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# DARPins: A new generation of protein therapeutics

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**DARPins (designed ankyrin repeat proteins) are a novel class of binding molecules with the potential to overcome limitations of monoclonal antibodies, hence allowing novel therapeutic approaches. DARPins are small, single domain proteins (14 kDa) which can be selected to bind any given target protein with high affinity and specificity. These characteristics make them ideal agonistic, antagonistic or inhibitory drug candidates. Furthermore, DARPins can be engineered to carry various effector functions or combine multiple binding specificities, enabling completely new drug formats. Taken together, DARPins are a prominent member of the next generation of protein therapeutics with the potential to surpass existing antibody drugs.**

## Introduction

Antibodies are, currently, a major driver of the pharmaceutical industry, with several blockbuster drugs on the market and many more in clinical development. The key for this success is that antibodies can be selected to bind to virtually any given target with high affinity and specificity, thereby displaying neutralizing or cytotoxic functions with very limited side effects [1,2]. Antibodies, however, suffer from clear limitations: they are expensive to produce, are difficult to formulate, show low tissue penetration, feature a complex architecture, bind their target bivalently, and their commercial use is often restricted by blocking intellectual property [3]. To address some of the shortcomings of antibodies, several antibody-engineering efforts have been started with considerable success [4]. These efforts, however, are mostly based on immunoglobulin scaffolds and, thus, the intrinsic limitations of this class of proteins will be difficult to correct.

In recent years, *in vitro* selection technologies, such as phage display and ribosome display, have been developed, allowing the selection of specific binders without immunization. This has opened the door for scaffolds other than immunoglobulins to be engineered as specific binding proteins, with the potential to overcome the shortcomings of antibodies. Several of these scaffolds, including DARPins (designed ankyrin repeat proteins), AdNectins, Affibodies, Anticalins, Avimers and others, are being developed with encouraging scientific success (for a review see refs. [5–7]) and high economic potential. This review focuses on DARPins as next generation protein drugs, with an emphasis on their advantages, such as facilitated production and formulation, increased tissue penetration and novel therapeutic molecular formats, including the ability to develop multispecific drugs.

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## DARPin design and characteristics

DARPins are derived from natural ankyrin repeat proteins, which are among the most abundant binding proteins found in the human genome [8]. Like all other repeat proteins, DARPins feature a protein architecture in which a varying number of structural motifs (repeats) stack to form the repeat protein domain (Figure 1). The natural biological function of repeat proteins is to bind to a target, which results in different modes of action, ranging from enzyme inhibition to simple anchoring of proteins to each other [9]. Recently, it was reported that the adaptive immune system of jawless vertebrates is not based on antibodies, but on leucine-rich repeat proteins, another group of repeat proteins [10]. Furthermore, the innate immune system of mammals and plants relies on repeat proteins [11]. The ability to display a large potential interaction surface for target binding, combined with the versatility that allows repeat shuffling and molecule length variations [12], has certainly contributed to nature's choice of repeat proteins for these important biologic functions.

