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[16.00–16.30]
Insights into molecular recognition from minimalist synthetic antibodies
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Antibodies can evolve to recognize essentially any protein with high specificity and affinity. While natural antigen binding sites utilize all 20 natural amino acids to some extent, analysis of functional antibodies reveals clear biases for or against some amino acids. Most significantly, tyrosine and serine are highly abundant in antigen binding sites in general and at antigen contact sites in particular.

As it is now possible to construct antibody-phage libraries with synthetic diversity, we have used synthetic antibody libraries to investigate the roles of different chemical diversity in antigen recognition. Using a tetranomial genetic code that allows for only four amino acids (tyrosine, serine, alanine, and aspartate) we were able to generate antibodies against vascular endothelial growth factor (VEGF) that bound with high affinity and specificity. Structural and mutational analyses indicated that tyrosine was the major mediator of binding energy at the antigen binding sites of the anti-VEGF Fabs, and the results suggested that it might be possible to further simplify the code for antigen recognition. To this end, we constructed Fab libraries in which combining sites were randomized with a binary genetic code that allowed for only tyrosine and serine. We envisioned that in these libraries, tyrosine might act as an effective "functional" amino acid with a large side chain that could provide significant binding contacts. In contrast, serine might act as an "auxiliary" amino acid with a small side chain that could provide space for the tyrosine side chains and also contribute to an overall hydrophilic surface.

Remarkably, naïve Fab libraries constructed with the binary code were extremely effective in generating highly specific antibodies against a wide array of antigens. Furthermore, the binary Fabs exhibited exquisite specificity in cell-based assays. The structures of binary Fabs in complex with antigen reveal that the highly homogenous binding surfaces are dominated by tyrosine, but nonetheless, recognize diverse chemical groups on the antigen. In ongoing studies, we are now adding back chemical and conformational diversity into the minimalist binary background in a precisely defined manner, and these highly controlled studies will enable the accurate assessment of the consequences of expanded diversity.

Our results suggest that certain amino acids possess features that increase the likelihood that they will be able to make productive contributions to binding affinity and specificity. Thus, biased libraries that favour such amino acids are likely to be much more effective in generating antibodies with high affinity and specificity. As a corollary, it seems that other amino acids are ill suited for productive contacts, and the absence or depletion of such amino acids will likely improve naïve antigen recognition. The results should aid the design and use of synthetic antibody libraries. In addition, and perhaps more importantly, the findings have significant implications for the fundamental principles and mechanisms that mediate molecular recognition at protein-protein interfaces.

[16.30–16.50]
Human CD32B (FcγRIIB): A multifaceted target for enhanced antibody therapeutics
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Receptors specific for the Fc portion of immunoglobulins link humoral responses to effector functions, both beneficial and idiopathic. CD32B (Fc-gamma-RIIB), the only inhibitory Fc receptor, is present at high levels

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on B cells and at lower levels on monocytes, dendritic cells, basophils and mast cells. CD32B counterbalances signaling through an activating receptor; including the BCR, activating Fc-gamma-R, or Fc-epsilon-R, on the corresponding cell types. We have generated, characterized and humanized several mAbs which bind with exquisite specificity to CD32B but not the closely related activating receptor, CD32A. This is the first time such selective antibodies have been described and their use has led to a greater understanding of the expression patterns and functional properties of these two receptors in normal and diseased tissues. We are also developing biologic agents to take advantage of the pattern of expression of CD32B and its inhibitory function therapeutically in oncology, autoimmunity and allergy. We have utilized Fc-engineering and a novel bispecific format to exploit several facets of CD32B as a therapeutic target. The resulting molecules are highly active in vitro and potent in vivo, as shown in several FcR-transgenic mouse models. A newly developed expression system allowing for secretion of antibody fragments by E. coli and full length mAbs or sFv-Fc fusion proteins in mammalian cells, without reformatting, will also be described.

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[16.50–17.10]

Humanization of a highly stable anti-lysozyme scFv endowed with intracellular functionality. A single framework residue dramatically affects protein expression and stability

