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Half-life extension using serum albumin-binding DARPin® domains

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Abstract

A long systemic half-life is key for therapeutic proteins. To that end we have generated serum albumin-binding designed ankyrin repeat domains. These domains bind serum albumin of different species with nanomolar affinities, and have significantly improved pharmacokinetic properties both in mouse and cynomolgus monkey compared to non-serum albumin-binding DARPin® domains. In addition, they exhibit high thermal stability and long storage stability, which is an essential feature for their use in drug development. Covalently linking a serum albumin-binding DARPin® domain to domains with other target specificities results in improvements of multiple orders of magnitude in exposure and terminal half-life, both in mouse and cynomolgus monkey. Pharmacokinetic assessment of such constructs revealed terminal half-life values ranging from 27 h to 80 h in mouse, and from 2.6 days to 20 days in cynomolgus monkey. Extrapolation by allometric scaling on these findings suggests terminal half-life values of 5–50 days in human, indicating that pharmacokinetic properties in the range of monoclonal antibodies can be achieved with DARPin® drug candidates. Such serum albumin-binding DARPin® domains are thus valuable tools for the generation of multi-functional drugs with an extended *in vivo* half-life.

Key words: designed ankyrin repeat protein, half-life, pharmacokinetics, serum albumin

Introduction

Small high-potency binding proteins are ideal building blocks for next-generation protein therapeutics with properties beyond conventional IgG antibodies (Binz *et al.*, 2005). Such proteins are particularly interesting due to their high potency, good tissue targeting (Zahnd *et al.*, 2010), the straight-forward generation of multi-functional molecules enabling novel therapeutic approaches, and the ease of manufacture using microbial hosts. In order to take full advantage of their potential, their pharmacokinetic properties needs to be tailored to the therapeutic needs. Naturally, such small proteins possess a short plasma half-life due to efficient kidney filtration (Zahnd *et al.*, 2010; Moeller and Tenten, 2013). Various pharmacokinetic engineering methods have previously been established, including PEGylation (Harris and Chess, 2003), polypeptide extension (Schellenberger *et al.*, 2009), HESylation (Liebner *et al.*, 2014), Fc fusions (Strohl, 2015) or albumin fusion (Sleep, 2015) amongst others (for review see Kontermann (2016)). While these methods enable pharmacokinetic modulation, they typically come with a caveat affecting the advantageous properties of the protein drug, e.g. affecting straight-forward manufacturing or affecting pharmacological activity. An alternative approach for pharmacokinetic engineering of therapeutic proteins is to use fusions to serum albumin-binding proteins or peptides. Serum albumin is a 66 kDa plasma protein serving colloidal pressure control and transporting various lipophilic substances and having a long half-life of about 19 days in human (Peters, 1985). Reaching a terminal half-life in that order of magnitude by

binding to serum albumin would enable the generation of drug candidates with attractive pharmacokinetic properties. Several such approaches are known from literature and comprise the use of natural albumin-binding proteins such as the streptococcal protein G-derived albumin-binding domain (Linhult *et al.*, 2002; Jonsson *et al.*, 2008), albumin-binding peptides (Dennis *et al.*, 2002; Revets and Boutton, 2011) or antibody fragments binding to serum albumin (Holt *et al.*, 2008; Müller *et al.*, 2012; Van Roy *et al.*, 2015), which were generated by library selection technologies.

Designed ankyrin repeat proteins are small proteins that can be selected to bind target proteins with high affinity and specificity (Binz *et al.*, 2004; Stumpp *et al.*, 2008). DARPin® domains exhibit favorable biophysical properties including high thermal and thermodynamic stability (Binz *et al.*, 2003; Plückthun, 2015), which translate to a high storage stability, and their amenability for the generation of multi-functional protein therapeutics. DARPin® domain-based therapeutics are currently being tested in clinical trials in ophthalmology and in oncology. Abicipar Pegol, an anti-vascular endothelial growth factor (VEGF) DARPin® drug candidate is currently being tested in multiple phase II and III clinical trials (https://clinicaltrials.gov/ct2/show/NCT02462486; https://clinicaltrials.gov/ct2/show/NCT02462928) for the treatment of wet age-related macular degeneration or for the treatment of diabetic macular edema. The molecule is PEGylated, leading to an optimal ocular pharmacokinetic profile upon intravitreal injection. Another DARPin® drug candidate is MP0250, a multi-domain protein inhibiting both VEGF and hepatocyte growth factor. MP0250 is currently in phase I clinical trials for the treatment of cancer (https://clinicaltrials.gov/ct2/show/NCT02194426).

Here, we describe the generation of serum albumin-binding DARPin® domains useful for pharmacokinetic engineering of protein therapeutics, including a detailed analysis of affinities, species cross reactivities, and pharmacokinetic analyses. Such a DARPin® domain provides MP0250 the needed long terminal half-live *in vivo* and thus an excellent systemic exposure of this drug candidate in human.

Results and discussion

Selection of well-behaved serum albumin-binding DARPin® domains

Ribosome display (Hanes *et al.*, 1998; Binz *et al.*, 2004; Zahnd *et al.*, 2007) was used to select specific albumin-binding DARPin® domains. After five selection rounds, pools of DNA were analyzed for individual serum albumin binders by crude extract ELISA screening. A number of serum albumin-specific binders were identified. In the following, individual serum albumin-specific DARPin® domains are described in detail, with a particular focus on N2C DARPin® domains, i.e. domains comprising one N-terminal capping repeat, two repeat modules, and one C-terminal capping repeat (see Binz *et al.*, (2003) for details on nomenclature). In particular, we focus on N2C domains belonging to the sequence family of the serum albumin-binding DARPin® domains currently being used in MP0250 in human and other DARPin® drugs in preclinical development. Sequences of individual domains and proteins consisting of multiple domains are given in the Supplementary Information to this publication and are schematically represented in Fig. 1.

