



DARPs against a functional IgE epitope

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ARTICLE INFO

Article history:

Received 30 May 2010

Received in revised form 13 July 2010

Accepted 22 July 2010

Available online 29 July 2010

Keywords:

DARPs
Designed ankyrin repeat proteins
Anti-IgE
Allergic diseases
Functional epitope
Omalizumab

ABSTRACT

The monoclonal anti-IgE antibody omalizumab (Xolair®) is mostly used for the treatment of severe allergic asthma. However, the requirement of high doses and suboptimal cost-effectiveness limits the use of the treatment. Here we propose to use a new drug format based on non-immunoglobulin structures, potentially offering increased clinical efficacy while being more cost-effective. For this purpose, DARPs™ (designed ankyrin repeat proteins) against the constant heavy chain region of IgE have been isolated. DARPs were binding to IgE with high specificity and affinities in the low nanomolar range. Selected DARPs antagonized the interaction between IgE and its high-affinity receptor in inhibition assays. Furthermore, anti-IgE DARPs were shown to inhibit proinflammatory mediator release from rat basophilic leukemia cells expressing human high-affinity IgE receptors with higher efficacy than the monoclonal anti-IgE antibody omalizumab. DARPs may thus represent promising future drug candidates for the treatment of allergy.

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1. Introduction

Since the identification of immunoglobulin E (IgE) as key mediator of immediate hypersensitivity in asthma and allergic disorders [1–4], controlling of the IgE response has become one of the main therapeutic objectives. Direct targeting of IgE interaction with its high-affinity receptor (FcεRI) [5–7] using monoclonal antibodies has been shown to be promising for the therapy of allergy [8,9]. Inhibition studies using ε-chain peptides [10,11] and the determination of the crystal structure of the IgE–FcεRI complex [12] mapped the two binding sites of FcεRI to the N-terminal region of the Cε3 domains of both IgE heavy chains. Consistently with this requirement, omalizumab as well as other non-anaphylactogenic anti-IgE antibodies have been shown to recognize epitopes overlapping with the binding site of FcεRI [13]. Indeed, binding of these epitopes is thought to be of key importance for any bivalent IgE inhibitor, as the binding of anti-IgE antibodies to other epitopes allows the cross-link of IgE molecules already bound by its receptors, leading to the activation and degranulation of the underlying effector cells [14].

Murine anti-IgE antibodies of such epitope specificity are capable to neutralize IgE *in vitro* [7,13,15]. One such antibody binding serum-free IgE is omalizumab (Xolair®, Novartis Pharmaceuticals, Germany; Genentech Inc., USA). It has been humanized [16] and was shown to efficiently decrease serum IgE levels *in vivo*

by 85–95%. The density of FcεRI on basophils was simultaneously declined [17]. Treatment led to lower exacerbations and reduced the need for systemic corticosteroid therapy in clinical trials [8,18–22]. However, as only 1000–1500 receptors need to be cross-linked to initiate an allergic reaction [17,23], high doses (ranging from 75 mg to 375 mg every two or four weeks, depending on the patient's body weight and total serum IgE levels) of omalizumab are required to achieve successful IgE neutralization, rendering this therapy useful and cost-effective only for a restricted group of patients with severe asthma [24]. Besides optimized cost-effectiveness, increased potency would not only allow the existing anti-IgE patients to be treated in a less-frequent and lower-dosing regimen, but also broaden the group of patients to those suffering from asthma but currently not matching the inclusion criteria for omalizumab treatment.

Numerous attempts have been made to create molecules with higher potency, either by modifying the immunoglobulin structure directly [25] or by developing alternative binding scaffolds based on non-immunoglobulin molecules [26]. DARPs (designed ankyrin repeat proteins) represent a promising approach of such novel binding scaffolds [26,27]. DARPs are designed proteins derived from natural ankyrin repeat proteins. They are composed of several repeat subunits each representing a binding domain. The assembled protein consists of two capping repeats intervened by two or more binding modules that are randomly modified on their surface. Combinatorial libraries with DARPs of varying repeat numbers have been constructed and have been used for selections against a wide range of targets [28–34], reviewed recently [35,36]. In the field of allergy, we have recently characterized bispecific DARPs that

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efficiently antagonized the IgE receptor [28]. The isolated DARPins exhibited high specificity, affinity and selectivity and were shown to bind their target via the designed randomized residues [37].

In this study we applied DARPIn libraries for the selection of binders to the constant region of IgE *in vitro* using ribosome display [38]. Specificity and affinity of the isolated DARPins were verified in enzyme-linked immunosorbent assays (ELISAs) and surface plasmon resonance (SPR) experiments, respectively. We produced bivalent and bispecific DARPins by cloning two DARPins joined by a glycine–serine linker. These constructs showed higher potency in inhibiting proinflammatory mediator release using *in vitro* functional assays compared to monoclonal anti-IgE antibodies. Further studies can now be performed to explore the therapeutic potential of DARPins in atopic diseases.

