthun A. 2006. Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries. *Protein Eng. Des. Sel.* 19:219–29
61. Kummer L, Parizek P, Rube P, Millgramm B, Prinz A, et al. 2012. Structural and functional analysis of phosphorylation-specific binders of the kinase ERK from designed ankyrin repeat protein libraries. *Proc. Natl. Acad. Sci. USA* 109:E2248–57
62. Parizek P, Kummer L, Rube P, Prinz A, Herberg FW, Plückthun A. 2012. Designed ankyrin repeat proteins (DARPins) as novel isoform-specific intracellular inhibitors of c-Jun N-terminal kinases. *ACS Chem. Biol.* 7:1356–66
63. Kummer L, Hsu CW, Dagliyan O, MacNevin C, Kaufholz M, et al. 2013. Knowledge-based design of a biosensor to quantify localized ERK activation in living cells. *Chem. Biol.* 20:847–56
64. Gilbreth RN, Koide S. 2012. Structural insights for engineering binding proteins based on non-antibody scaffolds. *Curr. Opin. Struct. Biol.* 22:413–20
65. Bukowska MA, Grütter MG. 2013. New concepts and aids to facilitate crystallization. *Curr. Opin. Struct. Biol.* 23:409–16
66. Theurillat JP, Dreier B, Nagy-Davidescu G, Seifert B, Behnke S, et al. 2010. Designed ankyrin repeat proteins: a novel tool for testing epidermal growth factor receptor 2 expression in breast cancer. *Mod. Pathol.* 23:1289–97
67. Kuo TT, Baker K, Yoshida M, Qiao SW, Aveson VG, et al. 2010. Neonatal Fc receptor: from immunity to therapeutics. *J. Clin. Immunol.* 30:777–89
68. Simon M, Frey R, Zangemeister-Wittke U, Plückthun A. 2013. Orthogonal assembly of a designed ankyrin repeat protein-cytotoxin conjugate with a clickable serum albumin module for half-life extension. *Bioconjug. Chem.* 24:1955–66

