DDT Vol. 8, No. 3 February 2003

Optimization of protein therapeutics by directed evolution

Alain P. Vasserot, Craig D. Dickinson, Ying Tang, William D. Huse, Keith S. Manchester and Jeffry D. Watkins

Directed evolution is a broadly applicable technology platform that is ideally suited to address the need for protein optimization and to fully exploit the therapeutic potential of biologics. The approach takes advantage of the remarkable structural and functional plasticity of proteins and permits the rapid remodeling of biologics into new entities with improved functions. The ability to ameliorate virtually any characteristic of a protein can translate into significant clinical benefits, including decreased immunogenicity, higher potency, greater efficacy and improved safety profile, and can considerably increase the probability of successfully developing and commercializing biotherapeutics.

Alain P. Vasserot*
Craig D. Dickinson
Ying Tang
William D. Huse
Keith S. Manchester
Jeffry D. Watkins
Applied Molecular Evolution (AME)
3520 Dunhill Street
San Diego, CA 92121, USA
*e-mail: avasserot@amevolution.com

▼ Modern day efforts to discover and develop small-molecule drugs inevitably entail a considerable amount of medicinal chemistry to optimize lead compounds that are identified in the screening process. Initial drug candidates are generally characterized using low stringency assays with the expectation that their potency can be increased after completion of the discovery process by following well-established SAR principles. Furthermore, it is also assumed that other crucial characteristics of the compounds, such as efficacy, toxicity and pharmacokinetic parameters, can be improved dramatically by introducing additional modifications. By contrast, biologics frequently enter clinical development with little or no optimization of their function, potency or safety profile. In addition, some biologics have pleiotropic effects that are precisely controlled in a normal cellular or corporeal context but that become liabilities when they are administered as therapeutics, raising concerns about treatment safety [1]. Although proteins have already been subjected to a natural evolutionary process, their use as human therapeutics poses a unique set of challenges [2] that can be addressed readily by further optimization efforts. Directed evolution is a powerful approach that can be used to optimize multiple characteristics of protein therapeutics. It is a three-step *in vitro* protein engineering process in which diversity is introduced into a gene of interest, followed by the expression of a collection of protein variants that are subsequently screened in a functional assay to identify molecules with improved characteristics. Directed evolution is significantly faster and more efficient than traditional protein engineering strategies and has been used to create new biological entities with optimized therapeutic properties.

Directed evolution strategies

Protein engineering often relies on structural approaches [3], empirical computer modeling [4] or mathematical predictions [5] to modify the sequence of a particular protein (Table 1). However, our limited understanding of how structure impacts function has, thus far, often confined the use of knowledge-based approaches to alterations that produce antagonists [6–8], a limitation that diminishes the usefulness of such strategies for optimizing therapeutic proteins. By contrast, directed evolution requires no previous knowledge about the structure of the target protein or its cognate because diversity is generally introduced in a stochastic manner followed by selection steps or screening procedures to direct the molecule's evolution towards functional improvements. The techniques used to identify improvements bear careful consideration as they often serve different purposes. Methods that display protein variants on phages [9], cells [10] or ribosomes [11] are generally based upon selection of the tightest binders from a large pool of variants followed by their subsequent identification through linkage of the

 1359-6446/02/$ – see front matter ©2002 Elsevier Science Ltd. All rights reserved. PII: S1359-6446(02)02590-4

Table 1. Engineered protein therapeutics

Engineered molecule	Product (organization)	Property engineered	Benefit	Ref
Anti-$\alpha_v\beta_3$ integrin antibody	Vitaxin™ (Applied Molecular Evolution/MedImmune)	Directed evolution used to humanize antibody, optimize affinity, and improve stability	Enhanced activity and stability	[15]
Anti-CD33 antibody	Mylotarg® (Wyeth)	Humanization by CDR grafting	Decreased immunigenicity	[30]
Anti-HER2 antibody	Herceptin® (Genentech)	Humanization by CDR grafting	Decreased immunigenicity	[32]
Anti-interleukin 2 receptor antibody	Zenapax® (Protein Design Laboratories/Roche)	Humanization by CDR grafting	Decreased immunigenicity	[31]
Anti-interleukin 8 Fab	(Genentech)	Free cysteine engineered for site specific PEGylation of Fab	Increased serum half-life	[47]
Anti-respiratory syncytial virus antibody	Synagis® (MedImmune)	Humanization by CDR grafting	Decreased immunigenicity	[33]
Anti-respiratory syncytial virus antibody	Numax™ (Applied Molecular Evolution/MedImmune)	Directed evolution used to improve association rate	Improved viral neutralzation	http://www.medimmune.com
Anti-TGFβ2 antibody	CAT-152 (CAT)	Human antibody discovered and optimized by phage display	Decreased immunigenicity	[48]
Epidermal growth factor	(Massachusetts Institute of Technology)	Single residue change reducing receptor affinity	Increased recycling after receptor binding and cell internalization; enhanced mitogenic potency	[49]
Erythropoietin	Aranesp™ (Amgen)	Structure aided design used to add glycosylation sites	Increased serum half-life resulting in increased efficacy	[50]
Granulocyte colony stimulating factor	(Massachusetts Institute of Technology)	Structure aided design used to add histidines to increase endocytic recycling	Increased half life in cell culture resulting in enhanced mitogenicity	[51]
Human growth hormone	(Genentech)	Phage display used to improve affinity for the receptor	No improvement reported	[52]
Heregulin	(Genentech)	Phage display used to improve affinity for the receptor	No improvement reported	[53]
Interferon α and β	Infergen® (Amgen)	Sequence of IFNs were aligned and consensus molecule created to enhance affinity and alter receptor specificity	Potential for reduced toxicity	[54]
Interferon α and β	(Maxygen)	Gene shuffling of interferon genes to enhance antiviral activity	Improved anti-viral activity shown on mouse cells	[55]
Interferon α	Albuferon™-Alpha (Human Genome Sciences)	Fusion of serum albumin with interferon α	Increased efficacy due to increased serum half-life	[56]
Interleukin 1 receptor	IL-1 Trap (Regeneron)	Fusion of IgG Fc region to soluble interleukin 1 receptor creating a receptor dimer	Increased affinity for interleukin 1resulting in more potent antagonist	http://www.regeneron.com
Interleukin 2	(Massachusetts Institute of Technology)	Combinatorial mutagenesis and screening for improved cell proliferation	Improved efficacy through enhanced recycling after receptor binding and internalization	[57]
Tissue factor	(Genentech)	Phage display used to increase affinity for Factor VIIa	Antagonist of Factor VIIa	[58]
Tumor necrosis factor α receptor	Enbrel® (Amgen/Wyeth)	Fusion of IgG Fc region to solubleTNF-α receptor creating a receptor dimer	Increased affinity for TNF-α resulting in more potent antagonist	[59]

