# Fragment-Based Screening Using Surface Plasmon Resonance Technology

Samantha Perspicace, David Banner, Jörg Benz, Francis Müller, Daniel Schlatter and Walter Huber

*J Biomol Screen* 2009 14: 337
DOI: 10.1177/1087057109332595

# Fragment-Based Screening Using Surface Plasmon Resonance Technology

SAMANTHA PERSPICACE, DAVID BANNER, JÖRG BENZ, FRANCIS MÜLLER, DANIEL SCHLATTER, and WALTER HUBER

Surface plasmon resonance (SPR) technology has emerged as a new and powerful technique to investigate the interaction between low-molecular-weight molecules and target proteins. In the present work, the authors assemble from a large compound collection a library of 2226 molecules (fragments having low molecular weights between 100 and 300 Da) to screen them for binding to chymase, a serine protease. Both the active chymase and a zymogen-like form of the protein were used in parallel to distinguish between specific and unspecific binding. The relative ligand-binding activity of the immobilized protein was periodically measured with a reference compound. The screening experiments were performed at 25 °C at a fragment concentration of 200 µM in the presence of 2% DMSO. Applying the filter cascade, affinity–selectivity–competition (competition with reference compounds and cross-competition with fragments), 80 compounds show up as positive screening hits. Competition experiments between fragments show that they bind to different parts of the active site. Of 36 fragments cocrystallized for X-ray studies, 12 could be located in the active site of the protein. These results validate the authors' library and demonstrate that the application of SPR technology as a filter in fragment screening can be achieved successfully. (*Journal of Biomolecular Screening*. 2009:337-349)

**Key words:** affinity, binding, selectivity, competition, high-throughput screening (HTS), surface plasmon resonance (SPR)

## INTRODUCTION

HIGH-THROUGHPUT SCREENING (HTS) is the tool most often used to carry out the search for new chemical compounds serving as lead structures for medicinal chemists.[1] Undoubtedly, there are many successfully performed HTS campaigns. However, the HTS strategy is complex and expensive and has, like every other method, a number of drawbacks that limit the probability of finding new compounds.[2,3] Therefore, the pharmaceutical industry is permanently exploring complementary and innovative technologies to improve the difficult process of the discovery of small-molecule drug candidates.[4] As one of these alternative methods, fragment-based screening has become a promising technology of pharmaceutical research.[5] Fragment-based screening is aimed at evolving new tight binder molecules in a step-by-step approach and is different from HTS, in which full-size compounds are screened in a 1-step procedure. Fragment-based screening involves the selection, screening, and optimization of so-called fragments. These are small, less complex molecules (sometimes also called scaffolds, needles, shapes, or binding elements) with much lower molecular weights (MW <200 Da) than those typically found in HTS compound collections.[6] The use of such fragments offers a number of attractive features compared with the use of classical compound libraries. Despite their low potency, fragments are always more efficient binders because, due to their lower complexity, they have higher binding energy per unit mass than the HTS hits. Without giving precise numbers, experts agree that a lower number of compounds is needed to fill the chemical space when working with fragments. The probability that fragments match the binding site of a target protein is higher, and it is easier to structurally validate and understand the binding interaction. However, at the end, precise information about the binding mode from X-ray structure analysis is a must for the subsequent chemical optimization process.

This chemical optimization process that comprises development of these fragments into leads and finally into drugs is, as a consequence of affinity enhancement, accompanied by an increase in molecular weight and most often in lipophilicity. The process can be done in several ways.[7] For example, fragments that bind in different parts of the target binding site can be rationally linked, promoted by innovative ideas from medicinal chemists and molecular modelers, to give larger and high-affinity ligands. Alternatively, the binding site can be used

F. Hoffmann-La Roche Ltd, Pharma Research, Discovery Technologies, Basel, Switzerland.

Received Oct 3, 2008, and in revised form Mon Dec 18, 2008. Accepted for publication Jan 6, 2009.

Journal of Biomolecular Screening 14(4); 2009
DOI:10.1177/1087057109332595

to guide self-assembling of fragments containing chemical groups that can react to link the fragments, an approach described as "click chemistry."[8,9] In a successful optimization process, the anchoring fragments that have been found to interact with the protein via discrete, specific contacts should preserve their binding modes even after linking them together into high-molecular-weight compounds.[5,10,11]

Special screening methods are generally needed for searching fragment hits because biological functional assays normally fail for compounds of very low affinity. Nuclear magnetic resonance (NMR) screening is one of the most productive fragment-based approaches through which it is possible to identify small-molecule inhibitors for a variety of targets.[10,11] On one hand, NMR is a beneficial method because it allows extracting structural information on the binding mode of the fragments.[10] On the other hand, the need for a significantly high protein concentration is rather disadvantageous. Moreover, the ligands are often not soluble at the required high concentrations. Mass spectroscopy is another method to discover weak binding ligands. It can offer the required sensitivity, but costs are high and interfacing problems such as polymer adsorption still have to be resolved satisfactorily.[12] X-ray crystallography can provide the most complete picture of fragment binding to a target. The binding orientation of the molecular fragments as determined in the crystal plays a critical role in guiding efficient lead optimization programs.[13] A drawback of applying X-ray to a fragment library is the low throughput of the technique. To circumvent this disadvantage, X-ray screening and NMR screening are often performed using compound cocktails.[14] The use of surface plasmon resonance (SPR) to screen for fragments has been recently demonstrated.[15,16]

The goal of this work is to present new selection criteria in such screens to reduce the number of false-positives. The use of an appropriate reference protein that enables testing of site-specific binding is demonstrated. Moreover, competition assays not only help to further reduce the number of false-positive hits but also give additional information of binding to different pockets of the active site.

We looked for fragments that bind in any of the pockets of chymase, a serine protease mainly found in mast cells.[17] Many secreted proteases have a pro-region attached to the N-terminus of the enzymes. Zymogen activation proceeds via proteolytic removal of these pro-regions. We demonstrate with our work that such a zymogen-like form (zymogen) can be used as a reference protein in a fragment screening to address the problem of selectivity of binding. Positive hits were identified according to selection criteria. In a 1st screen, these criteria were based on affinity and selectivity. The selectivity criterion was related to the binding of the compounds to the zymogen and to the active protein. The affinity filter was set to 3 times the noise level determined via standard deviation of negative controls. Compounds that were selected as positives in the 1st screen were further characterized by competition experiments using well-known inhibitors occupying most parts of the active site. Cross-competition of fragments indicated whether 2 given fragments bound to the same or different pockets in the active site. Finally, the fragment hits were also submitted for X-ray crystal structure determination.

## MATERIALS AND METHODS

### *Instruments*

Binding experiments were performed on a S51 Biacore instrument. The fragment solutions were prepared on a TECAN Genesis RSP 100 robot. Fluorescence measurements were performed on an SLM-AMINCO 8100 double-grating spectrofluorometer.

