Identification of MMP-12 Inhibitors by Using Biosensor-Based Screening of a Fragment Library

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Small inhibitors of matrix metalloproteinase 12 (MMP-12) have been identified with a biosensor-based screening strategy and a specifically designed fragment library. The interaction between fragments and three variants of the target and a reference protein with an active-site zinc ion was measured continuously by surface plasmon resonance. The developed experimental design overcame the inherent instability of MMP-12 and allowed the identification of fragments that interacted specifically with the active-site of MMP-12 but not with the reference protein. The interaction with MMP-12 for selected compounds were analyzed for concentration dependence and saturability. Compounds interacting distinctly with the target were further evaluated by an activity-based assay, verifying MMP-12 inhibition. Two effective inhibitors were identified, and the compound with highest affinity was confirmed to be a competitive inhibitor with an IC_{50} of 290 nM and a ligand efficiency of 0.7 kcal/mol heavy atom. This procedure integrates selectivity and binding site identification into the screening procedure and does not require structure determination.

Introduction

Matrix metalloproteinases (MMPs)[a] constitute a family of more than 20 structurally and functionally related enzymes in humans. They are all involved in remodeling of tissue, and many are recognized as potential drug targets (for a review, see ref 1). MMP-12 has been identified as a therapeutic target of emphysema and chronic obstructive pulmonary disease (COPD).[2,3] Like other MMPs, MMP-12 has both a catalytic and a structural zinc ion. In addition, three calcium ions, with different affinities for the enzyme, stabilize the active conformation and have been suggested to be of importance for the physiological regulation of the enzyme.[4] The catalytic zinc ion of MMPs has been exploited for drug design and inhibitors typically contain an effective zinc-binding group. Although this design has yielded potent inhibitors, clinical trials have shown that they are nonspecific and give unacceptable side effects such as musculoskeletal pain and inflammation.[5] Considering the high structural homology of this enzyme family and the catalytic zinc atom as a common feature, it is believed that the side effects of MMP inhibitors can be attributed to their interactions with other MMPs. Design of more specific inhibitors with reduced side effects has focused on the use of a nonpeptidic scaffold, a weaker zinc-chelating group, and by the introduction of groups that interact specifically with the S1′ pocket, the region with the lowest structural homology in the active site of MMPs.[6,7]

To better address the problems of low MMP inhibitor specificity, we have recently developed a biosensor assay for studying the interactions between potential inhibitors and MMP-12 (Gossas et al., submitted). Ilomastat (**1**, Figure 1) and other hydroxamate containing inhibitors were found to interact essentially irreversibly with MMP-12 as a result of a very slow dissociation rate. We therefore concluded that the slow dissociation of inhibitors may contribute to the low specificity of these types of inhibitors. As a consequence, we were interested in the discovery of new types of MMP-12 inhibitors with more favorable kinetics from a specificity perspective. To identify compounds that are good starting points for new lead series, we decided to explore the possibility of identifying low-molecular-weight fragments using our biosensor-based method. In the present study, the method was adapted to screening, and a strategy for identification of compounds interacting specifically with the active site of MMP-12 was devised. Moreover, a small fragment library was compiled for this purpose. The screening enabled the identification of fragments that interacted with MMP-12 but did not interact with carbonic anhydrase II, another

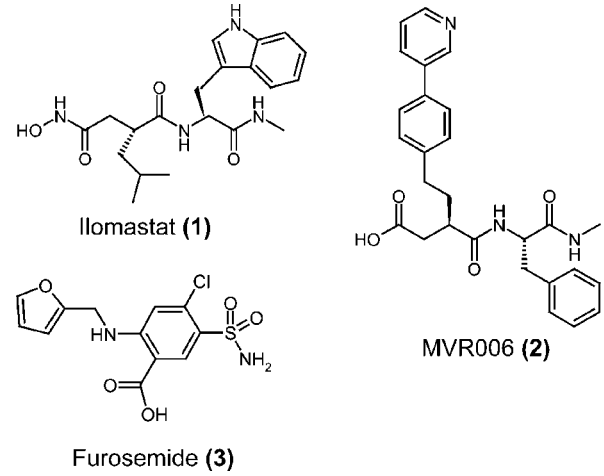

Figure 1. Structures of MMP-inhibitors (**1** and **2**) and carbonic anhydrase inhibitor (**3**) used as references.

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[a] Abbreviations: CA, Carbonic anhydrase; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; Hepes, 4-(2-hydroxyethyl 1-piperazine sulfonic acid); HBS-N, Hepes-buffered saline without detergent; MMP, matrix metalloproteinase; NHS, *N*-hydroxysuccinimide; SPR, surface plasmon resonance; Surfactant P20, Tween 20; Tris, (hydroxymethyl)aminomethane; RU, resonance units.

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enzyme with a catalytic zinc ion. The compound with the highest affinity was confirmed to be a competitive inhibitor of MMP-12. It was not possible to identify the actives in the library using virtual screening or docking approaches. Thus, at least for this library and target, the experimental strategy using biosensor technology was superior to virtual screening. Fragment library screening using biosensor technology with surface plasmon resonance (SPR) detection has clearly emerged as an interesting alternative for fragment-based drug discovery.

Results

Design and Characteristics of the Fragment Library. A partially focused fragment library, consisting of 245 compounds with a molecular weight in the range of 111−244, was compiled from three sets of compounds (see Experimental Section for details). Set A consisted of 149 compounds with structural similarities to S1′ substituents of known MMP inhibitors.[8] It was selected from commercially available benzenes, pyrazines, pyridazines, pyridines, and pyrimidines. An initial set was filtered by removing compounds with unwanted reactivity, e.g., acid chlorides, azides, aldehydes, hydrazones, etc. Compounds not meeting the following criteria were also eliminated: 130 < molecular weight < 250, −2 < log P < 3, rotatable bonds < 5, hydrogen bond donors < 3, hydrogen bond acceptors < 4, polar surface area < 85 Å^2, log S > 10^{-3} M. Finally, compounds with a Tanimoto similarity score >0.70 to any compound in set B or C were also eliminated. Set B consisted of 71 chemically diverse compounds originally compiled for another project at Medivir. It was also selected from commercially available compounds and filtered for unwanted reactivity as described above. Furthermore, compounds containing Cl, Br, or I or not meeting the following criteria were also eliminated: 100 < molecular weight < 250, log P < 3.5, rotatable bonds < 8, hydrogen bond donors < 6, hydrogen bond acceptors < 11, 0 < hetero atoms < 5. Finally, a diverse selection was performed. Set C consisted of 25 compounds with zinc-binding motifs that have previously been suggested to be potential useful tools in the design of metalloprotease inhibitors.[5,9] The molecular weight of the compounds in set C ranged from 111 to 242. Ilomastat (**1**) and MVR006 (**2**) (Gossas et al., submitted) were used as reference inhibitors for MMP-12 and furosemide (**3**) for carbonic anhydrase (Figure 1).

Design of Sensor Surfaces for Screening. Sensor surface layout and injection procedures suitable for the identification of compounds interacting with the active-site of MMP-12 were designed. They were based on an assay previously developed for the characterization of MMP-12 inhibitors (Gossas et al., submitted) and exploited the unique design of a biosensor instrument with four different flow cells on a single sensor chip (Figure 2a), with five detection areas for protein immobilization (spots) in each flow cell (Figure 2b). MMP-12 was immobilized to three of the spots. Because of the inherent instability of MMP-12, the wild-type enzyme was accompanied by two stabilized variants of the enzyme. Two methods for stabilization were chosen; one where MMP-12 had been made inactive by genetically substituting the catalytic glutamate with alanine, generating an E219A variant, and the other where the active-site-binding inhibitor ilomastat was present during immobilization.

One spot was used as a protein reference surface to detect nonspecific interactions with a nontarget protein. For this study, carbonic anhydrase II was found particularly suitable because it contains an active site zinc atom to which nonspecific zinc-binding fragments were expected to bind. It is not otherwise structurally or functionally related to MMPs. The fifth spot was used as a surface reference for the detection of nonspecific interactions between fragments and the dextran surface. It was activated and deactivated as when amine-coupling the enzymes to the sensor chip.