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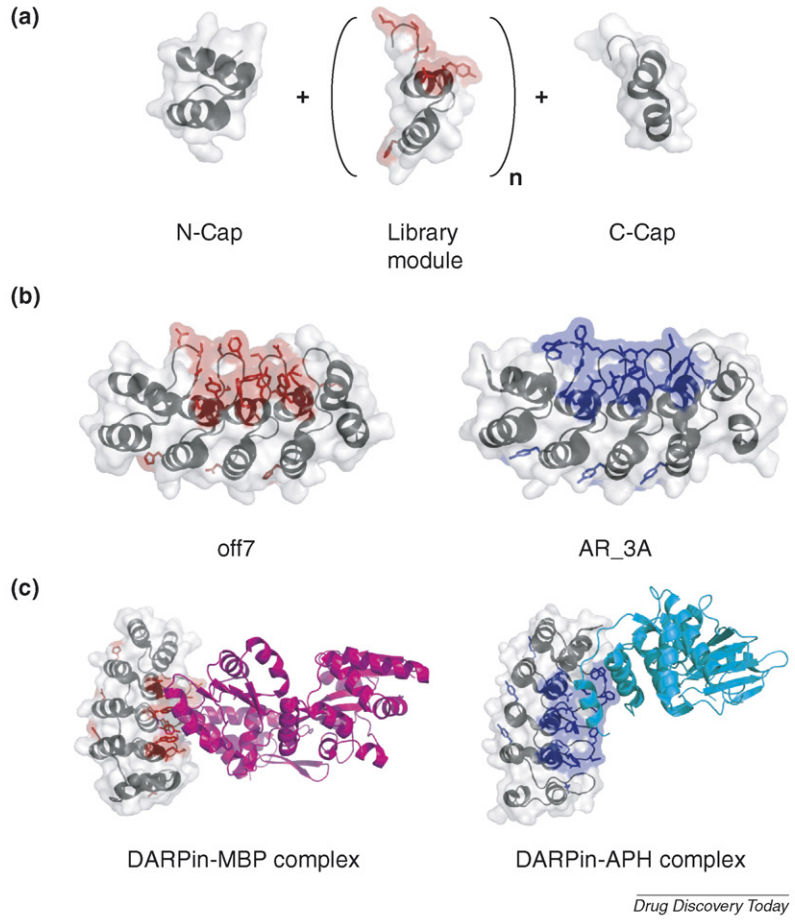


**FIGURE 1**

Scheme of the DARPin library design, the resulting DARPin molecules and the target protein binding. **(a)** DARPin libraries are composed of two caps (N- and C-) and a varying number of library modules (*n*), all shown as grey ribbon representation with a transparent space fill surface. Each library module corresponds to an ankyrin repeat of 33 amino acids, where seven positions are variable, displayed as red side chains (diversity $> 10^7$). Typically, two to four library modules ($n = 2–4$) are combined into one protein domain (diversity $> 10^{14}$), giving rise to DARPins between 14 and 21 kDa, respectively, which is about one tenth the size of a conventional antibody. **(b)** The three dimensional representation of two DARPins, both with three library modules, is shown. The blue and red colouring highlights the randomized surface for potential target interaction. **(c)** Two DARPin target protein complexes are shown (MBP, maltose binding protein and DARPin off7 PDB: 1SVX; APH aminoglycoside phosphotransferase and DARPin AR_3A, PDB: 2BKK). The targets are bound by the randomized target interaction surface of the DARPins (coloured in red and blue, respectively), thus resembling the natural target-binding mode, indistinguishable from natural ankyrin repeat proteins [18].

To take advantage of ankyrin repeat proteins as alternatives to antibody-based scaffolds, DARPin libraries were engineered by a consensus design approach [13]. Natural ankyrin repeat protein sequences, 229 ankyrin repeats for the initial design and another 2200 for subsequent refinement, were used in a consensus design approach to define a DARPin library module, which served as a building block for the DARPin libraries [14] (Figure 1). Since many sequences used for this approach were derived from the human genome, the designed library module closely resembled human sequences (~67% identity, ~71% similarity to human GA binding Protein, Uniprot ID Q06547). Different numbers of these modules are then genetically fused and flanked by capping repeats, shielding the continuous hydrophobic core of the repeat domain, giving rise to N×C DARPin libraries (Figure 1), where N stands for the N-terminal capping repeat, C for the C-terminal capping repeat and × for the number of library modules, typically ranging between two and four [14]. Since single library modules are built from 33 amino acids, of which seven are variable, resulting in a diversity of more than 10 million per module, the libraries carrying two of these repeat modules reach diversities of well above $10^{14}$. Since the molecular weight of one module is just above 3.5 kDa and DARPins are composed of four to six modules, their molecular weight ranges from 14 to 21 kDa, which is approximately one tenth of the size of a conventional IgG antibody, or one third of the size of the Fab fragment, the smallest antibody fragment currently approved for therapeutic use [15].