### *Proteins*

The synthetically made gene for chymase was purchased from GenScript Corp. (Piscataway, NJ). Chymase was cloned into the pET21a vector in a similar way as described by Takai and others.[18] The recombinant human chymase was expressed in BL21 (DE3) cells as an N-terminal fusion protein with a 19-amino-acid polypeptide carrying a 6xHis tag and a enterokinase cleavage site. Because the protein was expressed forming insoluble inclusion bodies, it had to be renatured. Isolated and purified inclusion bodies were modified under denatured conditions with glutathione and then dissolved in 6 M guanidine·HCl, 20 mM EDTA, pH 4.5. Refolding was done by a dilution of the protein solution into a large volume of refolding buffer: 50 mM Tris/HCl pH 8.0, 500 mM arginine, 1 mM EDTA, 0.5 mM cysteine. The preparation was kept at 4 °C for 2 days. The refolding solution was concentrated and dialyzed prior to a 1st chromatography. Zymogen chymase could be obtained by chromatography on Ni-NTA followed by a gel filtration. Enzymatically active chymase was obtained by cleaving off the 6xHis tag with enterokinase, whereas the zymogen form was further purified without enterokinase treatment. Both proteins were chromatographed on Superdex 75 in 50 mM MES, 150 mM NaCl, 1 mM TCEP, 10% glycerol at pH of 5.5. Finally, as shown by N-terminal sequencing and sodium dodecyl sulfate polyacrylamide gel electrophoresis, >98% pure protein was obtained. The purified proteins were monomeric as detected by analytical ultracentrifugation. Activated chymase was highly active in a fluorometric assay. The concentrations for the active and the zymogen protein were 450 μg/ml and 405 μg/ml, respectively.

### *Buffers*

The protein was dissolved in an MES buffer (50 mM MES pH 5.5, 150 mM NaCl, 1 mM TCEP, 10% glycerol). The immobilization buffer was 10 mM acetic acid pH 5.5. The

running buffer was 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, whereas the fluorescence titration buffer was 50 mM Tris pH 7.8, 150 mM NaCl, 1 mM TCEP.

### *Reference compounds*

For the binding experiments, we used 5 reference compounds (Ref I to Ref V) with different molecular weights. All of these reference compounds are active site binders.

### *Fragment library*

Compounds were selected from an internal Roche library containing about 149,000 fragments and from several libraries of external suppliers (BioNet, Maybridge, Fluka, Sigma-Aldrich, etc.) with a further 76,000 fragments. Criteria to select the compounds considered molecular weight, number of hydrogen bonds both as acceptors and as donors, number of rotatable bonds, and predicted log P values.

The fragment library itself consists of 2 sublibraries for which different criteria were applied. The filter applied for the 1st sublibrary was based on the rule of 3 reported by Congreve and others[19] to evaluate lead likeness. This is a modification of Lipinski's rule of 5 for drug likeness.[20,21] The 2nd sublibrary, with medium-sized fragments, was generated using the less stringent criteria of the Lipinski's rule of 5. From all this filtration, a fragment library was set up containing a final 2226 compounds dissolved as 10 mM solution in DMSO.

### *Immobilization of protein*

The proteins were immobilized on a CM5 sensor chip by amide coupling chemistry. The carboxylic acid groups in the adlayer were first activated by contacting them with a mixture of carbodiimide (750 mg/ml) and N-hydroxysuccinimide (115 mg/ml). The activated surface was then contacted with the protein solution at a concentration of 50 µg/ml. Approximately 6000 response units (RUs) of protein were immobilized, corresponding to a surface protein density of 6 ng/mm$^2$. Typically, the 3 spots of the S51 instrument were used as follows: 1 spot with active chymase, a 1st reference spot with zymogen, and a 2nd reference spot with uncoated dextran.

### *Determination of the amount of active protein*

Assuming linearity between molecular weight and refractive index, the percentage of active protein immobilized could be estimated from the saturation response of a reference compound according to equation 1.

$$R_{\max} = \frac{MW_{comp}}{MW_{prot}} \cdot R_{prot} \qquad (1)$$

$R_{max}$ is the saturation signal determined for the reference, $R_{prot}$ is the signal measured for the amount of immobilized protein [RU], and $MW_{comp}$ and $MW_{prot}$ are the respective molecular weights of reference compound and protein.

### *Binding experiments to test the binding activity of the immobilized protein*

The ligand-binding activity of the immobilized proteins was investigated with 2 known reference inhibitors, Ref I and Ref II. Primary sample solutions were prepared by diluting the DMSO stock solutions into the running buffer. Starting from the primary solutions, further dilutions were prepared on the Biacore instrument. Binding experiments were performed by injecting the solutions into the instrument over all channels in parallel (i.e., channels with active chymase, reference channel with zymogen, and reference channel with uncoated dextran). The time-dependent binding curves were monitored simultaneously. The flow rate was 40 µl/min. The surfaces were regenerated after each binding experiment by washing the surface with the running buffer. Kinetic rate constants and equilibrium dissociation constants (KD) were determined based on a 1:1 kinetic model using fitting procedures supplied by the instrument manufacturer (Biacore). Reference compound Ref II with its very fast kinetics and a KD value of ~10 µM was used during all screenings at a saturation concentration of 100 µM.

### *Sample preparation for screening*

Samples are obtained as 10 mM solution in a 384-well plate format. Two microliters of the DMSO samples were transferred into 96-well plates using a CiByWell Instrument equipped with a 96 pipette head. The 2-µl DMSO solutions were then diluted with HEPES buffer using a Multidrop instrument. The plates were then covered with a foil and mixed for 10 min on a shaker.

### *Sample preparation for competition assays*

Stock solutions were prepared in the same way as the solutions used for screening. Single compound solutions were prepared by diluting the stock solution by a factor of 2 with running buffer. Mixtures were prepared by mixing the 2 stock solutions in a 1:1 ratio. These operations were performed on a Tecan pipettor.

### *Binding experiments during screening*

For the screening, 10-mM compound solutions in DMSO were dispensed into the running buffer to obtain aqueous fragment solutions containing 2% DMSO. These solutions were injected over the protein sensor surface for 1 min. The flow rate was 40 µl/min, and after every 10 injections, the reference compound Ref II was injected as a control. This

control measurement was performed to check both the stability and ligand-binding activity of the immobilized proteins during a screen. All screenings were performed at 25 °C.

Two 96-well plates with 192 fragments and 16 control samples were screened per day. Regeneration of the surfaces between subsequent binding experiments was achieved by washing the surface extensively (30 s) with the running buffer plus 10% DMSO. To improve the quality of the initial screening data and to decrease the false-positive rate, the fragments were routinely screened in duplicate, always at 200 μM. Sensor responses were taken during each injection after a contact interval of 30 s. Report points were used for analysis. They were corrected for refractive index change using S51 Biacore software. The final responses were obtained by subtracting the corrected responses measured on the reference spot from the corrected responses from the measuring spot. Both signals were previously referenced against the uncoated dextran surface.

Positive hits were selected based on 2 criteria. The 1st was an affinity and the 2nd a selectivity criterion. Positive compounds at the given concentration generated an SPR signal that was larger than 3 times the standard deviation of the negative control. For ranking of the positive hits, the KD value could be estimated using equation 2 derived from the Langmuir adsorption isotherm.

$$KD = \frac{R_{\max} \cdot C}{R} - C \tag{2}$$

$R_{max,}$ R, and C correspond to the normalized saturation response of the reference compound, the normalized response of the test compound, and the concentration of the test solution, respectively. Equation 2 allows estimation of KD in only a limited range, that is, for KD values that are relatively close to the screening concentration ($0.1 \cdot KD < c < 10 \cdot KD$).

A selectivity factor was calculated by dividing the response of a given compound with the active protein by that obtained with the zymogen. A positive hit should not only fulfill the above affinity criterion but also have a selectivity factor >2.

### *Robustness of the screening*

To evaluate and validate the quality of HTS assays, the Z′ factor introduced by Zhang and others[22] was calculated according to equation 3:

$$Z' = 1 - \frac{3\sigma_s + 3\sigma_b}{\mu_s - \mu_b} \tag{3}$$

σ and μ are the standard deviation and the mean of the assay response, respectively. The indices s and b denote the positive (maximum assay response) and the negative (assay response in the absence of binding) control, respectively. The Z′ factor is a measure of the quality or power of the HTS assay. It compares the variations ($3\sigma_S + 3\sigma_b$) of the assay data with the signal window ($\mu_S - \mu_b$). The maximum value of the Z′ factor is 1. In this case, the standard deviations are negligible compared with the signal window. An assay with a Z′ factor >0.8 is considered as very good, and a Z′ factor >0.6 is good. For Z′ factors <0.5, the assay is not robust enough to be used for primary screening; that is, the test of compounds at 1 concentration will not lead to statistically relevant results.