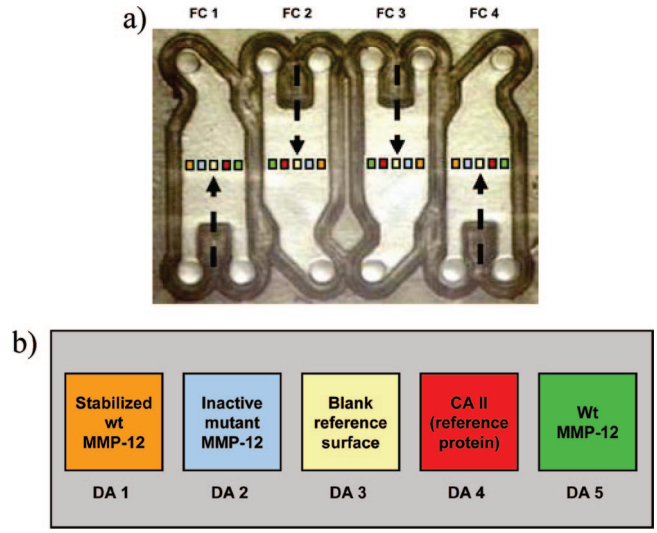

Figure 2. Layout of the four flow cells on a sensor chip (a) and the five detection areas used for protein immobilization on each chip (b), as used for the screening of the fragment library. The position of the reference and target surfaces are illustrated by the colored squares (b). The arrows in the individual flow cells indicate the flow direction (a).

The position of the proteins in the flow cells (Figure 2b) was defined by the optimal order in which the proteins could be immobilized using the hydrodynamic addressing of the different detection areas. It was primarily a consequence of the stability and the immobilization procedure for each particular protein. The protein concentration and injection time suitable for the immobilization was estimated from an experiment using a series of different concentrations of the enzymes (not shown). Conditions were selected so that the amount of immobilized protein gave a signal of at least 10 RU (resonance units) when injecting a saturating concentration (20 μM) of the reference compound (**2**) for MMP-12 or furosemide for carbonic anhydrase II. Even though essentially the same method was used for immobilizing the three variants of MMP-12, the amount of immobilized enzyme was significantly different. The highest signal after the immobilization step was attained with the inactive MMP-12 mutant (4240 ± 460 RU, n = 28), followed by the ilomastat stabilized wild-type MMP-12 (3960 ± 240 RU, n = 27). The lowest signal was reached with the wild-type MMP-12 (2250 ± 210 RU, n = 30). It was not possible to find any other conditions that resulted in higher amounts of immobilized wild-type MMP-12. The immobilization of carbonic anhydrase II resulted in a signal of 2570 ± 200 RU (n = 31).

Although 20 μM of the reference compounds saturated wild-type MMP-12 (irrespective of stabilization procedure) (Figure 3a,b) and carbonic anhydrase II (Figure 3d), saturation of mutant MMP-12 was not even achieved with 100 μM (Figure 3c). Nevertheless, to apply a simple and equivalent procedure for all surfaces, 20 μM of the reference compounds was used for estimating the amount of immobilized enzyme and the binding capacity also for this enzyme form.

Experimental Strategy. An overview of the complete screening and characterization strategy is shown in Figure 4. Two screening procedures were used in parallel: (1) a "direct screen" where fragments were injected alone, and (2) a "competition screen", where fragments were injected in mixture

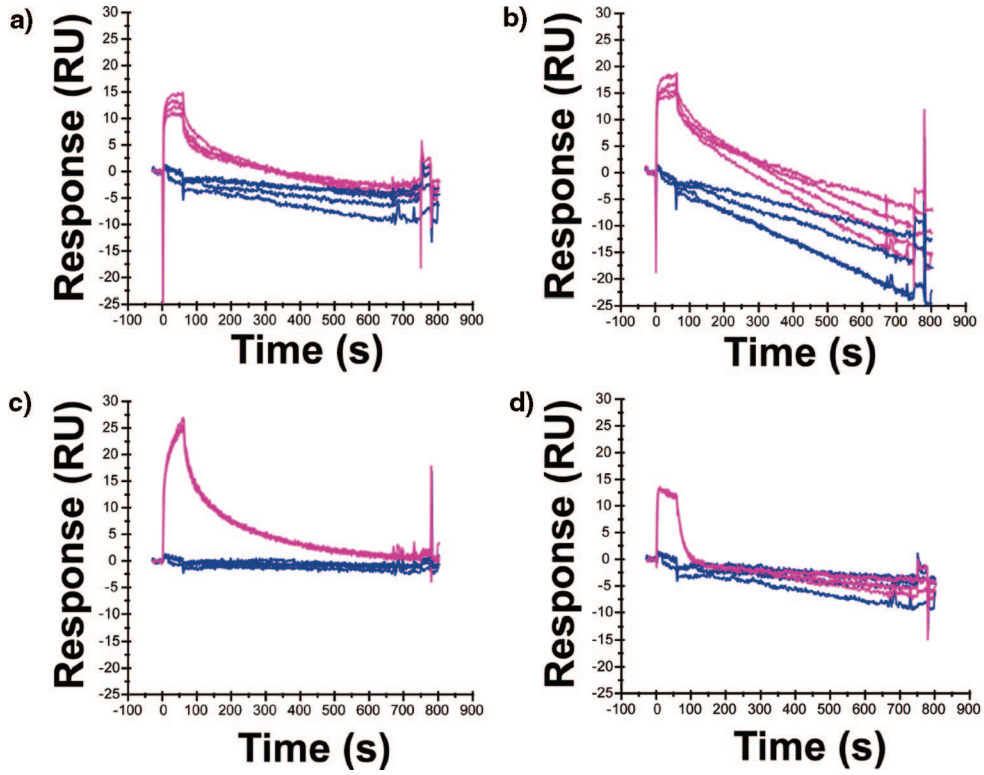

Figure 3. Verification of sensor surface functionality and definition of binding capacity. Signal for 20 μM of compound **2**, the MMP-12 reference, injected over wild-type MMP-12 (a), ilomastat stabilized MMP-12 (b), inactive MMP-12 mutant (c), or for 20 μM furosemide injected over carbonic anhydrase II (d). The graphs show four superimposed injections with reference compounds (top traces) and four injections of buffer alone (bottom traces).

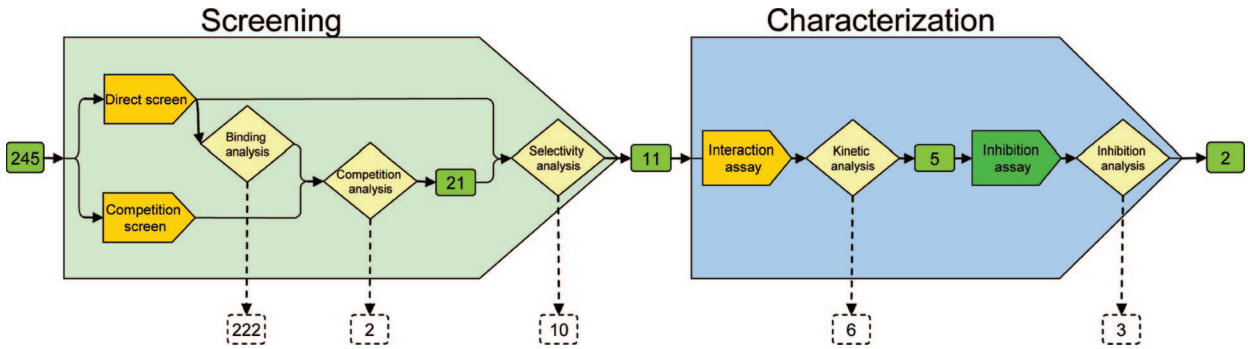

Figure 4. Overview of the strategy used for screening of fragment library and for characterization of fragments interacting with MMP-12. Numbers in green squares refer to the number of fragments selected for the following step and those in dashed squares show the number of excluded fragments. Arrows represent experimental assays or separate stages of the overall procedure, while diamonds represent analysis steps (see text for details).

with ilomastat. The fragments interacting with MMP-12, but not carbonic anhydrase, were identified and subsequently subjected to a kinetic analysis using a more extensive experimental setup.