The consensus design approach not only helped to design large libraries, but also yielded idealized DARPins with improved properties, such as very high expression levels, high stability and solubility [12,14]. Expression of functional DARPins can be performed in the cytoplasm of *Escherichia coli* (*E. coli*) with yields reaching 200 mg/l purified protein in simple shake flask cultures in the low expressor strain XL1-blue (14). Further, the production can be improved by using specialized expression strains (e.g. BL21) and

high cell density fermentation approaches. Since the molecular weight of the DARPin is around one tenth of an IgG antibody, this should translate into a molar increase over IgG of at least one order of magnitude. In addition, the stability of DARPins should allow for simple large-scale purification protocols, enabling rapid and low-cost production and purification. Purified DARPins can be concentrated to high concentrations as shown for several crystallization studies [16,17] and preliminary formulation experiments revealed that concentrations above 100 mg/ml DARPin are attainable (unpublished data). Thermal stabilities of DARPins are usually very high, with midpoints of denaturation between 66 and 95°C, with a clear trend that DARPins with more repeats are even more stable [14,16]. Recent studies with full consensus DARPins revealed that these proteins belong to the most stable proteins described to date with melting temperatures well above 100°C for longer constructs [16]. This stability and the fact that the DARPin fold shows no flexible peptidic domains, might explain why no proteolytic digestion has been detected in any experiment [18]. Indeed, DARPin binding activity was measured for DARPins at room temperature and in human serum at 37°C (*ex vivo*), both revealing half-lives of more than 60 days (unpublished data).

The ease of production, high stability and simple formulation impacts on different aspects of drug discovery and development. Samples may be miniaturized during the screening process as already small expression volumes allow production of sufficient quantities of DARPins. Hundreds of identified DARPins can be expressed in the mg scale for initial characterization and the most interesting ones can be produced in gram amounts in shake flasks within a few days for preclinical testing. This, together with the high stability and solubility, allows a different approach to efficacy models, as producing and purifying the protein in gram amounts is no longer a bottleneck. Different administration routes (topical, oral, nasal and inhaled), demanding high amounts of very stable protein, may thus be tested. Taken together, the consensus design with ankyrin repeat proteins yielded attractive DARPin libraries with many desired features of a protein therapeutic.

## DARPin selection and target binding

The most frequently used selection method used with DARPin libraries has been ribosome display [19]. This selection technology relies on the coupling of genotype (mRNA encoding DARPins) and phenotype (displayed DARPin protein) via the ribosome, resulting in ternary complexes (mRNA, ribosome, displayed DARPin) that are used for selection. The target protein is immobilized, the DARPin library of ternary complexes (up to $10^{12}$ are possible) is added, the non-binding complexes are washed away, and the genes of the target specific DARPins are recovered by RT-PCR [20]. This approach has yielded highly specific, high-affinity DARPins to many different types of proteins, including proteases, kinases, cytokines and membrane proteins (for a summary see ref. [21]). Usually, many different DARPins were selected per target with affinities in the pM to low nM range within only a few selection rounds [18,22]. The high target-binding affinities of the DARPins and the high diversity of sequences selected may be explained at different levels. First, the quality of the libraries seems to be very high, with many functional members. The high stability of the DARPin framework allows for more sequence diversity without destroying the protein structure than in the case of less stable framework libraries. Furthermore, ribosome display as a selection method allows sampling of large libraries. Finally, the interaction mode seems to be that of a rigid body (see below), meaning that only minimal entropy is lost in the binding event, a prerequisite for high affinities. As an alternative selection method, a phage display selection method was established, especially adapted for DARPins [23], that should allow for selection *in vivo* and at the tissue level, for example to select for DARPins capable of crossing the blood–brain barrier, applications not possible for ribosome display. As DARPins are also amenable to intracellular selection systems, such as the protein complementation assay [24], they appear to be compatible with most common selection methods used to date.

Several structures of DARPins in complex with their targets have been determined by X-ray crystallography (Figure 1), which help to understand the binding characteristics of DARPins [18,25–27]. As expected, the library modules with their randomized positions are responsible for the interaction with the target. These residues are on the concave DARPin molecular surface, forming an extended interaction interface, which binds structural epitopes on the target protein surface (Figure 1). Neither DARPins nor targets change their conformation upon binding. Some inhibitory DARPins [25,26], however, seem to freeze an inactive conformation of the target protein that is present in solution.