Individual such selected domains were expressed at levels between 0.2 g/l shakeflask culture in *E. coli* XL1 Blue as described previously (Binz *et al.*, 2003) and more than 0.5 g/l shakeflask culture in *E. coli* BL21 with rich medium. The MRGSH6-tagged

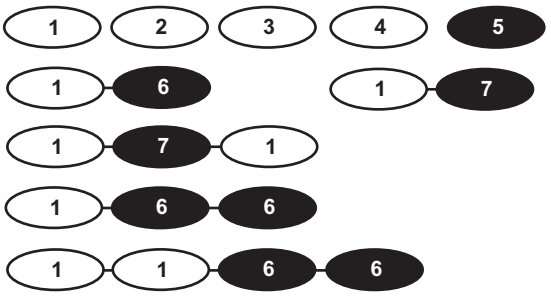

Fig. 1 Schematic representations of DARPin® domains and recombinant binding proteins used in the present study. White circles represent serum albumin-binding DARPin® domains (#1, #2, #3, #4). Black circles represent DARPin® domains with no binding specificity (#6) or a binding specificity for another target than serum albumin (#5 and #7). Lines between the circles indicate polypeptide linkers. Numbers indicate the domain number as listed in the Supplementary Information to this publication. The schematic representations used in this figure are used to illustrate Figs. 2–4.

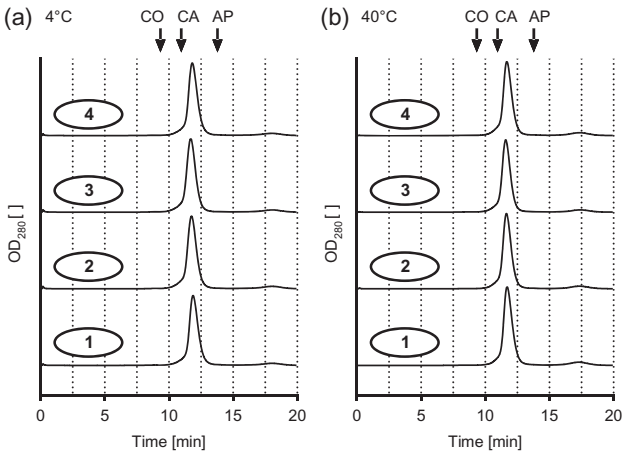

Fig. 2 Monomeric state and high storage stability of different serum albumin-binding DARPin® domains assessed by size-exclusion chromatography. (**a**) Size-exclusion chromatograms recorded after protein purification, indicating that the selected proteins are monomeric. (**b**) Size-exclusion chromatograms (equimolar) recorded after one month incubation at 40°C, indicating that the selected proteins exhibit high storage stability. Column: Superdex 200 5/150, Vo = 1.08 ml/5.4 min, Vt = 3.0 ml/15 min; 20 µl of 20–200 µM protein injected with 0.2 ml/min PBS flow. CO (conalbumin; 75 kDa), CA (carbonic anhydrase; 29 kDa) and AP (aprotinin; 6.5 kDa) marker elution volumes are indicated above the graphs. The schematic molecule representations used in this figure are analogous to the ones in Fig. 1.

domains could readily be purified to near homogeneity using a single IMAC step. In a first analysis step, selected DARPin® domains were subjected to biophysical and storage stability analyses. The results of these analyses are shown in Supplementary Table 1 and Fig. 2. In fluorescence-based thermal stability measurements, the domains exhibited mid-points of thermal denaturation (maxima of the derivative of the measured curve) ranging between Tm = 78°C and Tm = 81.5°C at pH 7.4 (Supplementary Table 1), and between Tm = 74.5°C and Tm = 79.5°C at pH 5.8 (Supplementary Table 1). These values are expected from previous thermal unfolding experiments for N2C molecules and confirm the high thermal stability of designed ankyrin repeat proteins. A high thermal stability in turn can be a good predictor for long storage stability. Storage stability was assessed by incubating the proteins for 1 month at 40°C in PBS, followed by a comparative assessment by size-exclusion

chromatography including pre-incubation samples. The results of this analysis are shown in Fig. 2. All four domains show overlapping size-exclusion chromatography elution profiles at Day 0 and Day 28 of this accelerated stability study, indicating sample stability under the conditions tested. Importantly, they elute as monomeric peaks and show no sign of aggregation, multimerization or degradation. This finding shows that all tested domains have favorable storage stability with no significant change in size-exclusion chromatography elution profile after thermal stress at 40°C for one month. Such storage stability behavior is a good predisposition for the use of such DARPin® domains as building blocks for multi-functional protein therapeutics.

