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Bioorganic & Medicinal Chemistry 12 (2004) 37–44
Bioorganic & Medicinal Chemistry

Structure–activity relationships by mass spectrometry: identification of novel MMP-3 inhibitors

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Received 30 January 2003; revised 20 October 2003; accepted 25 October 2003

Abstract—A novel class of nonpeptide inhibitors of stromelysin (MMP-3) has been discovered with the use of mass spectrometry. The method relies on the development of structure–activity relationships by mass spectrometry (SAR by MS) and utilizes information derived from the binding of known inhibitors to identify novel inhibitors of a target protein with a minimum of synthetic effort. Noncovalent complexes of known inhibitors with a target protein are analyzed; these inhibitors are deconstructed into sets of fragments which compete for common or overlapping binding sites on the target protein. The binding of each fragment set can be studied independently. With the use of competition studies, novel members of each fragment set are identified from compound libraries that bind to the same site on the target protein. A novel inhibitor of the target protein was then constructed by chemically linking a combination of members of each fragment set in a manner guided by the proximity and orientation of the fragments derived from the known inhibitors. In the case of stromelysin, a novel inhibitor composed of favorably linked fragments was observed to form a 1:1 complex with stromelysin. Compounds that were not linked appropriately formed higher order complexes with stoichiometries of 2:1 or greater. These linked molecules were subsequently assessed for their ability to block stromelysin function in a chromogenic substrate assay.

1. Introduction

Noncovalent macromolecular interactions are one of the most important aspects of many biological processes such as cell signaling, gene transcription, and translation.[1] The identification of molecules which bind to and block the function of biologically active proteins is the cornerstone of modern therapeutic intervention. Traditionally, lead compounds are identified through an iterative process of synthesis followed by in vitro biological evaluation. With the advent of high throughput screening (HTS) technologies, the process of lead identification is moving toward generating quality leads more quickly.[2] However, in vitro biological testing of small molecules' ability to block function is often fraught with false positives that can make it difficult to follow SAR trends.[3]

False positives can arise from the compounds interacting with the assay system rather than the biological target or from artifacts inherent in the detection system.[3] Because of these limitations, there is a need to incorporate other data sources for assessing potential small molecule leads.

Mass spectrometry is a valuable technique for the characterization of large biological macromolecules with advantages in both the speed and sensitivity of detection. Of the ionization techniques available, electrospray ionization (ESI) is most widely used to study macromolecular complexes because it is a mild form of ionization.[4,5] It has been demonstrated from many examples that the observations by ESI-MS for weakly bound noncovalent complexes to some extent reflect that which is observed in solution.

There have been a number of these examples in which mass spectrometry was used to study macromolecular interactions such as protein–peptide, peptide–peptide, protein–metal, and small molecule–RNA complexes.[6] Mass spectrometry has been used a screening tool for

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doi:10.1016/j.bmc.2003.10.053

combinatorial libraries of candidate peptides binding to the SRC SH2 domain.[7] We were interested in assessing further the capability of ESI-MS to identify not only potential small molecule inhibitors to various protein targets but to use it as a tool in developing a small molecule lead compound quickly with a minimum of synthetic effort.

Herein we describe our efforts to adapt standard ESI-MS methodologies to directly measure the binding affinity and stoichiometry of noncovalent complexes between known compounds and stromelysin (MMP-3). Further, we describe how these known molecules can be deconstructed into sets of fragment molecules where the members of each set compete for distinct binding sites on stromelysin.

A novel inhibitor of stromelysin is then constructed by combining a member of each set of fragments in a process guided by their connectivity in the known compounds. In this process optimal fragments are identified and linked chemically to form a lead compound that is shown to inhibit the function of stromelysin.

2. Results and discussion

2.1. Observation of the noncovalent complexes

With ESI-MS gas phase ions are produced directly from solution via a fine spray of droplets created in the presence of a strong electric field. Evaporation of solvent increases the droplet surface charge density. Expulsion of the ions from the droplet transfers them into the gas phase.[8] This gentle ionization technique produces gas phase ions that are typically <1 eV above their ground state and are generally not prone to additional unwanted fragmentation. This makes ESI-MS an attractive technique for studying noncovalent complexes. Unlike other spectroscopic techniques that provide only a time or weighted average of the species present in solution, data obtained from mass spectrometry provide relative populations of each species present for each charge state in the mass spectrum.[9] Noncovalent complexes of proteins and small molecules are typically observed under biologically relevant conditions that include water as the solvent at moderate pH ranges from 6 to 8.

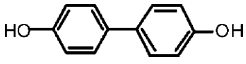
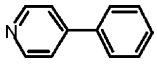
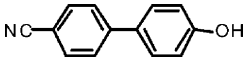
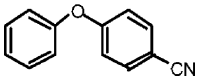
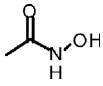
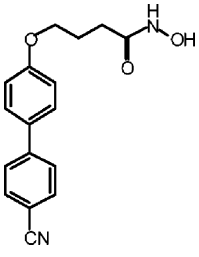
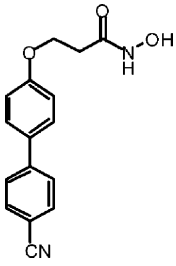
Typically, samples do not contain additional organic modifiers that can stabilize the signal but destabilize weak noncovalent complexes. Under ESI conditions care must be taken to avoid high concentrations of inorganic salts or nonvolatile buffers, which can mask the signal of the desired noncovalent complex of the target protein with a small molecule. In our case samples were diluted with water. The pH of the samples were 5.5–6.5—the optimal pH range for stromelysin activity.