Amgen (http://www.amgen.com); Applied Molecular Evolution (http://www.amevolution.com); Cambridge Antibody Technology (CAT; http://www.cambridgeantibody.com); Genentech (http://www.gene.com); Human Genome Sciences (http://www.hgsi.com); Maxygen (http://www.maxygen.com); MedImmune (http://www.medimmune.com); Protein Design Laboratories (http://www.pdl.com); Regeneron (http://www.regeneron.com); Roche (http://www.roche.com); Wyeth (http://www.wyeth.com).

variants to their nucleotide sequences. Consequently, surface display and selection strategies are frequently used in discovery efforts where, for instance, high affinity antibody variants are isolated from large combinatorial libraries [12]. However, the use of display technologies is limited to selections predicated on binding properties. Consequently, complex biological functions borne by therapeutic proteins, such as cell proliferation or inhibition, metabolic responses, signal cascades or enzymatic activities, can not be optimized by display strategies.

To address these limitations, we have developed a directed evolution platform [13–15] combined with highly predictive screening methods, that is ideally suited to meet the unique challenges posed by the optimization of biologics. By virtue of their ability to monitor the biological function of each individual variant in a library, screening methods are amenable to more sophisticated and more predictive assays that do not rely solely upon binding properties. Furthermore, this approach permits the use of multiple biological systems to generate protein variants, including mammalian expression systems necessary to evolve complex proteins whose post-translational modifications are required for biological activity. With an iterative potential for continual improvements until the desired characteristics are obtained, directed evolution driven by functional screening broadly mimics the process of natural evolution [15,16] and has enabled us to efficiently and rapidly evolve several biologics into new biological entities with optimized therapeutic properties.

Benefits of codon-based mutagenesis and focused libraries

Directed evolution technologies also differ in their approaches to generating protein diversity. Methods such as error-prone PCR [17], gene-shuffling [18], and *in vivo* mutagenesis [19] are geared toward broadly distributing mutations throughout a protein, but suffer from the inability to precisely control the location and number of mutations. Although the introduction of numerous amino acid mutations into a molecule at random locations might be acceptable for industrial applications, the accumulation of unnecessary changes is undesirable for pharmaceutical proteins, where immunogenicity is a primary concern. Moreover, by introducing changes at the nucleotide level, random mutagenesis can only explore a limited number of amino acid substitutions at a given position, which considerably diminishes the probability of identifying the appropriate combination(s) of changes required to optimize a specific biological function.

Most mutations have a neutral or detrimental effect on protein function [16], therefore, a selective and targeted mutagenesis process is crucial to the successful engineering of human therapeutics, where the objective is to significantly improve specific protein properties by changing the minimum number of amino acids. We have pioneered a mutagenesis procedure that introduces diversity into nucleic acids encoding the protein of interest through the targeted insertion of synthetic oligonucleotide pools generating changes at the codon level. This codon-based strategy enables full control over the location and extent of changes and permits the evaluation of the entire repertoire of natural amino acids at every position [13,20]. The procedure also facilitates the integration of structural data, when appropriate, while permitting the scanning of an entire protein in situations where no information is available. Mutagenesis oligonucleotides usually encoding all 20 amino acids at any given position are generated in a parallel synthesis process that creates multiple libraries of increasing diversity. Importantly, the procedure is designed to segregate the libraries into distinct pools enabling the number of amino acid changes to be predetermined and based upon the complexity of the engineering task and the biological assay used to monitor improvements.

Among the benefits of exerting precise control over library diversity is the ability to tailor the size of the libraries to ensure that the most predictive, functional assays can be used, irrespective of their throughput. For instance, while large combinatorial libraries with high diversity are screened in high throughput assays, more focused libraries with fewer randomized positions can be synthesized to take advantage of lower throughput, yet more predictive cell-based assays. Because of their small size and diversity, highly focused libraries can be screened extremely rapidly and the impact that each individual amino acid has on the function of the particular protein can be determined efficiently [15,21]. Beneficial mutations that emerge from this initial screening step can be combined in a new library and the iterative nature of the process permits the stepwise evolution of the target protein until a variant with the desired characteristics is created. Changes found at one position in a protein can often be combined with improvements found at other positions [22], thus significant ameliorations can be achieved extremely efficiently by combining beneficial changes identified in multiple, small, focused libraries [15] rather than exhaustively screening a single, large, fully randomized library. This unique directed evolution platform has been applied successfully to all major classes of biologics and the following examples detail some of the improvements achieved and their impact on the drug development process.