### *Reproducibility testing*

For the reproducibility testing, compounds were tested in duplicate. All of the steps, such as sample preparation, injection mode, washing procedures, and data evaluation, were included in this duplication.

### *Competition experiments with reference compounds*

The competition experiments were performed by sequentially injecting the solution of the test compound (fragment) at 200 μM, the reference compound at saturation concentration (100 μM), and a mixture of fragment (200 μM) and reference compound (100 μM) each for 1 min. A qualitative comparison of the signals observed for test compound, reference compound, and mixture was sufficient to distinguish between site-selective and non–site-selective binders. Compounds that bound to the same binding pocket as the reference compound showed a signal for the mixture with reference compound that was identical to the signal observed for the reference compound alone. Compounds that bound independently to an alternative site showed a signal for the mixture that corresponded to the sum of the signals determined individually for the reference and the test compound alone.

### *Cross-competition experiments between fragments*

Cross-competition experiments between fragments were performed in a similar way as the competitive assay. Instead of using a reference compound, another fragment was used to run the competition experiment. The evaluation of competitiveness was analogous to that when working with reference compound. When fragments occupied different pockets, the response observed for the mixture was the sum of the 2 individual responses observed for the fragments. Because saturation was seldom reached with low-affinity fragments, the response observed in the case of competition (binding to the same binding pocket) was given by the sum of the fractional occupancies (FO) of both compounds. Theoretically, such fractional occupancies and therefore the expected signal of the mixture of the 2 compounds can be calculated using equations 4.

$$FO(A) = \frac{1}{1 + \frac{KD_A}{C_A}\left(1 + \frac{C_B}{KD_B}\right)} \qquad FO\left(\underline{B}_{10}\right) = \frac{1}{1 + \frac{KD_B}{C_B}\left(1 + \frac{C_A}{KD_A}\right)} \quad (4)$$

$KD_A$ and $KD_B$ are the equilibrium dissociation constants of the competing compounds, and $C_A$ and $C_B$ are the respective concentrations. A more detailed discussion of this equation is given in the Results section.

### *Fluorescence quenching experiment*

Binding experiments in solution were carried out by fluorescence titration at 20 °C with reference compound Ref I. The protein concentration was 0.89 µM in the fluorescence titration buffer. Tryptophan fluorescence of the protein, excited at 280 nm, was recorded at 340 nm. Small aliquots of known concentration of compound Ref I, dissolved in DMSO, were added to the protein solution and each time the fluorescence intensity was measured. These fluorescence intensities, corrected for ligand adsorption,[23] were plotted against the total ligand concentration and fitted with a 4-parameter sigmoidal function. The KD value was obtained applying the law of mass action.[24]

### *X-ray crystallography*

Crystals of chymase were grown under standard conditions in the presence of ligand at 10-mM concentration or at saturation. Crystals were flash frozen and measured at the Swiss Light Source at the Paul Scherrer Institute, Villingen, Germany. Data were collected to a resolution of 1.75 Å or better.

## RESULTS

### *Binding of reference compound monitored by Biacore*

Binding experiments were generally performed with an amount of immobilized protein that corresponds to 6000 RU. **Figure 1** depicts typical binding curves monitored during the contact time with a solution of 2 reference compounds, Ref I and Ref II. Curves monitored from the channel with the active chymase (left) and from that with the zymogen (right) are shown. The reference compounds used bound only to the active protein. The nonactivated zymogen could therefore serve as an ideal reference protein during the screen to distinguish between specific and unspecific binding. For reference compound Ref I, the process of binding and dissociation could be resolved. Reference compound Ref II showed fast-on and fast-off kinetics that could not be resolved. Because the curves observed for the 2 reference compounds were reproducible over days, it was concluded that once immobilized, the protein is sufficiently stable to perform screening. The curves in **Figure 1** indicate that the saturation response for reference compound Ref I is approximately 60 RU. Using the 2 molecular weights of the immobilized protein and reference compound Ref I (30,000 Da and 456 Da, respectively) and the amount of immobilized protein ($R_{prot}$), the theoretical saturation response for 100% active protein is estimated to be 91 RU, according to equation 1. Comparing theoretical and experimental saturation response, the percentage of immobilized active protein is estimated to be about 66%. The experimental binding curves monitored from the channel with active protein for reference compound Ref I were fitted assuming a 1:1 complex. The equilibrium dissociation constant for the reference compound Ref I extracted by this procedure is 290 nM (**Figure 1, top, left**).

The kinetic behavior of reference compound Ref II was totally different from that of reference compound Ref I. The fast association and dissociation processes for reference compound Ref II did not allow for a fit of the curves with a kinetic model. Therefore, KD was determined via the analysis of a Langmuir adsorption isotherm or of a Scatchard plot. The adsorption isotherm for reference compound Ref II is shown in **Figure 2**. In this representation, the responses monitored at a given concentration of reference compound Ref II are plotted against the concentration. The plot can be fitted by a Langmuir adsorption isotherm describing the reversible formation of a 1:1 complex at the interface.

The fit of the adsorption isotherm of reference compound Ref II in **Figure 2** (**left**) gave a KD value of ~10 µM. The same KD resulted when subjecting the measured responses to a Scatchard-type analysis (**Figure 2, right**). The good linearity observed indicated a high homogeneity of the surface with respect to the protein-binding activity. It also indicated that a specific, low-affinity binding occurs with the reference compound Ref II. This compound represented an ideal control for the ligand-binding activity of the enzyme during the fragment screen because it dissociated quickly from the surface and the regeneration interval between fragment injections could be chosen to be very short. This sped up the whole screening process.

### *Fluorescence*

As a control, the affinity of reference compounds was also tested in homogeneous solution by an intrinsic fluorescence titration experiment. **Figure 3** shows a typical titration curve using reference compound Ref I. The measured fluorescence intensities, corrected for ligand absorption, were unambiguously fitted to a sigmoidal curve (solid line). Assuming a 1:1 binding model, a KD value of 300 nM was obtained, in agreement with the value of 290 nM determined by SPR. Thus, the immobilization did not influence the protein ligand-binding activity.

### *Determination of Z′ factor*

Z′ factors were determined for 4 reference compounds with different molecular weights. They were obtained by alternatively injecting solutions from a reference compound at saturation