Fragments were injected at a concentration of 200 μM across all flow cells and detection areas (Figure 2) in both screening procedures. Although this resulted in more data than was required for the purpose of the individual experiments, it was simpler to use the same surface design in all experiments and only extracting the information needed for the analysis. The additional data served as auxiliary information, useful for checking the performance of the procedure. Even though a fragment concentration of 100 μM had been used with success in a pilot screen (not shown), the concentration was increased to 200 μM in order to increase the probability of detecting low-affinity interactions.

The competition assay was designed so that fragments binding in the presence of ilomastat could be distinguished from those only binding in the absence of ilomastat, indicative of active site binding. The concentration of ilomastat required for blocking fragment interactions was determined by injecting a series of ilomastat concentrations (up to 10 μM) over all detection areas in a flow cell (Figure 5). A concentration of 2 μM appeared to essentially saturate MMP-12 and was chosen as the lowest concentration for the competition experiments. Compound **2** was confirmed not to bind to MMP-12 if this concentration of the slow dissociating inhibitor ilomastat had been injected previously. However, it had no effect on the binding of furosemide to carbonic anhydrase II.

To verify that the immobilized proteins were functional and had a sufficient binding capacity for the detection of weak interactions, reference compounds were injected before and

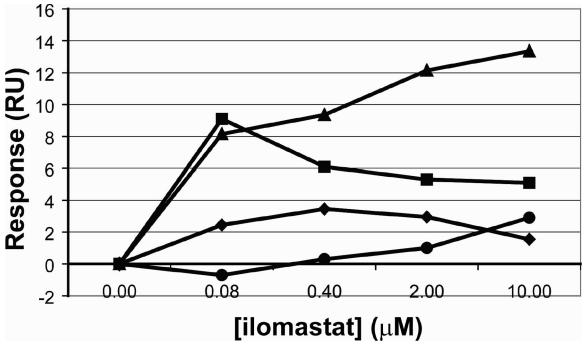

Figure 5. Identification of saturable ilomastat concentrations for sensor surfaces. Responses of varying concentrations of ilomastat interacting with wild-type MMP-12 (diamond), ilomastat wild-type MMP-12 (square), inactive MMP-12 mutant (triangle), and carbonic anhydrase II (circle). The responses were solvent corrected and normalized with respect to the response without ilomastat.

repeatedly during the course of the experiments. If the immobilized levels of enzymes were lower than expected to give a reference signal of more than 10 RU for one or more of the enzyme surfaces, the sensor chip was discarded and the screen was restarted with a new chip. In the competition screen, ilomastat was included in the buffer used for preparation of fragments and reference compounds in the interaction experiments. By injecting a sample of this buffer at defined intervals during the experiment, it was confirmed that any recorded signals, to any of the surfaces, were only a result of nonactive site interactions with the injected compounds. Furthermore, by injecting the reference compound, it was confirmed that ilomastat effectively blocked the active site of MMP-12.

Because of the relatively low stability of the sensor surfaces, multiple immobilizations and experimental series had to be used in order to screen the complete library. To facilitate the comparison of data from several experiments and varying conditions, a number of data processing procedures were used. These include correction for signals arising from differences in refractive index between buffer and the solvent used for dissolving the fragments, correction for slightly different immobilization levels in different experiments, and normalization of responses with respect to differences in the molecular weight of the fragments.

Screening of Fragment Library. 1. Selection of Fragments Binding to MMP-12. A direct screen of the complete fragment library (245 compounds) was performed in order to identify compounds that interacted with MMP-12 (Figure 6). Such compounds were identified by using two criteria for both wild-type and ilomastat-stabilized MMP-12. First they should give an *adjusted* response ≥30% of the *adjusted* response for the reference compound (**2**), and second, the actual solvent-corrected response should be >3 RU (see Experimental Section for details of the types of signals and how they are treated). These selection criteria resulted in the identification of 12 hits for wild-type MMP-12 and 19 for stabilized wild-type MMP-12 (Figure 6). Both target variants should ideally identify the same set of fragments, but due to a lower stability for the wild-type enzyme, it was not as sensitive. The stabilized surface did not bind some of the compounds as effectively as the unstabilized surface. A total of 23 compounds were selected in this step.

2. Identification of Fragments Binding to the Active Site of MMP-12. All fragments were also screened in competition with ilomastat (not shown). A reduced signal correction procedure was used for this data set because the response from the positive control was blocked by ilomastat and therefore useless for normalizing the signals from different experiments with respect to the signal of the controls. It was therefore assumed that the differences between the binding capacities of different sensor surfaces resulting from variations in the efficiencies of the immobilization procedures were insignificant. One surface was used for screening of compound set A and another for sets B and C. The data was analyzed separately for wild-type and ilomastat-stabilized wild-type MMP-12 (Figure 7). Two selection criteria were used in this screening step: As a first criterion, fragments were included if they gave a signal of at least 3 RU (line 1 in the graphs). The second criterion excluded fragments if their signal was additive to that for ilomastat. The cutoff for exclusion was the mean value for the signal of the positive control injected with ilomastat plus 3 times the standard deviation (signal + 3·SD), accounting for the error in determining additivity. The cutoff was set to 5 RU for wild-type MMP-12 and 8 RU for ilomastat-stabilized wild-type MMP-12 (line 2 in the graphs).

By comparing the signals from the two screening procedures (Figure 7), it was possible to identify fragments that could only bind to MMP-12 if ilomastat was not present, indicative of binding to the active site. This resulted in the identification of 8 hits for wild-type MMP-12 and 16 for stabilized wild-type MMP-12. A total of 21 fragments were selected from the combined analysis of the direct and competition screening procedures. (The two other fragments could perhaps bind to an allosteric site, but they were not analyzed further in this study.)

3. Elimination of Fragments Binding to Reference Enzyme. The specificity of the interactions between the selected fragments for MMP-12 relative to carbonic anhydrase II was analyzed. The signals for each compound and all four enzyme surfaces in the direct screen were compared (Figure 8). Fragments were regarded as unspecific if they resulted in higher signals for the carbonic anhydrase II surface than for any of the MMP-12 surfaces. This step eliminated 10 compounds, and only 11 fragments remained for the kinetic characterization.

Interaction Kinetic Characterization of "Hits". A kinetic analysis was carried out in order to investigate if the fragments interacted in a defined stoichiometric manner, as expected of a specific interaction to a defined binding site. Each of the 11 fragments were therefore injected over all four enzyme surfaces at several concentrations up to 500 μM. Five fragments gave signals higher than 3 RU for wild-type and/or stabilized wild-type MMP-12 and higher than the signal for carbonic anhydrase II (Figure 9). The saturation of the interaction was judged by extracting the signal at the end of the injection phase as a function of fragment concentration (Figure 9E). Fragment B7 showed the highest signal and clear saturation of ilomastat-stabilized MMP-12. Fragments A13 and B6 also appeared to saturate this surface, while fragment B1 showed a weak interaction that was not concentration dependent. Fragment B5 could not be properly evaluated due to lack of data for the highest concentration, but it appeared to give a high signal also with carbonic anhydrase II, making it less specific for MMP-12 than the other fragments.

The kinetic characterization revealed differences in the interactions of some compounds (e.g., B7) with the mutant enzyme as compared to that with the wild type (most notably seen in the stabilized form). This indicates that the active-site residue is directly or indirectly involved in the interaction with the fragment.