Relatively minor changes to the mass spectrometer were needed to observe the complexes with the most important parameter being the orifice potential. With our system it was found that stable positively charged complexes could be observed, and the measured K_d was constant if the orifice potential was maintained in the range of 55–70 V. Above 70 V fewer complexes were observed, and below 55 V a significant number of water-adducted ions were observed.

2.2. Binding of known inhibitors

We chose stromelysin and the hydroxamic acid inhibitors from Fesik's SAR by NMR study as a model system because of the availability of binding information. The two biphenyl hydroxamic acid inhibitors 6–7 (Table 1), were synthesized according to literature procedures.[4] When solutions of stromelysin with each of the inhibitors were analyzed by ESI-MS, we were able to detect the presence of stromelysin as the free enzyme as well as a complex with each of the biphenyl hydroxamic acids. We next determined these complexes were noncovalent in nature either by increasing the orifice potential or by adding small amounts of methanol to the sample

Table 1. Comparison of dissociation constants of MMP-3 fragments

Compd	Ligands	K_d (μM) Mass Spec.[a]	K_d (μM) NMR
1		223 ± 0.15	160 ± 0.15
2		300 ± 0.06	170 ± 0.03
3		20 ± 0.003	20 ± 0.01
4		460 ± 0.11	480 ± 0.27
5		10,000	17,000
6		23 ± 0.29	ND
7		8 ± 1.1	ND

[a] Uncertainties determined as the standard deviation of the mean of dissociation constants measured in different charge states of a mass spectrum.

solutions. Both methods eliminated the complexes of **6** or **7** with stromelysin from the mass spectra.

An interesting observation was made regarding the stoichiometry of the complexes of **6** and **7** with stromelysin. The more potent inhibitor **7** formed a 1:1 complex and no higher order complexes with the enzyme. On the other hand, compound **6** with an additional methylene unit linking the biphenyl and hydroxamate moieties formed both 1:1 and 2:1 complexes with stromelysin. Apparently, compound **7** with the optimized linker length bridging between the zinc and S1′ sites stabilizes a 1:1 complex with stromelysin (Fig. 1). Compound **6** appears to form two independent 1:1 complexes via the binding of the biphenyl group at the S1′ and/or the hydroxamic acid at the zinc sites, respectively. Bifunctional compounds that have the proper presentation of linked moieties should have a higher affinity for the target protein than each of the individual fragments. To test this hypothesis, we studied the competition of **6** and **7** for stromelysin. In the presence of equimolar amounts of **6** and **7**, which were in excess relative to the concentration of stromelysin, we could not determine the relative abundance of each complex because of the broadened peaks observed most likely due to the desolvation conditions necessary to observe the complexes. However, the 2:1 complex of **6** noted earlier was not observed in the presence of **7** indicating that the 1:1 complex of **7** is much higher affinity than the 2:1 complex of 6.

The presence of a 1:1 complex of small molecule to protein is an important observation from this initial work; and it is this observation that we will use to guide our efforts toward the development of novel compounds.

To obtain solution binding constants of the biphenyl hydroxamic acids, the assumption was made that the response factors such as ionizabilty of stromelysin (19,000 D) complexed with a small molecule (<300 D) were the same as that of the uncomplexed protein.[9a] Using ESI-MS the abundance of each species can be simultaneously determined.[10] The effective solution equilibrium concentrations of the individual species were calculated as the product of the relative peak intensities of the different species present and the original enzyme concentration.[11] Solution K_d values of 23 and 8 μM were obtained for **6** and **7**, respectively. These K_d values are surprisingly similar for inhibitors displaying such differential activities against stromelysin.4 Although care was taken to ensure that the pH of the samples were within the optimal 5.5–6.5 range for stromelysin activity, the $CaCl_2$ concentration was much lower in our ESI conditions than in typical stromelysin activity assay conditions.[12] It has been demonstrated that the calcium concentration can have a large affect on the observed K_d for stromelysin. This would explain why both inhibitors seem to have higher than expected K_d values. The relative dissociation constants that were measured accurately rank the two compounds.

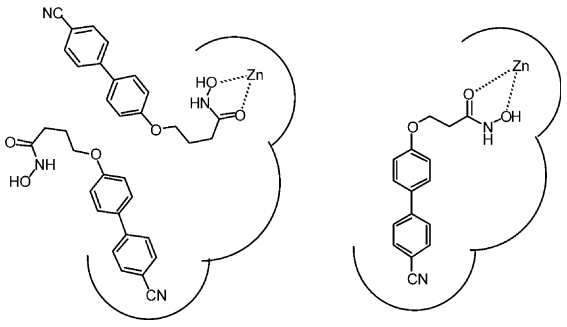


Figure 1. Optimally linked fragments bind as a 1:1 complex with the stromelysin. Fragments that are not linked properly bind as 1:1 and 2:1 complexes.

2.3. Known fragments from lead compounds

We were interested in exploring the use of ESI-MS as a tool for studying SAR trends for developing novel lead compounds. For the initial method development we used information that was readily available to explore the SAR.