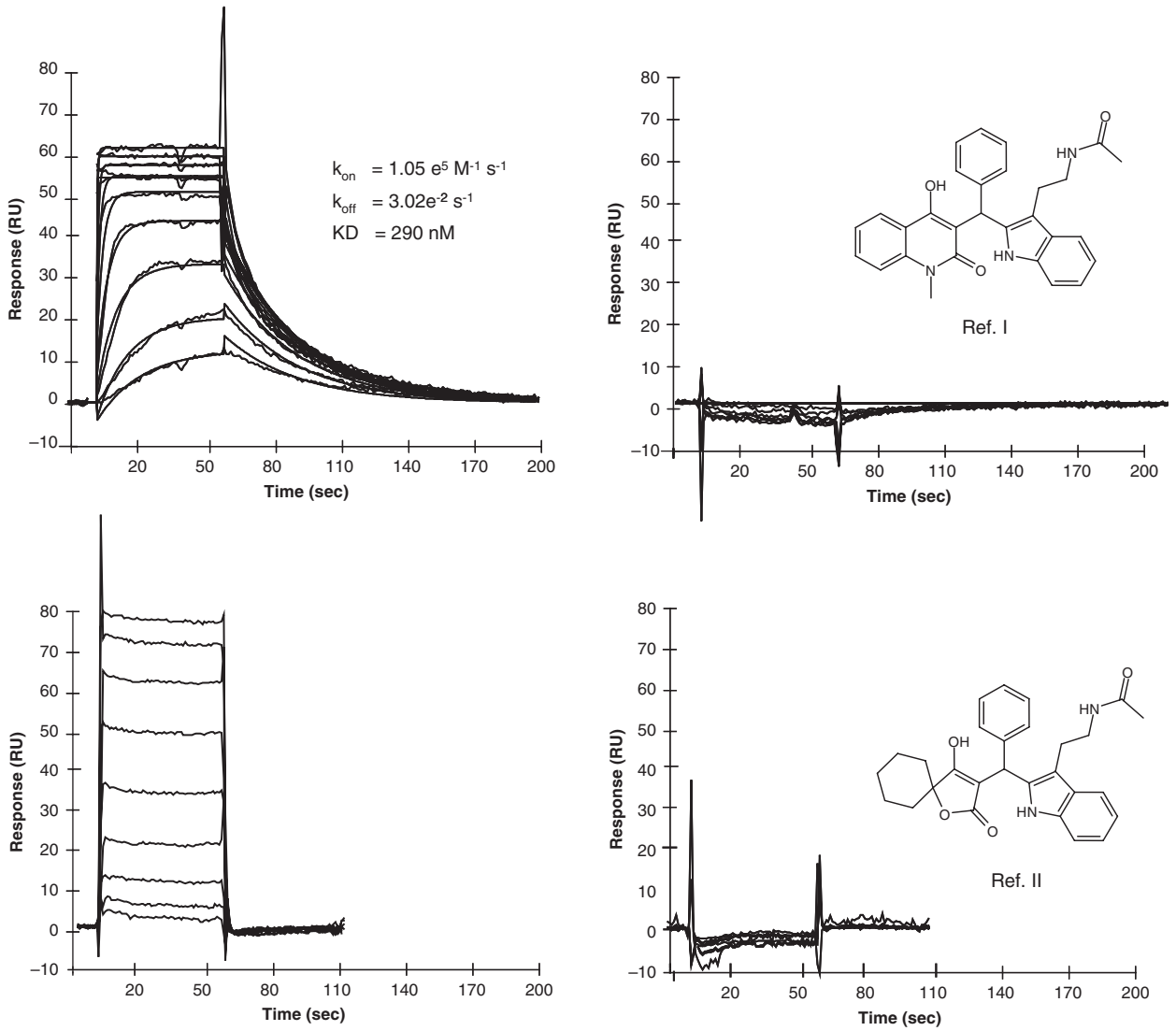


**FIG. 1.** (**Top**) Concentration-dependent binding curves monitored for reference compound Ref I in contact with activated chymase (**left**) and inactive zymogen (**right**). The black lines overlaid on the experimental curves (**left**) are the curves resulting from the fit of the curves with the mathematical function of a kinetic 1:1 binding model. (**Bottom**) Concentration-dependent binding curves of reference compound Ref II monitored in contact with active chymase (**left**) and in contact with inactive zymogen (**right**).

concentration and from a negative control. The solutions were prepared in the same way as the sample solutions during the screening by mixing the DMSO stock solution with the buffer solution. The Z′ factors were calculated from the monitored responses using equation 3. As expected from this equation, they are highly dependent on the saturation signal of the reference compound, which is related to the molecular weight of the compound. The Z′ factors are summarized in **Table 1**.

### *Reproducibility testing*

**Figure 4** shows the results from reproducibility testing for the samples of one 96-well plate. The statistical data of the correlation of the responses from each plate indicate that the measurements are highly reproducible. The slope of the correlation is as expected (1.0), and the standard error is about 2.4. The plate contained only a few very weak binders.

### *Fragment-based screening*

**Figure 5** shows the results of the screen of one 96-well plate in a graphical representation. The plate contained 96 solutions of test compounds and 8 solutions of the reference compound Ref II. In addition, 4 negative controls (buffer with DMSO) were injected during the run. The graph shows the height of the responses at the report points that were set during the association

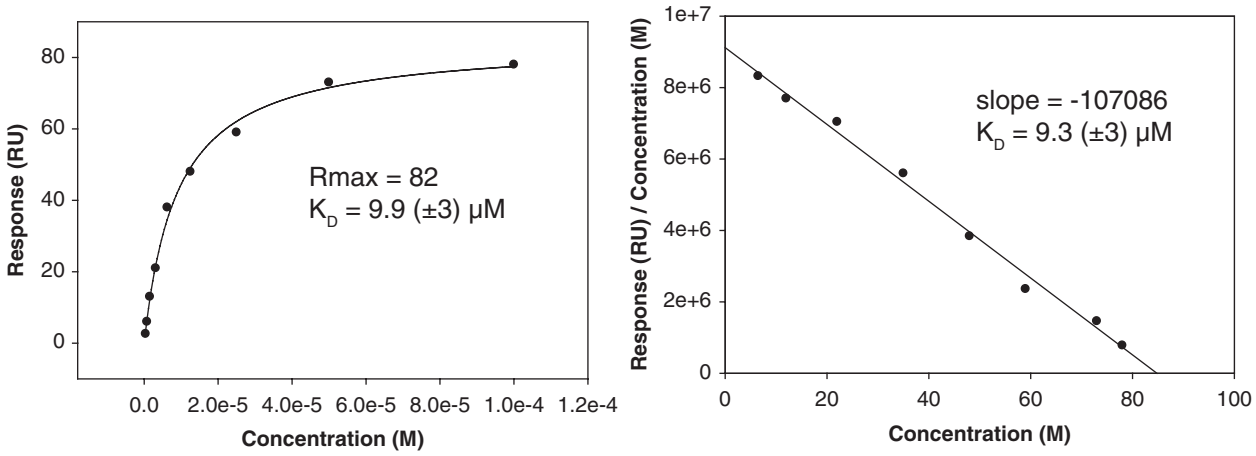


**FIG. 2.** Langmuir adsorption isotherm for the concentration-dependent binding of reference compound Ref II to the active chymase (**left**) and corresponding Scatchard plot (**right**).

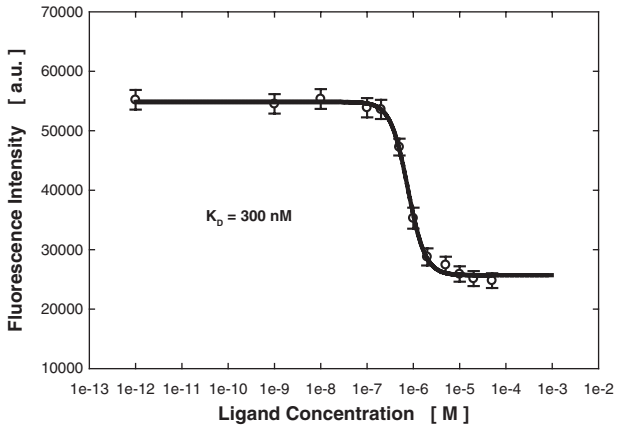


**FIG. 3.** Fluorescence titration of chymase in Tris buffer, pH 7.8, with reference compound Ref I. The measured intensity, corrected for ligand absorption, is plotted against the total concentration of added ligand. Excitation and emission wavelengths are 280 and 340 nm, respectively. The plot is fitted to a sigmoidal curve. The obtained dissociation constant KD is 300 nM.

