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Comparison of affinity ranking and immunochemical key data as measure for molecular antibody evolution

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Abstract. The evolutionary optimization of antibody binding properties *in vitro* opens new perspectives for immunochemistry, since the affinity and selectivity of a given antibody molecule can be tailored to meet the requirements of the envisaged analytical application. An efficient strategy for molecular antibody evolution is described that combines randomized point mutations and sequential recombination of variable antibody gene repertoires employing a group-selective library. This strategy enabled significant improvements in the binding of the model analyte atrazine that were monitored by both, kinetic measurements by the optical sensor BIAcore 2000™ and immunochemical key data obtained by enzyme-linked immunosorbent assay (ELISA). The K_D of the template antibody IPR-7 was improved by a factor of 17 from 1.27×10^{-8} M to 7.46×10^{-10} M for the optimized variant IPR-83. The enhanced K_D is well in line with the 15 fold lowered IC_{50} of the atrazine ELISA, which was shifted from 13.6 μg/l for IPR-7 to 0.9 μg/l for IPR-83. Once the analytical properties of antibody fragments are optimized, antibody functionality can be tailored for specific technical demands, e.g. the directed immobilization on microchip surfaces. As an example, the variable region encoding genes of the scFv variants were subcloned into the F_{ab} fragment expression vector pASK99, in order to reconstitute the antigen binding site of native antibody molecules. The expressed F_{ab} fragments provide a C-terminal affinity tag for functionalized sensor surfaces. Again, the evaluation by ELISA as well as by BIAcore revealed a consistent ratio of analyte binding enhancement for the engineered F_{ab} fragments.

1. Introduction

Antibody engineering is a powerful tool for modifying antibody properties [1–3]. Antibody variable (V) genes encoding the antigen binding domains are modified *in vivo* during the secondary immune response in the microenvironment of lymphoid germinal centers by somatic hypermutation. Appropriate variants are subsequently selected from this pool of mutant immunoglobulins upon their improved affinity to the antigen [4]. A strategy for mimicking antibody maturation *in vitro* is based on the evolutionary concept of "variation and selection". Similar to the *in vivo* process, the antibody gene is diversified in an initial step by mutational procedures such as PCR based sequence randomization (c.f. Fig. 1). The mutated antibody gene repertoire is subsequently cloned into an expression vector and displayed at the protein level for instance on the surface of filamentous phage [5]. The surface presentation of the antibody phenotype enables the selection of improved variants, for example by their enhanced affinity to the antigen. Similar to the natural process, the *in vitro* evolution is an iterative process. Hence, improved antibody variants can be utilized as template structures for subsequent evolutionary cycles, until they meet the requirements of their designated application.

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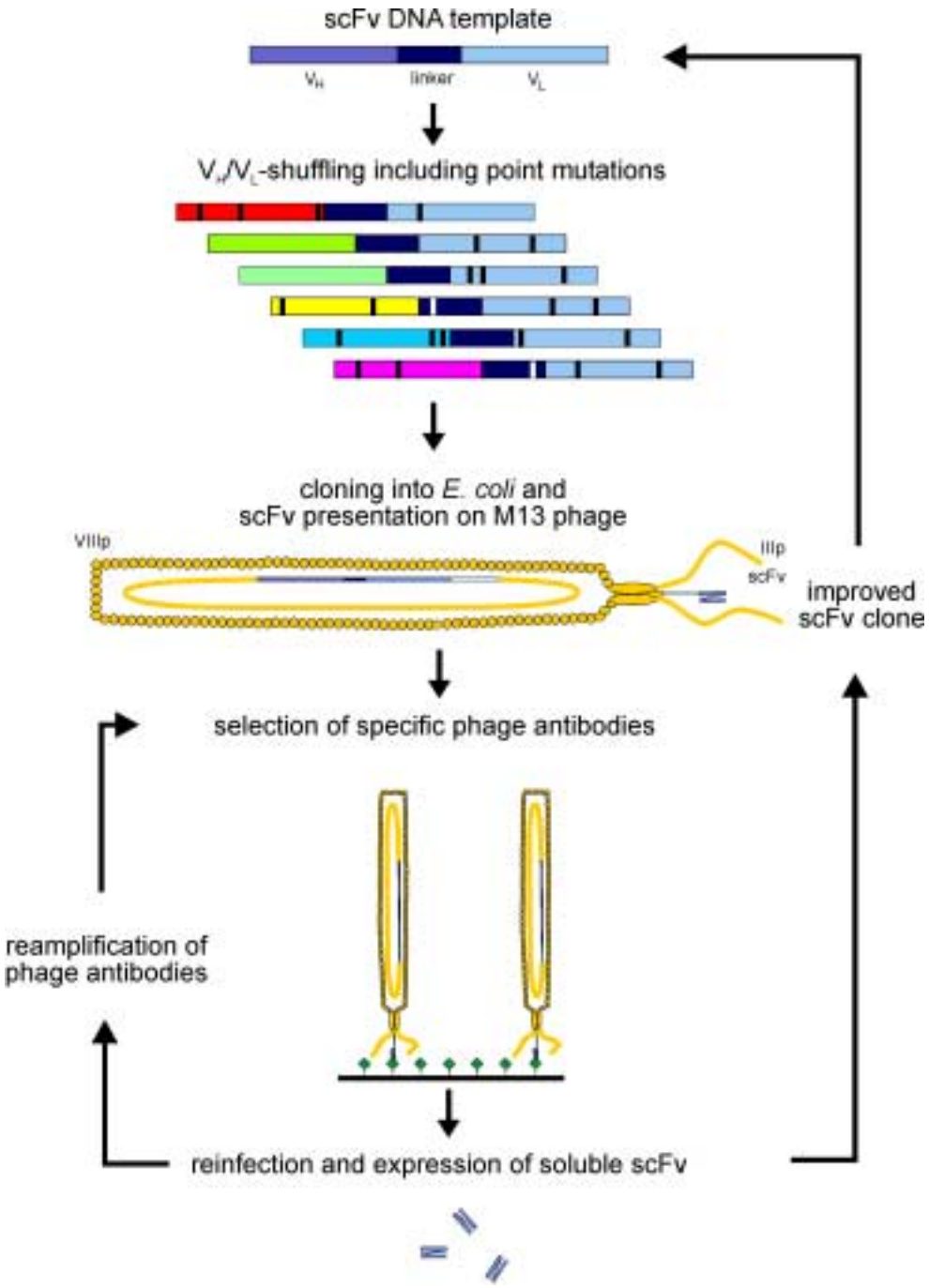