2. Materials and methods

2.1. IgE, anti-IgE and IgE receptor preparations

IgE-Sus11 is a monoclonal IgE from a hybridoma cell line [39,40]. The myelomas IgE-Zavazal and IgE-PS are kind gifts from Dr. V. Zavázal (Pilsen, Czech Republic) [41] and Drs. T. Ishizaka and K. Ishizaka (La Jolla, CA, USA), respectively. IgE-JW8 is a chimeric immunoglobulin, consisting of a human IgE constant and a mouse variable region, as previously described [42].

The following anti-human IgE antibodies were used: the murine monoclonal IgG BSW17 [7], the humanized monoclonal IgG omalizumab [16] (Xolair®, Novartis Pharmaceuticals, Germany; Genentech Inc., USA), and the murine monoclonal IgG Le27 [43].

Two different recombinant FcεR1α constructs were used: first, a fusion protein of human serum albumin flanked twice by the extracellular part of the alpha chain of the high-affinity IgE receptor (bivalent, bFcεR1α), a kind gift of Dr. H. Kocher (Novartis Pharma, Basel, Switzerland) [44], and second, a fusion protein of the extracellular part of FcεR1α to a human IgG₁ Fc (monovalent, mFcεR1α), a kind gift from Dr. M. Fux (Glenmark Pharmaceuticals S.A., La Chaux-de-Fonds, Switzerland).

2.2. DARPIn libraries

Libraries containing either two or three randomized internal repeats (designated as N2C or N3C, respectively) flanked by constant N- and C-terminal capping repeats were obtained from Molecular Partners AG (Zürich-Schlieren, Switzerland). The generation of these DARPIn libraries has been described elsewhere [27].

2.3. *In vitro* selection by ribosome display

The PCR-amplified N2C and N3C DARPIn libraries were transcribed and translated *in vitro* and subjected to four standard rounds of ribosome display selection as described earlier [26,28,38,45]. To avoid the selection of anti-idiotypic and anti-allotypic DARPins, subsequent selection rounds were performed with different IgE hybridomas and myelomas. After each round, the mRNA of the selected libraries was processed as described [28,33].

In the fifth ribosome display round, the libraries were subjected to off-rate selection, thereby increasing selection pressure. For this purpose, IgE-Sus11 was biotinylated using the EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce, MA, USA) according to the manufacturer's manual. 1 nM biotinylated IgE-Sus11 was incubated for 1 h with the translated libraries before the addition of a 500-fold molar excess of non-biotinylated IgE-Sus11 for a further hour. The libraries were then transferred to microtiter plates pre-coated with 0.5 μg neutravidin and incubated for 1 h at 4 °C in order to capture the

biotinylated binders complexed with IgE. After this last selection round, the libraries were processed as described above.

2.4. Expression and analysis of selected binders

From each library, single clone *Escherichia coli* crude extracts were generated as described previously by our group [28], and their IgE specificity was analyzed by ELISA (data not shown). Based on this assay, four DARPins were selected for further characterization.

2.5. Sequencing, protein production and purification of monovalent, bivalent and bispecific DARPins

DNA sequencing, expression in *E. coli* and immobilized metal affinity chromatography (IMAC) of the selected DARPins was performed as previously described [28]. For β-hexosaminidase release assay, the proteins were further purified by preparative size exclusion chromatography, on a HiLoad 26/60 Superdex-75 prep grade (Amersham Pharmacia Biotech, USA) column as described [27]. Cloning and production of bivalent and bispecific DARPins was carried out as previously published by our group [28].

2.6. Isotype specificity analysis of selected DARPins

To assess isotype specificity, the selected monovalent DARPins were tested on different immunoglobulin isotypes. IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, IgM (The Binding Site, product numbers BP078, BP080, BP082 and BP084, BP086 and BP091, respectively) and three different IgEs (IgE-Sus11, IgE-PS and IgE-Zavazal) were immobilized at 5 μg/ml in 100 μl PBS. Bound DARPins were detected with a biotinylated anti-polyhistidine antibody (R&D Systems, Minneapolis, USA) followed by a streptavidin–horseradish peroxidase (HRP) conjugate (DakoCytomation, Glostrup, Denmark).