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Introduction: In recent years the number of recombinant antibodies aimed at diagnostic and therapeutic applications has been increasing [1] and the single-chain variable domain fragment (scFvs) in particular, resulted one of the most successful antibody formats developed until now [2]. It is comprised of the variable regions of both the heavy and light chains, bearing the antigen binding site (ABS), joined together by a flexible linker that allows the expression as a single polypeptide sequence. The main advantages of these molecules over complete immunoglobulins include the ease of expression in different biological systems and the higher speed of tissue uptake and blood clearance. All of these properties are particularly useful in tumour targeting and for *in vivo* imaging purposes, so that several scFvs and scFv-containing molecular complexes are now undergoing clinical or preclinical studies [1]. The intrinsic thermodynamic stability of candidate therapeutic antibody fragments is considered an important requisite for *in vivo* applications since it correlates with favourable pharmacokinetic properties as well as with their functionality inside the cell [3,4,5], where they can modulate the function of proteins and intracellular pathogens [6] and target molecular processes associated with carcinogenesis [7]. Together with a high thermodynamic stability, another essential requisite for applications of antibody fragments in human therapy is that they should possess a low immunogenic potential. Indeed, a major obstacle hampering the use of antibody fragments of murine origin for human therapy concerns their potential to raise adverse immune responses in patients [8], such that most of the recombinant antibodies that reached pre-clinical or clinical trials are either of human origin or, in case they originated from different animal sources (usually rodent), they had been previously humanized, by replacing molecule regions that are not involved in antigen binding with homologous regions from human antibodies. In this

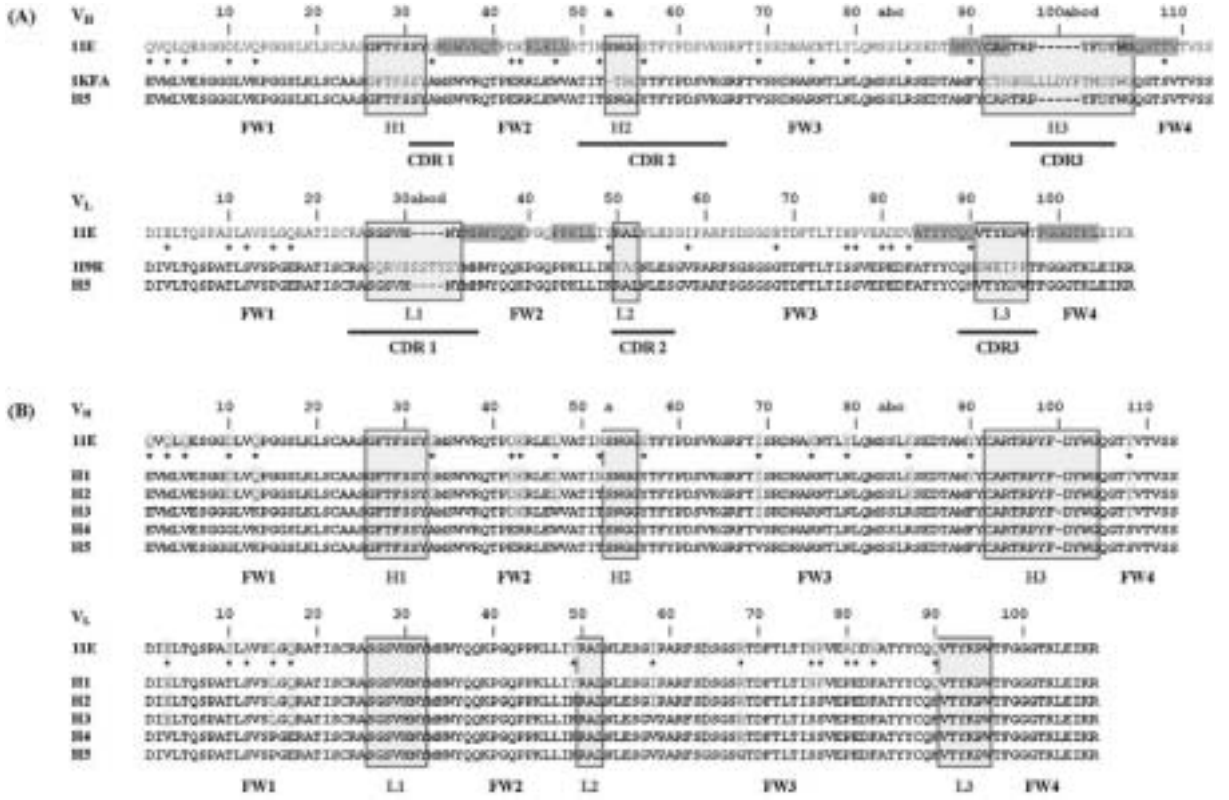


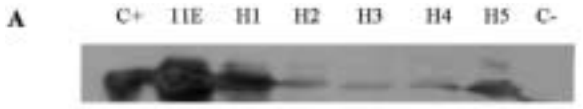
Fig. 1. Sequence alignment of V_H and V_L domains used for the humanisation of scFv(11E).(**A**) Sequence alignment between the V_H and V_L domains of the murine anti-HEL ABS-donor scFv(11E), the homologous ABS-acceptor domains from antibodies of human origin (PDB IDs: 1KFA and 1I9R, respectively) and the designed humanised scFv(H5). (**B**) Sequence alignment between the V_L and V_H domains of the murine anti-HEL ABS-donor scFv(11E), the intermediate scFv products scFv(H1), scFv(H2), scFv(H3) and scFv(H4), and the designed, fully humanised scFv(H5). Residue numbering, hypervariable loops, key-residues, CDRs and Fw regions are indicated as in Figure 1A. Non-identical Fw residues between scFv(11E) and scFv(H5) are indicated by asterisks. Residues identical to and different from those present in scFv(H5) are bold and highlighted yellow, respectively.