Engineering antibodies with fully human frameworks

Murine and chimeric antibodies used as human therapeutics frequently engender an immune response in patients

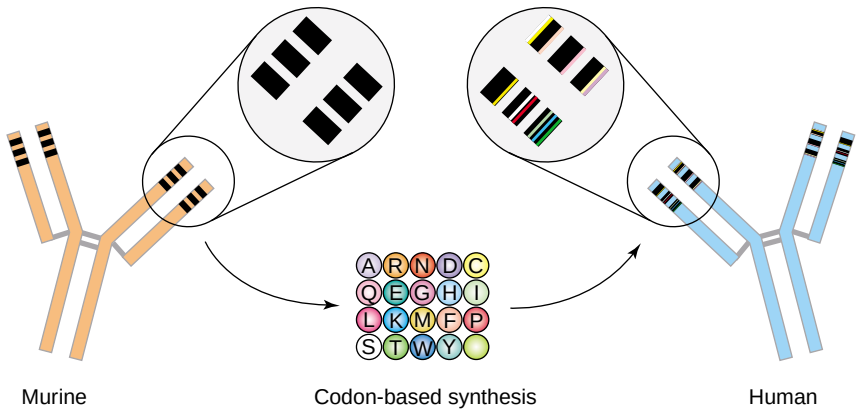

Figure 1. The frAMEworks™ humanization process. Libraries of CDR variants derived from the original murine or chimeric antibodies are inserted into a fully human, germline framework. Codon-based mutagenesis is used to create the variant libraries and to explore all possible amino acid changes at each position of the CDRs. A functional screen is then used to identify humanized variants that maintain or improve antigen binding. Subsequently, additional amino acid substitutions are introduced into the CDRs of the antibody and the process is reiterated until the desired improvements are reached. This approach generates antibodies with fully human frameworks and significantly enhanced affinity and/or activity.

that results in rapid elimination of the drug and, thus, severely limits their development as therapeutics [23]. In chronic disease indications where repeat administration is necessary, the problems posed by the development of neutralizing antibodies can be remedied either by humanization of the therapeutic antibody [24] or by *de novo* discovery of human antibodies using transgenic mice [25] or phage display libraries [26,27]. However, because of the distinct immune repertoires of mice and humans, these discovery platforms do not necessarily generate antibodies with the same epitope specificities.

Traditional antibody humanization [24,28] involves the direct transfer, or grafting, of murine complementarity-determining regions (CDRs) into a human framework, a process that usually results in a loss of activity. Structure-based computer modeling and algorithms are then used to predict which human residues in the new framework need to be reverted to the original mouse amino acids in an attempt to recover the affinity lost during the grafting process [29]. To date, four humanized antibodies [30–33] have received market approval (Table 1), while several others are in clinical development (Protein Design Laboratories: http://www.pdl.com).

By contrast, the frAMEworks™ (Applied Molecular Evolution; http://www.amevolution.com) approach (Fig. 1) combines fully human frameworks with synthetic CDRs and yields higher affinity antibodies that are devoid of any potentially immunogenic murine residues in the framework regions. Fully human, functional frameworks for both antibody chains are selected from among commonly occurring germline genes to maximize the occurrence of tolerance in the relevant patient populations. Libraries of synthetic CDR variants that are closely related to those of the parent molecule are then inserted into the selected frameworks and a functional assay is employed to identify amino acid changes that accommodate the new framework and simultaneously improve affinity or any other crucial property of the antibody. A concomitant increase in potency by simultaneous optimization has the potential to further minimize immunogenicity issues by enabling less frequent and/or lower dosing of the therapeutic protein. This approach has been successfully applied to numerous antibodies (Marquis, D. *et al.*, unpublished data) with a wide range of affinities. Whereas binding affinity lost during other humanization strategies, such as CDR grafting, might prove impossible to recover in the case of high affinity immunoglobulins, the frAMEworks™ approach has generated several antibodies with femtomolar affinities.

Increasing antibody potency by engineering binding affinity

Vitaxin™ (MedImmune; http://www.medimmune.com) is a humanized antibody that recognizes a conformational epitope on the $\alpha_v\beta_3$ integrin and functionally blocks ligand access to the cell surface receptor [15]. Interfering with $\alpha_v\beta_3$ integrin signaling prevents new blood vessel formation in a chick embryo chorioallantoic membrane model of angiogenesis [34] and could be of use in blocking osteoclast-mediated bone destruction, two properties that support the evaluation of this antibody in multiple, ongoing Phase I/II clinical trials in oncology and rheumatoid arthritis indications (MedImmune: http://www.medimmune.com). The original murine antibody was humanized and its affinity improved by approximately two orders of magnitude (Table 1). Mutations were introduced into each CDR of the antibody and multiple beneficial changes were identified, with H3 and L3 being the most productive regions. Among these mutations, several single amino acid substitutions resulted in a ≥10-fold improvement in affinity. Single mutations that significantly improved the binding properties of the antibody were combined in additional libraries to identify combinations of mutations that