2.7. Reactivity of DARPins to IgE in human serum

To investigate the binding efficacy of DARPins *in vivo* we tested whether they can recognize IgE in human serum. The bivalent anti-IgE DARPIn (E2.79/E2.79) and the controls (an unrelated non-specific bivalent DARPIn D2.7/D2.7, and the murine anti-human IgE specific antibody Le27) were coated at 5 μg/ml in 100 μl PBS. IgE-Sus11 was added at increasing concentrations diluted either in PBS/casein or in human serum. Bound IgE was detected using HRP-conjugated goat anti-human IgE (The Binding Site). TMB (3,3',5,5'-tetramethylbenzidine) was used as substrate to develop the ELISA. The reaction was stopped after 5 min with 1 M sulfuric acid and the absorbance was measured at OD 450 nm in a standard ELISA reader (BIO-TEK EL808, BioTek, Bad Friedrichshall, Germany).

2.8. Determination of kinetic parameters by SPR

SPR measurements were performed on a Biacore X instrument (Biacore AB, Uppsala, Sweden) using HBS-EP (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) as running buffer. IgE-Sus11 immobilized on one flow cell of a CM5 sensor surface chip to a final density of 2500 resonance units (RU) according to the manufacturer's protocol (amine coupling). The second flow cell was used as control for unspecific binding to the CM5 sensor surface. All measurements were done at a flow rate of 10 μl/min at room temperature. The DARPins E2.79, E3.54, E2.79/E2.79 and E2.79/E3.54 were injected for 3 min at concentrations ranging from 100 nM to 3.125 nM, including a HBS-EP buffer control. The off-rate was measured for additional 3 min. Regeneration of the sensor chip surface was performed by injection of 5 μl 25 mM NaOH. Kinetic data evaluation was performed using global fitting and the Lang-

muir 1:1 binding model of the BIAevaluation software (Biacore, Version 4.1).

2.9. Epitope mapping by SPR

Mapping of the epitopes of the DARPins on IgE was performed using the Biacore instrument with the same immobilization protocol, settings and buffers as described above. In an initial series of measurement cycles, the response of mFcεRIα (50 nM), omalizumab (10 nM) and Le27 (10 nM) to solid phase IgE was measured for 3 min and normalized to 100%. For each following measurement cycle, solid phase IgE was first saturated with multiple injections of different anti-IgE DARPins (E2_36, E2_71, E2_79 and E3_54). Each DARPin injection was used at a concentration of 100 nM and lasted for 3 min. Once saturation of the immobilized IgE was achieved, either mFcεRIα (50 nM), omalizumab (10 nM) or Le27 (10 nM) were co-injected with 100 nM anti-IgE DARPin for 3 min, and the additional response above the saturation level was measured and expressed as percentage of the response to unsaturated IgE.

2.10. Inhibition of IgE–FcεRIα interaction by monovalent, bivalent and bispecific DARPins

Using the Biacore X instrument with the same settings and buffers as described above, recombinant FcεRIα was immobilized on a CM5 sensor surface chip to a final density of about 2500 RU. IgE-Sus11 was injected at a concentration of 5 nM and the response after 2 min was measured and normalized to 100%. In subsequent measurement cycles, 5 nM IgE-Sus11 was pre-incubated for 5 min with equimolar, 2-fold, 5-fold and 10-fold molar excess of the anti-IgE DARPins (E2_79 and E3_54), in either monovalent, bivalent or bispecific format. The response of these pre-formed DARPin/IgE complexes on solid phase FcεRIα was measured after 2 min of injection and expressed as percentage of the response without DARPin.

2.11. Inhibition of proinflammatory mediator release from RBL-2H3-huα cells

Rat basophilic leukemia cells transfected with the alpha chain of the human high-affinity IgE receptor (FcεRIα), termed RBL-2H3-huα [46], were used to assess the release of the pre-formed mediator enzyme β-hexosaminidase. The experiment was performed according to previous described protocols [47–49] with minor modifications. Briefly, 1×10^5 cells were seeded in 96-well tissue-adherent cell culture plates in 100 μl antibiotic-free RPMI-1640 medium (Biochrom AG, Berlin, Germany) containing 10% fetal calf serum (FCS). IgE-Sus11 at a concentration of 0.54 nM was pre-incubated for 5 min with increasing concentrations (0.026–54 nM) of anti-IgE (DARPins in monovalent, bivalent and bispecific format; omalizumab; mFcεRIα) and then co-incubated overnight with the cells. On the next day, cells were washed once with HBSS (Hanks' balanced salt solution, Sigma, Buchs, Switzerland), once with HBSS containing 100 mg/l MgCl₂, and then triggered with 100 μl Le27 at a concentration of 5 μg/ml for 1 h at 37 °C. After incubation with the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (0.375 mg/ml; Sigma) for 1 h at 37 °C, the reaction was quenched using carbonate buffer pH 10. Secreted and stored β-hexosaminidase levels were measured as described, using a standard ELISA reader to determine OD₄₀₅. The percentage of the net mediator release was calculated as the ratio of secreted to stored β-hexosaminidase $\times$ 100. IC₅₀ values were calculated using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Selection of DARPins specific to IgE

Two DNA libraries coding for DARPins, with either two (N2C) or three (N3C) randomized ankyrin repeat modules, were used to select IgE isotype specific library members. Five ribosome display selection rounds followed by an off-rate selection round were carried out against different types of human IgE as described in Section 2. Based on the results of a screening ELISA after the off-rate selection round, four DARPins from the N2C library and one DARPin from the N3C library were chosen for further investigation and expressed in larger amount in *E. coli*.