Nanomolar affinity and broad species cross-reactivity

Human serum albumin is present in the blood at a concentration of about 600 µM (Peters, 1985). In circulation, the equilibrium between free serum albumin binder and binder complexed with serum albumin is expected to be almost completely at the side of the complex across a very broad range of affinities of the binder. Typical serum albumin binders do show serum albumin dissociation constants in the high nM to mid pM range (Dennis *et al.*, 2002; Linhult *et al.*, 2002; Holt *et al.*, 2008; Revets and Boutton, 2011; Müller *et al.*, 2012; Van Roy *et al.*, 2015), and they all exhibit attractive pharmacokinetic properties. The serum albumin-binding DARPin® domains presented here exhibit affinities ranging from 11nM to 21nM for human serum albumin at pH 7.4 (Table I). Our goal was to generate DARPin® domains that can bind serum albumin of different species. The selected domains showed binding to serum albumin of human, cynomolgus monkey, mouse, rat, and dog, with mid-nanomolar affinities (Table I). This broad species selectivity enables the use of the same serum albumin-binding DARPin® domains from research through to the application in man. Importantly, low nanomolar-affinity binding of serum albumin of the different species was confirmed for the domains at pH 6 (Table I). This is desired, as serum albumin-binding is particularly important during the FcRn-mediated endosomal recycling of serum albumin, where the pH is known to be lower. The serum albumin-binding DARPin® domains even bind slightly stronger to the serum albumin variants of different species at lower pH. The slightly higher affinity measured at pH 6 compared to pH 7.4 could hint at histidines being involved in the binding site either at the side of serum albumin or e.g. His48 in the designed ankyrin repeat domain. Interestingly, the latter is located just next to a Gly47Ser framework-mutation concerning the highly conserved glycine of the beta-turn of the ankyrin repeat. Further experiments would be needed to elucidate the role of these two residues. ELISA experiments indicate that DARPin® domain #1 is able to bind a protein consisting of domains 1 and 2 of HSA (data not shown), whereas the FcRn binding site is located on domain 3 of HSA. This indicates that its binding to serum albumin is unlikely to interfere with FcRn-mediated serum albumin recycling.

Serum albumin-binding DARPin® domains lead to improved pharmacokinetic properties

In mouse pharmacokinetic experiments the non-serum albumin-binding DARPin® domain #5 is eliminated within minutes with no measurable blood levels beyond 4 h. No terminal half-life was determined with the setup measured due to the very fast systemic clearance of the protein (Table II and Fig. 3). This is in line with a previously reported measurement of a non-serum albumin-binding DARPin® domain which exhibited a loss of 90% of the initial radioactivity after 7.5 min (Zahnd *et al.*, 2010). We chose to analyze the serum albumin-binding DARPin® domains #1 and #3, whose amino acid sequence differs by two amino acids, to evaluate the impact on the pharmacokinetic profile. Indeed, DARPin® domains binding to serum albumin exhibit clearly improved pharmacokinetic properties (Fig. 3), with domain #1 exhibiting a terminal half-life of 2.3 days (55 h; Table II and Fig. 3), and domain #3 exhibiting a terminal

Table I. Dissociation constant (Kd in [nM]) of selected serum albumin-binding DARPin® domains to serum albumin of different species at different pH measured by surface plasmon resonance

DARPin® domain #	pH 7.4					pH 6.0				
	Human	Cyno[a]	Mouse	Rat	Dog	Human	Cyno[a]	Mouse	Rat	Dog
1	11	63	56	91	77	6	9	8	8	11
2	21	110	124	242	185	12	23	20	15	18
3	21	110	142	266	180	8	14	3	8	10
4	11	74	68	109	81	5	8	8	8	6

[a]Cynomolgus monkey.

Table II. Terminal half-life of different serum albumin-binding constructs in comparison to non-serum albumin-binding protein #5

Construct #	$t_{1/2}$ mouse [d] ([h])	$t_{1/2}$ cynomolgus monkey [d]	$t_{1/2}$ human (extrapolated) [d]
5	0.00001 (0.0003)[a]	0.008	n.d.
1	2.3 (55)	12.0	*27.1*
3	2.1 (50)	16.0	*43.7*
1–6	1.8 (44)	10.6	*25.2*
1–6[b]	1.8 (42)	6.1	*11.3*
1–7	0.9 (21)	2.7	*4.7*
1–1	3.4 (82)	20.7	*50.3*

n.d., not determined.

Extrapolated values are shown in italic font.

[a]No terminal half-life value could be determined, the alpha half-life value is listed instead.

[b]In this construct, the two DARPin® domains are separated by a $(G_4S)_4$ linker.

