Improving the pharmacokinetic properties of biologics by fusion to an anti-HSA shark VNAR domain

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Abbreviations: IgNAR, Immunoglobulin like new antigen receptor; VNAR, variable domain of shark new antigen receptor; CDR, complementarity determining region; FW, framework; HV, hypervariable region; FcRn, neonatal Fc receptor; NHP, non-human primate; PK, pharmacokinetics

Introduction

The last decade has seen a substantial rise in the development of antibody-based biologics. An intrinsically high level of specificity for cognate target antigen reducing off-site toxicity, and amenability to in vitro molecular engineering make them extremely attractive candidates for clinical development. Unique to antibodies are the fragment crystallizable (Fc) related properties such as long serum half-life to optimise drug exposure, thereby increasing therapeutic efficacy. In this study, we adopted an immunization route to raise picomolar affinity shark immunoglobulin new antigen receptors (IgNARs) to target human serum albumin (HSA). From our model shark species, *Squalus acanthias*, a phage display library encompassing the variable binding domain of IgNAR (VNAR) was constructed, screened against target, and positive clones were characterized for affinity and specificity. N-terminal and C-terminal molecular fusions of our lead hit in complex with a naïve VNAR domain were expressed, purified and exhibited the retention of high affinity binding to HSA, but also cross-selectivity to mouse, rat and monkey serum albumin both in vitro and in vivo. Furthermore, the naïve VNAR had enhanced pharmacokinetic (PK) characteristics in both N- and C-terminal orientations and when tested as a three domain construct with naïve VNAR flanking the HSA binding domain at both the N and C termini. Molecules derived from this platform technology also demonstrated the potential for clinical utility by being available via the subcutaneous route of delivery. This study thus demonstrates the first in vivo functional efficacy of a VNAR binding domain with the ability to enhance PK properties and support delivery of multifunctional therapies.

Advances in recombinant antibody technology and protein engineering have provided the opportunity to reduce antibodies to their smallest binding domain components and have concomitantly driven the requirement for devising strategies to increase serum half-life to optimise drug exposure, thereby increasing therapeutic efficacy. In this study, we adopted an immunization route to raise picomolar affinity shark immunoglobulin new antigen receptors (IgNARs) to target human serum albumin (HSA). From our model shark species, *Squalus acanthias*, a phage display library encompassing the variable binding domain of IgNAR (VNAR) was constructed, screened against target, and positive clones were characterized for affinity and specificity. N-terminal and C-terminal molecular fusions of our lead hit in complex with a naïve VNAR domain were expressed, purified and exhibited the retention of high affinity binding to HSA, but also cross-selectivity to mouse, rat and monkey serum albumin both in vitro and in vivo. Furthermore, the naïve VNAR had enhanced pharmacokinetic (PK) characteristics in both N- and C-terminal orientations and when tested as a three domain construct with naïve VNAR flanking the HSA binding domain at both the N and C termini. Molecules derived from this platform technology also demonstrated the potential for clinical utility by being available via the subcutaneous route of delivery. This study thus demonstrates the first in vivo functional efficacy of a VNAR binding domain with the ability to enhance PK properties and support delivery of multifunctional therapies.

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deemed unsuitable for drug development, into effective therapeutic agents.

Naturally-occurring single chain antibodies offer the opportunity to reduce the size of binding domains further through their inherent lack of light chain partner. Convergent evolution has resulted in two very diverse classes of animal developing such single chain antibodies as an integral part of their immune repertoire: (1) IgNARs from cartilaginous fish,11 and (2) VHHs or nanobodies from the camelidae (camels, dromedaries and llamas).12 Both types bring great pharmaceutical promise through their stability, solubility and unique binding loop topography, but, with variable binding domains with an average molecular mass of 12–13 kDa, they are rapidly cleared in vivo by glomerular filtration.33 Substantial efforts to address the question of systemic half-life extension have included different strategies to counter unfavorable pharmacokinetic (PK) properties.44 Increasing the size of mAb and Fab antibody fragments to prevent glomerular clearance has been achieved by increasing the hydrodynamic size via chemical modification of random or directed conjugation to polyethylene glycol (PEG).15-18 Other re-formatting strategies such as alterations to site-specific glycosylation have shown moderate increased plasma half-life49 while exploitation of the FcRn recycling system by molecular Fc fusions have significantly extended circulating antibody fragment concentrations.50 Another strategy that hijacks this natural recycling system involves use of serum albumin binding to extend the circulating half-life of smaller proteins or peptides. Albumin is a large (67 kDa), abundant serum protein that plays multiple biological roles in the body, including osmotic hemostasis, fatty acid, lipid and metabolite transfer, metal ion binding and drug elimination.51 Interestingly, albumin has been shown to distribute to regions of inflammation, as illustrated in animal models of arthritis,22,23 and to accumulate in proliferating environments due to regions of inflammation, as illustrated in animal models.22,23