3.2. Isotype specificity analysis of selected DARPins

To assess immunoglobulin isotype specificity of the selected DARPins, they were purified via their His-tag and were tested for reactivity against all isotypes (purified IgE, IgG, IgA and IgM) by ELISA (Fig. 1). The DARPins E2_36, E2_60, E2_79 and E3_54 recognized all three immobilized types of IgE (IgE-Sus11 hybridoma, IgE-PS myeloma and IgE-Zavazal myeloma), whereas no detectable activity against any of the other immunoglobulin isotypes (IgG₁, IgG₂, IgG₃ and IgG₄, IgA₁ and IgM) was observed. In contrast, DARPin E2_71 only interacted with one (IgE-Sus11) of the three immobilized IgE sources and with none of the other antibody isotypes, suggesting that it recognizes idiotypic or allotypic determinants of the IgE-Sus11 hybridoma. Previously selected maltose binding protein specific DARPin off7 [37] was used as control and did not react with any of the immunoglobulins.

3.3. Determination of kinetic parameters of anti-IgE DARPins

Association and dissociation rate constants of IgE and selected DARPins were measured by surface plasmon resonance (SPR). DARPins E2_79 and E3_54 were chosen as representatives from their corresponding sequence groups and were tested for binding to IgE-Sus11. Both monovalent DARPins showed similar association and dissociation rates, resulting in nanomolar equilibrium dissociation constants (Table 1). The affinities of the monovalent DARPins were

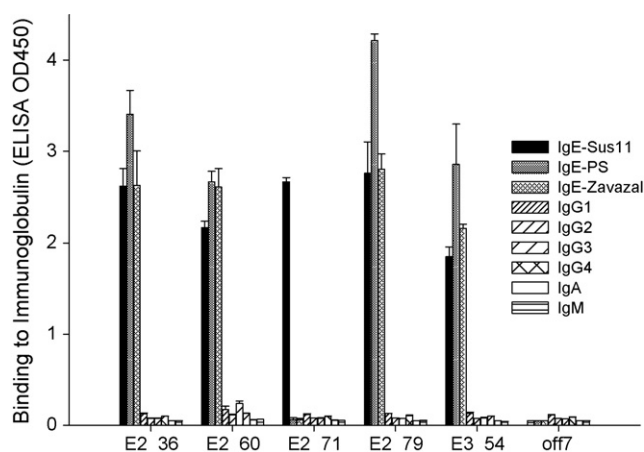


Fig. 1. High isotype specificity of selected anti-IgE DARPins. The interaction of DARPins with different immobilized human immunoglobulin classes and subclasses was compared by ELISA. Three different IgE idiotypes (IgE-Sus11 hybridoma, IgE-PS myeloma, and IgE-Zavazal myeloma), four human IgG myelomas of different subclasses (IgG₁, IgG₂, IgG₃ and IgG₄), as well as IgA₁ and IgM were coated on microtiter plates and were incubated with selected DARPins (E2_36, E2_60, E2_79, E2_71; and E3_54 off7 served as control). Bound DARPins were detected with biotinylated anti-polyhistidine antibody and horseradish peroxidase (HRP) conjugated streptavidin. The bars represent mean values from duplicates (indicated by error bars).

Table 1
Kinetic binding parameters of selected anti-IgE DARPs.

Anti-IgE binder (IMAC purified)	k_{on} [$10^5 \text{ M}^{-1} \text{ s}^{-1}$]	k_{off} [10^{-3} s^{-1}]	K_D [10^{-9} M]
E2_79	5.3	3.34	6.29
E3_54	4.44	3.03	6.83
E2_79/E2_79	8.49	0.75	0.88
E2_79/E3_54	2.05	0.46	2.27
Omalizumab	3.1	0.062	0.214

a hundred times lower than the affinities of the monoclonal anti-human IgE antibody omalizumab reported in the literature [50–52]. For better comparison to a bivalent antibody, bivalent and bispecific DARPs were produced as fusion proteins consisting of two monovalent DARPin genetically coupled by a 20-amino acid linker. The bivalent DARPin E2_79/E2_79 now showed a nearly 10-fold increase of its K_D , which was to be expected by an avidity increase of the bivalent construct (Table 1). Likewise, omalizumab shows a picomolar K_D .