Fig. 1. Principle of molecular antibody evolution. The scFv antibody gene template is randomized by chain shuffling as indicated by V genes in different grey scales. Additional point mutations introduced by error prone PCR are depicted as vertical lines.

The univocal and solid identification of improved variants is one of the key steps in molecular antibody evolution. In this paper, we compared two standard methods for the evaluation of the antibody binding properties applied in the field. The characterization of antibody variants for improved binding was performed in parallel by competitive enzyme-linked immunosorbent assay (ELISA) and by the surface plasmon resonance based optical sensor BIAcore 2000^{TM}. The ELISA was designed to deliver primarily analytical information such as the IC_{50} and detection limit of a calibration curve for a defined analyte, whereas the biosensor measurements provide kinetic rankings of the antibody variants.

The variable region encoding V genes of the single-chain Fv (scFv; c.f. Fig. 2) IPR-7 were utilized as template for affinity and selectivity maturation. IPR-7 has been isolated from a group-selective library that contains an increased percentage of *s*-triazine herbicide selective antibody genes [6]. This antibody fragment is characterized by its preferential binding to sebutylazine and a cross-reactivity of 74% to atrazine (c.f. Figs 3, 4). Sebutylazine is a herbicide of minor environmental relevance, whereas atrazine

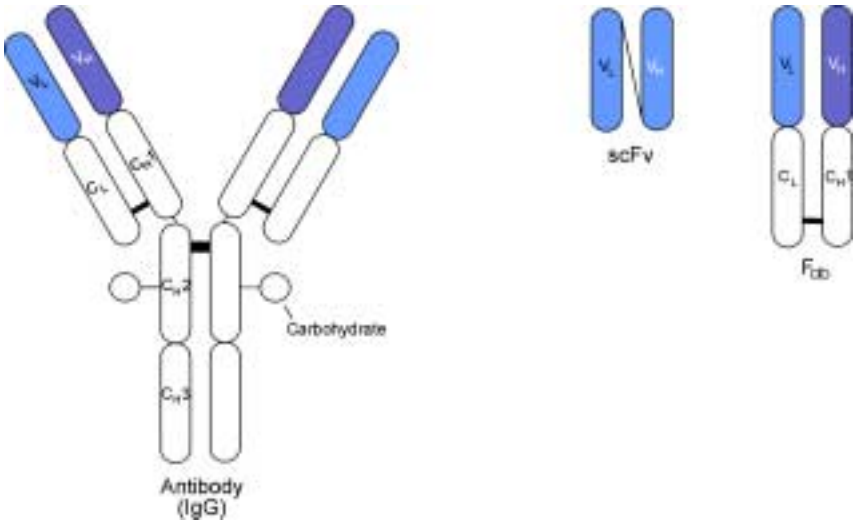

Fig. 2. Domain structure of a native antibody molecule (IgG), the corresponding recombinant single-chain Fv (scFv) and F_{ab} fragment. The antigen binding domains V_L and V_H are depicted in grey.