Results

Construction and screening of the immunized VNAR phage library. As the desired outcome of this study was the isolation of high affinity anti-albumin domains, immunization was the preferred route to drive an antigen-specific IgNAR response. *Squalus acanthias*, commonly called spiny dogfish, were immunized and boosted on a monthly basis with human serum albumin as described in the methods section. Over three different immunization campaigns, a total of 10 individual animals were challenged with human serum albumin. To measure antigen-specific IgNAR levels in the serum of these animals, each sample was serially diluted and tested by ELISA for binding to target. Background, non-specific responses were assessed in parallel with milk-coated plates. For detection, the anti-nurse shark IgNAR monoclonal antibody GA8, which cross-reacts with spiny dogfish IgNAR, was used.40 Figure 1A exemplifies a typical titer of shark sera binding to HSA over a five-month immunization campaign where binding to HSA was detectable over control (maximum levels depicted with dotted line in Fig. 1A) after 3 antigen challenges (bleed 3) and became much more pronounced after 2 further boosts forming a plateau in response (bleed 4 and 5, respectively). Of the ten animals immunized, 50% exhibited similar profiles to Figure 1A, 30% showed a response but in the presence of increased background levels to milk, and the final 20% showed a very weak IgNAR specific response above background. Based on the titer data set, an immune phage display library was constructed from VNAR transcripts isolated from bleed 5 of this animal, resulting in a library of approximately 2 × 107 clones. Sequence analyses of 179 clones from this library showed 88% in-frame full-length sequences with 92% unique sequences creating a hypothetical final library size of approximately 1.6 × 107 clones. Interestingly, there was a propensity toward VNAR domains lacking non-canonical cysteine residues compared with an equivalent analysis of naive whole blood sequences, which showed an approximate split between those containing none and those containing one CDR1 and one CDR3 cysteine residue defined as a Type II IgNAR based on nurse shark nomenclature.41 In addition, there was a decrease in the average length of CDR3 from 15 to 9.8 residues.46 The library was rescued and a solid phase sequential bio-panning strategy against 50 μg/ml, 5μg/ml and 0.5 μg/ml HSA was employed. Enrichment of antigen-specific phage during the selection campaign was observed by polyclonal phage ELISA (data not shown) against target (HSA) and control (hen egg lysozyme; HEL) proteins. Based on the positive enrichment for HSA binding VNARs (Fig. 1B), 92 individual colonies were picked from phage-selection 2 and 3 outputs for an ELISA based screening campaign using VNAR domains expressed periplasmically. As the intention of this study was to assess the utility of isolated albumin binders in an in vivo model of half-life extension, it was crucial to incorporate screening for both binding to ortholog albumin and binding to albumin at acidic pH to ensure retention via FcRn-mediated intracellular endosomal recycling29 (Fig. 2). Although the immunization and bio-panning strategy had been against HSA, good cross-reactivity was seen with multiple monoclonal VNAR proteins against both mouse and rat
albumin. Clones binding at both pH 7 and pH 6 and exhibiting cross-reactivity to murine albumin (clones marked black on the gray/black scale shown in the inset of Fig. 2) were selected for further in vitro characterization.

**Sequence analyses of selected and unselected clones.** Based on the criteria set to identify clones that cross-reacted with mouse and retained binding to albumin at lower pH, 25 clones were chosen from pan 2 (p2) and pan 3 (p3) of the library screen. The sequence of these clones was incredibly similar, collapsing to only 18 unique clones, based on single amino acid differences, as shown in the clustalW alignment in Figure 3A. All of these contained no non-canonical cysteine residues distinguishing them from the classical Type I, II and III described IgNARs in nurse shark. Only four different CDR3 sequences were identified and two different CDR1 sequences with a single substitute of S or R in position 33. HV2 also had a single amino acid change in position 48 that was either a Q or R, whereas HV4 remained consistent. The main differences were within the primer defined sequences and, interestingly, showed that changes at the N-terminus of these binding domains may have an influence on cross-reactivity, although this needs further investigation. From this panel of clones, six (p2-C06, p2-H08, p3-A08, p3-E06, p3-E07 and p3-F03; Fig. 3A, asterisks) were selected for further analysis. In addition to a more in-depth characterization of these clones, a retrospective analysis of unselected and pan 1-derived clones from the library was also performed to look for a more diverse set of potential binders. A total of 920 clones from pan 0 and pan 1 were screened for binding to HSA, resulting in the isolation of an additional 29 unique clones based on CDR3 diversity. Of these, 12 showed significant binding by periplasmic extract-based ELISA (OD450 ≥ average of blanks + 3 standard deviations of blanks) to MSA. In total, approximately 16% of the unselected library clones showed binding to HSA, increasing to ~88% in pan 1 and to ~100% thereon (Fig. 1B). The sequences of these 12 promising clones were aligned as shown in Figure 3B.