3.4. Epitope mapping by SPR

Functional anti-IgE antibodies such as omalizumab have been previously shown to recognize identical or overlapping epitopes with those of FcεRIα [53–56] in the Cε3 domain of IgE, thereby being able to prevent the interaction of IgE and FcεRIα initiating mast cell and basophil triggering. Thus, the capacity of the selected DARPs to react with the functionally active Cε3 domain of IgE was tested in a competition experiment using SPR. The experiment was set up to measure the potential of the DARPs to interfere with binding of FcεRIα, omalizumab and Le27 to IgE (Fig. 2).

To measure the maximal response without competitor DARPin, the binding of FcεRIα, omalizumab and Le27 to IgE-Sus11 immobilized on the sensor surface was analyzed and normalized to 100%. Then, solid phase IgE was pre-saturated with different DARPs (E2_36, E2_79, E2_71 and E3_54), and binding of FcεRIα, omalizumab and Le27 to the saturated IgE was re-measured and expressed as percentage of the response without prior saturation (Fig. 2). The DARPs E2_79 and E3_54 reduced binding of FcεRIα to IgE to a value of below 20%. Binding of omalizumab to IgE was reduced to a value of less than 40% (using E3_54 for saturation) or less than 20% (using E2_79). E2_36 also reduced FcεRIα and omalizumab binding to IgE to a value of around 40%. E2_71 was neither able to interfere with omalizumab nor with FcεRIα, which is con-

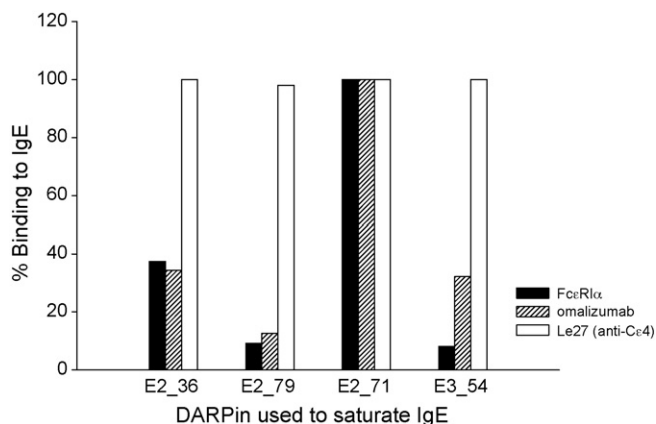


Fig. 2. Recognition of a functional IgE epitope. The capacity of the selected DARPs to interfere with the binding of soluble FcεRIα, omalizumab and Le27 to IgE-Sus11 was measured by SPR. Remaining binding of FcεRIα, omalizumab and Le27 after saturation of solid phase IgE-Sus11 using different DARPs is represented as percentage of binding without prior saturation.

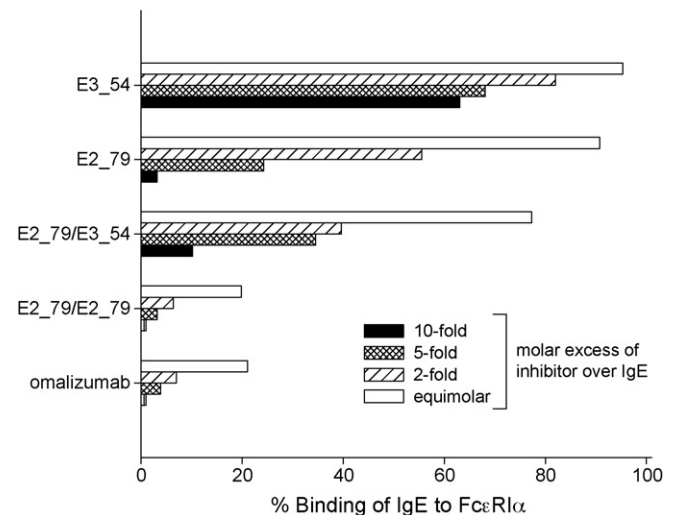


Fig. 3. Efficient inhibition of IgE binding to its receptor by monovalent and bivalent DARPs. The capacity of the DARPs E3_54 and E2_79 in monovalent, bivalent and bispecific form to inhibit the interaction of IgE with FcεRIα was assessed by SPR. IgE-Sus11 was pre-incubated with increasing molar excess of anti-IgE DARPs or omalizumab. The interaction of these pre-formed complexes with solid phase FcεRIα was then analyzed and expressed as percentage of the IgE binding without inhibitor.

sistent with our previous experiments and implies that this DARPin recognizes idiotypic or allotypic IgE determinants distant from the functional Cε3 epitope. None of the tested DARPs interfered with the binding of the Cε4-specific antibody Le27. These findings suggest that the DARPs E2_36, E2_79 and E3_54 are directed against a functional IgE epitope in or close to the Cε3 domain and may thus be used to interfere with the binding of IgE to its receptor.