work, our aim was to obtain a scFv scaffold endowed with the same favourable properties (i.e., high stability and intracellular functionality) of the scFv(F8) [5,9] and devoid of the potential immunogenicity associated with its murine origin. To this end, we humanized the anti-lysozyme scFv(11E) obtained from a phage-display library, which has the same framework regions and favourable properties as the scFv(F8) [10].

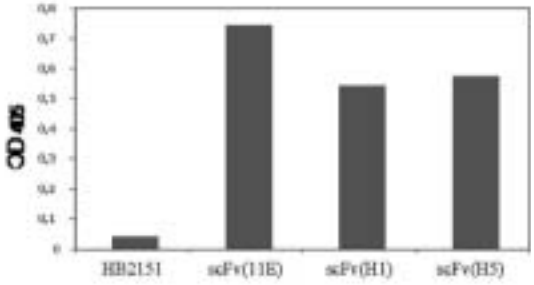
Humanization design: The humanized scFv(11E) was designed by joining the ABS of the scFv(11E) (ABS-donor) with the Fw regions of human origin (ABS-acceptors) showing the highest sequence identity with the Fw regions of scFv(11E).

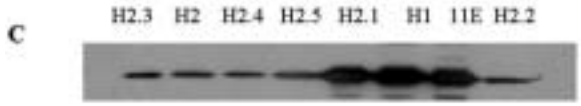
The selected ABS acceptors were the 4-B8(8)/E9 Fab (V_H3 fr1 subtype II, 82% sequence identity with scFv(11E) V_H domain) and humanized 5c8 Fab (V_kIII subgroup, 85% sequence identity with scFv(11E) V_L domain), respectively. For both Fabs a crystallographic structure was available from the Protein Data Bank (PDB), with identifiers (ID) 1KFA [11] Resolution = 2.80Å) and 1I9R [12], Resolution = 3.10Å), respectively. The high sequence identity between the murine ABS donor scFv(11E) and the selected ABS acceptors of human origin (higher than 80% for both V_L and V_H Fw regions) has been previously shown to correspond to a high structural conservation (i.e., RMSD < 1.0Å) in the core regions of homologous proteins [13]. Therefore, the main-chain conformation of the Fw regions of the ABS-donor scFv(11E) and ABS acceptor V_L and V_H domains is expected to be essentially the same and to be maintained in the fully humanized scFv(H5), comprising the same Fw regions as the ABS-acceptor V_L and V_H domains (Fig. 1).

Construction and analysis of the of the humanized scFv: The designed scFv(H5) sequence was obtained by stepwise mutagenesis of the scFv(11E) Fw regions. The mutagenesis and cloning strategy led to the generation of four intermediate scFv products



Clone	Relative expression levels
11E	1
H1	0.96 (±0.05)
H2	0.16 (±0.10)
H3	0.078 (±0.08)
H4	0.1 (±0.02)
H5	0.24 (±0.12)





Clone	Relative expression levels
11E	1
H1	0.95 (±0.05)
H2.1	0.99 (±0.02)
H2.2	0.37 (±0.09)
H2.3	0.37 (±0.14)
H2.4	0.33 (±0.10)
H2.5	0.12 (±0.10)
H2	0.21 (±0.15)

Fig. 2. Analysis of the mutagenised scFvs expressed in a soluble form in the periplasm. (A) Soluble periplasmic expression of the four intermediate clones scFv(H1)-(H4) and of the fully humanised scFv(H5). Extracts were normalized for total protein content and analysed by reducing SDS-PAGE. Relative expression levels of the analyzed clones were determined by densitometric analysis of three independent western blot results. Values reported in the tables represent the mean of three independent experiments with standard deviations. The scFv(11E) value was arbitrarily set to 1.Twenty nanograms of purified scFv(11E) were used as a positive control (C+), while untransformed *E. Coli* HB2151 periplasmic extract was used as a negative control (C−). (B) HEL binding activity of the scFv(11E), scFv(H1) and scFv(H5) periplasmic extracts were assayed by ELISA as described in materials and methods. Reported absorbance values were obtained after 30 min incubation. Extracts from untransformed *E. coli* HB2151 cells were used as a negative control. (C) Soluble periplasmic expression of the five intermediate scFv clones obtained following mutagenesis of scFv(H1). Extracts were normalized for total protein content and relative expression levels were determined by densitometric analysis of three independent western blot results. Twenty nanograms of purified scFv(11E) were used as a positive control (C+).