**Biophysical analysis of selected VNAR domains.** Some evidence suggests that PK can be tailored based on a direct correlation between affinity for albumin and serum half-life. As our main aim was to show unequivocally that we could isolate a VNAR domain capable of extending half-life, we were keen to prioritize our choice of in vivo candidate by means of greatest affinity and optimal binding kinetics across multiple species of albumin. All six lead clones demonstrated high affinities against a panel of mouse, human and rat albumin as measured by surface plasmon resonance (SPR). As with the initial selection criteria, it was crucial to identify a clone that not only bound albumin with high affinity, but also at acidic pH. From this analyses of all six clones, it was evident that clone E06 exhibited the most favorable binding kinetics (Fig. 4), followed by clone H08. As predicted by the similar sequences and borne out by the epitope mapping method on the BIACore (Fig. S1A), all six lead clones (pan 2 and 3) and all of the 12 unselected/selection round 1-derived clones (Fig. S1B) bound to the same, if not overlapping, epitope on HSA. E06 was chosen as the lead clone to take forward.

![Figure 1](image-url)

**Figure 1.** Measurement of anti-HSA IgNAR titer in immunized dogfish and library screening. (A) Bleeds were taken from immunized animals in anti-coagulant and centrifuged to separate plasma and blood cell components. Each bleed was serially diluted and added to an ELISA plate pre-coated with either HSA or control milk. Detection of bound IgNAR was achieved as described in the methods section. Bleeds are coded according to the key with pre-bleed highlighted with a dashed line; maximum backgrounds levels detected against milk are represented by the dotted horizontal line. Shark sera dilutions are shown on the x-axis. (B) Periplasmic protein ELISAs of each round of selection of the immunized library, Wy5k2, against HSA as described in the methods section. Each circle represents a single clone tested against binding to HSA (shown on the y-axis) and HEL (shown on the x-axis).

**Anti-HSA VNAR molecular fusions.** To determine the efficacy of the anti-HSA VNAR domains to increase the serum half-life of another unrelated VNAR domain, fusions consisting of both N-terminally and C-terminally linked HSA-binding E06 to a random naïve VNAR domain known as 2V (sequence shown in Fig. 3B) were constructed. 2V originated from the analyses of naïve spiny spleen tissue during the construction of the sequence database and was expressed as recombinant protein. This VNAR domain had no inherent affinity for HSA as determined by direct antigen binding ELISA and BIACore, and as such was a good candidate as a proof of concept to address the question of half-life extension. Three fusions were constructed: E06-2V, 2V-E06 and 2V-E06-2V with a flexible linker between the binding domains. E06 and E06 fusion proteins were expressed and purified as

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described in the methods section. SDS-PAGE gels showed each preparation consisted of a single protein band of the expected molecular mass (Fig. 5A) and each produced a single peak by analytical SEC (Fig. 5B). Based on preliminary data that had suggested that alterations to the N-terminus of the binding domains isolated affected their binding capacity, the N-terminal and C-terminal dimer fusions were designed to address the question of whether HSA binding was reduced in either orientation. Constructs were tested for their ability to retain high affinity for albumin as shown in Table 1 (representative sensorgrams shown in Fig. S2). E06-2V had a measured affinity for HSA of 0.071 nM and MSA of 0.279 nM compared with 0.189 nM and 0.740 nM, respectively, for the E06 domain alone. This decrease in affinity is most likely due to a lower on-rate measured by BIAcore, whereas the off-rates measured for all E06-constructs to HSA were relatively constant. Binding parameters of these constructs to CSA were very similar to those measured for HSA, whereas of all serum species tested, the affinity to RSA was the lowest. Overall, the fusion of 2V to either or both ends of the anti-HSA VNAR still exhibited high affinity for human, cynomolgus, mouse and rat serum albumin, suggesting no over-riding change in conformation or steric hindrance was caused by the partner constructs.

**Binding to FcRn is retained by HSA in complex with VNAR.** Any increase in serum half-life is dependent on the ability of HSA-VNAR complex to retain binding to FcRn. To measure the affinity of this interaction and indeed determine whether the epitopes were distinct, HSA alone and pre-complexed with E06 and E06-2V fusion were injected over immobilized human FcRn. Although multiple variations of this assay can be done, pre-complexing the VNAR and HSA was the best representation of what would occur in vivo. HSA, as expected, exhibited binding to immobilized FcRn, whereas E06 alone did not. When the two were pre-complexed at equimolar concentrations, binding to FcRn was retained (Fig. 5C), providing evidence that E06 (when fused to 2V) binds a distinct or non-overlapping epitope on HSA, enabling it to still bind FcRn when all three proteins are in complex.