3.5. Inhibition of IgE binding to its receptor by monovalent, bivalent and bispecific DARPs

Binding of IgE in the circulation and thereby preventing sensitization of mast cells and basophils has been shown to be the main mechanism of action of anti-IgE antibodies such as omalizumab [18,57,58]. Therefore, the capacity of the selected anti-IgE DARPs to block the IgE–FcεRIα interaction was assessed. Monovalent, bivalent and bispecific forms of E2_79 and E3_54 were used to carry out inhibition studies for the IgE–FcεRIα interaction (Fig. 3). The experiment was performed by pre-incubating IgE with increasing molar excess of either anti-IgE DARPs or omalizumab. The interaction of these pre-formed complexes with solid phase FcεRIα was then assessed by SPR. At 10-fold molar excess, E2_79 in monovalent and bivalent/bispecific form diminished the interaction of IgE with FcεRIα to below 10%. Compared to monovalent E2_79, however, bivalent E2_79 inhibited the binding of IgE to FcεRIα by around 80% already at 2-fold molar excess, and by over 90% at 5-fold molar excess, equally to omalizumab. The bispecific (E2_79/E3_54) form did not show significant enhancements compared to monovalent E2_79, suggesting that an avidity increase through bivalency may be able to enhance the inhibitory capacity.

3.6. Binding of DARPs to IgE in human serum

A potential use of DARPs *in vivo* would require that they neutralize soluble IgE in human serum. Therefore one selected anti-IgE DARPin produced as bivalent protein (E2_79/E2_79) was tested for binding to monoclonal human IgE (IgE-Sus11) diluted in human serum and as control in PBS/casein (Fig. 4). The DARPin recognized IgE to the same extent in both serum and in PBS/casein demonstrating that its binding capacity is not affected by the presence of

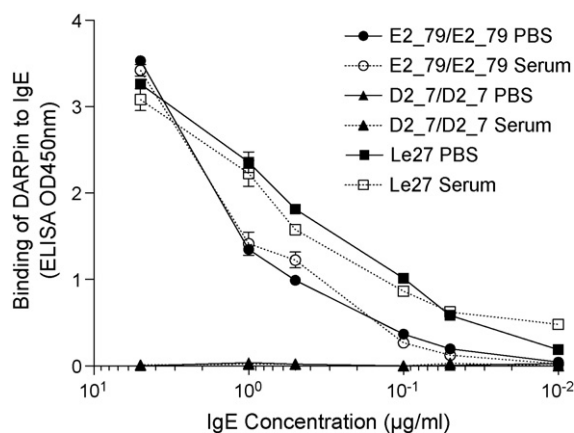


Fig. 4. Recognition of soluble IgE in human serum. The capacity of one selected DARPin to recognize IgE *in vivo* was tested with monoclonal IgE diluted in human serum by ELISA. The bivalent anti-IgE DARPin (E2.79/E2.79), an unrelated bivalent DARPin (D2.7/D2.7) and the monoclonal anti-human IgE antibody (Le27) were coated on microtiter plates and incubated with increasing concentrations of monoclonal IgE (IgE-Sus11). Bound IgE was detected with horseradish peroxidase (HRP) conjugated goat anti-human IgE. The symbols and bars represent mean values from duplicates.

other proteins in serum. The monoclonal anti-human IgE (Le27) showed a similar behavior as the anti-IgE DARPin, recognizing IgE equally well in serum as in PBS/casein. An unrelated bivalent DARPin (D2.7/D2.7) was used as control and did not react with IgE in any of the two settings.

3.7. Inhibition of proinflammatory mediator release from RBL-2H3-huα cells

Next we investigated the capacity of the DARPins to interfere functionally with the release of proinflammatory mediators. For this purpose, we tested whether they could inhibit mediator release from a rat basophilic cell line expressing human FcεRIα (Fig. 5 and Table 2). Such RBL-2H3-huα cells were co-cultured overnight with 0.54 nM IgE-Sus11 and increasing concentrations of DARPins, omalizumab or mFcεRIα, then triggered with an optimal concentration