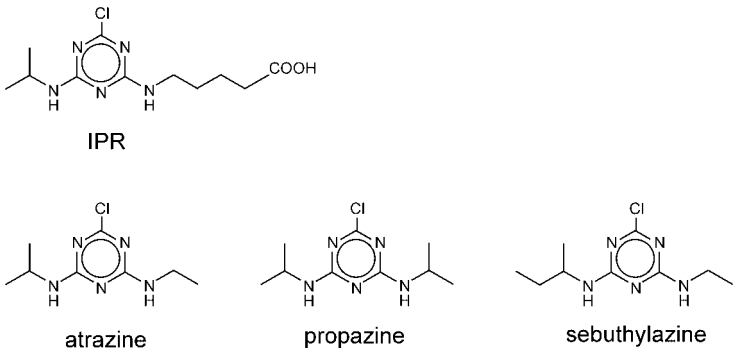

Fig. 3. Chemical structures of three *s*-triazine herbicides and the *s*-triazine derivative IPR (2-chloro-4-isopropylamino-6-aminocaproic acid-1,3,5-triazine) that was applied for phage selection, surface coating of the CM5 biosensor chip and horseradish peroxidase tracer conjugate in the competitive ELISA.

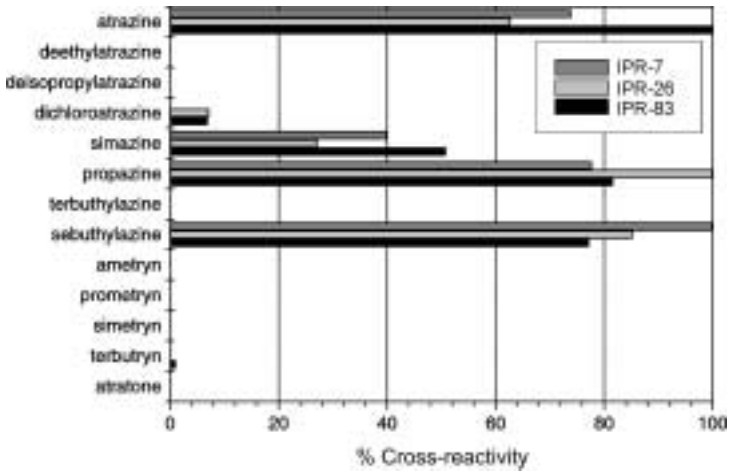

Fig. 4. Cross-reactivity of IPR-7 and $V_{H/L}$-shuffled clones against 13 different triazines. Cross-reactivity for each clone is based individually on the analyte that is bound with the highest affinity. Cross-reactivity was determined according to the formula: % cross-reactivity $= (A/C) \times 100\%$, where A is the concentration of the analyte at IC_{50} and C is the concentration of the cross-reacting triazine at IC_{50}. Thus, IPR-7 was based on sebuthylazine ($IC_{50} = 17.75$ μg/l), IPR-26 was based on propazine ($IC_{50} = 5.65$ μg/l) and IPR-83 was based on atrazine ($IC_{50} = 0.94$ μg/l). Dichloroatrazine: 2,4-dichloro-6-isopropylamino-1,3,5-triazine.

is one of the most widely used herbicides in industrial countries like the US with a worldwide production rate of 70 kilotons per year. Therefore, the goal of the molecular optimization was to modify the antibody binding site to achieve a preferential recognition of the priority s-triazine herbicide atrazine (c.f. Fig. 3). In addition, the affinity level of the resulting antibody fragment should be significantly elevated to enable the development of a sensitive assay for atrazine analysis. Finally, the optimized scFv variants should be transformed into the F_{ab} fragment format, in order to reconstitute the antigen binding site of native antibody molecules (c.f. Fig. 2).

2. Variation of template antibody IPR-7 and selection of improved clones

In order to randomize the antibody sequence of the template clone, the variable light chain (V_L) of IPR-7 was shuffled against the variable heavy chain (V_H) repertoire of a s-triazine group-selective antibody library [6] by PCR. Thus, the resulting scFvs comprised a broad panel of different V_H genes from the group-selective library combined with the IPR-7 V_L gene, both bearing additional point mutations that were generated by error prone PCR (c.f. Fig. 1). The mutated scFv gene repertoire was then cloned into the phagemid vector pCANTAB 5E [7]. Transformation of the *E. coli* strain TG1 with the recombinant phagemid yielded a library of 1.9×10^7 transformed colonies.

Hapten-specific clones of the V_H-shuffled library were enriched by three repetitive cycles of selection applying immunoaffinity chromatography [8]. Phage displaying selective scFv were separated from non-selective phage by a passage through columns, which were packed with the IPR derivative (c.f. Fig. 3) coupled to sepharose beads via an alkyl spacer. Unbound phage were removed by washing and the IPR-bound fraction was eluted thereafter by glycine-HCl.