Virtual Screening and Binding-Mode Modeling. For comparative purposes, virtual screening was applied in an

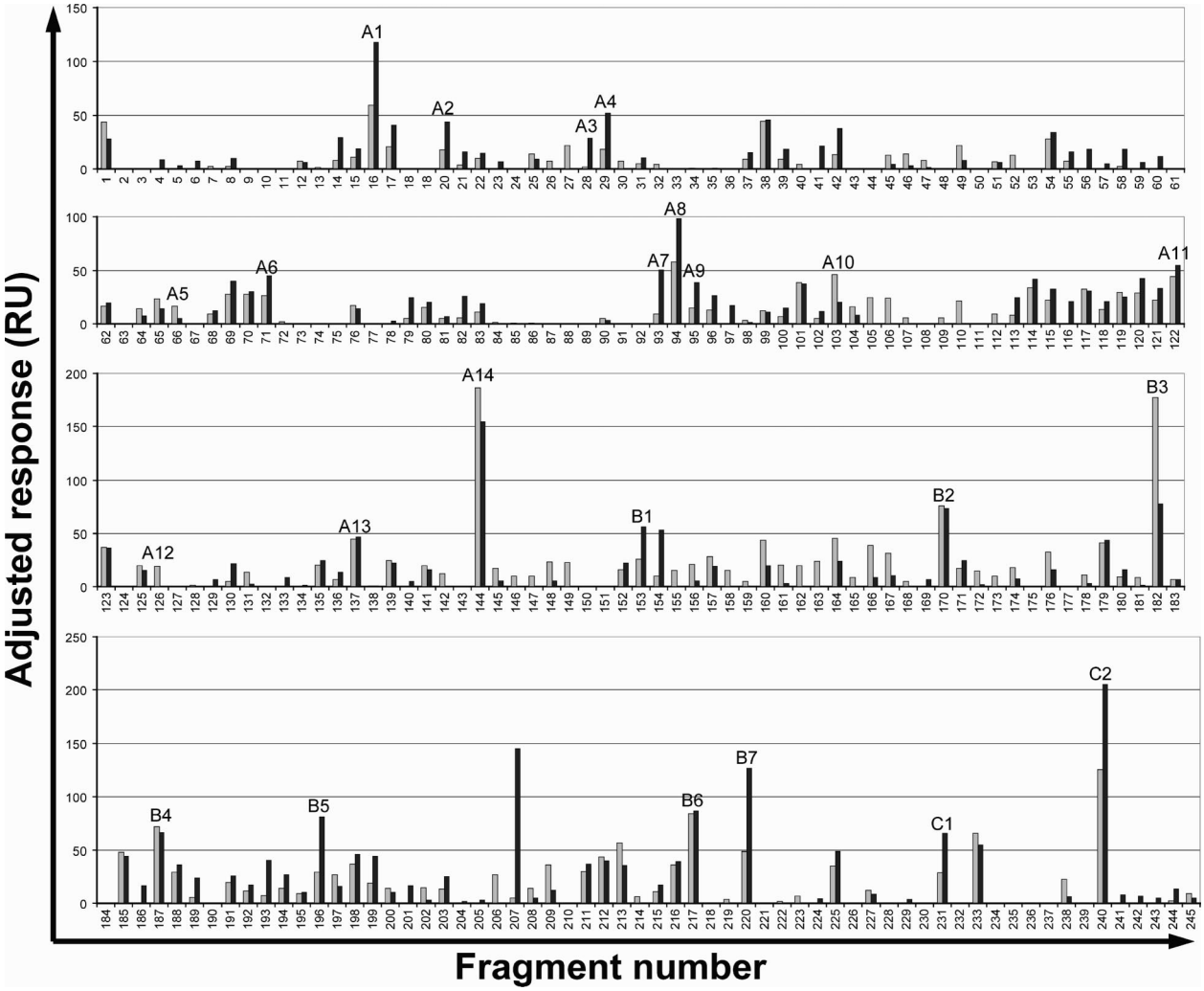

Figure 6. Binding analysis. Direct screen of fragment library using wild-type (light-grey) and ilomastat-stabilized (dark-grey) MMP-12. The signals were solvent corrected, adjusted for differences in binding capacity, and normalized with respect to differences in molecular weight of the samples. Note that selection of fragments required additional signal correction procedures and that simply identifying interacting fragments by the height of the bars can be misleading. The fragments selected for the next step are named in the graph; the letter indicates the set of compounds in the library from which it originates.

attempt to rank the fragments and thereby identify the most interesting compounds in the library. Two crystal structures of MMP-12 were used to mirror the experimental set up. One which is an active-site wild type (1JIZ)[10] and one with the E219A mutation (1JK3).[11] Each fragment of the library was docked to the protein structures using two different scoring functions in the program GOLD.[12] No correlation was found between the experimental results and the results of the virtual screen and for any combination of the two structures and the two scoring functions. Combining the scoring functions in a consensus score[13] did not improve the predictability of the virtual screen.

In the absence of structural data, molecular docking was applied to assess the binding mode and structural fit of the five fragments selected after the kinetic characterization. Visual inspection of the complete docking ensembles revealed complex patterns of interactions with multiple possible binding modes for all five fragments. Four of the five fragments only produced docking solutions located deep in the S1′ pocket at significant distance from the zinc atom.

Inhibition of MMP-12. Because the baseline drift of wild-type MMP-12 surfaces is a result of autoproteolysis, it is possible to detect inhibition of the enzyme from these experiments. For example, the injection of fragment **B7** clearly reduced the baseline drift of wild-type MMP-12, seen both in the steady-state and in the dissociation phases (compare the slope of analyte injections and the blank injection in Figure 9A,B). In contrast, fragment **A13** had little effect on the drift.

Inhibition of MMP-12 by the five fragments selected in the final step was confirmed in a steady-state activity assay. Initially, the compounds were assayed at 500 μM, the highest possible concentration for this assay. All compounds inhibited the enzyme, albeit **B1**, **B5**, and **A13** showed quite moderate inhibition ($\leq$30%), while **B7** and **B6**, on the other hand showed 100 and 60% inhibition, respectively (Table 1). Ilomastat was used as a reference and gave 75% inhibition at 2.5 nM.

The mode of inhibition of compound **B7** was estimated in an experiment where both substrate and inhibitor concentrations were varied (Figure 10a). A double reciprocal plot of the data illustrates that the compound was a competitive inhibitor of MMP-12. Because of a high inner filter effect of the substrate, it was not possible to determine K_m accurately and hence not K_i. Instead, IC_{50} values were used to compare the inhibitory effect of the fragments. The IC_{50} values for fragments **B6** and **B7** were determined from the data shown in Figure 10b. Fragment **B7** was clearly the most effective inhibitor, with an