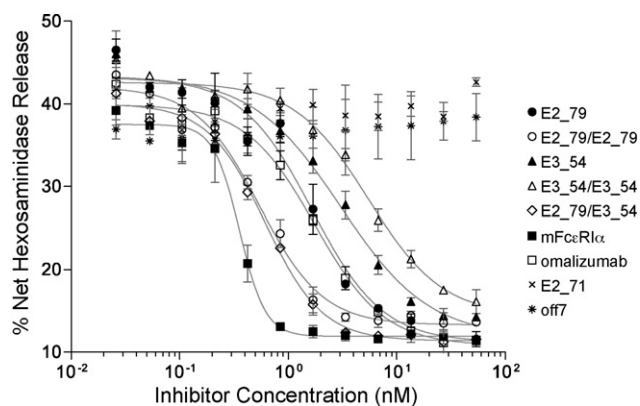


Fig. 5. Inhibition of mediator release from RBL-2H3-huα cells. The potential of the selected anti-IgE DARPins to inhibit proinflammatory mediator release from rat basophilic leukemia cells expressing human FcεRIα (RBL-2H3-huα) was compared by β-hexosaminidase release assay. The cells were co-cultured overnight with either only IgE-Sus11 (0.54 nM) as positive triggering control or with IgE-Sus11 and increasing concentrations (0.026–54 nM, corresponding to a 0.048-fold to 100-fold molar ratio) of monovalent, bivalent and bispecific DARPins E2.79 and E3.54, the monoclonal anti-IgE antibody omalizumab, the recombinant FcεRIα or the unrelated DARPin off7, respectively. Cells were triggered on the next day using a monoclonal anti-IgE antibody (Le27) and β-hexosaminidase release was measured. The symbols and bars represent mean values from duplicates. Grey lines represent curves fitted by GraphPad Prism.

Table 2

Inhibition of mediator release from RBL-2H3-huα cells.

Valency	Anti-IgE binder	IC ₅₀ [nM]
Monovalent	E2.79	1.66
	E3.54	3.04
	mFcεRIα	0.36
Bivalent	E2.79/E2.79	0.54
	E3.54/E3.54	5.47
	E2.79/E3.54	0.61
	Omalizumab	1.77

of the anaphylactogenic monoclonal anti-human IgE antibody Le27 on the next day. To measure maximal cellular degranulation, cells were incubated with IgE alone and then triggered with Le27, resulting in a net release in the range of 40–50% of the total amount of β-hexosaminidase.

Anti-IgE DARPins E2.79 and E3.54 in monovalent, bivalent and bispecific format as well as mFcεRIα and omalizumab were able to inhibit the release of β-hexosaminidase release in a dose-dependent manner. Consistent with our previous experiments, E2.71 did not show any functional activity, similar to the MBP-specific DARPin off7, which was used as negative control. Addition of the monovalent DARPin E2.79 at 50-fold molar excess induced a decrease of β-hexosaminidase release to the background level of 15% or below, which is comparable to the effect obtained with omalizumab at the same molar concentrations. Thus, E2.79 – being a monovalent molecule – was functionally equivalent to the intrinsically bivalent omalizumab. Albeit being of slightly lower affinity, E2.79 (half maximal inhibitory concentration IC₅₀ of approximately 1.66 nM, calculated from Fig. 5, see Table 2) was functionally superior to E3.54 (IC₅₀ of approximately 3.04 nM). Furthermore, bivalent E2.79 (IC₅₀ of about 540 pM) was able to completely inhibit mediator release already at about 5-fold molar excess over IgE, whereas omalizumab had to be added at 50-fold molar excess to achieve the same effect. Interestingly, also bispecific DARPin E2.79/E3.54 (IC₅₀ of about 610 pM) was able to inhibit mediator release to the same extent as bivalent E2.79. The calculated IC₅₀ for omalizumab is approximately 1.77 nM in this assay, being in the range of previously described values [51]. Taken together, these findings show that the selected anti-IgE DARPins might be superior inhibitors of proinflammatory mediator release in allergic reactions omalizumab, the sole biological currently used in asthma therapy.

4. Discussion

In the present work, we show the selection of DARPins, a novel class of scaffold proteins, against the constant region of IgE *in vitro* using ribosome display. The selected DARPins recognized IgE with exclusive specificity (Fig. 1) and did not cross-react with any other human serum proteins (Fig. 4). As IgE is the lowest abundant immunoglobulin class in humans [59], this lack of cross-reactivity with other isotypes is of particular therapeutic importance.