3. Characterization of triazine-selective clones

Soluble antibody fragments were expressed from an aliquot of 190 individual clones that were derived from the final, third phage selection cycle, and evaluated by ELISA for improved recognition of IPR-triazines. Six clones that exhibited improved binding and displacement by s-triazines containing isopropyl residues (i.e. atrazine and propazine, c.f. Fig. 3) were chosen for a detailed characterization, since these clones express mutant scFv with the highest sensitivity towards the target analyte as identified by ELISA. However, the six clones turned out to encode a single identical amino acid sequence designated as IPR-26. Alignment with germline genes (ImMunoGeneTics database [9]) revealed that the variable regions of IPR-26 belong to the V_H1 and $V\kappa1$ gene family.

The hapten-antibody binding affinities of the initial clone IPR-7 and the shuffled derivative IPR-26 to the s-triazine derivative IPR were determined by employing the surface plasmon resonance based optical sensor BIAcore 2000™ system. The chip surface was prepared with 400 RU covalently linked IPR-OVA. Ninety microliter of serial scFv concentrations (ranging from 1 to 50 nmol) of the individual antibody clones were injected and the sensorgrams were evaluated applying the BIA Evaluation 2.1 software. The binding was generally characterized by a low background signal that is due to small non-specific binding to OVA as observed in the control path during measurement. The dissociation equilibrium constant K_D of the affinity-purified scFv fragments was calculated from the association rate constant k_a and the dissociation rate constant k_d (Table 1). The equilibrium dissociation constant of 9.20×10^{-9} M of the scFv variant IPR-26 is approaching the typical K_D level in the lower nanomolar range of affinity matured antibodies *in vivo* [10].

In parallel to the kinetic characterization, IPR-26 was compared with IPR-7 by direct, competitive ELISA. Calibration curves were recorded with affinity-purified, monovalent scFv against a selection of 13 relevant triazines and utilized for the calculation of cross-reactivities (c.f. Fig. 4). The mutant scFv IPR-26 revealed a dominant recognition of propazine, a triazine herbicide containing two isopropyl residues (c.f. Fig. 3). The IC_{50} of IPR-26 for propazine was determined at 5.6 μg/l with a detection

Table 1

Association rate constant k_a, dissociation rate constant k_d and equilibrium dissociation constant K_D for IPR obtained from the scFv clones IPR-7, IPR-26 and IPR-83. The values for k_a, k_d and K_D were measured utilizing the BIAcore 2000™ system. Experimental: The IPR-ovalbumin (IPR-OVA) conjugate was immobilized on the CM5 chip by activating the carboxymethylated dextrane surface of the sensor with 35 μl of a mixture of equal volumes of N-hydroxysuccinimide (0.1 M in distilled water) and N-(3-dimethylaminopropyl-)-N′-ethyl-carbodiimide-hydrochloride (0.1 M in bidistilled water). IPR-OVA was dissolved in coupling buffer (0.1 mg/ml in 10 mM sodium acetate, pH 5) and injected at a constant flow of 10 μl/min until a specific surface of 400 resonance units (RU) was prepared (1 RU = 1 ng/mm^2). Uncoupled carrier protein OVA was injected as a control in another flow channel of the same chip until the amount of immobilized OVA in the control channel was equivalent to the specific IPR-OVA surface. Residual NHS esters were deactivated by injecting 30 μl of 1 M ethanolamine, pH 8.5. For interaction analyses, different concentrations of each scFv clone (in 10 mM PBS, pH 7.4) were injected for 3 min at a constant flow of 30 μl/min and 10 mM PBS as the buffer stream. The buffer used for the kinetic measurements was composed of 10 mM PBS, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 and 0.005% Tween 20. The measurements were performed at 24°C. Sensorgrams were evaluated with Bia Evaluation 2.1 software (BIAcore Ab, Uppsala, Sweden).

Clone	k_a ($M^{-1}\,s^{-1}$)	k_d (s^{-1})	$K_D = k_d/k_a$ (M)
IPR-7	1.38×10^5	1.75×10^{-3}	1.27×10^{-8}
IPR-26	2.10×10^5	1.93×10^{-3}	9.20×10^{-9}
IPR-83	6.73×10^5	5.02×10^{-4}	7.46×10^{-10}