**Table 1.** Z′ Factors Determined for 4 Reference Compounds With Different Molecular Weights[a]

| | *Ref II* | *Ref III* | *Ref IV* | *Ref V* |
|---|---|---|---|---|
| Molecular weight | 450 | 319 | 252 | 161 |
| Average response units (ref) | 51 | 43 | 21 | 11 |
| SD (ref) | 1.0 | 1.2 | 0.7 | 0.8 |
| Average response units (negative control) | 0 | 0 | 0 | 0 |
| SD (negative control) | 0.9 | 0.9 | 0.9 | 0.9 |
| Z′-factor | 0.88 | 0.85 | 0.77 | 0.54 |
| Normalized Z′ factor[b] | 0.80 | 0.82 | 0.77 | 0.72 |

a. Reference compounds were obtained by injecting alternatively solutions from a reference compound at saturation concentration and from a negative control. The Z′ factors were calculated from the monitored responses using equation 3. As expected from this equation, they are highly dependent on the saturation signal of the reference compound, which is related to the molecular weight of the compound.
b. Z′ factors calculated using molecular weight–normalized sensor responses.

phase. The responses measured for the zymogen are shown as white bars and the responses from the active protein as black bars. The responses measured for the 8 reference compounds injected as control during the run are marked with a black star. The black horizontal dashed line indicates 3 times the average noise level determined from responses observed for the negative controls. The low variation in the signal of the reference compound shows, on one hand, the high stability of the protein during the screen and, on the other hand, the high reproducibility of the assay. Comparison of white and black bars of the reference compound indicates that at the selected concentration, the selectivity factor is in the range of 8. The selection criteria for positive hits were based on the affinity and the selectivity of binding. In a 1st filtering, compounds were selected only if their affinity led to a sensor response that corresponded to 3 times the noise level (**Fig. 5**, black dashed line). It can be seen that only a few compounds from this representative set fulfilled this affinity criterion. This number was further reduced when the selectivity filter was applied. Applying a very loose filter, only compounds with a selectivity factor less then 2 were eliminated from the compounds that passed the affinity filter. Applying the affinity and selectivity criteria to the screening data in **Figure 5**, only 5 compounds (▼) fulfilled the 2 criteria. From the set of 2226 compounds selected for the fragment library, 24 compounds were eliminated as promiscuous compounds according to the criteria of over stoichiometric binding, precipitation, and/or

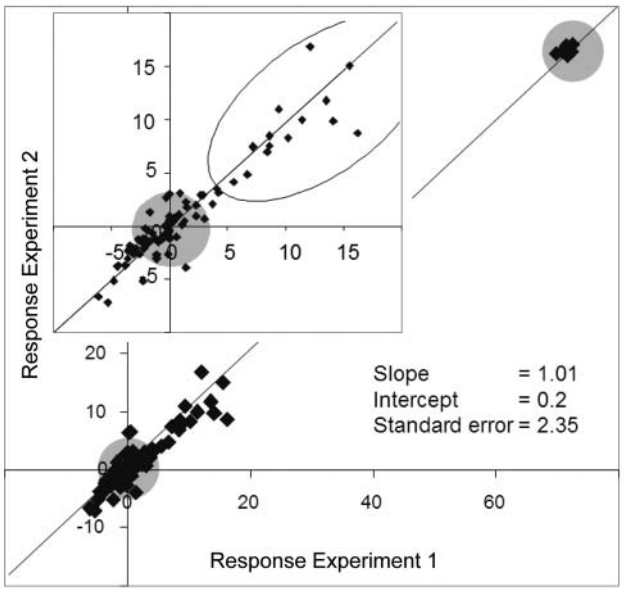


**FIG. 4.** Reproducibility testing for 96 compounds. Correlation of the responses measured from 2 independent experiments.

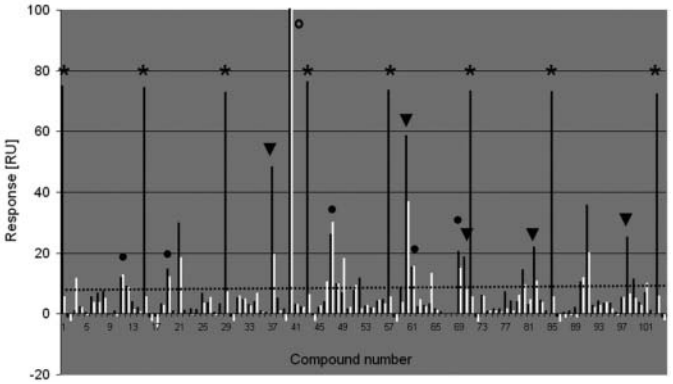


**FIG. 5.** Representation of the screening results of one 96-well plate. The signals of 96 test compounds and 8 reference compounds (marked with *) are shown. The responses are plotted as bars along the *x* axis. Bars are arranged pairwise; black bars represent the response monitored on the active protein, and white bars represent the response monitored on the zymogen. Typical promiscuous (○), nonselective (●), and selective binders (▼) are marked. The dashed black line represents the threshold value of 3 times the standard deviation of the negative control.

a sensogram that indicates nonequilibrium behavior.[25] A total of 734 compounds (33%) showed a sensor response that was higher than the threshold (i.e., higher than 3 times the standard deviation of the negative control). By applying the selectivity filter, the number of hits was reduced to 180 fragments (~8%).

### *Results of competition experiments*

**Figure 6** (**top**) summarizes graphically the results of the competition experiments that were performed with 4 fragments (A, B, C, D) and the reference compound Ref I. The figure shows the same behavior for all compounds. The response observed for all mixtures (light gray bars) is similar to the saturation signal of the reference. According to the theoretical background given in the Materials and Methods section, it was straightforward to conclude that all of these compounds bound to the active site. This was not surprising because these compounds were preselected based on their selectivity for the active protein. **Figure 6** (**bottom**) illustrates the results obtained when performing cross-competition experiments with fragments. In this graph, the observed signal for the mixture of 2 fragments (light gray bars) is compared to the calculated sum of the individual responses (dark gray bars). Two combinations, A+B and A+D, showed a similar behavior as that observed for the competition experiments with the reference compound Ref I. The response measured for the mixture was equal to the sum of the individual signals. For the 3rd combination, A+C, the measured response of the mixture was intermediate between the individual responses and their sum.

The interpretation of such a finding is complex and completely different from the situation described in the 1st experiment of **Figure 6**. Whereas a competing reference compound with high affinity that saturated all the binding sites was used in the 1st experiment (competition), the competitors in the 2nd experiment (cross-competition) were 2 fragments of low affinity. For these fragments, there is neither full occupancy nor full displacement of one compound by the other. The theoretical background to estimate the expected occupancies of binding sites by competing compounds for such a situation is given by equations 4. **Figure 7** shows graphically the results obtained by applying these equations to hypothetical KD values and concentrations. In the case of noncompetition (i.e., in the case of independent binding sites for each of the compounds), the occupancy of the binding sites, when injecting the mixture of both compounds, is always the sum of the individual occupancies. This holds also for the respective responses. It is therefore straightforward to conclude that compound Ref I occupies a different binding site than B and D because the response of their mixtures corresponds always to the sum of the individual responses.

In the case of competition between compounds of similar affinity and equal concentration, the occupancy of the binding sites by one of the components is heavily influenced by the presence of the other. This is clearly seen in **Figure 7** (**bottom**) by the shift of the curves 1 and 2 to 1a and 2a. The occupancy of the binding site, when injecting the mixture, lies always between the individual occupancies and the sum of the 2 individual occupancies. From the estimation of the occupancies, the expected signal of the 2 compounds could be estimated using equation 5.