We found distinct DARPin sequence families of which one (represented by the N2C DARPins E2.36, E2.60 and E2.79) seems to be able to bind a functional epitope in the Cε3 domain similar as the alpha chain of the high-affinity receptor for IgE (FcεRIα) and the therapeutic anti-IgE antibody omalizumab. Interestingly, only the DARPins belonging to this sequence family inhibited binding of FcεRIα to IgE to the same extent than binding of omalizumab to IgE. The DARPin E3.54 on the other hand – belonging to another distinct sequence family – inhibits the interaction of both omalizumab and FcεRIα with IgE, but with less efficiency. Its inhibition of the FcεRIα–IgE interaction is much stronger than its inhibition of the omalizumab–IgE interaction. This effect cannot be attributed to an affinity difference of the different DARPins – the monovalent

DARPin all have affinities in a very similar range (see Table 1). We thus concluded that the DARPins of the two sequence families recognize different epitopes on IgE. Also, comparing the different inhibition profiles of the DARPins for FcεRIα and omalizumab, it can be speculated that the epitopes recognized by FcεRI and omalizumab are not identical, but the epitope recognized by the DARPin E3.54 is identical or closely overlapping with that of FcεRIα. Taking into account that FcεRI binds two epitopes on IgE, it might also be possible that the DARPins use an allosteric mechanism to inhibit the IgE–FcεRI interaction.

The affinities of the selected monovalent anti-IgE DARPins were in the nanomolar range (Table 1). Direct comparison to the affinity of the intrinsically bivalent anti-IgE antibody omalizumab however is difficult, as the measured affinity of omalizumab appears to depend greatly on the experimental settings: equilibrium dissociation constants (K_D) ranging from 60 pM [50,52] to 2.8 nM [51] have been reported in different studies. Our own measurements support a picomolar K_D (Table 1). Genetically linking and producing the DARPins E2.79 and E3.54 in bivalent and bispecific format resulted in binders that maintained specificity for IgE and revealed a further improvement in affinity, with the bivalent DARPin E2.79/E2.79 revealing a K_D in the subnanomolar range. This affinity improvement is likely to be attributed to enhance avidity of the bivalent DARPin, similar to an intrinsically bivalent antibody.

Our results showed that the DARPin E2.79 – even though it has a significantly lower affinity and is monovalent compared to omalizumab – inhibits proinflammatory mediator release to the same extent (Fig. 5 and Table 2). Interestingly, bivalent/bispecific constructs that contain at least one E2.79 DARPin moiety have an inhibition capacity increased by 2–3 orders of magnitude compared to omalizumab. Different reasons may account for this better functionality. One might be linked to the fact that the DARPin E2.79 binds the same or a closely overlapping epitope on IgE as FcεRIα, thus being effective in inhibiting the IgE–FcεRI interaction. Another reason might stem from structural aspects of the IgE molecule – in particular of the Cε3 domain. In contrast to other immunoglobulin domains, the Cε3 domain was found to be in a molten globule conformation, holding a high degree of structural flexibility and adapting a well folded conformation upon binding to FcεRI [60,61]. Previous studies on the FcεRI–IgE interaction have revealed conformational changes upon binding in the IgE molecule, preventing binding of additional FcεRIα molecules [60,61]. In fact, in our competition assays, the DARPins were able to interfere with the binding of IgE to FcεRI (Figs. 3 and 5). This underlines our assumption that binding of the DARPins – in particular of DARPin E2.79 – may stabilize the Cε3 domain in a conformation preventing its interaction with FcεRI. However, further experiments will be needed to investigate the exact interaction residues and a potential allosteric inhibition mechanism of the described anti-IgE DARPins.

In conclusion, we have shown that anti-IgE DARPins inhibit proinflammatory mediator release *in vitro* with higher efficiency than the anti-IgE monoclonal antibody currently used in the therapy of allergy. Based on our data, we would expect that anti-IgE DARPins have a clear advantage over omalizumab as a lower amount of protein would be required to induce the same functional effect resulting in a better and more cost-effective drug. This potential advantage has to be confirmed by extensive efficacy studies *in vivo*. In that context, further questions relevant for therapeutic application of DARPins will need to be assessed, such as potential immunogenicity of DARPins in the human body. However, the problems linked to immunogenicity should be limited, as the natural origin of the DARPins, their high stability and solubility are most likely important factors contributing to low immunogenicity. Furthermore, even in the case of the development of antibodies against anti-IgE DARPins they will not be anaphylactogenic as anti-IgE DARPins only recognize soluble IgE and do not cross-link cell

surface IgE. Taken together, the described DARPins have favorable characteristics in binding and inhibiting IgE compared to the current anti-IgE therapeutic monoclonal antibody. Thus, DARPins may be considered a valuable option for further evaluation in the field of anti-IgE therapy.

Declaration of sources of funding

The work was supported by Swiss Commission of Technology and Innovation (KTI Grant 8803.1 LSPP-LS).

Acknowledgements

We thank Elsbeth Keller-Gautschi, Yvonne Fuhrer and Vreni Ramseyer for excellent technical assistance throughout the entire term of this project.

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