Acetohydroxamic acid was chosen as the ligand for the zinc binding site because of its good water solubility, and the mass of its binary complex with stromelysin would be distinct from the expected complexes of stromelysin and potential ligands. Several different concentrations of acetohydroxamic acid were run to determine an optimal concentration as well as the K_d. The dissociation constant obtained from ESI-MS was 10 mM which is in good agreement with the reported value (17 mM).[4] For binding studies on all ligands lacking a hydroxamic group the concentration of hydroxamic acid in the samples was kept constant at 2.25 mM. Details are given in the Experimental section.

Initially, samples of fragments were diluted from a 10 mM stock solution in 100% DMSO to final concentrations at their reported K_d values. This was to insure both species of complexed and uncomplexed protein would be observed. Figure 2 is an example of a typical mass spectrum. Peaks were observed in the mass spectrum that corresponds to stromelysin noncovalently bound to the ligands present in the sample. The peak at $m/z = 1142.0$ corresponded to the $(M + 17H)$ $+17$. The other three peaks in the $+17$ state corresponded to stromelysin complexed with the acetohydroxamic acid (1147.5), stromelysin complexed with biphenyl **3** (1153.2), and the ternary complex of stromelysin with

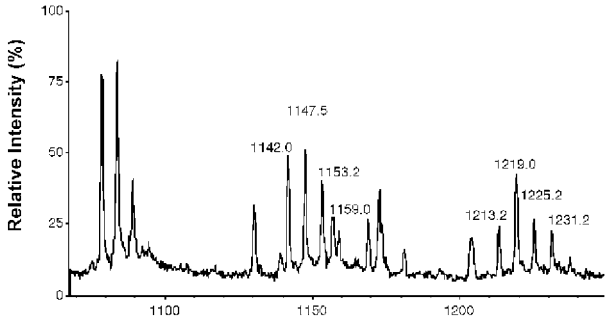


Figure 2. Mass spectrum of **3** and **5** with stromelysin.

acetohydroxamic acid and the biphenyl fragment (1159.0), respectively. No ions were detected that corresponded to more than a 1:1 complex of each ligand with stromelysin indicating the interactions with the protein were specific and not due a nonspecific binding of ligands to the protein as the solvent evaporated. The observed complexes were dissociated either by increasing the orifice potential or adding a declustering solvent such as methanol thus confirming that noncovalent complexes were observed.

In the initial screening of fragments known to bind in the S1′ binding site of stromelysin (**1**–**5**), a general agreement was observed between the K_d values measured by mass spectrometry as compared to reported values obtained in solution (see Table 1).

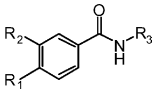
2.4. Development of novel fragments

Having determined mass spectrometry to be a quick and reliable method for studying small molecule protein interactions, we compiled a small library of novel fragments in order to identify alternative fragments that would bind as a 1:1 complex with stromelysin. From previous work it is known that the S1′ site is a deep largely hydrophobic pocket in which aromatic groups are preferred.[13] Based on their geometric similarities with the known S1′ biphenyl fragments a small library of fragments was compiled. These included compounds such as benzamides and benzophenone. In the initial screen, two compounds, benzophenone and amide **8** formed a 1:1 noncovalent complex with stromelysin. Through competition studies with 4-phenylpyridine, 2, both benzophenone and **8** were shown to compete for the same binding site on stromelysin. Benzamide **8** was the better binding fragment and had a measured K_d of 190 μM. We opted to pursue the benzamide class because of their ease of synthesis and greater availability of starting materials.

A library was designed to study structure activity relationships related to electronic differences between methoxy, cyano, and methyl substituted amides as well as geometric subtleties such as amide bond orientation, Tables 2 and 3. A small library of fifteen amides was synthesized utilizing standard methodologies. Eleven had detectable binding to stromelysin, and each of the amides was assessed for their ability to form a 1:1 complex with stromelysin. Those that were found to form a 1:1 complex were further assessed by a series of competition experiments designed to determine if they bound in the same binding site as the biphenyl ligands.

In initial competition experiments an equimolar mixture of 4-phenylpyridine and amide of interest was equilibrated with stromelysin, and the mixture was analyzed by mass spectrometry. Fragments were determined to compete if peaks corresponding to the masses of the individual binary complexes of amide or **2** with stromelysin but no ternary complex of stromelysin, amide, and **2** bound simultaneously could be detected (Fig. 3). Conversely, fragments that bound simultaneously to

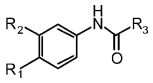
Table 2. Amide library



Compd	R_1	R_2	R_3	K_d (μM)
8	OCH_3	H	Bn	190
9	OCH_3	H	Cyclohexyl	230
10	OCH_3	H	Ph	435
11	OCH_3	H	$(CH_2)_2Ph$	[a]
12	OCH_3	H	*p*-Tolyl	30
13	CN	H	Ph	> 10,000
14	CN	H	$(CH_2)_2Ph$	300
15	CN	H	Bn	[a]
16	H	OCH_3	*p*-Tolyl	220
17	CH_3	H	Ph	310
18	*n*-Propyl	H	Ph	
19	H	CH_3	Ph	> 101,000

[a] Fragments did not compete with 4-phenylpyridine.