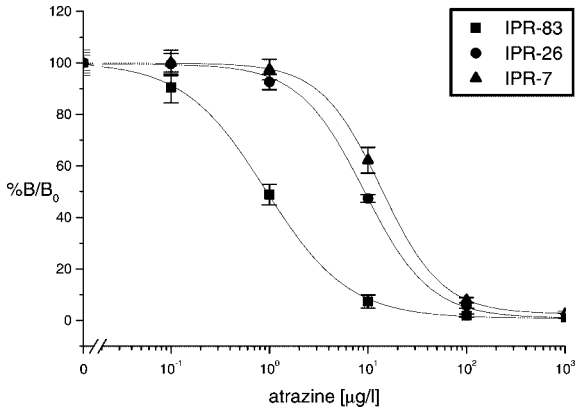

Fig. 5. Calibration curves of IPR-7 and $V_{H/L}$-shuffled clones for the analysis of atrazine by direct, heterogeneous, competitive ELISA. The original curves, which are based on relative luminescence units (RLU), were normalized according to $\%B/B_0 = (L - L_{\text{excess}})/(L_0 - L_{\text{excess}})$, where L is the luminescent signal, L_{excess} the luminescent signal at excess of atrazine and L_0 the luminescent signal at zero dose of atrazine. L_0 corresponds to the maximum achieved signal of the calibration curve and was determined as 362 RLU for IPR-7, 647 RLU for IPR-26 and 407 RLU for IPR-83. L_{excess} corresponds to the minimum achieved signal of the calibration curve and was determined as 61 RLU for IPR-7, 48 RLU for IPR-26 and 63 RLU for IPR-83. Experimental: Polystyrene microtiter plates (Greiner) were precoated overnight with 150 μl of anti-E-tag antibody (Pharmacia; 5 μg/ml in carbonate buffer: $NaHCO_3/Na_2CO_3$, 50 mM, pH 9.6) at 4°C. All further reactions were carried out at room temperature. Following three wash steps with PBS-Tween 20 (4 mM phosphate-buffered saline, pH 7.2, supplemented with 0.05% Tween 20), 150 μl affinity-purified scFv were filled into each well. Unbound scFv was removed by rinsing three times with PBS-Tween 20 after 2 h incubation. Hundred μl of triazine standard or PBS were added together with 50 μl enzyme tracer (IPR-horseradish peroxidase conjugate) and incubated for 1 h. After a final washing step, 150 μl of Super Signal ELISA Pico chemiluminescent substrate (Pierce) were pipetted into each well. The luminescence was measured after 5 min shaking incubation at 430 nm with a Tecan reader.

limit at 1.6 μg/l for this recombinant antibody fragment. Despite the preferential binding to propazine, IPR-26 exhibited a slightly improved assay sensitivity for the detection of atrazine as compared to the template antibody IPR-7 (c.f. Fig. 5). The IC_{50} for atrazine shifted from 13.6 μg/l for IPR-7 to 9.1 μg/l for IPR-26. The detection limit defined as the atrazine concentration at $B/B_0 = 80\%$ was thereby improved from 5.1 μg/l of IPR-7 to 3.2 μg/l of IPR-26. It should be noticed that the 1.4 fold improvement in K_D from IPR-7 to IPR-26 as determined by the kinetic measurements above is well in line with the 1.5 fold diminished IC_{50} and the 1.6 fold improved detection limit in the corresponding atrazine ELISAs.

4. Variation of antibody derivative IPR-26 and selection of further enhanced antibody clones

In order to further improve the affinity to s-triazines containing the isopropyl residue, the V_H encoding gene of the selected clone IPR-26 was shuffled in a second round of diversification against the V_L repertoire of the group-selective antibody library. Chain shuffling was performed essentially as outlined above. Again, V gene re-association was combined with point mutations that were introduced by error prone PCR. Thus, the resulting scFvs comprised a broad panel of different V_L genes from the group-selective library combined with the IPR-26 V_H gene bearing additional PCR generated point mutations. The mutated scFv gene repertoire was again cloned into the phagemid system yielding a library of 9.1×10^6 independent bacteria colonies.

5. Selection and characterization of improved, V_L-shuffled clones

Phage selection and identification of improved clones was performed as described above. A selection of seven clones that exhibited improved binding and displacement by s-triazines containing isopropyl residues in a primary screening by ELISA were chosen for sequencing. Two different antibody sequences designated as IPR-23 and IPR-83 could be distinguished for these clones. Alignment with germline genes [9] revealed that the variable regions of the IPR clones belonged to the V_H1 and $V\kappa1$ gene family.

Thus, all of the selected IPR clones were assigned to the identical germline V genes IGVH1S2 and IGKV1-110 according to the IMGT data base [9]. Comparison of these germline V genes with the IPR clones revealed an augmented number of mutations from 24 through 26 amino acid substitutions in the heavy chain and from 9 to 13 amino acid substitutions in the light chain with proceeding evolutionary mutation level. The majority of the germline mutations in the heavy chain are located in the complementarity-determining regions CDR1 and CDR2 as well as in the adjacent frame work regions. Further germline mutations accumulated in the FR1 and CDR1 of the light chain gene (data not shown).