69. Simon M, Stefan N, Borsig L, Plückthun A, Zangemeister-Wittke U. 2014. Increasing the antitumor effect of an EpCAM-targeting fusion toxin by facile click PEGylation. *Mol. Cancer Ther.* 13:375–85
70. Chapman AP. 2002. PEGylated antibodies and antibody fragments for improved therapy: a review. *Adv. Drug Deliv. Rev.* 54:531–45
71. Kubetzko S, Sarkar CA, Plückthun A. 2005. Protein PEGylation decreases observed target association rates via a dual blocking mechanism. *Mol. Pharmacol.* 68:1439–54
72. Zahnd C, Kawe M, Stumpp MT, de Pasquale C, Tamaskovic R, et al. 2010. Efficient tumor targeting with high-affinity designed ankyrin repeat proteins: effects of affinity and molecular size. *Cancer Res.* 70:1595–605
73. Willuda J, Kubetzko S, Waibel R, Schubiger PA, Zangemeister-Wittke U, Plückthun A. 2001. Tumor targeting of mono-, di- and tetravalent anti-p185^{HER-2} miniantibodies multimerized by self-associating peptides. *J. Biol. Chem.* 276:14385–92
74. Adams GP, McCartney JE, Tai MS, Oppermann H, Huston JS, et al. 1993. Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-*erb*B-2 single-chain Fv. *Cancer Res.* 53:4026–34
75. Maeda H, Nakamura H, Fang J. 2013. The EPR effect for macromolecular drug delivery to solid tumors: improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo. *Adv. Drug Deliv. Rev.* 65:71–79
76. Schmidt MM, Wittrup KD. 2009. A modeling analysis of the effects of molecular size and binding affinity on tumor targeting. *Mol. Cancer Ther.* 8:2861–71
77. Thurber GM, Schmidt MM, Wittrup KD. 2008. Antibody tumor penetration: transport opposed by systemic and antigen-mediated clearance. *Adv. Drug Deliv. Rev.* 60:1421–34
78. Adams GP, Schier R, McCall AM, Simmons HH, Horak EM, et al. 2001. High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules. *Cancer Res.* 61:4750–55
79. Rudnick SI, Lou J, Shaller CC, Tang Y, Klein-Szanto AJ, et al. 2011. Influence of affinity and antigen internalization on the uptake and penetration of anti-HER2 antibodies in solid tumors. *Cancer Res.* 71:2250–59
80. Waibel R, Alberto R, Willuda J, Finnern R, Schibli R, et al. 1999. Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)–carbonyl complex. *Nat. Biotechnol.* 17:897–901
81. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235:177–82
82. Moasser MM. 2007. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene* 26:6469–87
83. Verma S, Miles D, Gianni L, Krop IE, Welslau M, et al. 2012. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N. Engl. J. Med.* 367:1783–91
84. Burris HA III, Rugo HS, Vukelja SJ, Vogel CL, Borson RA, et al. 2011. Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)–positive breast cancer after prior HER2-directed therapy. *J. Clin. Oncol.* 29:398–405
85. Kumler I, Tuxen MK, Nielsen DL. 2014. A systematic review of dual targeting in HER2-positive breast cancer. *Cancer Treat. Rev.* 40:259–70
86. Lee SC, Srivastava RM, López-Albaitero A, Ferrone S, Ferris RL. 2011. Natural killer (NK):dendritic cell (DC) cross talk induced by therapeutic monoclonal antibody triggers tumor antigen-specific T cell immunity. *Immunol. Res.* 50:248–54
87. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. 2004. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* 5:317–28
88. Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick VD, et al. 1994. Coexpression of *erb*B2 and *erb*B3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.* 269:14661–65
89. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, et al. 2009. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell* 15:429–40
90. Schaefer G, Fitzpatrick VD, Sliwkowski MX. 1997. γ-Heregulin: a novel heregulin isoform that is an autocrine growth factor for the human breast cancer cell line, MDA-MB-175. *Oncogene* 15:1385–94

91. Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, et al. 2011. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. *Cancer Res.* 71:1871–82
92. Finn RS, Slamon DJ. 2003. Monoclonal antibody therapy for breast cancer: Herceptin. *Cancer Chemother. Biol. Response Modif.* 21:223–33
93. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, et al. 1999. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* 17:2639–48
94. Boersma YL, Chao G, Steiner D, Wittrup KD, Plückthun A. 2011. Bispecific designed ankyrin repeat proteins (DARPins) targeting epidermal growth factor receptor inhibit A431 cell proliferation and receptor recycling. *J. Biol. Chem.* 286:41273–85
95. Jost C, Schilling J, Tamaskovic R, Schwill M, Honegger A, Plückthun A. 2013. Structural basis for eliciting a cytotoxic effect in HER2-overexpressing cancer cells via binding to the extracellular domain of HER2. *Structure* 21:1979–91
96. Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, et al. 2003. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* 421:756–60
97. Dawson JP, Bu Z, Lemmon MA. 2007. Ligand-induced structural transitions in ErbB receptor extracellular domains. *Structure* 15:942–54
98. Endres NF, Das R, Smith AW, Arkhipov A, Kovacs E, et al. 2013. Conformational coupling across the plasma membrane in activation of the EGF receptor. *Cell* 152:543–56
99. Arkhipov A, Shan Y, Das R, Endres NF, Eastwood MP, et al. 2013. Architecture and membrane interactions of the EGF receptor. *Cell* 152:557–69
100. Zuppinger C, Suter TM. 2010. Cancer therapy-associated cardiotoxicity and signaling in the myocardium. *J. Cardiovasc. Pharmacol.* 56:141–46
101. van der Gun BT, Melchers LJ, Ruiters MH, de Leij LF, McLaughlin PM, Rots MG. 2010. EpCAM in carcinogenesis: the good, the bad or the ugly. *Carcinogenesis* 31:1913–21
102. Trzpis M, McLaughlin PM, de Leij LM, Harmsen MC. 2007. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. *Am. J. Pathol.* 171:386–95
103. Biggers K, Scheinfeld N. 2008. VB4-845, a conjugated recombinant antibody and immunotoxin for head and neck cancer and bladder cancer. *Curr. Opin. Mol. Ther.* 10:176–86
104. Di Paolo C, Willuda J, Kubetzko S, Lauffer I, Tschudi D, et al. 2003. A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. *Clin. Cancer. Res.* 9:2837–48
105. Hussain S, Plückthun A, Allen TM, Zangemeister-Wittke U. 2006. Chemosensitization of carcinoma cells using epithelial cell adhesion molecule-targeted liposomal antisense against bcl-2/bcl-xL. *Mol. Cancer Ther.* 5:3170–80
106. Hussain S, Plückthun A, Allen TM, Zangemeister-Wittke U. 2007. Antitumor activity of an epithelial cell adhesion molecule targeted nanovesicular drug delivery system. *Mol. Cancer Ther.* 6:3019–27
107. Martin-Killias P, Stefan N, Rothschild S, Plückthun A, Zangemeister-Wittke U. 2011. A novel fusion toxin derived from an EpCAM-specific designed ankyrin repeat protein has potent antitumor activity. *Clin. Cancer. Res.* 17:100–10
108. Alley SC, Okeley NM, Senter PD. 2010. Antibody-drug conjugates: targeted drug delivery for cancer. *Curr. Opin. Chem. Biol.* 14:529–37
109. Winkler J, Martin-Killias P, Plückthun A, Zangemeister-Wittke U. 2009. EpCAM-targeted delivery of nanocomplexed siRNA to tumor cells with designed ankyrin repeat proteins. *Mol. Cancer Ther.* 9:2674–83
110. Salmon F, Grosios K, Petry H. 2014. Safety profile of recombinant adeno-associated viral vectors: focus on alipogene tiparvovec (Glybera®). *Expert Rev. Clin. Pharmacol.* 7:53–65
111. Amalfitano A, Parks RJ. 2002. Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. *Curr. Gene Ther.* 2:111–33
112. Sullivan NJ, Geisbert TW, Geisbert JB, Xu L, Yang ZY, et al. 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* 424:681–84