Table 3. Reverse amide library



Compd	R_1	R_2	R_3	K_d (μM)
20	OCH_3	H	Bn	[a]
21	OCH_3	H	p-Tolyl	370
22	CN	H	Ph	[b]
23	CN	H	Bn	[b]

[a] No binding detected by mass spectrometry.
[b] Fragments did not compete with 4-phenylpyridine.

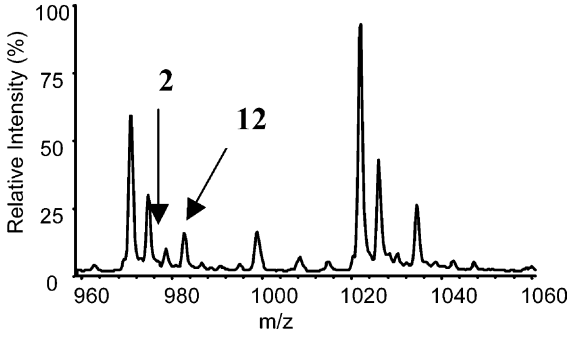


Figure 3. Competitive binding between **2** and **1**2. Peaks corresponding to each species bound to stromelysin while simultaneous binding of the fragments cannot be detected.

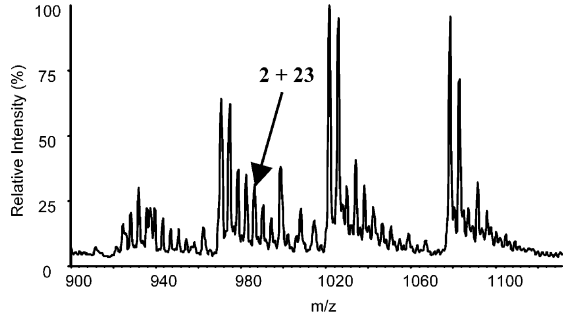


Figure 4. Noncompetitive binding between **2** and **2**3. Peaks corresponding to each species bound to stromelysin as well as both species bound simultaneously can be detected.

stromelysin (e.g., **2** and **23**, Fig. 4) were determined not to compete for the same binding site. Eight fragments from the library were found to compete with the phenylpyridine fragment for the S1′ binding site.

Generally, the *para*-methoxybezamides were preferred over the *para*-cyanobenzamides. In contrast Fesik observed the 4-methoxy substitution in the biphenyl fragments had a detrimental effect on binding. This observation indicated that although the two classes of fragments bound in the same binding site, their interactions with stromelysin were not identical. Reversal of the amide bond orientation generally had a detrimental effect on the binding of the fragment indicating there may be a specific hydrogen-bonding interaction between the protein and the amide. Benzamide **12** was determined to be the highest affinity fragment from the library exhibiting a 15-fold enhancement over compound **10**. Although the orientation of the amide fragments is not known, the most likely orientation for **12** is with the 4′-methyl oriented into the hydrophobic pocket. This would account for its enhanced activity versus the unsubstituted **10**. The dissociation constant for **12** was determined to be 30 μM at a number of different concentrations. This compares favorably with the best of the biphenyl fragments,[3] which had a K_d of 20 μM.

Through the use of ESI-MS we were able to identify a novel lead fragment very quickly with a minimal protein requirement. What remained to be done was to connect the novel benzamide fragment with the zinc-binding hydroxamic acid moiety. We were guided in this effort by the structure of the initial lead compound **7**.

2.5. Design and synthesis of linked fragments

Although the site of binding could be inferred through competition experiments, ESI-MS does not provide the same information about the distance or orientation of the diphenyl amides relative to acetohydroxamic acid moiety as was obtained by 2D NMR.[4] In addition, the orientation of the unsymmetrical amide fragments within the S1′ site was an uncertainty of the method. To address the issues of distance and orientation between the acetohydroxamic and **12**, a series of compounds were synthesized that varied the orientation of the diphenyl amide and the length and orientation of the methylene linker (Table 4). Both *meta* and *para* substituted phenylalkyl hydroxamic acids were synthesized. Based on a modeling comparison with Fesik's optimally linked biphenyl **7** and information about the structure of stromelysin's binding site, it was determined that *ortho* substituted benzamides would likely place the hydroxamic in a direction not conducive to binding.

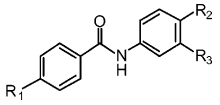
Issues about the relative orientation of the amide bond were addressed by synthesizing linked compounds that had either the methoxy or methyl groups *para* to the amide bond. The presence of the hydroxamic acid determined the orientation of the linked compounds relative to the S1′ binding site.

For speed and synthetic ease, linked compounds were synthesized with a methylene linker. Linked fragment compounds were synthesized by solid phase organic synthesis. Commercially available hydroxylamine resin was used under standard Fmoc synthesis conditions, Scheme 1. The desired Fmoc-protected aminobenzoic acids were loaded onto commercially available chlorotrityl hydroxylamine resin that was first deprotected with 20% piperidine in DMA. The desired amino acids were coupled to the resin utilizing standard HATU coupling conditions. Amines were deprotected and capped with either anisic or toluic acids. Linked compounds were cleaved from the resin under acidic conditions, and purified by reverse phase HPLC (Scheme 1).