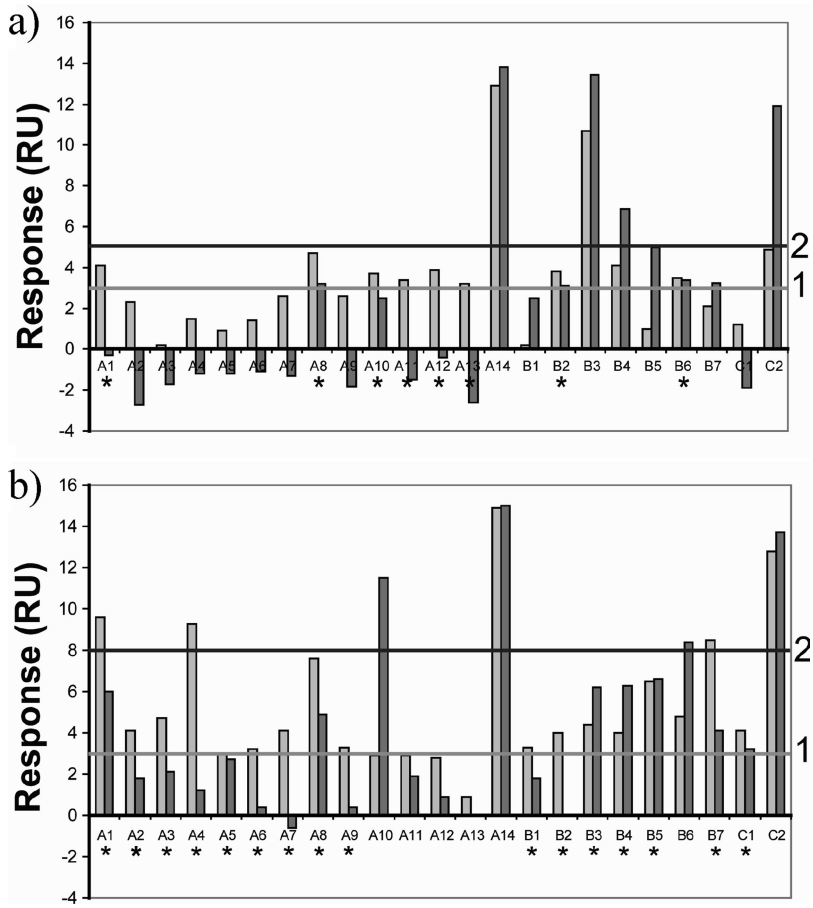

Figure 7. Competition analysis. Identification of fragments binding competitively with ilomastat to MMP-12, using (a) wt MMP-12 and (b) stabilized wild-type MMP-12. Data from the direct and competition screens were used to identify fragments that bound to the enzyme with greater than 3 RU in the direct screen (light grey bars and horizontal line 1) and that were additive with ilomastat in the competitive screen, i.e., fragments with lower than the mean value + 3 × SD of ilomastat blocked response of positive control (dark grey bars and horizontal line 2). The bars represent solvent-corrected responses (when multiple experiments were required, the mean values are shown). Selected fragments are marked by an asterisk.

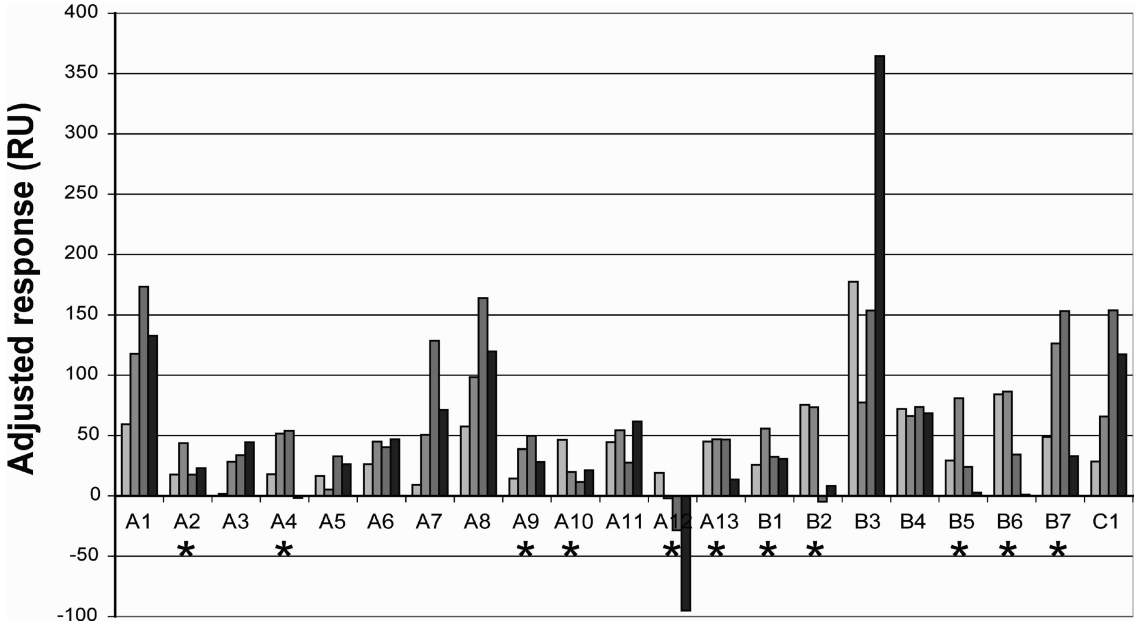

Figure 8. Selectivity analysis. Selection of fragments binding to MMP-12 but not to carbonic anhydrase II. The data for wild-type MMP-12, stabilized wild-type MMP-12, inactive mutant MMP-12, and carbonic anhydrase II (in the order from left to right) was extracted from the direct screen. The responses were solvent corrected, adjusted for differences in binding capacity, and normalized with respect to the molecular weight of the fragments. Fragments with higher signals for wild-type MMP-12 and/or stabilized wild-type MMP-12 than for carbonic anhydrase II are marked by an asterisk.