**Comparative pharmacokinetic profiles of single and anti-HSA fused VNAR domains.** A preliminary PK study in mice, performed with radio-labeled 2V and 2V-E06, indicated that addition of the albumin-binding E06 domain enhanced the PK characteristics of the NAR domain (Fig. 6A). The single 2V domain had a half-life of approximately nine minutes (0.15 h), compared with an estimated half-life of > 30 h for E06-2V. For subsequent studies, a highly sensitive LC-MS method was developed for the analysis of VNAR domains in biological fluids. This
method was applied to study the PK of the E06-2V and 2V-E06 constructs in the rat. The method included the analysis of peptides from both domains following capture with the C-terminal HIS-tag, demonstrating that the fused domains remained stably associated through the course of the experiment. Mean plasma concentrations after single intravenous dose of 1 mg/kg are shown in Figure 6B. Dosed proteins were detectable in all animals for the 168 h (7 d) duration of the study. Results of the non-compartmental PK analysis (Table 2) showed low clearances of 3.3 ml/h/kg for E06-2V and 2.7 ml/h/kg for 2V-E06. Volume of distribution was 105 ml/kg and 94 ml/kg for E06-2V and 2V-E06, respectively. The resultant half-lives of these molecules in the rat were 22 and 25 h, respectively.

Following development of the LC-MS assay with the relatively abundant samples from rats, further studies were performed in mice, using a slightly higher dose to ensure VNARs could be measured in the small volumes of plasma obtained at each time point. Mean plasma concentrations after a single intravenous (iv) or subcutaneous (sc) dose of 4 mg/kg 2V-E06 are shown in Figure 6C. Dosed protein was detectable for the duration of the study (168 h/7 d). This protein displayed good PK characteristics (Table 2), with a low clearance of 2.0 ml/h/kg, volume of distribution of 96 ml/kg and a half-life of 33 h.

Mean plasma concentrations after a single intravenous or subcutaneous dose of 2 mg/kg of the trimer 2V-E06-2V are shown in Figure 6D. Dosed protein was detectable for the duration of the study (168 h/7 d). This protein displayed good PK characteristics (Table 2), with a low clearance of 3.8 ml/h/kg, volume of distribution of 112 ml/kg and a half-life of 21 h. Subcutaneous bioavailability (F) after a dose of 2 mg/kg was approximately 46% relative to the intravenous dose of 2 mg/kg.

The LC-MS assay was also suitable for analyzing samples from the cynomolgus macaque study. Mean plasma concentrations of 2V-E06 and E06-2V after single intravenous or subcutaneous doses of 1 or 0.5 mg/kg are shown in Figure 6E. VNAR was detectable in all animals through the duration of the study (672 h iv and 336 h sc). Results of the non-compartmental PK analysis are provided in Table 2, and showed low clearances of 0.29 ml/h/kg for E06-2V and 0.25 ml/h/kg for 2V-E06. Volume of distribution was 69 ml/kg and 75 ml/kg for E06-2V and 2V-E06, respectively. The resultant half-lives of these molecules in the cynomolgus macaque were 164 and 210
We initiated our work by first compiling a comprehensive sequence database that acted as the foundation on which to design primers to build our phage display library, in addition to acting as an internal quality control to assess how good our library was with respect to sequence functionality. Cross reactivity of spiny IgNAR with a tool developed for nurse shark IgNAR (the monoclonal antibody GA8) facilitated the monitoring of samples from immunized animals during the program and enabled the choice of material for library build to be made based on the highest antigen titers measured. Similar temporal patterns of immune response have been measured in nurse sharks upon challenge with HEL, but this is the first report of an IgNAR response induced in this species of dogfish.43

It has been well established that IgNAR is amenable to phage display—immunized, naïve and semi-synthetic IgNAR phage display libraries have been reported in the literature.42,45-50 Here too, it proved an effective platform for the iterative enrichment and isolation of antigen specific binders. As serum albumin binds in a pH-dependent manner to FcRn, it was critical to incorporate this parameter in the post-selection characterization of lead clones in addition to cross-reactivity with MSA to fit with our initial murine PK model.29,51 As shown by the retrospective analysis of the unselected library, there was a good level of diversity of VNAR domains raised against HSA within this animal, which collapsed down rapidly after selection to essentially the high affinity E06 clone family differing in minor amino acid substitutions. Based on our binding data, the epitope recognized was present on human, monkey, mouse and rat albumin, but absent respectively. Subcutaneous bioavailability (F) after a dose of 0.5 mg/kg was at least 75% relative to the intravenous dose of 1 mg/kg. This dose normalized value is estimated on the basis of limited subcutaneous data. There was no indication of an anti-VNAR antibody response reducing exposure during the course of this study.

Discussion

In this study, we employed a novel shark immunization strategy to isolate a new class of half-life extension domains. IgNARs were first identified and characterized by Martin Flajnik and colleagues who functionally assigned these novel antigen receptors as a component of the adaptive immune system in nurse shark.11,42-44 We reasoned that, although these species are evolutionarily separated by approximately 200 million years, the presence of IgNAR in spiny dogfish could mean it arose from a common ancestor and may function in a similar way.45 Through immunization, we succeeded in isolating clones with picomolar affinity to target, which is the highest affinity reported in the absence of in vitro maturation, to our knowledge, for an IgNAR single domain. This illustrates the power of immunization for isolating high affinity binding domains and was the rationale for choosing this route. Another potential advantage of using sharks as a model species is that they are evolutionary distinct from mammals increasing the likelihood of raising a response to highly conserved mammalian proteins which may not elicit robust responses in other animals such as mice or rabbits.