2.6. Analysis of linked fragments

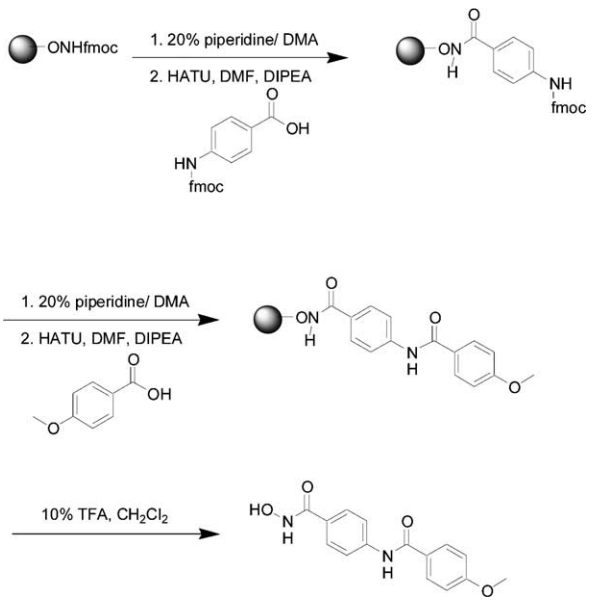
In a typical experiment linked compounds (ca. 20 μM) were equilibrated for one h with stromelysin (5 μM) prior to analysis. Each compound was analyzed for its ability to form a 1:1 complex with stromelysin. It was found that linked compounds **24**–**28** and **30**–**33** formed

Table 4. Linked fragment library



Compd	R_1	R_2	R_3	K_d μM	
24	OCH_3	CONH	H	ND	2:1
26	OCH_3	$CH_2CONHOH$	H	ND	2:1
27	OCH_3	H	$CH_2CONHOH$	ND	2:1
28	OCH_3	H	$(CH_2)_2CONHOH$	250	2:1
29	OCH_3	$(CH_2)_3CONHOH$	H	200	1:1[a]
30	CH_3	H	CONHOH	ND	2:1
31	CH_3	$CH_2CONHOH$	H	ND	2:1
32	CH_3	H	$CH_2CONHOH$	ND	2:1
33	CH_3	H	$(CH_2)_3CONHOH$	400	2:1
34	CH_3	$(CH_2)_3CONHOH$	H	ND	2:1

[a] Fragments that bind in more than on site on a protein can be chemically linked. Optimally linked fragments form a 1:1 complex with stromelysin. If the fragments are not linked properly, each of the fragments in the linked compounds act independently.



Scheme 1.

2:1 complexes of small molecule to protein indicating the linker was not optimal. Linked benzamide **29**, however, formed a 1:1 complex with stromelysin. The K_d for this compound was determined to be 200 μM, 4-fold higher than the benzamide **12** from which it was derived. The higher observed K_d may be due to some artifact related to the presence of the hydroxamic acid moeity. The higher than expected K_d values seemed to be consistent with all of the linked compounds. Never the less, we have been able to identify a structurally novel lead compound from a deconstruction and reconfiguration of an earlier lead molecule.

2.7. Biological activity of linked fragments

Linked fragments **24**–**33** were assayed for their ability to block the function of stromelysin in a chromagenic substrate assay using biphenyl hydroxamic acids **6** and **7** as internal standards. The IC_{50} for the two standards **6** and **7** were found to be 45 and 0.060 μM—slightly higher than the reported 3.4 and 0.025 μM, respectively. Many of the linked fragments were inactive within the limit of detection of this assay system. Three of the linked compounds—**28**, **29**, and **33**—did exhibit mild activity against stromelysin. Of these, only compound **29** formed a 1:1 complex with stromelysin and an IC_{50} of 200 μM which is similar to Fesik's compound **6**.

3. Conclusion

We have been able to demonstrate that mass spectrometry is a valuable tool for studying noncovalent protein small molecule interactions and that small fragments bound to a protein's surface can be detected by electrospray ionization mass spectrometry in a manner reflective of their affinities. The dissociation constants measured by this technique are comparable to those obtained from other methods with the possible exception of compound **7**. Dissociation constants ranging from low micromolar to millimolar were measured by this method. This is especially useful because this is a range of affinities, which is often difficult to measure by other techniques. While the absolute binding affinities may not appear to be accurate, general SAR trends can be obtained by this methodolgy. Compound **29** was the linked compound that formed a 1:1 complex and was also the compound that was the best inhibitor of stromelysin. The modest activity suggests that the binding of the linked compound was not optimal.

The activity can be increased through standard medicinal chemistry. However, we have demonstrated that a small molecule known to inhibit Stromelysin can be dissected into fragments and the binding of those fragments can be studied. In general, each of these fragments derived from the deconstruction of a known inhibitor defines a binding site on the protein, and these binding sites are proximal but not overlapping. Consequently, the fragments of a known inhibitor are capable of binding simultaneously to the target protein forming higher order complexes with the protein.

This has been confirmed in the case of stromelysin where a ternary complex of the enzyme with both a biphenyl and hydroxamic acid fragments was observed. Through the use of competition studies, novel fragments, which compete with the fragments of a known inhibitor for the same binding site can be identified. These novel fragments can subsequently be (re)combined to form novel small molecule lead compounds in a process guided by the connectivity of the original known inhibitor. Compounds in which the linking of the fragments is correct form a 1:1 complex with the target protein.