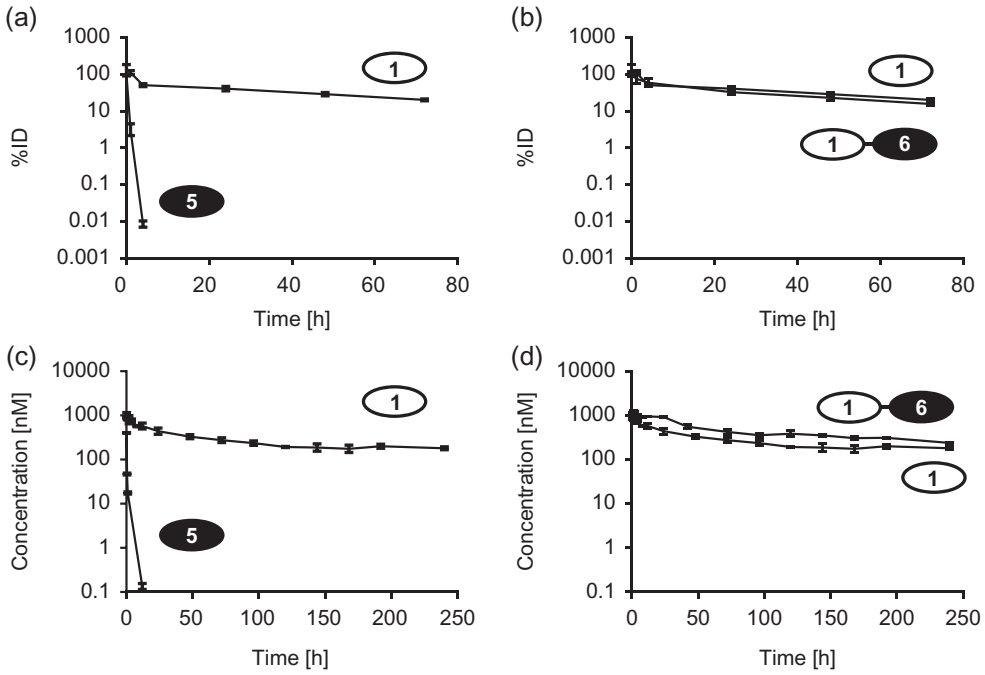

Fig. 3 Improved pharmacokinetic profiles of recombinant binding proteins comprising DARPin® domains binding serum albumin. (**a**) Mouse pharmacokinetic profile of serum albumin-binding DARPin® domain #1 in comparison to non-serum albumin-binding domain #5. (**b**) Mouse pharmacokinetic profile of serum albumin-binding DARPin® domain #1 in comparison to serum albumin-binding DARPin® domain #1 connected N-terminally to non-serum albumin-binding domain #6. (**c**) Cynomolgus monkey pharmacokinetic profile of non-serum albumin-binding DARPin® domain #5 in comparison to serum albumin-binding DARPin® domain #1. (**d**) Cynomolgus monkey pharmacokinetic profile of serum albumin-binding DARPin® domain #1 in comparison to serum albumin-binding DARPin® domain #1 connected N-terminally to non-serum albumin-binding domain #6. %ID: percent injected dose. Error bars indicate the standard deviation observed for the individual measurement points. The schematic molecule representations used in this figure are analogous to the ones in Fig. 1.

half-life of 2.1 days (50 h; Table II) in mouse. The half-life of serum albumin-binding DARPin® domains may thus be limited by the half-life of serum albumin, which exhibits a half-life of 1–2 days (35 h; 1.5 d; Chaudhury *et al.*, (2003)) in mice. In cynomolgus monkey pharmacokinetic studies domain #1 exhibits a terminal half-life of 12 days, compared to about 11 min of a DARPin® domain not binding to serum albumin (domain #5; Fig. 3, Table II). This corresponds to an about 1500-fold increase in terminal half-life. Interestingly, an albumin-binding DARPin® domains with 10-fold higher affinity for cynomolgus monkey serum albumin, exhibits comparable serum half-life as domain #1 in cynomolgus monkey (data not shown), suggesting that higher albumin-binding affinities of such a domain beyond a certain threshold, does not result in a longer terminal half-live. Also in cynomolgus monkeys it seems that the half-life reached with serum albumin-binding DARPin® domains is limited by the half-life of cynomolgus monkey serum albumin, which was reported to be about 7–9 days (Deo *et al.*, 1974). Overall, these results demonstrate that serum albumin-binding DARPin® domains have strongly improved pharmacokinetic properties compared to non-serum albumin-binding DARPin® domains. The pharmacokinetic traces measured in mouse and in cynomolgus monkey indicate *in vivo* stability of the constructs used. As the two serum albumin-binding DARPin® domains evaluated yielded comparable results, we decided to use domain #1 for further pharmacokinetic analyses.

Comparable pharmacokinetic analyses have previously been performed with other serum albumin-binding proteins. Serum albumin-binding antibody fragments exhibited 24 h terminal half-life in mouse compared to 42 min of non-serum albumin-binding antibody domains (Holt *et al.*, 2008). Another serum albumin-binding antibody fragment had a terminal half-life in mouse of 12 h (Hoefman *et al.*, 2015). These terminal half-life values appear to be slightly lower than the ones measured for the serum albumin-binding DARPin® domains. In rhesus monkeys, terminal half-lives of albumin-binding antibody fragments were claimed to be in a range of 8.0 days to 12.5 days (Beirnaert *et al.*, 2007), which is comparable to the half-life measured for DARPin® domains in cynomolgus monkey. A serum albumin-binding peptide exhibited a terminal half-life of 2.3 h in rabbits compared to a terminal half-life in the minute's range for a non-albumin-binding peptide (Dennis *et al.*, 2002).

Extending the half-life of protein therapeutics using serum albumin-binding DARPin® domains

As a further step we assessed the ability of the serum albumin-binding DARPin® domains in improving the pharmacokinetic properties of DARPin® domains with other functionalities. To that end, a set of fusion proteins was generated (Fig. 1; sequences see Supplementary Information). We chose to work with Pro-Thr-rich polypeptide linkers, as we believe the more rigid nature of Pro-Thr-rich polypeptide linkers leads to a good functionality of the individual domains (George and Heringa, 2002; Ruiz *et al.*, 2016) compared to the classical Gly-Ser-rich polypeptide linkers, which are more flexible and appear to be ideal to link normally associated domains in a single genetic construct, such as e.g. the immunoglobulin domains as used in scFvs (Chen *et al.*, 2013). The proteins were expressed and purified as described for the single-domain proteins. The proteins were tested

in mouse and cynomolgus monkey pharmacokinetic experiments (Fig. 3, Table II). In mouse pharmacokinetic experiments, different fusion proteins exhibited terminal half-lives in the range of 0.9 days to 1.8 days (Fig. 3, Table II). Similarly, these fusion proteins exhibited terminal half-lives in the range of 2.7 days to 10.6 days (Fig. 3, Table II) in cynomolgus monkeys, corresponding to an increase by a factor ranging from 338 to 1325 compared to a non-serum albumin-binding DARPin® domain. Importantly, the maximum values observed are in the range of the terminal half-lives of the serum albumin of the respective species. Constructs with a long terminal half-life in mouse also exhibit a long terminal half-life in cynomolgus monkey. Similarly, constructs with a short terminal half-life in mouse also exhibit a short terminal half-life in cynomolgus monkey. This finding indicates a good scalability of the pharmacokinetic parameters across species and supports the possibility for extrapolation to the situation in human. A construct with a Pro-Thr-rich polypeptide linker exhibited slightly improved terminal half-life compared to a construct comprising a Gly-Ser-rich polypeptide linker (Table II). A detailed analysis of DARPin® drug candidate MP0250 indicates that the target-binding activities of individual DARPin® domains are not affected upon transition to a multi-domain construct (Binz *et al.*, 2017).