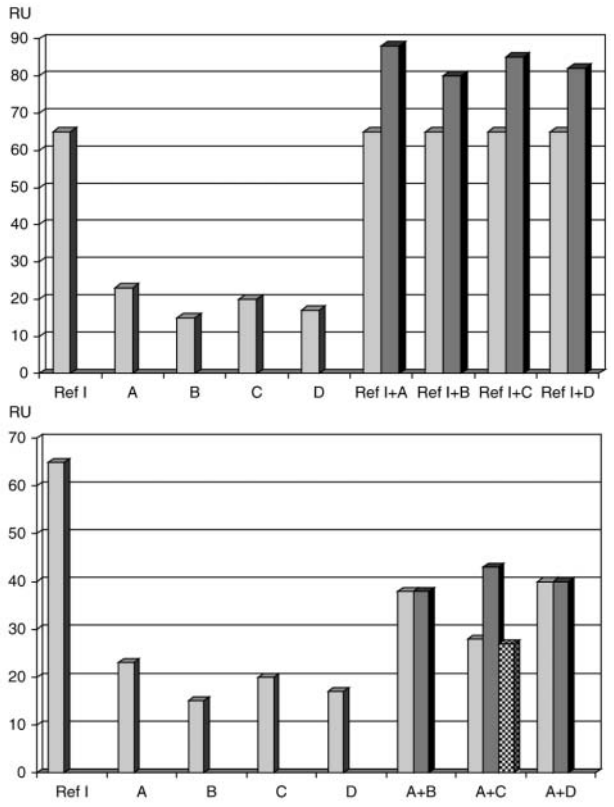


**FIG. 6.** (**Top**) Competition assay between fragments and reference compound Ref I. The measured responses for Ref I (100 μM) and compound (200 μM) are represented by a vertical light gray bar. The experimental responses measured for the mixture (Ref I + compound) are shown in light gray bars. The calculated signal for the mixture (Ref I + compound) expected for different binding sites is represented by dark gray bars. (**Bottom**) Cross-competition assay between fragments that occupy different or the same binding pockets of the protein. The measured responses for reference compound Ref I (100 μM) and compound (200 μM) are represented by a vertical light gray bar. The experimental responses measured for the mixture (A+B, A+C, A+D) are shown by a light gray bar, and a calculated signal for the mixture (A+B, A+C, A+D) expected for different binding pockets is represented by a dark gray bar. For the combination A+C, the measured response of the mixture was intermediate between the individual responses. Their sum calculated according to the fractional occupancies (equation 5) is represented by a dark gray bar with stars.

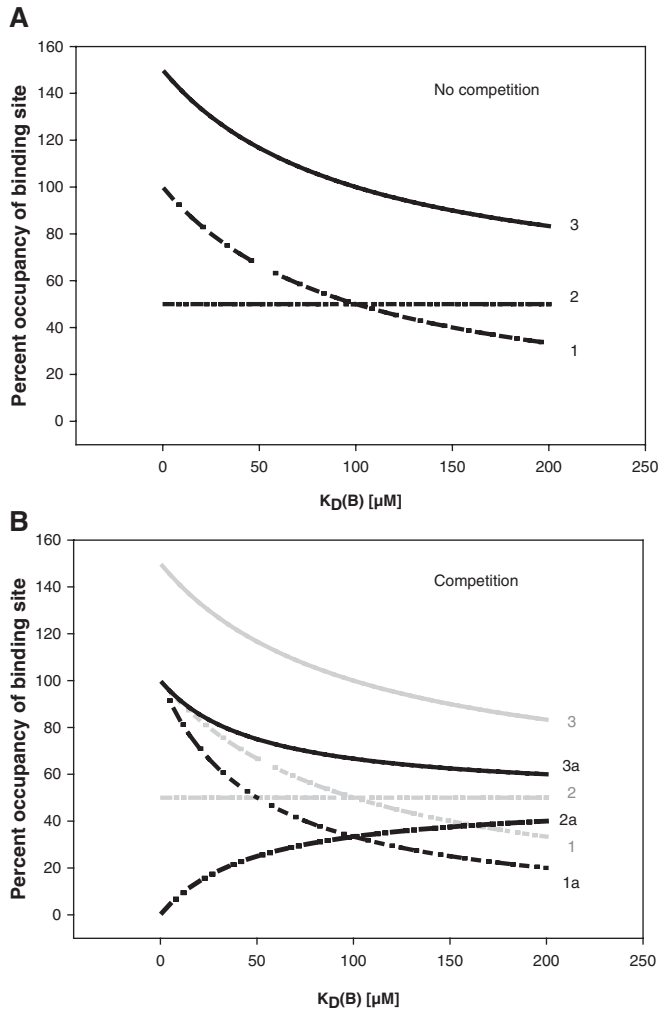


**FIG. 7.** Fractional occupancies for contacting an immobilized ligand with 2 analytes A and B depending on the equilibrium binding constant of analyte B. The parameters are chosen as follows: KD of test analyte (**A**): 100 μM; concentration of compound A: $C_A$ = 100 μM; KD of competitor (**B**): variable; concentration of compound B: $C_B$ = 100 μM. (**Top**) Curve 1: competitor alone; curve 2: analyte alone; curve 3: noncompetitive. (**Bottom**) Curve 1a: competitor (analyte present); curve 2a: analyte (competitor present); curve 3a: competitive binding, analyte + reference.

$$R_{observed} = FO(A) \cdot R_{max}(A) + FO(B) \cdot R_{max}(B) \quad (5)$$

in which $R_{max}$(A) and $R_{max}$(B) are the saturation responses of the 2 compounds and FO(A) and FO(B) are the respective fractional occupancies. **Table 2** contains the measured responses, the KD values, the $R_{max}$, and the concentrations to calculate the expected responses for different fragment mixtures. The estimation of the response for the signal of the mixture A+C gives a value of 27 RU for competition and a value of 43 RU for noncompetition, as shown in **Figure 6** (**bottom**) by the dark gray bar with stars and the dark gray bar, respectively. The result is clearly in favor of competition and identical binding sites for A and C.

From the 180 fragments found by the affinity and selectivity filter, 80 compounds showed such a competitive behavior. A statistically relevant correlation between degree of selectivity and competition behavior could not be extracted from these data.

**Table 2.** Observed and Calculated Responses of a Reference Compound, Some Fragments, and Their Mixture[a]

| Compound | Measured Response (RU) | MW (Da) | KD (μM) | $R_{max}$ Calculation (RU) | Response for Mixtures Calculation (RU) | |
|---|---|---|---|---|---|---|
| | | | | | No Competition | Competition |
| Ref I | 65 | 466 | 0.29 | 65 | | |
| A | 23 | 270 | 129 | 38 | | |
| B | 15 | 287 | 336 | 40 | | |
| C | 20 | 244 | 142 | 34 | | |
| D | 17 | 244 | 202 | 34 | | |
| A+B | 38 | | | | 38 | 26 |
| A+C | 28 | | | | 43 | 27 |
| A+D | 40 | | | | 40 | 26 |

a. The KD values were estimated from the Langmuir adsorption isotherm. The calculated responses of mixtures were obtained according to equation 5 (see text) in the case of competition and by just adding the individual observed responses in the case of no competition. Obviously, A and C compete for the same binding site.

Besides having confirmed the hits from the primary screen by competitive experiments, the fragment hits were also analyzed by X-ray crystallography. Of the first 36 crystals measured, 12 showed clear ligand electron density. **Figure 8** shows 2 examples.