named scFv(H1), scFv(H2), scFv(H3) and scFv(H4), as well as to the fully humanized scFv(H5) (Fig. 1B). All scFv variants were analysed for expression yields in a soluble form in the periplasm of *E. coli*. As shown in Fig. 2A, only scFv(H1) showed expression levels similar to those observed for scFv(11E), clones scFv(H2), scFv(H3), scFv(H4) and scFv(H5) showing expression yields 4 to 13 times lower. ScFv(H1) and scFv(H5) were purified from the periplasmic extract to further characterize their immunological properties. Purification yields for scFv(H5) proved to be very low (about 30–40 μg/L bacterial culture) while for scFv(H1) they were in the same range as scFv(11E) (about 1 mg/L). Functional analysis of the periplasmic extracts of scFv(H1) and scFv(H5) using ELISA showed that both possessed a specific binding activity for HEL (Fig. 2B).

Effects of a single mutation on the soluble expression levels of the fully humanized scFv(*H5*) *and of the scFv*(*H2*) *intermediate:* To identify residues potentially responsible for the drop in expression levels between scFv(H1) and scFv(H2), we analysed the expression profile of the five intermediate clones obtained by mutagenesis between these two molecules. As shown in Figure 2C, the expression levels of the intermediate clones decrease about three to eight-fold compared to scFv(H1), with the exception of scFv(H2.1), which differs from scFv(H1) by only two residues and

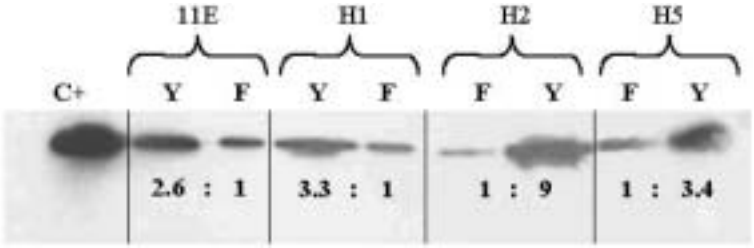


Fig. 3. Western blot analysis of the periplasmic expression levels of scFv(11E), scFv(H1), scFv(H2) and scFv(H5) and of their H90 mutants. F and Y indicate the amino acid residue present at position H90. Protein extracts of each clone were normalized for total protein content and analysed by reducing SDS-PAGE. Relative expression levels of the analyzed scFvs determined by densitometric analysis are indicated. Purified scFv(11E) (20 ng) was used as a positive control.