The binding of the target protein via conformational epitopes may also explain the exceptionally high specificities of the selected DARPins. For instance, a highly selective caspase-2 inhibitor was produced, showing no cross-inhibitory effect on any of the other caspases [26]. Also, kinase binders and inhibitors were obtained which could discriminate between highly similar isoforms [24]. With the rigid-body binding of the DARPins, small sequence or conformational differences are sufficient to account for high specificity, as revealed in the most recent example: DARPins were selected to bind AcrB, a bacterial membrane transporter responsible for drug resistance [27]. AcrB is a homotrimer and the X-ray structure of the DARPins in complex with AcrB revealed three asymmetric subunits. Indeed, only two of the three subunits were bound by a DARPin, because the third subunit was in a different conformation. This surprising asymmetry allowed structure determination and a more detailed understanding of the drug export mechanism of AcrB [27].

## Potential medical applications of DARPins

We will discuss here the range of possible applications and formats of DARPin-based drugs and work out where medical advantages may be expected. DARPin-based drugs can be based on either monovalent DARPins, that is simple DARPins without any modifications, or on conjugated DARPins, where the DARPin is chemically or genetically linked to other moieties. Conjugated DARPins can either be linked to low molecular weight cytotoxic agents for cell killing, polyethylene glycol (PEG) for serum half-life modulation, peptides or whole proteins, such as toxins, cytokines, antibody Fc-domains or even to other DARPins. DARPin-DARPin combinations can be either multivalent or multispecific. Obviously, the different approaches can be combined where useful.

DARPins have not yet been tested in clinical trials, but safety and efficacy has been shown in several preclinical models. Never-

theless, these experiments cannot fully rule out safety issues, such as potential immunogenicity in man [28]. Currently, it seems that DARPin drugs should not be limited by immunogenicity, as DARPins are characterized by high stability and no aggregation tendency, which are important prerequisites for low immunogenicity [29]. Furthermore, the abundance of the anykrin protein itself in the erythrocytes suggests that ankyrin protein fragments are constantly in the circulation, possibly inducing immunological tolerance. In the event that antibodies were raised to DARPin drugs, these are not expected to cross-react with human ankyrin repeat proteins, as these are not known to be extracellular.

## Monovalent DARPins as drugs

Monoclonal antibodies against soluble cytokines (e.g. TNFα or VEGF) are big commercial success stories. These antagonistic antibodies rely simply on neutralizing a cytokine, usually by preventing the cytokine from binding to its receptor, which subsequently stops receptor signalling. DARPins can be developed as antagonistic or even agonistic agents, offering several advantages over antibodies.

Owing to the much smaller size of DARPins, considerably improved tissue penetration is expected [30]. They could thus block ligands or toxins closer to their sites of action and lead to more potent inhibition. Antibodies, on the other hand, are known for their relatively poor tissue penetration [31,32] and are thus thought to act mainly on ligands present in the circulation. Obviously, increased tissue penetration of DARPins could also be a critical advantage if receptor molecules have to be antagonized, rather than their ligands sequestered.

Second, DARPins carry no Fc-moiety, avoiding a direct link to the immune system. The first generation of cytokine antibodies, however, are generally of the IgG1 type and, therefore, can directly activate the immune system, which might be one reason for some of the adverse events reported, especially in the case of membrane-associated forms of cytokines. As a result, the absence of effector functions in monovalent DARPin constructs might allow for very high dosing schemes.

The third advantage of DARPins lies in their ability to remove unwanted molecules rapidly from tissues or the circulation. Since DARPin–ligand complexes are typically cleared via the kidney, they are removed relatively rapidly from the circulation. This can be of great importance if DARPins were to be used for neutralizing toxins, accompanying some bacterial infections (e.g. *Clostridium difficile* toxin), factors involved in blood clotting (GP $\beta_{II}\alpha_{III}$ or vonWillebrand factor) or cytokines. By contrast, antibodies have long serum half-lives, potentially leading to accumulation of cytokine–antibody complexes in the serum such as seen with Avastin [33]. On the other hand, for some multimeric cytokines, it has been reported that at least some antibodies form large complexes and, thus, are preferentially excreted, which in turn can lead to reduced cytokine concentrations in the circulation [34].