## DISCUSSION

Three-dimensional structure information, most often extracted from X-ray structure analysis, is of utmost importance in fragment-based drug-discovery approaches. It is needed for the selection of the best fragments with respect to orientation in the binding pocket and for the determination of suitable exit vectors to start chemistry. Because X-ray structure analysis is a time- and material-consuming process, methods are needed to reduce the number of potential candidates from fragment libraries for X-ray trials. The present work introduces a fragment-screening approach that makes extensive use of several SPR-based binding assays. This leads to a reduction of the number of candidates from the library to 3.6%. A direct binding assay is used to determine the affinity of the fragments for active chymase. A 2nd direct binding assay that is performed in parallel with the inactive zymogen is used to test for active site-specific binding. This assay further reduced the number of candidates to 8%. Active site specificity of fragment binding is further confirmed with a competition assay using a competitor compound that fits and occupies the whole active site. Only 44% of the remaining compounds (3.6% of the total) passed this competition filter. The result of such extensive SPR work is a set of compounds with a relatively high positive hit rate (30%) in crystallization trials. The SPR screening cascade has proven to be an efficient tool to select fragments worth being submitted to crystallization trials.

A highly robust and reliable binding assay is a prerequisite for success of such SPR-driven filtering. In this work, the Z′ factor has been taken as a possible measure. During HTS campaigns, Z′ factors (see equation 3) are often used to check stability of the assay and goodness of the screen. Although Z′ factors used in SPR have some fundamental differences to Z′ factors used in HTS (see below), the use of such Z′ factors is also highly recommended for SPR screens, because it considers all relevant parameters that indicate instabilities of an assay during a screen such as change in the standard deviation of positive and negative controls and change in the ligand-binding activity of the immobilized protein. It represents therefore a single measure to compare assay quality and stability from plate to plate.

In contrast to Z′ factors in HTS, the Z′ factor of SPR-based binding assays should not be taken as an absolute quality criterion to compare different assays because Z′ factors of SPR binding assays are dependent on the saturation sensor response and hence from the optical mass of the compounds taken as references. This optical mass is roughly correlated with the molecular mass of compounds of similar elemental composition. Over the molecular weight range (451 Da ≥ MW ≥ 161 Da) covered by our reference compounds (Ref II to Ref IV), the Z′ factor changes from nearly 0.9 (450 Da) to about 0.5 (161 Da). **Table 1** shows that Z′ factors become similar within ±10% when using molecular weight-normalized sensor responses for the saturation signal of the positive control. To become a meaningful measure of quality that is comparable, assays and screens such Z′ factors should be adapted to the average molecular weight of the compound library screened. The average molecular weight of our fragment library is about 215 Da, and the relevant Z′ factor is about 0.77. This value indicates that testing the compounds of this library with this assay leads on average to statistically relevant results that enable

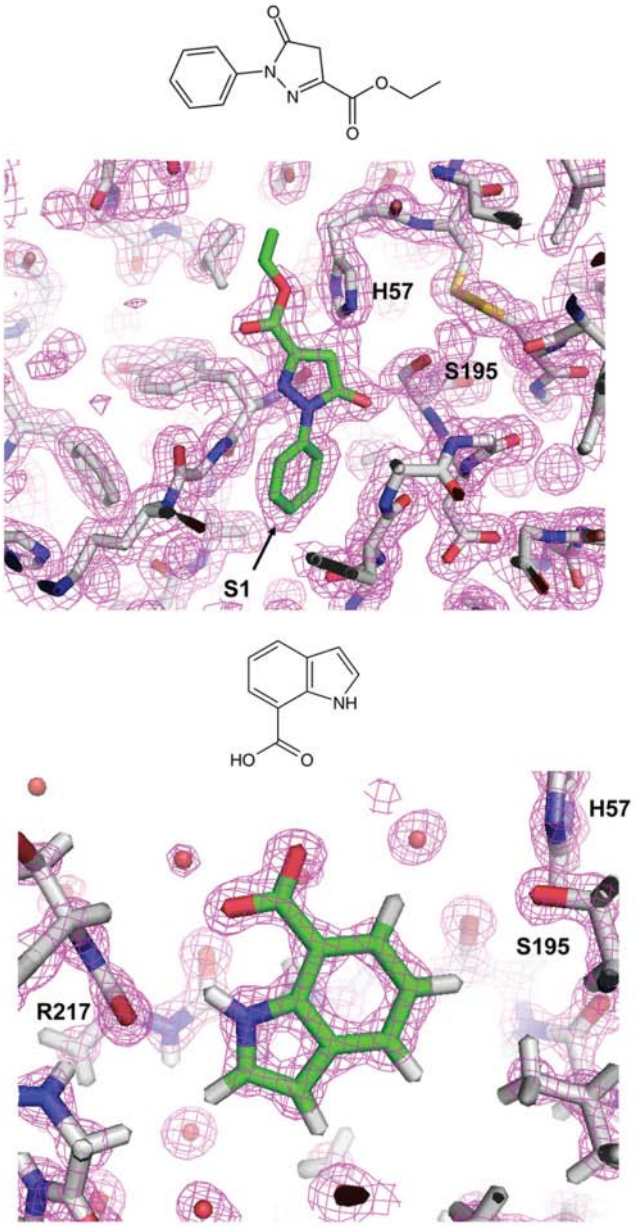


**FIG. 8.** Examples of fragment-binding modes determined by X-ray crystallography. (**Top**) A typical bound fragment: The phenyl group is inserted into the S1 pocket, and the keto oxygen is in the oxyanion hole. The electron density determined at 1.73 Å resolution is contoured at 1.0 rms. It may be seen that the ester group is largely degraded to the acid. (**Bottom**) A smaller fragment bound in the S1 pocket. The electron density determined at 1.0 Å resolution is contoured at 2.0 rms. Although the binding mode is extremely well determined, there appears to be no directed interaction, such as a hydrogen bond, which determines the precise position. All of the fragment structures determined show aromatic groups in S1, and many have acid groups or oxygen atoms in the oxyanion hole. This agrees with the known preference of chymase to cleave peptides after tyrosine, phenylalanine, or tryptophan.

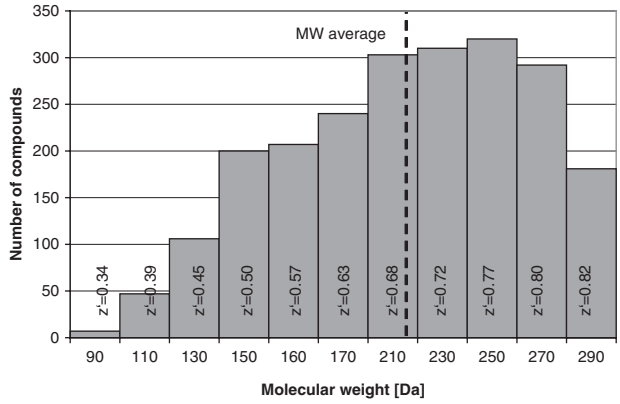


**FIG. 9.** Molecular weight distribution of the fragment library and the Z′ factor that are relevant for the different molecular weight segments. These Z′ factors have been estimated based on the molecular weight dependence shown in **Table 1**.

differentiation of positives and negatives. However, one should still keep in mind that libraries have a certain molecular weight distribution and that statistical relevance of SPR-assay results changes with the molecular weight of the tested compounds. **Figure 9** shows schematically the molecular weight distribution of our fragment library and the Z′ factors that are relevant for the different molecular weight segments. These Z′ factors have been estimated based on the molecular weight dependence shown in **Table 1**. The figure shows that, according to the Z′ factor, no statistically relevant results can be expected for 7% of our library with MW <140 Da. In addition to quality control, the Z′ factor can be used to determine the minimum molecular weight and the percentage of compounds of a given library for which statistically relevant data could be expected.