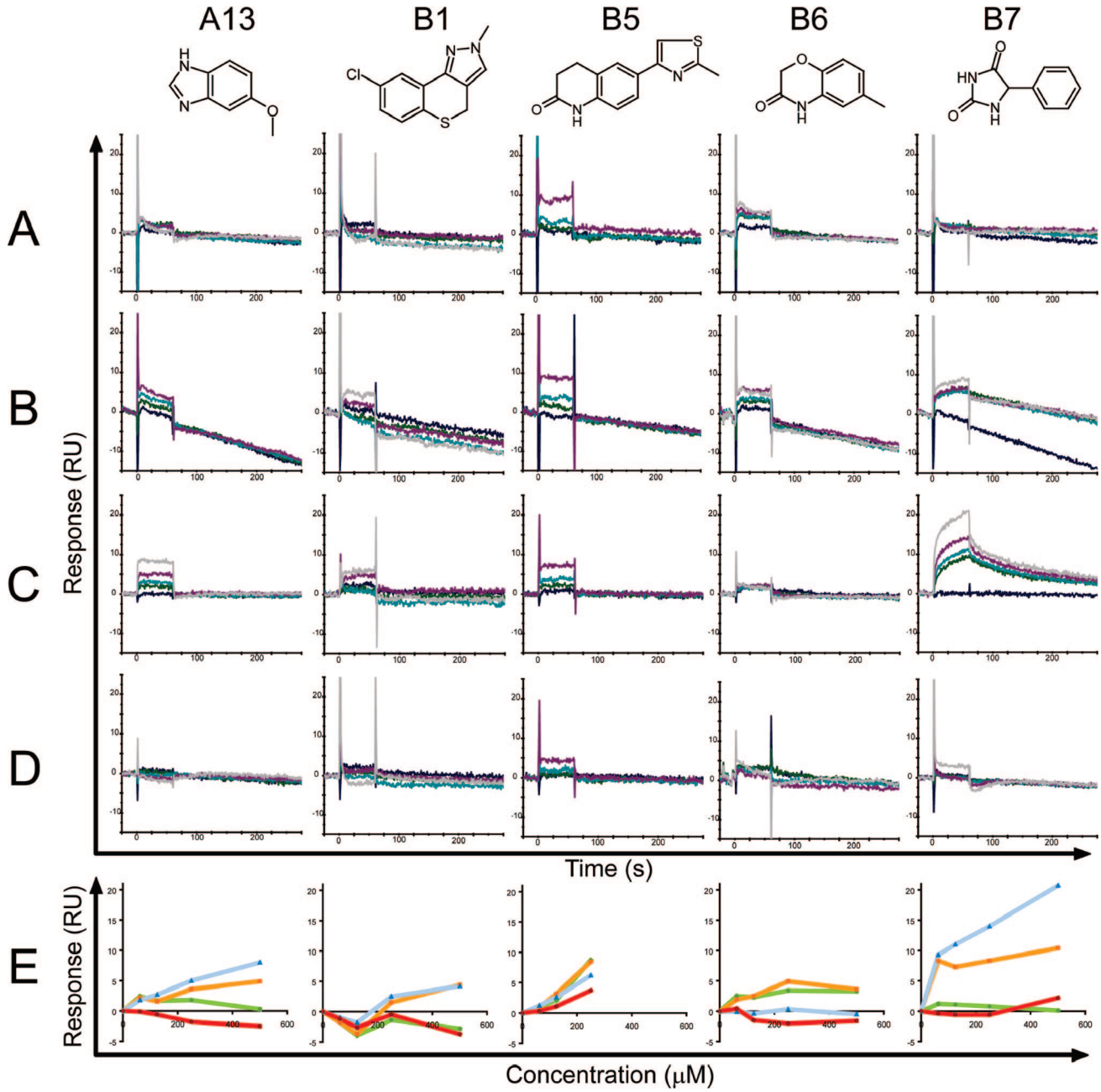

Figure 9. Kinetic analysis. Basic kinetic characterization of fragments **A13**, **B1**, **B5**, **B6**, and **B7** injected at concentrations 0, 63, 125, 250 and 500 μM. Overlay plots of solvent-corrected sensorgrams for (A) wild-type MMP-12, (B) stabilized wild-type MMP-12, (C) inactive mutant MMP-12, and (D) carbonic anhydrase II. (E) shows the highest responses for each sensorgram (i.e., at 500 μM) for wild-type MMP-12 (green), stabilized wild-type MMP-12 (orange), inactive mutant MMP-12 (blue), and carbonic anhydrase II (red). The responses in (E) have been normalized with the response from the concentration 0 μM, hence all series begin at 0 RU.

IC_{50} value of 0.29 μM (Table 1). The other three fragments exhibited too weak inhibition for IC_{50} values to be determined.

The ligand efficiency[14] was determined for the fragments that remained at the end of the screening and characterization (Table 1). Because of the difficulties in correcting for inner-filter effects, and hence the impracticability of accurate determination of K_i values, the ligand efficiency was calculated from IC_{50} values.

Discussion

As is often the case when new technologies are introduced and a single supplier dominates the market, the terminology used can be confusing or inappropriate. The term SPR is simply a biophysical detection method that can be used for many instruments even though it is often used to denote the instrumentation used in the present study. Another source of confusion is the possibility of using very different experimental designs with this type of biosensor. For example, the current experimental strategy should not be confused with chemical microarrays and SPR imaging, recently described in a review of "SPR-based fragment screening".[15] Although the immobilization of fragments may be an alternative for some projects, we have used a more general experimental design where the target is immobilized and fragments are used as analytes. The experimental design successfully exploited here for primary screening of a fragment library is similar to that implemented for the secondary screening of hits from primary screens performed with other techniques[16,17] and more recently implemented for the primary screening of thrombin binders.[18] The current study

Table 1. Inhibition and Ligand Efficiency of Selected Fragments[a]

compound	inhibition (%)	concentration (μM)	$IC_{50} \pm SD$ (μM)	heavy atoms (number)	ligand efficiency (kcal/mol heavy atoms)
A13	25	500	≈ 2000	11	≈ 0.3
B1	30	500	≈ 1000	15	≈ 0.3
B5	10	500	≈ 5000	17	≈ 0.2
B6	60	500	355 ± 14	12	0.39
B7	100	500	0.29 ± 0.01	13	0.69
ilomastat	75	0.0025	≈ 0.001	28	≈ 0.4

[a] Ligand efficiency was calculated according to Hopkins et al.[14] using the number of heavy atoms (i.e., non-hydrogen atoms) in the molecule and IC_{50} values. For compounds that resulted in less than 50% inhibition at 500 μM, and for the reference compound ilomastat, IC_{50} values were estimated from the degree of inhibition at 500 μM (2.5 nM for ilomastat).[26] These approximative IC_{50} values were used to estimate the ligand efficiency for these compounds.

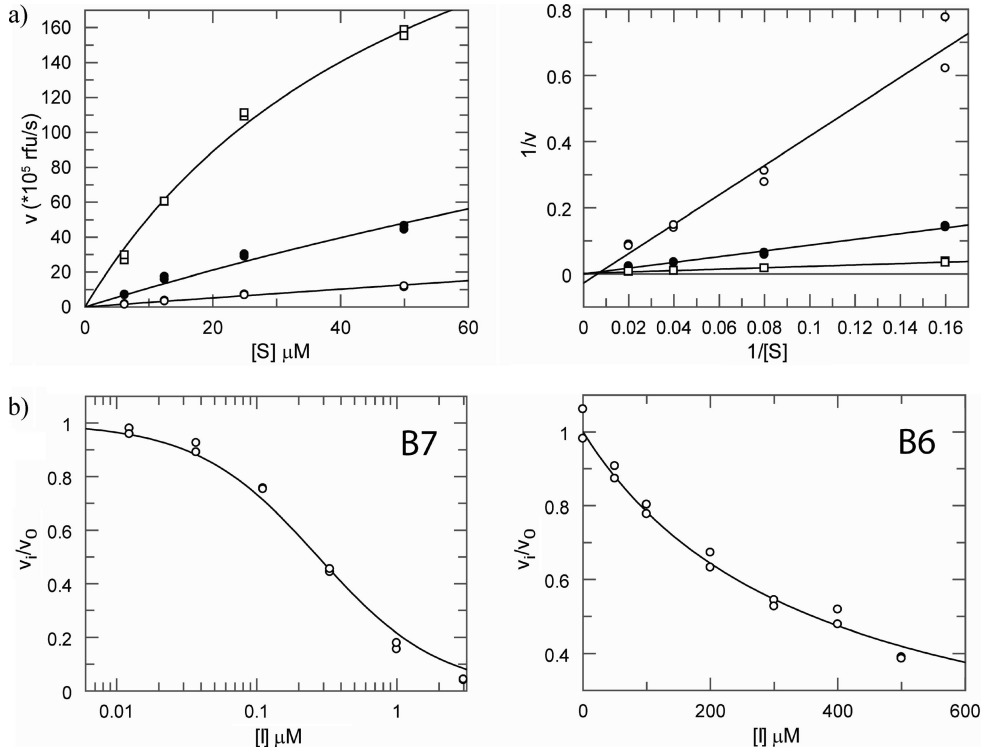

Figure 10. Inhibition analysis. Determination of inhibition mode and IC_{50} values for fragments. (a) Inhibition of MMP-12 by the compound **B7** at different substrate concentrations. □ [I] = 2.5 μM, ● [I] = 0.5 μM, ○ [I] = 0. (b) Initial velocity as a function of inhibitor concentration. The lines are a fit of an equation yielding the IC_{50} values in Table 1.

shows that this experimental design had adequate sensitivity also for fragment screening, and it was therefore not necessary to explore alternative experimental designs, for example, competition experiments using an immobilized effector, which are expected to give larger signals but which have other drawbacks.

Fragment screening using SPR biosensor technology and immobilized targets has long been thought to be out of reach, considering that the signal is correlated with the molecular weight of the analyte. In addition, the low SPR signals arising from the weak interaction between the fragments and the immobilized target protein make the data from a simple fragment screen more difficult to evaluate than data from secondary screening of hits identified in high-throughput screening programs. When working with notoriously structurally labile targets, such as MMP-12,[4] fragment screening becomes more challenging due to limitations in the amount of enzyme that can be immobilized, the fraction of immobilized enzyme that binds ligands, and baseline drift. Although the concentration of calcium was maximized with respect to instrument considerations, the enzyme was clearly subject to significant autoproteolysis. Degradation of the enzyme before and after immobilization resulted in a low initial binding capacity of the surface and a negative baseline drift, respectively. The lifetimes for the MMP-12 sensor surfaces were consequently very short and limited the number of compounds that could be screened per immobilization. By using an inactive mutant, it was possible to achieve a stable surface without baseline drift. However, the binding kinetics for this surface was slightly different from that of the wild-type enzyme, and a procedure for stabilizing the wild-type enzyme was therefore also devised. It involved inhibiting the enzyme with ilomastat before and during the immobilization and resulted in a surface with higher binding capacity but similar kinetics as the surface prepared with uninhibited wild-type MMP-12. Because the degree of autoproteolysis was proportional to the amount of immobilized active enzyme, the baseline drift was greater for the stabilized MMP-12 surface. However, it was compensated for by a higher binding capacity at the start of experiments. Nevertheless, even with these stabilization procedures, the short lifetime of the surface was a bottleneck in the screen and many immobilizations were required in order to screen the complete library.