The relatively fast clearance of monovalent DARPins can be reduced by PEGylation [35,36], fusion to serum protein-binding DARPins or slow release formulations [37]. This allows modulation of pharmacokinetics to achieve maximal biological effects.

Several applications of antagonistic DARPins have recently been reported. The selection of anti-idiotypic DARPins to an anti-IgE antibody has been achieved. These DARPins blocked the neutralizing activity of the anti-IgE antibody in cell-based assays demonstrating the highly specific antagonistic effect of the DARPins [38]. In a second study, DARPins binding both to IgE and the IgE-receptor and displaying antagonistic properties have been selected, inhibiting mast cell degranulation in cell-based assays with an efficacy comparable to Xolair. In another example, DARPins binding to CD4 with pM affinity were selected that blocked HIV entry into cells by competing with the binding of the viral protein gp120 to CD4 (Schweizer *et al.*, in press).

DARPins have not only been shown to inhibit a target protein by blocking receptor binding, but they can also exert allosteric inhibition mechanisms as shown for proteases, kinases and membrane proteins, for most of which the inhibition mechanism could be elucidated by X-ray crystallography (see above; for a review see [21]). The advantage of this type of inhibition compared to conventional competitive inhibition is that it is ligand/substrate independent. This principle has allowed the complete inhibition of enzymes even in the presence of large amounts of substrate, a situation that is found, for example, for enzymes in the extracellular matrix.

These examples underline the therapeutic potential of monovalent DARPins, especially since there is no need for effector functions, which could result in unwanted side effects.

## Conjugated DARPins as drugs

DARPins, as such, are simple single domain proteins and do not carry effector functions by default [14]. For many applications, especially in oncology, however, different effector functions may be desirable. There are many different ways to arm DARPins by adding effector function moieties, including coupling of small chemical entities or fusion to functional protein domains [39] (Figure 2). Owing to the very robust biophysical properties of DARPins, conjugation of such effector moieties is straightforward.

One advantage of the DARPin scaffold is the absence of cysteines, allowing the introduction of site-specific thiol groups for convenient chemical coupling without affecting the binding properties. In this way, functional chemical entities, such as cytotoxins, can be conjugated, or, alternatively chelating groups can be added to load DARPins with radioactive isotopes. The high disease-tissue specificity of DARPins and their short serum half-life are ideal in both cases, as the toxic moiety will accumulate rapidly in the disease tissue and unbound material will leave the body rapidly via the kidney. Certainly, kidney toxicity must remain in a tolerable range. Indeed, tumor targeting experiments with radioactively labeled DARPins showed very high accumulation of the radioactivity on the tumor with very favorable tumor to organ ratios very similar to what has been published for affibody molecules in the case of radioactive imaging or therapy [40,41]. The tumor accumulation of small scaffolds was significantly higher compared to antibody fragments described to date. It may be speculated that these small proteins can extravasate from the bloodstream and penetrate the tumor more efficiently than IgG-type antibody formats [31].

DARPins, genetically coupled to effector proteins, can enable targeted delivery of cytokines, enzymes (enabling antibody-directed enzyme pro-drug therapy (ADEPT)), natural or engineered Fc domains and others (Figure 2). Neri and co-workers have published