This discussion of the Z′ factor also points at which parameters have to be optimized to increase the Z′ factor for compounds of the low-molecular-weight segment of a library. Often, the standard deviations of positive and negative controls in the numerator of equation 3 are quite constant from assay to assay because they are mainly determined by instrumental parameters (temperature stability, fluidic system, etc.) and buffer conditions. Z′ factor and thus assay quality can therefore be improved by making the denominator in equation 3 larger (i.e., by optimizing the immobilization procedure for maximum response). The theoretically calculated ligand-binding activity of the protein (66% of the immobilized protein active) is already quite high, and room for improvement along this line is limited for this assay. Protein-engineering techniques would be required that enable directed immobilization and 100% preservation of binding activity of the protein upon immobilization.

Beside assay robustness and quality, testing of primary hits with assays enabling selectivity filtering is another important

prerequisite for efficient selection of candidates from fragment libraries for crystallization trials. Affinity filtering based on responses monitored at a single concentration would have eliminated in the present case only 67% of the compounds as negatives. Especially in this case, where protein/ligand crystals had to be prepared by co-crystallization, the remaining 730 compounds would have been by far too many. Only the application of the 1st selectivity filter using the inactive zymogen as a reference protein and the selectivity filter using competition experiments reduced the library to a manageable number of 80 compounds. A hit rate of 30% for such crystallization experiments is excellent, considering that there are severe differences in buffer conditions between SPR binding experiments and crystallization experiments.

In this work, the potential of competition experiments is further exploited by performing cross-competition experiments between fragments. In the present case, 2 pockets that are located in the active site of the protein, but that can bind independently different fragments, have been identified by cross-competition experiments and by crystallography. Finding such fragments can significantly influence the follow-up strategy of a fragment screen. Such independently binding fragments could in principle be directly linked to give larger high-affinity compounds. In the absence of such independently binding fragments, a "fragment evolution" strategy has to be applied.

In summary, the present work shows the efficient use of a SPR-based screening cascade for the selection of fragments from a library for crystallization trials. As already mentioned in the introduction, many other techniques (NMR, mass spectroscopy, and thermal shift experiments) described in the literature are suitable for fragment screenings. The main advantages of the SPR technology compared with others reside in low material consumption and relatively high throughput. Only a few micrograms of protein were needed for immobilization on the surface. Such a surface could be used for up to 600 binding experiments.

## ACKNOWLEDGMENTS

We thank Eric Kusznir, Josiane Kohler, and Martine Stihle for their skilled experimental work.

## REFERENCES

1. Bohacek RS, McMartin C, Guida WC: The art and practice of structure-based drug design: a molecular modeling perspective. *Med Res Rev* 1996;16(1):3-50.
2. Fox S, Wang H, Sopchak L, Khoury R: High throughput screening: early successes indicate a promising future. *J Biomol Screen* 2001;6(3):137-140.
3. Hann MM, Leach AR, Harper G: Molecular complexity and its impact on the probability of finding leads for drug discovery. *J Chem Inf Comput Sci* 2001;41(3):856-864.
4. Johnston KA: Chemical attraction: affinity techniques aid in new drug discovery. *Pharma Genomics* 2001;28:30-39.
5. Albert JS, Blomberg N, Breeze AL, Brown AJ, Burrows JN, Edwards PD, et al: An integrated approach to fragment-based lead generation: philosophy, strategy and case studies from AstraZeneca's drug discovery programmes. *Curr Top Med Chem* 2007;7(16):1600-1629.
6. Gribbon P, Sewing A: Fluorescence readouts in HTS: no gain without pain? *Drug Discov Today* 2003;8(22):1035-1043.
7. Huc I, Lehn JM: Virtual combinatorial libraries: dynamic generation of molecular and supramolecular diversity by self-assembly. *Proc Natl Acad Sci U S A* 1997;94(6):2106-2110.
8. Erlanson DA, Lam JW, Wiesmann C, Luong TN, Simmons RL, DeLano WL, et al: In situ assembly of enzyme inhibitors using extended tethering. *Nat Biotechnol* 2003;21(3):308-314.
9. Krasinski A, Radic Z, Manetsch R, Raushel J, Taylor P, Sharpless KB, et al: In situ selection of lead compounds by click chemistry: target-guided optimization of acetylcholinesterase inhibitors. *J Am Chem Soc* 2005;127(18):6686-6692.
10. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW: Discovering high-affinity ligands for proteins: SAR by NMR. *Science* 1996;274(5292):1531-1534.
11. Boehm HJ, Boehringer M, Bur D, Gmuender H, Huber W, Klaus W, et al: Novel inhibitors of DNA gyrase: 3D structure based biased needle screening, hit validation by biophysical methods, and 3D guided optimization. A promising alternative to random screening. *J Med Chem* 2000;43(14):2664-2674.
12. Hofstadler SA, Griffey RH: Analysis of noncovalent complexes of DNA and RNA by mass spectrometry. *Chem Rev* 2001;101(2):377-390.
13. Nienaber VL, Richardson PL, Klighofer V, Bouska JJ, Giranda VL, Greer J: Discovering novel ligands for macromolecules using X-ray crystallographic screening. *Nat Biotechnol* 2000;18(10):1105-1108.
14. Hartshorn MJ, Murray CW, Cleasby A, Frederickson M, Tickle IJ, Jhoti H: Fragment-based lead discovery using X-ray crystallography. *J Med Chem* 2005;48(2):403-413.
15. Hämäläinen MD, Zhukov A, Ivarsson M, Fex T, Gottfries J, Karlsson R, et al: Label-free primary screening and affinity ranking of fragment libraries using parallel analysis of protein panels. *J Biomol Screen* 2008;13(3):202-209.
16. Nordström H, Gossas T, Hämäläinen M, Källblad P, Nyström S, Wallberg H, et al: Identification of MMP-12 inhibitors by using biosensor-based screening of a fragment library. *J Med Chem* 2008;51:3449-3459.
17. Johnson SL, Pellecchia M: Structure- and fragment-based approaches to protease inhibition. *Curr Top Med Chem* 2006;6(4):317-329.
18. Takai S, Sumi S, Aoike M, Sakaguchi M, Itoh Y, Jin D, et al: Characterization of recombinant human chymase expressed in *Escherichia coli*. *Jpn J Pharmacol* 2000;82(2):144-149.
19. Congreve M, Carr R, Murray C, Jhoti H: A "rule of three" for fragment-based lead discovery? *Drug Discov Today* 2003;8(19):876-877.
20. Lipinski CA: Chris Lipinski discusses life and chemistry after the Rule of Five. *Drug Discov Today* 2003;8(1):12-16.
21. Lipinski CA: Drug-like properties and the causes of poor solubility and poor permeability. *J Pharmacol Toxicol Methods* 2000;44(1):235-249.
22. Zhang JH, Chung TD, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4(2):67-73.

23. Birdsall B, King R, Lewis CA Jr, Goode SR, Dunlap RB, Roberts GC: Correction for light absorption in fluorescence studies of protein-ligand interactions. *Anal Biochem* 1983;22(24):353-361.
24. Eftink MR: Fluorescence methods for studying equilibrium macromolecule-ligand interactions. *Methods Enzymol* 1997;278:221-257.
25. Giannetti AM, Koch BD, Browner MF: Surface plasmon resonance based assay for the detection and characterization of promiscuous inhibitors. *J Med Chem* 2008;51(3):574-580.

Address correspondence to:
*Samantha Perspicace*
*Biozentrum*
*Klingerbergstrasse 50/70*
*CH-4056 Basel*
*Switzerland*

*E-mail:* samantha.perspicace@unibas.ch