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from bovine and rabbit albumin (data not shown). Preliminary genomic sequence information from this species of shark (unpublished results) reveals a high level of homology and very little sequence deviation in the lead clones from germline. In an extensive immunization campaign with HEL in nurse shark, an ancestral family clone isolated bound with nanomolar affinity to antigen only one order of magnitude less than the isolated affinity matured clones.\textsuperscript{44} It was surmised that although evidence of in vivo maturation exists, in the absence of a defined germinal center the extensive primary repertoire may compensate. Whether this is true in this model is debatable at this juncture and would demand a more detailed program of immunization and thorough characterization of clones to elucidate fully.

As the mechanism for extended half-life of albumin revolves round the binding to and re-cycling via the FcRn, it was critical to determine whether the binding of the VNAR clones to HSA was in any way detrimental to its affinity for FcRn. If the epitopes overlapped or if VNAR binding induced a conformational change within this large globular protein to obliterate FcRn binding, then the chances of any of these clones succeeding as tools for half-life extension would be undermined. Albumin consists of three homologous domains (I, II and III) and within each, two further subdomains.\textsuperscript{52,53} Of the three main domains, III is the region responsible for the interaction between it and FcRn.\textsuperscript{4,54,55} By pre-complexing our lead VNAR domains with HSA, we showed that the epitope recognized by the lead VNARs was either completely distinct from this region or at least did not sterically prevent the binding of HSA either directly or indirectly by inducing conformational change to FcRn. An in depth analysis of the interaction of E06 with HSA where target and binding domain have been co-crystallized clearly shows the epitope on HSA is within domain II, distinct from that of FcRn (unpublished data), which is in agreement with this study. Further evidence was borne out by the in vivo PK study where the spiny 2V binding domain was cleared very rapidly, presumably via glomerular filtration and excretion through the kidneys. Fusing this domain to the lead VNAR domain, E06, increased the plasma half-life of 2V approximately 220-fold from just less than 9 min to 33 h, which is in good agreement with the expected endogenous MSA value of approximately 35 h.\textsuperscript{29} This increase in half-life would not be explained purely by an increase in the size of the protein as the cut-off for glomerular filtration is approximately 45 kDa.\textsuperscript{56} The extension of serum half-life in the NHP model was that expected of albumin, and, taken in concert with the murine and rat data, can be extrapolated to an approximate half-life of 19 d in humans (unpublished data). It is also apparent that dosing across the range of 0.5 mg/kg to 4.0 mg/kg gives consistent PK characteristics based on albumin binding. Given the abundance of albumin, it is likely that a therapeutic protein delivered with an albumin-binding domain would not saturate this mechanism at therapeutically relevant doses. Other approaches involving albumin binding peptides have fallen short of equaling the natural half-life of albumin achieving a terminal $t_{1/2}$ of approximately 10.4 h and as a comparison of using a single binding domain strategy, albumin specific dAbs achieved a half-life of 24 h in a murine model.\textsuperscript{36,38}
The anti-HSA VNAR retains albumin binding and concomitant PK enhancement when flanked on either side with NAR domains of other specificity, therefore making this an attractive modality for designing single molecules with combined specificities.

As the origin of these domains is so divergent from mammals the question of potential immunogenicity undoubtedly arises. Although not the direct subject of this work, the NHP study that incorporated consecutive administration of 2V-E06 (iv and sc) showed no evidence of anti-VNAR antibody production. However, being of critical importance in the development of human therapeutics, the question of potential immunogenicity of VNAR domains does require further investigation.

Here, for the first time, we described the isolation of picomolar affinity shark binding domains from a dogfish species using an immunization strategy and showed in vivo efficacy across three species models of PK. The lead clone isolated exhibited the ability to improve the PK parameters of a fused tandem VNAR domain exhibiting serum half-life values equating to that of albumin itself. This study provides the potential of these single binding domains to achieve picomolar affinities for target in the absence of in vitro maturation and the capacity to act as tools to improve the PK properties of other biologic proteins.