Cross-reactivities of IPR-23 and IPR-83 were again calculated from calibration curves obtained by ELISA. Since both clones did not reveal significant differences in their binding behavior to the tested triazines, only the cross-reactivity values of IPR-83 are detailed in Fig. 4. In the competitive ELISA this clone is preferentially displaced by atrazine, the target triazine herbicide that contains a single isopropyl residue. Thus, the optimization goal for the antibody selectivity was fully achieved. The IC_{50} of IPR-83 for atrazine was determined at 0.9 μg/l with a detection limit at 0.2 μg/l for this scFv (c.f. Fig. 5). The co-selected variant IPR-23 with an IC_{50} of 1.0 μg/l and a detection limit at 0.2 μg/l showed no significant deviation from the values obtained with IPR-83 within the experimental error. Thereby, the detection limit of the mutant scFv IPR-83 and IPR-23 was improved 25-fold as compared to the initial template antibody IPR-7. Simultaneously, the cross-reactivity pattern to s-triazines indicates a shift towards atrazine as the dominant analyte, followed by propazine.

Kinetic characteristics of IPR-83 were determined by surface plasmon resonance (BIAcore 2000) according to the procedure described above. The kinetic data of this scFv variant are detailed in Table 1. The molecular evolution from IPR-26 to IPR-83 resulted in a 12.3 fold reduction of the K_D, which is mirrored by a 10.1 fold decrease of the IC_{50} atrazine concentration and a 16.0 fold decline of the detection limit in the corresponding ELISA (c.f. Fig. 5). The overall improvement in the K_D from the template antibody IPR-7 to the optimized variant IPR-83 by a factor of 17 is almost consistent with the 15 fold lowered atrazine concentration for the IC_{50}. The detection limit was even 25 fold diminished.

The slight alteration in K_D from IPR-7 to IPR-26 is mainly due to an increased association rate constant k_a. In contrast, the significant shift in K_D from IPR-26 to IPR-83 is caused by an increase in k_a and a concomitant decrease of k_d. This result might be surprising in the first instance, since improved antibody affinity is frequently caused by a reduced dissociation rate constant k_d. However, the alteration of the binding kinetics of the antibody variants described in this paper are most likely a consequence of the applied selection strategy. In order to isolate improved clones from the shuffled pool of antibody variants, the stringency was successively raised for both, faster association and slower dissociation rate of the antibody fragments to the IPR-derivative.

6. Molecular transformation from scFv into F_{ab} antibody fragment format

In order to obtain recombinant antibody fragments lacking the scFv peptide linker and thus reconstituting an antigen binding site of natural antibody molecules, the variable heavy (V_H) and light chain (V_L)

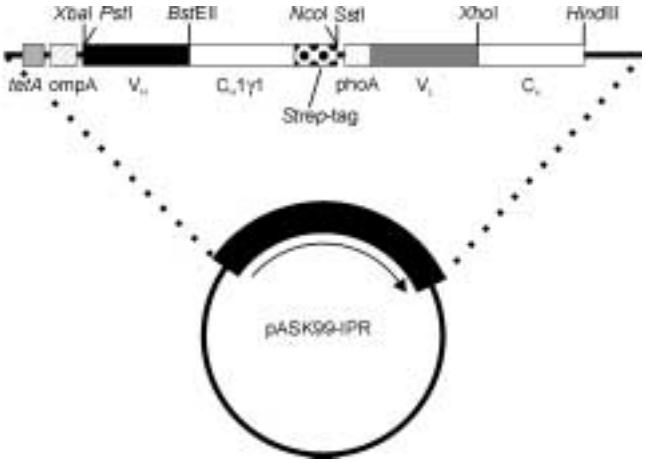

Fig. 6. pASK99-IPR expression plasmid. The plasmid was kindly provided by Prof. A. Skerra, Lehrstuhl für Biologische Chemie, Center of Life Sciences Weihenstephan, Technische Universität München, Germany. Schematic representation of the vector pASK99 after the transfer of genes for the variable light (V_L) and heavy chain (V_H) regions from scFv encoding pCANTAB 5E. The vector incorporates the genes for the constant heavy ($C_H1\gamma1$) and light chain ($C\kappa$) regions for construction and expression of F_{ab} fragments in the periplasm of *E. coli*. F_{ab} expression is under transcriptional control of the *tet*A promoter. *Omp*A and phoA indicate the ompA leader and the phoA leader, respectively. *Strep* tag represents the peptide encoding sequence for the streptavidin affinity tag [11]. The restriction enzymes *Pst*I, *Bst*EII, *Sst*I and *Xho*I were used for inserting the variable region genes and *Xba*I, *Nco*I, *Sst*I and *Hind*III for ligating the heavy and light chain fragments with pASK99.