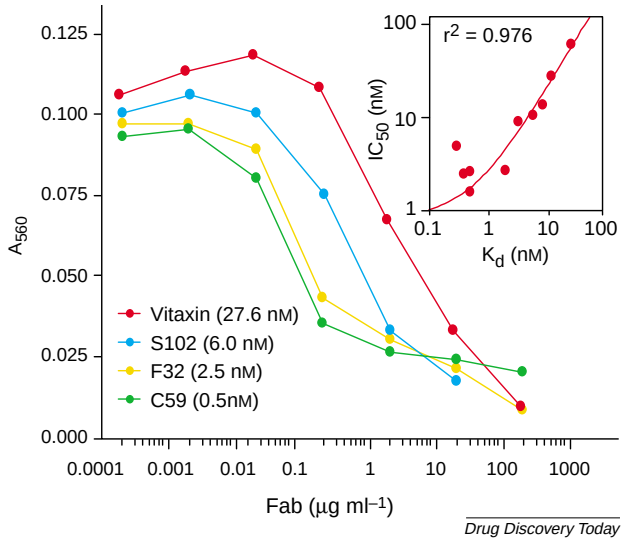

Figure 2. Inhibition of ligand binding. Fibrogen binding to $\alpha_v\beta_3$ integrin was measured in the presence of increasing concentrations of three engineered Vitaxin™ variants displaying gradual increases in binding affinity. The inset shows the correlation between affinity (K_d) and inhibition of ligand binding (IC_{50}). Higher affinity variants identified in the initial engineering process display the same extent of functional inhibition at significantly lower concentrations. Reproduced, with permission, from Ref. [15].

further enhance binding. Several antibody molecules from these combinatorial libraries were extensively characterized and revealed improvements in affinity ranging from one to two orders of magnitude. Moreover, in a cell adhesion assay, the improved variants were able to inhibit ligand binding at significantly lower concentrations than the parent antibody [15] and affinity improvements correlated almost linearly with an increase in potency (Fig. 2).

In several instances, particularly where antigen binding is driven predominantly by monovalent interactions rather than by avidity, an increase in potency can be achieved by creating antibodies with higher binding affinity. Screening is generally performed under prolonged incubation conditions that favor the discovery of variants with slower dissociation rates resulting in tighter, protracted binding to a particular target. This, in turn, translates into more efficacious ligand blocking and the ability to achieve comparable therapeutic results at lower doses. Importantly, the identification of several more potent anti-$\alpha_v\beta_3$ integrin antibodies was successfully completed in a two-step process after screening less than 2600 variants and in the absence of any structural information on the antibody or the conformational epitope that it recognizes. However, even if this information had been available, simultaneously randomizing the five beneficial positions found in one of the highest affinity variants would have necessitated the construction of a library of over three million members, an arduous and time-consuming alternative that clearly demonstrates the superiority of a method that combines small, highly focused libraries with functional screening. It is also noteworthy that, in the course of the first clinical trials, no evidence of immune response to Vitaxin™ was observed in the treated patient population [35].

Increasing potency by selectively engineering association rate

Increases in binding affinity often translate linearly into higher potency antibodies, but in certain cases, the correlation is more tenuous. In efforts to create a more potent version of Synagis® (MedImmune; http://www.medimmune.com), a humanized monoclonal antibody (Table 1) directed against the respiratory syncytial virus (RSV) and currently marketed to prevent respiratory infection in neonates [33], binding affinity was improved by more than 2000-fold, predominantly through decreases in the dissociation rate constant of the antibody. However, when a panel of improved affinity immunoglobulin variants was further tested in a microneutralization assay measuring the ability of the antibody to prevent RSV infection of target cells [36], no significant correlation was observed between binding affinity and viral neutralization. We reasoned that, not unlike the situation encountered in the inhibition of barnase activity by barstar [37], faster docking of the antibody to the virus particle might confer superior anti-viral properties to Synagis®, and new assays were developed to specifically monitor improvements in the association rate of the antibody with the F protein of RSV. Single mutations proved to have a rather small impact on the association rate constant and, consequently, designing assays that are capable of reliably measuring marginal improvements was crucial to the success of this project. By combining specific beneficial amino acid changes in the CDRs of the antibody, several variants with significantly improved association rates were created that exhibited a 10 to 35-fold increase in potency in the microneutralization assay. The exact mechanism(s) by which the association rate of an antibody ultimately influences its efficacy as a therapeutic are not well understood. Assuming that RSV neutralization is a function of the availability and degree of occupancy of the docking protein on the viral particle, the improvement achieved in the association rate constant of Synagis® would proportionately decrease the concentration of antibody required to block infection and enable the therapeutic agent to compete more effectively with the cellular receptor used by the virus for productive infection.

Interestingly, mutations that affected the association rate constant were distinct from those that predominantly

impacted the dissociation rate of the antibody. This emphasizes the power of an engineering approach that has the capability to identify and selectively modify predetermined amino acid positions that have dramatically different impacts on protein performance or function. The new variants, termed Numax™ (MedImmune; http://www.medimmune.com), are being evaluated in animal models to determine which molecule will enter clinical development (MedImmune: http://www.medimmune.com).