**Materials and Methods**

Detailed protocols for immunization, library build, selections and screening can be found in Müller et al.61

### Table 1. Kinetic measurements for E06 and E06 fusion proteins

| Sample | Ligand | Mean \( k_a \pm SE \) \((x 10^6 \text{ M}^{-1} \text{s}^{-1})\) | Mean \( k_d \pm SE \) \((x 10^{-4} \text{ s}^{-1})\) | Mean \( K_D \pm SE \) \((\text{nM})\) |
|--------|--------|---------------------------------|---------------------------------|-----------------|
| E06    | HSA    | 3.092 ± 0.034                   | 5.825 ± 0.103                   | 0.189 ± 0.005   |
|        | CSA    | 2.675 ± 0.023                   | 5.834 ± 0.185                   | 0.219 ± 0.005   |
|        | MSA    | 2.316 ± 0.010                   | 17.130 ± 0.360                  | 0.740 ± 0.012   |
|        | RSA    | 2.240 ± 0.058                   | 30.110 ± 0.110                  | 1.345 ± 0.030   |
|        | HEL    | -                               | -                               | -               |
| E06-2V | HSA    | 7.464 ± 0.384                   | 5.307 ± 0.147                   | 0.071 ± 0.002   |
|        | CSA    | 8.996 ± 0.841                   | 5.915 ± 0.370                   | 0.066 ± 0.002   |
|        | MSA    | 5.299 ± 0.463                   | 14.695 ± 0.415                  | 0.279 ± 0.017   |
|        | RSA    | 5.553 ± 0.452                   | 27.510 ± 0.310                  | 0.498 ± 0.035   |
|        | HEL    | -                               | -                               | -               |
| 2V-E06 | HSA    | 0.953 ± 0.097                   | 6.412 ± 0.116                   | 0.721 ± 0.095   |
|        | CSA    | 0.820 ± 0.138                   | 6.821 ± 0.163                   | 0.910 ± 0.157   |
|        | MSA    | 0.862 ± 0.018                   | 21.420 ± 0.150                  | 2.486 ± 0.036   |
|        | RSA    | 0.623 ± 0.091                   | 34.418 ± 0.550                  | 5.949 ± 0.984   |
|        | HEL    | -                               | -                               | -               |
| 2V-E06-2V| HSA  | 0.689 ± 0.064                   | 5.762 ± 0.068                   | 0.896 ± 0.091   |
|         | CSA    | 0.592 ± 0.069                   | 5.904 ± 0.186                   | 1.052 ± 0.155   |
|         | MSA    | 0.507 ± 0.047                   | 19.783 ± 0.473                  | 4.032 ± 0.472   |
|         | RSA    | 0.448 ± 0.041                   | 31.353 ± 0.919                  | 7.245 ± 0.934   |
|         | HEL    | -                               | -                               | -               |
| 2V     | HSA    | -                               | -                               | -               |
|        | CSA    | -                               | -                               | -               |
|        | MSA    | -                               | -                               | -               |
|        | RSA    | -                               | -                               | -               |
|        | HEL    | -                               | -                               | -               |
| 5A7    | CSA    | N.A                            | N.A                            | N.A             |
|        | MSA    | -                               | -                               | -               |
|        | RSA    | N.A                            | N.A                            | N.A             |
|        | HEL    | 1.543 ± 0.025                  | 468.450 ± 11.350               | 30.365 ± 0.255  |

BIAcore analysis of purified E06 alone, as an N-terminal fusion, C-terminal fusion and dual fusion with 2V against human (HSA), cynomolagus (CSA), mouse (MSA) and rat (RSA) albumins. Hen egg lysozyme (HEL) is used as a negative control. 5A7 (anti-HEL VNAR) was used as a positive control for the HEL coated chip. Values are expressed as an average of the number (n) of independent repeat experiments.
administered intravenously via the caudal vein. Bleeds were taken between each boost using sodium citrate as an anticoagulant and separated into plasma and whole blood cell pellets stored in RNA stabilization buffer (Qiagen, 76104).

**Plasma titer measurements.** The measurements were essentially as described in Müller et al. Briefly, HSA or 2% milk was coated onto MaxiSorp plates (NUNC, 439454) at 10 μg/ml in carbonate coating buffer for 1 h at 37°C, followed by washing,
blocking and repeat washes. Dogfish plasma was serially diluted and incubated overnight (100 μl/well) at 4°C then washed before the addition of the anti-nurse shark monoclonal antibody GA8 (diluted 1:500 and added at 100 μl/well). Signal was generated by the addition of anti-mouse IgG antibody HRP conjugate (Sigma, A9917) diluted 1:1000 in PBS for 1 h at room temperature followed by washing and the addition of SureBlue TMB (Sigma) diluted 1:500 and added at 100 μl/well. The TMB blue color was stopped with 1 M H2SO4 and the absorbance measured at 450 nm.

Cloning of VNAR fusion proteins. E06-2V and 2V-E06 fusion dimers were cloned using a standard PCR overlapping extension assembly method joining E06 and 2V via the linker sequence (GGGGS)4GAHS; GAHS resulted from the BsuHII restriction site used. The trimer 2V-E06-2V was cloned via cutting the two different orientated dimer constructs by the use of the restriction sites Eco RV (cuts in E06) and EcoRI (cuts C-terminal of the dimers) and reassembling the fragments EcoRI-'backbone'-2V-E06'-E07 with Eco RV-E06-2V-backbone'-EcoRI.