domains of the three scFv variants IPR-7, IPR-26 and Ipr-83 were cloned into the F_{ab} expression vector pASK99 (Fig. 6). This vector contains the murine heavy ($C_H1\gamma1$) and light chain (C_κ) constant regions of mouse immunoglobulin G (IgG). The V_H and V_L genes were isolated from scFv harbouring pCANTAB 5E phagemids by PCR. The corresponding primers were designed to anneal within the flanking regions of a whole set of murine variable genes – thus providing a generic approach. Subcloning was facilitated by using three restriction enzyme recognition sites, which are frequently conserved in antibody framework regions. The fourth restriction site *Xho*I that was needed for insertion into pASK99, was introduced at the 3′-end of V_L by the corresponding PCR primer. The amplified V genes were digested and inserted into the plasmid vector pASK99 in order to obtain full-length F_{ab} genes. Functional F_{ab} fragments were expressed in transformed *E. coli* and purified via streptavidin-affinity chromatography [11].

7. Characterization of F_{ab} antibody fragments

Following *Strep*-tag affinity purification, the eluted fractions were analysed by SDS-PAGE under reducing and non reducing conditions (c.f. Fig. 7). The heavy and light chains of the reduced F_{ab} fragments appeared in equimolar amounts with a size of c. 20–25 kD. The non-reduced F_{ab} fragments revealed a size about 40 kD. F_{ab} containing fractions were pooled and characterized according to the parent scFv fragments.

The functionality of the generated F_{ab} variants were again examined in a direct, competitive ELISA (c.f. Fig. 8). The key values of the atrazine calibration curves are as follows: The IC_{50} of the F_{ab} variants were calculated at 60.5 μg/l for F_{ab} IPR-7, 15.2 μg/l for F_{ab} IPR-26 and 2.7 μg/l for F_{ab} IPR-83. The corresponding detection limits (IC_{80}) were determined at 14.9 μg/l, 5.7 μg/l and 0.13 μg/l for F_{ab} IPR-7, F_{ab} IPR-26 and F_{ab} IPR-83, respectively. The IC_{50} and the IC_{80} values reflected the evolutionary

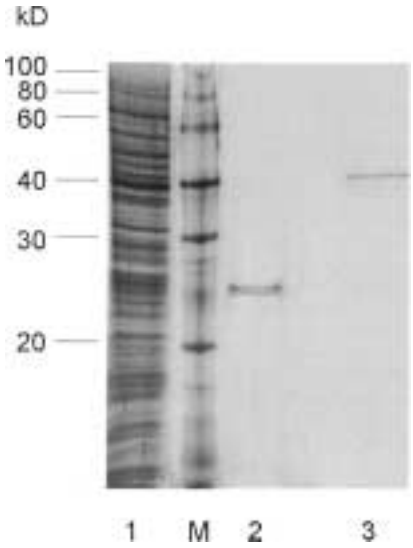

Fig. 7. SDS-PAGE of bacterial protein fractions. Lane 1: Periplasmic cell fraction. M: Molecular mass marker. Lane 2: F_{ab} fragment eluted during streptavidin-affinity chromatography, reduced, revealing its light and heavy chain. Lane 3: F_{ab} fragment eluted during streptavidin-affinity chromatography, with the disulfide bond between the two chains not reduced prior to gel electrophoresis.

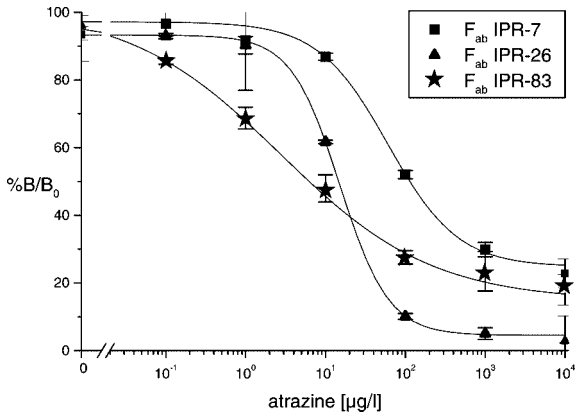

Fig. 8. Calibration curves of the s-triazine selective F_{ab} variants F_{ab} IPR-7, F_{ab} IPR-26 and F_{ab} IPR-83. The curves were determined in a direct, competitive ELISA basically according to the scFv calibration curves shown in Fig. 4. However, F_{ab} fragments were immobilized at the microtiter plate by goat-anti-mouse IgG instead of the anti-E-tag antibody.

modified binding characteristics of the F_{ab} mutants as also indicated by the IC_{50} and the IC_{80} values of the corresponding scFv fragments (c.f. Table 1).