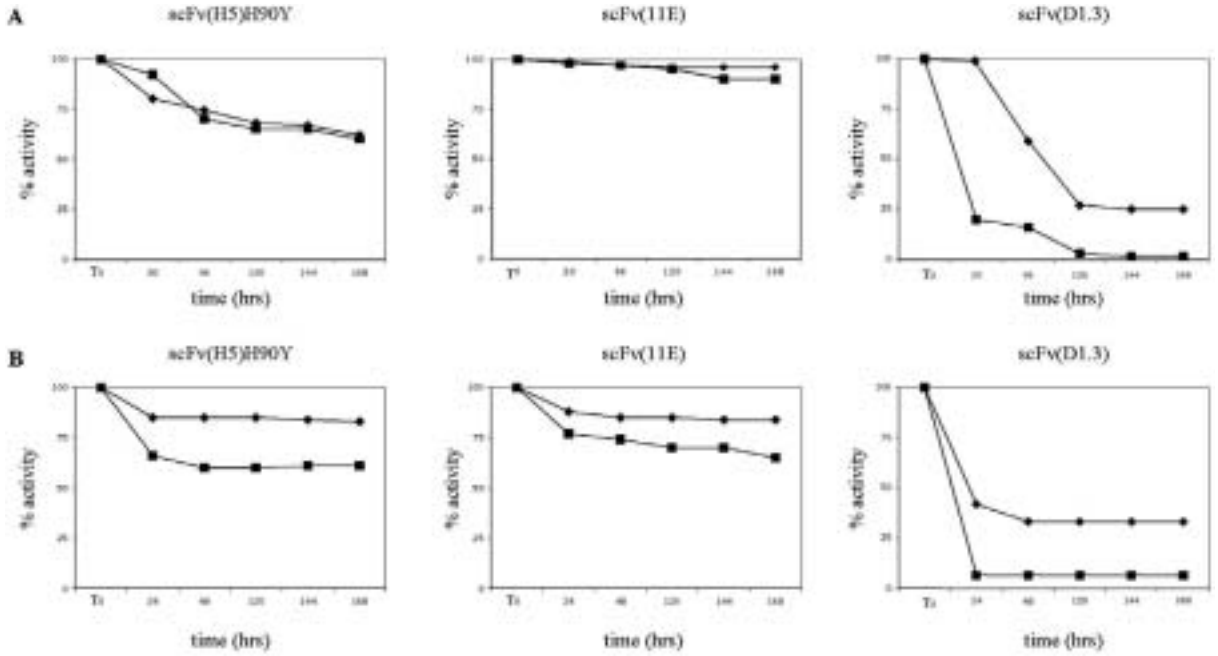


Fig. 4. Stability of the final humanized scFv(H5)H90Y, scFv(11E) (used as a highly stable scFv control) and scFv(D1.3) (used as a poorly stable scFv control) in serum and phosphate buffer. The scFvs were incubated at 4°C and 37°C for various time period (up to 168 hours) either in PBS (**A**) or in human serum (**B**). Antigen binding activity (RU) was determined by SPR on a HEL coated chip. Residual activity was determined at each time point as follows: RU of the scFv sample treated with PBS or human serum at 37°C or 4°C/RU of the scFv at T_0. Diamonds: percentage of activity observed after incubation at 4°C. Squares: percentage of activity observed after incubation at 37°C. Lines connecting the data points are used to guide the eye of the reader.

whose expression level is the same of scFv(11E) and scFv(H1). Comparison of the amino acid substitutions occurring between all the scFv intermediates showed that all clones had Phe in position H90, except for scFv(H2.1) that maintained the original Tyr residue. Therefore, we investigated the role of the residue occurring at position H90 on the expression levels of soluble scFv molecules. To this aim, we constructed a panel of mutants. In scFv(H2) and scFv(H5), showing low expression levels, we mutated Phe H90 to Tyr, obtaining scFv(H2)H90Y and scFv(H5)H90Y, respectively. In scFv(11E) and scFv(H1), showing high expression levels, we mutated Tyr H90 to Phe, obtaining scFv(11E)H90F and scFv(H1)H90F, respectively. The resulting expression levels of these mutants in the periplasm of *E. coli* are shown in Fig. 3. ScFv(H2)H90Y and scFv(H5)H90Y showed a 9 and 3.4 fold increase in the expression levels compared to the original scFv(H2) and scFv(H5), respectively, while scFv(11E)H90F and scFv(H1)H90F showed a 2.6 and 3.3 fold decrease compared to scFv(11E) and scFv(H1), respectively. The purification yield of scFv(H2)H90Y

was about 600 μg/L of bacterial culture, and the yield of scFv(H5)H90Y about 450 μg/L of bacterial culture, i.e. more than 10-fold higher than that of scFv(H5).

Affinity measurements of the humanized scFv(H5) H90Y towards HEL by surface plasmon resonance: The binding activity of the humanized scFv(H5)H90Y for HEL was analysed by surface plasmon resonance (SPR). The calculated association and dissociation rate constants were 1.45×10^4 $M^{-1}s^{-1}$ and 1.05×10^{-3} s^{-1} respectively, giving a K_D of 72 nM, similar to, and even better than, that measured for the cognate scFv(11E) ($K_D = 105$ nM) [10]. The binding activity of the reduced scFv(H5)H90Y was also calculated by SPR. The scFv was subjected to strongly reducing conditions (DTT 12.5 mM) and its reduced state was confirmed by gel-shift analysis on a non reducing SDS-PAGE. The calculated association and dissociation rate constants were 2.83×10^4 $M^{-1}s^{-1}$ and 2.71×10^{-3} s^{-1}, respectively, giving a K_D of 96 nM, which is essentially the same as that calculated for the non reduced form.

Serum stability of the humanized scFv: For clinical applications recombinant antibodies must be resistant towards human serum proteases and retain their activity at 37°C (body temperature). We assessed the stability of the scFv(H5)H90Y by measuring its binding to HEL by SPR after incubation at 37°C in human serum or in PBS, for various time periods. As reference molecules we used the scFv(11E), previously shown to be endowed with high thermodynamic stability [10], and the scFv(D1.3) having standard thermodynamic properties. As shown in Fig. 4, scFv(11E) retained 90% and 65% of its binding activity when incubated for seven days at 37°C in PBS (panel A) and in human serum (panel B) respectively, whereas scFv(D1.3) lost more than 90% of its activity after just one day of incubation at 37°C in both media. The scFv(H5)H90Y retained 66% of its binding activity after seven days incubation at 37°C both in PBS (Fig. 4A) and in human serum (Fig. 4B), indicating that its stability in human serum is essentially the same of the highly thermodynamically stable parent scFv(11E).