Expression and purification of VNAR proteins. High-throughput prokaryotic expression, screening and purification of the VNARs were performed essentially as described in Müller et al. and Cummins et al. For in vitro protein characterization, 6-anti-HSA clones (p2-C06, p2-H08, p3-A08, p3-E06, p3-E07 and p3-F03) were cloned into a proprietary mammalian expression vector containing a C-terminal 6-HIS tag facilitating IMAC purification of expressed proteins post PEI-mediated transient expression in HEK 293 suspension culture or COS-7 monolayers. Expression levels of VNAR monomeric and fusion proteins were generally in the region of 1.5–3 mg per liter using serum free conditioned media. VNAR proteins were purified from conditioned media after an initial 0.2 μm filtration step using IMAC followed by cation exchange chromatography with buffer exchange as appropriate between steps. Proteins were then subjected to a final polishing step by passage over a Superdex 200 26/60 size-exclusion column equilibrated with PBS. Eluted peaks from SEC were concentrated using GE Healthcare’s BIAcore T200 and 2000 biosensors.

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BIAcore analyses. Kinetics measurements. BIAcore analysis of the VNAR monomers and fusion proteins were performed using GE Healthcare’s BIAcore T200 and 2000 biosensors.
The ligand was immobilized on a series S CM5 carboxymethyl dextran-coated chip via standard amine coupling using NHS/EDC chemistry. Human, rat and mouse serum albumin (Sigma, A5843: 5 g, A4538: 25 mg, A3139: 10 mg), cytomolgus serum albumin (isolated from plasma using SwellGel Blue Albumin Removal Kit; Pierce, 89845) and hen (chicken) egg lysozyme (HEL; Sigma, 6876) were diluted in 10 mM sodium acetate immobilization buffer (pH 4 or 4.5 for albumins, and pH 5 for HEL). Approximately 3000 response units (Fig. 4, BIAcore 2000) or 600–700 RU (Table 1 and Fig. S2, BIAcore T200) of the ligand, serum albumin, was immobilized using the BIAcore target ligand immobilization program (immobilization level for HEL was approximately 350 RU). One of the flow cells was blank immobilized and used for reference subtraction. Samples were 2-, 3- or 4-fold serial diluted from 10 nM in HEPES-buffered running buffer including 150 mM NaCl, 3 mM EDTA and 0.05% w/v Surfactant P20, pH 7.4 (HBS EP+, GE Healthcare, BR-1006-69) or 50 mM MES, 150 mM NaCl, pH 5.5, and were injected for 2 min at a flow rate of 30 μl/min, 60 μl/min or 80 μl/min and the dissociation phase monitored for 5 to 6 min, followed by a 30 sec or two 10 μl regeneration pulses using 10 mM glycine pH 2 or 1.5, respectively, at a flow rate of 10 μl/min or 100 μl/min. Association and dissociation rates of the reference- and blank-subtracted sensorgrams were calculated using the 1:1 global Langmuir binding model fit analysis (BIAcore Evaluation Software).

**Dual binding of HSA to FcRn and E06.** A BIAcore T100 biosensor instrument was used to assess if HSA binds both FcRn and E06 simultaneously. Biotinylated FcRn was affinity captured on the surface of a streptavidin (SA) chip (GE Healthcare, BR-1005-31) to a final surface density of approximately 600 RU. Protein samples were prepared and all experiments performed using a running buffer of 20 mM MES with 150 nM NaCl, 3 mM EDTA, 0.5% P20, pH 6.0. One micromolar HSA was injected or pre-complexed with a 1.5 molar excess (1.5 μM) of E06 or E06-2V for one hour and injected. 1.5 μM E06-2V was injected as a control. Samples were injected at 10 μl/min for 3 min, with a 3 min dissociation time, and the surface was regenerated between cycles by a 20 sec injection of 20 mM Tris, pH 8.1 at a flow rate of 30 μl/min. Sensorgrams were referenced by subtracting blank flow cell data from each sample injected, and a buffer blank was included in the main data set to illustrate buffer artifacts.

**Epitope mapping on HSA.** The six pan 2 and 3 derived clones were mapped using a BIAcore 2000 instrument. 3000 RU of the anti-HSA VNARs E06, E07 and H08 were immobilized directly via standard amine coupling onto a CM5 BIAcore chip (Fc2, Fc3 and Fc4, respectively). Fc1 was used as blank reference. 100 nM HSA was flown over all four flow cells for 3 min at 40 μl/min, followed by a 10 sec wash, and then 10 μg/ml E06, E07 or H08 was injected over the Fc2, Fc3 or Fc4 to allow the saturation of HSA binding site at 40 μl/min for 30 sec. Finally 10 μg/ml of E06, E07 or H08 was injected over all four flow cells for 3 min at 40 μl/min. This was repeated with the anti-HSA VNARs A08, C06 and F03 over all four flow cells. All experimental steps were performed at 25°C in HBS EP+ buffer. Blank and buffer effects were subtracted for each sensorgram using blank and buffer reference subtraction.