Simultaneous class switching, humanization and optimization of potency

Antibody discovery approaches often favor the identification of high affinity immunoglobulins against dominant epitopes, perhaps limiting the diversity of antigen recognition and binding specificities. In some cases, despite repeat discovery efforts, the only antibodies exhibiting the desired biological properties are IgM molecules [38,39] whose pentameric structure and inherently low affinity not only make it difficult to achieve the desired level of potency or efficacy, but also create significant manufacturing challenges. *In vitro* affinity maturation by directed evolution provides an alternative to rediscovery and enables research efforts to focus on therapeutically relevant epitopes, irrespective of the class or the affinity of the original immunoglobulin. For instance, in a discovery effort aimed at identifying antibodies against tumor-specific antigens, *in vitro* immunization of human lymphocytes was used to generate several antibodies, all of which were eventually determined to be IgM molecules. Because these IgMs appeared to have the required tumor-specificity, simultaneous class switch to IgG1 and *in vitro* affinity maturation was chosen to obviate the need for rediscovery and to transform these molecules into high affinity IgG antibodies (Fig. 3) appropriate for clinical development [38]. Directed evolution was also applied to murine IgM antibodies that recognize cryptic epitopes on human collagen generated during the proteolytic degradation of the extracellular matrix that accompanies the angiogenesis process [39]. These epitopes are not as accessible on native collagen, and antibodies against these neoantigens displayed properties and unique specificities warranting further development as potential anti-cancer agents. During the engineering process, the CDRs of two mouse antibodies were optimized to support fully human frameworks and to substantially improve antigen binding, thereby facilitating class switching to IgG molecules. In this case, the inherently low affinities of the original mouse IgM antibodies were increased by three orders of magnitude while preserving the required epitope specificities.

Examples abound in the scientific literature where multiple antibodies can be discovered against a specific target,

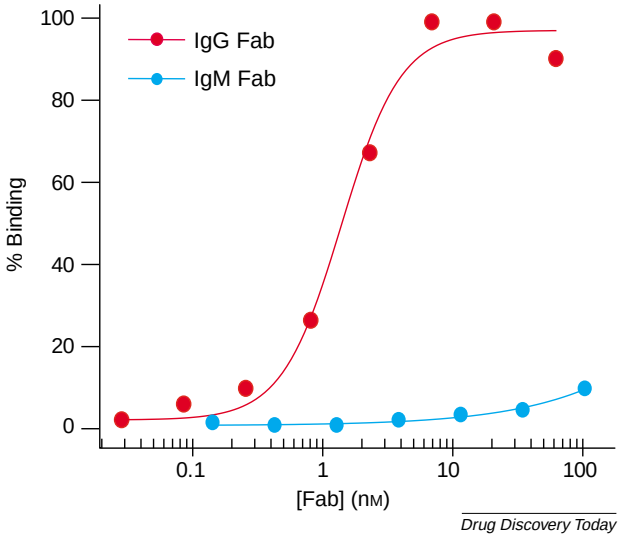

Figure 3. Optimization of a low affinity IgM antibody reactive with a tumor-associated antigen. The heavy and light chain variable regions of a human IgM reactive with cultured tumor cells were cloned into a bacterial expression vector to allow class switching to an IgG molecule and affinity maturation by directed evolution. To characterize the optimized variants, soluble Fab fragments were titrated against the LH13 tumor-associated antigen in an ELISA format. The intrinsically low affinity (K_d > 100 nM) of the non-engineered monovalent IgM fragment is demonstrated by the weak signal obtained at high concentrations (blue). By contrast, an affinity-matured variant containing two amino acid changes in the light chain CDR3 and one change in the heavy chain CDR3 displays a considerably higher affinity for the antigen (red). Reproduced, with permission, from Ref. [38].

but only a few of them display unique specificities for a particular epitope that justify further clinical development. Often, the early humoral response to a particular antigen results in the production of interesting IgM antibodies that exhibit a multifarious antigen recognition profile subsequently narrowed during the later stages of the maturation process. In most cases, the impracticalities associated with the development and commercialization of IgM antibodies prompt rediscovery efforts, an onerous and time-consuming process that can be readily avoided by optimization.

Engineering broader reactivity of antibodies

Antibodies have emerged as useful therapeutics in the treatment of multi-drug resistant infectious agents. However, the degree of neutralization observed toward clinical isolates of a pathogen can vary considerably. For instance, a murine monoclonal antibody developed to prevent nosocomial infections in premature neonates displayed sub-optimal reactivity toward several bacterial isolates, a limitation that might have required the development of an antibody