Conclusions: Because of its stability in human serum, both under oxidising and reducing conditions, and of its potentially low immunogenic properties, the scFv(H5)H90Y scaffold obtained in this work represents an ideal candidate to produce highly stable chimeric scFv molecules for extra- and intra-cellular applications in human therapy and *in vivo* diagnosis, by either combinatorial methods (i.e., construction of new libraries based on this scaffold) or rationally designed ABS grafting from an ABS-donor Ab or Ab fragment of known V_L and V_H sequence against a required antigen.

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[17.10–17.30]

Human antibodies selected by phage display as potent and selective protease inhibitors

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Introduction: The control of proteolysis in an organism is achieved under normal circumstances through a balance of protease production, degradation and inactivation by inhibitors. Unregulated or dysregulated protease activities can result in the onset or progression of disease. A number of proteases, such as members of the matrix metalloproteinase (MMPs) and serine protease families, have been validated as potential therapeutic targets in inflammatory disorders, tumor growth and metastasis [1,2].

Matrix metalloproteinases (*MMPs*)*:* Belong to the metzincin superfamily that includes more than 20 zinc-dependent enzymes sharing common features [3]. Their structure is composed of three domains, a prodomain that is removed upon activation, a catalytic domain responsible for hydrolytic activity, and a hemopoxin-like domain that is probably playing a role in substrate recognition. The regulation of MMPs occurs at both a level of expression, activation, localization, and TIMP inhibition [4]. MMPs recognize diverse substrates that extend beyond extracellular matrix components, also regulating other pro-MMPs. Since their substrates are diverse, MMPs are involved in variety of homeostatic functions, such as bone remodeling, wound healing, and several aspects of immunity. However, overexpressed or misregulated MMPs are involved in a number of pathological processes, such as tumor progression, fibrosis, chronic inflammation, tissue destruction [5,6].

Here, we will describe the work on two MMP members, the secreted MMP-26 and the membrane-type MMP-14. MMP-26 (also know as endometase and matrilysin-2) has not yet been extensively studied, except for its tissue localization and substrate identification [7]. Interest in this protease as a potential therapeutic candidate has emerged from work showing its overexpression in cancers of epithelial origin of the endometrium, prostate, and mammary gland [8,9,10]. MMP-14 (MT1-MMP) is the prototypic member of the membrane-type MMP (MT-MMP) subgroup [11]. MMP-14 regulates the activation of pro-MMP2 and pro-MMP13. Its overexpression has been associated with various pathological conditions and MMP-14 is proposed to play important roles in tumor progression by promoting cell invasion, angiogenesis and matrix degradation [6].

Serine proteases are a class of peptidases characterized by the presence of a serine residue in the active site of the enzyme. They participate in a wide range of functions in the body, including blood clotting, immunity, and inflammation, as well as contributing to digestive enzymes. Serine proteases are naturally inhibited by proteins called "serpins" by forming a covalent bond with the serine protease. The best-studied serpins are antithrombin and A1-antitrypsin, known for their role in coagulation/thrombosis and emphysema respectively [12]. In this paper, we will report work on human kallikrein 1 (hK1), a member of the kallikrein serine protease subgroup. The role of hK1 is demonstrated in establishement of preclinical models of asthma and as such is a potential target in the treatment of airway inflammatory diseases [13].

Protease inhibitors as potential drugs: Although interest in protease inhibition started about 15 years ago, there are relatively few marketed protease inhibitors. The majority of drug discovery efforts in this field were devoted to small molecule drugs. There has been success with HIV protease and ACE inhibition, but many others such as MMP- inhibitors failed to give expected results either because of lack of efficacy or severe side-effects observed during clinical trials [2]. Structural similarities within the active site of most proteolytic enzyme families result in the majority of small molecule protease inhibitors being able to inhibit some related family members of the target protease. The key challenge in future discovery of protease inhibitor drugs will be to identify potent and specific inhibitors of proteases. The next generation of therapeutic drug candidates against proteases is likely to consist of neutralizing antibodies identified from phage display libraries of human antibody fragments. The large surface area of interaction of an antibody with its antigen provides the molecular basis for obtaining highly selective protease inhibitors.