A number of similar approaches of fusing albumin-binding polypeptides to other protein domains are known, including fusion approaches with peptides (Dennis *et al.*, 2002; Nguyen *et al.*, 2006; Revets and Boutton, 2011), albumin-binding domains (Stork *et al.*, 2007; Tolmachev *et al.*, 2007; Hopp *et al.*, 2010; Frejd, 2012; Orlova *et al.*, 2013) and antibody fragments (Holt *et al.*, 2008; Walker *et al.*, 2010; Müller *et al.*, 2012; O'Connor-Semmes *et al.*, 2014; Goodall *et al.*, 2015; Hoefman *et al.*, 2015; Van Roy *et al.*, 2015). Serum albumin-binding peptide fusion were used to improve the terminal half-life of antibody fragments from 24 min (bare antibody fragment) to 10.4 h (fusion) (Dennis *et al.*, 2002) in mouse, or from 1.28 h to 19.7 h in mouse (Nguyen *et al.*, 2006), or from 31.2 h to 37.6 h in cynomolgus monkey (Revets and Boutton, 2011). The albumin-binding domain of streptococcal protein G (ABD) was used to improve the pharmacokinetic profiles of the Fab fragment of Herceptin (2.1 h to 20.9 h improvement in terminal half-life in mouse) (Schlapschy *et al.*, 2007), a diabody (5.6 h up to 47.5 h in mouse) (Stork *et al.*, 2007; Hopp *et al.*, 2010), or small helical proteins (38 min to 35.8 h, or 30 min to 41 h, in mouse) (Tolmachev *et al.*, 2007; Orlova *et al.*, 2013), amongst others (Frejd, 2012). Similar effects were achieved using serum albumin-binding shark antibody fragments (Müller *et al.*, 2012), other serum albumin-binding antibody fragments (Holt *et al.*, 2008; Walker *et al.*, 2010; O'Connor-Semmes *et al.*, 2014; Goodall *et al.*, 2015; Hoefman *et al.*, 2015; Van Roy *et al.*, 2015). Overall, the serum albumin-binding DARPin® domains can be used to create multi-domain DARPin® domains with pharmacokinetic properties that resemble the ones of serum albumin.

The approach of using serum albumin-binding polypeptides as genetic fusion partner has various advantages over other serum half-life extension techniques. Direct fusion to serum albumin yields similar pharmacokinetic properties, yet does not allow for bacterial production. PEGylation, HESylation and polypeptide extension approaches typically are less efficient in improving pharmacokinetic properties and PEGylation and HESylation require chemical modification complicating the production process. The half-lives reported here indicate that the albumin-binding approach yields similar pharmacokinetic properties as seen for monoclonal antibodies. In addition this technology allows to take advantage of the high stability of DARPin® domains and the possibility of producing therapeutic proteins efficiently in bacteria.

Maximizing terminal half-life – surprising avidity effect

There are contradicting data on the effect of serum albumin affinity/avidity and half-life extension. Studies with peptides (Dennis *et al.*, 2002; Nguyen *et al.*, 2006; Revets and Boutton, 2011) suggest a correlation between serum albumin affinity and half-life extension. The peptides used in these studies were of mid nM affinities. Interestingly, a study comparing a construct comprising one albumin-binding domain (Kd = 11.2 nM) with a construct comprising two albumin-binding domains (apparent Kd = 2.5 nM) revealed no change in pharmacokinetic properties of the construct in mouse, indicating that nM affinity is sufficient for albumin-binding proteins, and no benefit is gained when using higher affinity or valency (Hopp *et al.*, 2010). Despite these findings we generated proteins comprising more than one albumin-binding DARPin® domain. Surprisingly, we find that the use of two albumin-binding DARPin® domains with binding specificity for serum albumin further improves the pharmacokinetic profile (Fig. 4, Table II). Comparing a construct comprising one serum albumin-binding

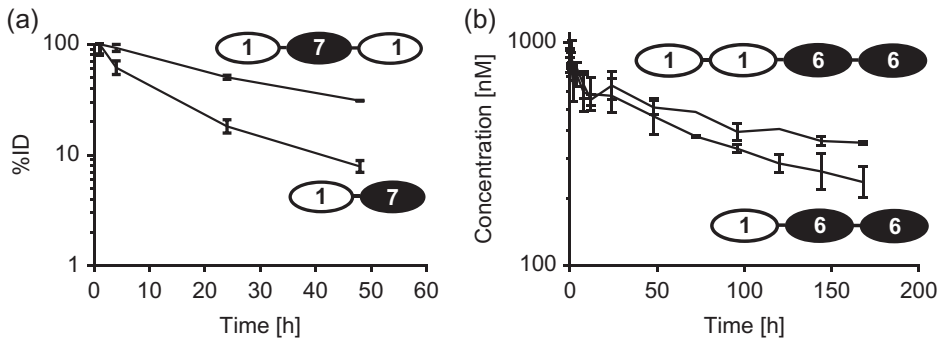

Fig. 4 Pharmacokinetic engineering using more than one serum albumin-binding. (**a**) Mouse pharmacokinetic profile comparison of a recombinant protein comprising one serum albumin-binding DARPin® domain #1 and one domain #7 with a recombinant binding protein comprising twice serum albumin-binding DARPin® domain #1 and one domain #7. (**b**) Cynomolgus monkey pharmacokinetic profile comparison of a recombinant protein comprising one serum albumin-binding DARPin® domain #1 and twice domain #6 with a recombinant binding protein comprising twice serum albumin-binding DARPin® domains #1 and twice domains #6. Terminal half-lives were determined, using the values in the range of 0.33 days to 7 days, to be $T_{1/2}$ = 4.7 days and $T_{1/2}$ = 7.7 days, respectively. Error bars indicate the standard deviation observed for the individual measurement points. The schematic molecule representations used in this figure are analogous to the ones in Fig. 1. Experimental details see Materials and Methods.