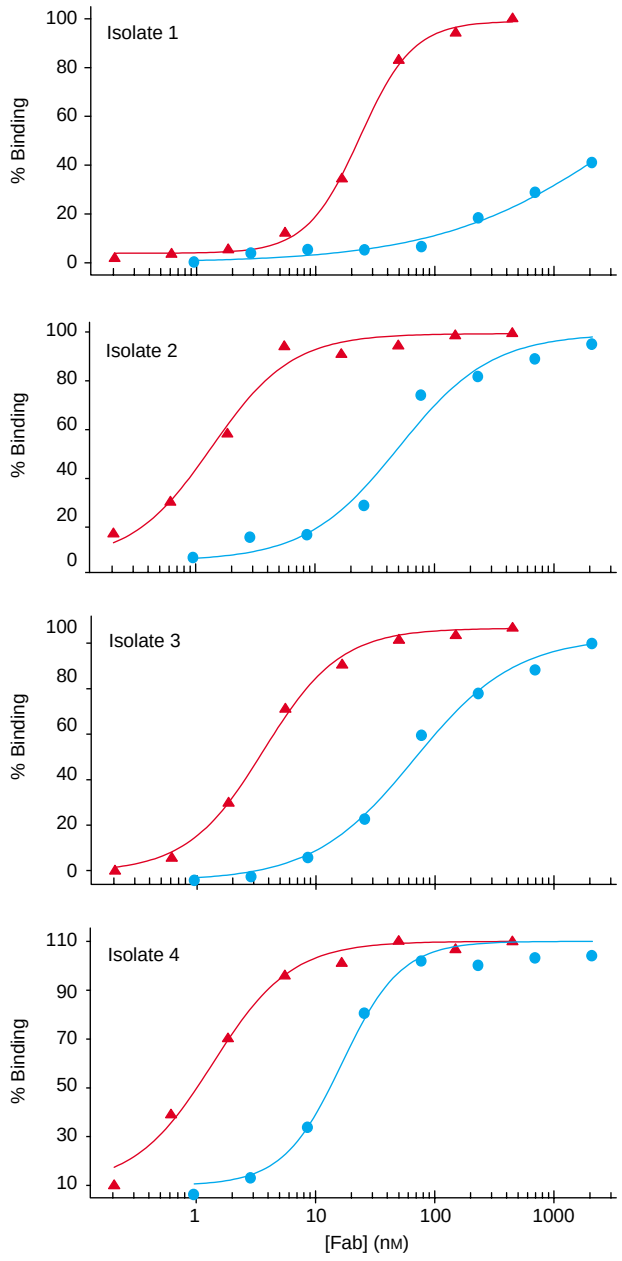

Figure 4. Enhancing the reactivity of a monoclonal antibody to a broader range of clinical isolates. The heavy and light chain variable regions of a murine IgG that displayed selective reactivity toward clinical isolate 4 of a bacterial pathogen were cloned into a bacterial expression vector to enable engineering by directed evolution. Antibody variants with enhanced binding to a broader range of clinical isolates were identified by quantitation of Fab fragment binding to the bacterial pathogen immobilized in a 96-well plate. The binding profile of a variant with optimized reactivity is shown for four representative clinical isolates (red), including those that were the most (isolate 4) and least (isolate 1) reactive toward the original murine antibody (blue). Enhanced efficacy of the variants (neutralization of the pathogen) requires the expression of intact IgG (not shown).

cocktail as a viable clinical alternative. We were interested in demonstrating that directed evolution could broaden the recognition and neutralization properties of this antibody to all relevant isolates of the pathogen and generate a superior therapeutic candidate. The strategy was to engineer individual CDRs to substantially increase the binding characteristics of the antibody toward non-reactive isolates while preserving its existing neutralization properties. The parent antibody was simultaneously engineered against a panel of numerous clinical isolates, a process that led to the identification of several variants whose binding affinities exceeded the threshold necessary for recognition and neutralization of all isolates, including those that were previously classified as non-reactive (Fig. 4).

Here again, the optimization process was successfully completed in the absence of any underlying information regarding the structural basis for the differential recognition of these isolates by the antibody. This further demonstrates the power of a function-based approach to protein engineering and augurs a possible generalization of this strategy to other antibodies against infectious agents, such as anti-HIV antibodies [40], whose limited recognition of specific viral clades might impede their potential clinical development.

Engineering catalytic activity and substrate specificity of enzymes

Although the previous examples describe the engineering of antibodies, directed evolution can be applied to virtually any protein, including cytokines, hormones, growth factors and enzymes. Butyrylcholinesterase (BChE) is a serum enzyme that displays a broad range of detoxification properties, including the catalysis and breakdown of organophosphate compounds that are present in nerve gases and pesticides [41]. BChE also metabolizes and inactivates cocaine, albeit in an extremely inefficient manner [42]. Because the administration of plasma-derived BChE provides some protection against cocaine toxicity *in vivo* [43], we were interested in engineering the catalytic efficiency (k_{cat}/K_m) of its cocaine hydrolase activity to support further development as a viable therapeutic for treating acute cocaine toxicity and addiction [44,45]. Libraries of BChE variants, designed to evaluate multiple regions of the protein including the highly conserved catalytic domain, were screened for hydrolase activity at substrate concentrations substantially below the K_m of the enzyme to identify mutations that either increased the k_{cat} or decreased the K_m of BChE. Beneficial mutations were identified throughout the protein, including mutations spatially distant from the proposed active site of the enzyme, and several single amino acid substitutions were characterized that increased the cocaine hydrolase activity by as much as

10-fold. Subsequently, the catalytic efficiency of the enzyme was further improved through the synthesis and screening of new libraries combining these initial mutations. Variants displaying ≥100-fold improvement in hydrolase activity are currently being tested in animal models of acute cocaine toxicity (Pancook, J.D. *et al.*, unpublished data).

This project necessitated the development of a mammalian expression system capable of generating thousands of individual variants properly glycosylated and assembled in a tetrameric structure, a technology that facilitated the subsequent engineering of other complex proteins whose post-translational modifications are required for biological activity. Furthermore, the engineering of BChE expanded the applicability of our directed evolution approach beyond the optimization of protein–protein interfaces and into the engineering of small molecule recognition and catalysis by proteins, reinforcing the notion that efficient optimization of complex biologics requires a dedicated strategy.

Improving protein stability by removal of deamidation sites

A common problem encountered during the development and commercialization of biologics is chemical instability, such as susceptibility to deamidation at asparagine residues or methionine oxydation [46]. In its worst manifestations, deamidation of proteins results in the loss of biological activity and undesirable heterogeneity that can significantly impact the purification process and the overall costs of production. Often, simple replacement of the affected asparagine detrimentally impacts function and, consequently, additional engineering is required to recover biological activity. By excluding the susceptible asparagine residue and simultaneously randomizing amino acids at adjacent and/or distant positions in the protein, compensatory changes can be identified that completely eliminate deamidation while preserving or even improving activity. In the case of the aforementioned Vitaxin™ antibody, a deamidation site was eliminated from a CDR position, further increasing its affinity and resulting in a 300% improvement in purification yields (Wu, H. *et al.*, unpublished data).