Phage display technology: Phage display allows rapid isolation of fully human target-specific antibodies from a library of billions of different antibodies. In addition since it is an in vitro process, it is possible to obtain antibodies to human targets that are toxic, non immunogenic, immunosuppressive, or identical to mouse proteins. The antibody phage display libraries used here are a phagemid or a phage system (known as the Dyax FAB-310 and FAB-410 libraries)

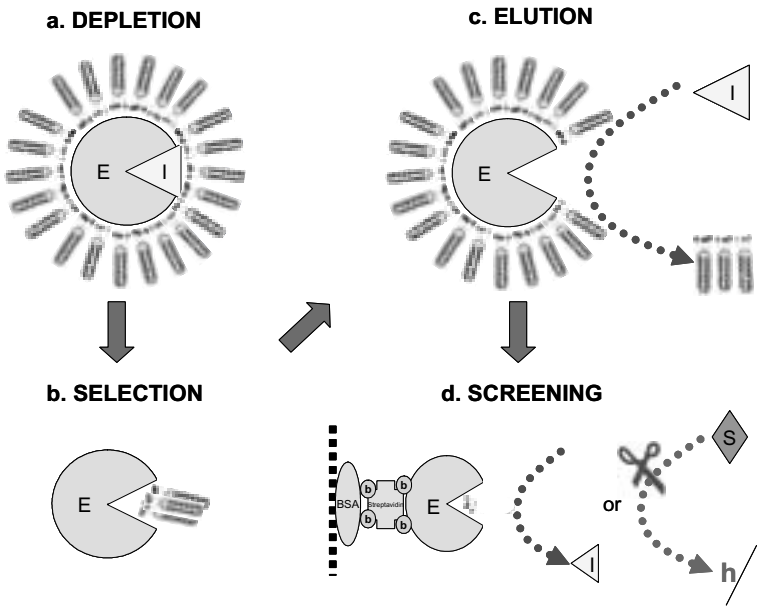

Fig. 1. Selection and screening strategies designed to obtain FAB protease inhibitors. (A) Depletion with enzyme (E): Inhibitor (I) Complexes is followed by (B) Selection on the active enzyme; (C) Elution with inhibitors (I) (D) Screening FABS by competition with inhibitors. B = Biotin group, I = inhibitor, S = substrate.

that display antibody Fab fragments fused to the M13 gene III protein. The antibody repertoire contains VL sequences, captured from a diverse population of normal and autoimmune human donors, randomly paired with VH sequences that combine within one V-gene as scaffold (VH3-23) synthetic diversity at functional sites within the VH-CDR1/2 and a non-immune VH-CDR3 diversity. This library has been shown to be successful on multiple targets and routinely produces high-affinity antibodies (KD<1nM) without the need for time-consuming affinity maturation [14]. The vector system used in this library has a modular design that allows a straightforward transfer of Fab cassettes into different expression vectors for soluble Fab and IgG of various classes [15].

Using Dyax's phage and phagemid: Fab libraries, we have identified selective and potent inhibitors of both serine and metalloproteinases. Their discovery and characterization are described below in more detail.

Discovery of protease inhibitor: Selection strategy design -¤C The standard selection process involves the exposure of the antibody phage library to the targeted protein, resulting in a collection of different antibodies that recognize a large panel of epitopes at the antigen surface. Driving the selection to a specific epitope on the target might help homing in to antibodies with the desired specificity. In the case of enzyme inhibition, the specific epitope to recognize is the active site of

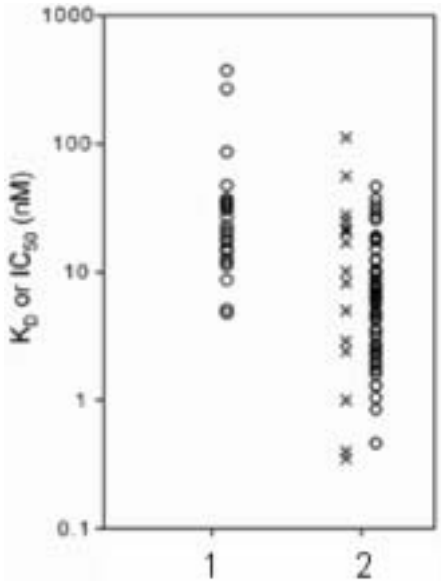

Fig. 2. Range of FAB antibody fragment affinities inhibiting the HK1 sering protease after selecting without (1) Or with (2) A depletion procedure using the HK1-Inhibitor complex. Open circles are K_D values and crosses are IC_{50} estimates.

the protein, known as the catalytic domain. It is well known that an enzyme is regulated in vivo by binding to its natural inhibitor, preventing the substrate from binding the active catalytic domain. Based on this con-

cept, we have designed selection strategies to target only the active catalytic domain (Fig. 1a,b,c). Using an enzyme-inhibitor complex in first instance of the selection depletes the library for non-inhibiting antibodies that recognize surface structural epitopes of the target but not the active site; the inhibitor (I) prevents the binding of antibodies to the catalytic domain of the enzyme (E) (Fig. 1a). After library depletion for non-inhibiting binders, the library can then be selected on the active enzyme only to capture antibodies recognizing the remaining epitopes, i.e. present on the catalytic domain (Fig. 1b). This strategy followed for the hK1 enzyme was shown to provide more antibodies with inhibition properties than straightforward standard selection methods as presented in Figure 2. Target expression and detailed selection procedure are described elsewhere [16]. The probability of finding inhibitors with improved KD or IC50 is consequently increased in such selection outputs. A similar strategy was followed to select Fab using MMPs in complex with their natural inhibitor TIMP-2 in an initial depletion step. Another strategy followed for the recovery of antibodies specific for the catalytic domain can be achieved through an elution step that uses a known inhibitor as a binding competitor (Fig. 1c). This strategy was also used for hK1 (aprotinin) and MMP-14 (TIMP-2) but was found to be less effective that the strategy using the E-I complex in depletion (data not shown).