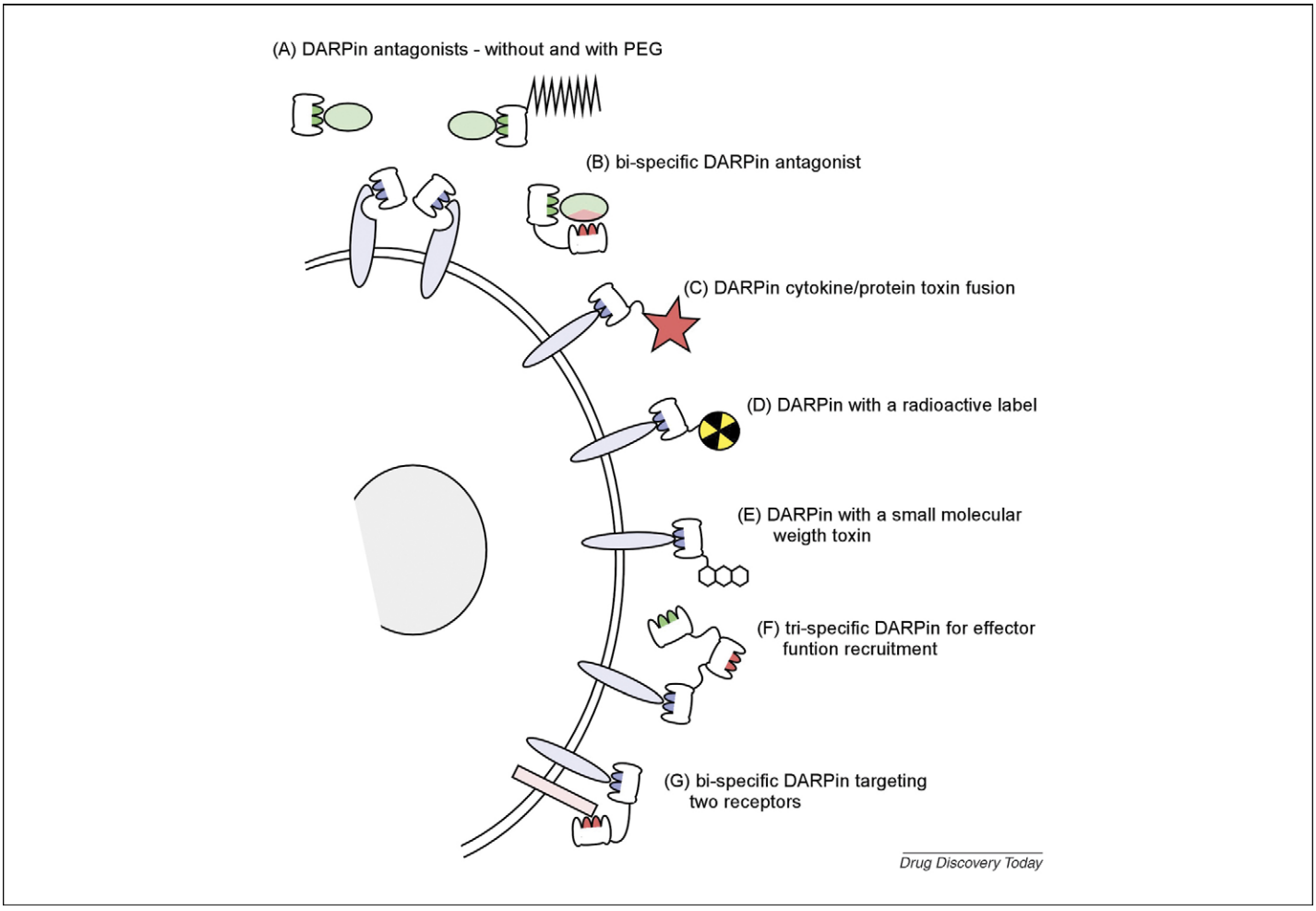


**FIGURE 2**

Possible therapeutic modes of actions of DARPins. An overview of DARPin applications (white boxes with modular architecture and coloured target binding surface) is given. **(A)** Monovalent DARPins as antagonists are shown, binding either a receptor (blue) or a ligand (green), of which one DARPin is schematically PEGylated for half-life engineering. **(B)** Multispecifc DARPins are depicted, binding the target at two sites for additional efficacy. **(C)** DARPins binding a cell surface antigen of target cell, for example tumor cell, are armed with a fusion agent, which can be a protein (toxin, cytokine or enzyme), **(D)** a radioactive ligand, or **(E)** a small molecular weight toxin. **(F)** A tri-specific DARPin may carry specificity for a tumor antigen, another for recruitment of effector cells, while a third DARPin, binding a serum protein, may be added for serum half-life engineering. **(G)** A bi-specific DARPin composed of two different medium-affinity tumor target binders may show enhanced tumor selectivity over monovalent high-affinity binders, as only the combination of the two antigens is exclusively present on the tumor surface adding to high affinity binding of the bi-specific construct, even if the individual antigens alone are expressed also in healthy tissue.

studies with a plethora of fusion proteins (for review see ref. [42]). These studies were performed with an antibody single chain variable fragment (scFv) specifically targeting the EDB domain of fibronectin, an antigen that is expressed exclusively on growing blood vessels. A range of cytokines, including IL-2, IL-12, IL-15, GM-CSF and TNF, could successfully be delivered by genetically fusing the scFv to the effector protein. In principle, the same applications are possible for DARPins, probably with advantages in protein production and stability.