Conclusion

Directed evolution is a powerful approach for improving virtually any property of a therapeutic protein for which an appropriate screen can be devised. It is a broadly applicable technology designed to remodel a wide range of proteins and to ameliorate the clinical profile of approved biologics or the therapeutic use of molecules under development. Strategies to improve biologics are generally based on the particular function of the protein and might involve several approaches including, for instance, engineering enhanced neutralization properties, altered specificity for cellular receptors or recruitment of cellular functions. As the previous examples demonstrate, significant improvements in potency, efficacy, enzymatic activity, antigen recognition profile and manufacturing have been achieved by using our directed evolution platform. Substantial clinical benefits can also be realized through modifications that impact safety by reducing immunogenicity of antibody therapeutics or by narrowing the receptor specificity of a hormone or cytokine to reduce side effects. The ability to improve such crucial parameters could ultimately determine whether a drug is commercialized because optimization of biologics paves the way for less frequent injections, new routes of administration, decreased manufacturing costs and expanded therapeutic applications. Finally, by creating new functional entities, optimization applied to existing pharmaceuticals can broaden and extend intellectual property as second-generation therapeutics.

Acknowledgements

We would like to acknowledge the contribution of many colleagues, especially Drs James F. Young, Oksana Lockridge and James D. Pancook, as well as Greg Beuerlein, Ying Nie, Gerlinde Pecht, and Max Ader. We also would like to thank Vicki Campbell for help in preparing the figures. Part of the work described here is supported by NIH grant 5 R44 GM60106–03 to J.D.W.

References

1 Dayan, A.D. (1995) Safety evaluation of biological and biotechnology-derived medicines. *Toxicology* 105, 59–68
2 Serabian, M.A. and Pilaro, A.M. (1999) Safety assessment of biotechnology-derived pharmaceuticals: ICH and beyond. *Toxicol. Pathol.* 27, 27–31
3 Wells, J.A. (1996) Binding in the growth hormone receptor complex. *Proc. Natl. Acad. Sci. U. S. A.* 93, 1–6
4 Riechmann, L. *et al.* (1988) Reshaping human antibodies for therapy. *Nature* 332, 323–327
5 Selzer, T. *et al.* (2000) Rational design of faster associating and tighter binding protein complexes. *Nat. Struct. Biol.* 7, 537–541
6 Kopchick, J.J. *et al.* (2002) Growth hormone receptor antagonists: discovery, development, and use in patients with acromegaly. *Endocr. Rev.* 23, 623–646
7 Altmann, S.W. and Kastelein, R.A. (1995) Rational design of a mouse granulocyte macrophage-colony-stimulating factor receptor antagonist. *J. Biol. Chem.* 270, 2233–2240
8 Tavernier, J. *et al.* (1995) Identification of receptor-binding domains on human interleukin 5 and design of an interleukin 5-derived receptor antagonist. *Proc. Natl. Acad. Sci. U. S. A.* 92, 5194–5198
9 Smothers, J.F. *et al.* (2002) Affinity selection from biological libraries. *Science* 298, 621–622
10 Boder, E.T. *et al.* (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10701–10705
11 Pluckthun, A. *et al.* (2000) *In vitro* selection and evolution of proteins. *Adv. Protein Chem.* 55, 367–403