In the case of stromelysin, novel benzamides were shown to compete with known biphenyl fragments at the S1′ site. Guided by the structure of the known lead, compound **7**, a simple strategy for linking the novel benzamide fragment to the known hydroxamic acid fragment identified a novel inhibitor of Stromelysin. From a small library of linked fragments, the proper linker in compound **29** was readily recognized by the formation of a 1:1 complex with stromelysin. In a focused library containing only **28** small molecules, we were able to quickly identify a novel lead compound, **29**. This would be a departure point for subsequent medicinal chemistry studies to identify a high affinity inhibitor of stromelysin from this novel structural class. We have demonstrated the utility of mass spectrometry as a tool in a lead identification process targeting an enzyme with known inhibitors. Future studies will attempt to extend this methodology to the identification of inhibitors of novel protein targets without the aid provided by existing inhibitors.

4. Experimental

NMR Spectroscopy. All NMR spectra were recorded on a Varian Mercury VX 300 mHz instrument at 25 °C. Chemical shift assigned relative to TMS.

4.1. General amide synthesis

Benzoyl chloride (2.15 mmol) was dissolved in 30 mL of CH_2Cl_2. To the solution was added 2.15 mmol of aniline and an excess amount of Hunig's base. The reaction was stirred at room temperature until complete as analyzed by TLC (3:2 hexane/ethyl acetate). Standard aqueous work up was employed followed by removal of the solvent by rotary evaporation. All compounds were purified by reversed phase HPLC. Commercially available acid chlorides were used whenever available. Standard DCC coupling conditions were utilized for amide synthesis from commercially available carboxylic acids, or the acids were converted to the acid chloride prior to coupling by treatment with oxalyl chloride.

4.2. General synthesis of linked compounds

4.2.1. Sample purification. Amides were purified on a LCMS API150EX mass spectrometer equipped with a 1×10 cm Amicon C18 reversed phase column eluting with 10 mM NH_4OAc/CH_3CN (0–90% in 20 min). Fractions were collected with a Gilson 215 liquid handler. Hydroxamic acids were purified with a Waters HPLC equipped with a Waters 490E UV–vis detector on an Amicon C18 column (1×50 cm) using a gradient elution with 10 mM NH_4OAc/CH_3CN (0–75% CH3CN in 25 min).

4.3. Mass spectrometry sample preparation

For mass spectrometry experiments with fragments all samples were prepared as follows. To a 5 μL aliquot of stromelysin catalytic domain (19.6 mg/mL, 20 mM Tris, pH 7.5, 0.02% NaN_3, 5 mM $CaCl_2$) was added 5 μL of 450 mM acetohydroxamic acid (pH 6.0). A 2 μL aliquot of this solution was placed in an eppendorf tube. To this sample was added 100 μL of water followed by the desired fragment (2–4 μL) depending on the desired final concentration. This mixture was allowed to equilibrate at room temperature for 1 h. Sample were diluted to a final volume of 200 μL. The final pH of the sample was 5.5–6.5.

4.4. Mass spectrometry

Mass Spectra were recorded on a Perkin Elmer Sciex API III mass spectrometer. Samples were introduced via 50 μM i.d. fused silica capillary by a 50 μL syringe using a Harvard Apparatus Model 22 syringe pump at a flow rate of 1.50 μL/min. The orifice potential was optimized to 60 V, and the interface heater was at 56 °C. Mass spectra were obtained by averaging a sufficient number of scans to gain adequate signal-to-noise.

4.5. Determination of stromelysin inhibition

Stromelysin inhibition was measured using commercially available MMP-3 Activity Assay Kit (Chemicon International ECM481) in which activity is related to cleavage of FITC labeled substrate. The reported conditions were modified to allow assays to be run in a 96-well microtitre plate. The stromelysin used for the assays was the same protein used for the mass spectrometry experiments. Samples were diluted to various concentrations with assay buffer from stock solutions in 100% DMSO; total concentration of DMSO was kept below 20%.

4.5.1. Cyclohexyl-4-methoxybenzamide (9). ^{1}H NMR (DMSO-d_6) δ = 1.13–1.78 (m, 10H), 3.73 (m, 1H), 3.80 (s, 3H), 6.98 (dt, 2H, J = 2.0, 9.0 Hz), 8.05 (d, 1H, J = 8.1 Hz).

4.5.2. 4-Methoxy-N-phenylbenzamide (10). ^{1}H NMR ($CDCl_3$) δ = 3.88 (s, 3H), 6.98 (dt, 2H, J = 2.1, 8.7 Hz), 7.14 (t, 1H, J = 6.3 Hz), 7.39 (t, 2H, J = 8.4 Hz), 7.65 (dd, 2H, J = 1.5, 7.5 Hz), 7.72 (bs, 1H), 7.84 (dt, 2H, J = 1.8, 8.7 Hz); ^{13}C ($CDCl_3$) δ = 55.50, 113.92, 120.06, 124.24, 128.77, 128.76, 128.96, 138.00, 162.34.

4.5.3. *N*-(2-Phenethyl)-4-methoxybenzamide (11). ^{1}H NMR ($CDCl_3$) δ = 2.93 (t, 2H, J = 6.6 Hz), 3.72 (q, 2H, J = 5.7 Hz), 3.84 (s, 3H), 6.02 (bs, 1H), 6.89 (dt, 2H, J = 1.8, 8.7 Hz), 7.23–7.34 (m, 5H), 7.65 (dt, 2H, J = 2.4, 9.0 Hz); ^{13}C ($CDCl_3$) δ = 35.87, 41.12, 55.41, 113.68, 126.46, 128.46, 128.60, 128.72, 138.91.