DARPin® domain with a construct comprising two serum albumin-binding DARPin® domains, a clear improvement in terminal half-life can be observed in mouse (2.3 days vs. 3.4 days) as well as in cynomolgus monkey (12 d vs. 20.7 d; Table II), corresponding to an increase of 48% and 73%, respectively. The half-live values observed for constructs with a single serum albumin-binding DARPin® domain appear to reach half-life values similar to those postulated for mouse and cynomolgus monkey serum albumin (Deo *et al.*, 1974; Chaudhury *et al.*, 2003). Interestingly, the terminal half-life values observed for the constructs comprising two serum albumin-binding DARPin® domains appear to be beyond the reported half-life values of mouse and cynomolgus monkey serum albumin. The increase of terminal half-life in mouse and cynomolgus monkey upon fusion of multiple serum albumin-binding DARPin® domains was confirmed by the analysis of additional proteins comprising multiple domains (Fig. 4). A construct comprising two serum albumin-binding DARPin® domains and one non-serum albumin-binding domain exhibits improved pharmacokinetics in mouse compared to a construct comprising only one serum albumin-binding DARPin® domain (Fig. 4a). Similarly, a construct comprising two serum albumin-binding DARPin® domains and two non-serum albumin-binding domains exhibits an improved terminal half-life in cynomolgus monkey compared to a construct comprising only one serum albumin-binding DARPin® domain (see Fig. 4b; $T_{1/2}$ = 7.7 days and $T_{1/2}$ = 4.7 days, respectively). As domain #6 has no explicit binding specificity, specific target-mediated effects can be ruled out to cause the effect. Size-exclusion chromatography analyses coupled to multi-angle light scattering show that two serum albumin molecules can be bound at the same time in constructs comprising two serum albumin-binding DARPin® domains (Fig. 5), which was further confirmed by surface plasmon resonance (SPR) (data not shown). Interestingly, no effects on pharmacokinetic properties of corresponding constructs in mice were reported in previous studies using two albumin-binding domains of streptococcal protein G (Hopp *et al.*, 2010). Additional preclinical data on MP0250, a four-domain DARPin® drug candidate comprising two DARPin® domains with binding specificity for serum albumin are described elsewhere and confirm that the use of two serum albumin-binding DARPin® domains is a useful approach to maximize terminal half-life of therapeutic proteins (Binz *et al.*, 2017). Further studies will be needed to elucidate the cause for the unexpected gain in terminal half-life length by adding a second albumin-binding DARPin® domain. The Stokes radius of the construct is increasing when binding two serum albumin-binding molecules (Fig. 5), which could reduce the amount of protein that leaves the blood by kidney filtration. This could lead to an increased apparent half-life of the bound serum albumin molecules beyond the one known for free serum albumin. Alternatively, valency could play an important role during endosomal recycling protecting the protein in case serum albumin is being degraded, by offering an escape via interaction to an intact second serum albumin molecule. This would also explain the possibility of reaching half-lives beyond the half-life of serum albumin. Further studies will be needed to describe the cause of the effect observed in more detail, in particular considering the data of Hopp *et al.* (2010) that suggest no effect of increased affinity, increased valency, or increased stokes radius.

Extrapolation to human – favorable pharmacokinetic profile expected

In mouse and cynomolgus monkey pharmacokinetic studies, half-lives matching the one of the respective species serum albumin are achieved using the serum albumin-binding DARPin® domain technology (Table II). By scaling using the data shown here, a terminal half-life in human would be predicted in the range varying from 4.7 days to 50.3 days considering mouse (18 g), cynomolgus monkey (5 kg), and human (80 kg) (Table II). Similar to the situation in mice and cynomolgus monkey, it can be assumed that the terminal half-life of a monovalent serum albumin-binding DARPin® domain is limited to that of serum albumin in humans, i.e. approximately 19 days (Peters, 1985). A favorable terminal half-life in human is thus to be expected, enabling low-frequency dosing of drug candidates, comparable to human IgG antibodies. This favorable pharmacokinetic feature potentially enables dosing regimens comparable to the ones of monoclonal antibodies. Indeed, the analysis of the pharmacokinetic properties of MP0250 in cynomolgus monkey recently confirmed that drug candidates with favorable pharmacokinetic profiles can be achieved (Binz *et al.*, 2017).

Conclusion

Serum albumin-binding DARPin® domains are valuable tools to prolong the terminal half-life of next-generation protein therapeutics. Our results suggest that such domains can harness the pharmacokinetic profile of serum albumin, enabling the generation of long-acting multi-functional DARPin® drugs. This adds an additional important pillar to the DARPin® drug platform, which allows the efficient generation of multi-functional drugs. Overall, this platform has the potential to enable novel therapeutic approaches beyond the possibilities of monoclonal antibodies. With the multi-functional DARPin® drug MP0250 comprising such an albumin-binding DARPin® domain, this approach is currently undergoing clinical validation.