The 12 unselected and pan 1 derived clones were mapped using a BIAcore T200 instrument. Approximately 700 RU of E06 was immobilized on CM5 chip Fc1+2 using 10 mM sodium acetate immobilization buffer pH 5.5 via BIAcore’s target ligand immobilization program. Fc1 served as the reference cell. 100 nM HSA was injected and captured in Fc2 via E06 for 1 min at 30 μl/min. After a short wash period, all different VNAR clones and a goat polyclonal anti-HSA antibody (Sigma, A1151) or buffer were run in individual cycles over both flow-cells of the chip for 1 min at a flow rate of 30 μl/min, followed by a 1 min dissociation phase, before the chip was regenerated via a 30 sec regeneration pulse using 10 mM glycine pH 2 at 10 μl/min. All steps were performed at 25°C in HBS EP+ buffer. Reference-subtracted sensorgrams were analyzed.

**Pharmacokinetic models.** Mouse. For preliminary PK studies, the proteins were iodinated using IODO-BEADS method (Pierce, 28665) and were injected intravenously into the tail vein of male C57BL/6 mice, with 4–6 mice per timepoint. The dose was 1 and 0.3 mg/kg for 2V and E06-2V, respectively, and the dosing volume 4 ml/kg based on the most recent body weight prior to dosing. Blood sampling was performed at pre-dose and then over the 0.03–216 h period and processed for plasma by centrifugation. Radioactive equivalent concentrations in plasma were determined by gamma-counting, as previously described.66 Purified 2V-E06 and 2V-E06-2V were injected at a dose of 4 and 2 mg/kg, respectively, both iv and sc into groups of 12 CD1 mice. Two blood samples plus terminal bleeds were taken from each animal at intervals to provide duplicate samples to cover time points from 1–168 h. Rat; E06-2V and 2V-E06 were injected iv at 1 mg/kg into groups of 3 Wistar rats and blood samples taken from 0.25–168 h. NHP; A PK study in cynomolgus monkeys was performed with E06-2V and 2V-E06 dosed at 1 mg/kg iv to groups of two animals. Blood sampling was performed at from 0.25 h–28 d. After 28 d, the E06-2V group were injected sc with 2 mg/kg, respectively, both iv and sc into groups of 12 CD1 mice. Blood sampling was performed at pre-dose and then over the 0.03–216 h period and processed for plasma by centrifugation. Radioactive equivalent concentrations in plasma were determined by gamma-counting, as previously described. Reference-subtracted sensorgrams were analyzed.

**LC-MS analyses of PK samples.** VNAR concentrations in plasma were analyzed by quantitative LC-MS (unpublished data). Briefly, plasma samples were treated as follows: 50 μl plasma was added to 50 μl 6 M guanidine containing the peptide internal standard and reduced with 20 μl of 32 mM Tris (2-carboxy-ethyl) phosphine-hydrochloride (TCEP) at 56°C for 45 min. Samples were alkylated by addition of 10 μl of 128 mM iodoacetamide at 37°C for 60 min. Samples were diluted by the addition of 150 μl 100 mM phosphate, pH8, 0.1% CHAPS. Using a Kingfisher magnetic bead processor, magnetic Ni-beads (25 μl/sample) were washed in 100 mM phosphate, pH8, 0.1% CHAPS before being transferred to plasma sample plate and incubated for 1 h. Three washes were performed: first and second wash: transfer beads to plate containing 100 μl phosphate, pH8, 0.1% CHAPS; third wash: transfer beads to plate containing 100 μl phosphate, pH8, 0.1% CHAPS + 20 mM imidazole. Bound VNAR was then eluted by transferring the beads to a plate containing 100 μl phosphate, pH8, + 250 mM imidazole. Beads were removed and 100 μl 100 mM TRIS, pH 8 containing...
20 μg/ml trypsin was added and incubated for 4 h at 37°C. Following this, 20 μL 100 mM TRIS, pH 8 containing 100 μg/ml trypsin was added and incubated overnight at 37°C. Samples were then loaded into a CTC PAL auto-sampler and analyzed using LC-MS. Signature peptides were separated on an Agilent 1100 HPLC system using an Onyx monolithic RP C18 guard trapping cartridge and a Waters XBridge BEH130 C18 Column, 3.5 μM, 2.1 x 10 mm analytical column. Peptides were eluted with a gradient of 5% to 45% acetonitrile in water with 0.1% formic acid. Signature peptides within each partner were analyzed independently: E06 signature peptide—EQISISGR and 2V signature peptide—AQSLAISTR. The analytes were detected by atmospheric pressure electro spray ionisation MS/MS using an AB Sciex API5500 QTRAP triple quadrupole mass spectrometer. The ion chromatograms were quantified by reference to standards spiked into fresh control plasma and analyzed over the range 0.04 to 50 μg/ml. The ion chromatograms were integrated and quantified by interpolation of the standard curve with a 1/y weighting using AB Sciex Analyst 1.5.1 software.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental material may be downloaded here: www.landesbioscience.com/journals/mabs/article/22242
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