4.5.4. 4-Methoxy-*N*-*p*-tolylbenzamide (12). ^{1}H NMR ($CDCl_3$) δ = 2.34 (s, 3H), 3.88 (s, 3H), 6.98 (dt, 2H, J = 2.1, 9.0 Hz), 7.18 (d, 2H, J = 8.7 Hz), 7.52 (d, 2H, J = 8.4 Hz), 7.68 (bs, 1H), 7.85 (dt, 2H, J = 1.8, 8.7 Hz); 13C ($CDCl_3$) δ = 20.96, 55.47, 113.85, 120.18, 127.19, 128.75, 129.41, 133.83, 135.45, 162.22, 164.97.

4.5.5. 4-Cyano-*N*-phenylbenzamide (13). ^{1}H NMR (DMSO-d_6) δ = 7.16 (t, 1H, J = 7.5 Hz), 7.38 (t, 2H, J = 8.4 Hz), 7.78 (d, 2H, J = 7.8 Hz), 8.05 (d, 2H, J = 6.3 Hz), 8.12 (d, 2H, J = 8.1 Hz), 10.49 (s, 1H).

4.5.6. 4-Cyano-*N*-phenethylbenzamide (14). ^{1}H NMR ($CDCl_3$) δ = 2.95 (t, 2H, J = 6.6 Hz), 3.76 (q, 2H, J = 6.6 Hz), 6.10 (bs, 1H), 7.20–7.37 (m, 5H), 7.73 (q, 4H, J = 8.2 Hz).

4.5.7. *N*-Benzyl-4-cyanobenzamide (15). ^{1}H NMR (DMSO-d_6) δ = 4.51 (d, 2H, J = 6.0 Hz), 7.23–7.34 (m, 5H), 7.99 (dd, 2H, J = 2.4, 8.7 Hz), 8.03 (dd, 2H, J = 1.8, 8.4 Hz), 9.32 (t, 1H, J = 6.3 Hz).

4.5.8. 3-Methoxy-*N*-*p*-tolylbenzamide (16). ^{1}H NMR (DMSO-d_6) δ = 2.28 (s, 3H), 3.84 (s, 3H), 7.14–7.17 (m, 3H), 7.44 (d, 1H, J = 7.8 Hz), 7.54 (d, 1H J = 7.8 Hz), 7.66 (d, 2H, H = 8.4 Hz), 10.14 (s, 1H).

4.5.9. 4-Methyl-*N*-phenylbenzamide (17). ^{1}H NMR (DMSO-d_6) δ = 2.38 (s, 3H), 7.09 (t, 1H, J = 7.4 Hz), 7.35 (d, 2H, J = 6.6 Hz), 7.78 (d, 2H, J = 7.4 Hz), 7.89 (d, 2H, J = 8.1 Hz), 10.15 (s, 1H).

4.5.10. *N*-(4-Methoxyphenyl)-4-methylbenzamide (21). ^{1}H NMR (DMSO-d_6) δ = 2.49 (s, 3H), 3.74 (s, 3H), 6.93 (d, 2H, J = 9.0 Hz), 7.34 (d, 2H, J = 8.4 Hz), 7.68 (d, 2H, J = 9.3 Hz), 7.87, (d, 2H, J = 8.4 Hz), 10.04 (s, 1H).

4.5.11. *N*-(4-Cyanophenyl)benzamide (22). ^{1}H NMR (DMSO-d_6) δ = 7.54–7.64 (m, 3H), 7.82 (dt, 2H, J = 1.9, 8.8 Hz), 7.94–8.01 (m, 4H), 10.65 (s, 1H).

4.5.12. *N*-(4-Cyanophenyl)-2-Phenylacetamide (23). ^{1}H NMR (DMSO-d_6) δ = 7.13 (t, 1H, J = 7.5 Hz), 7.38, (t, 2H, J = 8.4 Hz), 7.76 (d, 2H, J = 8.4 Hz), 8.05 (dt, 2H, J = 1.8, 8.4 Hz), 8.12 (d, 2H, J = 8.1 Hz), 10.49 (s, 1H).

4.5.13. 4-(4 - Methoxybenzoylamino)benzohydroxamic acid (24). ^{1}H NMR (DMSO-d_6) δ = 3.95 (s, 3H); 7.16 (d, 2H, J = 9.0 Hz); 7.83 (d, 2H, J = 8.7 Hz); 7.93 (d, 2H, J = 9.0 Hz); 8.06 (d, 2H, J = 9.0 Hz); 10.38 (s, 1H). HRMS: calcd 286.0954; found 285.0999 (M−H).

4.5.14. 3 - (4 - Methoxybenzoylamino)benzohydroxamic acid (25). ^{1}H NMR (DMSO-d_6) δ = 3.85 (s, 3H); 7.05 (d, 2H, J = 9.0 Hz); 7.39 (m, 2H); 7.91–8.00 (m, 3H); 8.17 (s, 1H); 9.00 (s, 1H); 10.21 (s, 1H); 11.17 (s, 1H); HRMS: calcd 286.0954; found 286.0957.