Materials and Methods

Ribosome display selections, protein engineering, and initial characterization

The selection of serum albumin-binding DARPin® domains was performed by ribosome display (Hanes *et al.*, 1998) using biotinylated HSA, libraries of designed ankyrin repeat proteins and protocols as described (Binz *et al.*, 2003, 2004; Zahnd *et al.*, 2007). Five selection rounds on biotinylated HSA (EZ-Link™ Sulfo-NHS-Biotin, ThermoFisher; HSA from CSL Bering or Sigma), immobilized via neutravidin, were performed during which the number of reverse transcription PCR cycles constantly reduced from 40 to 30, adjusting to the yield due to enrichment of binders. DNA pools were ligated into expression vector pQE30 (Qiagen) and *E. coli* XL1-Blue (Stratagene) were transformed with the ligation mixes. Individual colonies were grown overnight at 37°C in 96-deep-well plates containing 1 ml/well growth medium (2YT containing 1% glucose and 100 µg/ml ampicillin). One ml/well of fresh 2YT containing 50 µg/ml ampicillin was inoculated with 100 µl of the overnight culture in fresh 96-deep-well plates. After incubation for 2 h at 37°C, expression was induced with IPTG (1 mM final concentration) and continued for 3 h. Cells were harvested, resuspended in 100 µl B-PERII (Pierce) and incubated for 15 min at room temperature with shaking. About 900 µl PBS-TC (PBS supplemented with 0.25% Casein hyrolysate, 0.1% Tween 20, pH 7.4) were added and cell debris were removed by centrifugation. About 100 µl/well of each lysate were applied to NeutrAvidin coated MaxiSorp plates containing either HSA, MSA, or CSA or the unrelated maltose-binding protein immobilized via their biotin moiety and incubated for 1 h at room

temperature. After extensive washing with PBS-T (PBS supplemented with 0.1% Tween 20, pH 7.4) ELISAs were developed using the monoclonal anti-RGS(His)$_4$ antibody (34 650, Qiagen) as primary antibody and a polyclonal goat anti-mouse antibody conjugated with alkaline phosphatase (A3562, Sigma) as secondary reagent. Binding was then detected by using disodium 4-nitrophenyl phosphate (4NPP, Fluka) as a substrate for alkaline phosphatase. The color development was measured at 405 nm. The DNA sequences of colonies resulting in an ELISA signal were determined using standard methods. This study focusses on one sequence family. In this family, the C-terminal capping repeat steming from the DARPin® libraries (Binz *et al.*, 2003) has been exchanged as described (Interlandi *et al.*, 2008; Steiner *et al.*, 2012), and the four variants shown here comprise different combinations of framework mutations that have been observed in that sequence family. The proteins were modified using site-directed mutagenesis or DNA synthesis.

Cloning of proteins consisting of multiple DARPin® domains

Proteins comprising more than one DARPin® domain were either generated by conventional PCR-cloning methods or by standard gene synthesis. The non-serum albumin-binding domains #5 and #7 have picomolar affinity to VEGF (mouse, cynomolgus monkey, and human; data not shown), whereas domain #6 is a full consensus designed ankyrin repeat domain with no known binding specificity (Wetzel *et al.*, 2008).

Protein expression, purification and stability measurements

Selected clones were expressed in *E. coli* BL21 or XL1-Blue cells as described (Binz *et al.*, 2003) and purified either using immobilized metal ion affinity chromatography as described (Binz *et al.*, 2003) or using standard anion exchange chromatography (Q-Sepharose FF, GE Healthcare) followed by size-exclusion chromatography (Superdex 200, GE Healthcare). Protein purity >95% was verified using SDS-15% PAGE. For stability studies, proteins were concentrated to approximately 30 mg/ml (approximately 2 mM) in PBS and stored for 28 days at 40°C (stability study). Samples were taken at study start and Day 28. Samples were diluted to 500 µM and analyzed by size-exclusion chromatography using a HPLC system (Agilent 1200 series) using a Superdex 200 5/150 column (GE Healthcare) at 20°C and 0.2 ml/min flow in PBS. Aprotinin (M_W 6.5 kDa), Carbonic Anhydrase (M_W 29 kDa) and Conalbumin (M_W 75 kDa) were used as standards for apparent molecular weight determination of the sample proteins. Thermal stability of proteins was analyzed in a fluorescence-based thermal stability assay (Niesen *et al.*, 2007) by mixing 80 µM protein (either in PBS pH 7.4 or MES buffer pH 5.8) with 1x SYPRO orange (Invitrogen) in 50 µl volume and using a C1000 thermal cycler with a CFX96 optical system (BioRad; ramp 20°C to 95°C at 0.5°C increments including a 25 s hold step after each temperature increment; excitation 515–535 nm, detection 560–580 nm; buffer signal subtraction).

Surface Plasmon Resonance

SPR was measured using a ProteOn instrument (BioRad). Running buffer was PBS, pH 7.4, containing 0.01% Tween 20. Approximately 2000 resonance units (RU) anti-RGS-His antibody (Qiagen, cat. no. 34 650) were immobilized covalently on a GLC chip (BioRad). 150 µl of 1 µM protein solution were then injected in 300 s (flow rate = 30 µl/min) for protein immobilization via the antibody. Serum albumin of different species (400, 200, 100, and 50 nM each; human, cynomolgus monkey, mouse, rat, rabbit or dog serum albumin) in PBS containing 0.01% Tween were injected for 1 min at 100 µl/min, followed by a running buffer flow for 10 to 30 min. The signals of an uncoated reference cell and a reference injection were subtracted from the signals (double-referencing). Kinetic interaction parameters were determined using the ProteOn software (BioRad). The experiments at pH 6.0 were performed as described above, using a phosphate/citrate buffer (PBS pH-adjusted using 1 M citric acid).