One simple type of protein constructs are DARPin–DARPin fusions, similar to what has been shown for antibody fragments (as reviewed by Marvin and Zhu [43]). Multimeric or multispecific DARPins are useful for many different applications: Several receptors can be inhibited with one molecule [43], immune system effector cells can be recruited [44], receptors can be targeted to, for example transport antagonistic DARPins through the blood–brain barrier [45] or binding to serum proteins (United States Patent Application 20070093651) can be used to modulate the pharmacokinetics as described above (Figure 2). Many multispecific and mutlivalent DARPins have already been successfully combined and showed unaltered high expression levels, high stability and target binding to all cognate targets of the individual DARPin subdomains in the construct. An attractive bispecific DARPin could be composed of two mid-affinity DARPins of two different tumor markers which are simultaneously present on one tumor. This bispecific DARPin drug would display high tumor selectivity, with limited binding to healthy tissue expressing only one of the two tumor antigens [43].

While multispecific antibody fragments have given promising preclinical results, limitations in production and formulation appear to have slowed down their further development. DARPins are highly attractive in this respect, owing to their favorable production and formulation properties.

**BOX 1**

### Advantages of Therapeutic DARPin

Monovalent DARPins

- High tissue penetration: DARPins as comparatively small proteins offer a much higher tissue penetration compared to antibodies. This facilitates reaching targets outside the blood circulation.
- Absence of effector function: Unlike antibodies having an Fc-domain, DARPins do not contain effector functions. DARPins are hence ideally suited for neutralization of soluble targets and undesirable side-effects emerging from binding membrane-associated variants of the target are minimized.
- Adjustable pharmacokinetics (PK): Unmodified DARPins offer fast PK with predicted half-lives in the range of hours, which can be used to rapidly remove unwanted molecules from the blood stream. The half-life can be prolonged by fusion to PEG or serum protein binding molecules.
- Allosteric Inhibition: DARPins bind their target proteins usually on a conformational epitope. This allows them to freeze a specific conformation of the target protein, which can be used to generate allosteric inhibitors.
- New administration routes: The high stability of DARPins and their simple production enable new administration routes in which very large amounts of drug is needed

Conjugated DARPins

- Targeted delivery of payloads possible: DARPins are highly suitable to deliver active moieties to sites of disease tissue. This could be in oncology where DARPins are used to deliver toxins to tumors or inflammation where DARPins inhibit cytokines in inflamed sites.
- Multispecificity: Several DARPins with different specificities can be fused to result in at least trivalent molecules. This would allow the combination of various functions in one molecule, for example to hijack a transcytosis receptor, binding a target in a disease tissue and recruit effector molecules in that tissue.
- Combination of payload and multispecificity: Both strategies can be combined, for example to deliver payloads site-specifically after transcytosis.

## Conclusions

The success of protein drugs has revolutionized drug development. While this field was pioneered by biotech companies, most large pharmaceutical companies have now started initiatives for protein-based therapeutics and have active antibody programs to fill their pipelines. With the growing understanding of how these proteins achieve their clinical efficacy, the demand for more specialized drugs rises. We believe that DARPins are a very promising and valuable addition to protein based drugs, combining many advantages into one molecule family (Box 1), overcoming limitations of current protein-based therapies and providing unique advantages and novel medical opportunities.

## Conflicts of interest

The authors declare competing financial interests. The DARPin technology is covered by a patent application owned by the University of Zürich and licensed to Molecular Partners on an exclusive basis. All authors are shareholders of Molecular Partners.

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