The binding properties of the F_{ab} fragments to the s-triazine derivative IPR were further examined by employing the BIAcore 2000^{TM} system as already described above. The association rate constant k_a, dissociation rate constant k_d and equilibrium dissociation constant K_D of the three F_{ab} variants are detailed in Table 2. The equilibrium dissociation constants of the F_{ab} variants are roughly approaching the K_D level of the corresponding parent scFv fragments. The 1.8 fold improvement in K_D from IPR-7 to IPR-26 meets the order of magnitude of the 4 fold diminished IC_{50} and the 2.6 fold decreased detection limit in the atrazine ELISA. The subsequent molecular evolution from IPR-26 to IPR-83 resulted in a 13.7

Table 2
Association rate constant k_a, dissociation rate constant k_d and equilibrium dissociation constant K_D of the s-triazine selective F_{ab} fragments obtained from the clones IPR-7, IPR-26 and IPR-83. The values for k_a, k_d and K_D were measured by BIAcore 2000™ as described in the legend of Table 1

Clone	k_a ($M^{-1}\,s^{-1}$)	k_d (s^{-1})	$K_D = k_d/k_a$ (M)
IPR-7	9.72×10^4	2.65×10^{-3}	2.73×10^{-8}
IPR-26	1.85×10^5	2.83×10^{-3}	1.53×10^{-8}
IPR-83	5.49×10^5	6.17×10^{-4}	1.12×10^{-9}

fold reduction of the K_D, which is in the order of magnitude of the 5.6 fold decrease of the IC_{50} atrazine concentration and the 43.8 fold decline of the detection limit. In contrast, the overall improvement in the K_D from IPR-7 to IPR-83 by a factor of 24 is well compared to the 22 fold lowered atrazine concentration for the IC_{50}. The detection limit was even 115 fold diminished. The deviations revealed here are mainly due to the significantly altered steepness of the F_{ab} IPR-83 calibration curve for the detection of atrazine (c.f. Fig. 8).

The biosensor set-ups showed that the F_{ab} antibody fragments bind IPR-OVA with a high selectivity. The comparison of the kinetic data of the IPR clones in the scFv with the F_{ab} format did generally reveal a bias towards slightly improved K_D values for the scFv fragments. This is consistent with the ELISA data, where the variants were characterized by lower IC_{50} concentrations in the scFv format as compared to the F_{ab} fragments.

8. Conclusions

Molecular evolution of antibody fragments as well as the transformation from one molecular format to another paves the way for tailored binding molecules, that can be optimised for various analytical applications. The molecular evolution of antibody affinity can be monitored by various techniques. The degree of the individual levels of evolutionary affinity enhancement was evaluated by competitive ELISA as well as by the BIAcore 2000™ system with appropriate consistency – at least in a relative manner. Therefore, both evaluation methods seem to provide adequate measures for the evolutionary optimisation of antibody molecules.

Acknowledgements

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References

[1] E.T. Boder, K.S. Midelfort and K.D. Wittrup, Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity, *Proc. Natl. Acad. Sci. USA* **97** (2000), 10 701–10 705.
[2] P.S. Daugherty, G. Chen, B.L. Iverson and G. Georgiou, Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies, *Proc. Natl. Acad. Sci. USA* **97** (2000) 2029–2034.

[3] J. Hanes, C. Schaffitzel, A. Knappik and A. Plückthun, Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display, *Nature Biotechnol.* **18** (2000), 1287–1292.
[4] K. Rajewsky, Clonal selection and learning in the antibody system, *Nature* **381** (1996), 751–758.
[5] G.P. Smith and J.K. Scott, Libraries of peptides and proteins displayed on filamentous phage, *Methods Enzymol.* **217** (1993), 228–257.
[6] K. Kramer, Synthesis of a group-selective antibody library against haptens, *J. Immunol. Meth.* **266** (2002), 209–220.
[7] J.A. Malone, pCANTAB 5E: A vector designed for the expression of antibody F variable regions as phage displayed or soluble single-chain proteins (scFv), Direct submission, Pharmacia Biotech Inc., 1994.
[8] T. Kretschmar, C. Zimmermann and M. Geiser, Selection procedures for nonmatured phage antibodies: A quantitative comparison and optimization strategies, *Anal. Biochem.* **224** (1995), 413–419.
[9] M.-P. Lefranc, Unique database numbering for immunoglobulins, T cell receptors and Ig-like domains, *The Immunologist* **7** (1999), 132–136.
[10] G. Winter, A.D. Griffths, R.E. Hawkins and H.R. Hoogenboom, Making antibodies by phage display technology, *Annu. Rev. Immunol.* **12** (1994), 433–455.
[11] T.G.M. Schmidt and A. Skerra, One-step affinity purification of bacterially produced proteins by means of the "strep tag" and immobilized recombinant core streptavidin, *J. Chromatog. A* **676** (1994), 337–345.