4.5.15. [4-(4-Methoxybenzoylamino)phenyl]aceto-hydroxamic acid (26). ^{1}H NMR (DMSO-d_6) δ = 3.56 (s, 2H); 3.62 (s, 3H); 6.84 (d, 2H, J = 9.7 Hz); 7.32, (m, 4H); 7.48 (d, 2H, J = 9.1 Hz); 10.02 (s, 1H); HRMS: calcd 300.1110; found 300.1120.

4.5.16. [3-(4-Methoxybenzoylamino)phenyl]aceto-hydroxamic acid (27). ^{1}H NMR (DMSO-d_6) δ = 3.27 (s, 3H); 3.84 (s, 2H); 6.97 (d, 1H, J = 7.2 Hz); 7.05 (d, 2H, J = 8.7 Hz); 7.26 (t, 1H, J = 7.8 Hz); 7.62 (d, 1H, J = 7.8 Hz); 7.69 (s, 1H); 7.95 (d, 2H, J = 8.7 Hz); 8.81 (bs, 1H); 10.09 (s, 1H); HRMS: calcd 300.1110; found 300.1122.

4.5.17. [3-(4-Methoxybenzoylamino)phenyl]propano-hydroxamic acid (28). ^{1}H NMR (DMSO-d_6) δ = 2.26 (t, 2H, J = 7.2 Hz); 2.80 (t, 2H, J = 7.5 Hz); 3.84 (s, 3H); 6.91 (d, 1H, J = 7.8 Hz); 7.05 (d, 2H, J = 9.0 Hz); 7.24 (t, 1H, J = 7.8 Hz); 7.58 (m, 2H); 7.95 (d, 2H, J = 8.7 Hz); 8.71 (d, 2H, J = 1.8 Hz); 10.03 (s, 1H); 10.39 (s, 1H); HRMS: calcd 314.1267; found 314.1262.

4.5.18. [4 - (4 - Methoxybenzoylamino)phenyl]butano-hydroxamic acid (29). ^{1}H NMR (DMSO-d_6) δ = 1.79–1.80 (m, 2H); 1.93–2.00 (m, 2H); 2.72 (m, 2H); 7.04 (d, 2H, J = 8.7 Hz); 7.13 (d, 2H, J = 8.4 Hz); 7.65 (d, 2H, J = 8.4 Hz); 7.93 (d, 2H, J = 8.7 Hz); 8.69 (s, 1H); 10.02 (s, 1H); 10.37 (s, 1H); HRMS: calcd 328.1423; found 328.1437.

4.5.19. 3-(4-Methylbenzoylamino)benzohydroxamic acid (30). ^{1}H NMR (DMSO-d_6) δ = 2.39 (s, 3H); 7.33 (d, 2H, J = 7.8 Hz); 7.40 (d, 2H, J = 7.5 Hz); 7.89–7.95 (m, 3H); 8.19 (s, 1H); 10.31 (s, 1H); HRMS: calcd 270.1004; found 270.1012.

4.5.20. [4-(4-Methylbenzoylamino)phenyl]acetohydroxamic acid (31). ^{1}H NMR (DMSO-d_6) δ = 2.38 (s, 3H); 3.25 (s, 2H); 7.20 (d, 2H, J = 8.4 Hz); 7.32 (d, 2H, J = 8.4 Hz); 7.67 (d, 2H, J = 8.7 Hz); 7.86 (d, 2H, J = 8.1 Hz); 8.82 (d, 1H, J = 1.8 Hz); 10.12 (s, 1H); 10.63 (s, 1H); HRMS: calcd 284.1161; found .1162.

4.5.21. [3-(4-Methylbenzoylamino)phenyl]acetohydroxamic acid (32). ^{1}H NMR (DMSO-d_6) δ = 2.26 (t, 2H, J = 6.9 Hz); 2.39 (s, 3H); 2.80 (t, 2H, J = 8.4 Hz); 6.92 (d, 1H, J = 7.8 Hz); 7.32 (d, 2H, J = 8.1 Hz); 7.58 (d, 1H, J = 8.1 Hz); 7.63 (s, 1H); 7.86 (d, 2H, J = 8.1 Hz); 8.71 (d, 1H, J = 1.8 Hz); 10.10 (s, 1H); 10.39 (s, 1H); HRMS: calculated 284.1161; found 284.1158.

4.5.22. [3-(4-Methylbenzoylamino)phenyl]propano-hydroxamic acid (33). ^{1}H NMR (DMSO-d_6) δ = 1.78 (m, 2H); 2.00 (t, 2H, J = 7.2 Hz); 2.38 (s, 3H); 3.11 (q, 2H, J = 7.2 Hz); 7.14 (d, 2H, J = 8.4 Hz); 7.32 (d, 2H, J = 7.8 Hz); 7.64 (d, 2H, J = 8.1 Hz); 7.85 (d, 2H, J = 8.4 Hz); 8.69 (d, 1H, J = 1.2 Hz), 10.10 (s, 1H); 10.37 (s, 1H); HRMS: calculated 298.1317; found 298.1330.

Acknowledgements

We wish to thank Wayne Levin from Roche-Nutley for generously supplying the stromelysin used for this study. We would also like to thank John Nicholas for his helpful modeling suggestions.

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