12 Clackson, T. *et al.* (1991) Making antibody fragments using phage display libraries. *Nature* 352, 624–628
13 Huse, W.D. *et al.* (1993) Increased antibody affinity and specificity by codon-based mutagenesis. *Int. Rev. Immunol.* 10, 129–137
14 Yelton, D.E. *et al.* (1995) Affinity maturation of the BR96 anti-carcinoma antibody by codon-based mutagenesis. *J. Immunol.* 155, 1994–2004
15 Wu, H. *et al.* (1998) Stepwise *in vitro* affinity maturation of Vitaxin, an $\alpha_v\beta_3$-specific humanized mAb. *Proc. Natl. Acad. Sci. U. S. A.* 95, 6037–6042
16 Arnold, F.H. *et al.* (2001) How enzymes adapt: lessons from directed evolution. *Trends Biochem. Sci.* 2, 100–106
17 Henke, E. and Bornscheuer, U.T. (1999) Directed evolution of an esterase from Pseudomonas fluorescens. Random mutagenesis by error-prone PCR or a mutator strain and identification of mutants showing enhanced enantioselectivity by a resorufin-based fluorescence assay. *Biol. Chem.* 380, 1029–1033
18 Kolkman, J.A. and Stemmer, W.P. (2001) Directed evolution of proteins by exon shuffling. *Nat. Biotechnol.* 19, 423–428
19 Foster, P.L. (1991) *In vivo* mutagenesis. *Methods Enzymol.* 204, 114–125
20 Glaser, S.M. *et al.* (1992) Antibody engineering by codon-based mutagenesis in a filamentous phage vector system. *J. Immunol.* 149, 3903–3913
21 Wu, H. *et al.* (1999) Humanization of a murine monoclonal antibody by simultaneous optimization of framework and CDR residues. *J. Mol. Biol.* 294, 151–162
22 Wells, J.A. (1990) Additivity of mutational effects in proteins. *Biochemistry* 29, 8509–8517
23 Chester, K.A. *et al.* (1995) Clinical issues in antibody design. *Trends Biotechnol.* 8, 294–300
24 Maynard, J. and Georgiou, G. (2000) Antibody engineering. *Annu. Rev. Biomed. Eng.* 2, 339–376
25 Mendez, M.J. *et al.* (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat. Genet.* 15, 146–156
26 Griffiths, A.D. *et al.* (1994) Isolation of high affinity human antibodies directly from larger synthetic repertoires. *EMBO J.* 13, 3245–3260
27 Knappik, A. *et al.* (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* 296, 57–86
28 Jones, P.T. *et al.* ((1986)) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321, 522–525
29 Foote, J. and Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224, 487–499
30 Co, M.S. *et al.* (1992) Chimeric and humanized antibodies with specificity for the CD33 antigen. *J. Immunol.* 148, 1149–1154
31 Hakimi, J. *et al.* (1997) Development of Zenapax®: a humanized anti-Tac antibody. In *Antibody Therapeutics* (Harris W.J. and Adair J.R., eds.), pp. 277–300, CRC Press
32 Goldenberg, M.M. (1999) Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. *Clin. Ther.* 21, 309–318
33 Johnson, S. *et al.* ((1997)) Development of a humanized monoclonal antibody (MEDI-493) with potent *in vitro* and *in vivo* activity against respiratory syncytial virus. *J. Infect. Dis.* 176, 1215–1224
34 Brooks, P.C. *et al.* (1994) Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* 264, 569–571
35 Gutheil, J.C. *et al.* (2000) Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin $\alpha_v\beta_3$. *Clin. Cancer Res.* 6, 3056–3061
36 Cheng, X. *et al.* (2002) Expression of β-galactosidase by recombinant respiratory syncytial viruses for microneutralization assay. *J. Virol. Methods* 105, 287–296
37 Schreiber, G. and Fersht, A.R. (1996) Rapid, electrostatically assisted association of proteins. *Nat. Struct. Biol.* 3, 427–431
38 Pancook, J.D. *et al.* (2001) *In vitro* affinity maturation of human IgM antibodies reactive with tumor-associated antigens. *Hybrid. Hybridomics* 20, 383–396
39 Xu, J. *et al.* (2000) Generation of monoclonal antibodies to cryptic collagen sites by using subtractive immunization. *Hybridoma* 19, 375–385
40 Barbas, C.F. *et al.* ((1994)) *In vitro* evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. *Proc. Natl. Acad. Sci. U. S. A.* 91, 3809–3813
41 Schwarz, M. *et al.* ((1995)) Engineering of human cholinesterases explains and predicts diverse consequences of administration of various drugs and poisons. *Pharmacol. Ther.* 67, 283–322
42 Xie, W. *et al.* ((1999)) An improved cocaine hydrolase: the A328Y mutant of human butyrylcholinesterase is 4-fold more efficient. *Mol. Pharmacol.* 1, 83–91
43 Lynch, T.J. *et al.* (1997) Cocaine detoxification by human plasma butyrylcholinesterase. *Toxicol. Appl. Pharmacol.* 145, 363–371
44 Duysen, E.G. *et al.* ((2002)) Wild-type and A328W mutant human butyrylcholinesterase tetramers expressed in Chinese hamster ovary cells have a 16-hour half-life in the circulation and protect mice from cocaine toxicity. *J. Pharmacol. Exp. Ther.* 302, 751–758
45 Sun, H. *et al.* (2002) Cocaine metabolism accelerated by a re-engineered human butyrylcholinesterase. *J. Pharmacol. Exp. Ther.* 302, 710–716
46 Volkin, D.B. *et al.* ((1997)) Degradative covalent reactions important to protein stability. *Mol. Biotechnol.* 2, 105–122
47 Leong, S.R. *et al.* (2001) Adapting pharmacokinetic properties of a humanized anti-interleukin-8 antibody for therapeutic applications using site-specific pegylation. *Cytokine* 16, 106–119
48 Thompson, J.E. *et al.* (1999) A fully human antibody neutralising biologically active human TGFβ2 for use in therapy. *J. Immunol. Methods* 227, 17–29
49 Reddy, C.C. *et al.* (1996) Engineering epidermal growth factor for enhanced mitogenic potency. *Nat. Biotechnol.* 14, 1696–1699
50 Egrie, J.C. and Browne, J.K. (2001) Development and characterization of novel erythropoiesis stimulating protein (NESP). *Br. J. Cancer* 84(Suppl 1), 3–10
51 Sarkar, C.A. *et al.* (2002) Rational cytokine design for increased lifetime and enhanced potency using pH-activated "histidine switching". *Nat. Biotechnol.* 20, 908–913
52 Pearce, K.H. *et al.* (1999) Growth hormone binding affinity for its receptor surpasses the requirements for cellular activity. *Biochemistry* 38, 81–89
53 Ballinger, M.D. *et al.* (1998) Selection of heregulin variants having higher affinity for the ErbB3 receptor by monovalent phage display. *J. Biol. Chem.* 273, 11675–11684
54 Blatt, L.M. *et al.* (1996) The biologic activity and molecular characterization of a novel synthetic interferon-alpha species, consensus interferon. *J. Interferon Cytokine Res.* 16, 489–499
55 Chang, C.C. *et al.* (1999) Evolution of a cytokine using DNA family shuffling. *Nat. Biotechnol.* 17, 793–797
56 Osborn, B.L. *et al.* (2002) Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys. *J. Pharmacol. Exp. Ther.* 303, 540–548
57 Fallon, E.M. *et al.* (2000) Increased endosomal sorting of ligand to recycling enhances potency of an interleukin-2 analog. *J. Biol. Chem.* 275, 6790–6797
58 Yang, J. *et al.* (2002) Enhancing the anticoagulant potency of soluble tissue factor mutants by increasing their affinity to factor VIIa. *Thromb. Haemost.* 87, 450–458
59 Moreland, L.W. *et al.* (1997) Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N. Engl. J. Med.* 337, 141–147