A fragment concentration of 200 μM was used in the screening. Although we had tested 100 μM with some success, we decided to use a higher concentration because it was expected to facilitate the detection of low affinity interactions.

However, higher concentrations typically also resulted in a higher signal for the reference surfaces, indicating that there is no real benefit in increasing the fragment concentration. No regeneration was required in the screen because fragments typically dissociate very rapidly. However, it was important to use a reference compound with a sufficiently rapid dissociation for complete dissociation within the defined dissociation phase. In this case, we compensated a somewhat limiting dissociation rate by having a long dissociation time.

The screening strategy was designed so that the fragments interacting with the active site of MMP-12 could be identified. This was accomplished by comparing signals for fragments alone (direct screen) with those for fragments mixed with ilomastat (competition screen). Fragments interacting competitively with ilomastat were regarded as active-site binding, although it cannot be excluded that they are competitive allosteric inhibitors. However, the structural reasoning (see below) supports the conclusion that the fragments interact with the active site.

A basic kinetic characterization was used to obtain more detailed information about the mechanism and affinity of the interaction for the 11 active-site-binding compounds identified in the screen. Three fragments were found to saturate the MMP-12 surface and allowed the estimation of the affinities of the interaction and the degree of inhibition. **B7** was clearly the most effective inhibitor identified, although **A13** and **B6** also interacted with MMP-12 in a saturable manner. The ligand efficiency for **B7** and **B6** were 0.7 and 0.4 kcal/mol heavy atoms, respectively. The disruption of the baseline drift by some of the selected fragments indicated that they inhibited the enzyme. A separate inhibition analysis confirmed that they inhibited the activity of the enzyme in a steady-state kinetic assay.

Interestingly, **B7** served as an important internal control of the library because it contains a hydantoin group, a previously recognized substructure for MMP-12 inhibitors.[19] An amide motif in **B5** and **B6** suggests that interaction with the catalytic zinc atom might be a common denominator for the identified fragments. The active-site glutamate residue may also be a significant determinant for binding of these fragments because **B6**, in contrast to the other two fragments, did not interact with the E219A substituted variant of MMP-12. Moreover, the kinetics of **B7** and the reference compound **2** were also influenced by substitution of this residue.

The comparative virtual screen performed did not produce results that enabled the identification of the active fragments through such means. This lack of correlation between experimental results and in silico data highlights the limitation of computational methods to quantitatively assess molecular interactions. The more qualitative binding-mode modeling yielded interesting albeit complex results that require detailed interpretation. Although methods for assessing multiple binding modes in structure-based design has been explored,[20] further work is necessary. Alternatively, combination studies with SPR and docking using expanded analogue series could facilitate the interpretability of the models. High-quality structural information is optimally obtained through experimental techniques such as X-ray diffraction.

It was interesting that none of the fragments from set C were selected in the study and that only one made it to the selectivity analysis. After all, they were originally selected for the fragment library based on their zinc-binding motifs. Such motifs have been suggested to be potentially useful in the design of metalloprotease inhibitors.[5,9] The validity of the zinc-binding motifs was, however, confirmed through the identification of compounds **B6** and **B7**. (**B5** was also found to interact with MMP-12 but was excluded due to unspecific interactions with carbonic anhydrase II and was later found to be a poor inhibitor.)

An advantage with NMR and X-ray crystallography for fragment screening is that both methods give useful structural knowledge about the binding site and the orientation of the fragment.[14,21–24] However, although the biosensor-based method used here does not give direct structural information, it is possible to obtain such information indirectly. For example, the location of the binding site for fragments can be identified by using a combination of a direct screen and a competition screen as implemented in this study. Further structural details of the interaction may be obtained by the use of structural variants of the target enzyme.

Importantly, there are several additional benefits of the biosensor-based method. In comparison to the above techniques, the consumption of both fragments and enzymes is relatively low. When used in a strategy as the one implemented here, it is possible to obtain kinetic and mechanistic information about the interaction. Moreover, it is relatively simple to identify promiscuous binders and false positives by characterizing the basic kinetic features of interacting compounds and by using suitable reference enzymes.[25]

Conclusions

The strategy and library used in the present study identified several compounds that provide good starting points for novel lead development. This study consequently illustrates the usefulness of SPR-biosensor technology in fragment screening despite the considerable experimental challenges when using MMP-12 as a target. Furthermore, the results show that it is sufficient to use a library in the order of 200−300 fragments, providing that it is carefully designed. Fragment screening using biosensor technology and immobilized targets obviously presents an important complement to NMR and X-ray crystallography, the two experimental techniques primarily used for fragment-based drug discovery today.

Experimental Section

Enzymes and Compounds. The fragment library compounds originated from three distinct sets, all obtained from commercial sources. All compounds were selected from the Daylight Available Chemicals Directory (ACD) (Daylight Chemical Information Systems Inc., CA). Ilomastat ((2*R*)-*N*′-hydroxy-*N*-[(2*S*)-3-(1*H*-indol-3-yl)-1-methylamino-1-oxopropan-2-yl]-2-(2-methylpropyl)butanediamide) was purchased from Chemicon, and the reference compound MVR006 (**2**) was synthesized by Medivir (to be published).

Mutant MMP-12 was constructed as described by Lang et al.[11] Wild-type and mutant MMP-12 were expressed and purified essentially according to Morales and co-workers.[7] Briefly, the cell pellet was homogenized in lysis buffer and broken by a cell disruptor (Constant Systems, UK) at 1.7 kbar. Inclusion bodies were collected by centrifugation at 20000*g* for 15 min at 4 °C. The inclusion bodies were washed 4 times with wash buffer (10 mL/g cell pellet) and thereafter dissolved in 10 mL of denaturing buffer/g cell pellet and centrifuged at 75000*g* for 90 min. The supernatant was adjusted to pH 10 with 10 M NaOH and loaded at 3 mL/min on a 6 mL Resource Q column (GE Healthcare Bio-Sciences, Uppsala, Sweden) equilibrated with buffer. The sample was eluted from the column by a 0−20% gradient (15 column volumes) of elution buffer. Protein fractions eluting from the column between 8−16% of elution buffer were pooled and the protein concentration was adjusted to ≤1 mg/mL. MMP-12 was refolded by three successive dialysis steps for 17.5, 4.5, and 3.5 h respectively at 4 °C (sample/buffer volume 1/20). Protein precipitated during refolding was removed by centrifugation at 3000*g* for 10 min. Usually the yield was 2−3 mg pure MMP-12 per gram cell pellet.

Bovine carbonic anhydrase II and furosemide (4-chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid) were purchased from Sigma-Aldrich (Stockholm, Sweden).

Interaction Analysis. All experiments were performed with a Biacore A100 instrument (GE Healthcare Bio-Sciences, Uppsala, Sweden). Biacore CM5 sensor chips and reagents were all from Biacore (now GE Healthcare Biosciences).

Immobilization. Immediately before immobilization, the storage buffer for the enzymes was changed to 5 mM maleate (Sigma-Aldrich, Stockholm, Sweden), pH 6.0, and 10 mM $CaCl_2$ (Merck, Stockholm, Sweden) with protein-desalting spin columns (Pierce, Rockford, USA). The enzymes were then further diluted with the maleate buffer to give a concentration of about 4.0 μM for wild-type MMP-12 and 2.3 μM for the inactive MMP-12 mutant. Stabilization of wild-type MMP-12 by inhibition was achieved by adding 4.0 μM ilomastat to 17 μM wild-type MMP-12 when thawing the enzyme.