113. Yeh HH, Ogawa K, Balatoni J, Mukhapadhyay U, Pal A, et al. 2011. Molecular imaging of active mutant L858R EGF receptor (EGFR) kinase-expressing nonsmall cell lung carcinomas using PET/CT. *Proc. Natl. Acad. Sci. USA* 108:1603–8
114. Beatty MS, Curiel DT. 2012. Chapter two–adenovirus strategies for tissue-specific targeting. *Adv. Cancer Res.* 115:39–67
115. Dreier B, Honegger A, Hess C, Nagy-Davidescu G, Mittl PR, et al. 2013. Development of a generic adenovirus delivery system based on structure-guided design of bispecific trimeric DARPin adapters. *Proc. Natl. Acad. Sci. USA* 110:E869–77
116. Forrer P, Chang C, Ott D, Wlodawer A, Plückthun A. 2004. Kinetic stability and crystal structure of the viral capsid protein SHP. *J. Mol. Biol.* 344:179–93
117. Yanagi Y, Takeda M, Ohno S, Seki F. 2006. Measles virus receptors and tropism. *Jpn. J. Infect. Dis.* 59:1–5
118. Münch RC, Mühlebach MD, Schaser T, Kneissl S, Jost C, et al. 2011. DARPins: an efficient targeting domain for lentiviral vectors. *Mol. Ther.* 19:686–93
119. Friedrich K, Hanauer JR, Prüfer S, Münch RC, Volker I, et al. 2013. DARPin-targeting of measles virus: unique bispecificity, effective oncolysis, and enhanced safety. *Mol. Ther.* 21:849–59
120. Münch RC, Janicki H, Volker I, Rasbach A, Hallek M, et al. 2013. Displaying high-affinity ligands on adeno-associated viral vectors enables tumor cell-specific and safe gene transfer. *Mol. Ther.* 21:109–18
121. Stahl A, Stumpp MT, Schlegel A, Ekawardhani S, Lehrling C, et al. 2013. Highly potent VEGF-A-antagonistic DARPins as anti-angiogenic agents for topical and intravitreal applications. *Angiogenesis* 16:101–11
122. Syed BA, Evans JB, Bielory L. 2012. Wet AMD market. *Nat. Rev. Drug Discov.* 11:827
123. Campochiaro PA, Channa R, Berger BB, Heier JS, Brown DM, et al. 2013. Treatment of diabetic macular edema with a designed ankyrin repeat protein that binds vascular endothelial growth factor: a phase I/II study. *Am. J. Ophthalmol.* 155:697–704.e2
124. Wolf S, Souied EH, Mauget-Faysse M, Devin F, Patel M, et al. 2011. *Phase I MP0112 Wet AMD study: results of a single escalating dose study with DARPin MP0112 in Wet AMD.* Presented at Annu. Meet. Assoc. Res. Vis. Ophthalmology, May 2, Fort Lauderdale, FL, poster 1655
125. Souied EH, Devin F, Mauget-Faÿsse M, Kolář P, Wolf-Schnurrbusch U, et al. 2014. Treatment of exudative age-related macular degeneration with a designed ankyrin repeat protein that binds vascular endothelial growth factor: a Phase I/II study. *Am. J. Ophthalmol.* 158:724–32.e2
126. Schilling J, Schoeppe J, Sauer E, Plückthun A. 2014. Co-crystallization with conformation-specific designed ankyrin repeat proteins explains the conformational flexibility of BCL-W. *J. Mol. Biol.* 426:2346–61
127. Schroeder T, Barandun J, Flütsch A, Briand C, Mittl PR, Grütter MG. 2013. Specific inhibition of caspase-3 by a competitive DARPin: molecular mimicry between native and designed inhibitors. *Structure* 21:277–89
128. Pecqueur L, Duellberg C, Dreier B, Jiang Q, Wang C, et al. 2012. A designed ankyrin repeat protein selected to bind to tubulin caps the microtubule plus end. *Proc. Natl. Acad. Sci. USA* 109:12011–16
129. Gigant B, Wang W, Dreier B, Jiang Q, Pecqueur L, et al. 2013. Structure of a kinesin-tubulin complex and implications for kinesin motility. *Nat. Struct. Mol. Biol.* 20:1001–7
130. Eggel A, Baumann MJ, Amstutz P, Stadler BM, Vogel M. 2009. DARPins as bispecific receptor antagonists analyzed for immunoglobulin E receptor blockage. *J. Mol. Biol.* 393:598–607
131. Eggel A, Buschor P, Baumann MJ, Amstutz P, Stadler BM, Vogel M. 2011. Inhibition of ongoing allergic reactions using a novel anti-IgE DARPin-Fc fusion protein. *Allergy* 66:961–68
132. Kim B, Eggel A, Tarchevskaya SS, Vogel M, Prinz H, Jardetzky TS. 2012. Accelerated disassembly of IgE-receptor complexes by a disruptive macromolecular inhibitor. *Nature* 491:613–17
133. Mann A, Friedrich N, Krarup A, Weber J, Stiegeler E, et al. 2013. Conformation-dependent recognition of HIV gp120 by designed ankyrin repeat proteins provides access to novel HIV entry inhibitors. *J. Virol.* 87:5868–81
134. Bender NK, Heilig CE, Droll B, Wohlgemuth J, Armbruster FP, Heilig B. 2007. Immunogenicity, efficacy and adverse events of adalimumab in RA patients. *Rheumatol. Int.* 27:269–74

Annual Review of Pharmacology and Toxicology

Volume 55, 2015

Contents

Indexes

Errata

An online log of corrections to *Annual Review of Pharmacology and Toxicology* articles may be found at http://www.annualreviews.org/errata/pharmtox