Screening strategies for binding activity: The introduction of automated primary screening methods to the phage display process provides the opportunity to evaluate hundreds of Fabs in downstream assays [17,18]. In this study, the initial screening step was performed by automated enzyme-linked immunosorbent assay (ELISA) in medium (MMP-26) or high throughput mode (MMP-14, hK1). Sorting for binders to the catalytic domain can also be done by ELISA using the natural inhibitor as competitor in the assay (Fig. 1d). Further characterization of the antibodies is initiated once a set of unique clones is defined.

Cell-based and indirect screening strategies for inhibition: A total of 68 unique Fabs were converted to the huIgG4 format and characterized for their properties to inhibit the activity of the MMP-26 enzyme. To achieve this, two indirect assays were developed. The first one monitored the invasion of a cancer cell line in presence or absence of the anti-MMP-26 Fabs. Nine Fab antibodies were shown to decrease the JEG-3 cell invasion *in vitro* with IC50<50nM. The second assay exploited the property of MMP-26 to cleave pro-MMP-9 into active MMP-9, monitoring the cleavage of a peptide substrate. Two antibodies were shown to be able to block MMP-26-induced MMP-9 activity.

Screening strategies for inhibition and potency: The rapid discovery of the most potent antibodies identified in the primary screen depends on the development and use of suitable secondary assays. For MMP-14, steady-state enzyme inhibition assays using Phytip purified Fabs were directly performed as secondary screen on 70 unique Fabs, looking for cleavage inhibition of a small fluorescent quenched peptide substrate (Fig. 1d). A total of 16 inhibitors were identified. Further characterization of enzyme inhibitors was performed by the measurement of their IC50 towards the enzyme using IgGs. This step is a lenghtly process compared to other automated steps developed for phage display since it requires the evaluation of the antibody inhibition property using different antibody concentrations. Two out of the 16 MMP-14 inhibitors were found to have IC50 in the subnanomolar range.

In case of hK1, 355 unique Fab binders were first ranked using surface plasmon resonance microarrays prior to IC50 determination [16]. Thirty five inhibitors were found with two of them having IC50 $<$1 nM.

IC50 ranking of the different antibodies early in drug discovery accelerates the lead discovery. We have used a procedure that combines semi-automated Fab purification with medium throughtput IC50 determination of Fabs. The VH3-23 scaffold used in this library binds to protein A. This property was exploited to develop a semi-automated protein purification procedure of Fab fragments. Fab fragments are produced in 5 mL bacterial culture supernatants in 24-well plates and sequentially purified in solution using protein A coupled to magnetic beads. The recovered protein amount average is about 5 μg per sample. The concentration of the set of Fab protein preparations is first assessed by Biacore using a protein A coated chip [19], then normalized among the samples. The screening for inhibitors is subsequently performed as described above using a fluorescent quenched substrate.

Screening for selectivity and specificity: Depending on the need for crossreactive antibodies to other species counterparts of the target (most often mouse), the selection strategies can include the use of the mouse protein, driving the selections to antibodies that recognize common epitopes present on both targets. In case the targets are homologous, alternation of the targets during selection might not be necessary. The specificity to each target is analyzed by ELISA and inhibition assays. Finally, the antibodies are verified for their selectivity by measuring their inhibition of a panel of related proteases.

Conclusions: The combination of human Fab phage display libraries and automation provides a means to identify rapidly high affinity lead candidates. A variety of techniques can be used in the selection process to enrich the Fab output for protease inhibitors. Antibodies represent a new generation of selective protease inhibitors.

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Trends in scientific & patent production on antibodies and related technologies

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Journals, industry reports, company communications, and conferences such as Human Antibodies & Hybridomas, regularly show the growth in the number of antibodies that old and new technologies allow to generate, identify, produce, and/or characterize. Many databases and search techologies give the possibility to extract, from the large body of scientific & patent publications on these subjects, the information that can be potentially relevant for taking decisions of scientific, technical, commercial, or legal importance. The presentation will provide some examples on how trends in scientific & patent production on antibody-related technologies and products can be generated using appropriate search strategies and what such analyses can tell on the state of the industry.