The enzymes were covalently immobilized to the sensor chip surface by amine coupling: MMP-12 was immobilized with 10 mM Hepes, pH 7.4, 90 mM NaCl, 50 mM $CaCl_2$, 0.05% Surfactant P20 as running buffer. The CM5 surface was activated with a 10 min injection of EDC/NHS (200 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/50 mM *N*-hydroxysuccinimide) (GE Healthcare Bio-Sciences, Uppsala, Sweden). The enzyme was injected for 7 min at flow rate of 10 μL/min over the activated surface and was thereafter cross-linked with a 2 min pulse of EDC/NHS with 10 mM $CaCl_2$. The enzyme stabilized with ilomastat was treated essentially in the same way, but 2.5 μM ilomastat was present in the coupling buffer and the enzyme was immobilized at a concentration of 2.0 μM. After amine coupling and cross-linking, remaining active succinimides on the surface were deactivated by an injection of 10 mM ethanolamine with 10 mM $CaCl_2$ for 6 min for wild-type MMP-12 or 7 min for stabilized wild-type and mutant MMP-12.

A stock solution of 5.0 mg/mL carbonic anhydrase II in HBS-N buffer (10 mM Hepes, pH 7.4, and 0.15 M NaCl, GE Healthcare Biosciences, Uppsala, Sweden) was diluted in water to 1.6 mg/mL prior to immobilization. It was further diluted in the instrument to 0.16 mg/mL in 10 mM acetate, pH 5.0, with 5 mM $CaCl_2$ just prior to injection over the activated surface. The enzyme was immobilized for 12 min at a flow rate of 10 μL/min; deactivation was performed with a 7 min injection of 10 mM ethanolamine with 10 mM $CaCl_2$.

Immobilization of proteins in the A100 instrument flow cell is done in one-half of the flow cell at a time. In these experiments, the steps for the first half-were: (1) injection of wild-type MMP-12, (2) cross-linking, (3) deactivation, (4) injection of carbonic anhydrase, and (5) deactivation. The steps for the second half-were: (1) injection of stabilized wild-type MMP-12, (2) injection of mutant MMP-12, (3) cross-linking, and (4) deactivation. This resulted in the surface design as illustrated in Figure 2b.

Even though the calcium concentration in the running buffer was reduced to 50 mM from optimal 200 mM,[4] calcium precipitation was observed in the experiments. This was overcome by washing the flow cells and tubings before each immobilization with 1% acetic acid using the *desorb* method in the A100 instrument software.

Screening Conditions. With minor deviations, the same procedures and conditions were used in all screening experiments. Screening was performed with a flow rate of 30 μL/min, and cycles encompassed sample injection for 1 min followed by dissociation for 10 min. No regeneration was required. The running buffer was 10 mM Hepes pH 7.4, 50 mM $CaCl_2$, 90 mM NaCl, 0.05% surfactant P20, and 5% DMSO (Riedel-de Haën, Seelze, Germany). Fragments and positive controls (**2** and **3**) were stored as 4 mM stock solutions in 100% DMSO. Prior to screening, fragment samples were diluted to 200 μM in running buffer without DMSO, using a robot (MultiProbe II^{HT}, Perkin-Elmer, USA). Thus, the final concentration of DMSO in the analysis was defined to be 5%. Nevertheless, in order to be able to correct for small variations in DMSO between samples, injection of eight solvent samples ranging from 4.5% to 5.8% DMSO were performed every 15th cycle for correction purposes. To monitor the binding capacity of the enzyme surfaces during the screening, injections of the reference compound **2** and furosemide (330.7 g/mol) were used as positive controls (20 μM) for MMP-12 and carbonic anhydrase II, respectively. Injections of buffer in the beginning and at the end of the screen, and every 15th cycle, served as negative controls.

Direct Screen. The direct screen was performed as described above. The experiment was initiated by a capture injection of 2 or 10 μM ilomastat for 1 min, directed over only spot 1 in all four flow cells in order to enhance initial inhibition of enzyme activity of stabilized MMP-12.

Competition Screen. The conditions for this screen was as above, but with the exception that the screening was initiated by injecting 2 μM ilomastat over all enzyme surfaces before screening in order to saturate the surfaces with the competitor before screening. Fragments and control samples were diluted with 2 μM ilomastat (10 μM in later runs) in 10 mM Hepes, pH 7.4, 90 mM NaCl, 50 mM $CaCl_2$, 0.05% surfactant P20 prior to screening.

The amount of competitor used in this screen was determined by injecting a dilution series of ilomastat over the four enzymes in one flow cell. The concentrations were 0.04, 0.8, 2, and 10 μM ilomastat in 10 mM Hepes, pH 7.4, 90 mM NaCl, 50 mM $CaCl_2$, 0.05% surfactant P20 with 5% DMSO. The flow rate was 30 μL/min, and the samples were injected for 2 min and left to dissociate for 10 min.

Kinetic Characterization. A series of fragment concentrations of 0, 63, 125, 250, and 500 μM were injected with the same experimental set up as described for the screening.

Signal Processing. Signals were corrected for nonspecific binding to the surface by subtracting signals from the reference surface from those of the enzyme surfaces (reference subtraction). In addition, correction for other minor differences between enzyme and reference surface interactions with DMSO was performed by using a series of solvent standards (solvent correction). Moreover, signals were normalized with respect to the molecular weight of each injected sample because the signal is correlated to the molecular weight of the analyte. In addition, signals were corrected for differences in the binding capacity of the surface due to differences in the amount of immobilized enzyme or degree of degradation at different time points in the screening. Thus, responses for the positive controls were set to 100 and the negative to 0, and signals for fragments were expressed relatively to the signal of the controls. The correction procedures for the presented experiments are specified in the figure legends.

Inhibition of MMP-12 Activity. Measurements of MMP-12 activity were performed in 50 mM Tris, pH 7.5, 200 mM $CaCl_2$, 6% DMSO at 30 °C. Cleavage of the fluorogenic peptide substrate Mca-K-P-L-G-L-Dpa-AR-NH_2 (R&D Systems, Abingdon, UK) was followed as an increase in fluorescence, as measured with a Fluoroscan Ascent fluorescence plate reader (Thermo Electron OY, Helsinki, Finland).

Inhibition was determined at a single substrate concentration but varying inhibitor concentrations. IC_{50} values for the compounds **B6** and **B7** were determined by fitting the equation $v_i/v_0 = 1/(1+[I]/IC_{50})$ to initial velocity data as a function of inhibitor concentration, where v_i and v_0 are the rates of the inhibited and uninhibited reactions, respectively. IC_{50} values for the compounds **A13**, **B1**, and **B5** were estimated from determinations of the fraction of inhibited enzyme at a fixed inhibitor concentration of 500 μM and using the equation $IC_{50} = [I] \times (1/(1 - v_i/v_0) - 1)$.[26] Grafit 5.0 was used for the nonlinear regression analysis. Ilomastat was used as a reference in all inhibition experiments.

Computational Chemistry. The computational methods used for this work include protein and ligand structure preparation, virtual screening, and binding-mode modeling through docking. The protein was prepared from crystal structure 1JK3[11] (E219A) and 1JIZ[10] (wt) of MMP-12. Waters and the synthetic inhibitor were removed. Hydrogens were added in SYBYL 7.1 (Tripos International, St. Louis, MO). The positions of the hydrogens were subject to 500 iterations of steepest descent minimization (convergent criteria: 0.1), followed by 500 steps of conjugate gradient minimization (convergent criteria: 0.01) using the Merck molecular force

field (MMFF).[27] All non-hydrogen atoms were held rigid during these minimizations. The ligands were also built using SYBYL 7.1 and subject to 1000 iterations of steepest descent minimization (convergent criteria: 0.01) using the Merck molecular force field (MMFF).[27] Both stereoisomers (*R*,*S*) of compound **B7** were modeled because the experimental testing was performed using a racemic mixture. Virtual screen and binding-mode modeling were performed using the program GOLD[12] at standard default settings. The GOLD Fitness scoring function and the ChemScore[28] scoring function were explored in individual experiments. The site was defined using a 10 Å radius around an atom positioned at the mouth of the S1′ pocket. Each compound was docked 100 times. The binding-mode modeling and the results were assessed through visual inspection of the complete docking ensembles.

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