Multi-angle static light scattering coupled to size-exclusion chromatography (SEC-MALS)

MP0250 (30 µM; for amino acid sequence see Bakker *et al.*, (2016)), purified HSA (60 µM), as well as MP0250/HSA mixture (30 µM/60 µM) were analyzed. SEC-MALS was performed on a Agilent 1200 system (Life Technologies, USA) connected to a Wyatt (USA) MALS and refractive index detector (flow rate: 0.6 ml/min; injection volume: 100 µl; column: GE Healthcare (USA) Superdex200 10/300GL). The MP0250/HSA mixture was pre-incubated for 3 h at 20°C prior injection. The chromatograms are shown in Fig. 5 and the molecular masses of the eluates were determined and compared to the theoretical molecular masses (see figure legend of Fig. 5). For this experiment, 100 mg HSA (CSL Behring 20% solution) were purified using a Superdex 200_26.60 column on a AEKTA prime system (GE Healthcare; 2.0 ml/min, PBS, isocratic flow, injection

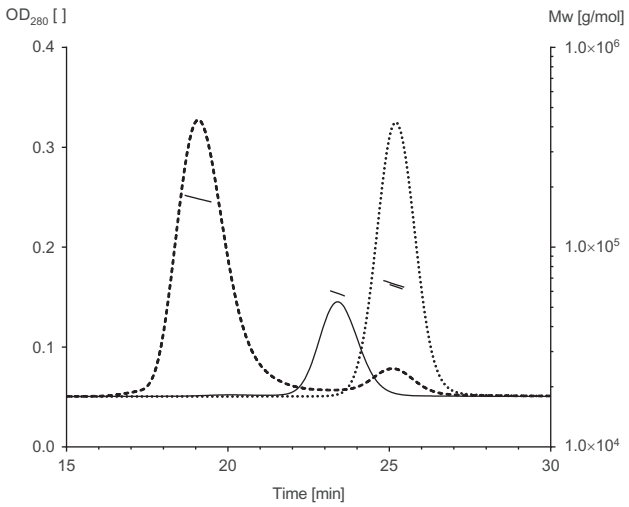

Fig. 5 Serum albumin-binding monitored by static multi-angle light scattering coupled to size-exclusion chromatography (SEC-MALS). The UV signals as well as the SEC-MALS signals of MP250 (solid line; a recombinant binding protein consisting of two serum albumin-binding DARPin® domains as well as two target-binding DARPin® domains), HSA (dotted line) and the 1:2 mixture of MP0250 with HSA (dashed line) are shown. For HSA, a molecular weight of 63′350 Da was measured (theoretical molecular weight: 69′366.6 Da) and for MP0250 a molecular weight of 58′700 Da was measured (theoretical molecular weight: 62′397.0 Da). For the MP0250/HSA mixture, the elution peak comprised 1:2 complexes with a measured molecular weight of 197′500 Da (theoretical molecular weight: 201′139.2 Da; front of the main peak), 1:1 complexes with a measured molecular weight of 132′500 Da (theoretical molecular weight: 131′763.6 Da; tail of the main peak), and mixtures of the two (middle of the main peak). Additionally a fraction of free HSA is observed. Mw: molecular weight [Da]; OD: optical density at 280 nm; t: time [min].

volume 10 ml of 1:20 in PBS diluted HSA, collecting 4 ml fractions). The peak fraction of the main peak was used for performance of the SEC-MALS experiment.

Mouse pharmacokinetic studies

The studies were performed as described previously (Waibel *et al.*, 1999). In brief, proteins (40 µg) were incubated with ^{99m}Tc-carbonyl (0.8–1.6 m Ci) for 1 h before dilution to 400 µl in PBS (pH 7.4). The pharmacokinetic profile was measured in one mouse per construct for ethical reasons. Each mouse was injected intravenously with 100 µl of the labeled protein solution (equivalent to 10 µg protein and 0.2–0.4 m Ci; tail vein). Blood samples of the mice were collected at 1 h, 4 h, 24 h and 48 h after the initial injection, the radioactivity of the samples was measured and the percentage injected dose (%ID) was ploted over time. The pharmacokinetic parameters including terminal half-life values were derived.

Cynomolgus monkey pharmacokinetic studies

Proteins diluted in PBS were injected as a bolus injection in the cephalic vein of cynomolgus monkeys at doses of 0.5 mg/kg to 1 mg/kg. The pharmacokinetic profile was measured in one cynomolgus monkey per construct for ethical reasons. Blood was collected from the femoral vein at various time points followed by plasma generation. Protein concentration was determined using a sandwich ELISA. Plasma samples as well as standards were serially diluted in PBS-C (PBS containing 0.25% casein, pH 7.4) and added to MaxiSorp plates that were coated with an anti-DARPin® domain specific rabbit monoclonal antibody (generated using conventional immunization and hybridoma technologies). Binding protein was detected using an anti-RGS(His)4 antibody HRP conjugate (Qiagen) and a standard ELISA development using 100 µl BM-Blue POD substrate (Roche Diagnostics), stopped by adding 50 µl of 1 M H_2SO_4, followed by measurement of the absorbance at 450 nm (and subtracting the absorbance at 620 nm). The concentration of the protein in the plasma sample was calculated by performing a monoexponential regression on a standard curve of the protein diluted in monkey serum using the software Prism (GraphPad Software). Pharmacokinetic parameters were determined by non-compartmental analyses using the software Phoenix WinNonLin (Certara) or by non-linear regressions (two-phase decay; Prism), using concentration values up to 240 h after injection. In case of two-phase decay, the half-life value of the second (beta) phase was taken as terminal plasma half-life.

Alometric scaling

Allometric scaling (Ings, 1990; Ling *et al.*, 2009) to human (80 kg) listed in Table II was calculated asuming mouse (18 g) and cynomolgus monkey (5 kg) weights and using the values for half-life listed in Table II, ploting log(half-life) as a function of log(weight) and extrapolating to the human body weight.

Supplementary data

Supplementary data are available at *Protein Engineering, Design & Selection* online.

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DARPin® is a registered trademark of Molecular Partners AG.

Conflict of interest

All authors hold options or shares of Molecular Partners AG.

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